IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

HUVEPHARMA EOOD and HUVEPHARMA, INC.,	)	
Plaintiffs,	)	
v.	)	C.A. No. 1:18-cv-914-RGA
E.I. DU PONT DE NEMOURS AND COMPANY, DUPONT INDUSTRIAL BIOSCIENCES USA, LLC, DANISCO USA INC., and DANISCO US INC.,	)	JURY TRIAL DEMANDED
Defendants.	)	

AMENDED COMPLAINT

Plaintiffs Huvepharma EOOD (formerly Huvepharma AD) and Huvepharma, Inc. (collectively, "Plaintiffs" or "Huvepharma"), for their amended complaint against E.I. Du Pont De Nemours and Company, DuPont Industrial Biosciences USA, LLC, Danisco USA, Inc., and Danisco US Inc. (collectively, "Defendants"), allege the following:

NATURE OF THE ACTION

1. This is an action for patent infringement arising under the United States Patent Act, 35 U.S.C §§ 1, et seq., including 35 U.S.C § 271.

2. Huvepharma brings this action to obtain relief for Defendants' infringement of Huvepharma's rights under the Patent Laws of the United States 35 U.S.C §§ 1, et seq., which arise from U.S. Patent Nos. 7,026,150 (the "'150 Patent," attached as Exhibit 1); 7,312,063 (the "'063 Patent," attached as Exhibit 2); and 8,455,232 (the "'232 Patent," attached as Exhibit 3).

3. Huvepharma EOOD is a private company incorporated and existing under the laws of the Republic of Bulgaria, registered with the Commercial Register under Unified Identity Code (UIC) 203631745, having its headquarters at 5th floor, 3a, Nikolay Haytov Str., 1113 Sofia, Bulgaria. Huvepharma's wholly-owned United States subsidiary, Huvepharma, Inc., has an

address at 525 Westpark Dr. # 230, Peachtree City, Georgia 30269. Huvepharma, Inc. operates six production facilities in the United States, and commercializes Plaintiffs' phytase product OptiPhos® in the United States under the terms of an agreement with Huvepharma EOOD.

4. Huvepharma is a global biotech and pharmaceutical company that develops, manufactures, and commercializes human and animal health products, including enzymes for food and animal feed. One of Huvepharma's products that it successfully sells in the United States is OptiPhos®, which is an additive to feed for animals, including swine and poultry, and in particular an *Escherichia coli* ("*E.coli*") derived 6-phytase, which is recombinantly produced in the heterologous yeast host *Pichia pastoris*, in a submerged fermentation process. OptiPhos® is available in three different forms, each suitable for a specific application: 1) a coated form used for pelleted feeds, 2) a liquid form used for post pelleting liquid application processes, and 3) a granular form used in mash and pelleted feeds.

5. Previously competing phytase products were less effective than OptiPhos® because they only operate effectively within a limited pH range and are thermally intolerant during the feed manufacturing process when the phytase is combined with animal feed. These previous competing phytase products were also inferior to OptiPhos® because they degrade when exposed to pepsin, which is a naturally present (endogenous) enzyme produced in the stomach of animals.

6. Huvepharma's OptiPhos® is more effective in animal diets for poultry and swine than these previously competing products because it works effectively at a broad pH range (between pH 1 and 5), is thermally tolerant during the manufacturing process when combined with animal feed and is relatively insensitive to degradation by pepsin. OptiPhos® also operates faster than others previously used in releasing phosphorus from its indigestible form phytate,

which is the natural form in which most of the phosphorus is stored in grains and seeds, and thus enables the poultry and swine ingesting the product to grow faster and to receive other health benefits.

7. The method of manufacturing Huvepharma's OptiPhos® was invented and initially developed in the 1998-time frame by Dr. Xingen Lei, a researcher at Cornell University, and constituted a publicly recognized breakthrough in the field of phytase enzymes for integration into animal feed. Cornell Research Foundation, Inc. obtained the '150, '063, and '232 Patents (the "Patents") that disclose, claim, and otherwise protect Dr. Lei's inventive method of producing OptiPhos®.

8. Ultimately, Cornell Research Foundation, Inc. entered into an exclusive license with Huvepharma in return for Huvepharma commercializing OptiPhos® in the United States. However, as explained below, Huvepharma's commercialization efforts have been negatively impacted, and the patent rights have been infringed, by the actions of Defendants, and in particular based on Defendants' manufacture, importation, sale, distribution, and commercialization in the United States of a product line that infringes claims of the patents, *i.e.*, the Phyzyme phytase product line.

9. Upon information and belief, the Phyzyme phytase product line includes products that are and have been commercialized in the United States under various trade names, including at least Phyzyme, Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, and Phyzyme XP Liquid. The products in the Phyzyme phytase product line are also manufactured and sold with different concentration designations, for example Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000G, Phyzyme XP 15000L, Phyzyme XP 10000 TPT, etc. These Phyzyme phytase products are or have been manufactured outside of the United States and imported into and

commercialized by Defendants in the United States. For example, Phyzyme XP 10000 TPT is or has been manufactured overseas, imported into the United States, and commercialized in the United States by Defendants.

10. Defendant E. I. du Pont de Nemours and Company ("DuPont") is a corporation organized under the laws of Delaware, with a registered office at Corporation Trust Center, 1209 Orange Street, Wilmington, Delaware, 19801. DuPont is a wholly owned subsidiary of DowDuPont Inc., a corporation organized under the laws of Delaware, with registered offices at 2211 H.H. Dow Way, Midland, Michigan 48674.

11. Defendant DuPont Industrial Biosciences USA, LLC ("DuPont I.B.") is a limited liability company organized under the laws of Delaware, with its principal place of business at Dupont Experimental Station, 200 Powder Mill Road, Wilmington, Delaware 19803. DuPont I.B. is a wholly-owned subsidiary of DuPont, and effectively controlled by DuPont.

12. Defendant Danisco USA Inc. ("Danisco USA") is a corporation organized under the laws of Delaware, with its principal place of business at 10994 Three Mile Road, Thomson, Illinois, 61285. Danisco USA is a wholly owned subsidiary of DuPont, and effectively controlled by DuPont.

13. Defendant Danisco US Inc. ("Danisco US") is a corporation organized under the laws of Delaware, with its principal place of business at 925 Page Mill Road, Palo Alto, California 94304. Danisco US is a wholly owned subsidiary of DuPont, and effectively controlled by DuPont. Danisco US operates and/or has operated as DuPont I.B.

14. Genencor International, B.V. ("Genencor") is a corporation organized under the laws of The Netherlands, with its principal place of business at Archimedesweg 30, 2333 CN

Leiden, The Netherlands. Genencor is a wholly owned subsidiary of DuPont, and effectively controlled by DuPont.

15. Danisco Argentina S.A. ("Danisco Argentina") is a corporation organized under the laws of Argentina, with its principal place of business at Ingeniero E. Butty 240, Buenos Aires, Argentina. Danisco Argentina is a wholly owned subsidiary of DuPont, and effectively controlled by DuPont.

16. Danisco Mexico S.A. de C.V. ("Danisco Mexico") is a corporation organized under the laws of Mexico, with its principal place of business at KM 37 Carr. Colima-Manzanillo, MEX-28100 Tecoman, Mexico. Danisco Mexico is a wholly owned subsidiary of DuPont, and effectively controlled by DuPont.

17. Finnfeeds Finland Oy ("Finnfeeds"), formerly Finnfeeds Oy Danisco Animal Nutrition Finland, is a corporation organized under the laws of Finland, with its principal place of business at Satamatie 2, FI-21100 Naantalij, Finland. Finnfeeds is a wholly owned subsidiary of DuPont, and effectively controlled by DuPont.

18. Upon information and belief, Phyzyme XP phytase was developed in the 2003 time frame under a strategic alliance with Danisco Animal Nutrition, a subsidiary of Dansico AS and Verenium[1]. Danisco Animal Nutrition was the feed ingredients business unit of Danisco AS, Denmark, both of which defendant DuPont acquired in 2010 and now operate under DuPont I.B. Under the collaboration, Diversa was responsible for manufacturing Phyzyme phytase products, while Danisco Animal Nutrition held exclusive worldwide marketing rights.

19. Upon information and belief, beginning in the 2003 time frame, Verenium and/or Danisco Animal Products contracted with Fermic S.A. de C.V. in Mexico ("Fermic") to

[1] In 2013, BASF Corporation acquired Verenium Corporation ("Verenium"). Verenium's predecessor-in-interest is Diversa Corporation ("Diversa").

manufacture Phyzyme phytase products in Mexico, which was then imported by Defendants and/or their predecessors-in-interest into the United States.[2] Defendants have a long and close relationship with Fermic. When DuPont acquired Danisco AS in 2010, DuPont and/or co-defendants or their subsidiaries continued to use Fermic to manufacture Phyzyme phytase products in Mexico. DuPont, alone or through co-defendants or other subsidiaries, controls Fermic's practice of Huvepharma's patented processes claimed in the '150 Patent, the '063 Patent, and/or the '232 Patent relating to the manufacture of Phyzyme phytase products, at least some of which is then imported into the United States and used, offered for sale, sold, and/or distributed by Defendants in the United States.

20. Upon information and belief, beginning in the 2008 time frame, DuPont's subsidiaries Genencor and/or Finnfeeds have been engaged in the manufacture of Phyzyme phytase products overseas, including the use of *E.coli* derived phytase enzymes that are developed for use in animal feeds, at least some of which Defendants then imported into the United States. Beginning in the 2010 time frame, Genencor and/or Finnfeeds began manufacturing Phyzyme phytase products at a second location in Europe including the use of *E.coli* derived phytase enzymes that are developed for use in animal feeds, at least some of which was then imported by defendants into the United States.

21. Upon information and belief, DuPont is and/or has been engaged in the importation into the United States of *E.coli* derived phytase enzymes developed for use in animal feeds, including at least Phyzyme phytase products, which are used, offered for sale, sold, and/or distributed by Defendants in the United States.

[2] Fermic S.A. de C.V. is a corporation organized under the laws of Mexico, with its principal place of business at Reforma #873 Col. San Nicolas Tolentino, Del Iztapalapa, 09850, Mexico. Fermic is a custom manufacturer of biotechnological products. Upon information and belief, Fermic is and/or has been engaged in the manufacture of Phyzyme phytase products in Mexico, including the use of E.coli derived phytase enzymes that are developed for use in animal feeds.

22. Upon information and belief, Danisco Argentina is and/or has been engaged in the importation into the United States of *E.coli* derived phytase enzymes developed for use in animal feeds, including at least Phyzyme phytase products manufactured by Fermic, which are used, offered for sale, sold, and/or distributed by Defendants in the United States.

23. Upon information and belief, Danisco Mexico is and/or has been engaged in the importation into the United States of *E.coli* derived phytase enzymes developed for use in animal feeds, including at least Phyzyme phytase products manufactured by Fermic and Phyzyme phytase products manufactured by Genencor and/or Finnfeeds, which are used, offered for sale, sold, and/or distributed by Defendants in the United States.

24. Upon information and belief, Finnfeeds is and/or has been engaged in the importation into the United States of *E.coli* derived phytase enzymes developed for use in animal feeds, including at least Phyzyme phytase products manufactured by Fermic and Phyzyme phytase products manufactured by Finnfeeds consigned to Danisco Mexico, which are used, offered for sale, sold, and/or distributed by Defendants in the United States.

25. Upon information and belief, Genencor has been engaged in the importation into the United States of *E.coli* derived phytase enzymes developed for use in animal feeds, including at least Phyzyme phytase products consigned to Danisco Mexico, which are used, offered for sale, sold, and/or distributed by Defendants in the United States.

26. Upon information and belief, Danisco US is and/or has been engaged in the importation into the United States of *E.coli* derived phytase enzymes developed for use in animal feeds, including at least Phyzyme phytase products manufactured by Finnfeeds, which are used, offered for sale, sold, and/or distributed by Defendants in the United States.

27. Upon information and belief, Danisco USA is and/or has been engaged in the importation into the United States *E.coli* derived phytase enzymes developed for use in animal feeds, including at least Phyzyme phytase products manufactured by Finnfeeds, which are used, offered for sale, sold, and/or distributed by Defendants in the United States.

28. Upon information and belief, Defendants have and/or are using, advertising, offering for sale, selling, distributing and/or otherwise commercializing at least the imported Phyzyme phytase products within the United States. Defendants perform at least some of the commercialization of Phyzyme phytase products in the United States under the trade name DuPont Danisco Animal Products.

29. Upon information and belief, Defendants manufacture, use, and commercialize phytase products for applications other than animal nutrition. For example, Defendants manufacture and commercialize phytase products that are used by ethanol producers as beneficial additives for various stages in production of fuel ethanol. (Exs. 23–27.)

30. Upon information and belief, DuPont has and/or is using, advertising, offering for sale, selling, distributing and/or otherwise commercializing at least the imported Phyzyme phytase products within the United States.

31. Upon information and belief, DuPont I.B. has and/or is using, advertising, offering for sale, selling, importing, distributing and/or otherwise commercializing at least the imported Phyzyme phytase products within the United States.

32. Upon information and belief, Danisco USA has and/or is using, advertising, offering for sale, selling, distributing and/or otherwise commercializing at least the imported Phyzyme phytase products within the United States.

33. Upon information and belief, Danisco US has and/or is using, advertising, offering for sale, selling, distributing and/or otherwise commercializing at least the imported Phyzyme phytase products within the United States.

34. Upon information and belief, DuPont wholly owns defendants DuPont I.B., Danisco US, and Danisco USA, each of which has engaged at least in infringing activities described herein. (Ex. 20.)

JURISDICTION AND VENUE

35. This action arises under the Patent Laws of the United States, Title 35, United States Code, §§ 1 *et seq*., including 35 U.S.C. §§ 271 and 281.

36. This Court has jurisdiction over the subject matter of this action pursuant to 28 U.S.C. §§ 1331 and 1338(a).

37. This Court has personal jurisdiction over defendant DuPont, at least because DuPont has purposefully availed itself of the benefits and protections of Delaware state law by incorporating in Delaware.

38. This Court has personal jurisdiction over defendant DuPont I.B., at least because DuPont I.B. has purposefully availed itself of the benefits and protections of Delaware state law by incorporating in Delaware as a limited liability company.

39. This Court has personal jurisdiction over defendant Danisco USA, at least because Danisco USA has purposefully availed itself of the benefits and protections of Delaware state law by incorporating in Delaware.

40. This Court has personal jurisdiction over defendant Danisco US, at least because Danisco US has purposefully availed itself of the benefits and protections of Delaware state law by incorporating in Delaware.

41. Venue is proper in this District under 28 U.S.C. §§ 1391(b) and (c), and 1400(b) because defendants DuPont, Danisco USA, and Danisco US are Delaware corporations, DuPont I.B. is a Delaware company, and Delaware is a convenient forum for resolution of the parties' disputes set forth herein.

BACKGROUND

PHYTASE PRODUCTS FOR ANIMAL NUTRITION

42. The ingestion by certain animals, such as poultry and swine, of phosphate ("P") helps to accelerate growth and provides other health benefits. Phytate (myo-inositol hexophosphate), which includes P, is often included in animal feed for this purpose, *i.e.*, to enable the animals to ingest P. Phytases, which are a specific group of monoester phosphatases, initiate the release of P from the phytate. (Ex. 1: Column 1, Lines 10-31.)

43. Swine and poultry have little natural phytase in their gastrointestinal tracts. Thus, these animals naturally fail to effectively release P from the phytate in their food, and thus fail to benefit thereby. Under these circumstances, the phytate with P passes through the animals' gastrointestinal tracts and excretes as manure, which unfortunately pollutes the environment. In addition, the diet of the swine and poultry needs to be supplemented with inorganic P, which is a non-renewable nutrient, such as in the form of a vitamin. Phytase has therefore been added to animal feed to enable the animals to initiate the release of P from the phytate.

44. Two phytases, PhyA and PhyB, were used prior to the inventions that are the subject of the patents-in-suit. PhyA and PhyB were extracted from *Aspergillus niger* NRRL3135 (*A. niger*), and cloned and sequenced. (Ex. 1: Column 1, Lines 32-40.) As an example, a PhyA gene was introduced into A. niger, *i.e.*, a homologous host, and this phytase was to a certain degree effective in releasing P from phytate in animal feed. In particular, supplemental

microbial phytase of this source in the diets for swine and poultry was shown to be effective in allowing the animals to release P from the phytate in their feed. However, PhyA and PhyB were subject to problems. For example, PhyA and PhyB were expensive to produce. In addition, certain aspects of PhyA and PhyB made them difficult to manufacture and incorporate effectively as functional enzymes into animal feed. For example, the manufacturing process of feed pellets involves the application of a certain amount of heat (*i.e.*, increase in temperature), but unfortunately PhyA and PhyB are subject to being destroyed when exposed to this heat. In other words, the PhyA and PhyB phytases are not sufficiently thermotolerant for this manufacturing process to avoid degradation. (Ex. 1: Column 2, Lines 5-33.)

45. To solve the shortcomings and problems of producing a viable, *i.e.*, functional, phytase enzyme for use in animal feed, Dr. Lei discovered the inventions that are the subject of the patents-in-suit while he was a professor in the Department of Animal Science and Department of Horticultural Sciences at Cornell University. The production methods Dr. Lei invented produced phytases that were at least as effective as, yet more thermostable than, the existing PhyA and PhyB phytases, and therefore were more effective in the animal feed industry.

46. The patents-in-suit involve producing phytases that are encoded by genes isolated from bacterial cells, *i.e.*, from *E.coli*. These encoded genes are not expressed in their homologous bacterial host, but instead are expressed in either a fungal strain or a yeast strain, *i.e.*, a heterologous host. Isolating the expressed, encoded genes leads to an *E.coli* phytase that catalyzes the release of P from phytate. The heterologous host phytase production methods advantageously create phytases, which along with other improved biochemical properties, are characterized with improved thermal stability.

47. Cornell Research Foundation ("Cornell"), wishing to commercialize Dr. Lei's breakthrough discoveries, collaborated with Phytex, LLC, which was a company formed to produce and commercialize Dr. Lei's new thermostable phytase. On September 1, 2001, Cornell entered into an exclusive license agreement with Phytex in return for Phytex producing and commercializing the thermostable phytase. Phytex commercialized the phytase product under the trademarked name "OptiPhos®," which it began manufacturing and selling in the United States in 2006.

48. In 2013, Huvepharma acquired all of Phytex's rights in the thermostable phytase, *i.e.*, OptiPhos®. In particular, Huvepharma acquired Phytex's exclusive license agreement with Cornell, which gave Huvepharma the exclusive rights to produce and commercialize OptiPhos®, and an exclusive license to Cornell's patents. Huvepharma has been manufacturing and commercializing OptiPhos®, which is recognized as the most efficient and stable phytase available in the market with a track record of proven effectiveness. Huvepharma has continually produced and sold OptiPhos® in the United States since acquiring the rights discussed above.

49. Upon information and belief, beginning in the 2010 time frame, DuPont, by and through the other Defendants in this action, began manufacturing, importing, selling, offering to sell, distributing, and otherwise commercializing phytase products marketed under the trade name brand Phyzyme XP. At least the accused products Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT (hereinafter "Phyzyme phytase products", are and/or have been manufactured outside of the United States, and then imported into and commercialized in the United States by

Defendants, and used, offered for sale, distributed, and/or sold by Defendants in the United States.

50. Upon information and belief, the accused Phyzyme phytase products are produced using the same methods in the context of and as claimed in the patents. Thus, the evidence and descriptions below describing the method of producing Phyzyme XP are applicable to any one or more of the other accused products Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT.

51. Upon information and belief, beginning in the 2010 time frame, DuPont acquired the corporation Danisco AS ("Danisco"), headquartered in Belgium, and began operating its manufacturing plant in Finland that was previously owned by Danisco subsidiary Finnfeeds, which is now a wholly owned subsidiary of DuPont and DuPont I.B. or one of DuPont's wholly-owned subsidiaries, to produce Phyzyme phytase products.

52. Upon information and belief, DuPont's subsidiary Genencor at least operates as DuPont's Phyzyme phytase products manufacturer, sales and/or distribution subsidiary business in Europe.

53. Upon information and belief, beginning in the 2010 time frame, at least the defendants DuPont, Danisco USA and Danisco US imported Phyzyme phytase products that were manufactured by Finnfeeds and/or Genencor in Finland into the United States.

54. Upon information and belief, beginning in the 2010 time frame, at least the defendant DuPont I.B., through its wholly-owned subsidiary Finnfeeds, imported Phyzyme phytase products that were manufactured by Fermic in Mexico into the United States.

55. Additionally, upon information and belief, the wholly-owned DuPont foreign subsidiaries Danisco Argentina and Danisco Mexicana import and/or have imported Phyzyme phytase products that were manufactured by Fermic in Mexico into the United States.

56. Upon information and belief, after importation of the Phyzyme phytase products including Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, the Phyzyme phytase products were transferred to at least DuPont, Danisco USA, and/or Danisco US which entities then used, offered for sale, distributed, and/or sold the Phyzyme phytase products in the United States under the company trade name DuPont Danisco Animal Products.

57. Upon information and belief, each use, offer for sale, and/or sale of Phyzyme phytase products by Defendants is a use, offer for sale, and/or sale of phytase imported into the United States by Defendants that was produced and/or is being produced in yeast. (Ex. 4 at 1-3; Ex. 5 at 1-3, Ex. 6 at 1-3.)

58. For example, Phyzyme XP phytase is sourced from an *E.coli* species bacterium and is expressed in the yeast Saccharomyces pombe. (Ex. 7 at 2.) For example, Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified heterologous yeast host *Schizosacchoaromyces pombe* ("*S.pombe*"). (Ex. 10 at 1.) In an example, Genencor operates biorefineries that manufacture Phyzyme phytase products as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme phytase products for animal feed. (Ex. 9 at 1-6; Ex. 8 at 1; Ex. 18 at 2, 4-6.) For example, the additive Phyzyme XP 10000 (TPT/L) phytase is a preparation of 6-phytase produced by the genetically

modified heterologous yeast host *Schizosacchoaromyces pombe* and available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. (Ex. 12 at 4; Ex. 13 at 3.) The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G and varies only in the final formulation. (Ex. 11 at 5; Ex. 12 at 4.)

59. In another example, the packaging label of an order of Phyzyme XP 10000 TPT acquired in the United States by Huvepharma demonstrates that Phyzyme XP 10000 TPT was manufactured in Finland and sold by Genencor. (Ex. 8 at 1.) Analytical testing of the Phyzyme XP 10000 TPT sample in Exhibit 8 by Plaintiffs confirmed that the sample contained a yeast. In another example, a Phyzyme XP 10000 TPT phytase sample acquired by Huvepharma in Belgium was manufactured by Genencor in Finland. (Ex. 14 at 1-7.) Analytical testing of the Phyzyme XP 10000 TPT sample in Exhibit 8 by Plaintiffs confirmed that the sample contained a yeast.

60. Upon information and belief, each use, offer for sale, and/or sale of Phyzyme phytase products by Defendants is a use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States by Defendants that was produced and/or is being produced by providing a heterologous polynucleotide from non-yeast organism that encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase, and that was produced and/or is being produced by providing a polynucleotide encoding a phytase from *E.coli*. (Ex. 4 at 4-5; Ex. 5 at 4-5; Ex. 6 at 4-5.)

61. For example, Phyzyme phytase products advertised and sold by DuPont Danisco Animal Nutrition was sourced from *E.coli* species bacterium and expressed in the heterologous

yeast host *Saccharomyces pombe*. (Ex. 7 at 2, 4.) As an example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity, and the production strain for Phyzyme XP is derived from a heterologous strain of the fission yeast host *Schizosacchoaromyces pombe*. (Ex. 11 at 5; Ex. 12 at 4.) In an example, Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified heterologous micro-organism yeast host *Schizosacchoaromyces pombe*. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. (Ex. 12 at 4; Ex. 13 at 3.)

62. For example, the donor organism for Phyzyme XP is *E.coli* B ATCC 11303. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native *E.coli* leader peptide sequence that was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. (Ex. 10 at 6.) The final production strain ATCC5233 expresses the 6-phytase appA gene from *E.coli* B. It contains the expression cassette integrated into the chromosome. (Ex. 10 at 9.)

63. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified heterologous micro-organism yeast host *Schizosacchoaromyces pombe*. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. (Ex. 12 at 4; Ex. 13 at 3.)

64. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of an AppA *E.coli* bacterium; and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA *E.coli* bacterium.

65. Upon information and belief, each use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase products phytase imported into the United States that was produced and/or is being produced by expressing the protein or polypeptide in a yeast.

66. For example, Phyzyme XP is sourced from an *E.coli* species bacterium and is expressed in the yeast Saccharomyces pombe. (Ex. 7 at 2.)

67. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity, and the production strain for Phyzyme XP is derived from a strain of the fission yeast *Schizosacchoaromyces pombe*. (Ex. 11 at 5; Ex. 12 at 4.)

68. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified heterologous micro-organism yeast host *Schizosacchoaromyces pombe*. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. (Ex. 12 at 4; Ex. 13 at 3.)

69. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is the heterologous yeast *Schizosaccaromyces pombe* ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. (Ex. 11 at 5.) The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Phyzyme XP is

produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. (Ex. 10 at 6.)

70. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a heterologous yeast host *Schizosaccharomyces pombe*. (Ex. 9 at 5-6; Ex. 14 at 1-2.)

71. In another example, the packaging label of an order of Phyzyme XP 10000 TPT acquired in the United States by Huvepharma demonstrates that Phyzyme XP 10000 TPT was manufactured in Finland and sold by Genencor. (Ex. 8 at 1.) Analytical testing of the Phyzyme XP 10000 TPT sample in Exhibit 8 by Plaintiffs confirmed that the sample contained a yeast. In another example, a Phyzyme XP 10000 TPT phytase sample acquired by Huvepharma in Belgium was manufactured by Genencor in Finland. (Ex. 14 at 1-7.) Analytical testing of the Phyzyme XP 10000 TPT sample in Exhibit 8 by Plaintiffs confirmed that the sample contained a yeast.

72. Each use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States that was produced by isolating the expressed protein or polypeptide.

73. For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides. (Ex. 9 at 8.) The production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., >95%) are removed. In the clarification step, either conventional (depth) filtration or tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration

step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. (Ex. 10 at 6.)

74. Upon information and belief, each use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States that was produced and/or is being produced by a method wherein said protein or polypeptide catalyzes the release of phosphate from phytate.

75. For example, Phyzyme XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects. (Ex. 7 at 2.)

76. For example, the FEEDAP Panel considers that the change in the formulation (of Phyzyme XP 10000 L/TPT) will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion (relating to Phyzyme XP 5000 L/G). (Ex. 13 at 7.)

77. For example, Phyzyme XP for Pigs and Chickens is a phytase designed specifically to improve the availability of phosphorus, calcium and amino acids contained in cereal grains, oil seed meals and their by-products. (Ex. 15 at 1.)

78. For example, a Phyzyme XP 10000 TPT product insert states the product is phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig diets. (Ex. 14 at 2.)

79. In a further example, Phyzyme XP is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This

would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in manure. (Ex. 10 at 5.)

80. Upon information and belief, each use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States that was produced and/or is being produced by a method wherein said protein or polypeptide catalyzes the release of phosphate from phytate.

81. For example, Phyzyme XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects. (Ex. 7 at 2.)

82. In a further example, Phyzyme XP is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in the manure. (Ex. 10 at 5.)

83. For example, the FEEDAP Panel (The Panel on Additives and Products or Substances used in Animal Feed) considers that the change in the formulation (of Phyzyme XP 10000 L/TPT) will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion (relating to Phyzyme XP 5000 L/G). (Ex. 13 at 7.)

84. For example, Phyzyme XP for Pigs and Chickens is a phytase designed specifically to improve the availability of phosphorus, calcium and amino acids contained in cereal grains, oil seed meals and their by-products. (Ex. 15 at 1.)

85. For example, a Phyzyme XP 10000 TPT product insert states the product is phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig diets. (Ex. 14 at 2.)

86. Upon information and belief, each use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase products imported into the United States that was produced and/or is being produced by a method that provides increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell.

87. For example, Phyzyme XP TPT has "unrivalled heat stability, superior performance," and "in a series of trials conducted at the Technological Institute, Kolding, Denmark, Phyzyme XP TPT is shown to be more heat stable than other leading phytase products." (Ex. 17 at 1-2.)

88. For example, significant loss of phytase activity during steam conditioning and subsequent pelleting of feed negatively impacts profitability. Phyzyme XP incorporates unique TPT coating technology, which offers heat stability up to 95°C (203°F). The TPT coating also allows rapid release of phytase in the upper part of the gut to achieve optimum bio-efficacy. (Ex. 16 at 2.)

89. For example, Phyzyme XP 5000L is also stable after mixing with feedingstuffs with approximately 80% of activity remaining after six months storage in a feed mash at both 20°C and 35°C. Following pelleting of feeds at 80C, 82% of the initial phytase activity was still present, thus Phyzyme XP 5000G is considered to be stable when pelleted up to this temperature. (Ex. 11 at 5.)

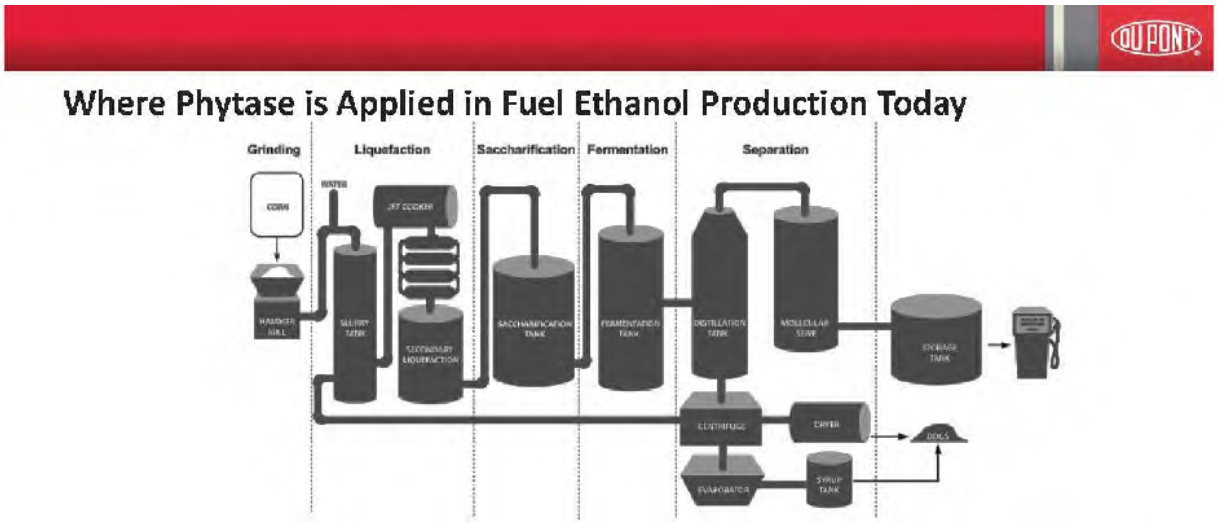
90. For example, Phyzyme XP 10000 TPT stability in pelleting, when tested with a maize-based diet and was conditioned for 30 seconds at 90°C or 95°C and pelleted, resulted in a percentage of recovery of the initial enzymatic activity of 99% and 96%, respectively. It is not expected that the double concentration of the Phyzyme XP 10000 L enzyme (compared to Phyzyme XP 5000L) will modify the stability of the product in feedingstuffs. Phyzyme XP 5000 showed an 80% of the activity remaining when added to feedingstuffs and stored for six months at 20°C and 35°C. (Ex.13 at 7.)

91. For example, Phyzyme XP is a phytase protected by unique Thermal Protection Technology coating from Danisco Animal Nutrition. Phyzyme XP TPT is heat stable to 95°C/203°F during pelleting, and Phyzyme XP G should not exceed conditioning and pelleting temperatures of 70°C/158°F. (Ex. 7 at 3-4.)

DEFENDANTS' OTHER PHYTASE PRODUCTS FOR APPLICATIONS OTHER THAN ANIMAL NUTRITION, SUCH AS FOR FUEL ETHANOL PRODUCTION

92. Upon information and belief, Defendants manufacture, import, sell, distribute, and commercialize in the United States products containing phytase under various trade names that are used in the production of fuel ethanol, including, but not limited to, the Optimash Phytase product. (Exs. 23–27.)

93. Upon information and belief, Defendants use, and advertise for customers to purchase and use, in the United States products containing phytase under various trade names including, but not limited to, Optimash Phytase in various stages of fuel ethanol production, including grinding, liquefaction, saccharification, fermentation, and separation processes, and corn oil production. (Exs. 23-25, Ex. 26 at 8-12; Ex. 27 at 28-29.)



(Ex. 26 at 11.)

94. Upon information and belief, Optimash Phytase is a phytase used for dry grind ethanol production in the saccharification and fermentation stages. (Ex. 23 at 1-2; Ex. 27 at 29-30.) Optimash Phytase is a concentrated phytase solution characterized by its ability to efficiently hydrolize phytate under ethanol fermentation conditions, which benefits ethanol producers by generating inositol, an important yeast nutrient. (Ex. 23 at 1.)

95. Upon information and belief, Defendants use, or have used, Optimash Phytase in various stages of fuel ethanol production at the DuPont cellulosic fuel ethanol production plant in Nevada, Iowa. The Nevada, Iowa plant is the world's largest cellulosic ethanol plant. (Ex. 27 at 13-16.)

96. On May 14, 2014, DuPont I.B. published the presentation "How Ethanol Production Technology Impacts DDGS Value in Animal Nutrition." (Ex. 26 at 1.) Upon information and belief, that presentation shows that Phyzyme XP was the only phytase product in production and use for DuPont Danisco Animal Nutrition Phytases. (Ex. 26 at 5.) The remainder of the presentation describes the need for, and use of, phytase in fuel ethanol production processes. (Ex. 26 at 6-32.)

97. Upon information and belief, Defendants' Optimash Phytase product is produced according to production methods of Defendants' Phyzyme XP phytase product lines. Thus, the evidence describing the method of producing the Phyzyme XP product lines are applicable to Defendants' Optimash Phytase product. (Exs. 20-22.)

DEFENDANTS' WILLFUL INFRINGEMENT

98. Upon information and belief, two entities of which Danisco is intimately affiliated Diversa and Royal DSM AS ("DSM") had actual knowledge during the period of infringement of Huvepharma's patent family that is the subject of the present litigation. Upon information and belief, Danisco would also have been aware of Huvepharma's asserted patents based on this close affiliation with Diversa and DSM.

99. First, according to Diversa's Form 10-K filing for 2006, Diversa launched Phyzyme XP phytase (one of the infringing products) in 2003 with "our partner Danisco Animal Nutrition." According to a 2003 press release, Diversa developed Phyzyme XP phytase enzyme in collaboration with Danisco Animal Nutrition.

100. Upon information and belief, in the same time frame, Diversa filed and prosecuted patent applications directed to thermotolerant phytase. For example, in 2001, Diversa filed a provisional patent application disclosing microbially-expressed thermotolerant phytase for animal feed. Diversa received at least two granted patents claiming priority to the 2001 provisional patent application: U.S. Patent No. 7,135,323 (the "'323 Patent") and U.S. Patent No. 7,138,260 (the "'260 Patent," which is a divisional of the '323 Patent).

101. During prosecution of Diversa's '323 Patent, Diversa cited and submitted as relevant prior art (via an Information Disclosure Statement) Huvepharma's U.S. Patent No.

6,451,572 (the "'572 Patent") to Dr. Xingen Lei, which is the parent patent of the presently asserted '150 Patent, '063 Patent, and '232 Patent. Because Diversa's '260 Patent was a divisional of its '323 Patent, Huvepharma's '572 Patent is also of record to Diversa's '260 Patent. In fact, in the sole Office Action issued during USPTO examination of Diversa's '323 Patent, the Examiner discussed in the context of both rejections Huvepharma's '572 Patent. Thus, Diversa was clearly aware of Huvepharma's Patents.

102. Upon information and belief, due to the partnership and development collaboration between Diversa and Danisco, Defendants were privy to Diversa's phytase efforts, and in particular privy to the same body of information as Diversa with regard to the infringing Phyzyme XP phytase and Diversa's efforts to patent aspects of its phytase products. Importantly, Defendants would have had knowledge of Diversa's patent applications and granted patents citing Huvepharma's parent '572 Patent, and therefore would have had knowledge of Huvepharma's asserted '150 Patent, '063 Patent, and '232 Patent during the period of infringement.

103. Second, Defendants also had pre-suit knowledge of Huvepharma's asserted '150 Patent, '063 Patent, and '232 Patent via one of Danisco's major shareholders, the Dutch chemicals company DSM. In 2011, DSM held a 4.95% stake in Danisco, which amounted to 2.36 million shares. DSM's stake was so significant that Danisco was required to seek DSM's approval prior to moving forward with DuPont's 2011 acquisition of Danisco, and thus significant collaboration occurred between Danisco and DSM at least during the acquisition process and due diligence thereof.

104. In November 2006, DSM initiated a European Opposition Proceeding against two of Huvepharma's European patents that are European counterparts of Huvepharma's '572 patent,

which as discussed above is the parent patent of Huvepharma's patents asserted in the present litigation.[3] In February 2013, the European Opposition was settled with DSM. Thus, the EU opposition was prosecuted during the due diligence that resulted in Dupont's 2011 acquisition of Danisco. Because of the collaboration between DSM and Danisco during the due diligence period, Danisco would have been aware of DSM's European Opposition proceeding, and thus Danisco would have had knowledge of Huvepharma's '572 patent family, which includes Huvepharma's asserted '150 Patent, '063 Patent, and '232 Patent.

105. Despite having knowledge of Huvepharma's '150 Patent, '063 Patent, and '232 Patent from sources identified above, and despite the fact that infringement of the claims of the Huvepharma patents was readily determinable by Defendants, Defendants continued to sell their Phyzyme XP phytase products and Optimash Phytase products. Defendants were aware that, as a direct competitor, Huvepharma had been harmed by such sales. Nevertheless, Defendants did not cease selling infringing products, nor did Defendants enter into good-faith licensing negotiations with Huvepharma. Therefore, Defendants' actions have been both deliberate and malicious. Accordingly, Defendants' infringement of Huvepharma's '150 Patent, '063 Patent, and '232 Patent has been willful.

COUNT I
(Infringement of U.S. Patent No. 7,026,150)

106. Plaintiffs repeat and re-allege each and every allegation contained in the preceding paragraphs of this Complaint as if stated in their entirety herein, and incorporate them herein by reference.

[3] DSM Nutritional Products AG, DSM Nutritional Products, Ltd., and Novozymes A/S filed the opposition to European Patent 1090129B1 against Cornell Research Foundation, Inc. and Phytex LLC.

107. On April 11, 2006, the United States Patent and Trademark Office duly and legally issued the ’150 Patent, entitled “Overexpression of Phytase Genes in Yeast Systems,” to inventor Xingen Lei. The ’150 Patent was assigned at issuance to Cornell Research Foundation, Inc., Ithaca, New York. Cornell Research Foundation, Inc. is the owner of the ’150 Patent by virtue of that assignment, which was duly recorded at the United States Patent and Trademark Office at Reel 013294 and Frame 0133, and continues to be the owner of the ’150 Patent. (Ex. 3.)

108. Pursuant to an agreement entered into with Cornell Research Foundation, Inc., Huvepharma obtained an exclusive license to the ’150 Patent and has the right to sue for infringement of that patent and recover damages for such infringement.

109. Upon information and belief, Defendants have infringed and continue to infringe at least claims 1–3, 6–8, 28–29, and 30–33 of the ’150 Patent pursuant to 35 U.S.C. § 271(g), literally or under the doctrine of equivalents, at least by importing into the United States, offering to sell, selling, and/or using without authority Phyzyme phytase products, including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, and the Optimash Phytase product.

110. Upon information and belief, Defendants have infringed and continue to infringe at least claims 1–3, 6–8, 28–29, and 30–33 of the ’150 Patent pursuant to 35 U.S.C. § 271(g), literally or under the doctrine of equivalents, by offering to sell, selling, and/or using within the United States without authority Phyzyme phytase products, including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or

Phyzyme XP 10000TPT, and the Optimash Phytase product, which were imported into the United States.

111. Upon information and belief, the Phyzyme phytase products are produced using the same methods in the context of the ’150 Patent claims. Thus, the evidence and descriptions herein describing the method of producing Phyzyme XP is applicable to any one or more of the other Phyzyme phytase products, including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, and the Optimash Phytase product.

112. As examples, Exhibit 4 and Exhibit 20 are preliminary and exemplary claim charts detailing Defendants’ infringement of claims 1–3, 6–8, 28–29, and 30–33 of the ’150 Patent. These charts are not intended to limit Huvepharma’s right to modify the charts or allege that other of Defendants’ products and/or activities infringe the identified claims or any other claims of the ’150 Patent or any other patent. Exhibit 4 and Exhibit 20 are hereby incorporated by reference in their entirety. Each claim element in Exhibit 4 that is mapped to the accused Phyzyme phytase products, including Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT shall be considered an allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required. Each claim element in Exhibit 20 that is mapped to the accused phytase products, including the Optimash Phytase product, shall be considered an allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required.

113. Defendants' infringement of the '150 Patent has injured Plaintiffs in their business and property rights. Plaintiffs are entitled to recover monetary damages based on the injuries arising from Defendants' infringement pursuant to 35 U.S.C. § 284 in an amount to be determined at trial.

COUNT II
(Infringement of U.S. Patent No. 7,312,063)

114. Plaintiffs repeat and re-allege each and every allegation contained in the preceding paragraphs of this Complaint as if stated in their entirety herein, and incorporate them herein by reference.

115. On December 25, 2007, the United States Patent and Trademark Office duly and legally issued the '063 Patent, entitled "Overexpression of Phytase Genes in Yeast Systems," to inventor Xingen Lei. The '063 Patent was assigned at issuance to Cornell Research Foundation, Inc., Ithaca, New York. Cornell Research Foundation, Inc. is the owner of the '063 Patent by virtue of that assignment, which was duly recorded at the United States Patent and Trademark Office at Reel 009457 and Frame 0350, and continues to be the owner of the '063 Patent.

116. Pursuant to an agreement entered into with Cornell Research Foundation, Inc., Huvepharma obtained an exclusive license to the '063 Patent and has the right to sue for infringement of that patent and recover damages for such infringement.

117. Upon information and belief, Defendants have infringed and continue to infringe at least claims 1-2 and 5-7 of the '063 Patent pursuant to 35 U.S.C. § 271(g), literally or under the doctrine of equivalents, at least by importing into the United States, offering to sell, selling, and/or using without authority Phyzyme phytase products, including at least Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500,

Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, and the Optimash Phytase product.

118. Upon information and belief, Defendants have infringed and continue to infringe at least claims 1-2 and 5-7 of the ’063 Patent pursuant to 35 U.S.C. § 271(g), literally or under the doctrine of equivalents, by offering to sell, selling, and/or using within the United States without authority Phyzyme phytase products, including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, and the Optimash Phytase product, which were imported into the United States.

119. Upon information and belief, the Phyzyme phytase products are produced using the same methods in the context of the ’063 Patent claims. Thus, the evidence and descriptions herein describing the method of producing Phyzyme XP is applicable to any one or more of the other Phyzyme phytase products, including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, and the Optimash Phytase product.

120. As examples, Exhibit 5 and Exhibit 21 are preliminary and exemplary claim charts detailing Defendants’ infringement of claims 1-2 and 5-7 of the ’063 Patent. These charts are not intended to limit Huvepharma’s right to modify the charts or allege that other products and/or activities of Defendants infringe the above identified claims or any other claims of the ’063 Patent or any other patent. Exhibit 5 and Exhibit 21 are hereby incorporated by reference in their entirety. Each claim element in Exhibit 5 that is mapped to the accused

Phyzyme phytase products, including Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, shall be considered an allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required. Each claim element in Exhibit 21 that is mapped to the accused phytase products, including the Optimash Phytase product, shall be considered an allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required.

121. Defendants' infringement of the '063 Patent has injured Plaintiffs in their business and property rights. Plaintiffs are entitled to recover monetary damages based on the injuries arising from Defendants' infringement pursuant to 35 U.S.C. § 284 in an amount to be determined at trial.

COUNT III
(Infringement of U.S. Patent No. 8,455,232)

122. Plaintiffs repeat and re-allege each and every allegation contained in the preceding paragraphs of this Complaint as if stated in their entirety herein, and incorporate them herein by reference.

123. On June 4, 2013, the United States Patent and Trademark Office duly and legally issued the '232 Patent, entitled "Overexpression of Phytase Genes in Yeast Systems," to inventor Xingen Lei. The '232 Patent was assigned at issuance to Cornell Research Foundation, Inc., Ithaca, New York. Cornell Research Foundation, Inc. is the owner of the '232 Patent by virtue of that assignment, which was duly recorded at the United States Patent and Trademark Office at Reel 009457 and Frame 0350, and continues to be the owner of the '232 Patent. (Ex. 6.)

124. Pursuant to an agreement entered into with Cornell Research Foundation, Inc., Huvepharma obtained an exclusive license to the ’232 Patent and has the right to sue for infringement of that patent and recover damages for such infringement.

125. Upon information and belief, Defendants have infringed and continue to infringe at least claims 1-2 of the ’232 Patent pursuant to 35 U.S.C. § 271(g), literally or under the doctrine of equivalents, at least by importing into the United States, offering to sell, selling, and/or using without authority Phyzyme phytase products, including but not limited to at least Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, and the Optimash Phytase product.

126. Upon information and belief, Defendants have infringed and continue to infringe at least claims 1-2 of the ’232 Patent pursuant to 35 U.S.C. § 271(g), literally or under the doctrine of equivalents, by offering to sell, selling, and/or using within the United States without authority Phyzyme phytase products, including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, and the Optimash Phytase product, which were imported into the United States.

127. Upon information and belief, the Phyzyme phytase products are produced using the same methods in the context of the ’232 Patent claims. Thus, the evidence and descriptions herein describing the method of producing Phyzyme XP is applicable to any one or more of the other Phyzyme phytase products, including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G,

Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT, and the Optimash Phytase product.

128. As examples, Exhibit 6 and Exhibit 22 are preliminary and exemplary claim charts detailing Defendants' infringement of claims 1-2 of the '232 Patent. These charts are not intended to limit Huvepharma's right to modify the chart or allege that other products and/or activities of Defendants infringe the above identified claims or any other claims of the '232 Patent or any other patent. Exhibit 6 and Exhibit 22 are hereby incorporated by reference in their entirety. Each claim element in Exhibit 6 that is mapped to the accused Phyzyme phytase products, including Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT shall be considered an allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required. Each claim element in Exhibit 22 that is mapped to the accused Optimash Phytase product shall be considered an allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required.

129. Defendants' infringement of the '232 Patent has injured Plaintiffs in their business and property rights. Plaintiffs are entitled to recover monetary damages based on the injuries arising from Defendants' infringement pursuant to 35 U.S.C. § 284 in an amount to be determined at trial.

PRAYER FOR RELIEF

WHEREFORE, Plaintiffs pray for relief as follows:

A. Judgment that Defendants have infringed one or more claims of the '150 Patent, the '063 Patent, and/or the '232 Patent;

B. An award of damages pursuant to 35 U.S.C. § 284;

C. Judgment that Defendants' acts were willful;

D. An award to Plaintiffs of their costs and reasonable expenses to the fullest extent permitted by law; and

E. An award of such other and further relief as the Court may deem just and proper.

DEMAND FOR JURY TRIAL

Pursuant to Federal Rule of Civil Procedure 38(b), Plaintiff demands trial by jury on all issues so triable.

October 9, 2018

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EXHIBIT 1

US007026150B2

(12) **United States Patent**
Lei

(10) **Patent No.: US 7,026,150 B2**
(45) **Date of Patent: *Apr. 11, 2006**

(54) **OVEREXPRESSION OF PHYTASE GENES IN YEAST SYSTEMS**

(75) Inventor: **Xingen Lei**, Ithaca, NY (US)

(73) Assignee: **Cornell Research Foundation, Inc.**, Ithaca, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 6 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **10/094,693**

(22) Filed: **Mar. 8, 2002**

(65) **Prior Publication Data**

US 2002/0192791 A1 Dec. 19, 2002

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/104,769, filed on Jun. 25, 1998, now Pat. No. 6,451,572.

(51) **Int. Cl.**

C12N 9/16	(2006.01)
C12N 1/20	(2006.01)
C12N 1/14	(2006.01)
C12N 15/00	(2006.01)
C07H 21/04	(2006.01)

(52) **U.S. Cl.** **435/196**; 435/252.3; 435/320.1; 435/254.1; 435/254.2; 435/254.21; 435/254.23; 435/254.3; 536/23.2; 536/23.4; 536/23.7

(58) **Field of Classification Search** 435/196, 435/252.3, 320.1, 254.1, 254.2, 254.21, 254.23; 536/23.4, 23.7

See application file for complete search history.

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(74) *Attorney, Agent, or Firm*—Nixon Peabody LLP

(57) **ABSTRACT**

The present invention relates to a method of producing a heterologous protein or polypeptide having phytase activity in a yeast system. The invention also provides proteins having phytase activity which have increased thermostability. Yeast strains which produce a heterologous phytase and the vectors used to produce the phytase are also provided.

40 Claims, 23 Drawing Sheets

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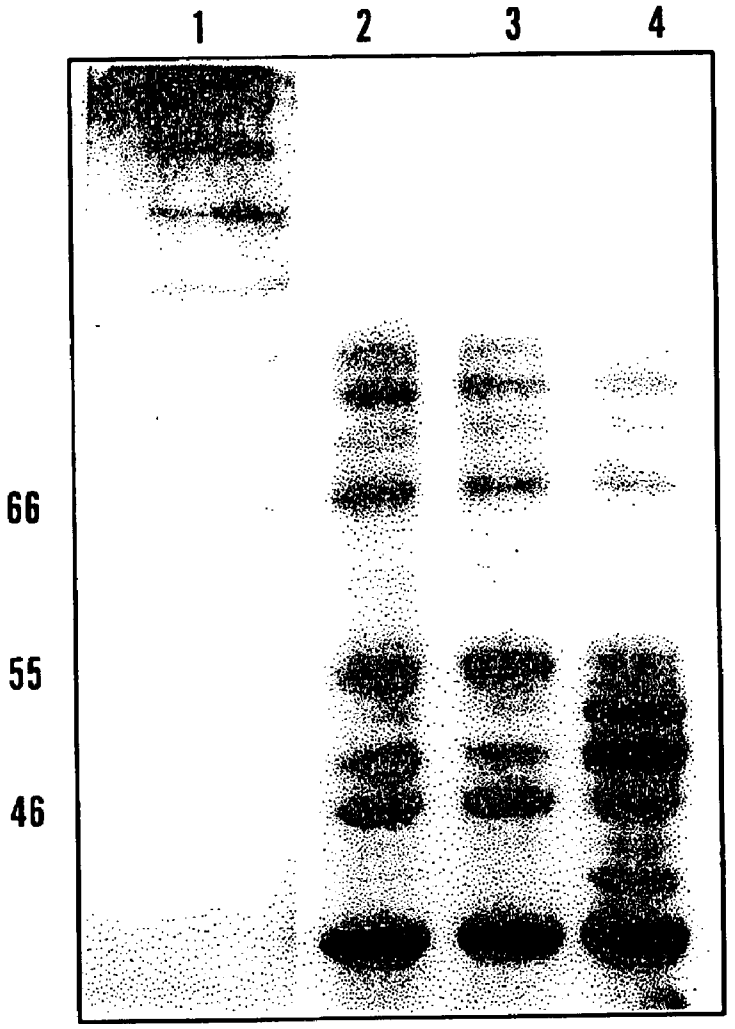

FIG. 1

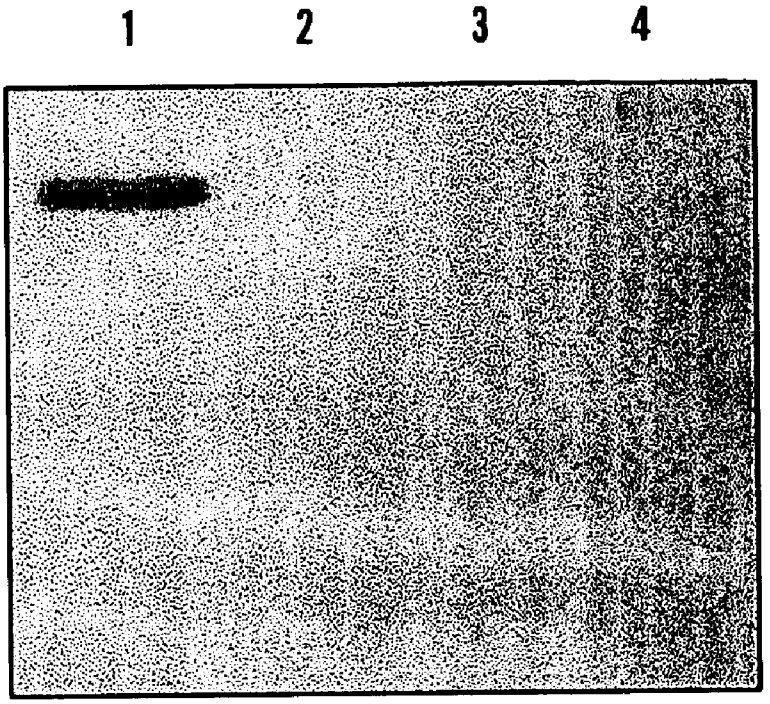

FIG. 2

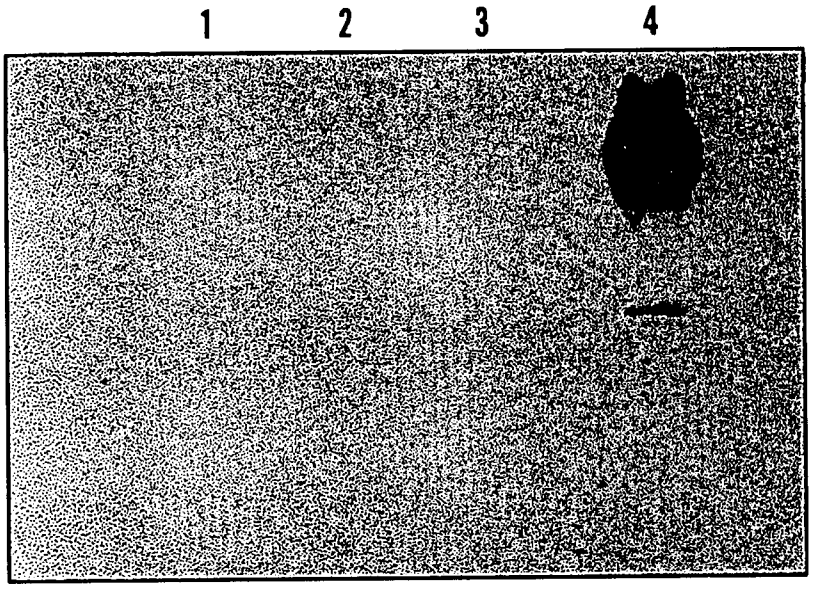

FIG. 3

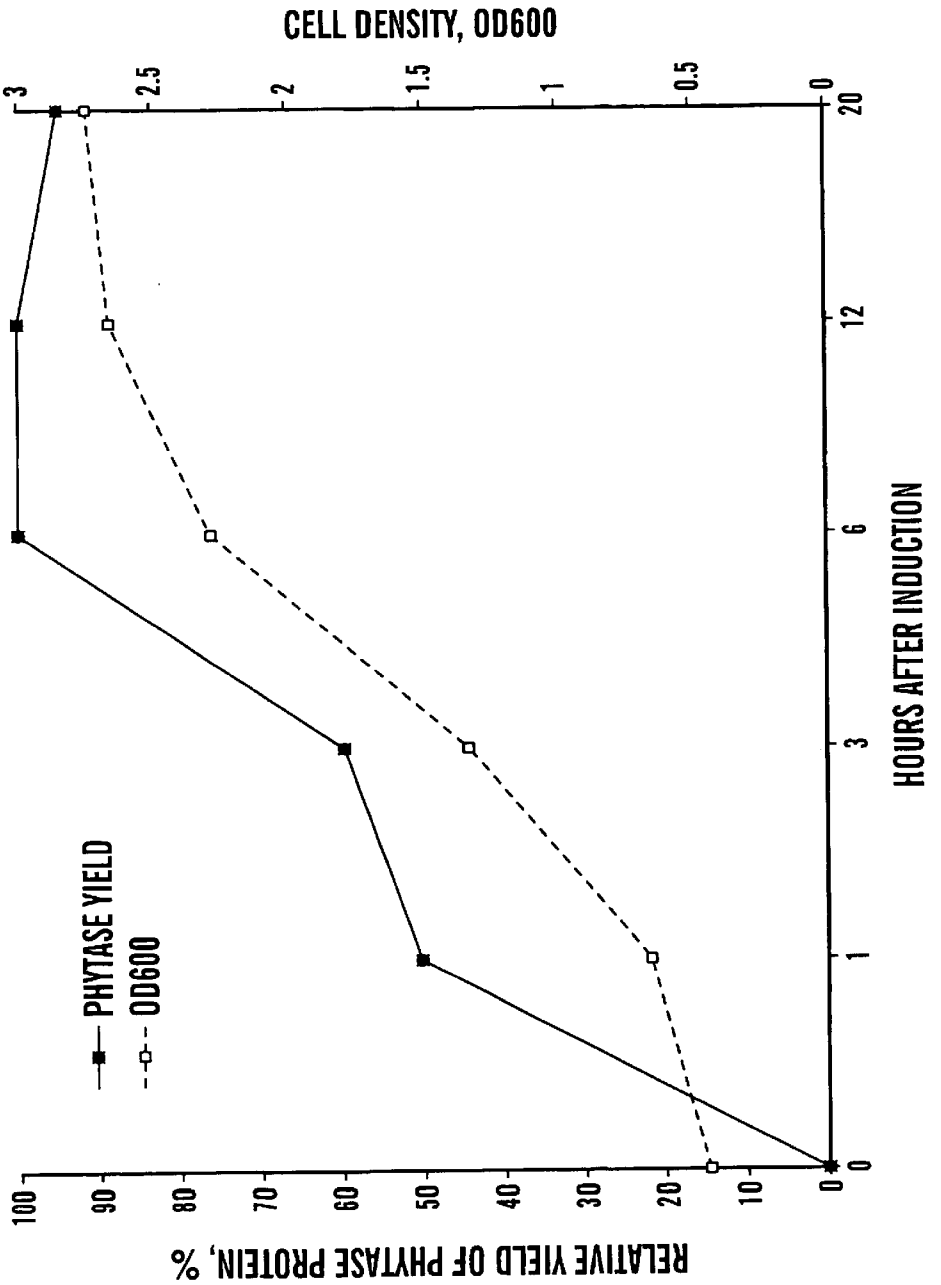
CELL DENSITY, OD600
3
2.5
2
1.5
1
0.5
0
20
12
6
3
1
0
HOURS AFTER INDUCTION
PHYTASE YIELD
OD600
100
90
80
70
60
50
40
30
20
10
0
RELATIVE YIELD OF PHYTASE PROTEIN, %

FIG. 4

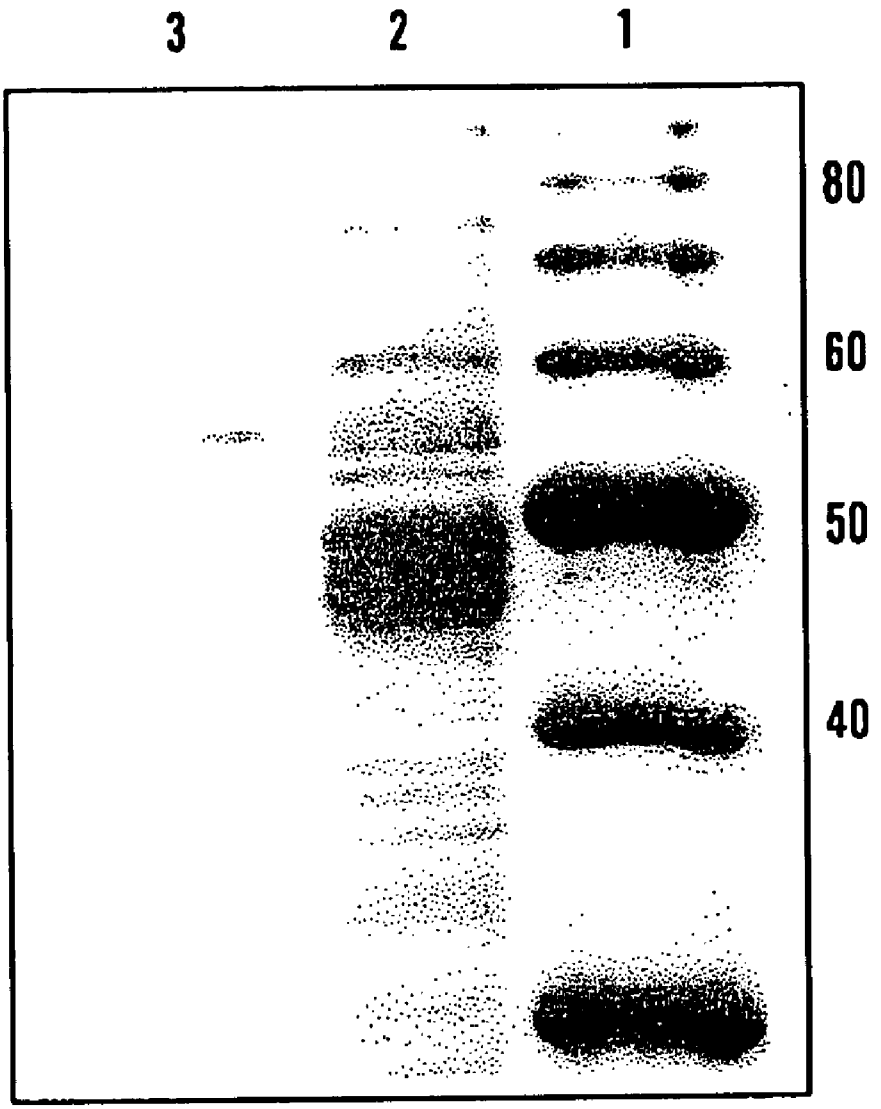

FIG. 5

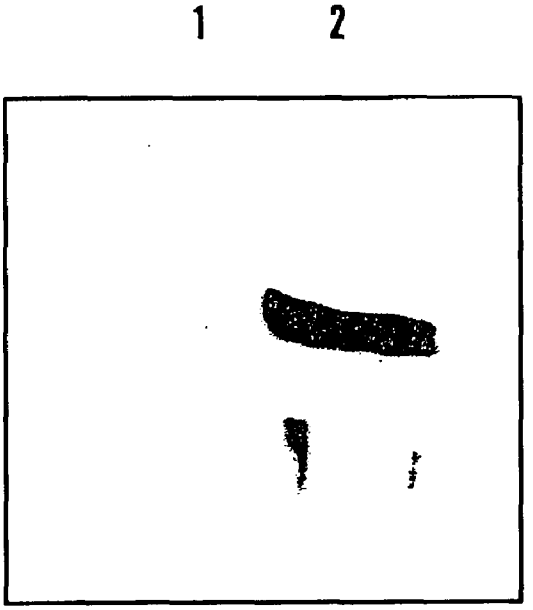

FIG. 6

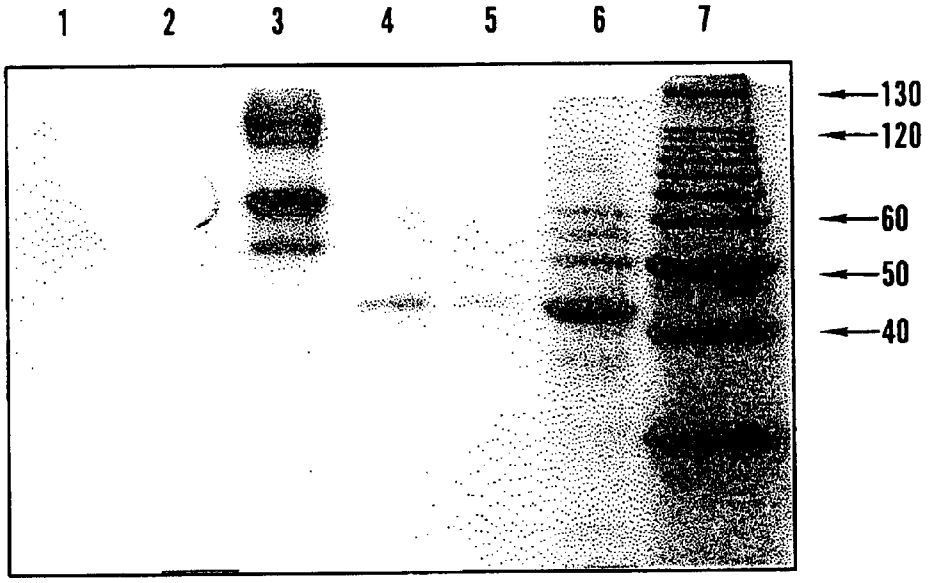

FIG. 7

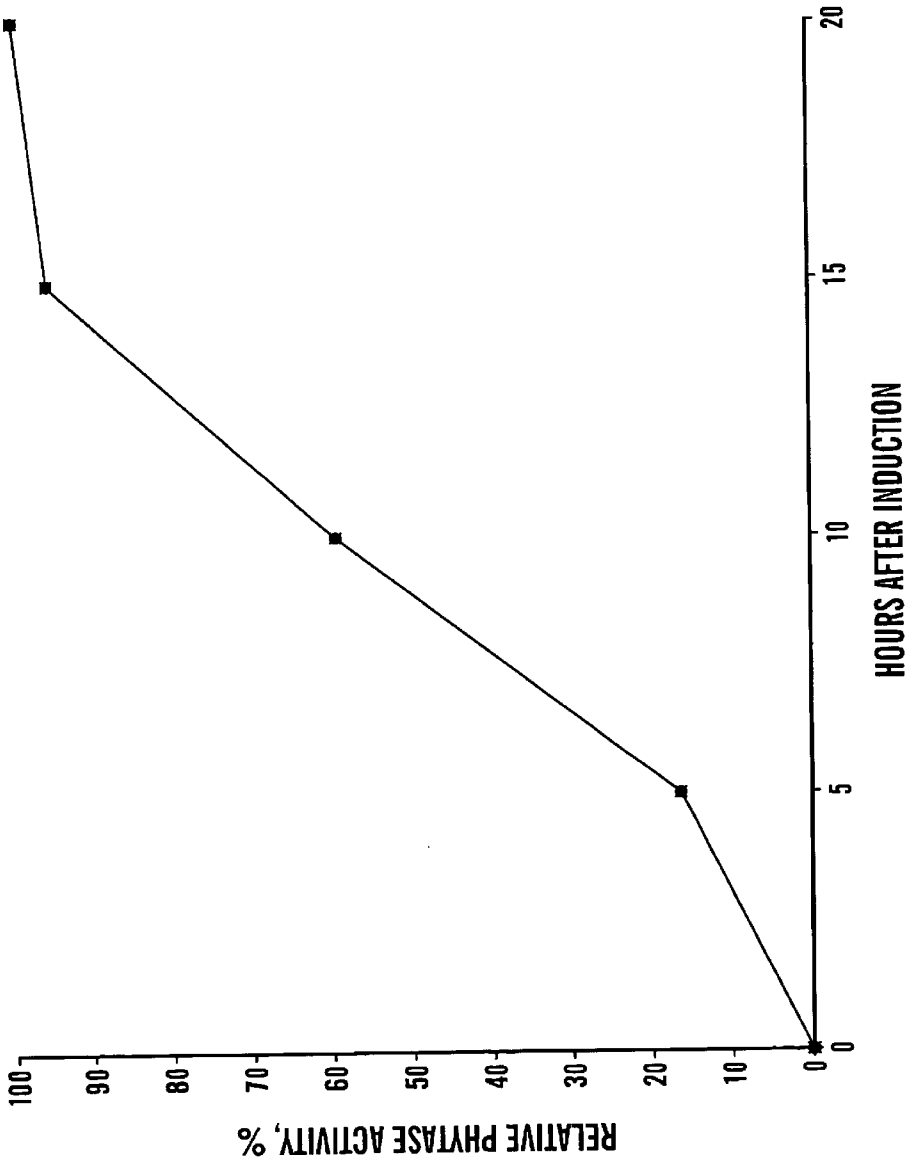
RELATIVE PHYTASE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
HOURS AFTER INDUCTION

FIG. 8

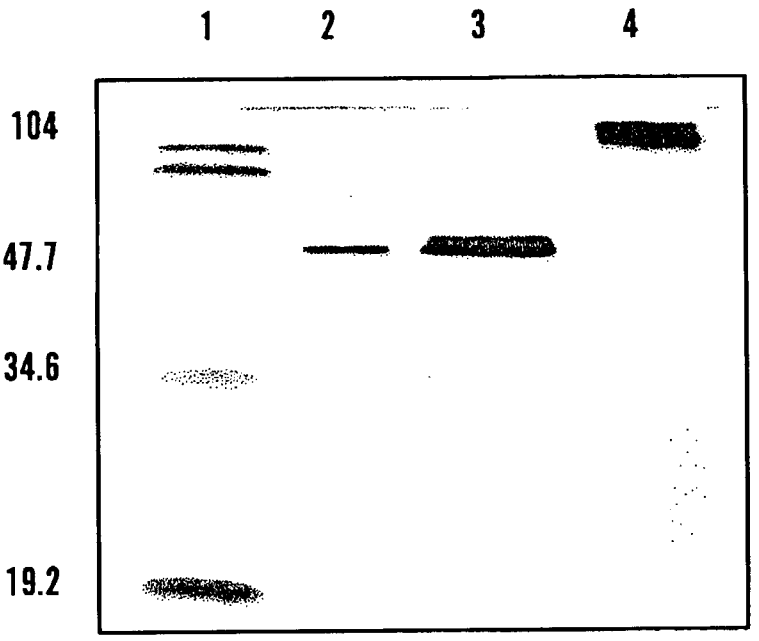

FIG. 9

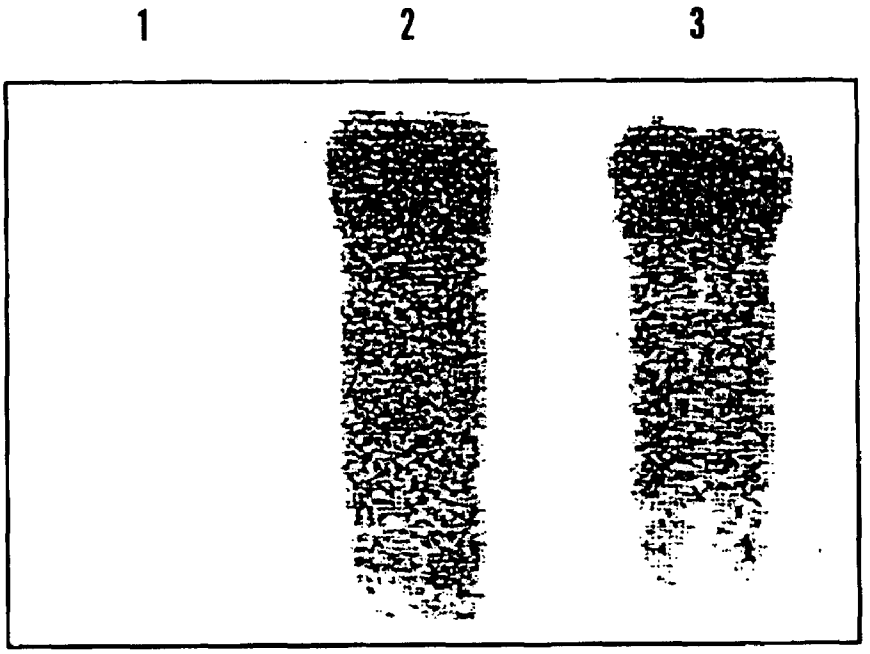

FIG. 10

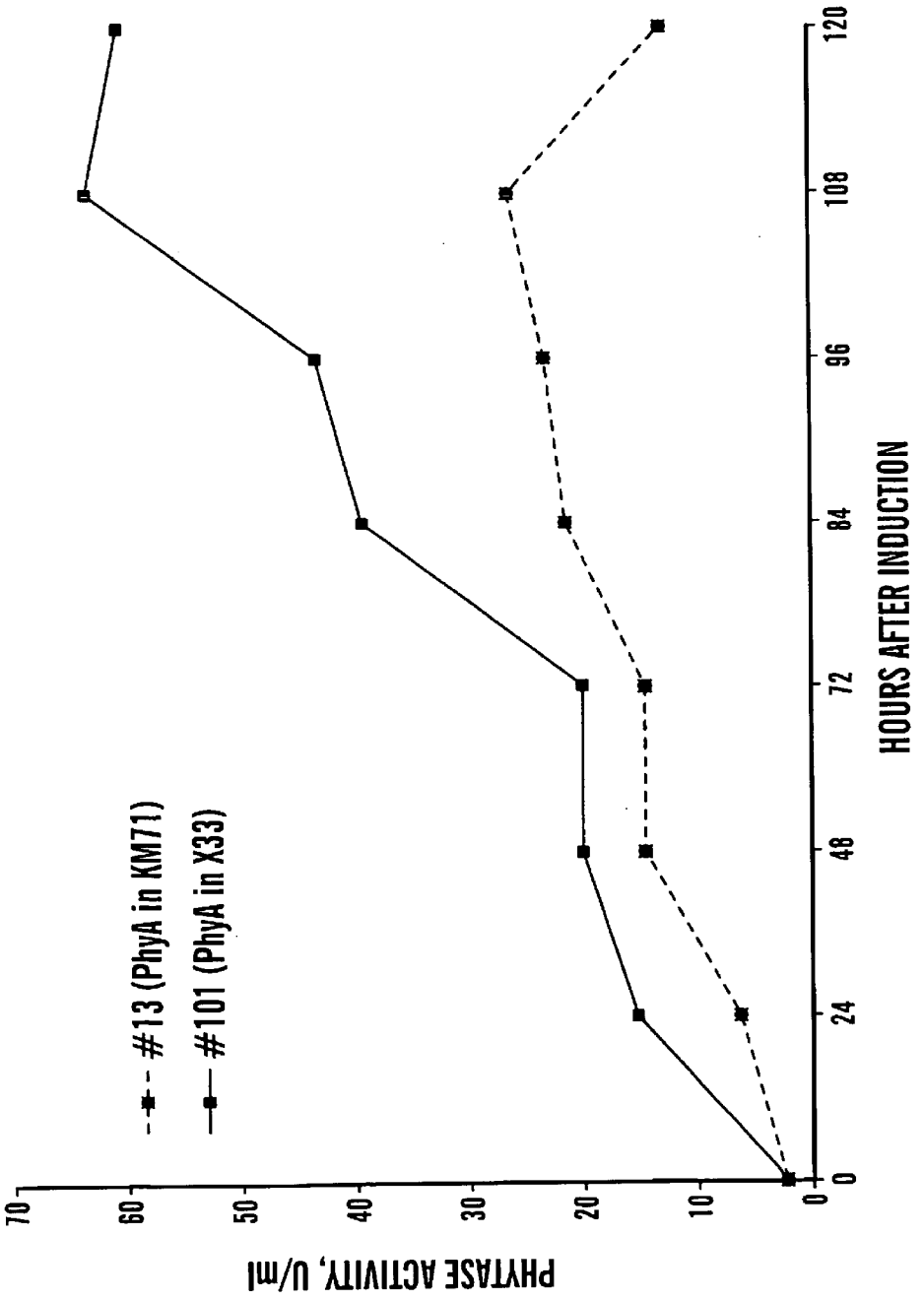
PHYTASE ACTIVITY, U/ml
HOURS AFTER INDUCTION
#13 (PhyA in KM71)
#101 (PhyA in X33)
0
10
20
30
40
50
60
70
0
24
48
72
84
96
108
120

FIG. 11

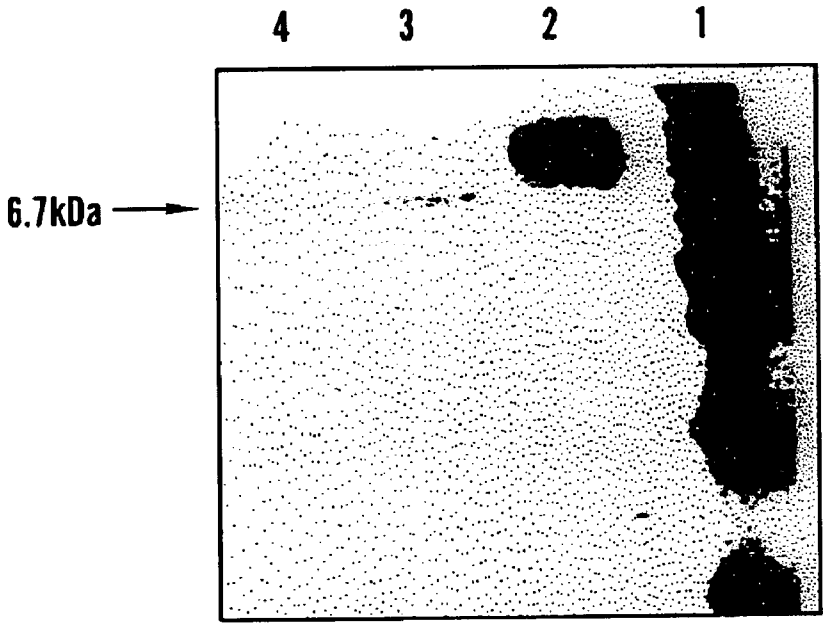

FIG. 12

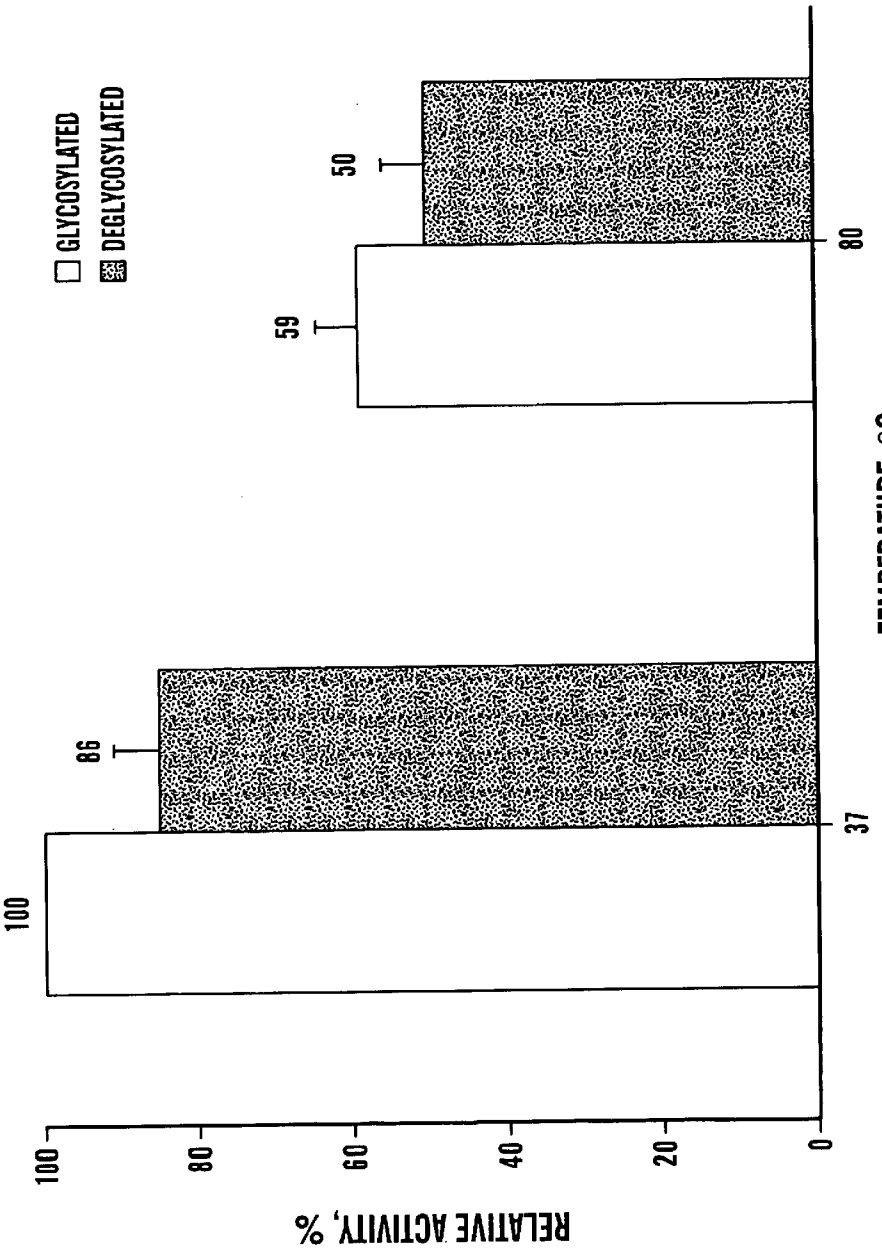
GLYCOSYLATED
DEGLYCOSYLATED
RELATIVE ACTIVITY, %
100
80
60
40
20
0
100
86
59
50
37
80
TEMPERATURE, °C

FIG. 13

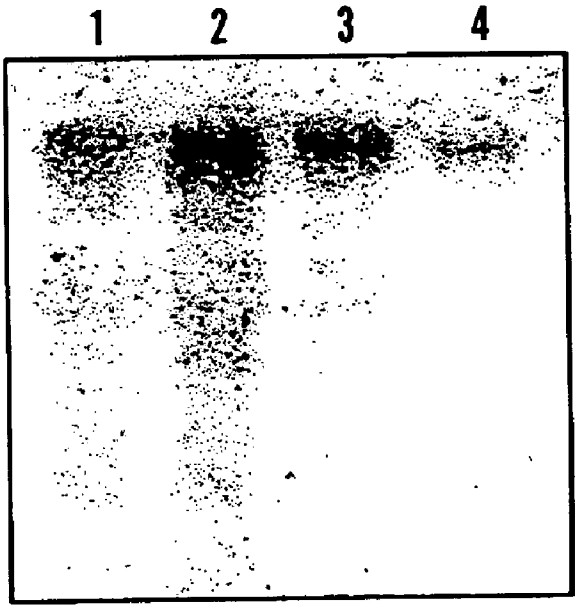

FIG. 14

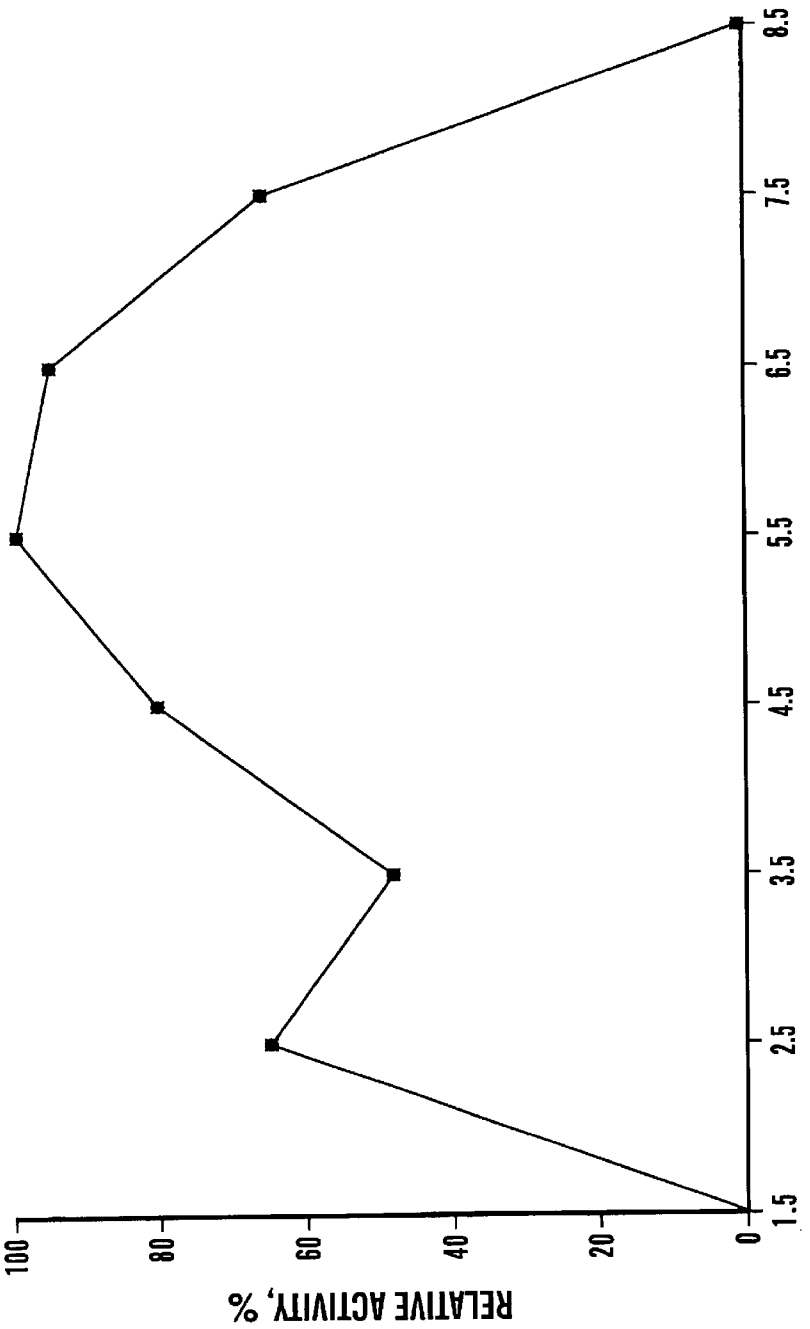

FIG. 15

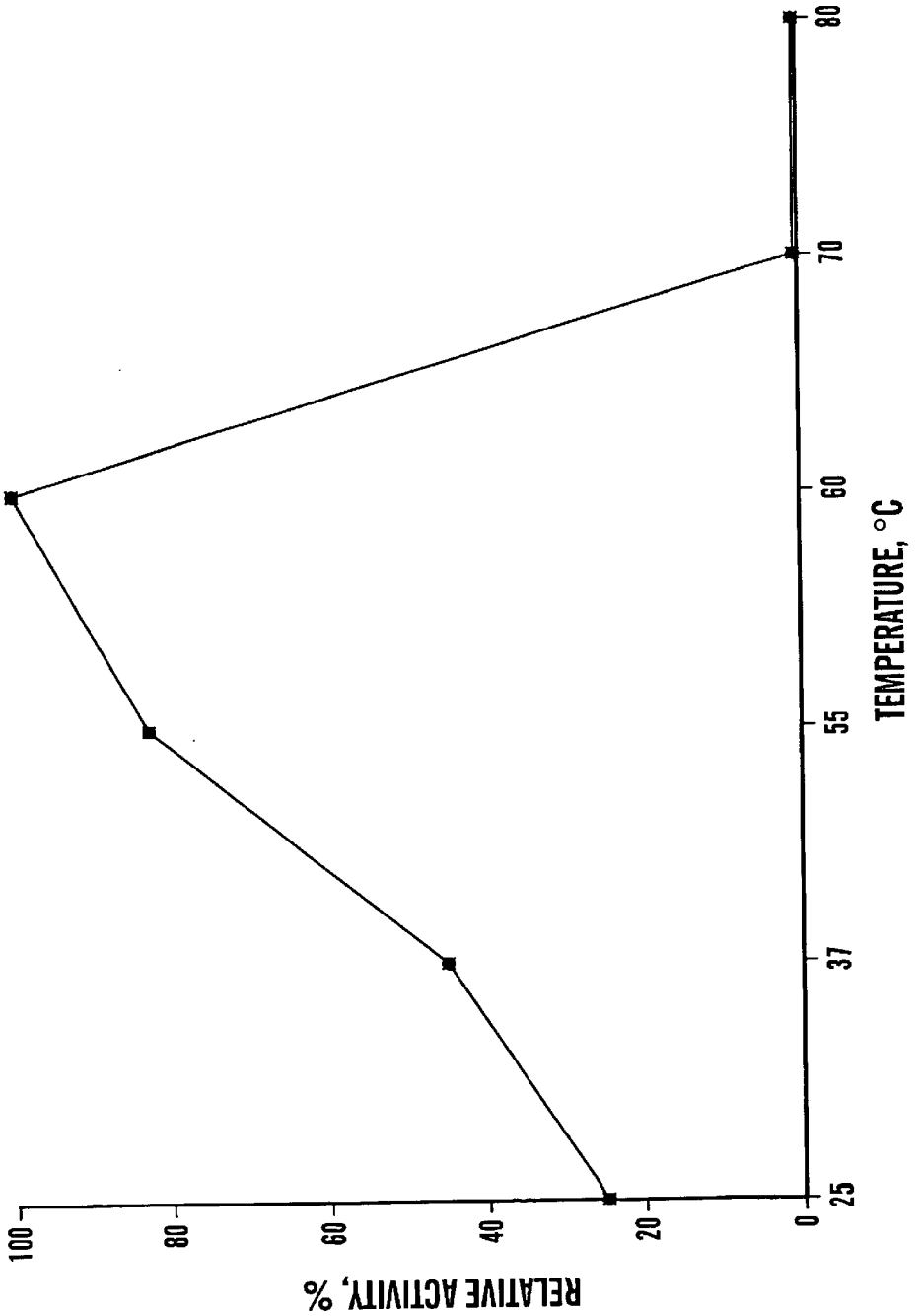
RELATIVE ACTIVITY, %
100
80
60
40
20
0
25
37
55
60
70
80
TEMPERATURE, °C

FIG. 16

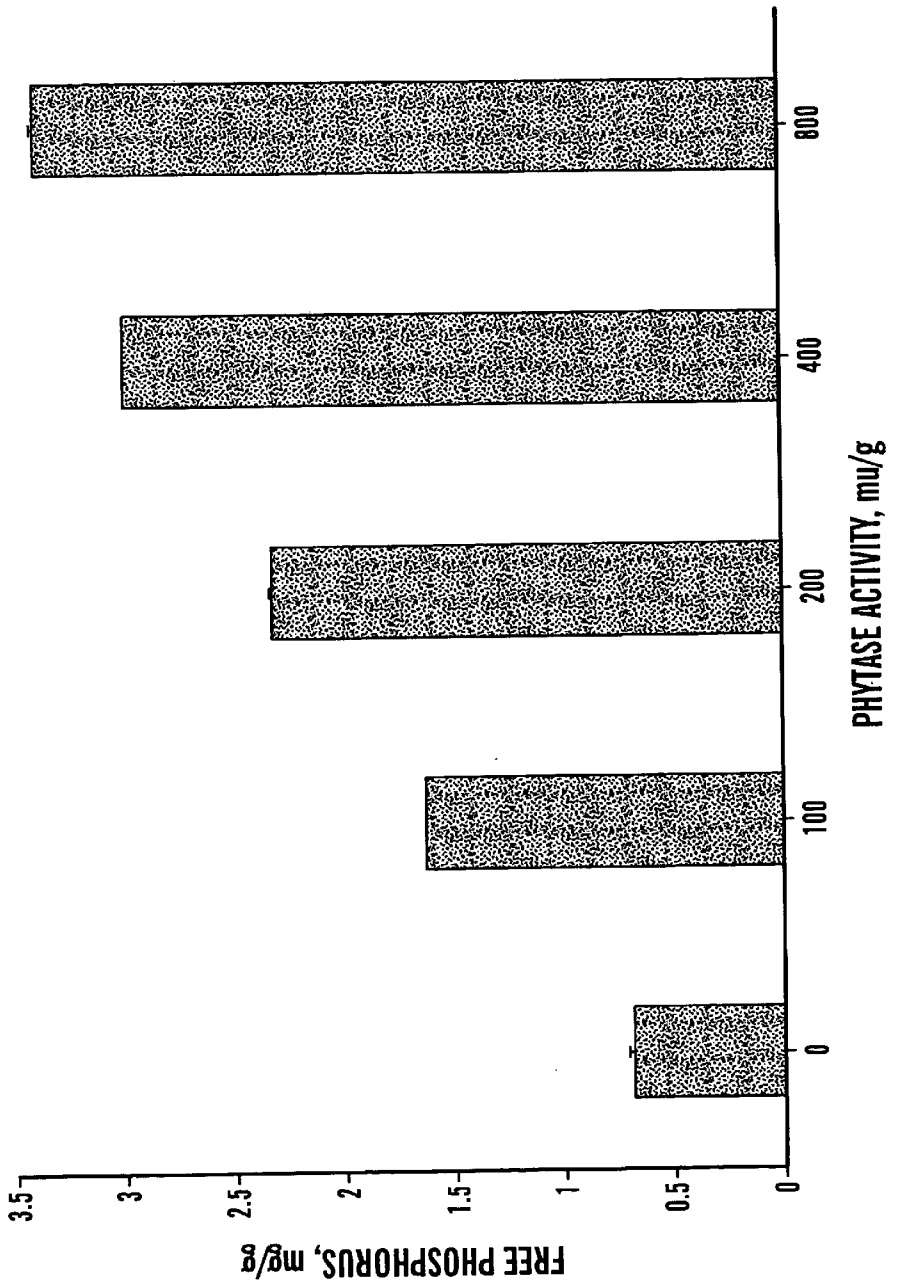
FREE PHOSPHORUS, mg/g
3.5
3
2.5
2
1.5
1
0.5
0
0
100
200
400
800
PHYTASE ACTIVITY, mu/g

FIG. 17

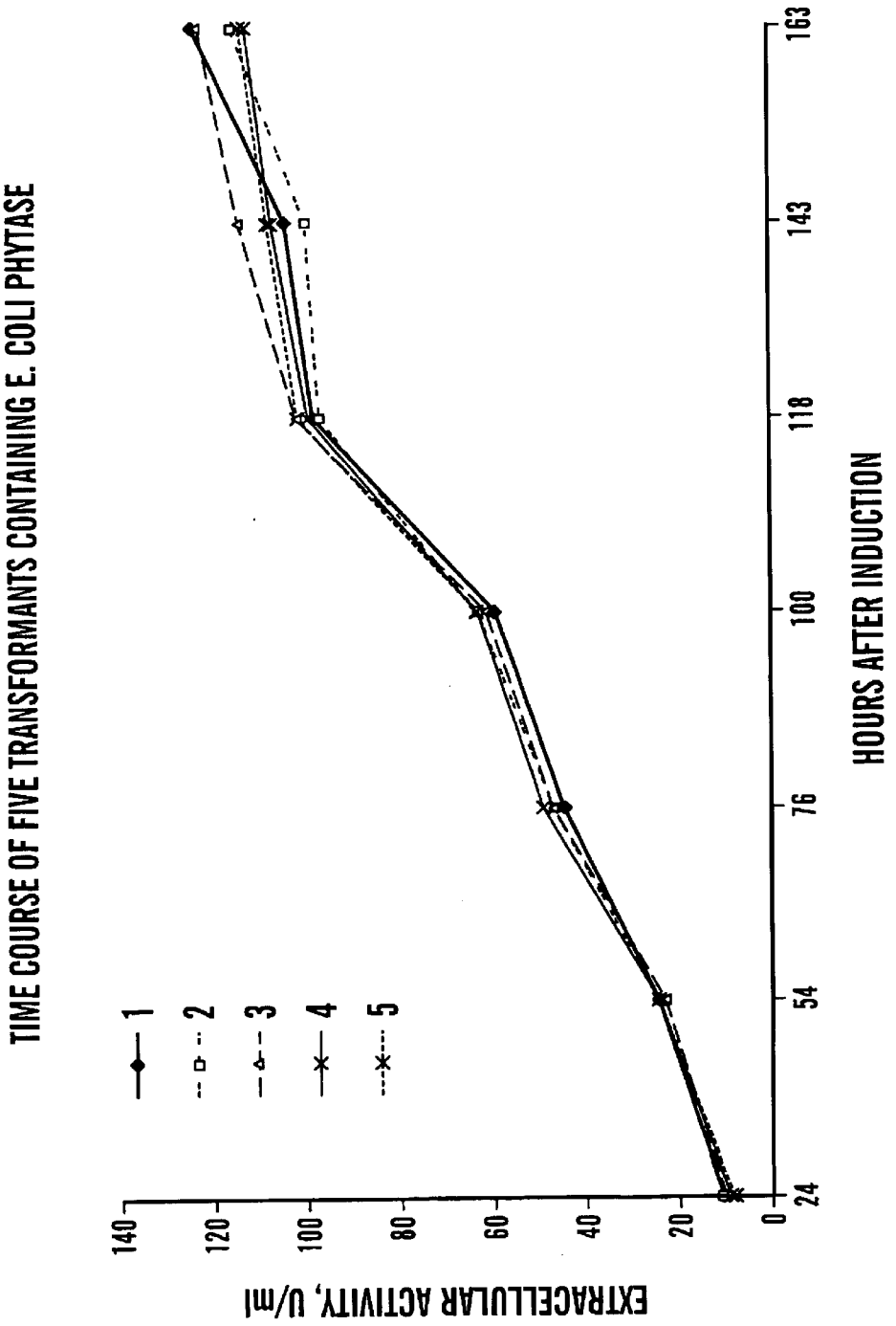
TIME COURSE OF FIVE TRANSFORMANTS CONTAINING E. COLI PHYTASE
EXTRACELLULAR ACTIVITY, U/ml
HOURS AFTER INDUCTION
1
2
3
4
5
0
20
40
60
80
100
120
140
24
54
76
100
118
143
163

FIG. 18

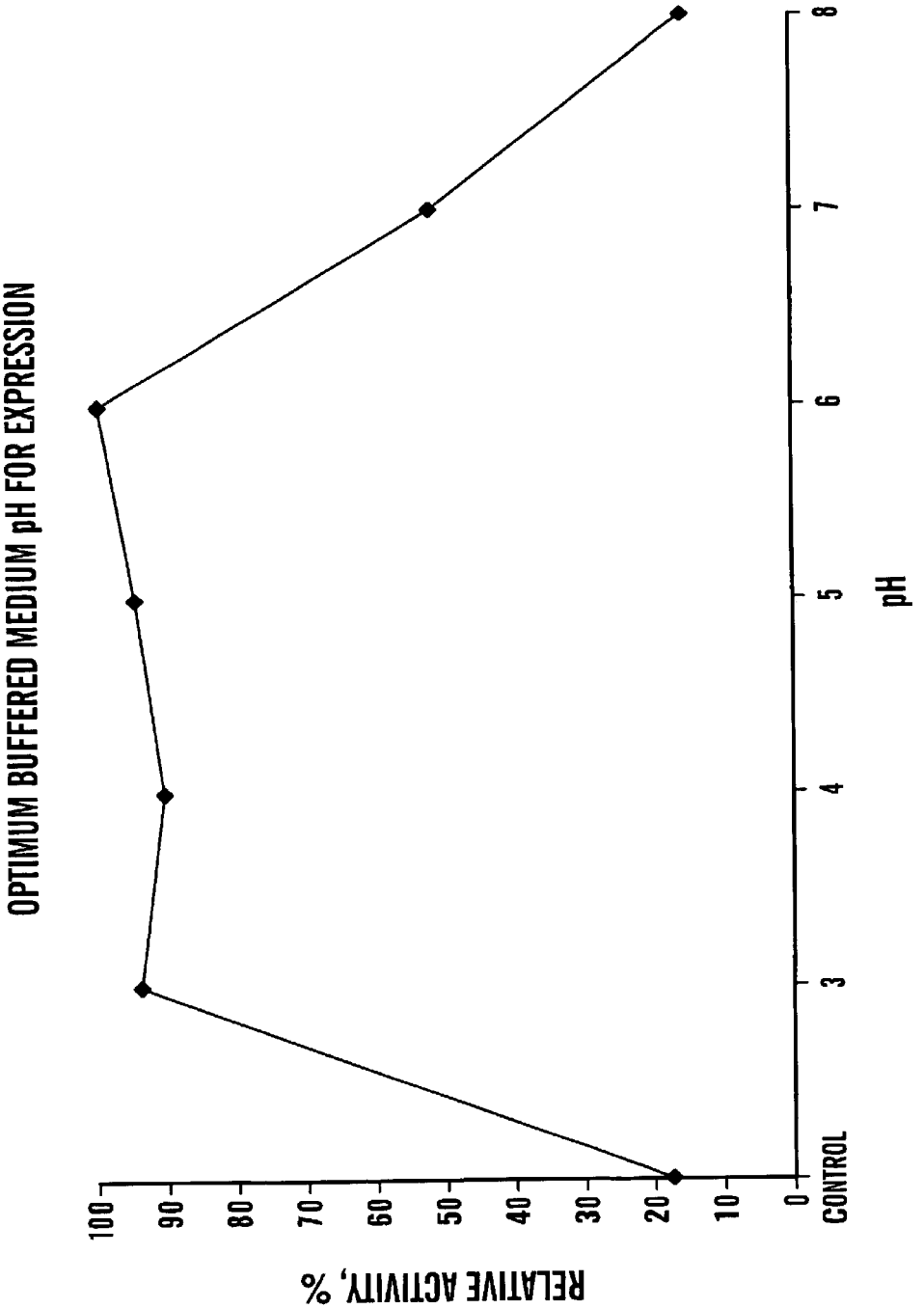
OPTIMUM BUFFERED MEDIUM pH FOR EXPRESSION
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
CONTROL
3
4
5
6
7
8
pH

FIG. 19

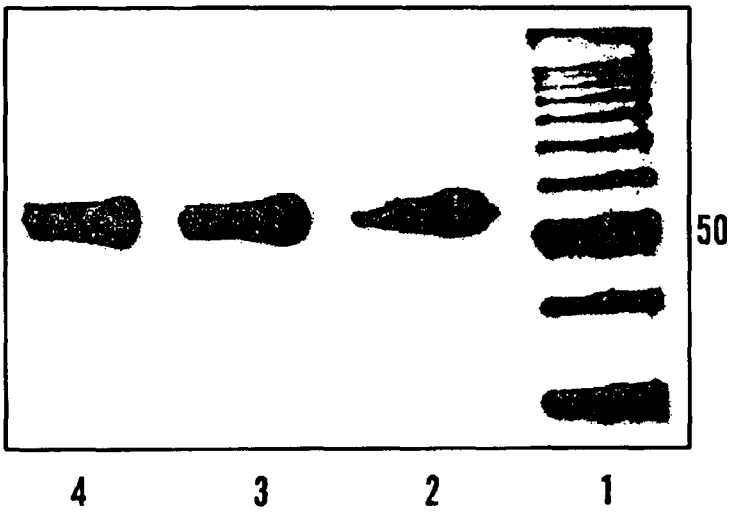

FIG. 20

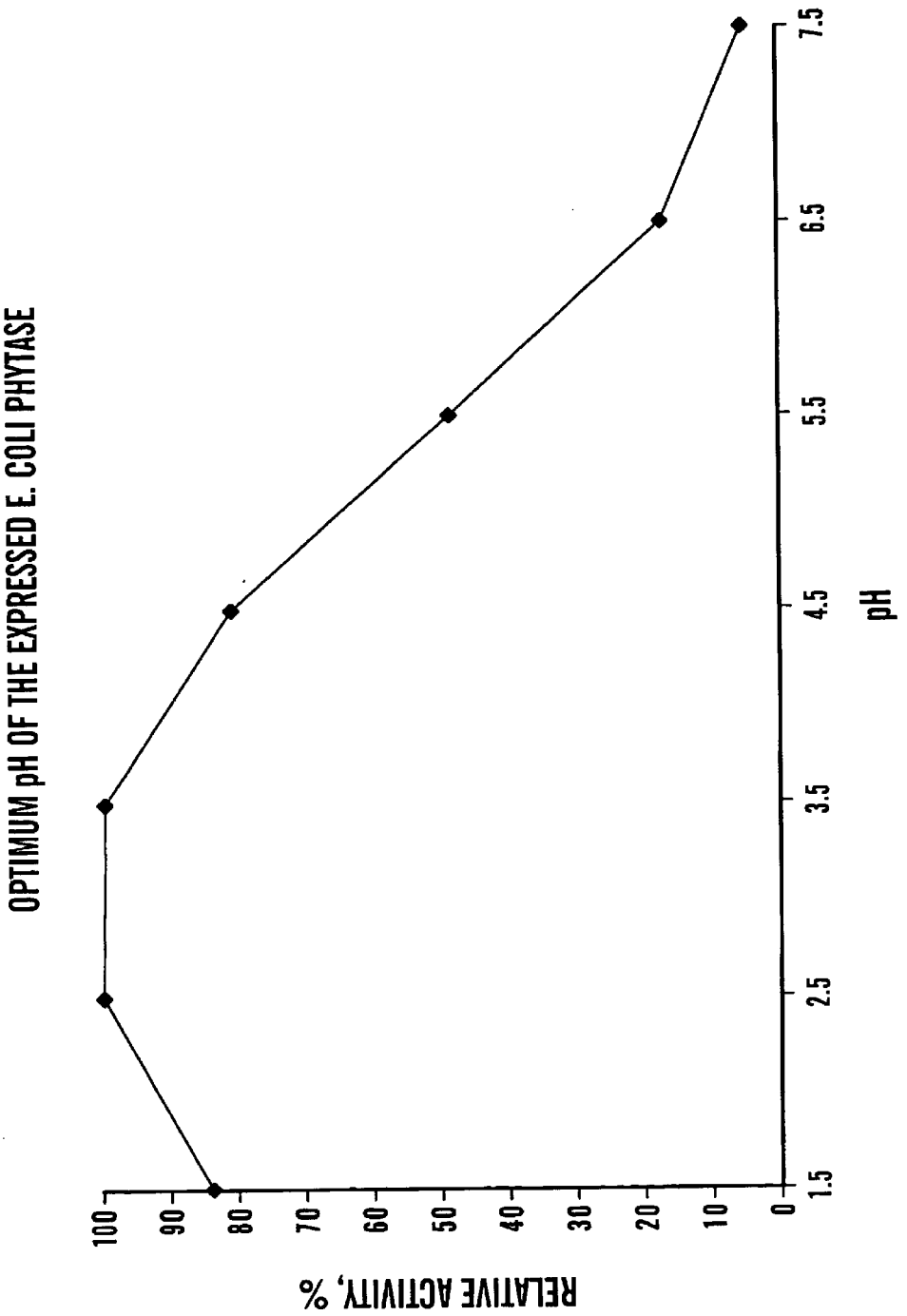
OPTIMUM pH OF THE EXPRESSED E. COLI PHYTASE
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
pH

FIG. 21

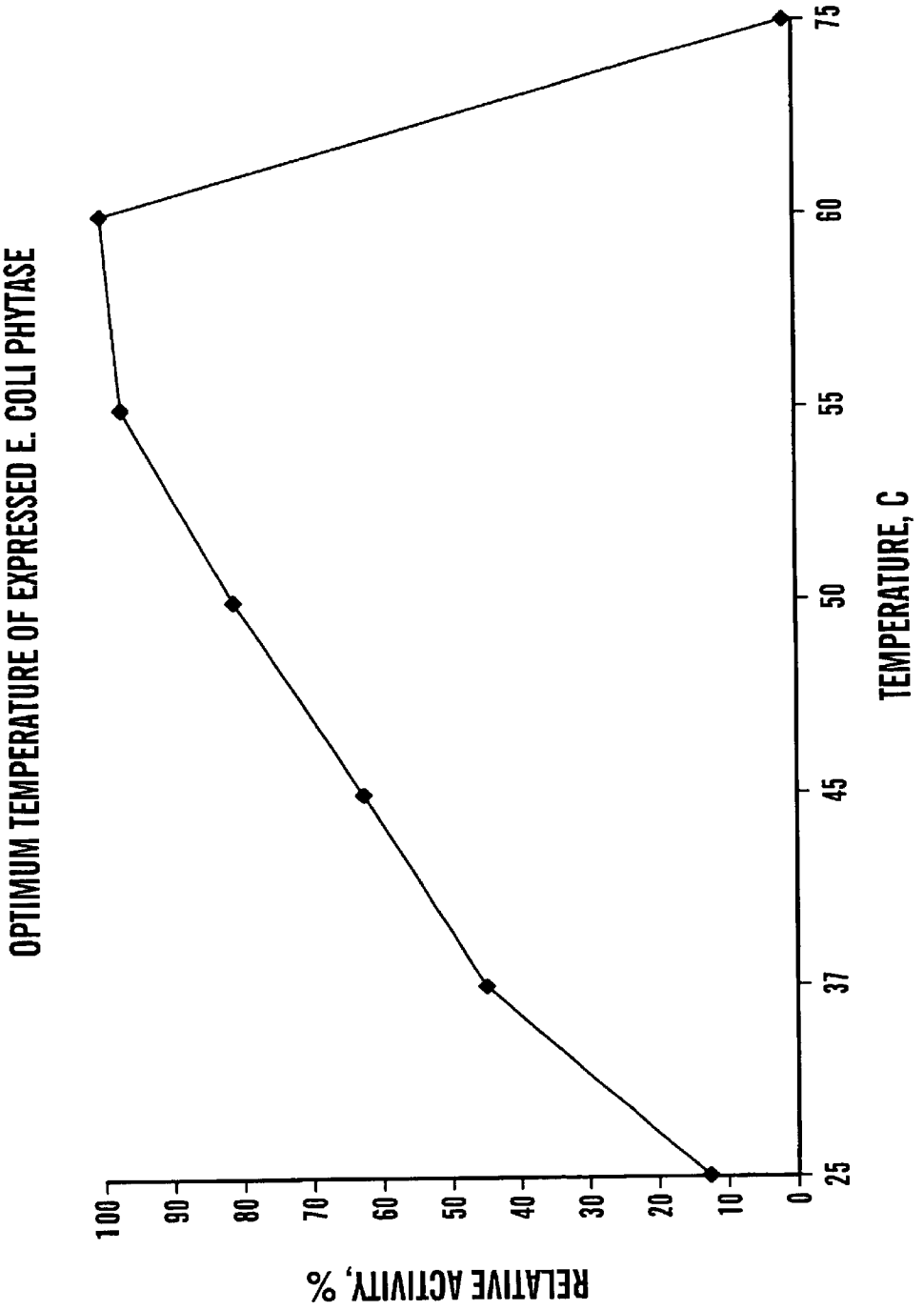
OPTIMUM TEMPERATURE OF EXPRESSED E. COLI PHYTASE
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
25
37
45
50
55
60
75
TEMPERATURE, C

FIG. 22

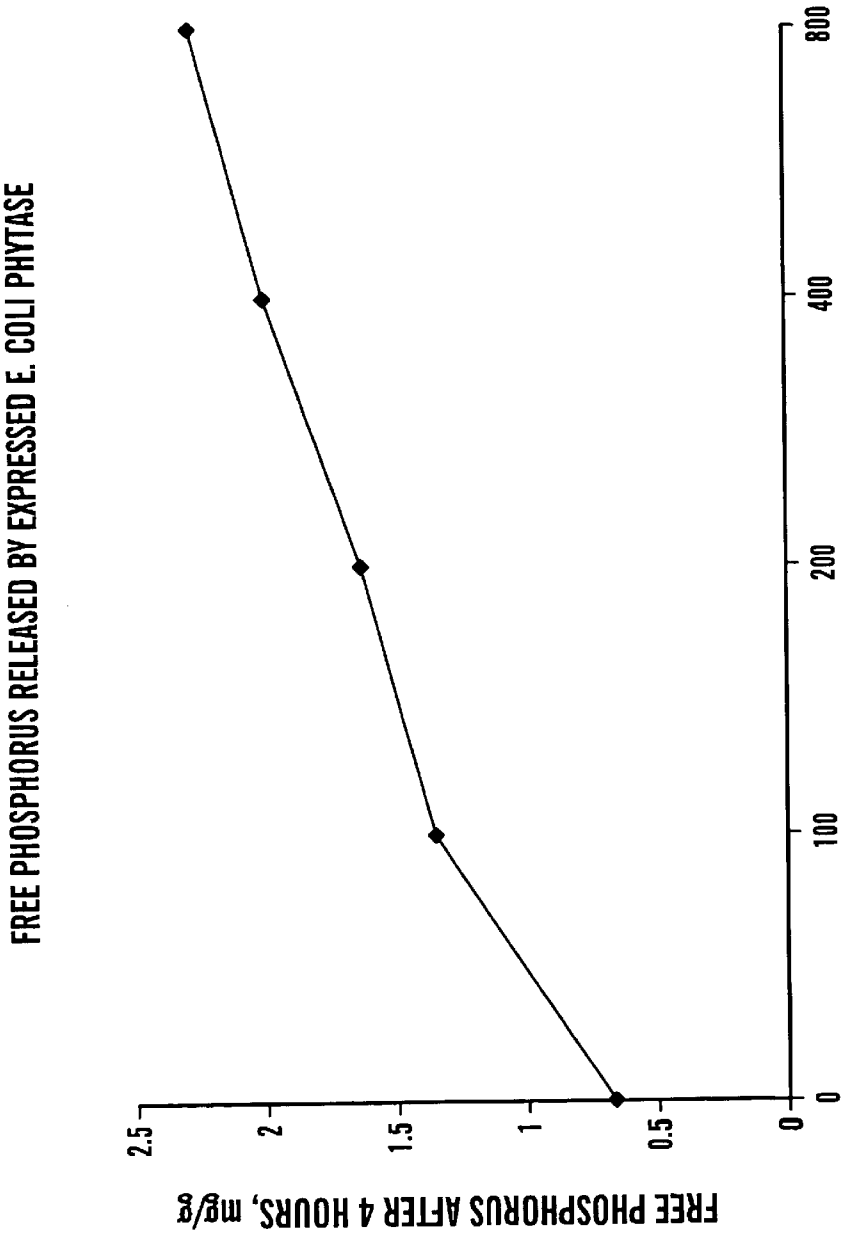
FREE PHOSPHORUS RELEASED BY EXPRESSED E. COLI PHYTASE
FREE PHOSPHORUS AFTER 4 HOURS, mg/g
2.5
2
1.5
1
0.5
0
0
100
200
400
800
PHYTASE, mU/g

FIG. 23

OVEREXPRESSION OF PHYTASE GENES IN YEAST SYSTEMS

This is a continuation-in-part of U.S. patent application Ser. No. 09/104,769, filed Jun. 25, 1998 now U.S. Pat. No 6,451,572.

FIELD OF THE INVENTION

The present invention relates to a method of producing phytase in yeast, yeast strains which express heterologous phytase, and the heterologous phytase produced by yeast.

BACKGROUND OF THE INVENTION

Phytases, a specific group of monoester phosphatases, are required to initiate the release of phosphate ("P") from phytate (myo-inositol hexophosphate), the major storage form of P in cereal foods or feeds (Reddy, N. R. et al., "Phytates in Legumes and Cereals," *Advances in Food Research,* 28:1 (1982)). Because simple-stomached animals like swine and poultry as well as humans have little phytase activity in their gastrointestinal tracts, nearly all of the ingested phytate P is indigestible. This results in the need for supplementation of inorganic P, an expensive and non-renewable nutrient, in diets for these animals. More undesirably, the unutilized phytate-P excreted through manure of these animals becomes P pollution of the environment (Cromwell, G. L. et al., "P—A Key Essential Nutrient, Yet a Possible Major Pollutant—Its Central Role in Animal Nutrition," *Biotechnology In the Feed Industry*; Proceedings Alltech 7th Annual Symposium, p. 133 (1991)). Furthermore, phytate chelates with essential trace elements like zinc and produces nutrient deficiencies such as growth and mental retardation in children ingesting mainly plant origin foods without removal of phytate.

Two phytases, phyA and phyB, from *Aspergillus niger* NRRL3135 have been cloned and sequenced (Ehrlich, K. C. et al., "Identification and Cloning of a Second Phytase Gene (phys) from *Aspergillus niger* (ficuum)," *Biochem. Biophys. Res. Commun.,* 195:53–57 (1993); Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori," Gene,* 133:56–62 (1993)). Recently, new phytase genes have been isolated from *Aspergillus terreus* and *Myceliophthora thermophila* (Mitchell et al., "The Phytase Subfamily of Histidine Acid Phosphatases: Isolation of Genes for Two Novel Phytases From the Fungi *Aspergillus terreus* and *Myceliophthora thermophila," Microbiology* 143:245–252, (1997)), *Aspergillus fumigatus* (Pasamontes et al., "Gene Cloning, Purification, and Characterization of a Heat-Stable Phytase from the Fungus *Aspergillus fumigatus" Appl. Environ. Microbiol.,* 63:1696–1700 (1997)), *Emericella nidulans* and *Talaromyces thermophilus* (Pasamontes et al., "Cloning of the Phytase from *Emericella nidulans* and the Thermnophilic Fungus *Talaromyces thermophilus," Biochim. Biophys. Acta.,* 1353:217–223 (1997)), and maize (Maugenest et al., "Cloning and Characterization of a cDNA Encoding a Maize Seedling Phytase," *Biochem. J.* 322:511–517, 1997)).

Various types of phytase enzymes have been isolated and/or purified from *Enterobacter* sp. 4 (Yoon et al., "Isolation and Identification of Phytase-Producing Bacterium, *Enterobacter* sp. 4, and Enzymatic Properties of Phytase Enzyme.," *Enzyme and Microbial Technology* 18:449–454 (1996)), *Klebsiella terrigena* (Greiner et al., "Purification and Characterization of a Phytase from *Klebsiella terrigena.," Arch. Biochem. Biophys.* 341:201–206 (1997)),

and *Bacillus* sp. DS11 (Kim et al., "Purification and Properties of a Thernostable Phytase from *Bacillus* sp. DS11," *Enzyme and Microbial Technology* 22:2–7 (1998)). Properties of these enzyme have been studied. In addition, the crystal structure of phy A from *Aspergillus ficuum* has been reported (Kostrewa et al., "Crystal Structure of Phytase from *Aspergillus ficuum* at 2.5 A Resolution," *Nature Structure Biology* 4:185–190 (1997)).

Hartingsveldt et al. introduced phyA gene into *A. niger* and obtained a ten-fold increase of phytase activity compared to the wild type. ("Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87–94 (1993)). Supplemental microbial phytase of this source in the diets for pigs and poultry has been shown to be effective in improving utilization of phytate-P and zinc (Simons et al., "Improvement of Phosphorus Availability By Microbial Phytase in Broilers and Pigs," *Br. J. Nutr.,* 64:525 (1990); Lei, X. G. et al., "Supplementing Corn-Soybean Meal Diets With Microbial Phytase Linearly Improves Phytate P Utilization by Weaning Pigs," *J. Anim. Sci.,* 71:3359 (1993); Lei, X. G. et al., "Supplementing Corn-Soybean Meal Diets With Microbial Phytase Maximizes Phytate P Utilization by Weaning Pigs," *J. Anim. Sci.,* 71:3368 (1993); Cromwell, G. L. et al., "P—A Key Essential Nutrient, Yet a Possible Major Pollutant—Its Central Role in Animal Nutrition," *Biotechnology In the Feed Industry*; Proceedings Alltech 7th Annual Symposium, p. 133 (1991)). But, expenses of the limited available commercial phytase supply and the activity instability of the enzyme to heat of feed pelleting preclude its practical use in animal industry (Jongbloed, A. W. et al., "Effect of Pelleting Mixed Feeds on Phytase Activity and Apparent Absorbability of Phosphorus and Calcium in Pigs," *Animal Feed Science and Technology,* 28:233–242 (1990)). Moreover, phytase produced from *A. niger* is presumably not the safest source for human food manufacturing.

Yeast can be used to produce enzymes effectively while grown on simple and inexpensive media. With a proper signal sequence, the enzyme can be secreted into the media for convenient collection. Some yeast expression systems have the added advantage of being well accepted in the food industry and are safe and effective producers of food products.

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. This system is well-known for its ability to express high levels of heterologous proteins. Because it is an eukaryote, *Pichia* has many of the advantages of higher eukaryotic expression systems such as protein processing, folding, and post-transcriptional modification.

Thus, there is a need to develop an efficient and simple system to produce phytase economically for the application of food and feed industry.

SUMMARY OF THE INVENTION

The present invention relates to a method of producing phytase in yeast by introducing a heterologous gene which encodes a protein or polypeptide with phytase/acid phosphatase activity into a yeast strain and expressing that gene.

The present invention also relates to a protein or polypeptide having phytase activity with optimum activity in a temperature range of 57–65° C. at pH of 2.5 to 3.5 or of 5.5. Optimal pH at 2.5 to 3.5 is particularly important for phytase, because that is the stomach pH of animals.

The invention further provides a yeast cell carrying a heterologous gene which encodes a protein or polypeptide

with phytase activity and which is functionally linked to a promoter capable of expressing phytase in yeast.

Yet another aspect of the invention is a vector having a gene from a non-yeast organism which encodes a protein or polypeptide with phytase activity, a promoter which is capable of initiating transcription in yeast functionally linked to the gene encoding a peptide with phytase activity, and with an origin of replication capable of maintaining the vector in yeast or being capable of integrating into the host genome.

The invention also provides a method for producing a protein or polypeptide having phytase activity. An isolated appA gene, which encodes a protein or polypeptide with phytase activity, is expressed in a host cell.

The invention also includes a method of converting phytate to inositol and inorganic phosphate. The appA gene expresses a protein of polypeptide with phytase activity in a host cell. The protein or polypeptide is then contacted with phytate to catalyze the conversion of phytate to inositol and inorganic phosphate.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows an SDS-PAGE analysis of soluble protein prepared from the phytase gene transformed *E. coli* induced with IPTG. The cells were grown 4 hours before harvesting. Lane 1: Marker; Lanes 2 and 3: Transformants of pEP1 (the expressed protein was approximately 55 kDa); Lane 4: Transformant with only the expression vector pET25b(+).

FIG. **2** shows the Western blot analysis of the expressed phytase protein in *E. coli*. The antibody was raised against purified native phytase of *A. niger*. Each lane contained 50 μg total intracellular protein. Lanes 1 and 2: Recombinants after and before induction. Lanes 3 and 4: control (only the expressing vector) after and before induction.

FIG. **3** is a scan image of Northern blotting analysis of the mRNA of PhlyA in *E. coli*. A 1.4 kb PhyA probe was used. Each lane contained 20 μg of total RNA. Lanes 1 and 2: RNA isolated from the control cells (only the expression vector) before and after induction. Lanes 3 and 4: RNA isolated from the recombinants containing PhyA before and after induction.

FIG. **4** is a time course of the induced expression of phytase (pEP1) in *E. coli* BL21(DE3). The cells were induced when the OD_{600} reached 0.5. The soluble protein, prepared at each time point, was quantified by SDS-PAGE analysis.

FIG. **5** shows an SDS-PAGE analysis of the expressed extracellular phytase protein by the phytase transformed *S. lividans* after growing for 72 hours. Cells were spun for 15 minutes at 8,000×g, and the supernatant was subjected to gel electrophoresis. Lane 1: Marker; Lane 2: Control with the only expression vector; Lane 3: Positive colony expressed phytase and the size was approximately 57 kDa.

FIG. **6** shows the Western blot analysis of the phytase expressed by *S. lividans*, using a phytase antibody raised against purified native phytase of *A. niger*. Each lane was loaded with 20 μg medium (supernatant) protein. Lane 1: Supernatant from the vector transformed control cell culture; Lane 2: Supernatant from the culture inoculated with the positive colony.

FIG. **7** depicts an SDS-PAGE analysis of the extracellular phytase expressed by *S. cerevisiae*. Each lane was loaded with 50 μg medium (supernatant) protein. Lanes 1 to 3: Supernatant from the culture inoculated with the positive colony harvested at 5, 10, and 25 hours after induction, respectively; Lanes 4 to 6: Supernatant from the vector-transformed control cell culture harvested at 5, 10, and 25 hours after induction, respectively; Lane 7: Marker (kDa). The expressed phytase was approximately 110 kDa (confirmed by Western blot).

FIG. **8** is a time course of the extracellular phytase activity expressed by the pYPP1 construct transformed *S. cerevisiae* after induced by galactose. The activity was analyzed in the supernatant of the collected medium.

FIG. **9** shows the Western blot analysis of the extracellular phytase expressed by *S. cerevisiae* before and after deglycosylation (Endo H), using a phytase antibody raised against purified native phytase in *A. niger*. Lane 1: Prestained SDS-PAGE standards (kDa) from Bio-Rad; Lanes 2 and 3: deglycosylated 10 and 20 μg phytase protein, respectively; Lane 4: glycosylated phytase (20 μg protein).

FIG. **10** is a scan image of Northern blot analysis for total RNA isolated from transformed *S. cerevisiae* cells. Lane 1: Control (with only the expression vector pYES2); Lanes 2 and 3: Tranformants of pYPP1.

FIG. **11** is a time course of the extracellular phyA phytase activity produced by *Pichia pastoris* transformants of Mut^s (KM71) and Mut+ (X33) after induction.

FIG. **12** depicts an SDS-PAGE analysis of the overexpressed phytase in *Pichia,* with construct of pPICZαA-PhyA in KM71 (MUT^s). Lane 1: protein ladder. Lane 2: 40 μl of the supernatant of AK1 (a colony showed 21,700 mU/ml of extracellular phytase), collected 108 hours after induction. Lane 3: 40 μl of the supernatant of a control strain overexpressing human serum albumin (HAS, 6.7 kDa) at a level of 1 g/L. Lane 4: 40 μl of the supernatant of the KM71 control.

FIG. **13** depicts effects of deglycosylation by Endo H on the thermostability of the expressed phytase in *Pichia.* Phytase activity was measured after the enzymes were heated for 15 minutes under 37 or 80° C. in 0.2 M citrate buffer, pH 5.5.

FIG. **14** is a scan image of Northern analysis of the expressed phyA mRNA by the transformed *Pichia pastoris* strains (KM71 and X33). A 1.3 kb phyA probe was used for blotting. Lanes 1 and 2: the transformant of KM71 before and after induction; Lanes 3 and 4: the transformant of X33 after and before induction.

FIG. **15** shows the optimum pH of the expressed extracellular phytase by *Pichia* (X33). Buffers of 0.2 M glycine-HCl for pH 1.5, 2.5, 3.5; 0.2 M sodium citrate for pH 4.5, 5.5, 6.5, and 0.2 M Tris-HCl for pH 7.5 and 8.5 were used.

FIG. **16** shows the optimum temperature of the expressed extracellular phytase by *Pichia* (X33). The assays were conducted in 0.2 M citrate buffer, pH 5.5.

FIG. **17** depicts the release of free phosphorus from soybean by the expressed phytase in *Pichia* (X33). Five grams of soybean were suspended in 25 ml of 0.2 M citrate, pH 5.5, with different amounts of the enzyme. The incubation was conducted for 4 hours under 37° C. and the free phosphorus released in the supernatant was determined.

FIG. **18** shows a time course of the expression of the extracellular phytase activity from five transformants of *Pichia pastoris* containing the *E. coli* appA gene.

FIG. **19** graphically shows the relationship between medium pH and the expression of phytase activity by *Pichia pastoris.*

FIG. **20** is an SDS-PAGE analysis of the *E. coli* phytase overexpressed in *Pichia pastoris*. Lane 1: Protein ladder; Lanes 2 to 4: Supernatants collected from the cultures of positive colonies 23, 22, and 11, respectively, at 118 hours after induction.

FIG. **21** graphically shows the optimum pH of the overexpressed *E. coli* phytase by *Pichia pastoris.*

FIG. **22** graphically shows the optimum temperature of the overexpressed *E. coli* phytase by *Pichia pastoris.*

FIG. **23** shows the amount of free phosphorus released from soybean meal by the overexpressed *E. coli* phytase from *Pichia pastoris* after four hours treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing phytase in yeast. According to this method, a heterologous gene which encodes a protein or polypeptide with phytase activity is expressed in a yeast strain.

The enzymes which catalyze the conversion of phytate to inositol and inorganic phosphorus are broadly known as phytases. Phytase producing microorganisms comprise bacteria such as *Bacillus subtilis* (Paver et al., J. Bacteriol. 151, 1102 (1982), which is hereby incorporated by reference) and *Pseudomonas* (Cosgrove, *Austral. J. Biol. Sci.* 23:1207 (1970), which is hereby incorporated by reference); yeasts, such as *Saccharomyces cerevisiae* (Nayini et al., *Lebensmittel Wissenschaft und Technologie* 17:24 (1984), which is hereby incorporated by reference); and fungi, such as *Aspergillus terreus* (Yamada et al., *Agric. Biol. Chem.* 32:1275 (1986), which is hereby incorporated by reference), and *Aspergillus ficuum* (van Gorcom et al., European Patent Application 89/202,436, which is hereby incorporated by reference).

Phytases are also endogenously present in many plant species. Loewus, In: *Plant Biology* vol. 9: "Inositol Metabolism in Plants" (eds. D. J. Morre, W. F. Boss, F. A. Loewus) 13 (1990); and Gellatly, et al., *Plant Physiology* (supplement) 93:562 (1990), which are hereby incorporated by reference, mention the isolation and characterization of a phytase cDNA clone obtained from potato tubers. Gibson, et al., *J. Cell Biochem.,* 12C:L407 (1988) and Christen, et al., *J. Cell Biochem.,* 12C:L402 (1988), which are hereby incorporated by reference, mentions the synthesis of endogenous phytase during the germination of soybean seeds.

Preferably, the protein or polypeptide with phytase activity is secreted by the cell into growth media. This allows for higher expression levels and easier isolation of the product. The protein or polypeptide with phytase activity is coupled to a signal sequence capable of directing the protein out of the cell. Preferably, the signal sequence is cleaved from the protein.

In a preferred embodiment, the heterologous gene, which encodes a protein or polypeptide with phytase activity, is spliced in frame with a transcriptional enhancer element.

Preferred heterologous genes encoding proteins with phytase activity are isolated from a bacterial cell. A more preferred gene is the phyA gene of *Aspergillus niger*. A gene encoding phytase, phyA, from *Aspergillus niger* NRRL3135 has been cloned and sequenced (Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori,*" *Gene,* 133:56–62 (1993), which are hereby incorporated by reference). Hartingsveldt et al. introduced phyA gene into *A. niger*, and obtained a tenfold increase of phytase activity compared to the wild type. (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger,*" *Gene* 127:87–94 (1993), which is hereby incorporated by reference.)

Another preferred heterologous gene is the appA gene of *E. coli*. The gene, originally defined as *E. coli* periplasmic phosphoanhydride phosphohydrolase (appA) gene, contains 1,298 nucleotides (GeneBank accession number: M58708). The gene was first found to code for an acid phosphatase protein of optimal pH of 2.5 (EcAP) in *E. coli*. The acid phosphatase is a monomer with a molecular mass of 44,644 daltons. Mature EcAP contains 410 amino acids (Dassa, J. et al., "The Complete Nucleotide Sequence of the *Escherichia Coli* Gene AppA Reveals Significant Homology Between Ph 2.5 Acid Phosphatase and Glucose-1-Phosphatase," *J. Bacteriology,* 172:5497–5500 (1990), which is hereby incorporated by reference). Ostanin, et al. overexpressed appA in *E. coli* BL21 using a pT7 vector and increased its acid phosphatase activity by approximately 400-folds (440 mU/mg protein), (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia Coli* Acid Phosphatase," *J. Biol. Chem.,* 267:22830–36 (1992), which is hereby incorporated by reference). The product of the appA gene was not previously known to have phytase activity.

The appA or phyA gene can be expressed in any prokaryotic or eukaryotic expression system. A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Preferred vectors include a viral vector, plasmid, cosmid or an oligonucleotide. Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Preferred hosts for expressing appA or phyA include fungal cells, including species of yeast or filamentous fungi, may be used as host cells in accordance with the present invention. Preferred yeast host cells include different strains of *Saccharomyces cerevisiae*. Other yeasts like *Kluyveromyces, Torulaspora,* and *Schizosaccharomyces* can also be used. In a preferred embodiment, the yeast strain used to overexpress the protein is *Saccharomyces cerevisiae*. Preferred filamentous fungi host cells include *Aspergillus* and *Neurospora.* A more preferred strain of *Aspergillus* is *Aspergillus niger.*

In another preferred embodiment of the present invention, the yeast strain is a methylotrophic yeast strain. Methylotrophic yeast are those yeast genera capable of utilizing methanol as a carbon source for the production of the energy resources necessary to maintain cellular function and containing a gene for the expression of alcohol oxidase. Typical methylotrophic yeasts include members of the genera *Pichia, Hansenula, Torulopsis, Candida,* and *Karwinskia.* These yeast genera can use methanol as a sole carbon source. In a more preferred embodiment, the methylotrophic yeast strain is *Pichia pastoris.*

The present invention also provides a protein or polypeptide with phytase activity. PhyA is expressed in *Pichia* and the resulting protein produced has much higher extracellular activity (~65 mU/ml). The phytase activity yield was approximately 30-fold greater than that in phyA transformed *Saccharomyces cerevisiae,* 21-fold greater than that in wild type of *Aspergillus niger*, and 65,000-fold greater than that in the untransformed *Pichia.* The optimal pH of the expressed phytase was 2.5 and 5.5, and the optimal tem-

perature was 60° C. Similarly, appA is expressed in *Pichia* and *Saccharomyces cerevisiae* with the resulting protein having much higher extracellular activity and a much preferred optimal pH of 2.5 to 3.5.

A preferred embodiment of the invention is a protein or polypeptide having phytase activity with optimum activity in a temperature range of 57 to 65° C. A more preferred embodiment is a protein or polypeptide having phytase activity, where its temperature range for optimum activity is from 58 to 62° C.

Yet another preferred embodiment is a protein or polypeptide having phytase activity where the protein retains at least 40% of its activity after heating the protein for 15 minutes at 80° C. More preferred is a protein or polypeptide having phytase activity where the protein retains at least 60% of its activity after heating the protein for 15 minutes at 60° C.

Purified protein may be obtained by several methods. The protein or polypeptide of the present invention is preferably produced in purified form (preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is secreted into the growth medium of recombinant host cells. Alternatively, the protein or polypeptide of the present invention is produced but not secreted into growth medium. In such cases, to isolate the protein, the host cell carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove cell debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the polypeptide or protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

The present invention also provides a yeast strain having a heterologous gene which encodes a protein or polypeptide with phytase activity. The heterologous gene should be functionally linked to a promoter capable of expressing phytase in yeast.

Yet another aspect of the invention is a vector for expressing phytase in yeast. The vector carries a gene from a non-yeast organism which encodes a protein or polypeptide with phytase activity. The phytase gene can be cloned into any vector which replicates autonomously or integrates into the genome of yeast. The copy number of autonomously replicating plasmids, e.g. YEp plasmids may be high, but their mitotic stability may be insufficient (Bitter et al., "Expression and Secretion Vectors for Yeast," *Meth. Enzymol.* 153:516–44 (1987), which is hereby incorporated by reference). They may contain the 2 mu-plasmid sequence responsible for autonomous replication, and an *E. coli* sequence responsible for replication in *E. coli*. The vectors preferably contain a genetic marker for selection of yeast transformants, and an antibiotic resistance gene for selection in *E. coli*. The episomal vectors containing the ARS and CEN sequences occur as a single copy per cell, and they are more stable than the YEp vectors. Integrative vectors are used when a DNA fragment is integrated as one or multiple copies into the yeast genome. In this case, the recombinant DNA is stable and no selection is needed (Struhl et al., "High-Frequency Transformation of Yeast: Autonomous Replication of Hybrid DNA Molecules," *Proc. Nat'l Acad. Sci. USA* 76:1035–39 (1979); Powels et al., *Cloning Vectors*, I–IV, et seq. Elsevier, (1985); Sakai et al., "Enhanced Secretion of Human Nerve Growth Factor from *Saccharomyces Cerevisiae* Using an Advanced δ-Integration System," *Biotechnology* 9:1382–85 (1991), which are hereby incorporated by reference). Some vectors have an origin of replication, which functions in the selected host cell. Suitable origins of replication include 2μ, ARS1, and 25 μM. The vectors have restriction endonuclease sites for insertion of the fusion gene and promoter sequences, and selection markers. The vectors may be modified by removal or addition of restriction sites, or removal of other unwanted nucleotides.

The phytase gene can be placed under the control of any promoter (Stetler et al., "Secretion of Active, Full- and Half-Length Human Secretory Leukocyte Protease Inhibitor by *Saccharomyces cerevisiae*," *Biotechnology* 7:55–60, (1989), which is hereby incorporated by reference). One can choose a constitutive or regulated yeast promoter. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073 (1980), which is hereby incorporated by reference) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149 (1968); and Holland et al., *Biochem.* 17:4900, (1978), which are hereby incorporated by reference), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in EP A-73,657 to Hitzeman, which is hereby incorporated by reference. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al., *J. Biol. Chem.* 258:2674 (1982) and Beier et al., *Nature* 300:724 (1982), which are hereby incorporated by reference.

One can choose a constitutive or regulated yeast promoter. The strong promoters of e.g., phosphoglycerate kinase (PGK) gene, other genes encoding glycolytic enzymes, and the alpha -factor gene, are constitutive. When a constitutive promoter is used, the product is synthesized during cell growth. The ADH2 promoter is regulated with ethanol and glucose, the GAL-1-10 and GAL7 promoters with galactose and glucose, the PHO5 promoter with phosphate, and the metallothionine promoter with copper. The heat shock promoters, to which the HSP150 promoter belongs, are regulated by temperature. Hybrid promoters can also be used. A regulated promoter is used when continuous expression of the desired product is harmful for the host cells. Instead of yeast promoters, a strong prokaryotic promoter such as the T7 promoter, can be used, but in this case the yeast strain has to be transformed with a gene encoding the respective polymerase. For transcription termination, the HSP150 terminator, or any other functional terminator is used. Here, promoters and terminators are called control elements. The present invention is not restricted to any specific vector, promoter, or terminator.

The vector may also carry a selectable marker. Selectable markers are often antibiotic resistance genes or genes capable of complementing strains of yeast having well characterized metabolic deficiencies, such as tryptophan or histidine deficient mutants. Preferred selectable markers include URA3, LEU2, HIS3, TRP1, HIS4, ARG4, or antibiotic-resistance genes.

The vector may also have an origin of replication capable of replication in a bacterial cell. Manipulation of vectors is more efficient in bacterial strains. Preferred bacterial origin of replications are ColE1, Ori, or oriT.

A leader sequence either from the yeast or from phytase genes or other sources can be used to support the secretion

of expressed phytase enzyme into the medium. The present invention is not restricted to any specific type of leader sequence or signal peptide.

Suitable leader sequences include the yeast alpha factor leader sequence, which may be employed to direct secretion of the phytase. The alpha factor leader sequence is often inserted between the promoter sequence and the structural gene sequence (Kurjan et al., *Cell* 30:933, (1982); Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, (1984); U.S. Pat. No. 4,546,082; and European published patent application No. 324,274, which are hereby incorporated by reference). Another suitable leader sequence is the *S. cerevisiae* MF alpha 1 (alpha-factor) is synthesized as a prepro form of 165 amino acids comprising signal-or prepeptide of 19 amino acids followed by a "leader" or propeptide of 64 amino acids, encompassing three N-linked glycosylation sites followed by (LysArg(Asp/Glu, Ala)2-3 alpha-factor)4 (Kudjan, et al., *Cell* 30:933–43 (1982), which is hereby incorporated by reference). The signal-leader part of the preproMF alpha 1 has been widely employed to obtain synthesis and secretion of heterologous proteins in *S. cerivisiae*. Use of signal/leader peptides homologous to yeast is known from. U.S. Pat. No. 4,546,082, European Patent Applications Nos. 116,201; 123,294; 123,544; 163,529; and 123,289 and DK Patent Application No. 3614/83, which arc hereby incorporated by reference. In European Patent Application No. 123,289, which is hereby incorporated by reference, utilization of the *S. cerevisiae* a-factor precursor is described whereas WO 84/01153, which is hereby incorporated by reference, indicates utilization of the *Saccharomyces cerevisiae* invertase signal peptide, and German Patent Application DK 3614/83, which is hereby incorporated by reference, indicates utilization of the *Saccharomyces cerevisiae* PH05 signal peptide for secretion of foreign proteins.

The alpha -factor signal-leader from *Saccharomyces cerevisiae* (MF alpha 1 or MF alpha 2) may also be utilized in the secretion process of expressed heterologous proteins in yeast (U.S. Pat. No. 4,546,082, European Patent Applications Nos. 16,201; 123,294; 123 544; and 163,529, which are hereby incorporated by reference). By fusing a DNA sequence encoding the *S. cerevisiea* MF alpha 1 signal/leader sequence at the 5' end of the gene for the desired protein secretion and processing of the desired protein was demonstrated. The use of the mouse salivary amylase signal peptide (or a mutant thereof) to provide secretion of heterologous proteins expressed in yeast has been described in Published PCT Applications Nos. WO 89/02463 and WO 90/10075, which are hereby incorporated by reference.

U.S. Pat. No. 5,726,038 describes the use of the signal peptide of the yeast aspartic protease 3, which is capable of providing improved secretion of proteins expressed in yeast. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978), which is hereby incorporated by reference. The Hinnen et al. protocol selects for Trp transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

The gene may be maintained on stable expression vector, an artificial chromosome, or by integration into the yeast host cell chromosome. Integration into the chromosome may be accomplished by cloning the phytase gene into a vector which will recombine into a yeast chromosome. Suitable vectors may include nucleotide sequences which are homologous to nucleotide sequences in the yeast chromosome. Alternatively, the phytase gene may be located between recombination sites, such as transposable elements, which can mobilize the gene into the chromosome.

The present invention also provides a method of producing phytase by providing an isolated appA gene, which encodes a protein or polypeptide with phytase activity, and expressing the gene in host cell. Preferably the appA gene is isolated from *Escherichia coli*. Preferred host cells include yeast or filamentous fungi. The preferred filamentous fungi is *Aspergillus niger* and the preferred yeast are *Saccharomyces, Kluyveromyces, Torulaspora,* and *Schizosaccharomyces,* in particular, the yeast strain, *Saccromyces cerivesia.*

A method of converting phytate to inositol and inorganic phosphorus is also provided. An appA gene is isolated from an organism, using techniques well known in the art. A protein or polypeptide with phytase activity is then expressed from the gene in a host cell. The resulting protein or polypeptide is mixed or contacted with phyate. This technique is especially useful for treating phytate in food or animal feed.

The preferred appA gene is isolated from *Escherichia coli.*

While the phytase enzyme produced in a yeast system released phytate-P from corn and soy as effectively as the currently commercial phytase, it appeared to be more thermostable. This phytase overexpression system in yeast can be used to provide thermostable phytase for use in the food and feed industries.

EXAMPLES

Example 1

Materials and Methods for Overexpressing PhtyA in *E. Coli, S. Lividans*, and a *Saccharouiyces* System

Phytase gene, host strains, and expression plasmids. Phytase gene, phyA, was kindly provided by Dr. E. J. Mullaney of the USDA. The gene (GenBank Accession number M94550) was contained in plasmid pMD4.21 in *E. coli* strain HB101. A 2.7 kb SphI fragment of *A. niger* DNA contained the coding region of the deglycosylated phytase and its 5' and 3' flanking sequences. A plasmid containing the signal peptide sequence, Spxy, of the active xylanase gene of *Aureobasidum pullulans* (GenBank Accession number U10298) was kindly provided by Dr. X. L. Li of the University of Georgia. The *E. coli* strain DH5α was used as an initial host for all the recombinant plasmids. In order to express phyA in *E. coli*, the expression vector, pET25b(+) (Novagen, Madison, Wis.) and the expression host, BL21 (DE3)pLysS, were used. In order to express phyA in *S. lividans* TK 24, plasmid pSES1 (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividansoglucanase* Gene and an Endoglucanase Gene from *Thermornonospora Fusca,*" *Appl. Environ. Microbiol.,* 59:3032–43 (1993), which is hereby incorporated by reference), was used to construct the shuttle plasmid (from Dr. D. B. Wilson of Cornell University and he obtained it from Dr. D. A. Hopwood, John Innes Institute, Norwich, England). In order to express phyA in yeast, the expression vector pYES2 and

the host *S. cerevisiae* strain, INVSc1 (Invitrogen, San Diego, Calif.) were used.

Plasmid cassette constructions and transformations. All the constructed plasmids and the correspondent hosts are listed in Table 1. A 1.4 kb PCR fragment of phyA gene was amplified from the pMD4.21 by using two primers: upstream 5'-CGG AAT TCG TCA CCT CCG GAC T-3' (SEQ ID No. 1) and downstream 5'-CCC AAG CTT CTA AGC AAA ACA CTC-3' (SEQ ID No. 2). The resulting fragment contained the sequence coding for the deglycosylated phytase of *A. niger*, PhyA, and EcoRI and HindIII restriction site upstream and downstream, respectively. After purification with Geneclean II kit (Bio101, Inc., La Jolla, Calif.), the fragment was inserted into pET25b(+), and the resulting construct pEP1 (6893 bp) was transformed into BL21(DE3)pLysS after initial confirmation in DH5α cells. The expression was under the control of T7 promoter followed by the lead sequence (pel B) encoding 21 amino acids, and phyA. The host transformed with the pET25(+) vector only was used as the control.

TABLE 1

Expression vectors, constructs, and their host strains used in the study

Plasmid	Host	Description[1]	Reference[2]
pET25b(+)	*E. coli* DH5α and BL21 (DE3)pLysS	Expression vector	Novagen
pEP1	*E. coli* BL21 (DE3)pLysS	pET25bα(+) + phyA gene	This paper
PSES2	*E. coli* DH5α and *S. lividans* TK24	Expression vector	Jung et al.[2], 1993
PSPP1	*E. coli* DH5α and *S. lividans* TK24	pSES2 + Spe2 + phyA	This paper
PYES2	*E. coli* DH5α and *S. cerevisiae* INVSc1	Expression vector	Invitrogen
PYEP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Spe2 + phyA	This paper
PYXP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Spxy + phyA	This paper
PYPP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Sphy + phyA	This paper

[1]Spe2 is the signal peptide for endoglucanase E2 of *T. fusca* (Wilson, D. B., "Biochemistry and Genetics of Actinomycete Cellulases," Crit. Rev. Biotechnol., 12:45–63 (1992), which is hereby incorporated by reference); Spxy is the signal peptide for xylanase of *A. pullulans* (Li and Ljungdahl, "Cloning, Sequencing, and Regulation of a Xylanase Gene from the Fungus *Aureobasidium pullulans* Y-2331-1," Appl. Environ. Microbiol., 60:3160–66 (1994); Li and Ljungdahl, "Expression of *Aureobasidium pullulans* xynA in, and Secretion of the Xylanase from, *Saccharomyces cerevisiae*," Appl. Environ. Microbiol., 62:209–13 (1996), which are hereby incorporated by reference); and Sphy is the signal peptide for phyA of *A. niger* (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," Gene 127:87–94 (1993), which is hereby incorporated by reference).
[2]Jung, E.D. et al., "DNA Sequences and Expression in *Streptomyces Lividansoglucanase* Gene and an Endoglucanase Gene from *Thermomonospora Fusca*," Appl. Environ. Microbiol., 59:3032–43 (1993), which is hereby incorporated by reference).

The construction of the plasmid for phyA expression in *S. lividans* started with the synthesis of a fragment containing pLT1 promoter and Spe2 signal peptide (Lao, G. et al., "DNA Sequences of Three Beta-1,4-endoglucanase Genes From *Thermomonospora Fusca," J. Bacteriol.*, 173:3397–407 (1991), which is hereby incorporated by reference) by PCR. An upstream primer, 5'-CAG CTA TGA CCA TGA TTA CGC C-3' (SEQ ID No. 3), and a downstream primer, 5'-CCT AGA ACG GGA ATT CAT TGG CCG CC-3' (SEQ ID No. 4), contained PstI and EcoRI restriction sites, respectively. The fragment was amplified from pBW2 (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene From *Thermomonospora Fusca," Appl. Environ. Microbiol.*, 59:3032–43 (1993), which is hereby incorporated by reference) and then digested with PstI and EcoRI, while the construct pEP1 and plasmid pBluescript SK+ (Strategene, La Jolla, Calif.) were digested with EcoRI and HindIII, and PstI and HindIII, respectively. The three digested fragments were subsequently purified using Geneclean II kit and ligated into a single recombinant construct that contained the desired restriction sites of PstI and KpnI (from pBluescript SK*), pLT1 promoter and Spe2 leading peptide of endoglucanase E2 (551 bp, Lao, G. et al., "DNA Sequences of Three Beta-1,4-endoglucanase Genes From *Thermomonospora Fusca," J. Bacteriol.*, 173:3397–407 (1991), which is hereby incorporated by reference), and phyA gene (1365 bp). After the construct was digested with PstI and KpnI, the resulting fragment was inserted into the expression vector pSES1, and the formed shuttle plasmid (pSPP1, 9131 bp) was transformed into the host *S. lividans* protoplasts according to Hopwood et al. (Hopwood, D. A., et al., *Genetic Manipulation of Streptomyces—A Laboratory Manual*, The John Innes Foundation, Norwich, England (1985), which is hereby incorporated by reference). Likewise, a control was prepared by transforming *S. lividans* with expression vector pSES2.

Three shuttle plasmids with three different signal peptide sequences were constructed to express phyA in the yeast system (See Table 2). The first plasmid was originated from a HindIII digested fragment of pSPp1, including the promoter pLT1, lead sequence Spe2, and the coding region sequence of phyA. The fragment was ligated into the HindIII site of pYES2 treated with calf intestinal alkaline phosphatase and the plasmid was named pYEP1 (7783 bp) after its right orientation was confirmed. The second plasmid contained Spxy, a signal peptide sequence of xylanase gene from *A. pullulans* (Li, X. L. et al., "Cloning, Sequencing, and Regulation of a Xylanase Gene From the Fungus *Aureobasidium Pullulans*) Y-2311-1," *Appl. Environ. Microbiol.*, 60:3160–166 (1994); Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea," Appl. Environ. Microbiol.*, 62:209–13 (1996), which are hereby incorporated by reference), and phyA gene. Spxy was spliced with phyA by overlap extension (Horton, R. M., "In Vitro Recombination and Mutagenesis of DNA: SOEing Together Tailor-Made Genes," *PCR Protocols: Current Methods and Applications,* 251–61 (1993), which is hereby incorporated by reference) with two successive steps of PCR. One was to amplify Spxy sequence from pCE4 (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea," Appl. Environ. Microbiol.*, 62:209–13 (1996), which is hereby incorporated by reference) using upstream primer (5'-CCC AAG CTT GAT CAC ATC CAT TCA-3') (SEQ ID No. 5) with a HindIII restriction site (primer 1) and overlapping downstream primer (5'-CGG GGA CTG CTA GCG CAC GTT CGA T-3', primer 2) (SEQ ID No. 6). The other PCR was to amplify the coding region of phyA from pEP1 using overlapping upstream primer (5'-ATC GAA CGT GCG CTA GCA GCA GTC CCC G-3', primer 3) (SEQ ID No. 7) and downstream primer (5'-GCT CTA GAC TAA GCA AAA CAC TCC-3', primer 4) (SEQ ID No. 8) with a XbaI restriction site. The second step of PCR was conducted to merge the two fragments generated from the above two PCR by using the two purified fragments as the templates and primers 1 and 4. The resulting fragment contained HindIII and XbaI restriction sites and was cloned into pSES2. This plasmid was named pYXP1 (7219 bp). The

third plasmid contained the signal peptide (Sphy) sequence of phyA and the coding region of phyA, excluding the intron between them (Hartingsveldt, W. van., et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87–94 (1993), which is hereby incorporated by reference). Two primers, including a 70 bp of upstream primer contained the signal peptide with an engineered KpnI restriction site and a downstream primer that was the same one used for pYXP1 construction (primer 4) were used to amplify the desired fragment from pEP1. The PCR product was digested with KpnI and XbaI and cloned into pSES2, resulting in a plasmid named pYPP1 (7176 bp). All the above three constructs were transformed into *S. cerevisiae* by the method of Ito et al., "Transformation of Intact Yeast Cells Treated with Alkali Cations," *J. Bacteriol.,* 153:163–68 (1983), which is hereby incorporated by reference.

TABLE 2

Signal peptides used for expression of phyA in *S. cerevisiae*

Construct Size (bp)	Peptide	Gene	Organism	Phytase activity[1] (mPU/ml)
pYEP1 7783	Spe2 (93 bp)	Cellulase E2	*T. fusca*	.80
pYXP1 7219	Spxy (102 bp)	Xylanase A	*A. pullulans*	Non-detectable
pYPP1 7176	Sphy (57 bp)	PhyA Phytase	*A. niger*	146
pSES1[2] 7224			*S. cerevisiae*	Non-detectable

[1]The phytase activity was detected in the supernatant of cell culture of Sabouraud-raffinose medium 15 hours after induced by adding galactose. See text for definition of phytase units.
[2]Expression vector for *S. cerevisiae*, used as a control.

Growth medium and induction of the gene expression. In the *E. coli* system, the transformants were grown in 50 ml of LB medium containing 50 μg/ml of ampicillin at 30° C. After the OD_{600} value of the medium reached 0.5 to 0.6, phytase gene expression was induced by adding IPTG (isopropyl b-D-thiogalactopyranoside) into the medium to a final concentration of 1 mM. Three hours after the induction, cells were collected by spinning down at 8000×g for 15 minutes, washed with 1×PBS, and lysed by lysozyme. Soluble and insoluble fractions of the cells were prepared, and a sample containing 500 μg of total protein (Lowry, O. H. et al., "Protein Measurement With the Folin Phenol Reagent," *J. Biol. Chem.,* 193:265–75 (1951), which is hereby incorporated by reference) was suspended in the same volume of 2×SDS buffer and analyzed by SDS-PAGE (Laemmli, U. K., "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4," *Nature (London)*, 227:680–85 (1970), which is hereby incorporated by reference).

Recombinant *S. lividans* was grown in TSB broth with 5 μg/ml of thiostrepton at 30° C. (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene from *Thermomonospora Fusca," Appl. Environ. Microbiol.,* 59:3032–43 (1993), which is hereby incorporated by reference). After 72 hours incubation, the cells and medium were harvested and prepared for SDS-PAGE (Wilson, D. B., "Biochemistry and Genetics of Actinomycete Cellulases," *Crit. Rev. Biotechnol.,* 12:45–63 (1992), which is hereby incorporated by reference).

Transformants of *S. cerevisiae* were initially grown in Sabouraud-raffinose (4%) medium (100 ml) without uracil for 48 hours, sterile galactose was then added into the medium (2%) to induce phytase expression. Samples of media and cells were collected at various time points, and extracellular and intracellular samples were prepared as described by Li and Ljungdahl, "Expression of *Aureobasidium pullulans* xynA in, and Secretion of the Xylanase from, *Saccharomyces cerevisiae," Appl. Environ. Microbiol.,* 62:209–13 (1996), which is hereby incorporated by reference. When needed, the supernatant of the expressed cell culture fractions was concentrated with Stirred Cells of Amicon (Beverly, Mass.) by using YM10 membranes (MW cutoff of 10,000). Other media were tested accordingly.

Enzyme protein and activity assay. Amounts of expressed phytase protein under various conditions were quantified by the relative densitometry of specific bands in SDS-PAGE, using IS-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, Calif.). Phytase activity in the samples of media and cells was determined as previously described (Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori," Gene,* 133:56–62 (1993), which is hereby incorporated by reference) and the inorganic phosphate released was assayed by the method of Chen, P. S. et al., "Microdetermination of P," *Anal. Chem.,* 28:1756–58 (1956), which is hereby incorporated by reference. One phytase unit (PU) was defined as the amount of enzyme that releases one μmol of inorganic phosphate from sodium phytate per minute at 37° C.

Western blotting (immunoblot) analysis. The soluble fraction of the cell mass of the phytase transformed *E. coli* and the medium supernatant of *S. lividans* and *S. cerevisiae* transformants were collected as for SDS-PAGE. After electrophoresis, the proteins were then transferred onto Protran® nitrocellulose membrane (Schleicher & Schuell, Keene, N.H., USA) in 20 mM Tris-HCl (pH 8.3), 20% methanol, and 0.1% SDS, by using a Mini Trans-Blot cell (Bio-Rad Laboratories). Transfer was done overnight at a constant 50 V and the initial buffer temperature was 4° C. The membranes were then subjected to Western blot analysis. A rabbit polyclonal IgG (Kindly provided by Dr. A. H. J. Ullah of USDA. Dilution, 1: 5,000) against purified native *A. niger* phytase was used as the first antibody. The blotting was finalized using Immuno-Blot Assay Kit (Bio-Rad Laboratories) containing a second antibody conjugated with horseradish peroxidase.

Total RNA isolation and analysis. Total RNA was isolated with TRIzol™ Reagent (GIBCO BRL, Gaithersburg, Md.) from *E. coli* and *S. cerevisiae* transformants 3 and 15 hours after induction, respectively. RNA samples (10 μg per lane) were then separated by formaldehyde agarose (1.5%, wt/vol) gel electrophoresis and transferred to Hyblot membranes (National Labnet, Woodbridge, N.J.) (Davis et al., *Basic Methods in Molecular Biology,* 2nd Ed., Appleton and Lange, Norwalle, Conn. (1994), which is hereby incorporated by reference). A 1.4 kb EcoRI-HindIII fragment in plasmid pEP1 was prepared and was random-primed labeled with ^{32}P using a DNA labeling kit followed by G-50 column purification (Pharmacia Biotech., Piscataway, N.J.) and then hybridized with the blotted RNA membranes in a hybridization oven (Hybaid, Middlesex, UK). The hybridized membranes were exposed to screens in Fuji Imaging Plate and analyzed by Bio-Imaging Analyzer (Kohshin Graphic Systems, Fuji, Japan).

Example 2

Expression of PhyA in *E. coli*

Four hours after the induction, a specific band (~55 kDa) was viewed in SDS-PAGE (12.5%) of the soluble cell

fraction, compared to the only expression vector transformed control (See FIGS. **1** and **2**). This band represented 3.8% of the total soluble protein of this fraction. Correspondingly, northern analysis showed overcxpression of phyA mRNA in these phytase gene transformants and no signal was viewed in the control cells (See FIG. **3**).

In order to optimize phytase protein expression, the time course and the effects of a series of factors on the expression were studied. These factors included incubation temperature (30 and 37° C.), medium pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), anaerobiosis (adding sterile mineral oil on the top of the growing cells), inorganic phosphate level in the medium (Dassa, E. et al., "The Acid Phosphatases with Optimum pH of 2.5 of *Escherichia coli*," *J. Bio. Chem.*, 257:6669–76 (1982), which is hereby incorporated by reference), and sodium phytate (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM). Results indicated that expression of phytase protein was accumulated linearly with time for the first six hours after induction (See FIG. **4**). Thereafter, the expression remained relatively unchanged although bacterial cells continued to grow. Only medium pH and sodium phytate concentration significantly affected the phytase protein expression. Maximum protein was shown at pH 6.0 and 0.3 mM of sodium phytate, in which phytase protein was increased from 3.8 to 9.6% of the total soluble protein.

No phytase activity was detected extracellularly or intracellularly. This may not be completely unexpected, because the native phytase from *A. niger* is a glycoprotein with a size of 70–80 kDa (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," *Gene* 127:87–94 (1993), which is hereby incorporated by reference). The protein expressed in the *E. coli* system of this study had a size of approximately 55 kDa. Presumably, the lack of glycosylation of the protein and other necessary post-translational modifications during secretion would preclude phytase activity.

Example 3

Expression of PhyA in *S. lividans*

Heterologous genes have been expressed in *S. lividans*, and the resulting products secreted into the medium with enzymatic activity (Ghangas, G. S. et al., "Cloning of the *Thermomonospora Fusca* Endoglucanase E2 Gene in *Streptomyces Lividans*: Affinity Purification and Functional Domains of the Cloned Gene Product," *Appl. Environ. Microbiol.*, 54:2521–26 (1988); Wilson, D. B., "Biochemistry and Genctics of Actinomycete Cellulases," *Crit. Rev. Biotechnol.*, 12:45–63 (1992); Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene from *Thermomonospora Fusca*," *Environ. Microbiol.*, 59:3032–43 (1993), which are hereby incorporated by reference). Similarly, phyA gene was expressed in *S. lividans* and the protein was introduced into the medium, as shown in a specific band in the SDS-PAGE analyzed medium samples (See FIGS. **5** and **6**). This suggested that the signal peptide from endoglucanase E2 gene of *T. fusca* was able to lead phytase protein out of the cell. This protein was 57 kDa and represented 16.2% of the total protein in the medium. Changing medium pH to 6.0 and adding 0.3 mM of sodium phytate in the medium improved the protein yield to 20.3% of the total protein. Because phytase protein was secreted into the medium in such a high level, it should be easy to purify and used effectively for a variety of purposes such as producing phytase antibody. Once again, no increased phytase activity was found either in the medium or in the lysed cells. Although the protein size increased a little bit (2–3%) compared to the one expressed in *E. coli*, presumably due to glycosylation of phytase protein in this expression system, there was still no phytase activity.

Example 4

Expression of PhyA in *S. cerevisiae*

Three different signal peptides were used to compare the efficiency in leading the expressed protein out of the cells (See Table 2). The phytase activity was substantially increased in the Sabouraud-raffinose medium growing the transformants of pYEP1 and pYPP1, but not pYXP1. Visible phytase protein was shown by SDS-PAGE 20 hours after induction (FIG. **7**).

The expression of transformants of pYEP1 and pYPP1 were determined in three different types of medium: Sabouraud-raffinose (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea*," *Appl. Environ. Microbiol.*, 62:209–13 (1996), which is hereby incorporated by reference), Sabouraud-glycerol, and a modified general-purposed YEPD medium. As to transformants of pYEP1, similar phytase activity was expressed in the Sabouraud-raffinose and Sabouraud-medium, but there was no activity detected in the YEPD medium. In contrast, phytase activity in the medium cultured with transformants of pYPP1 varied greatly with the different types of medium. The activity was enhanced to 375 mU/ml when Sabouraud-glycerol medium was used. The activity was further increased to 1675 mU/ml, when the medium was changed to YEPD (See Table 3). While the YEPD medium was much cheaper than the Sabouraud-raffinose medium, the phytase yield was increased more than ten-folds. Thus, the putative signal peptide from the fungal phytase gene achieved the most efficient expression of the extracellular phytase activity. Nearly all the protein produced was secreted into the YEPD medium, because very little activity was detected in the yeast cells. The time course of the phytase expression in this system was shown in FIG. **8**.

TABLE 3

Phytase activity expressed from transformant with pYPP1 in different media

	Hours after induction (mPU/ml)[1]		
Medium	0	10	15
Sabouraud-raffinose	22	136	146
Sabouraud-glycerol	6	174	375
YEPD	18	1238	1675

[1]The phytase activity was detected in the supernatant of cell culture of the three media 0, 10, and 15 hours after induced by adding galactose. See text for definition of phytase units.

A variety of microorganisms including bacilli, yeasts, and filamentous fungi have phytase activity, while *A. niger* NRRL3135 strain produces the highest activity (340 mU/ml, Shieh, T. R. et al., "Survey of Microorganisms for the Production of Extracellular Phytase," *Appl. Environ. Microbiol.*, 16:1348–51 (1968), which is hereby incorporated by reference). *Schwanniomyces castellii* CBS 2863 has the highest phytase activity among 21 yeast strains (140 mU/ml, Lambrechts, C. et al., "Utilization of Phytate by Some Yeasts," *Biotechnology Letters*, 14:61–6 (1992), which is hereby incorporated by reference). Clearly, the recombinant yeast strain transformed with pYPP1 in the present study produced much higher phytase activity (1675 mU/ml) than *A. niger* (4-fold) and *S. castellii* CBS 2863 (11-fold). Maximum phytase production can be obtained in the system by optimizing the incubation conditions and modifying the plasmid cassettes (Demolder, J. W. et al.,

"Efficient Synthesis of Secreted Murine Interleukin-2 by *Saccharomyces Cerevisiae*: Influence of 3'-Untranslated Regions and Codon Usage," *Gene,* 111:207–13 (1992), which is hereby incorporated by reference).

The high level of phytase activity expression in *S. cerevisiae* was most likely due to the sufficient glycosylation of phytase protein and other post-translational modifications by yeast. After the medium supernatant was concentrated and subjected to SDS-PAGE analysis, there was a band with approximately 110 kDa (See FIGS. **7** and **9**), which was larger than the size of the native protein from *A. niger* (Hartingsveldt, W. van. et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87–94 (1993), which is hereby incorporated by reference). Northern analysis confirmed the specific overexpression of phyA mRNA (See FIG. **10**). These results indicated that the yeast system was efficient to overexpress actively extracellular phytase enzyme. Yeast system has several advantages over bacteria or other systems such as *A. niger* (Hartingsveldt, W. van. et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87–94 (1993), which is hereby incorporated by reference). It carries out post-translational modifications, including proper folding, glycosylation, disulfide bond formation, and proteolysis, during the translocation of proteins through the endoplasmic reticulum and the cell membrane. The secretion of proteins is facilitated by hydrophobic short signal peptides at the N-terminal regions of the protein precursors (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea," Appl. Environ. Microbiol.,* 62:209–13 (1996), which is hereby incorporated by reference). Proteins secreted by yeast cells are protected from aggregation and protease degradation. Most importantly, enzyme proteins produced by *S. cerevisiae* are easily purified, because it secretes only a few proteins. Considering the well-known safety of yeast products to both human beings and animals, this system is of great potential for human food and animal feed industry.

Example 5

Properties of the PhyA Phytase Overexpressed in *Saccharoiiyces cerevisiae*

The overexpressed phytase from transfon-ants of pYPP1 plasmid was concentrated and used to study its property (See Table 4). The enzyme showed two optimum pH ranges: 2 to 2.5 and 5.0 to 5.5. However, enzyme activity at pH 2 to 2.5 was only 60% of the activity at pH 5 to 5.5. There was no activity detected at either pH 1 or 8. The optimum pH was virtually the same as the phytase from *A. niger* (Simons et al., "Improvement of Phosphorus Availability by Microbial Phytase in Broilers and Pigs," *Br. J. Nutr.,* 64:525 (1990), which is hereby incorporated by reference), thus active function in hydrolysis of phytate-P in the gastrointestinal tracts would certainly be expected. The optimum temperature of the enzyme was 60° C., while the current one on the market produced by Gist-Brocades is 55° C. (BASF, 1996). More than 80% of the activity remained at 50 to 55° C., but little activity was detected at 75 or 80° C. Heating the enzyme for 15 min at 37 and 80° C., the remaining activity for the expressed yeast phytase of the present invention was 100 and 63%, respectively, and for BASF Gist-Brocades phytase was 100 and 52%, respectively. The differences between the two enzyme sources at any given temperature were significant (See Table 5). Thus, the yeast phytase appeared to be more heat stable than the current commercial phytase product.

TABLE 4

Characteristics of the overexpressed phytase in yeast[1]

Optimum pH[2]									
pH	1.0	2.0	2.5	3.0	4.0	5.0	5.5	6.0	8.0
Relative Activity (%)	.5^{e} ± .2	59.7^{c} ± 3	64.8^{c} ± 6	48.1^{d} ± 4	81.0^{b} ± 5	100.0^{a} ± 1	95.0^{a} ± 6	66.3^{c} ± 1	.8^{e} ± .4
Optimum Temperature[3]									
° C.	25	37	45	50	55	60	75	80	
Relative activity (%)	24.2^{e} ± .8	44.6^{d} ± 3	63.9^{c} ± 8	83.6^{b} ± 2	89.8^{b} ± 4	100.0^{a} ± 4	.6^{f} ± .1	.9^{f} ± .2	

[1]Data are means of relative activity ± standard deviation (n = 4). Means in a row with different superscript letters differ (P < 0.05). The general linear model of the statistical analysis system (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multiple treatment mean comparison. Significance level was set as P < 0.05.
[2]The activity was assayed at 37° C. (see context for phytase unit definition). Different buffers were used: 0.2 mM glycine-HCI buffer for pH 1.0 to 3.5; 0.2 mM sodium citrate buffer for pH 4.0 to 6.5; and 0.2 mM Tris-HCI buffer for pH over 7.
[3]Optimum temperature was determined at pH 5.5 (0.2 mM sodium citrate buffer).

TABLE 5

Comparison of the thermostability of overexpressed phytase in yeast and Gist-Brocades phytase produced by *A. niger*[1,2]

Relative activity, %	37° C.	80° C.
Yeast phytase	100^{a} ± 1	63^{b} ± 1
A. niger phytase	100^{x} ± 3	52^{y} ± 2
P[3] <		.03

[1]Data are means of relative activity ± standard deviation (n = 3). Means in a row with the different superscript letters differ (P < 0.05). The general linear model of the statistical analysis system (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multiple treatment mean comparison. Significance level was set as P < 0.05.
[2]The enzyme was heated for 15 minutes at different temperatures before reacting at 37° C. and pH 5.5.
[3]Significance (P values) of t-test between the activity of the two phytases at each temperature setting.

Although it is unclear how such improvement in thermostability is related to different post-translational modifications (folding, cleavage, glycosylation, etc.), (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea," Appl. Environ. Microbiol.*, 62:209–13 (1996), which is hereby incorporated by reference), it is certainly advantageous to have more thermostable phytase enzyme that can hopefully be resistant to the heat during feed pelleting, which is a problem with the current Gist-Brocades phytase.

Example 6

In Vitro Hydrolysis of Phytate-P from Corn, Soy, and Wheat Middlings by the Expressed Yeast Phytase

The expressed yeast phytase released phytate-P from corn and soybean meal as effectively as the Gist-Brocades phytase based on per unit activity (See Table 6). As expected, the hydrolysis of phytate-P was a function of time and activity dosage. The expressed yeast phytase was also effective in releasing phytate-P from wheat middling, indicating its great potential in bread fermentation. Because the wheat middling used in this study contained much higher intrinsic phytase activity than commonly used wheat flour, much greater effect of the expressed yeast phytase on improving flour phytate-P hydrolysis and in trace clement releasing would be expected, when it is used in a bakery (Hall, M. N. et al., "The Early Days of Yeast Genetics," *Cold Spring Harbor Laboratory Press* (1993), which is hereby incorporated by reference).

TABLE 6

	Free phosphorus released from corn, soybean meal (SBM), and wheat middlings by overexpressed yeast phytase and fungus *A. niger* phytase in vitro[1]					
Yeast phytase (PU/kg)	0	100	250	500	1000	250 (fungus phytase)
	Free phosphorus (mg/g)					
Corn:						
1 hour	$.23^{d} \pm .03$	$.64^{c} \pm .08$	$1.14^{b} \pm .18$	$1.46^{a} \pm .04$	$1.54^{a} \pm .04$	$1.16^{b} \pm .15$
4 hour	$.36^{c} \pm .02$	$1.26^{b} \pm .04$	$1.60^{a} \pm .03$	$1.66^{a} \pm .06$	$1.72^{a} \pm .04$	$1.68^{a} \pm .04$
SBM:						
1 hour	$.68^{d} \pm .01$	$1.18^{cd} \pm .02$	$1.62^{c} \pm .18$	$2.48^{b} \pm .32$	$3.13^{a} \pm .19$	$1.68^{c} \pm .2$
4 hour	$.73^{d}$	1.67^{c}	2.69^{b}	3.41^{a}	3.71^{a}	2.78^{b}
Wheat Middlings:						
1 hour	$3.56 \pm .39$		$4.11 \pm .64$	$4.67^{2} \pm .05$		
4 hour	$5.63 \pm .5$		$6.02 \pm .48$	$6.38^{2} \pm .07$		

[1]Each sample of 5 g was stirred in 20 ml of 0.2 mM sodium citrate buffer at 37° C. for 1 or 4 hours. The supernatant was obtained by spinning for 15 minutes at 8000 g. After going through Whatman 541 filter paper, the sample was subjected to free P assay by the method of Chen, P.S. et al., "Microdetermination of P," Anal. Chem., 28:1756–58 (1956), which is hereby incorporated by reference.Data in the table are means of relative activity ± standard deviation (n = 4). The General Linear Model of the Statistical Analysis System (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multiple treatment mean comparison. Significance level was set as $P < 0.05$. A significant difference existed between 1 and 4 hour for every feed at each doseof enzyme as analyzed by t-test. Means in a row with different superscript letters differ ($P < 0.05$).
[2]n = 2

The overexpression of *Aspergillus niger* phytase (phyA) in *Escherichia coli, Streptoinyces lividans*, and *Saccharomyces cerevisiae* were compared to develop an efficient and simple system to produce phytase economically. A 55 kDa soluble intracellular protein, representing 9.6% of the total soluble protein, was expressed in *E. coli* by using pET25b (+) system. A 57 kDa extracellular protein, representing 20.3% of the total protein in the medium, was expressed in *S. lividans* by using a shuttle plasmid containing the pLT1 promoter and SpeII leading peptide of endoglucanase E2. No increase in phytase activity was shown in either expression system, presumably due to the lack of glycosylation and other necessary post-translational modification. In contrast, high extracellular phytase activity was produced in *S. cerevisiae* transformed with phyA gene. Three different signal peptides and three different types of medium were compared to identify the best expression vector and condition. Use of the signal peptide Sphy from phyA gene and YEPD medium produced the highest extracellular phytase activity. The overexpressed phytase in yeast was approximately 110 kDa, had two pH optima: 2.0 to 2.5 and 5.5 to 6.0, and the optimum temperature was at 60° C.

Example 7

Methods and Materials for Expression of phyA in *Pichia*

Host and vector. An EasySelect™ *Pichia* Expression Kit was purchased from Invitrogen (San Diego, Calif.). The kit provides hosts and vectors to express the gene either intracellularly or extracellularly, in strains of either Mut^{+} or Mut^{s} (Methanol utilization normal or slow). X33 was used as a Mut^{+} strain and KM71 as a Mut^{s} strain. Two vectors were used, pPICZ B (3.3 kb) and pPICZαA (3.6 kb), both use AOX1 as the promoter.

Construction of the Expressing Vectors. To compare the effect of different signal peptides on the expression of PhyA in *Pichia* system, two constructs were prepared. First, a 1.4

kb EcoRI-KpnI fragment, containing the PhyA sequence encoding the mature phytase protein, was ligated into pPICZαA. In this plasmid (pPICZα-phyA), PhyA was led by an alpha-factor, a very general-used signal peptide from *Saccharomyces cerevisiae*. Second, a 1.4 kb KpnI-XbaI fragment of pYPP1 was ligated into the vector (the coding region of phyA was led by its own signal peptide that was very effective in secreting the expressed phytase in *Saccharomyces cerevisiae*.)

Transformation and expression. The confirmed constructs were linearized by PmeI and transformed into GS115 and KM71, by EasyComp™ provided by the kit. Neocin™ was used to select the positive colonies. After a single colony was inoculated into 10 ml of MGY medium and grown to OD_{600} of 2–6 at 30° C., the cells were collected by centrifugation and resuspended into 10 ml of MMY medium (containing 0.5% of methanol). The samples were collected every 12 or 24 h after induction, The cells were separated from the supernatant and lysed with glass beads in breaking buffer. Phytase activity in the supernatant and cells was assayed as described previously. SDS-PAGE and Western blot were conducted to determine the size and relative amount of the expressed protein.

Example 8

PhyA Phytase Activity in *Pichia*

The expression construct using alpha-factor as the signal peptide for phyA was transformed into two *Pichia* strains. KM71 is a methanol utilization slow strain, while X33 is a *Pichia* wild-type utilizing methanol efficiently. The screening and incubation were conducted in 10 ml shake flasks under 29–30° C. For the transformants of KM71, 19 out of 20 picked colonies had extracellular phytase activity greater than 6 units/ml of culture supernatant after induction for 24 hours. Colony No. 13 showed the highest activity of 26 units/ml after incubated for 108 hours. For the transformants of X33, all colonies (20/20) had more than 10 units/ml after induced for 24 hours. One of the colonies (#101) produced phytase activity of 65 units/ml of supernatant. A time course study of the phytase expression in KM71 and X33 was summarized in FIG. **11**. Despite the difference of these two strains in utilizing methanol and, therefore, the ability in expressing phytase, it was found that alpha-factor was correctly processed by yeast cells. Besides, almost all of the expressed protein was secreted into the medium since not more than 5% of the total activity expressed was found intracellularly.

Effects of inorganic phosphorus and pH of media on the phytase expression were studied in the media (BMGY and BMMY) using a phyA recombinant of X33 (#101). The medium containing 50 mM phosphate produced the highest phytase activity, 66 units/ml at 168 hours after induction. By including 50 mM phosphatc in the media, the effect of different pH of this buffer (3, 4, 5, 6, 7, and 8) on expression was also studied. When the pH was 6, this X33 transformant produced 75 units phytase/ml supernatant. Based on the protein concentration and SDS-PAGE analysis, the expression phytase protein yield was estimated to be between 3 to 4 mg/ml.

Example 9

Properties of the PhyA Phytase Expressed in *Pichia*

Molecular size and deglycosylation of the expressed phytase. After the supernatant of the medium inoculated with the phyA transformant was subjected to SDS-PAGE, a strong band around 95 kDa was seen (FIG. **12**). This was almost the only viewed protein in the supernatant. The expressed phytase reacted efficiently with the rabbit polyclonal antibody raised against purified native *A. niger* phytase. This indicated that the immunoreactivity of the expressed phytase was essentially the same as that of the native phytase from *A. niger*. The size was decreased to 50 kDa by deglycosylation using Endo H. The phyA antibody also reacted with the deglycosylated phytase. In addition, deglycosylation, conducted under native conditions, reduced the phytase activity about 15%, indicating that glycosylation was important for the activity of the phytases. Moreover, glycosylation affected the thermostability of the enzymes (FIG. **13**).

Northern analysis. As showed in FIG. **14**, a 1.3 kb phyA DNA probe hybridized with the mRNA of the induced transformants from both KM71 (#13) and X33 (#101). Response was also seen from the transformants prior to induction. Probably, the expression of phyA in this system was not controlled strictly at the level of transcription.

Optimal pH and temperature and phytate-phosphorus hydrolysis. Similar to *A. niger* phytase, the expressed phytase had two optimum pH, 2.5 and 5.5 (FIG. **15**). The optimum temperature of the expressed phytase was 60° C. (FIG. **16**). When the expressed phytase was incubated with soy samples at 100, 200, 400, 800 mU/g of sample at 37° C., phosphorus was released in a linear fashion with the phytase dose (FIG. **17**).

Example 10

Methods and Materials for Overexpression of *E. coli* appA Gene in *Saccharontyces cerevisiae*

Gene and Protein. This gene, originally defined as *E. coli* periplasmic phosphoanhydride phosphohydrolase (appA) gene, contains 1,298 nucleotides (GeneBank accession number: M58708). The gene was first found to code for an acid phosphatase protein of optimal pH of 2.5 (EcAP) in *E. coli*. The acid phosphatase is a monomer with a molecular mass of 44,644 daltons. Mature EcAP contains 410 amino acids (Dassa, J. et al., "The Complete Nucleotide Sequence of the *Escherichia coli* Gene appA Reveals Significant Homology Between pH 2.5 Acid Phosphatase and Glucose-1-Phosphatase," *J. Bacteriology,* 172:5497–5500 (1990), which is hereby incorporated by reference). Ostanin, K. et al. ("Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.,* 267:22830–36 (1992), which is hereby incorporated by reference), overexpressed appA in *E. coli* BL21 using a pT7 vector and increased its acid phosphatase activity by approximately 400-fold (440 mU/mg protein).

The gene and a host *E. coli* strain CU 1869 (No. 47092) were purchased from ATCC. The gene, an insert of 1.3 kb, was transformed into *E. coli* strain BL21 (no. 87441) using an expression vector pAPPA1 (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.,* 267:22830–36 (1992), which is hereby incorporated by reference).

Host and Vector. The vector for overexpressing appA gene in *Saccharomyces cerevisiae* was pYES2 and the host was INVScI (Invitrogen, San Diego, Calif.).

Construction of the Expression Vector. Initially, a 1.3 kb XbaI fragment was isolated from pAPPA1. This fragment contained the appA gene with its own signal peptide. After

being ligated into the XbaI site of pYES2, the construct (PYES2-appA) was transformed into *Saccharomyces cerevisiae*. But, no phytase activity was increased in either extra- or intra-cellular parts compared to the controls. pAPPA1 and pYPP1 (PhyA and its signal peptide in pYES2) were cotransformed into the yeast strain. Again, no increase in phytase activity due to pAPPA1 was detected in the media or the yeast cells.

Two primers were synthesized to construct the signal peptide of PhyA gene with the coding region of appA gene. One was 80 bp long containing the PhyA signal peptide and a KpnI site at 5' end: GGG GTA CCA TGG GCG TCT CTG CTG TTC TAC TTC CTT TGT ATC TCC TGT CTG GAG TCA CCT CCG GAC AGA GTG AGC CGG AG (SEQ ID No. 9). The other primer was 24 bp long, with an EcoRI site at its 3' end: GGG AAT TCA TTA CAA ACT GCA GGC (SEQ ID No. 10). The PCR was run for 25 cycles, with 1 min denaturing at 95° C., 1 min annealing at 58° C., and 1 min chain extending at 72° C. A 1.3 kb fragment was amplified, digested, and ligated into pYES2. After the insert was confirmed by restriction mapping, the construct (pYES2-SphyA-appA) was transformed into INVScI by lithium acetate method.

Expression. The selected transformants were inoculated into YEPD medium. The expression was induced by adding galactose into the culture after OD_{600} reached 2, as described previously. The cells were harvested 15 or 20 h after induction.

Activity Assay. Acid phosphatase activity was assayed at 37° C. in 25 mM glycine-HCl buffer (pH 2.5), using p-nitrophenyl phosphate as the substrate (stock 250 mM). Reaction buffer of 1.7 ml was added into 0.1ml samples. After they were incubated for 5 min in a 37° C. waterbath, 0.2 ml of prewarmed substrate was added and mixed. The reaction solution was transferred into a prewarmed cuvette and incubated for 2 min in a 37° C. spectrophotometric compartment. The released p-nitrophenol was read continuously for 5 min at 405 nm for enzyme activity calculation.

In vitro study. Soybean meal (5.0 g) was suspended into 20 ml of 20 mM citrate buffer, pH 5.5, mixed with 200 mU of phytase, incubated at 37° C. for 4 h with continuous shaking. After chilling on ice for 10 min, the slurry was transferred into a centrifuge tube and spun for 15 min at 15,000×g. The supernatant was used to determine free phosphorus.

Example 11

Quantitation of Phytase Activity from Overexpression of *E. coli* appA Gene in *Saccharonmyces cerevisiae*

The intracellular acid phosphatase activity in the appA overexpressed *E. coli* (pAPPA1) was 440 mU/mg protein. Unprecedently, an intracellular phytase activity greater than 4900 mU/mg protein was found in the transformed strain. But, there was only minimal phytase activity in the control (BL21). Thus, this acid phosphatase gene also codes for a phytase. The appA gene sequence was aligned with that of PhyA and found that these two genes shared 23% of identity.

Transforming INVScI with the construct of pYES2-Sphy-appA (led by the signal peptide of PhyA) produced extracellular phytase activity in the supernatant that was 2,000-fold greater than those of the wild type or of the transformant containing appA gene plus its own signal peptide (See Table 7).

TABLE 7

Extracellular phytase activity in transformants of appA gene with different signal peptides

Construct	Signal	Activity (mU/ml)	Activity (mU/mg protein)
PYES-appA	appA	Undetectable	Undetectable
pYES2-SphyA-appA	PhyA	1,158	445

The effects of medium (YEPD) inorganic phosphorus, phytate, pH, and temperature on the expression of phytase activity by pYES2-Sphy-A-appA are presented in Table 8. The highest phytase activity was 2,286 mU/ml (633 mU/mg protein) at the optimal condition.

TABLE 8

Effect of different conditions in the YEPD medium on phytase activity expression of pYES2-SphyA-appA in yeast.

Medium Conditions	Activity (mU/ml)
Phosphorus, mg/100 ml	
0	1402
1	714
5	722
10	456
Sodium phytate, g/100 ml	
0	870
0.1	1019
1.0	1748
pH	
5.0	892
7.0	996
8.0	2286
Temperature, ° C.	
25	312
30	1036
37	996

The thermostability of the overexpressed extracellular phytase activity produced by the yeast transformant was greater than that of the intracellular phytase produced by *E. coli* transformed with pAPPA1 (See Table 9). Heating the extracellular phytase for 15 min at 80° C. resulted in 30% of loss of its phytase activity, while almost all the phytase activity from *E. coli* was lost under the same condition.

TABLE 9

Effect of heating different sources of phytases under 80° C. for 15 min on their activities

Phytase	Relative activity after heating, %
appA in *E. coli*	0.1
appA in *S. cerevisiae*	69
PhyA in *S. cerevisiae*	66
BASF phytase	50

Comparisons of the effect on releasing phosphorus from soybean meal by phytases (200 mU) of *E. coli*, overexpressed AppA in yeast, and BASF are presented in Table 10. The results indicate that all three sources of phytases released phytate-phosphorus effectively from soybean meal.

TABLE 10

Free phosphorus released from soybean meal by different sources of phytases

Phytase	Phosphorus (mg/g)
appA in *E. coli*	1.11
appA in *S. cerevisiae*	0.69
BASF	0.87

E. coli appA (acid phosphatase) gene when expressed in *Sacchacromyces cerevisiae* produces extracellular phytase activity in the media that was more than 2,000-fold greater than the control. The overexpressed phytase effectively releases phytate-phosphorus from soybean meal, and seems to be more thermostable than the presently available commercial phytase or the intracellular phytase produced in *E. coli* by the same gene (appA).

Example 12

Methods and Materials for Overexpressing the *E. coli* appA Gene Encoding an Acid Phosphatase/ Phytase in *Pichia pastoris*

Gene and Protein. The appA gene and the host *E. coli* strain CU1867 (No. 47092) were obtained from ATCC. The gene, an insert of 1.3 kb, was transformed into *E. coli* strain BL21 (No. 87441) using an expression vector pAPPA1 (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.*, 267:22830–36 (1992), which is hereby incorporated by reference).

Host and Vector. An EasySelect™ *Pichia* Expression Kit was obtained from Invitrogen (San Diego, Calif.). The kit provides hosts and vectors to express the gene either intracellularly or extracellularly in a wild-type strain (X-33). Two vectors were used, pPICZ B (3.3 kb) and pPICZαA (3.6 kb), both use AOX1 as the promoter.

Construction of the Expression Vector. Two primers were used to amplify the appA gene from pAPPA1 and two restriction sites EcoRi and KpnI were produced at the 5' and 3' ends, respectively.

```
Upstream primer:    GGA ATT CCA GAG TGA GCC GGA   (SEQ ID No. 11)

Downstream primer:  GGG GTA CCT TAC AAA CTG CAC G (SEQ ID No. 12)
```

Template: pAPPA1 DNA isolated from ATCC 87441

PCR was run for 30 cycles, with 1 min denaturing at 94° C., 1 min annealing at 55° C., and 1 min chain extending at 72° C. A 1,245 base-pair fragment was amplified, digested with EcoRI and KpnI, and ligated (16° C. overnight) into pPICZ B (3.3 kb) and pPICZαA (3.6 kb). The ligation was confirmed by restriction mapping after transforming the constructs into DH5α.

Transformation of the construct into *Pichia* (X33). For each transformation, 100 μg of plasmid DNA was prepared and linearized by digesting with PmeI. After linearization, the DNA was purified and resuspended into 10 μL of sterile, deionized water. Half amount of the DNA was actually used for each transformation. Electroporation and the EasyComp chemical kit (Invitrogen) were both used to transform the DNA into X33. In the case of electroporation, an Electro Cell Manipulator (ECM 600, Gentromics, BTX Instrument Division, San Diego, Calif. 92121) and 2 mm cuvettes were used. The resistance was 186 Ohm, the charging voltage was 1.5 kilovolts, and the actual charging length was approximately 7 milliseconds. The electroporated cells were incubated on YPD agar plates containing 100 mg Zeocin/mL at 30° C. for 2–4 days for colony growth. In the case of chemical transformation, cells were grown on YPDS agar plates containing 100 mg Zeocin/mL. Compared with the electroporation, the chemical method had lower transformation efficiency.

Expression. Single colonies were inoculated into 10 ml of MGY medium (30 ml tube) and grown (16–18 h) to OD_{600} of 5–6 at 28–30° C. in a shaking incubator (200 rpm). The cells were collected by centrifugation (2,000 rpm) and resuspended into 10 ml of BMMY medium (containing 0.5% of methanol) to induce the expression. The samples (200 μL) were collected every 12 or 24 h after induction. Methanol (100%) was added at 100 μL every 24 to maintain a concentration of 0.5–1% in the media.

Assays. The cells were separated from the media (supernatant) and lysed with glass beads in breaking buffer. Extracellular phytase activity in the supernatant and intracellular phytase activity in the lysed cells were assayed as described previously (0.2 M citrate buffer, pH 5.5 under 37° C. using 10 mM sodium phytate). Acid phosphatase activity was assayed at 37° C. in 25 mM glycine-HCl buffer (pH 2.5), using p-nitrophenyl phosphate as the substrate (stock 250 mM). Reaction buffer of 1.7 ml was added into 0.1 ml samples. The released p-nitrophenol was read continuously for 5 min at 405 nm for enzyme activity calculation. SDS-PAGE (12%) was conducted to determine the size and relative amount of the expressed protein. The optimal pH and temperature of the expressed phytase were determined as described in the results.

In vitro study. Soybean meal (5.0 g) was suspended into 20 ml of 20 mM citrate buffer, pH 5.5, mixed with different levels of phytase, and incubated at 37° C. for 4 h with continuous shaking. After being chilled on ice for 10 min, the slurry was transferred into a centrifuge tube and spun for 15 min at 15,000×g. The supernatant was used to determine free phosphorus.

Example 13

Colony Phytase Activity Screening for *Phicia pastoris* Overexpressing the *E coli* appA Gene

Wild-type *Pichia* X33 produces minimal phytase activity intracellularly (<0.03 U/mg protein) or extracellularly (<0.05 U/mL). The X33 cells transformed with the appA gene inserted into pPICZB (without the α-factor and presumably produces intracellular phytase) did not show any increase in phytase activity (extracellular, 0.2 U/mL and intracellular, 0.05 U/mg protein).

Transforming X33 cells with the construct of pPIZαA-appA (led by the signal peptide of α-factor) produced extracellular phytase activity in the media. Initially, 72 colonies were screened. Only two colonies had activity <1 U/mL 40 hours after induction. Most of the colonies had activity ranging from 10 to 20 U/mL 40 hours after induction. All of the 70 colonies had phytase activity >80 U/mL 118 hours after induction. The highest phytase activity so far detected was 215 U/mL, 192 hours after the induction (See Table 11).

TABLE 11

Range of extracellular phytase activity in X33 colonies transformed with pPIZαA-appA 40 and 118 hours after induction.

Number of Colonies	40 hours after induction	118 hours after induction
2	<1 U/mL	
6	1 to 10 U/mL	
36	11 to 20 U/mL	
28	>20 U/mL	
70		>80 U/mL

Phytase and acid phosphatase activities in the transformant expressing 215U phytase activity /mL were compared with those of the wild-type of X33 (192 hours after induction) (See Table 12). Almost all of the expressed phytase protein was secreted from the cells, indicating that α-factor was a very effective signal peptide for phytase secretion.

TABLE 12

Phytase and acid phosphatase activities in the pPIZαA-appA transformant and the wild-type of X33 192 hours after induction.

	Wild-type X33		pPIZαA-appA transformant	
	Extracellular U/mL	Intracellular U/mg protein	Extracellular U/mL	Intracellular U/mg protein
Phytase	0.05	0.03	215	0.5
Acid phosphatase	0.01	0.002	5.88	0.9

Transformants of *E. coli* with the same acid phosphatase appA gene had intracellular phytase activity of 5 U/mg protein (Ostanin et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.,* 267:22830–36 (1992), which is hereby incorporated by reference). Transforming PhyA gene in *A. niger* produced an extracellular activity of 7.6 U/ml (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87–94 (1993), which is hereby incorporated by reference). Compared with these results, the phytase expression system in *Pichia* is a very efficient expression system.

Example 14

Time-Course of Phytase Expression

There was a linear increase in extracellular phytase activity in the media almost in all of the selected colonies up to 192 hours after induction. FIG. **18** summarized the activity changes of five selected colonies from 24 to 163 hours after induction.

Example 15

Effects of Medium pH on the Expression of Phytase (Colony #23, Activity 136 U/mL at 186 h)

Using 0.1 M phosphate buffered media, the effects of different pH on the production of extracellular phytase in the transformants were studied against a control medium without buffer (pH 7.0). The medium buffered to pH 6 produced the highest phytase activity (See FIG. **19**).

Example 16

Size of the Expressed Extracellular Phytase

Using SDS-PAGE (12% gel) analysis, a clear band was noticed in the medium supernatant of culture inoculated with three different colonies (See FIG. **20**). The size was around 55 kDa, probably partially glycosylated. Because the expressed protein represented almost the only visible band in the supernatant, it would be convenient to collect the enzyme product without the need for a tedious purification.

Example 17

Optimum pH and Temperature of the Expressed Extracellular Phytase (Colony #23)

The optimum pH of the expressed phytase was 2.5 to 3.5 (See FIG. **21**). This is significantly different from that of phyA phytase either from *A. niger* (BASF) or our other expression systems. It is ideal for phytase function at the stomach pH.

The optimum temperature of the expressed enzyme was 60° C. (See FIG. **22**).

Example 18

Effect of the Expressed Phytase on Phytate-Phosphorus Hydrolysis from Soybean Meal

This overexpressed *E. coli* phytase (Colony #23) effectively hydrolyzed phytate-phosphorus from soybean meal (See FIG. **23**). The release of free phosphorus in the mixture was linear from 0 to 800 mU of phytase/g of feed.

Example 19

Effects of the Expressed *E. coli* AppA Phytase by *Pichia pastoris* on Phytate Phosphorus Bioavailability to Weanling Pigs

To determine the nutritional values of the expressed *E. coli* phytase by *Pichia* in swine diets, the efficacy of this new phytase was compared with those of inorganic phosphorus or the commercially available microbial phytase (Natuphos™, BASF Corp., Mt. Olive, N.J.). Forty-eight weanling pigs were selected from multiparous sows at Cornell Swine Research Farm. The pigs were weaned at 21 days of age and fed a commercial creep feed until day 28. They were then placed two per pen with six pens assigned randomly per treatment. The pigs were given two weeks to adjust to the corn-soybean meal basal diet (Table 13).

TABLE 13

Formulation of the Experiment Diets for Pigs.

Ingredient	+C % diet	−C % diet	YP % diet	MP % diet
Corn	60.5	61.57	61.07	61.07
Whey Protein Concentrate	3	3	3	3
SBM 44%	30	30	30	30
ComOil	3	3	3	3
Lime	0.8	0.93	0.93	0.93
Di-calcium phosphate	1.2	0	0	0
Vitamin and Mineral premix	0.5	0.5	0.5	0.5
ECAP premix	0	0	0.5	0
MP premix	0	0	0	0.5
Salt	0.5	0.5	0.5	0.5
CSP 250	0.5	0.5	0.5	0.5
Total	100	100	100	100
CP	20.6	20.6	20.6	20.6

TABLE 13-continued

Formulation of the Experiment Diets for Pigs.

Ingredient	+C % diet	−C % diet	YP % diet	MP % diet
Ca	0.73	0.47	0.47	0.47
P_{total}	0.6	0.39	0.39	0.39

Note:
All premixes use corn as the carrier
Vitamin and Mineral Premix supplies: 2,540 IU Vit. A, 660 IU Vit. D, 15 IU Vit. E, 2.2 mg Vit. K, 3.3 mg Riboflavin, 13.2 mg Pantothenic acid, 17.6 mg Niacin, 110.1 mg Choline, 1.98 ug B-12, 37.4 mg Mn, 0.6 mg I, 10 mg Cu, 0.3 mg Se, 100 mg Zn, and 100 mg Fe per Kg of diet

Then, each pen received one of the four treatment diets. The positive control group (+C) received the basal diet supplemented with dicalcium phosphate. The negative control group (−C) received just the basal diet. The yeast phytase group (YP) received the basal diet supplemented with the expressed *E. coli* phytase at 1,200 U/kg of feed. The microbial phytase group (MP) received the basal diet supplemented with the BASF phytase at 1,200 U/kg of feed. Pigs were given free access to feed and water. Body weight gain of individual pigs was recorded weekly. Daily feed intake of individual pens was recorded daily. Blood samples from each of the individual pigs were taken weekly to assay plasma inorganic phosphorus concentrations. The results of body weight (BW), average daily gain (ADG), average feed intake (ADFI), and feed/gain ratio (F:G), and plasma inorganic phosphorus (PP) are presented in Table 14.

TABLE 14

Summary of PP, BW, ADG, ADFI, and F:G of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
Initial				
PP	12.99	13.02	13.07	13.54
BW	11.54	11.63	12	11.5
Week 1				
PP	10.83^{A}	6.48^{C}	8.59^{B}	8.35^{B}
BW	14	13.83	14.29	13.92
ADG	.351	.316	.327	.345
ADFI	.700	.684	.697	.697
F:G	2.04	2.20	2.18	2.13
Week 2				
PP	9.76^{A}	5.64^{D}	8.72^{B}	7.84^{C}
BW	18.04	17.42	17.83	17.71
ADG	.578	.512	.506	.542
ADFI	.833	.855	.784	.837
F:G	1.46^{B}	1.67^{A}	1.56^{AB}	1.55^{AB}
Week 3				
PP	11^{A}	6.26^{C}	8.64^{B}	8.13^{B}
BW	22.58	21.17	22	22.21
ADG	$.649^{A}$	$.536^{B}$	$.595^{AB}$	$.643^{AB}$
ADFI	1.166	1.02	1.001	1.003
F:G	1.8	1.92	1.71	1.36
Week 4				
PP	10.94^{A}	6.31^{C}	9.65^{B}	9.2
BW	27.54	25.29	27.79	27.38
ADG	$.708^{AB}$	$.589^{B}$	$.827^{A}$	$.738^{AB}$
ADFI	1.395^{A}	1.049^{B}	1.309^{A}	1.273^{AB}
F:G	1.98	1.87	1.59	1.73

[1]Numbers in the same row without sharing a common letter are significantly different.
Analysis of difference was conducted with the Bonferroni (Dunn) T-tests with alpha = 0.05 and df = 20

In addition, there was severe phosphorus deficiency in the negative control group in the end of the four-week experiment. But, there was no sign of phosphorus deficiency in the other three groups. Clearly, the expressed *E. coli* phytase by *Pichia* was at least, if not more, effective as the commercial microbial phytase in improving bioavailability of phytate-phosphorus from the corn-soybean meal diets for weanling pigs. It can be used to replace inorganic phosphorus supplementation to weanling pigs.

Example 20

Effects of the Expressed *E. coli* AppA Phytase by *Piclia pastoris* on Iron (Fe) and Phytate Phosphorus Bioavailability to Weanling Pigs

To determnine the effect of the overexpressed *E. coli* phytase by *Pichia* on dietary phytate—bound Fe bioavailability to weanling pigs, 20 anemic pigs (21 days old and 7.3 g hemoglobin (Hb)/dL blood) were selected. The pigs were fed an Fe-deficient creep feed for 7 days and housed in metabolic cages at the age of 28 days old. The pigs were then fed the experimental diets at the age of 35 days old for 5 weeks. The treatment diets were as follows: Fe-deficient basal diet (−C, with added inorganic phosphorus), Fe-supplemented diet (+C), the Fe- and phosphorus-deficient diet supplemented with the expressed *E. coli* phytase (YP), or the commercial microbial phytase (BASF, MP) at 1,200 U/kg of feed. Body weight (BW), packed cell volume (PCV), Hb, and plasma inorganic phosphorus (PP) were determined weekly. The results are presented in Table 15.

TABLE 15

Summary of PCV, Hb, BW, and PP of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
Initial				
PCV	25	25	26	24
Hb	7.73	7.22	7.85	7.08
BW	8.14	8.27	8.17	7.45
PP	7.92	7.76	7.21	7.36
Week 1				
PCV	25	26	29	27
Hb	7.62	8.3	8.77	7.88
BW	9.44	8.84	9.63	8.57
PP	8.41	8.45	8.48	8.22
Week 2				
PCV	29	26	30	28
Hb	8.6	7.34	8.93	8.27
BW	12.32	10.13	11.91	10.84
PP	10.28^{a}	9.05^{ab}	8.89^{ab}	8.22^{a}

TABLE 15-continued

Summary of PCV, Hb, BW, and PP of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
Week 3				
PCV	36[a]	29[b]	34[a]	33[a]
Hb	11.55[a]	8.2[b]	10.84[a]	9.96[ab]
BW	16.77[a]	13[b]	15.62[ab]	14.62[ab]
PP	12.14[a]	11.37[ab]	10.25[bc]	9.71[c]
Week 4				
PCV	39	34	38	36
Hb	12.99[a]	10.11[b]	12.27[a]	11.35[ab]
BW	21.36[a]	17.37[b]	19.44[ab]	18.56[ab]
PP	10.19[a]	9.34[ab]	9.49[ab]	8.8[b]
Week 5				
PCV	40	38	40	39
Hb	13.52[a]	12.24[b]	13.64[a]	13.13[ab]

TABLE 15-continued

Summary of PCV, Hb, BW, and PP of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
BW	26.53	22.59	24.27	23.43
PP	9.27[a]	8.95[ab]	8.79[ab]	8.02[n]

[1]Values are means (n = 5). Means within the same row without sharing a common superscript ltter are significantly different (P < 0.10).

In conclusion, the overexpressed *E. coli* phytase by *Pichia* was at least as effective as the BASF phytase in improving phytate-phosphorus and Fe utilization in corn-soy diets for weanling pigs.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these therefore are considered within the scope of the invention as defined in the claims which follow.

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What is claimed:

1. A method of producing phytase in yeast comprising:

providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase;

expressing the protein or polypeptide in a yeast; and

isolating the expressed protein or polypeptide, wherein said protein or polypeptide catalyzes the release of phosphate from phytate and has increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell.

2. The method according to claim **1**, wherein the heterologous polynucleotide is an isolated appA polynucleotide.

3. The method according to claim **1**, wherein the yeast is selected from the group consisting of *Saccharomyces* species *Pichia* species, *Kluyveromyces* species, *Hansenula* species, *Candida* species, *Torulaspora* species, and *Schizosaccharomyces* species.

4. The method according to claim **3**, wherein the yeast is *Pichia*.

5. The method according to claim **1**, wherein the protein or polypeptide has an optimal phytase activity at a pH of less than about 4.

6. The method according to claim **1**, wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted.

7. The method according to claim **6**, wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.

8. The method according to claim **1**, wherein the heterologous polynucleotide which encodes a protein or polypeptide with phytase activity is spliced in frame with a transcriptional enhancer element.

9. The method according to claim **1**, wherein the heterologous polynucleotide is carried on a vector for stable transformation.

10. The method according to claim **1**, wherein the heterologous polynucloetide is carried on an artifical chromosome.

11. The method according to claim **1**, wherein the heterologous polynucleotide is integrated into a chromosome of the yeast.

12. A yeast strain comprising:

a heterologous polynucleotide from a non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase and is functionally linked to a promoter, wherein said protein or polypeptide catalyzes the release of phosphate from phylate and has increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell.

13. The yeast strain according to claim **12**, wherein the heterologous polynucleotide is an isolated appA polynucleotide.

14. The yeast strain according to claim **12**, wherein the yeast is selected from the group consisting of *Saccharomyces* species, *Pichia* species, *Kluyveromyces* species, *Hansenula* species, *candida* species, *Torulaspora* species, and *Schizosaccharomyces* species.

15. The yeast strain according to claim **14**, wherein the yeast is *Pichia*.

16. The yeast strain according to claim **12**, wherein the heterologous polynucleotide which encodes a protein or polypeptide with phytase activity is spliced in frame with a transcriptional enhancer element.

17. The yeast strain according to claim **12**, wherein the heterologous polynucleotide is carried on a vector for stable transformation.

18. The yeast strain according to claim **12**, wherein the heterologous polynucleotide is carried on an artifical chromosome.

19. The yeast strain according to claim **12**, wherein the heterologous polynucleotide is integrated into a chromosome of the yeast.

20. The yeast srain according to claim **12**, wherein the protein or polypeptide is preceded by a signal peptide.

21. A vector comprising:

a polynucleotide from a non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase, wherein said protein or polypeptide catalyzes the release of phosphate from phytate and has increased thermostability when expressed in a yeast host cell compared to a non-yeast host cell;

a promoter functionally linked to the polynucleotide encoding the protein or polypeptide; and

an origin of replication to direct replication of the vector in yeast.

22. The vector according to claim **21**, further comprising:

a selectable marker.

23. The vector according to claim **22**, wherein the selectable marker is selected from the group consisting of URA3, LEU2, TRP1, HIS3, ARG4, and an antibiotic resistance gene.

24. The vector according to claim **21**, further comprising:

an origin of replication to direct replication of the vector in a bacterial cell.

25. The vector according to claim **24**, wherein the origin of replication is selected from the group consisting of ColE1, Ori, and oriT.

26. The vector according to claim **21**, wherein the protein or polypeptide is preceded by a signal peptide.

27. The vector according to claim **21**, wherein the polynucleotide which encodes a protein or polypeptide is spliced in frame with a transcriptional enhancer element.

28. A method of producing a protein or polypeptide having phytase activity comprising:

providing an isolated appA polynucleotide, which encodes a protein or polypeptide with phytase activity;

expressing said polynucleotide in a yeast host cell under conditions effective to produce the protein or polypeptide having phytase activity; and

isolating the expressed protein or polypeptide.

29. The method according to claim **28**, wherein the yeast host cell is selected from the group consisting of *Saccaromyces* species, *Pichia* species, *Kluyveromyces* species, *Hansenula* species, *Candida* species, *Torulaspora* species, and *Schizosaccharomyces* species.

30. The method according to claim **29**, wherein the yeast host cell is *Pichia*.

31. The method according to claim **28**, wherein the protein or polypeptide, preceded by a signal peptide, is secreted by the cell into a growth medium or is not secreted.

32. The method according to claim **31**, wherein the protein or polypeptide is secreted by the yeast host cell into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.

33. The method according to claim **28**, wherein the appA polynucleotide is spliced in frame witha transcriptional enhancer element.

34. The method according to claim **28**, wherein the appA polynucleotide is carried on a vector for stable transformation.

35. The method according to claim **28**, wherein the appA polynucleotide is carried on an artifical chromosome.

36. The method according to claim **28**, wherein the appA polynucleotide is integrated into a chromosome of the yeast host cell.

37. The method according to claim **1**, wherein the heterologous polynucleotide is an isolated phyA polynucleotide.

38. The yeast strain according to claim **12**, wherein the heterologous polynucleotide is an isolated phyA polynucleotide.

39. The vector according to claim **21**, wherein the polynucleotide is an isolated appA polynucleotide.

40. The vector according to claim **21**, wherein the polynucleotide is an isolated phyA polynucleotide.

* * * * *

EXHIBIT 2

US007312063B2

(12) **United States Patent**
Lei

(10) **Patent No.: US 7,312,063 B2**
(45) **Date of Patent: Dec. 25, 2007**

(54) **OVEREXPRESSION OF PHYTASE GENES IN YEAST SYSTEMS**

(75) Inventor: **Xingen Lei**, Ithaca, NY (US)

(73) Assignee: **Cornell Research Foundation, Inc.**, Ithaca, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **11/372,851**

(22) Filed: **Mar. 10, 2006**

(65) **Prior Publication Data**

US 2006/0153902 A1 Jul. 13, 2006

Related U.S. Application Data

(63) Continuation of application No. 10/094,693, filed on Mar. 8, 2002, now Pat. No. 7,026,150, which is a continuation-in-part of application No. 09/104,769, filed on Jun. 25, 1998, now Pat. No. 6,451,572.

(51) **Int. Cl.**

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C12N 1/20	(2006.01)
C12N 1/14	(2006.01)
C12N 15/00	(2006.01)
C07H 21/04	(2006.01)

(52) **U.S. Cl.** **435/196**; 435/252.3; 435/320.1; 435/254.2; 435/254.21; 435/254.1; 435/254.23; 435/254.3; 536/23.2; 536/23.4; 536/23.7

(58) **Field of Classification Search** 435/196, 435/252.3, 320.1, 254.1, 254.2, 254.21, 254.23, 435/254.3; 536/23.2, 23.4, 23.7
See application file for complete search history.

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Primary Examiner—Tekchand Saidha
(74) *Attorney, Agent, or Firm*—Nixon Peabody LLP

(57) **ABSTRACT**

The present invention relates to a method of producing a heterologous protein or polypeptide having phytase activity in a yeast system. The invention also provides proteins having phytase activity which have increased thermostability. Yeast strains which produce a heterologous phytase and the vectors used to produce the phytase are also provided.

27 Claims, 23 Drawing Sheets

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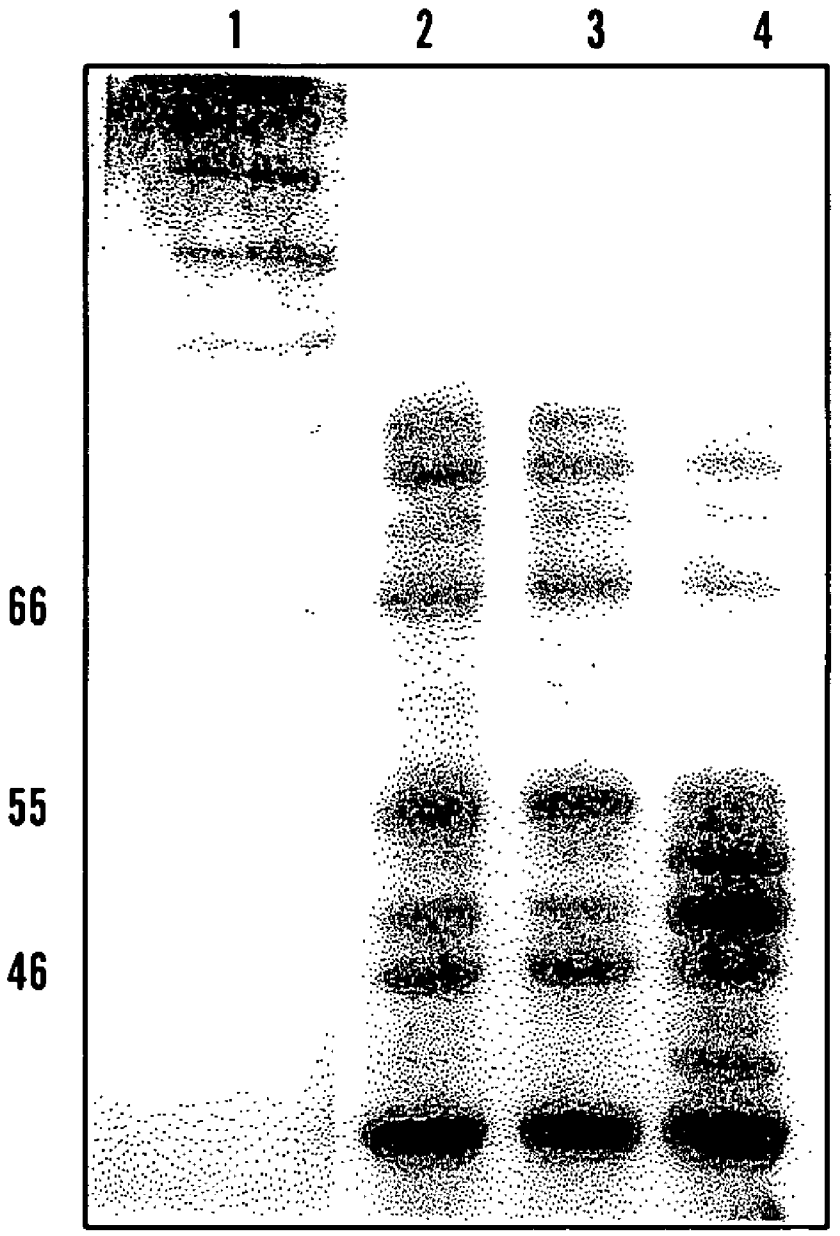

FIG. 1

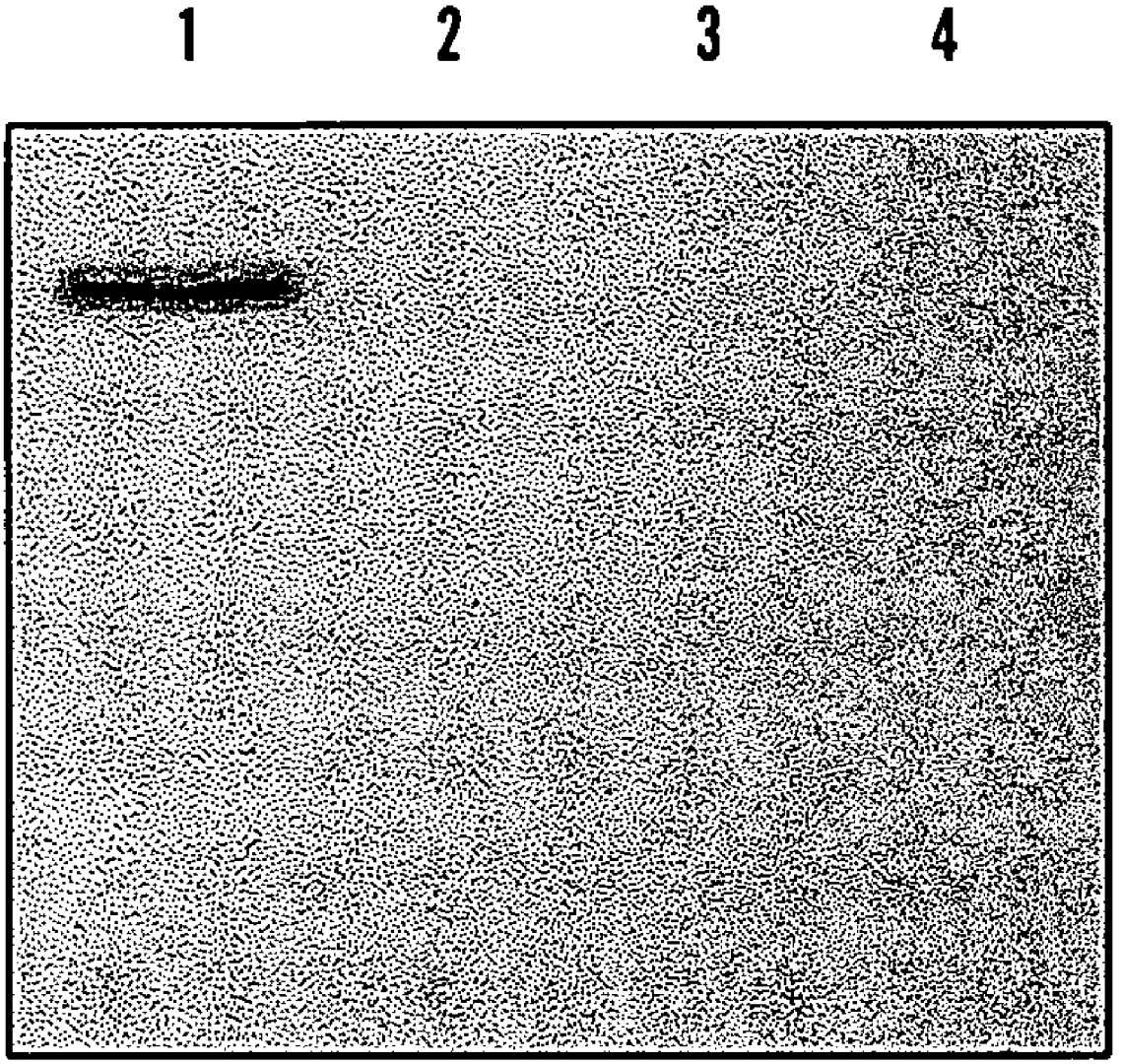

FIG. 2

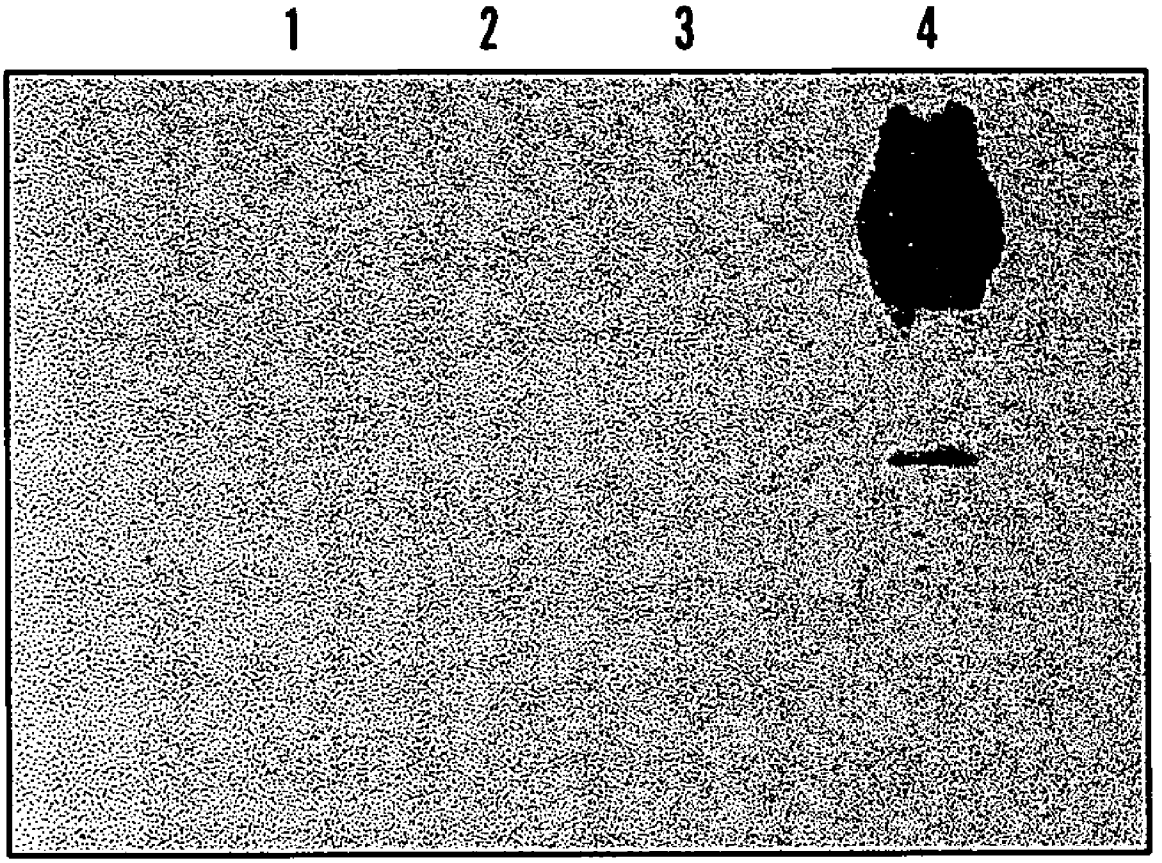

FIG. 3

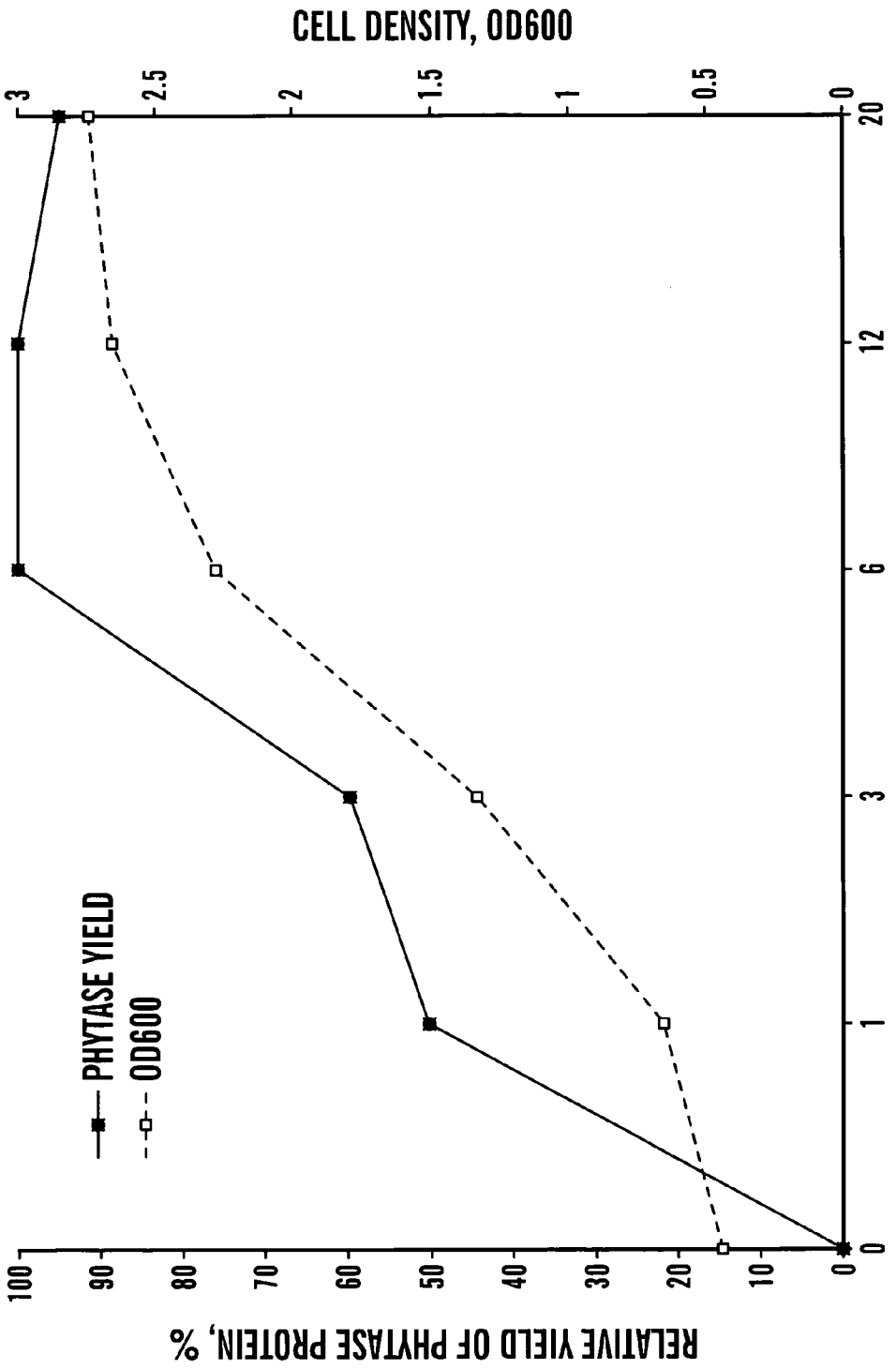
CELL DENSITY, OD600
3
2.5
2
1.5
1
0.5
0
20
12
6
3
1
0
HOURS AFTER INDUCTION
PHYTASE YIELD
OD600
100
90
80
70
60
50
40
30
20
10
0
RELATIVE YIELD OF PHYTASE PROTEIN, %

FIG. 4

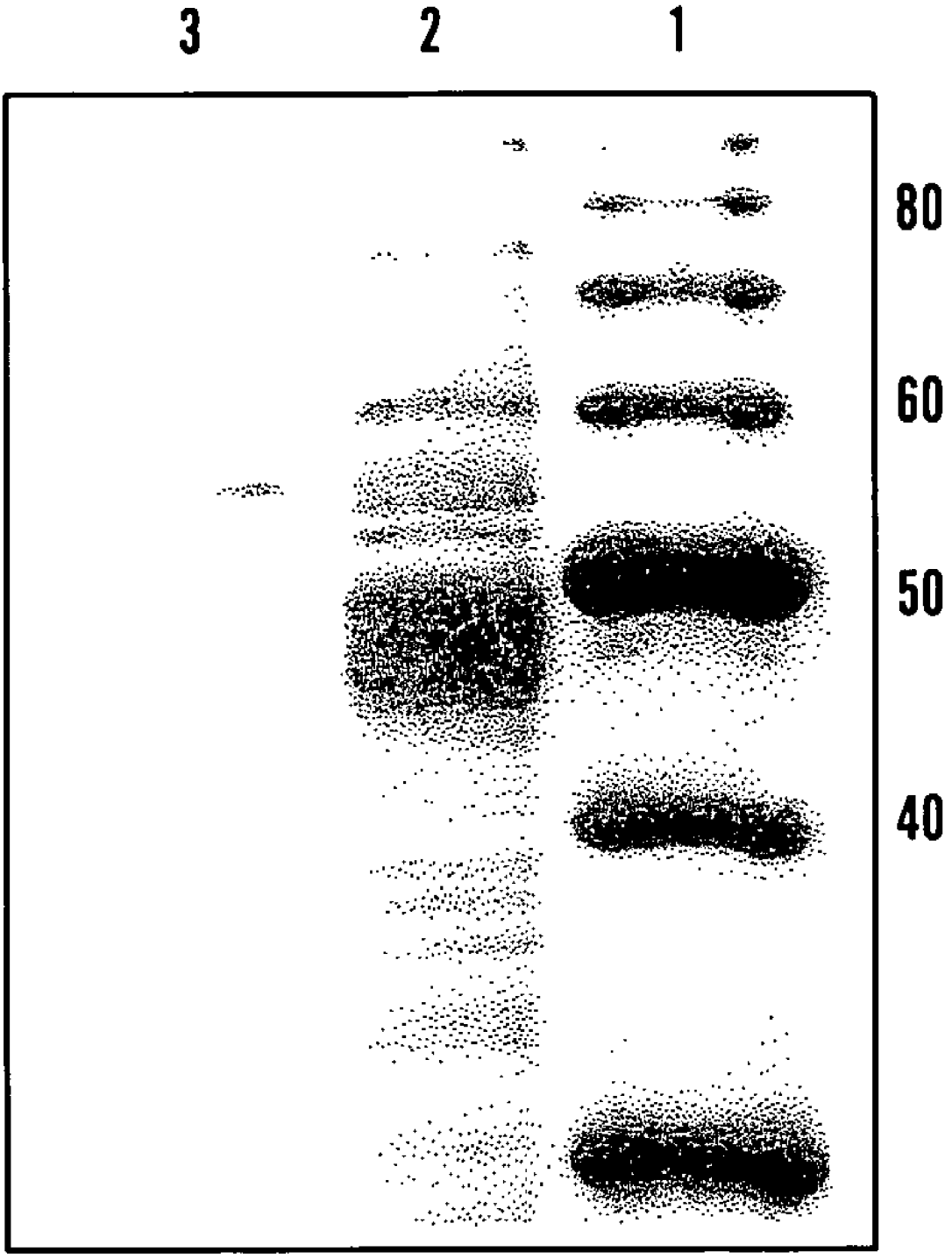

FIG. 5

1 2

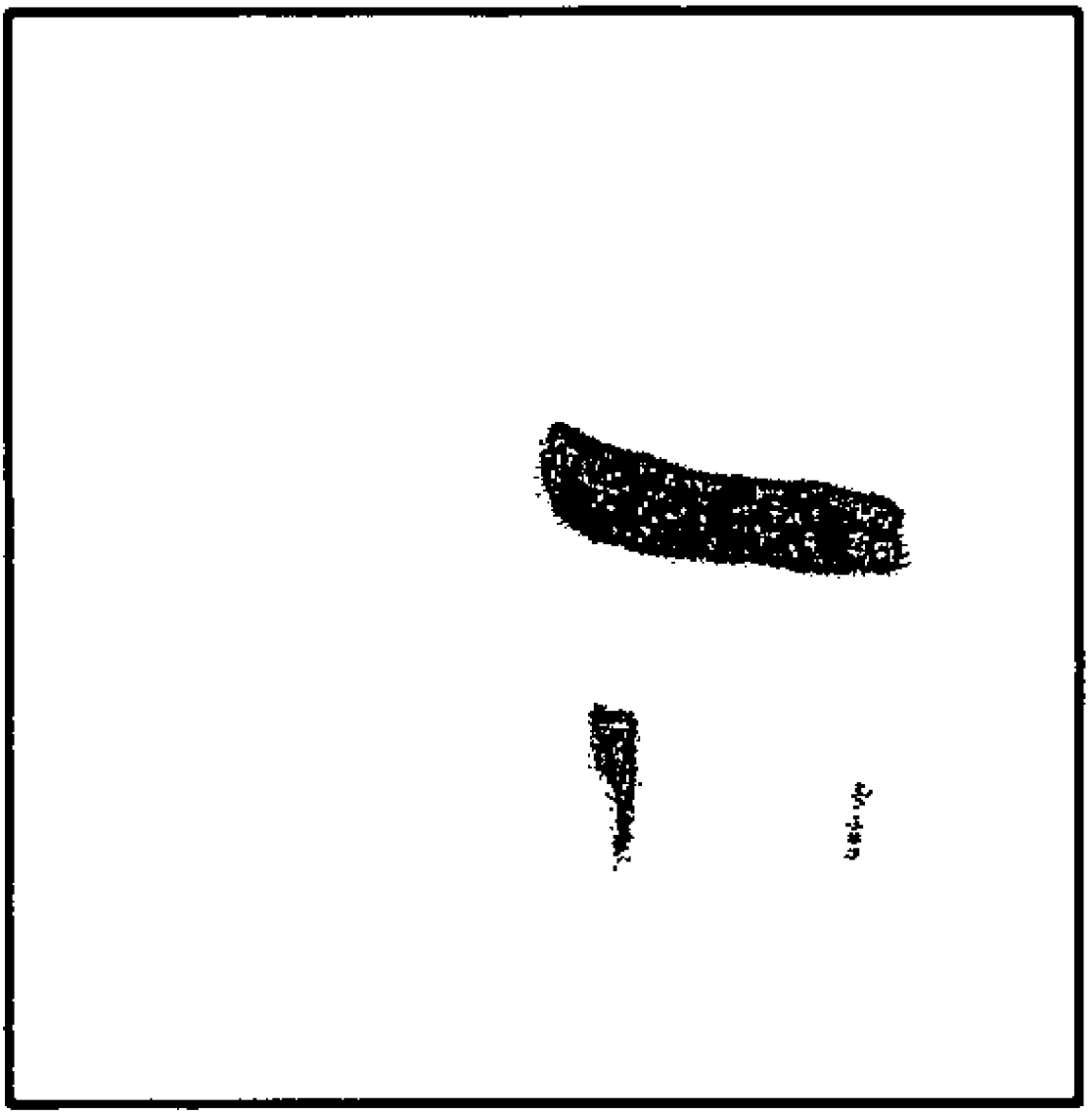

FIG. 6

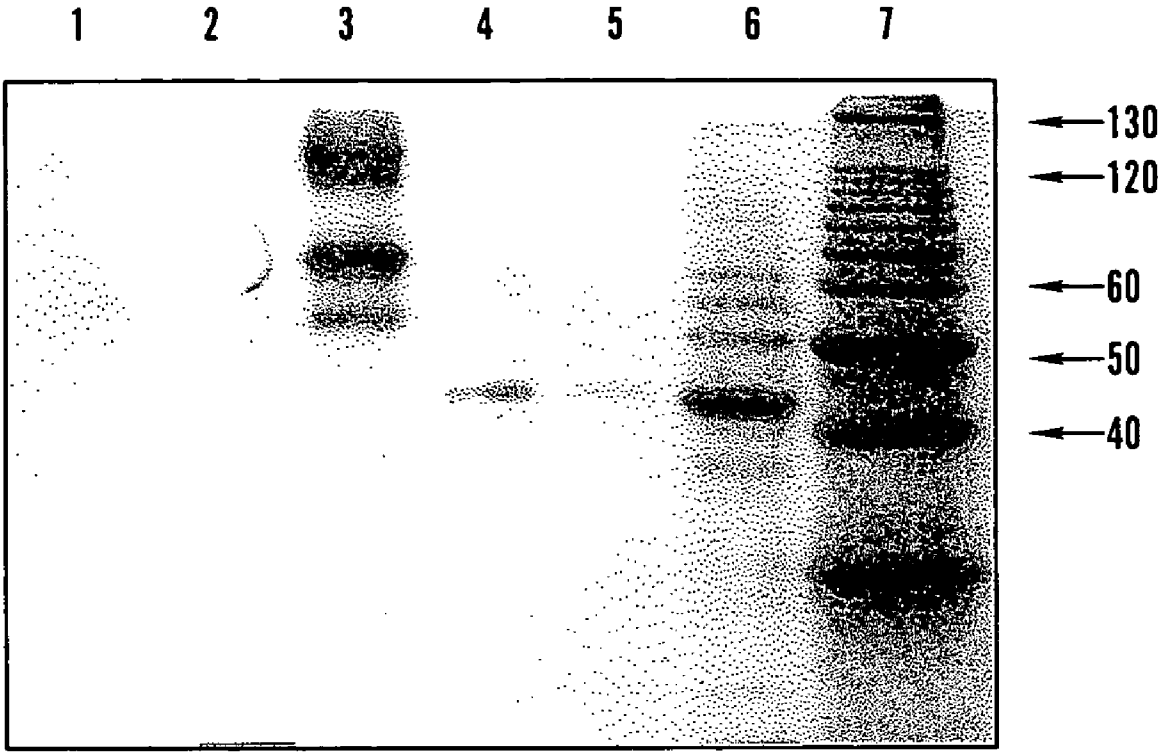

FIG. 7

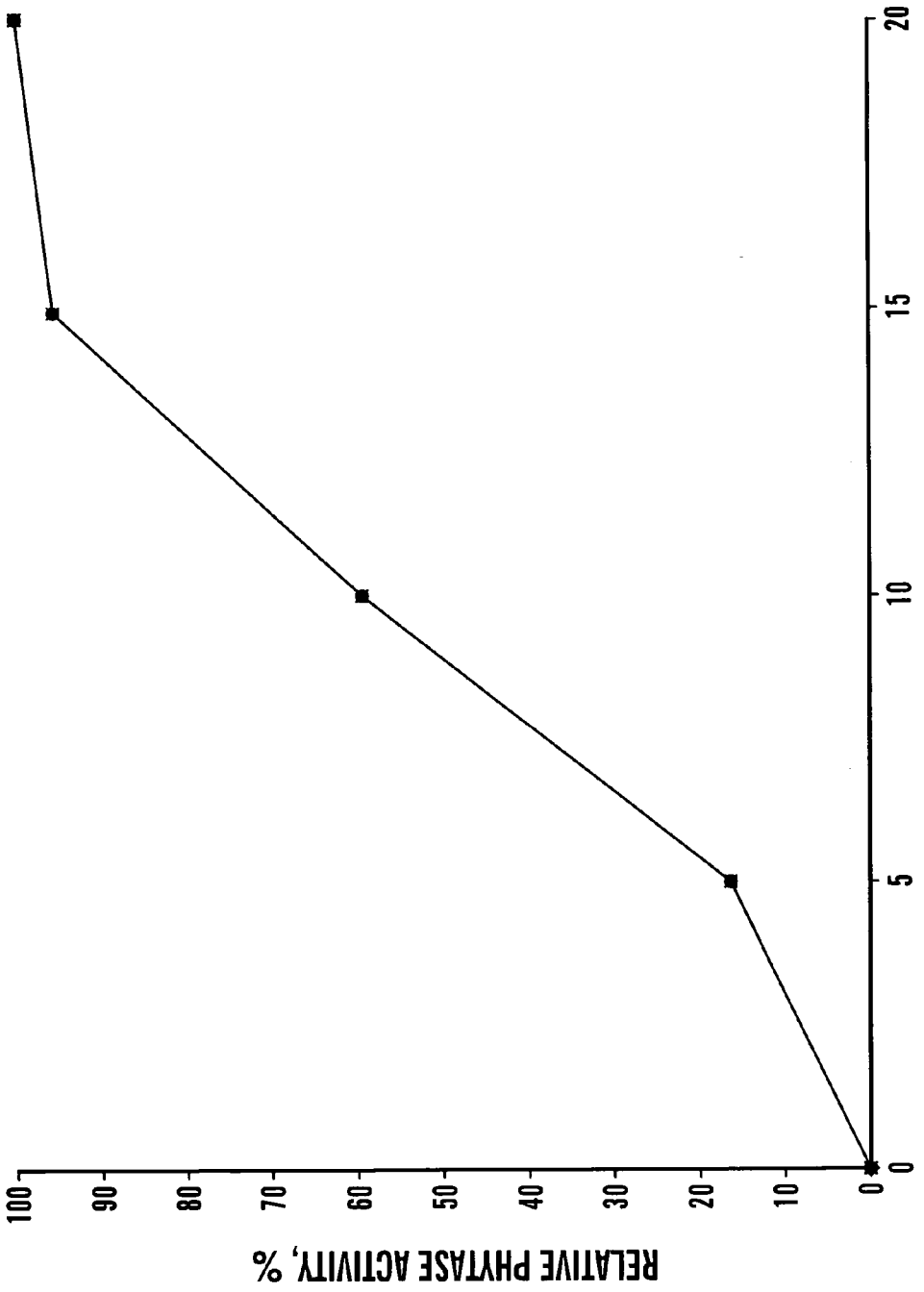
RELATIVE PHYTASE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
HOURS AFTER INDUCTION

FIG. 8

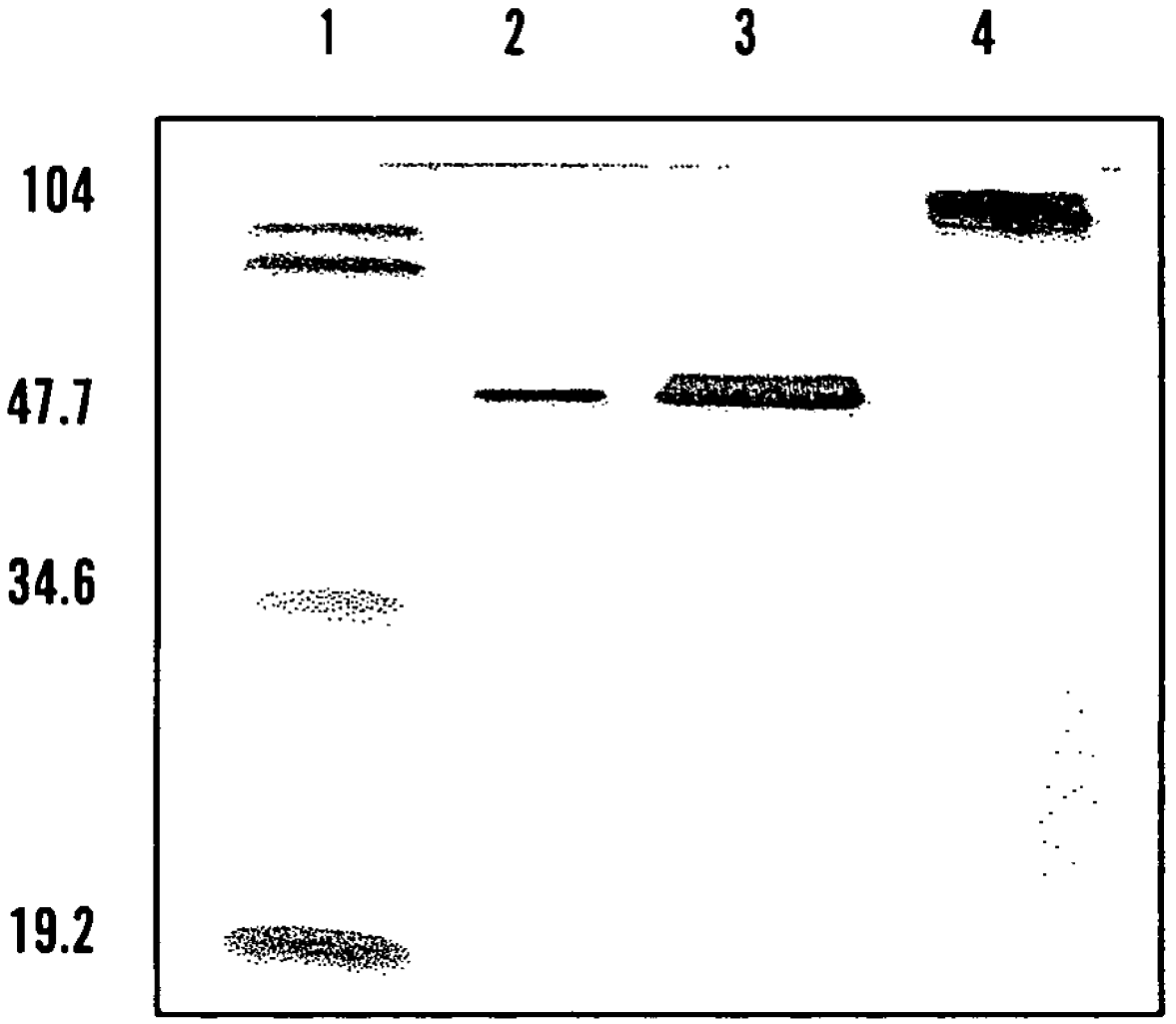

FIG. 9

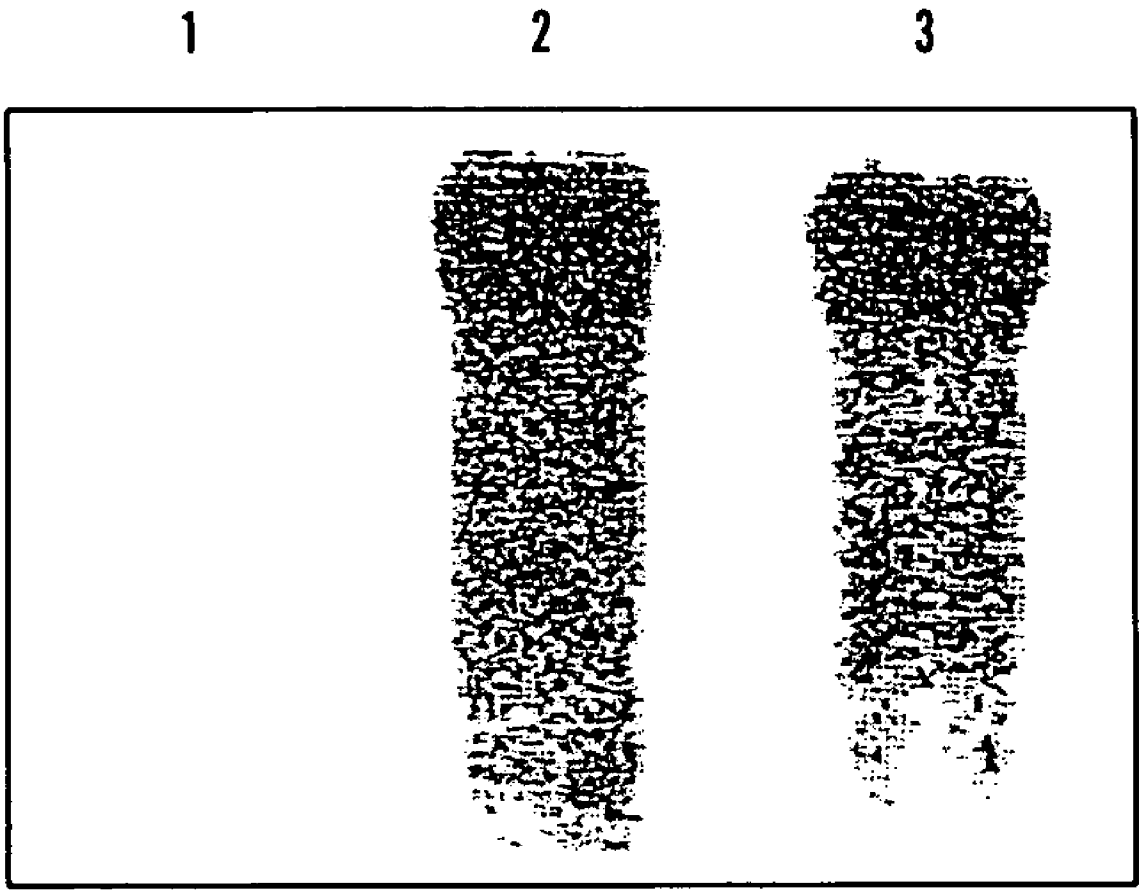

FIG. 10

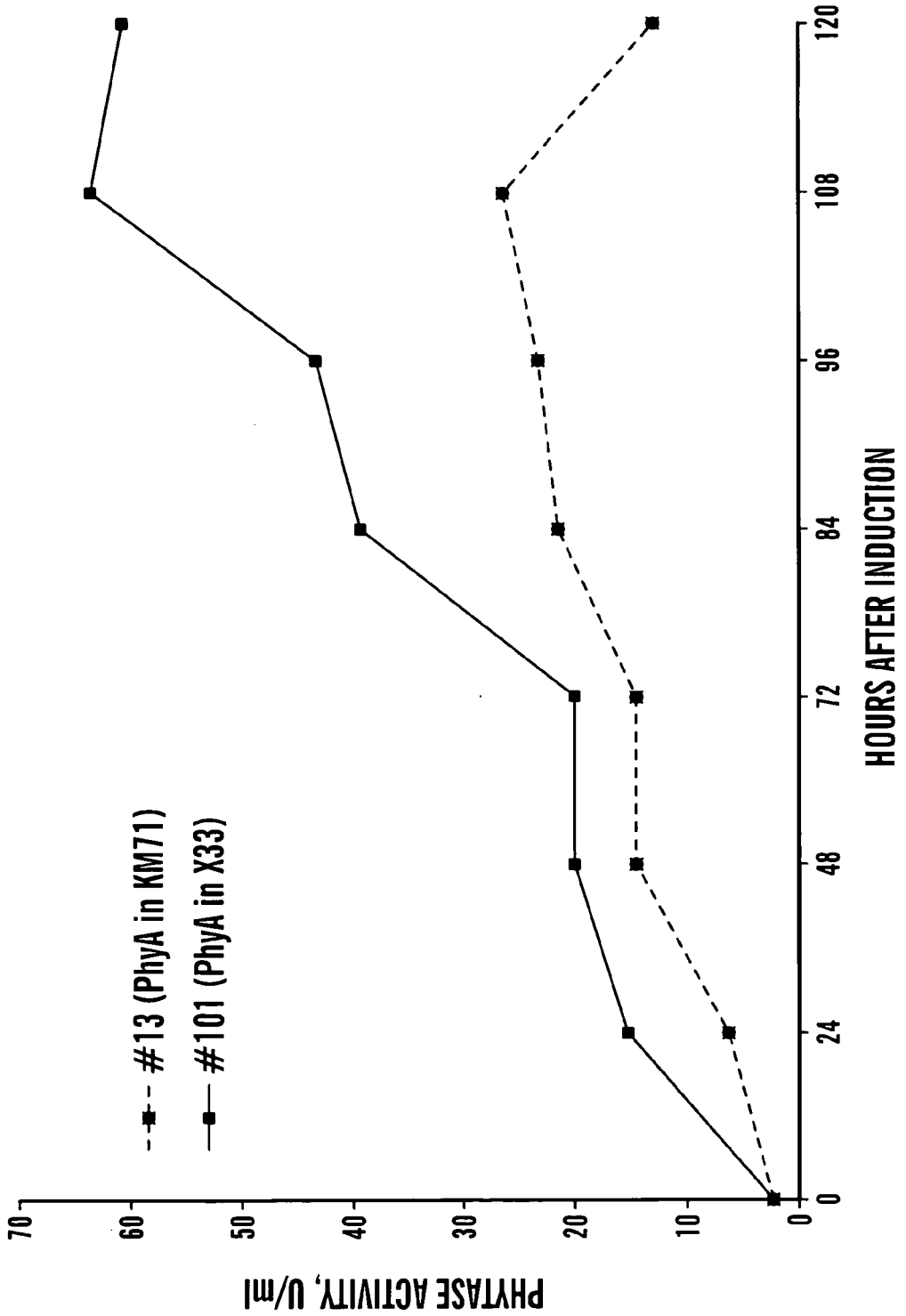
PHYTASE ACTIVITY, U/ml
70
60
50
40
30
20
10
0
0
24
48
72
84
96
108
120
HOURS AFTER INDUCTION
#13 (PhyA in KM71)
#101 (PhyA in X33)

FIG. 11

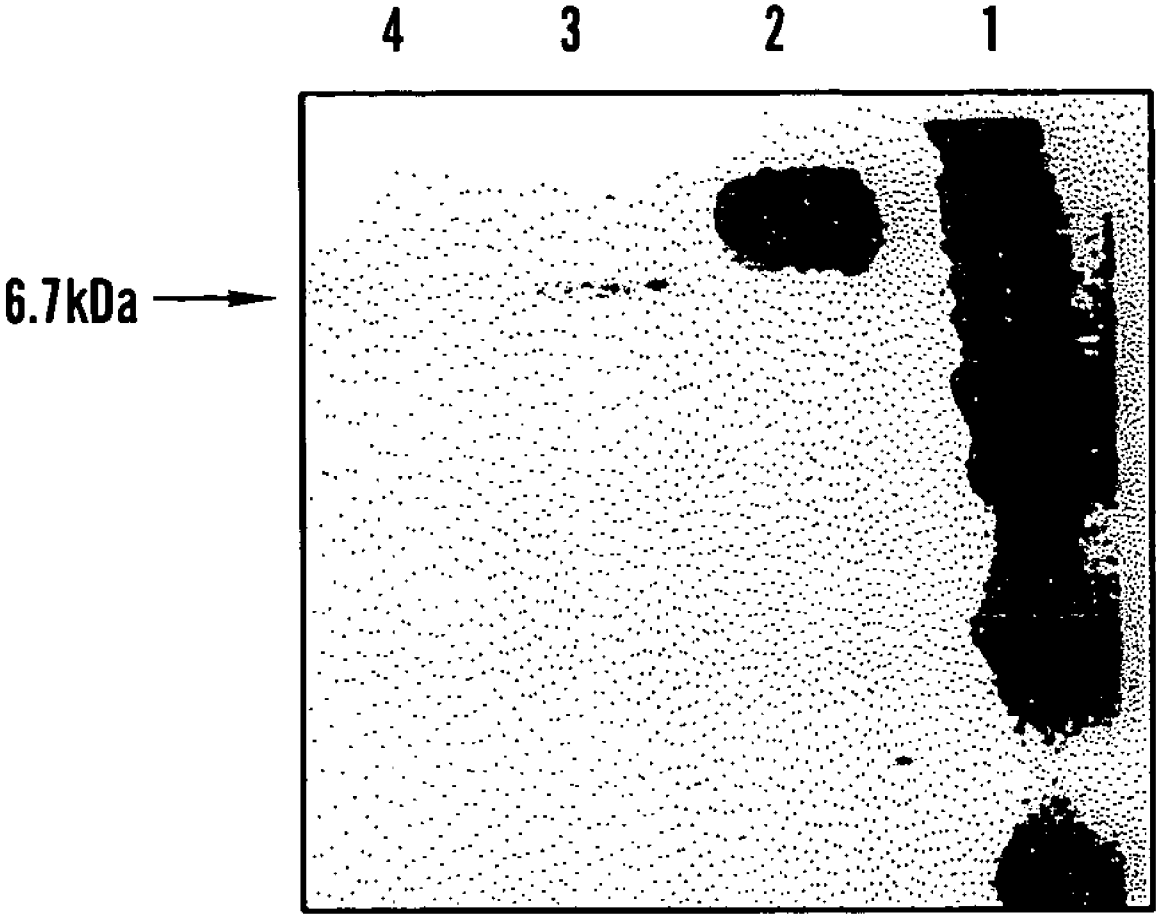

FIG. 12

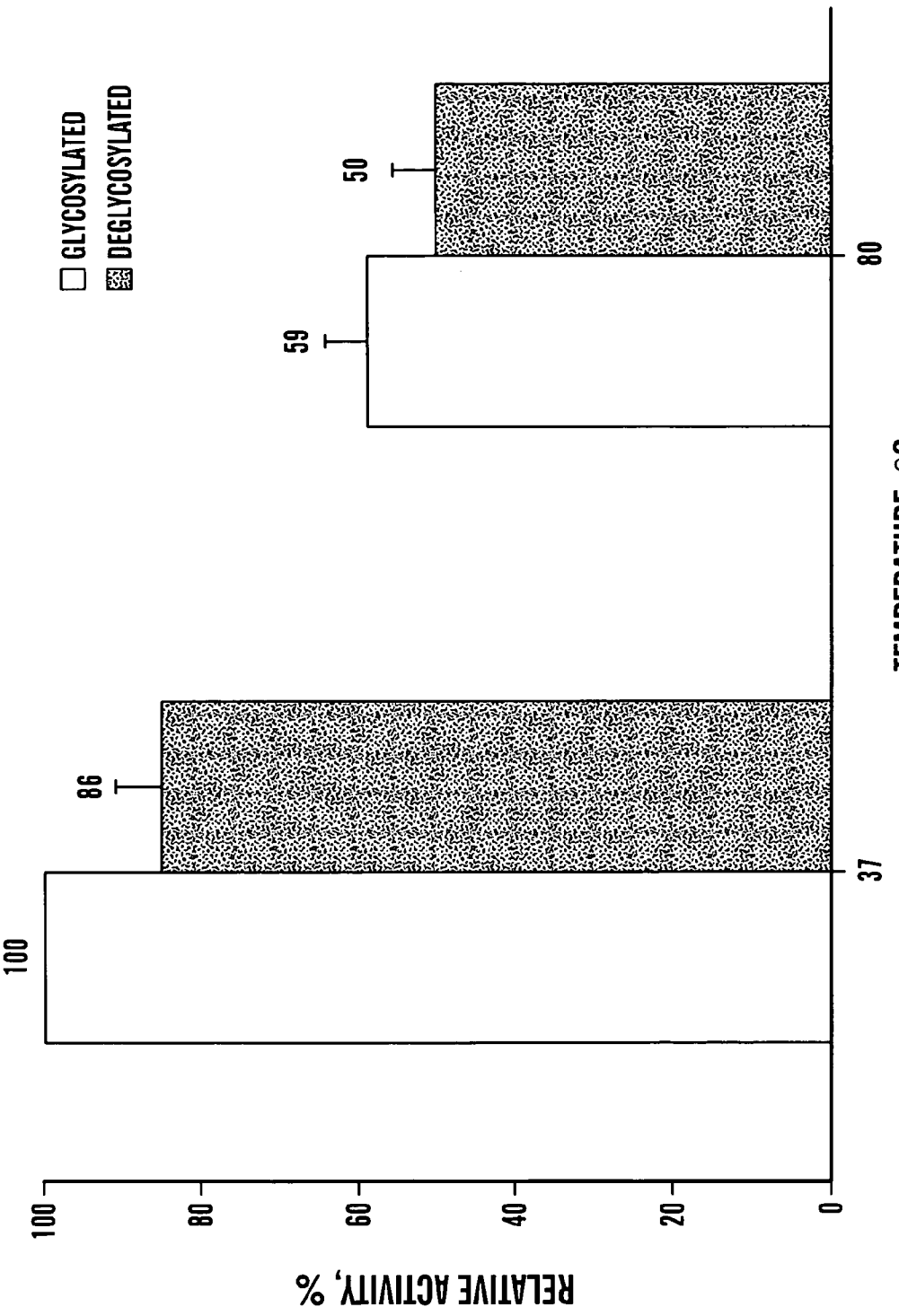
GLYCOSYLATED
DEGLYCOSYLATED
RELATIVE ACTIVITY, %
0
20
40
60
80
100
100
86
59
50
37
80
TEMPERATURE, °C

FIG. 13

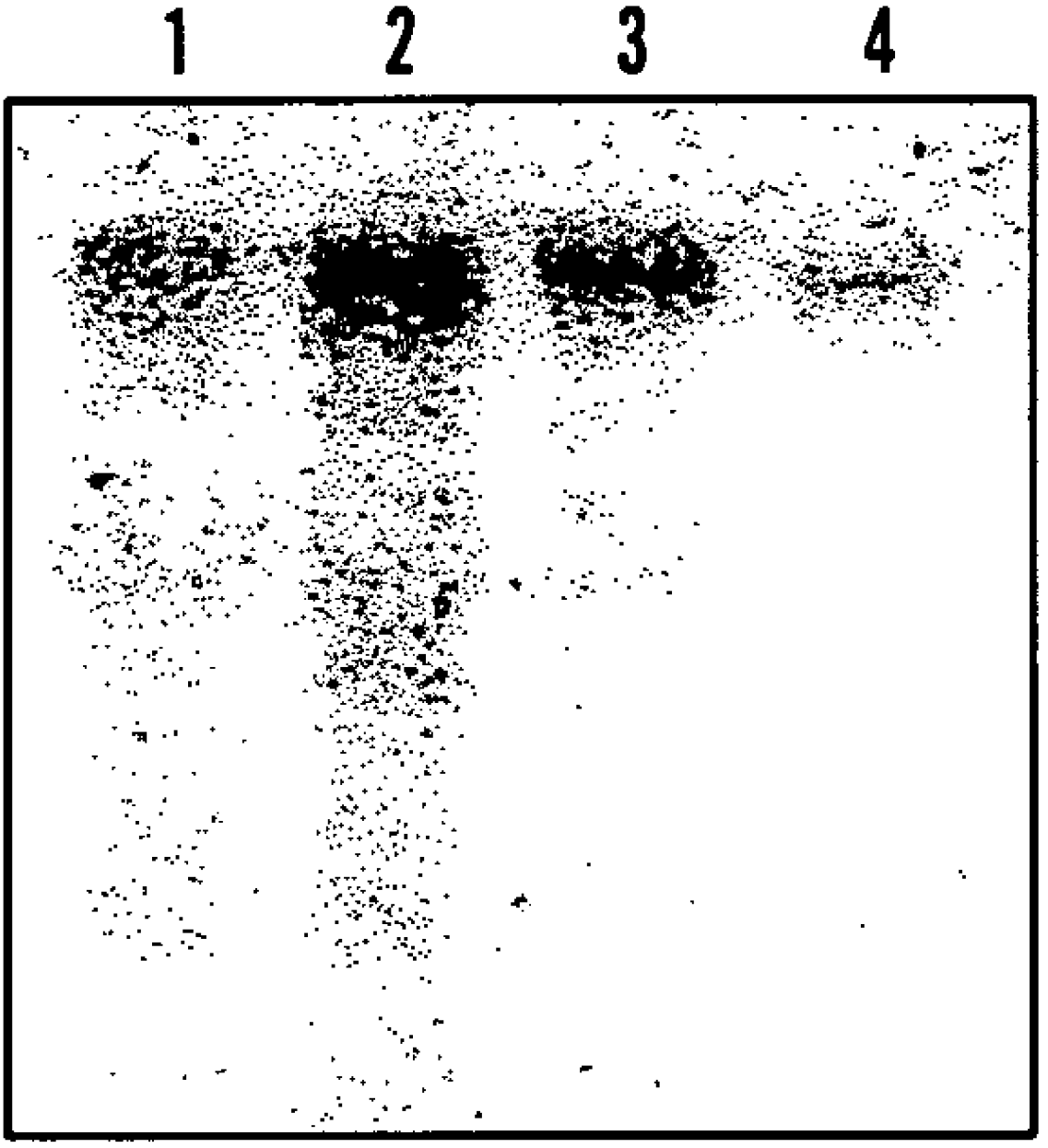

FIG. 14

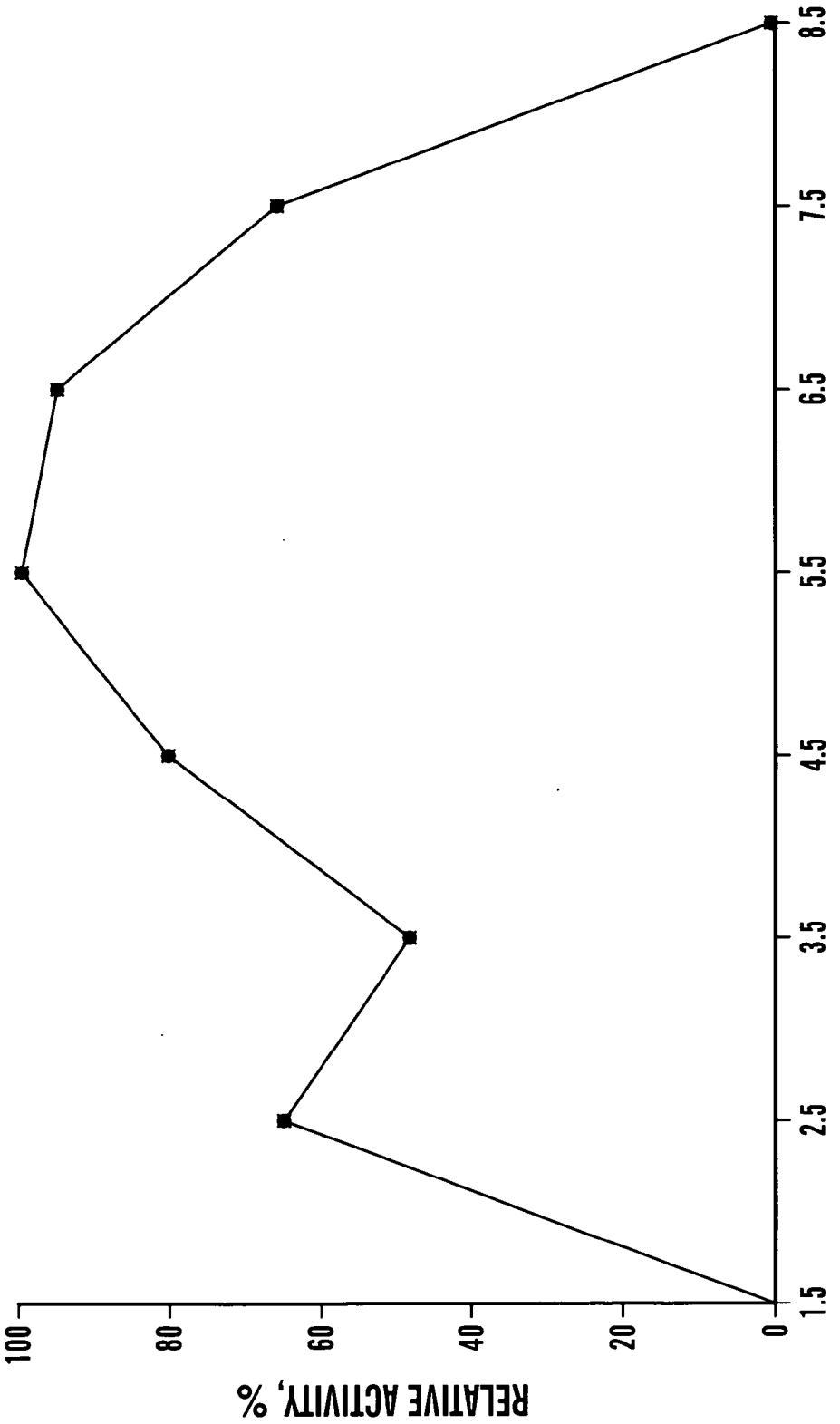

FIG. 15

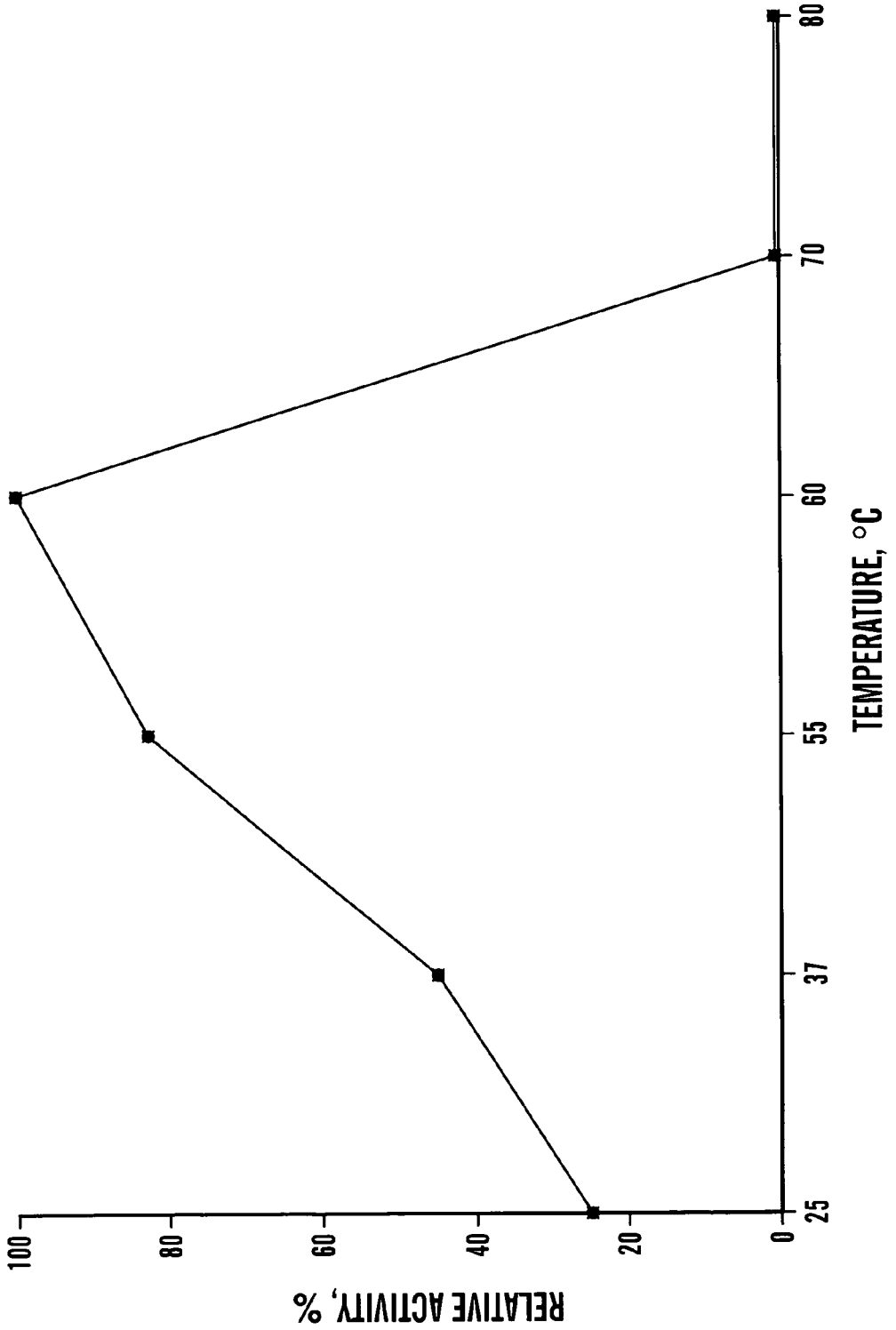
RELATIVE ACTIVITY, %
100
80
60
40
20
0
25
37
55
60
70
80
TEMPERATURE, °C

FIG. 16

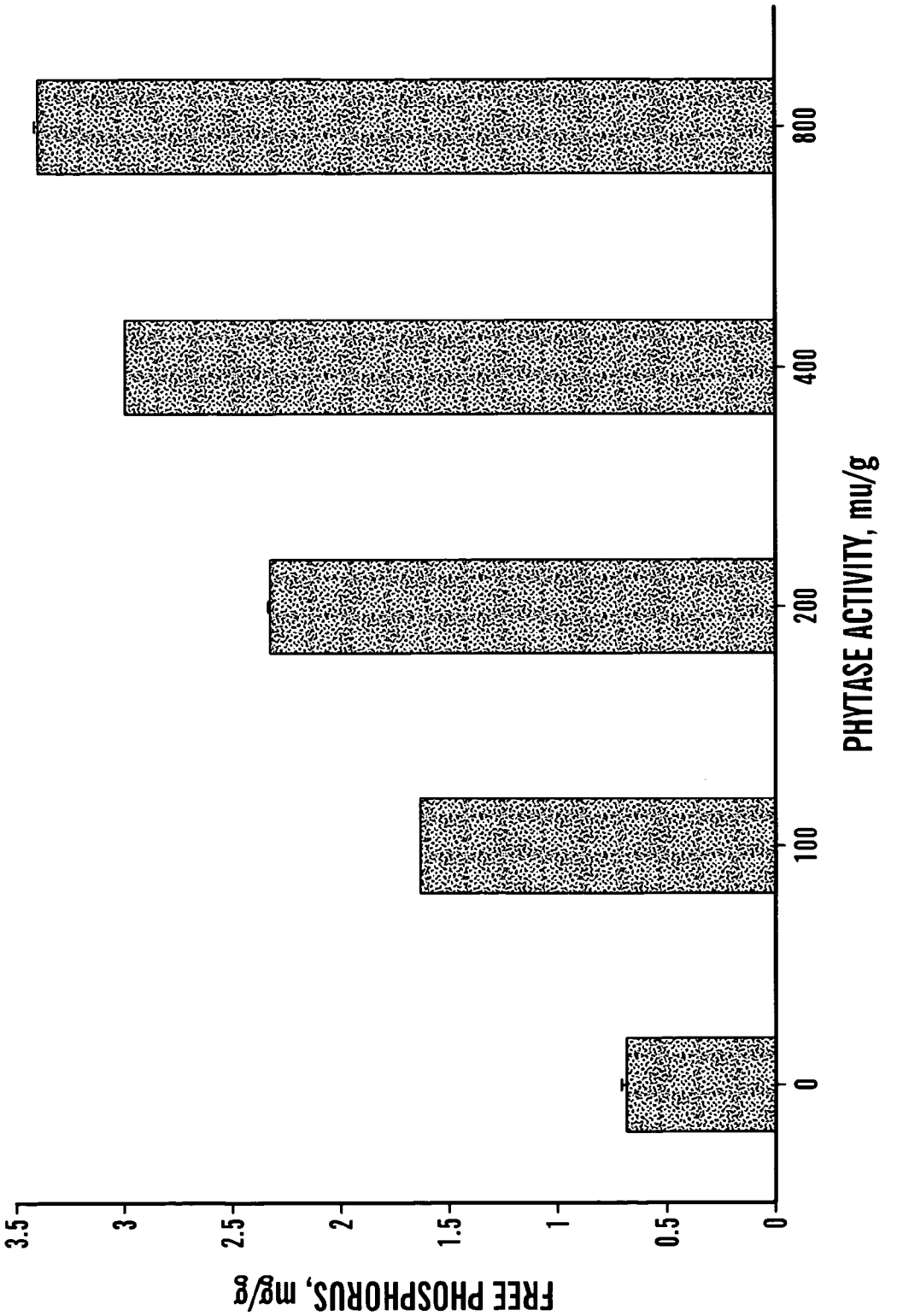
FREE PHOSPHORUS, mg/g
3.5
3
2.5
2
1.5
1
0.5
0
0
100
200
400
800
PHYTASE ACTIVITY, mu/g

FIG. 17

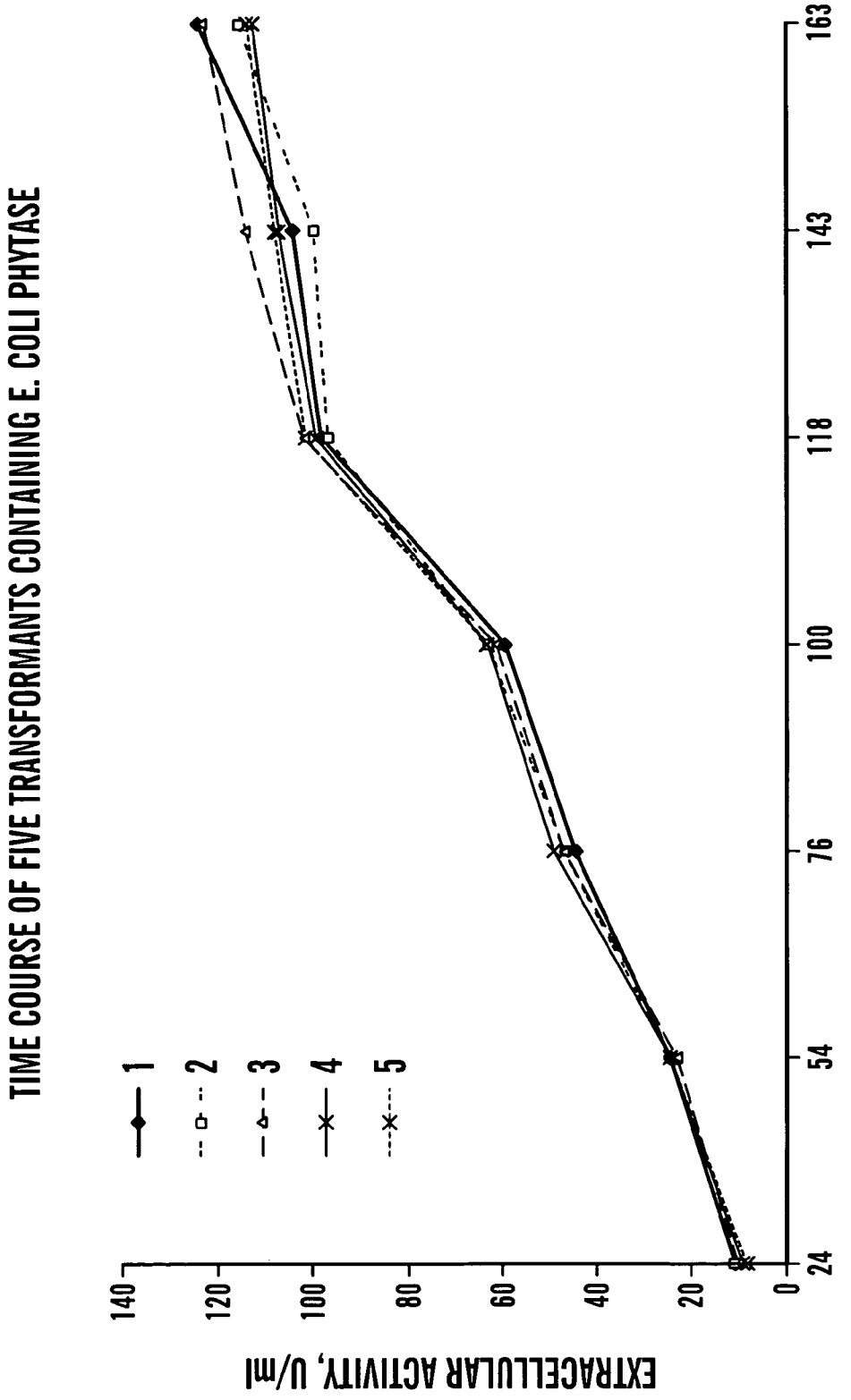
TIME COURSE OF FIVE TRANSFORMANTS CONTAINING E. COLI PHYTASE
EXTRACELLULAR ACTIVITY, U/ml
HOURS AFTER INDUCTION
1
2
3
4
5
0
20
40
60
80
100
120
140
24
54
76
100
118
143
163

FIG. 18

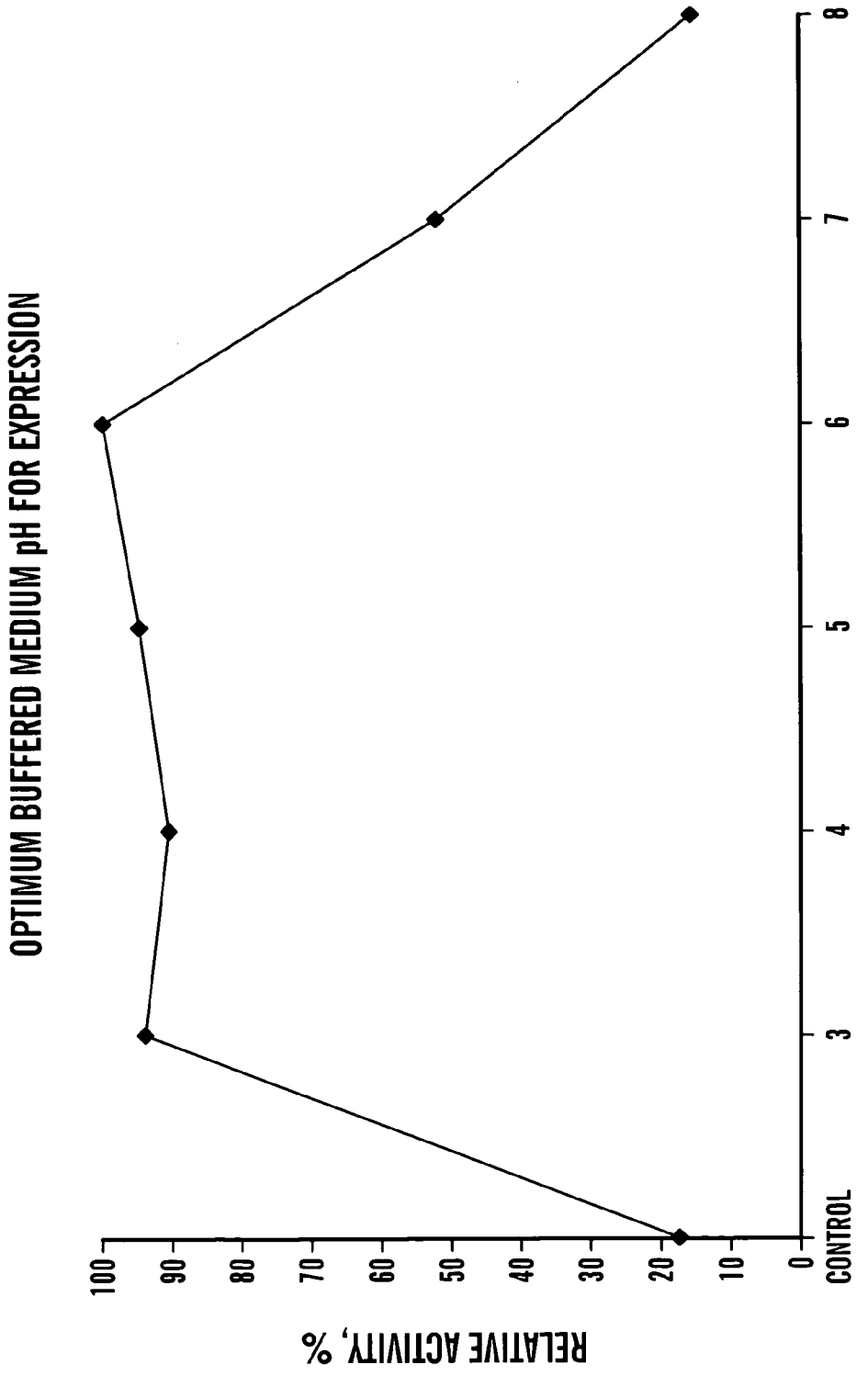
OPTIMUM BUFFERED MEDIUM pH FOR EXPRESSION
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
CONTROL
3
4
5
6
7
8
pH

FIG. 19

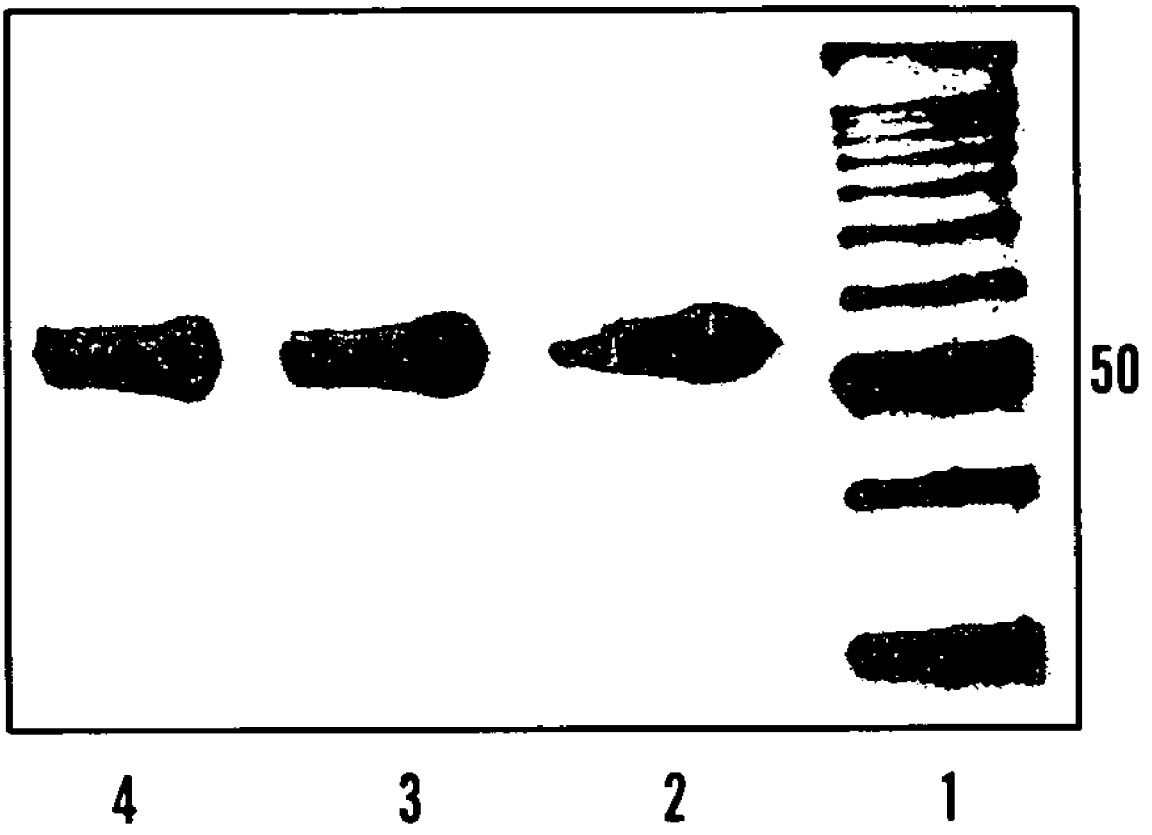

FIG. 20

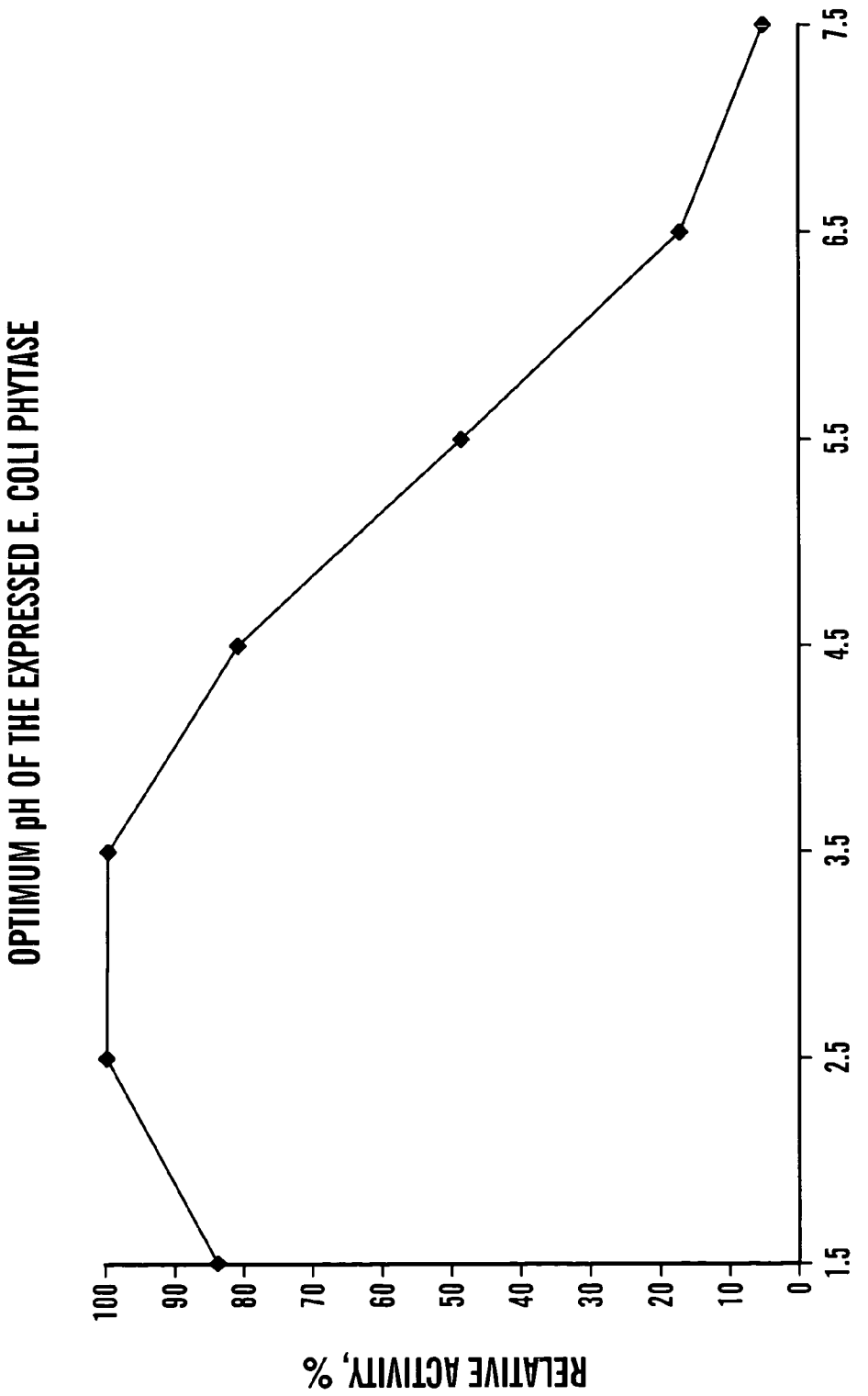
OPTIMUM pH OF THE EXPRESSED E. COLI PHYTASE
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
pH

FIG. 21

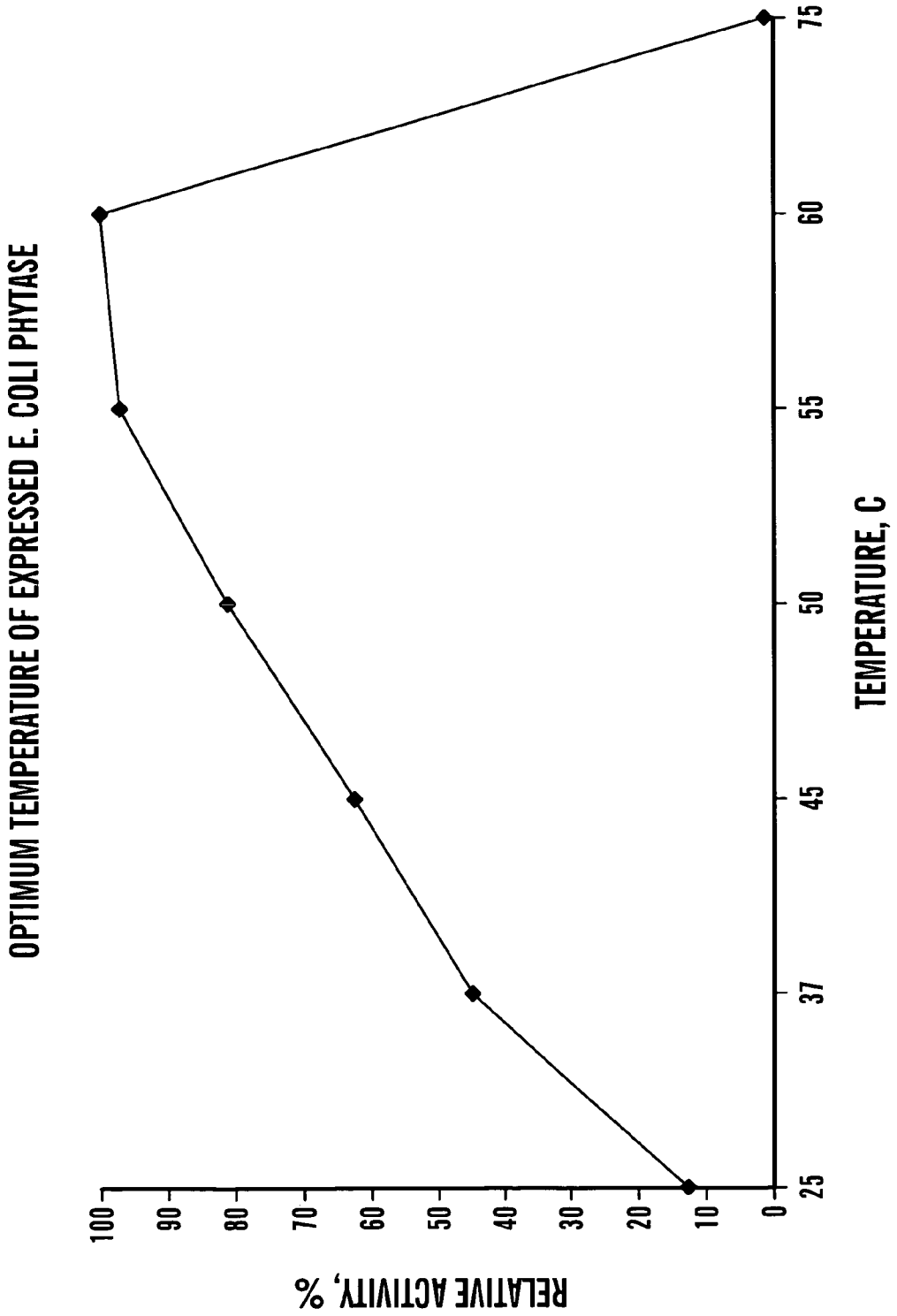
OPTIMUM TEMPERATURE OF EXPRESSED E. COLI PHYTASE
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
25
37
45
50
55
60
75
TEMPERATURE, C

FIG. 22

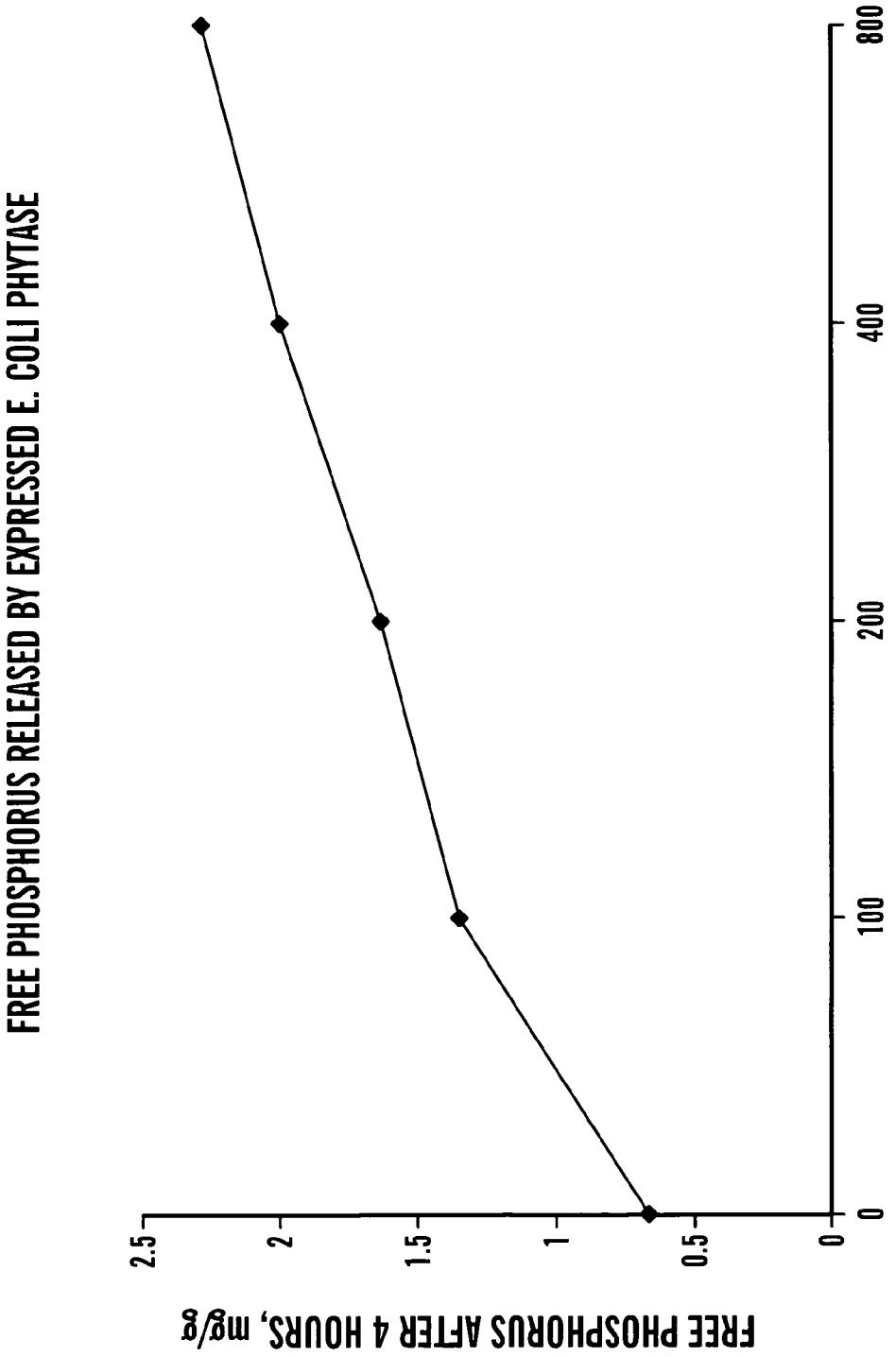
FREE PHOSPHORUS RELEASED BY EXPRESSED E. COLI PHYTASE
FREE PHOSPHORUS AFTER 4 HOURS, mg/g
2.5
2
1.5
1
0.5
0
0
100
200
400
800
PHYTASE, mU/g

FIG. 23

OVEREXPRESSION OF PHYTASE GENES IN YEAST SYSTEMS

This application is a continuation of U.S. patent application Ser. No. 10/094,693, filed Mar. 8, 2002, now U.S. Pat. No. 7,026,150, issued Apr. 11, 2006, which is a continuation-in-part of U.S. patent application Ser. No. 09/104,769, filed Jun. 25, 1998, now U.S. Pat. No. 6,451,572, issued Sep. 17, 2002, which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to a method of producing phytase in yeast, yeast strains which express heterologous phytase, and the heterologous phytase produced by yeast.

BACKGROUND OF THE INVENTION

Phytases, a specific group of monoester phosphatases, are required to initiate the release of phosphate ("P") from phytate (myo-inositol hexophosphate), the major storage form of P in cereal foods or feeds (Reddy, N. R. et al., "Phytates in Legumes and Cereals," *Advances in Food Research,* 28:1 (1982)). Because simple-stomached animals like swine and poultry as well as humans have little phytase activity in their gastrointestinal tracts, nearly all of the ingested phytate P is indigestible. This results in the need for supplementation of inorganic P, an expensive and non-renewable nutrient, in diets for these animals. More undesirably, the unutilized phytate-P excreted through manure of these animals becomes P pollution of the environment (Cromwell, G. L. et al., "P-A Key Essential Nutrient, Yet a Possible Major Pollutant—Its Central Role in Animal Nutrition," *Biotechnology In the Feed Industry;* Proceedings Alltech 7th Annual Symposium, p. 133 (1991)). Furthermore, phytate chelates with essential trace elements like zinc and produces nutrient deficiencies such as growth and mental retardation in children ingesting mainly plant origin foods without removal of phytate.

Two phytases, phyA and phyB, from *Aspergillus niger* NRRL3135 have been cloned and sequenced (Ehrlich, K. C. et al., "Identification and Cloning of a Second Phytase Gene (phys) from *Aspergillus niger (ficuum)*," *Biochem. Biophys. Res. Commun.,* 195:53–57 (1993); Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori,*" *Gene,* 133:56–62 (1993)). Recently, new phytase genes have been isolated from *Aspergillus terreus* and *Myceliophthora thermophila* (Mitchell et al., "The Phytase Subfamily of Histidine Acid Phosphatases: Isolation of Genes for Two Novel Phytases From the Fungi *Aspergillus terreus* and *Myceliophthora thermophila,*" *Microbiology* 143:245–252, (1997)), *Aspergillus fumigatus* (Pasamontes et al., "Gene Cloning, Purification, and Characterization of a Heat-Stable Phytase from the Fungus *Aspergillus fumigatus*" *Appl. Environ. Microbiol.,* 63:1696–1700 (1997)), *Emericella nidulans* and *Talaromyces thermophilus* (Pasamontes et al., "Cloning of the Phytase from *Emericella nidulans* and the Thermophilic Fungus *Talaromyces thermophilus,*" *Biochim. Biophys. Acta.,* 1353:217–223 (1997)), and maize (Maugenest et al., "Cloning and Characterization of a cDNA Encoding a Maize Seedling Phytase," *Biochem. J.* 322:511–517, 1997)).

Various types of phytase enzymes have been isolated and/or purified from *Enterobacter* sp. 4 (Yoon et al., "Isolation and Identification of Phytase-Producing Bacterium, *Enterobacter* sp. 4, and Enzymatic Properties of Phytase Enzyme," *Enzyme and Microbial Technology* 18:449–454 (1996)), *Klebsiella terrigena* (Greiner et al., "Purification and Characterization of a Phytase from *Klebsiella terrigena.,*" *Arch. Biochem. Biophys.* 341:201–206 (1997)), and *Bacillus* sp. DS11 (Kim et al., "Purification and Properties of a Thermostable Phytase from *Bacillus* sp. DS11," *Enzyme and Microbial Technology* 22:2–7 (1998)). Properties of these enzyme have been studied. In addition, the crystal structure of phy A from *Aspergillus ficuum* has been reported (Kostrewa et al., "Crystal Structure of Phytase from *Aspergillus ficuum* at 2.5 A Resolution," *Nature Structure Biology* 4:185–190 (1997)).

Hartingsveldt et al. introduced phyA gene into *A. niger* and obtained a ten-fold increase of phytase activity compared to the wild type. ("Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger,*" *Gene* 127:87–94 (1993)). Supplemental microbial phytase of this source in the diets for pigs and poultry has been shown to be effective in improving utilization of phytate-P and zinc (Simons et al., "Improvement of Phosphorus Availability By Microbial Phytase in Broilers and Pigs," *Br. J. Nutr.,* 64:525 (1990); Lei, X. G. et al., "Supplementing Corn-Soybean Meal Diets With Microbial Phytase Linearly Improves Phytate P Utilization by Weaning Pigs," *J. Anim. Sci.,* 71:3359 (1993); Lei, X. G. et al., "Supplementing Corn-Soybean Meal Diets With Microbial Phytase Maximizes Phytate P Utilization by Weaning Pigs," *J. Anim. Sci.,* 71:3368 (1993); Cromwell, G. L. et al., "P-A Key Essential Nutrient, Yet a Possible Major Pollutant—Its Central Role in Animal Nutrition," *Biotechnology In the Feed Industry;* Proceedings Alltech 7th Annual Symposium, p. 133 (1991)). But, expenses of the limited available commercial phytase supply and the activity instability of the enzyme to heat of feed pelleting preclude its practical use in animal industry (Jongbloed, A. W. et al., "Effect of Pelleting Mixed Feeds on Phytase Activity and Apparent Absorbability of Phosphorus and Calcium in Pigs," *Animal Feed Science and Technology,* 28:233–242 (1990)). Moreover, phytase produced from *A. niger* is presumably not the safest source for human food manufacturing.

Yeast can be used to produce enzymes effectively while grown on simple and inexpensive media. With a proper signal sequence, the enzyme can be secreted into the media for convenient collection. Some yeast expression systems have the added advantage of being well accepted in the food industry and are safe and effective producers of food products.

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. This system is well-known for its ability to express high levels of heterologous proteins. Because it is an eukaryote, *Pichia* has many of the advantages of higher eukaryotic expression systems such as protein processing, folding, and post-transcriptional modification.

Thus, there is a need to develop an efficient and simple system to produce phytase economically for the application of food and feed industry.

SUMMARY OF THE INVENTION

The present invention relates to a method of producing phytase in yeast by introducing a heterologous gene which encodes a protein or polypeptide with phytase/acid phosphatase activity into a yeast strain and expressing that gene.

The present invention also relates to a protein or polypeptide having phytase activity with optimum activity in a

temperature range of 57–65° C. at pH of 2.5 to 3.5 or of 5.5. Optimal pH at 2.5 to 3.5 is particularly important for phytase, because that is the stomach pH of animals.

The invention further provides a yeast cell carrying a heterologous gene which encodes a protein or polypeptide with phytase activity and which is functionally linked to a promoter capable of expressing phytase in yeast.

Yet another aspect of the invention is a vector having a gene from a non-yeast organism which encodes a protein or polypeptide with phytase activity, a promoter which is capable of initiating transcription in yeast functionally linked to the gene encoding a peptide with phytase activity, and with an origin of replication capable of maintaining the vector in yeast or being capable of integrating into the host genome.

The invention also provides a method for producing a protein or polypeptide having phytase activity. An isolated appA gene, which encodes a protein or polypeptide with phytase activity, is expressed in a host cell.

The invention also includes a method of converting phytate to inositol and inorganic phosphate. The appA gene expresses a protein of polypeptide with phytase activity in a host cell. The protein or polypeptide is then contacted with phytate to catalyze the conversion of phytate to inositol and inorganic phosphate.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows an SDS-PAGE analysis of soluble protein prepared from the phytase gene transformed *E. coli* induced with IPTG. The cells were grown 4 hours before harvesting. Lane 1: Marker; Lanes 2 and 3: Transformants of pEP1 (the expressed protein was approximately 55 kDa); Lane 4: Transformant with only the expression vector pET25b(+).

FIG. **2** shows the Western blot analysis of the expressed phytase protein in *E. coli.* The antibody was raised against purified native phytase of *A. niger.* Each lane contained 50 μg total intracellular protein. Lanes 1 and 2: Recombinants after and before induction. Lanes 3 and 4: control (only the expressing vector) after and before induction.

FIG. **3** is a scan image of Northern blotting analysis of the mRNA of PhyA in *E. coli.* A 1.4 kb PhyA probe was used. Each lane contained 20 μg of total RNA. Lanes 1 and 2: RNA isolated from the control cells (only the expression vector) before and after induction. Lanes 3 and 4: RNA isolated from the recombinants containing PhyA before and after induction.

FIG. **4** is a time course of the induced expression of phytase (pEP1) in *E. coli* BL21(DE3). The cells were induced when the OD_{600} reached 0.5. The soluble protein, prepared at each time point, was quantified by SDS-PAGE analysis.

FIG. **5** shows an SDS-PAGE analysis of the expressed extracellular phytase protein by the phytase transformed *S. lividans* after growing for 72 hours. Cells were spun for 15 minutes at 8,000×g, and the supernatant was subjected to gel electrophoresis. Lane 1: Marker; Lane 2: Control with the only expression vector; Lane 3: Positive colony expressed phytase and the size was approximately 57 kDa.

FIG. **6** shows the Western blot analysis of the phytase expressed by *S. lividans,* using a phytase antibody raised against purified native phytase of *A. niger.* Each lane was loaded with 20 μg medium (supernatant) protein. Lane 1: Supernatant from the vector transformed control cell culture; Lane 2: Supernatant from the culture inoculated with the positive colony.

FIG. **7** depicts an SDS-PAGE analysis of the extracellular phytase expressed by *S. cerevisiae.* Each lane was loaded with 50 μg medium (supernatant) protein. Lanes 1 to 3: Supernatant from the culture inoculated with the positive colony harvested at 5, 10, and 25 hours after induction, respectively; Lanes 4 to 6: Supernatant from the vector-transformed control cell culture harvested at 5, 10, and 25 hours after induction, respectively; Lane 7: Marker (kDa). The expressed phytase was approximately 110 kDa (confirmed by Western blot).

FIG. **8** is a time course of the extracellular phytase activity expressed by the pYPP1 construct transformed *S. cerevisiae* after induced by galactose. The activity was analyzed in the supernatant of the collected medium.

FIG. **9** shows the Western blot analysis of the extracellular phytase expressed by *S. cerevisiae* before and after deglycosylation (Endo H), using a phytase antibody raised against purified native phytase in *A. niger.* Lane 1: Prestained SDS-PAGE standards (kDa) from Bio-Rad; Lanes 2 and 3: deglycosylated 10 and 20 μg phytase protein, respectively; Lane 4: glycosylated phytase (20 μg protein).

FIG. **10** is a scan image of Northern blot analysis for total RNA isolated from transformed *S. cerevisiae* cells. Lane 1: Control (with only the expression vector pYES2); Lanes 2 and 3: Tranformants of pYPP1.

FIG. **11** is a time course of the extracellular phyA phytase activity produced by *Pichia pastoris* transformants of Mut^s (KM71) and Mut+ (X33) after induction.

FIG. **12** depicts an SDS-PAGE analysis of the overexpressed phytase in *Pichia,* with construct of pPICZαA-PhyA in KM71 (MUT^s). Lane 1: protein ladder. Lane 2: 40 μl of the supernatant of AK1 (a colony showed 21,700 mU/ml of extracellular phytase), collected 108 hours after induction. Lane 3: 40 μl of the supernatant of a control strain overexpressing human serum albumin (HAS, 6.7 kDa) at a level of 1 g/L. Lane 4: 40 μl of the supernatant of the KM71 control.

FIG. **13** depicts effects of deglycosylation by Endo H on the thermostability of the expressed phytase in *Pichia.* Phytase activity was measured after the enzymes were heated for 15 minutes under 37 or 80° C. in 0.2 M citrate buffer, pH 5.5.

FIG. **14** is a scan image of Northern analysis of the expressed phyA mRNA by the transformed *Pichia pastoris* strains (KM71 and X33). A 1.3 kb phyA probe was used for blotting. Lanes 1 and 2: the transformant of KM71 before and after induction; Lanes 3 and 4: the transformant of X33 after and before induction.

FIG. **15** shows the optimum pH of the expressed extracellular phytase by *Pichia* (X33). Buffers of 0.2 M glycine-HCl for pH 1.5, 2.5, 3.5; 0.2 M sodium citrate for pH 4.5, 5.5, 6.5, and 0.2 M Tris-HCl for pH 7.5 and 8.5 were used.

FIG. **16** shows the optimum temperature of the expressed extracellular phytase by *Pichia* (X33). The assays were conducted in 0.2 M citrate buffer, pH 5.5.

FIG. **17** depicts the release of free phosphorus from soybean by the expressed phytase in *Pichia* (X33). Five grams of soybean were suspended in 25 ml of 0.2 M citrate, pH 5.5, with different amounts of the enzyme. The incubation was conducted for 4 hours under 37° C. and the free phosphorus released in the supernatant was determined.

FIG. **18** shows a time course of the expression of the extracellular phytase activity from five transformants of *Pichia pastoris* containing the *E. coli* appA gene.

FIG. **19** graphically shows the relationship between medium pH and the expression of phytase activity by *Pichia pastoris.*

FIG. **20** is an SDS-PAGE analysis of the *E. coli* phytase overexpressed in *Pichia pastoris*. Lane 1: Protein ladder; Lanes 2 to 4: Supernatants collected from the cultures of positive colonies 23, 22, and 11, respectively, at 118 hours after induction.

FIG. **21** graphically shows the optimum pH of the overexpressed *E. coli* phytase by *Pichia pastoris*.

FIG. **22** graphically shows the optimum temperature of the overexpressed *E. coli* phytase by *Pichia pastoris*.

FIG. **23** shows the amount of free phosphorus released from soybean meal by the overexpressed *E. coli* phytase from *Pichia pastoris* after four hours treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing phytase in yeast. According to this method, a heterologous gene which encodes a protein or polypeptide with phytase activity is expressed in a yeast strain.

The enzymes which catalyze the conversion of phytate to inositol and inorganic phosphorus are broadly known as phytases. Phytase producing microorganisms comprise bacteria such as *Bacillus subtilis* (Paver et al., J. Bacteriol. 151, 1102 (1982), which is hereby incorporated by reference) and *Pseudomonas* (Cosgrove, *Austral. J. Biol. Sci.* 23:1207 (1970), which is hereby incorporated by reference); yeasts, such as *Saccharomyces cerevisiae* (Nayini et al., *Lebensmittel Wissenschaft und Technologie* 17:24 (1984), which is hereby incorporated by reference); and fungi, such as *Aspergillus terreus* (Yamada et al., *Agric. Biol. Chem.* 32:1275 (1986), which is hereby incorporated by reference), and *Aspergillus ficuum* (van Gorcom et al., European Patent Application 89/202,436, which is hereby incorporated by reference).

Phytases are also endogenously present in many plant species. Loewus, In: *Plant Biology* vol. 9: "Inositol Metabolism in Plants" (eds. D. J. Morre, W. F. Boss, F. A. Loewus) 13 (1990); and Gellatly, et al., *Plant Physiology* (supplement) 93:562 (1990), which are hereby incorporated by reference, mention the isolation and characterization of a phytase cDNA clone obtained from potato tubers. Gibson, et al., *J. Cell Biochem.*, 12C:L407 (1988) and Christen, et al., *J. Cell Biochem.*, 12C:L402 (1988), which are hereby incorporated by reference, mentions the synthesis of endogenous phytase during the germination of soybean seeds.

Preferably, the protein or polypeptide with phytase activity is secreted by the cell into growth media. This allows for higher expression levels and easier isolation of the product. The protein or polypeptide with phytase activity is coupled to a signal sequence capable of directing the protein out of the cell. Preferably, the signal sequence is cleaved from the protein.

In a preferred embodiment, the heterologous gene, which encodes a protein or polypeptide with phytase activity, is spliced in frame with a transcriptional enhancer element.

Preferred heterologous genes encoding proteins with phytase activity are isolated from a bacterial cell. A more preferred gene is the phyA gene of *Aspergillus niger*. A gene encoding phytase, phyA, from *Aspergillus niger* NRRL3135 has been cloned and sequenced (Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori*," *Gene*, 133:56–62 (1993), which are hereby incorporated by reference). Hartingsveldt et al. introduced phyA gene into *A. niger*, and obtained a tenfold increase of phytase activity compared to the wild type. (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," *Gene* 127:87–94 (1993), which is hereby incorporated by reference.)

Another preferred heterologous gene is the appA gene of *E. coli*. The gene, originally defined as *E. coli* periplasmic phosphoanhydride phosphohydrolase (appA) gene, contains 1,298 nucleotides (GeneBank accession number: M58708). The gene was first found to code for an acid phosphatase protein of optimal pH of 2.5 (EcAP) in *E. coli*. The acid phosphatase is a monomer with a molecular mass of 44,644 daltons. Mature EcAP contains 410 amino acids (Dassa, J. et al., "The Complete Nucleotide Sequence of the *Escherichia Coli* Gene AppA Reveals Significant Homology Between Ph 2.5 Acid Phosphatase and Glucose-1-Phosphatase," *J. Bacteriology*, 172:5497–5500 (1990), which is hereby incorporated by reference). Ostanin, et al. overexpressed appA in *E. coli* BL21 using a pT7 vector and increased its acid phosphatase activity by approximately 400-folds (440 mU/mg protein) (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia Coli* Acid Phosphatase," *J. Biol. Chem.*, 267:22830–36 (1992), which is hereby incorporated by reference). The product of the appA gene was not previously known to have phytase activity.

The appA or phyA gene can be expressed in any prokaryotic or eukaryotic expression system. A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Preferred vectors include a viral vector, plasmid, cosmid or an oligonucleotide. Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Preferred hosts for expressing appA or phyA include fungal cells, including species of yeast or filamentous fungi, may be used as host cells in accordance with the present invention. Preferred yeast host cells include different strains of *Saccharomyces cerevisiae*. Other yeasts like *Kluyveromyces, Torulaspora,* and *Schizosaccharomyces* can also be used. In a preferred embodiment, the yeast strain used to overexpress the protein is *Saccharomyces cerevisiae*. Preferred filamentous fungi host cells include *Aspergillus* and *Neurospora*. A more preferred strain of *Aspergillus* is *Aspergillus niger*.

In another preferred embodiment of the present invention, the yeast strain is a methylotrophic yeast strain. Methylotrophic yeast are those yeast genera capable of utilizing methanol as a carbon source for the production of the energy resources necessary to maintain cellular function and containing a gene for the expression of alcohol oxidase. Typical methylotrophic yeasts include members of the genera *Pichia, Hansenula, Torulopsis, Candida,* and *Karwinskia*. These yeast genera can use methanol as a sole carbon source. In a more preferred embodiment, the methylotrophic yeast strain is *Pichia pastoris*.

The present invention also provides a protein or polypeptide with phytase activity. PhyA is expressed in *Pichia* and the resulting protein produced has much higher extracellular

activity (~65 mU/ml). The phytase activity yield was approximately 30-fold greater than that in phyA transformed *Saccharomyces cerevisiae,* 21-fold greater than that in wild type of *Aspergillus niger,* and 65,000-fold greater than that in the untransformed *Pichia.* The optimal pH of the expressed phytase was 2.5 and 5.5, and the optimal temperature was 60° C. Similarly, appA is expressed in *Pichia* and *Saccharomyces cerevisiae* with the resulting protein having much higher extracellular activity and a much preferred optimal pH of 2.5 to 3.5.

A preferred embodiment of the invention is a protein or polypeptide having phytase activity with optimum activity in a temperature range of 57 to 65° C. A more preferred embodiment is a protein or polypeptide having phytase activity, where its temperature range for optimum activity is from 58 to 62° C.

Yet another preferred embodiment is a protein or polypeptide having phytase activity where the protein retains at least 40% of its activity after heating the protein for 15 minutes at 80° C. More preferred is a protein or polypeptide having phytase activity where the protein retains at least 60% of its activity after heating the protein for 15 minutes at 60° C.

Purified protein may be obtained by several methods. The protein or polypeptide of the present invention is preferably produced in purified form (preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is secreted into the growth medium of recombinant host cells. Alternatively, the protein or polypeptide of the present invention is produced but not secreted into growth medium. In such cases, to isolate the protein, the host cell carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove cell debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the polypeptide or protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

The present invention also provides a yeast strain having a heterologous gene which encodes a protein or polypeptide with phytase activity. The heterologous gene should be functionally linked to a promoter capable of expressing phytase in yeast.

Yet another aspect of the invention is a vector for expressing phytase in yeast. The vector carries a gene from a non-yeast organism which encodes a protein or polypeptide with phytase activity. The phytase gene can be cloned into any vector which replicates autonomously or integrates into the genome of yeast. The copy number of autonomously replicating plasmids, e.g. YEp plasmids may be high, but their mitotic stability may be insufficient (Bitter et al., "Expression and Secretion Vectors for Yeast," *Meth. Enzymol.* 153:516–44 (1987), which is hereby incorporated by reference). They may contain the 2 mu-plasmid sequence responsible for autonomous replication, and an *E. coli* sequence responsible for replication in *E. coli.* The vectors preferably contain a genetic marker for selection of yeast transformants, and an antibiotic resistance gene for selection in *E. coli.* The episomal vectors containing the ARS and CEN sequences occur as a single copy per cell, and they are more stable than the YEp vectors. Integrative vectors are used when a DNA fragment is integrated as one or multiple copies into the yeast genome. In this case, the recombinant DNA is stable and no selection is needed (Struhl et al., "High-Frequency Transformation of Yeast: Autonomous Replication of Hybrid DNA Molecules," *Proc. Nat'l Acad. Sci. USA* 76:1035–39 (1979); Powels et al., *Cloning Vectors, I-IV,* et seq. Elsevier, (1985); Sakai et al., "Enhanced Secretion of Human Nerve Growth Factor from *Saccharomyces Cerevisiae* Using an Advanced δ-Integration System," *Biotechnology* 9:1382–85 (1991), which are hereby incorporated by reference). Some vectors have an origin of replication, which functions in the selected host cell. Suitable origins of replication include 2μ, ARS1, and 25 μM. The vectors have restriction endonuclease sites for insertion of the fusion gene and promoter sequences, and selection markers. The vectors may be modified by removal or addition of restriction sites, or removal of other unwanted nucleotides.

The phytase gene can be placed under the control of any promoter (Stetler et al., "Secretion of Active, Full- and Half-Length Human Secretory Leukocyte Protease Inhibitor by *Saccharomyces cerevisiae,*" *Biotechnology* 7:55–60, (1989), which is hereby incorporated by reference). One can choose a constitutive or regulated yeast promoter. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073 (1980), which is hereby incorporated by reference) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149 (1968); and Holland et al., *Biochem.* 17:4900, (1978), which are hereby incorporated by reference), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in EP A-73,657 to Hitzeman, which is hereby incorporated by reference. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al., *J. Biol. Chem.* 258:2674 (1982) and Beier et al., *Nature* 300:724 (1982), which are hereby incorporated by reference.

One can choose a constitutive or regulated yeast promoter. The strong promoters of e.g., phosphoglycerate kinase (PGK) gene, other genes encoding glycolytic enzymes, and the alpha -factor gene, are constitutive. When a constitutive promoter is used, the product is synthesized during cell growth. The ADH2 promoter is regulated with ethanol and glucose, the GAL-1-10 and GAL7 promoters with galactose and glucose, the PHO5 promoter with phosphate, and the metallothionine promoter with copper. The heat shock promoters, to which the HSP150 promoter belongs, are regulated by temperature. Hybrid promoters can also be used. A regulated promoter is used when continuous expression of the desired product is harmful for the host cells. Instead of yeast promoters, a strong prokaryotic promoter such as the T7 promoter, can be used, but in this case the yeast strain has to be transformed with a gene encoding the respective polymerase. For transcription termination, the HSP150 terminator, or any other functional terminator is used. Here, promoters and terminators are called control elements. The present invention is not restricted to any specific vector, promoter, or terminator.

The vector may also carry a selectable marker. Selectable markers are often antibiotic resistance genes or genes capable of complementing strains of yeast having well characterized metabolic deficiencies, such as tryptophan or histidine deficient mutants. Preferred selectable markers include URA3, LEU2, HIS3, TRP1, HIS4, ARG4, or antibiotic resistance genes.

The vector may also have an origin of replication capable of replication in a bacterial cell. Manipulation of vectors is more efficient in bacterial strains. Preferred bacterial origin of replications are ColE1, Ori, or oriT.

A leader sequence either from the yeast or from phytase genes or other sources can be used to support the secretion of expressed phytase enzyme into the medium. The present invention is not restricted to any specific type of leader sequence or signal peptide.

Suitable leader sequences include the yeast alpha factor leader sequence, which may be employed to direct secretion of the phytase. The alpha factor leader sequence is often inserted between the promoter sequence and the structural gene sequence (Kurjan et al., *Cell* 30:933, (1982); Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, (1984); U.S. Pat. No. 4,546,082; and European published patent application No. 324,274, which are hereby incorporated by reference). Another suitable leader sequence is the *S. cerevisiae* MF alpha 1 (alpha-factor) is synthesized as a prepro form of 165 amino acids comprising signal-or prepeptide of 19 amino acids followed by a "leader" or propeptide of 64 amino acids, encompassing three N-linked glycosylation sites followed by (LysArg(Asp/Glu, Ala)2-3 alpha-factor)4 (Kurjan, et al., *Cell* 30:933–43 (1982), which is hereby incorporated by reference). The signal-leader part of the preproMF alpha 1 has been widely employed to obtain synthesis and secretion of heterologous proteins in *S. cerivisiae*. Use of signal/leader peptides homologous to yeast is known from. U.S. Pat. No. 4,546,082, European Patent Applications Nos. 116,201; 123,294; 123,544; 163,529; and 123,289 and DK Patent Application No. 3614/83, which are hereby incorporated by reference. In European Patent Application No. 123,289, which is hereby incorporated by reference, utilization of the *S. cerevisiae* a-factor precursor is described whereas WO 84/01153, which is hereby incorporated by reference, indicates utilization of the *Saccharomyces cerevisiae* invertase signal peptide, and German Patent Application DK 3614/83, which is hereby incorporated by reference, indicates utilization of the *Saccharomyces cerevisiae* PH05 signal peptide for secretion of foreign proteins.

The alpha-factor signal-leader from *Saccharomyces cerevisiae* (MF alpha 1 or MF alpha 2) may also be utilized in the secretion process of expressed heterologous proteins in yeast (U.S. Pat. No. 4,546,082, European Patent Applications Nos. 16,201; 123,294; 123 544; and 163,529, which are hereby incorporated by reference). By fusing a DNA sequence encoding the *S. cerevisiea* MF alpha 1 signal/leader sequence at the 5' end of the gene for the desired protein secretion and processing of the desired protein was demonstrated. The use of the mouse salivary amylase signal peptide (or a mutant thereof) to provide secretion of heterologous proteins expressed in yeast has been described in Published PCT Applications Nos. WO 89/02463 and WO 90/10075, which are hereby incorporated by reference.

U.S. Pat. No. 5,726,038 describes the use of the signal peptide of the yeast aspartic protease 3, which is capable of providing improved secretion of proteins expressed in yeast. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978), which is hereby incorporated by reference. The Hinnen et al. protocol selects for Trp transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

The gene may be maintained on stable expression vector, an artificial chromosome, or by integration into the yeast host cell chromosome. Integration into the chromosome may be accomplished by cloning the phytase gene into a vector which will recombine into a yeast chromosome. Suitable vectors may include nucleotide sequences which are homologous to nucleotide sequences in the yeast chromosome. Alternatively, the phytase gene may be located between recombination sites, such as transposable elements, which can mobilize the gene into the chromosome.

The present invention also provides a method of producing phytase by providing an isolated appA gene, which encodes a protein or polypeptide with phytase activity, and expressing the gene in host cell. Preferably the appA gene is isolated from *Escherichia coli.* Preferred host cells include yeast or filamentous fungi. The preferred filamentous fungi is *Aspergillus niger* and the preferred yeast are *Saccharomyces, Kluyveromyces, Torulaspora,* and *Schizosaccharomyces,* in particular, the yeast strain, *Saccromyces cerivesia.*

A method of converting phytate to inositol and inorganic phosphorus is also provided. An appA gene is isolated from an organism, using techniques well known in the art. A protein or polypeptide with phytase activity is then expressed from the gene in a host cell. The resulting protein or polypeptide is mixed or contacted with phyate. This technique is especially useful for treating phytate in food or animal feed.

The preferred appA gene is isolated from *Escherichia coli.*

While the phytase enzyme produced in a yeast system released phytate-P from corn and soy as effectively as the currently commercial phytase, it appeared to be more thermostable. This phytase overexpression system in yeast can be used to provide thermostable phytase for use in the food and feed industries.

EXAMPLES

Example 1

Materials and Methods for Overexpressing PhyA in *E. Coli, S. Lividans,* and a *Saccharomyces* System

Phytase gene, host strains, and expression plasmids. Phytase gene, phyA, was kindly provided by Dr. E. J. Mullaney of the USDA. The gene (GenBank Accession number M94550) was contained in plasmid pMD4.21 in *E. coli* strain HB101. A 2.7 kb SphI fragment of *A. niger* DNA contained the coding region of the deglycosylated phytase and its 5' and 3' flanking sequences. A plasmid containing the signal peptide sequence, Spxy, of the active xylanase gene of *Aureobasidum pullulans* (GenBank Accession number U10298) was kindly provided by Dr. X. L. Li of the University of Georgia. The *E. coli* strain DH5α was used as an initial host for all the recombinant plasmids. In order to express phyA in *E. coli,* the expression vector, pET25b(+) (Novagen, Madison, Wis.) and the expression host, BL21 (DE3)pLysS, were used. In order to express phyA in *S. lividans* TK 24, plasmid pSES1 (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividansoglucanase* Gene and an Endoglucanase Gene from *Thermomonospora Fusca,*" *Appl. Environ. Microbiol.,* 59:3032–43 (1993), which is hereby incorporated by reference), was used to construct the shuttle plasmid (from Dr. D. B. Wilson of Cornell University and he obtained it from Dr. D. A. Hopwood, John Innes Institute, Norwich, England). In order

to express phyA in yeast, the expression vector pYES2 and the host *S. cerevisiae* strain, INVSc1 (Invitrogen, San Diego, Calif.) were used.

Plasmid cassette constructions and transformations. All the constructed plasmids and the correspondent hosts are listed in Table 1. A 1.4 kb PCR fragment of phyA gene was amplified from the pMD4.21 by using two primers: upstream 5'-CGG AAT TCG TCA CCT CCG GAC T-3' (SEQ ID NO:1) and downstream 5'-CCC AAG CTT CTA AGC AAA ACA CTC-3' (SEQ ID NO:2). The resulting fragment contained the sequence coding for the deglycosylated phytase of *A. niger,* PhyA, and EcoRI and HindIII restriction site upstream and downstream, respectively. After purification with Geneclean II kit (Bio101, Inc., La Jolla, Calif.), the fragment was inserted into pET25b(+), and the resulting construct pEP1 (6893 bp) was transformed into BL21(DE3)pLysS after initial confirmation in DH5α cells. The expression was under the control of T7 promoter followed by the lead sequence (pel B) encoding 21 amino acids, and phyA. The host transformed with the pET25(+) vector only was used as the control.

TABLE 1

Expression vectors, constructs, and their host strains used in the study

Plasmid	Host	Description[1]	Reference[2]
pET25b(+)	*E. coli* DH5α and BL21 (DE3)pLysS	Expression vector	Novagen
pEP1	*E. coli* BL21 (DE3)pLysS	pET25bα(+) + phyA gene	This paper
PSES2	*E. coli* DH5α and *S. lividans* TK24	Expression vector	Jung et al.[2], 1993
PSPP1	*E. coli* DH5α and *S. lividans* TK24	pSES2 + Spe2 + phyA	This paper
PYES2	*E. coli* DH5α and *S. cerevisiae* INVSc1	Expression vector	Invitrogen
PYEP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Spe2 + phyA	This paper
PYXP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Spxy + phyA	This paper
PYPP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Sphy + phyA	This paper

[1]Spe2 is the signal peptide for endoglucanase E2 of *T. fusca* (Wilson, D. B., "Biochemistry and Genetics of Actinomycete Cellulases," Crit. Rev. Biotechnol, 12: 45–63 (1992), which is hereby incorporated by reference); Spxy is the signal peptide for xylanase of *A. pullulans* (Liand Ljungdahl, "Cloning, Sequencing, and Regulation of a Xylanase Gene fromthe Fungus *Aureobasidium pullulans* Y-2331-1," Appl. Environ. Microbiol., 60: 3160–66 (1994); Li and Ljungdahl, "Expression of *Aureobasidium pullulans* xynA in, and Secretion of the Xylanase from, *Saccharomyces cerevisiae*," Appl. Environ. Microbiol., 62: 209–13 (1996),which are hereby incorporated by reference); and Sphy is the signal peptide for phyA of *A. niger* (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," Gene 127: 87–94 (1993), which is hereby incorporated by reference).

[2]Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces* Lividansoglucanase Gene and an Endoglucanase Gene from *Thermomonospora Fusca*," Appl. Environ. Microbiol., 59: 3032–43 (1993), which is hereby incorporated by reference).

The construction of the plasmid for phyA expression in *S. lividans* started with the synthesis of a fragment containing pLT1 promoter and Spe2 signal peptide (Lao, G. et al., "DNA Sequences of Three Beta-1,4-endoglucanase Genes From *Thermomonospora Fusca," J. Bacteriol.,* 173:3397–407 (1991), which is hereby incorporated by reference) by PCR. An upstream primer, 5'-CAG CTA TGA CCA TGA TTA CGC C-3' (SEQ ID NO:3), and a downstream primer, 5'-CCT AGA ACG GGA ATT CAT TGG CCG CC-3' (SEQ ID NO:4), contained PstI and EcoRI restriction sites, respectively. The fragment was amplified from pBW2 (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene From *Thermomonospora Fusca," Appl. Environ. Microbiol.,* 59:3032–43 (1993), which is hereby incorporated by reference) and then digested with PstI and EcoRI, while the construct pEP1 and plasmid pBluescript SK+ (Strategene, La Jolla, Calif.) were digested with EcoRI and HindIII, and PstI and HindIII, respectively. The three digested fragments were subsequently purified using Geneclean II kit and ligated into a single recombinant construct that contained the desired restriction sites of PstI and KpnI (from pBluescript SK*), pLT1 promoter and Spe2 leading peptide of endoglucanase E2 (551 bp, Lao, G. et al., "DNA Sequences of Three Beta-1,4-endoglucanase Genes From *Thermomonospora Fusca," J. Bacteriol.,* 173:3397–407 (1991), which is hereby incorporated by reference), and phyA gene (1365 bp). After the construct was digested with PstI and KpnI, the resulting fragment was inserted into the expression vector pSES1, and the formed shuttle plasmid (pSPP1, 9131 bp) was transformed into the host *S. lividans* protoplasts according to Hopwood et al. (Hopwood, D. A., et al., *Genetic Manipulation of Streptomyces—A Laboratory Manual,* The John Innes Foundation, Norwich, England (1985), which is hereby incorporated by reference). Likewise, a control was prepared by transforming *S. lividans* with expression vector pSES2.

Three shuttle plasmids with three different signal peptide sequences were constructed to express phyA in the yeast system (See Table 2). The first plasmid was originated from a HindIII digested fragment of pSPp1, including the promoter pLT1, lead sequence Spe2, and the coding region sequence of phyA. The fragment was ligated into the HindIII site of pYES2 treated with calf intestinal alkaline phosphatase and the plasmid was named pYEP1 (7783 bp) after its right orientation was confirmed. The second plasmid contained Spxy, a signal peptide sequence of xylanase gene from *A. pullulans* (Li, X. L. et al., "Cloning, Sequencing, and Regulation of a Xylanase Gene From the Fungus *Aureobasidium Pullulans*) Y-2311-1," *Appl. Environ. Microbiol.,* 60:3160–166 (1994); Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiae*a," *Appl. Environ. Microbiol.,* 62:209–13 (1996), which are hereby incorporated by reference), and phyA gene. Spxy was spliced with phyA by overlap extension (Horton, R. M., "In Vitro Recombination and Mutagenesis of DNA: SOEing Together Tailor-Made Genes," *PCR Protocols: Current Methods and Applications,* 251–61 (1993), which is hereby incorporated by reference) with two successive steps of PCR. One was to amplify Spxy sequence from pCE4 (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiae*a," *Appl. Environ. Microbiol.,* 62:209–13 (1996), which is hereby incorporated by reference) using upstream primer (5'-CCC AAG CTT GAT CAC ATC CAT TCA-3') (SEQ ID NO:5) with a HindIII restriction site (primer 1) and overlapping downstream primer (5'-CGG GGA CTG CTA GCG CAC GTT CGA T-3', primer 2) (SEQ ID NO:6). The other PCR was to amplify the coding region of phyA from pEP1 using overlapping upstream primer (5'-ATC GAA CGT GCG CTA GCA GCA GTC CCC G-3', primer 3) (SEQ ID NO:7) and downstream primer (5'-GCT CTA GAC TAA GCA AAA CAC TCC-3', primer 4) (SEQ ID NO:8) with a XbaI restriction site. The second step of PCR was conducted to merge the two fragments generated from the above two PCR by using the two purified fragments as the templates and primers 1 and 4. The resulting fragment contained HindIII and XbaI restriction sites and was cloned into pSES2. This plasmid was named pYXP1 (7219 bp). The third plasmid contained the signal peptide (Sphy) sequence of phyA and the coding region of phyA, excluding the intron between

them (Hartingsveldt, W. van., et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger,*" *Gene* 127:87–94 (1993), which is hereby incorporated by reference). Two primers, including a 70 bp of upstream primer contained the signal peptide with an engineered KpnI restriction site and a downstream primer that was the same one used for pYXP1 construction (primer 4) were used to amplify the desired fragment from pEP1. The PCR product was digested with KpnI and XbaI and cloned into pSES2, resulting in a plasmid named pYPP1 (7176 bp). All the above three constructs were transformed into *S. cerevisiae* by the method of Ito et al., "Transformation of Intact Yeast Cells Treated with Alkali Cations," *J. Bacteriol.,* 153:163–68 (1983), which is hereby incorporated by reference.

TABLE 2

Signal peptides used for expression of phyA in *S. cerevisiae*

Construct Size (bp)	Peptide	Gene	Organism	Phytase activity[1] (mPU/ml)
pYEP1 7783	Spe2 (93 bp)	Cellulase E2	*T. fusca*	.80
pYXP1 7219	Spxy (102 bp)	Xylanase A	*A. pullulans*	Non-detectable
pYPP1 7176	Sphy (57 bp)	PhyA Phytase	*A. niger*	146
pSES1[2] 7224			*S. cerevisiae*	Non-detectable

[1]The phytase activity was detected in the supernatant of cell culture of Sabouraud-raffinose medium 15 hours after induced by adding galactose. See text for definition of phytase units.
[2]Expression vector for *S. cerevisiae*, used as a control.

Growth medium and induction of the gene expression. In the *E. coli* system, the transformants were grown in 50 ml of LB medium containing 50 μg/ml of ampicillin at 30° C. After the OD_{600} value of the medium reached 0.5 to 0.6, phytase gene expression was induced by adding IPTG (isopropyl b-D-thiogalactopyranoside) into the medium to a final concentration of 1 mM. Three hours after the induction, cells were collected by spinning down at 8000×g for 15 minutes, washed with 1×PBS, and lysed by lysozyme. Soluble and insoluble fractions of the cells were prepared, and a sample containing 500 μg of total protein (Lowry, O. H. et al., "Protein Measurement With the Folin Phenol Reagent," *J. Biol. Chem.,* 193:265–75 (1951), which is hereby incorporated by reference) was suspended in the same volume of 2×SDS buffer and analyzed by SDS-PAGE (Laemmli, U. K., "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4," *Nature* (London), 227:680–85 (1970), which is hereby incorporated by reference).

Recombinant *S. lividans* was grown in TSB broth with 5 μg/ml of *thiostrepton* at 30° C. (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene from *Thermomonospora Fusca,*" *Appl. Environ. Microbiol.,* 59:3032–43 (1993), which is hereby incorporated by reference). After 72 hours incubation, the cells and medium were harvested and prepared for SDS-PAGE (Wilson, D. B., "Biochemistry and Genetics of Actinomycete Cellulases," *Crit. Rev. Biotechnol.,* 12:45–63 (1992), which is hereby incorporated by reference).

Transformants of *S. cerevisiae* were initially grown in Sabouraud-raffinose (4%) medium (100 ml) without uracil for 48 hours, sterile galactose was then added into the medium (2%) to induce phytase expression. Samples of media and cells were collected at various time points, and extracellular and intracellular samples were prepared as described by Li and Ljungdahl, "Expression of *Aureobasidium pullulans* xynA in, and Secretion of the Xylanase from, *Saccharomyces cerevisiae,*" *Appl. Environ. Microbiol.,* 62:209–13 (1996), which is hereby incorporated by reference. When needed, the supernatant of the expressed cell culture fractions was concentrated with Stirred Cells of Amicon (Beverly, Mass.) by using YM10 membranes (MW cutoff of 10,000). Other media were tested accordingly.

Enzyme protein and activity assay. Amounts of expressed phytase protein under various conditions were quantified by the relative densitometry of specific bands in SDS-PAGE, using IS-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, Calif.). Phytase activity in the samples of media and cells was determined as previously described (Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori,*" *Gene,* 133:56–62 (1993), which is hereby incorporated by reference) and the inorganic phosphate released was assayed by the method of Chen, P. S. et al., "Microdetermination of P," *Anal. Chem.,* 28:1756–58 (1956), which is hereby incorporated by reference. One phytase unit (PU) was defined as the amount of enzyme that releases one pmol of inorganic phosphate from sodium phytate per minute at 37° C.

Western blotting (immunoblot) analysis. The soluble fraction of the cell mass of the phytase transformed *E. coli* and the medium supernatant of *S. lividans* and *S. cerevisiae* transformants were collected as for SDS-PAGE. After electrophoresis, the proteins were then transferred onto Protran® nitrocellulose membrane (Schleicher & Schuell, Keene, N.H., USA) in 20 mM Tris-HCl (pH 8.3), 20% methanol, and 0.1% SDS, by using a Mini Trans-Blot cell (Bio-Rad Laboratories). Transfer was done overnight at a constant 50 V and the initial buffer temperature was 4° C. The membranes were then subjected to Western blot analysis. A rabbit polyclonal IgG (Kindly provided by Dr. A. H. J. Ullah of USDA. Dilution, 1: 5,000) against purified native *A. niger* phytase was used as the first antibody. The blotting was finalized using Immuno-Blot Assay Kit (Bio-Rad Laboratories) containing a second antibody conjugated with horseradish peroxidase.

Total RNA isolation and analysis. Total RNA was isolated with TRIzol™ Reagent (GIBCO BRL, Gaithersburg, Md.) from *E. coli* and *S. cerevisiae* transformants 3 and 15 hours after induction, respectively. RNA samples (10 μg per lane) were then separated by formaldehyde agarose (1.5%, wt/vol) gel electrophoresis and transferred to Hyblot membranes (National Labnet, Woodbridge, N.J.) (Davis et al., *Basic Methods in Molecular Biology,* 2nd Ed., Appleton and Lange, Norwalle, Conn. (1994), which is hereby incorporated by reference). A 1.4 kb EcoRI-HindIII fragment in plasmid pEP1 was prepared and was random-primed labeled with ^{32}P using a DNA labeling kit followed by G-50 column purification (Pharmacia Biotech., Piscataway, N.J.) and then hybridized with the blotted RNA membranes in a hybridization oven (Hybaid, Middlesex, UK). The hybridized membranes were exposed to screens in Fuji Imaging Plate and analyzed by Bio-Imaging Analyzer (Kohshin Graphic Systems, Fuji, Japan).

Example 2

Expression of PhyA in *E. coli*

Four hours after the induction, a specific band (~55 kDa) was viewed in SDS-PAGE (12.5%) of the soluble cell fraction, compared to the only expression vector transformed control (See FIGS. **1** and **2**). This band represented 3.8% of the total soluble protein of this fraction. Corre-

spondingly, northern analysis showed overexpression of phyA mRNA in these phytase gene transformants and no signal was viewed in the control cells (See FIG. **3**).

In order to optimize phytase protein expression, the time course and the effects of a series of factors on the expression were studied. These factors included incubation temperature (30 and 37° C.), medium pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), anaerobiosis (adding sterile mineral oil on the top of the growing cells), inorganic phosphate level in the medium (Dassa, E. et al., "The Acid Phosphatases with Optimum pH of 2.5 of *Escherichia coli*," *J. Bio. Chem.*, 257:6669–76 (1982), which is hereby incorporated by reference), and sodium phytate (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM). Results indicated that expression of phytase protein was accumulated linearly with time for the first six hours after induction (See FIG. **4**). Thereafter, the expression remained relatively unchanged although bacterial cells continued to grow. Only medium pH and sodium phytate concentration significantly affected the phytase protein expression. Maximum protein was shown at pH 6.0 and 0.3 mM of sodium phytate, in which phytase protein was increased from 3.8 to 9.6% of the total soluble protein.

No phytase activity was detected extracellularly or intracellularly. This may not be completely unexpected, because the native phytase from *A. niger* is a glycoprotein with a size of 70–80 kDa (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," *Gene* 127:87–94 (1993), which is hereby incorporated by reference). The protein expressed in the *E. coli* system of this study had a size of approximately 55 kDa. Presumably, the lack of glycosylation of the protein and other necessary post-translational modifications during secretion would preclude phytase activity.

Example 3

Expression of PhyA in *S. lividans*

Heterologous genes have been expressed in *S. lividans*, and the resulting products secreted into the medium with enzymatic activity (Ghangas, G. S. et al., "Cloning of the *Thermomonospora Fusca* Endoglucanase E2 Gene in *Streptomyces Lividans:* Affinity Purification and Functional Domains of the Cloned Gene Product," *Appl. Environ. Microbiol.*, 54:2521–26 (1988); Wilson, D. B., "Biochemistry and Genetics of Actinomycete Cellulases," *Crit. Rev. Biotechnol.*, 12:45–63 (1992); Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene from *Thermomonospora Fusca*," *Environ. Microbiol.*, 59:3032–43 (1993), which are hereby incorporated by reference). Similarly, phyA gene was expressed in *S. lividans* and the protein was introduced into the medium, as shown in a specific band in the SDS-PAGE analyzed medium samples (See FIGS. **5** and **6**). This suggested that the signal peptide from endoglucanase E2 gene of *T. fisca* was able to lead phytase protein out of the cell. This protein was 57 kDa and represented 16.2% of the total protein in the medium. Changing medium pH to 6.0 and adding 0.3 mM of sodium phytate in the medium improved the protein yield to 20.3% of the total protein. Because phytase protein was secreted into the medium in such a high level, it should be easy to purify and used effectively for a variety of purposes such as producing phytase antibody. Once again, no increased phytase activity was found either in the medium or in the lysed cells. Although the protein size increased a little bit (2–3%) compared to the one expressed in *E. coli*, presumably due to glycosylation of phytase protein in this expression system, there was still no phytase activity.

Example 4

Expression of PhyA in *S. cerevisiae*

Three different signal peptides were used to compare the efficiency in leading the expressed protein out of the cells (See Table 2). The phytase activity was substantially increased in the Sabouraud-raffinose medium growing the transformants of pYEP1 and pYPP1, but not pYXP1. Visible phytase protein was shown by SDS-PAGE 20 hours after induction (FIG. **7**).

The expression of transformants of pYEP1 and pYPP1 were determined in three different types of medium: Sabouraud-raffinose (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiae*a," *Appl. Environ. Microbiol.*, 62:209–13 (1996), which is hereby incorporated by reference), Sabouraud-glycerol, and a modified general-purposed YEPD medium. As to transformants of pYEP1, similar phytase activity was expressed in the Sabouraud-raffinose and Sabouraud-medium, but there was no activity detected in the YEPD medium. In contrast, phytase activity in the medium cultured with transformants of pYPP1 varied greatly with the different types of medium. The activity was enhanced to 375 mU/ml when Sabouraud-glycerol medium was used. The activity was further increased to 1675 mU/ml, when the medium was changed to YEPD (See Table 3). While the YEPD medium was much cheaper than the Sabouraud-raffinose medium, the phytase yield was increased more than ten-folds. Thus, the putative signal peptide from the fungal phytase gene achieved the most efficient expression of the extracellular phytase activity. Nearly all the protein produced was secreted into the YEPD medium, because very little activity was detected in the yeast cells. The time course of the phytase expression in this system was shown in FIG. **8**.

TABLE 3

Phytase activity expressed from transformant with pYPP1 in different media

	Hours after induction (mPU/ml)[1]		
Medium	0	10	15
Sabouraud-raffinose	22	136	146
Sabouraud-glycerol	6	174	375
YEPD	18	1238	1675

[1]The phytase activity was detected in the supernatant of cell culture of the three media 0, 10, and 15 hours after induced by adding galactose. See text for definition of phytase units.

A variety of microorganisms including bacilli, yeasts, and filamentous fungi have phytase activity, while *A. niger* NRRL3135 strain produces the highest activity (340 mU/ml, Shieh, T. R. et al., "Survey of Microorganisms for the Production of Extracellular Phytase," *Appl. Environ. Microbiol.*, 16:1348–51 (1968), which is hereby incorporated by reference). *Schwanniomyces castellii* CBS 2863 has the highest phytase activity among 21 yeast strains (140 mU/ml, Lambrechts, C. et al., "Utilization of Phytate by Some Yeasts," *Biotechnology Letters*, 14:61–6 (1992), which is hereby incorporated by reference). Clearly, the recombinant yeast strain transformed with pYPP1 in the present study produced much higher phytase activity (1675 mU/ml) than *A. niger* (4-fold) and *S. castellii* CBS 2863 (11-fold). Maximum phytase production can be obtained in the system by optimizing the incubation conditions and modifying the plasmid cassettes (Demolder, J. W. et al., "Efficient Synthesis of Secreted Murine Interleukin-2 by *Saccharomyces*

Cerevisiae: Influence of 3'-Untranslated Regions and Codon Usage," *Gene,* 111:207–13 (1992), which is hereby incorporated by reference).

The high level of phytase activity expression in *S. cerevisiae* was most likely due to the sufficient glycosylation of phytase protein and other post-translational modifications by yeast. After the medium supernatant was concentrated and subjected to SDS-PAGE analysis, there was a band with approximately 110 kDa (See FIGS. **7** and **9**), which was larger than the size of the native protein from *A. niger* (Hartingsveldt, W. van. et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87–94 (1993), which is hereby incorporated by reference). Northern analysis confirmed the specific overexpression of phyA mRNA (See FIG. **10**). These results indicated that the yeast system was efficient to overexpress actively extracellular phytase enzyme. Yeast system has several advantages over bacteria or other systems such as *A. niger* (Hartingsveldt, W. van. et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87–94 (1993), which is hereby incorporated by reference). It carries out post-translational modifications, including proper folding, glycosylation, disulfide bond formation, and proteolysis, during the translocation of proteins through the endoplasmic reticulum and the cell membrane. The secretion of proteins is facilitated by hydrophobic short signal peptides at the N-terminal regions of the protein precursors (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea*," *Appl. Environ. Microbiol.,* 62:209–13 (1996), which is hereby incorporated by reference). Proteins secreted by yeast cells are protected from aggregation and protease degradation. Most importantly, enzyme proteins produced by *S. cerevisiae* are easily purified, because it secretes only a few proteins. Considering the well-known safety of yeast products to both human beings and animals, this system is of great potential for human food and animal feed industry.

Example 5

Properties of the PhyA Phytase Overexpressed in *Saccharomyces cerevisiae*

The overexpressed phytase from transformants of pYPP1 plasmid was concentrated and used to study its property (See Table 4). The enzyme showed two optimum pH ranges: 2 to 2.5 and 5.0 to 5.5. However, enzyme activity at pH 2 to 2.5 was only 60% of the activity at pH 5 to 5.5. There was no activity detected at either pH 1 or 8. The optimum pH was virtually the same as the phytase from *A. niger* (Simons et al., "Improvement of Phosphorus Availability by Microbial Phytase in Broilers and Pigs," *Br. J. Nutr.,* 64:525 (1990), which is hereby incorporated by reference), thus active function in hydrolysis of phytate-P in the gastrointestinal tracts would certainly be expected. The optimum temperature of the enzyme was 60° C., while the current one on the market produced by Gist-Brocades is 55° C. (BASF, 1996). More than 80% of the activity remained at 50 to 55° C., but little activity was detected at 75 or 80° C. Heating the enzyme for 15 min at 37 and 80° C., the remaining activity for the expressed yeast phytase of the present invention was 100 and 63%, respectively, and for BASF Gist-Brocades phytase was 100 and 52%, respectively. The differences between the two enzyme sources at any given temperature were significant (See Table 5). Thus, the yeast phytase appeared to be more heat stable than the current commercial phytase product.

TABLE 4

Characteristics of the overexpressed phytase in yeast[1]

				Optimum pH[2]					
pH	1.0	2.0	2.5	3.0	4.0	5.0	5.5	6.0	8.0
Relative Activity (%)	.5^{e} ± .2	59.7^{c} ± 3	64.8^{c} ± 6	48.1^{d} ± 4	81.0^{b} ± 5	100.0^{a} ± 1	95.0^{a} ± 6	66.3^{c} ± 1	.8^{e} ± .4
				Optimum Temperature[3]					
° C.	25	37	45	50	55	60	75	80	
Relative activity (%)	24.2^{e} ± .8	44.6^{d} ± 3	63.9^{c} ± 8	83.6^{b} ± 2	89.8^{b} ± 4	100.0^{a} ± 4	.6^{f} ± .1	.9^{f} ± .2	

[1]Data are means of relative activity ± standard deviation (n = 4). Means in a row with different superscript letters differ (P < 0.05). The general linear model of the statistical analysis system (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multiple treatment mean comparison. Significance level was set as P < 0.05.
[2]The activity was assayed at 37° C. (see context for phytase unit definition). Different buffers were used: 0.2 mM glycine-HCl buffer for pH 1.0 to 3.5; 0.2 mM sodium citrate buffer for pH 4.0 to 6.5; and 0.2 mM Tris-HCl buffer for pH over 7.
[3]Optimum temperature was determined at pH 5.5 (0.2 mM sodium citrate buffer).

TABLE 5

Comparison of the thermostability of overexpressed phytase in yeast and Gist-Brocades phytase produced by *A. niger* [1,2]

Relative activity, %	37° C.	80° C.
Yeast phytase	100^{a} ± 1	63^{b} ± 1
A. niger phytase	100^{x} ± 3	52^{y} ± 2
P [3] <		.03

[1] Data are means of relative activity ± standard deviation (n = 3). Means in a row with the different superscript letters differ (P < 0.05). The general linear model of the statistical analysis system (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multiple treatment mean comparison. Significance level was set as P < 0.05.
[2] The enzyme was heated for 15 minutes at different temperatures before reacting at 37° C. and pH 5.5.
[3] Significance (P values) of t-test between the activity of the two phytases at each temperature setting.

Although it is unclear how such improvement in thermostability is related to different post-translational modifications (folding, cleavage, glycosylation, etc.), (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea*," *Appl. Environ. Microbiol.,* 62:209–13 (1996), which is hereby incorporated by reference), it is certainly advantageous to have more thermostable phytase enzyme that can

hopefully be resistant to the heat during feed pelleting, which is a problem with the current Gist-Brocades phytase.

Example 6

In Vitro Hydrolysis of Phytate-P from Corn, Soy, and Wheat Middlings by the Expressed Yeast Phytase

The expressed yeast phytase released phytate-P from corn and soybean meal as effectively as the Gist-Brocades phytase based on per unit activity (See Table 6). As expected, the hydrolysis of phytate-P was a function of time and activity dosage. The expressed yeast phytase was also effective in releasing phytate-P from wheat middling, indicating its great potential in bread fermentation. Because the wheat middling used in this study contained much higher intrinsic phytase activity than commonly used wheat flour, much greater effect of the expressed yeast phytase on improving flour phytate-P hydrolysis and in trace element releasing would be expected, when it is used in a bakery (Hall, M. N. et al., "The Early Days of Yeast Genetics," Cold Spring Harbor Laboratory Press (1993), which is hereby incorporated by reference).

TABLE 6

Free phosphorus released from corn, soybean meal (SBM), and wheat middlings by overexpressed yeast phytase and fungus *A. niger* phytase in vitro[1]

Yeast phytase (PU/kg)		0	100	250	500	1000	250 (fungus phytase)
		Free phosphorus (mg/g)					
Corn:	1 hour	$.23^{d} \pm .03$	$.64^{c} \pm .08$	$1.14^{b} \pm .18$	$1.46^{a} \pm .04$	$1.54^{a} \pm .04$	$1.16^{b} \pm .15$
	4 hour	$.36^{c} \pm .02$	$1.26^{b} \pm .04$	$1.60^{a} \pm .03$	$1.66^{a} \pm .06$	$1.72^{a} \pm .04$	$1.68^{a} \pm .04$
SBM:	1 hour	$.68^{d} \pm .01$	$1.18^{cd} \pm .02$	$1.62^{c} \pm .18$	$2.48^{b} \pm .32$	$3.13^{a} \pm .19$	$1.68^{c} \pm .2$
	4 hour	$.73^{d}$	1.67^{c}	2.69^{b}	3.41^{a}	3.71^{a}	2.78^{b}
Wheat	1 hour	$3.56 \pm .39$		$4.11 \pm .64$	$4.67^{2} \pm .05$		
Middlings:	4 hour	$5.63 \pm .5$		$6.02 \pm .48$	$6.38^{2} \pm .07$		

[1]Each sample of 5 g was stirred in 20 ml of 0.2 mM sodium citrate buffer at 37° C. for 1 or 4 hours. The supernatant was obtained by spinning for 15 minutes at 8000 g. After going through Whatman 541 filter paper, the sample was subjected to free P assay by the method of Chen, P. S. et al., "Microdetermination of P," Anal.Chem., 28: 1756–58 (1956), which is hereby incorporated by reference. Data in the table are means of relative activity ± standard deviation (n = 4). The General Linear Model of the Statistical Analysis System (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multipletreatment mean comparison. Significance level was set as $P < 0.05$. A significant difference existed between 1 and 4 hour for every feed at each dose of enzyme as analyzed by t-test. Means in a row with different superscript letters differ ($P < 0.05$).
[2]n = 2

The overexpression of *Aspergillus niger* phytase (phyA) in *Escherichia coli, Streptomyces lividans,* and *Saccharomyces cerevisiae* were compared to develop an efficient and simple system to produce phytase economically. A 55 kDa soluble intracellular protein, representing 9.6% of the total soluble protein, was expressed in *E. coli* by using pET25b (+) system. A 57 kDa extracellular protein, representing 20.3% of the total protein in the medium, was expressed in *S. lividans* by using a shuttle plasmid containing the pLT1 promoter and SpeII leading peptide of endoglucanase E2. No increase in phytase activity was shown in either expression system, presumably due to the lack of glycosylation and other necessary post-translational modification. In contrast, high extracellular phytase activity was produced in *S. cerevisiae* transformed with phyA gene. Three different signal peptides and three different types of medium were compared to identify the best expression vector and condition. Use of the signal peptide Sphy from phyA gene and YEPD medium produced the highest extracellular phytase activity. The overexpressed phytase in yeast was approximately 110 kDa, had two pH optima: 2.0 to 2.5 and 5.5 to 6.0, and the optimum temperature was at 60° C.

Example 7

Methods and Materials for Expression of phyA in *Pichia*

Host and vector. An EasySelect™ *Pichia* Expression Kit was purchased from Invitrogen (San Diego, Calif.). The kit provides hosts and vectors to express the gene either intracellularly or extracellularly, in strains of either Mut^{+} or Mut^{s} (Methanol utilization normal or slow). X33 was used as a Mut^{+} strain and KM71 as a Mut^{s} strain. Two vectors were used, pPICZ B (3.3 kb) and pPICZαA (3.6 kb), both use AOX1 as the promoter.

Construction of the Expressing Vectors. To compare the effect of different signal peptides on the expression of PhyA in *Pichia* system, two constructs were prepared. First, a 1.4 kb EcoRI-KpnI fragment, containing the PhyA sequence encoding the mature phytase protein, was ligated into pPICZαA. In this plasmid (pPICZα-phyA), PhyA was led by an alpha-factor, a very general-used signal peptide from *Saccharomyces cerevisiae.* Second, a 1.4 kb KpnI-XbaI fragment of pYPP1 was ligated into the vector (the coding region of phyA was led by its own signal peptide that was very effective in secreting the expressed phytase in *Saccharomyces cerevisiae.*)

Transformation and expression. The confirmed constructs were linearized by PmeI and transformed into GS115 and KM71, by EasyComp™ provided by the kit. Neocin™ was used to select the positive colonies. After a single colony was inoculated into 10 ml of MGY medium and grown to OD_{600} of 2–6 at 30° C., the cells were collected by centrifugation and resuspended into 10 ml of MMY medium (containing 0.5% of methanol). The samples were collected every 12 or 24 h after induction. The cells were separated from the supernatant and lysed with glass beads in breaking buffer. Phytase activity in the supernatant and cells was assayed as described previously. SDS-PAGE and Western

blot were conducted to determine the size and relative amount of the expressed protein.

Example 8

PhyA Phytase Activity in *Pichia*

The expression construct using alpha-factor as the signal peptide for phyA was transformed into two *Pichia* strains. KM71 is a methanol utilization slow strain, while X33 is a *Pichia* wild-type utilizing methanol efficiently. The screening and incubation were conducted in 10 ml shake flasks under 29–30° C. For the transformants of KM71, 19 out of 20 picked colonies had extracellular phytase activity greater than 6 units/ml of culture supernatant after induction for 24 hours. Colony No. 13 showed the highest activity of 26 units/ml after incubated for 108 hours. For the transformants of X33, all colonies (20/20) had more than 10 units/ml after induced for 24 hours. One of the colonies (#101) produced phytase activity of 65 units/ml of supernatant. A time course study of the phytase expression in KM71 and X33 was summarized in FIG. **11**. Despite the difference of these two strains in utilizing methanol and, therefore, the ability in expressing phytase, it was found that alpha-factor was correctly processed by yeast cells. Besides, almost all of the expressed protein was secreted into the medium since not more than 5% of the total activity expressed was found intracellularly.

Effects of inorganic phosphorus and pH of media on the phytase expression were studied in the media (BMGY and BMMY) using a phyA recombinant of X33 (#101). The medium containing 50 mM phosphate produced the highest phytase activity, 66 units/ml at 168 hours after induction. By including 50 mM phosphate in the media, the effect of different pH of this buffer (3, 4, 5, 6, 7, and 8) on expression was also studied. When the pH was 6, this X33 transformant produced 75 units phytase/ml supernatant. Based on the protein concentration and SDS-PAGE analysis, the expression phytase protein yield was estimated to be between 3 to 4 mg/ml.

Example 9

Properties of the PhyA Phytase Expressed in *Pichia*

Molecular size and deglycosylation of the expressed phytase. After the supernatant of the medium inoculated with the phyA transformant was subjected to SDS-PAGE, a strong band around 95 kDa was seen (FIG. **12**). This was almost the only viewed protein in the supernatant. The expressed phytase reacted efficiently with the rabbit polyclonal antibody raised against purified native *A. niger* phytase. This indicated that the immunoreactivity of the expressed phytase was essentially the same as that of the native phytase from *A. niger*. The size was decreased to 50 kDa by deglycosylation using Endo H. The phyA antibody also reacted with the deglycosylated phytase. In addition, deglycosylation, conducted under native conditions, reduced the phytase activity about 15%, indicating that glycosylation was important for the activity of the phytases. Moreover, glycosylation affected the thermostability of the enzymes (FIG. **13**).

Northern analysis. As showed in FIG. **14**, a 1.3 kb phyA DNA probe hybridized with the mRNA of the induced transformants from both KM71 (#13) and X33 (#101). Response was also seen from the transformants prior to induction. Probably, the expression of phyA in this system was not controlled strictly at the level of transcription.

Optimal pH and temperature and phytate-phosphorus hydrolysis. Similar to *A. niger* phytase, the expressed phytase had two optimum pH, 2.5 and 5.5 (FIG. **15**). The optimum temperature of the expressed phytase was 60° C. (FIG. **16**). When the expressed phytase was incubated with soy samples at 100, 200, 400, 800 mU/g of sample at 37° C., phosphorus was released in a linear fashion with the phytase dose (FIG. **17**).

Example 10

Methods and Materials for Overexpression of *E. coli* appA Gene in *Saccharomyces cerevisiae*

Gene and Protein. This gene, originally defined as *E. coli* periplasmic phosphoanhydride phosphohydrolase (appA) gene, contains 1,298 nucleotides (GeneBank accession number: M58708). The gene was first found to code for an acid phosphatase protein of optimal pH of 2.5 (EcAP) in *E. coli*. The acid phosphatase is a monomer with a molecular mass of 44,644 daltons. Mature EcAP contains 410 amino acids (Dassa, J. et al., "The Complete Nucleotide Sequence of the *Escherichia coli* Gene appA Reveals Significant Homology Between pH 2.5 Acid Phosphatase and Glucose-1-Phosphatase," *J. Bacteriology,* 172:5497–5500 (1990), which is hereby incorporated by reference). Ostanin, K. et al. ("Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.,* 267: 22830–36 (1992), which is hereby incorporated by reference), overexpressed appA in *E. coli* BL21 using a pT7 vector and increased its acid phosphatase activity by approximately 400-fold (440 mU/mg protein).

The gene and a host *E. coli* strain CU 1869 (No. 47092) were purchased from ATCC. The gene, an insert of 1.3 kb, was transformed into *E. coli* strain BL21 (no. 87441) using an expression vector pAPPA1 (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.,* 267: 22830–36 (1992), which is hereby incorporated by reference).

Host and Vector. The vector for overexpressing appA gene in *Saccharomyces cerevisiae* was pYES2 and the host was INVScI (Invitrogen, San Diego, Calif.).

Construction of the Expression Vector. Initially, a 1.3 kb XbaI fragment was isolated from pAPPA1. This fragment contained the appA gene with its own signal peptide. After being ligated into the XbaI site of pYES2, the construct (PYES2-appA) was transformed into *Saccharomyces cerevisiae*. But, no phytase activity was increased in either extra- or intra-cellular parts compared to the controls. pAPPA1 and pYPP1 (PhyA and its signal peptide in pYES2) were cotransformed into the yeast strain. Again, no increase in phytase activity due to pAPPA1 was detected in the media or the yeast cells.

Two primers were synthesized to construct the signal peptide of PhyA gene with the coding region of appA gene. One was 80 bp long containing the PhyA signal peptide and a KpnI site at 5' end: GGG GTA CCA TGG GCG TCT CTG CTG TTC TAC TTC CTT TGT ATC TCC TGT CTG GAG TCA CCT CCG GAC AGA GTG AGC CGG AG (SEQ ID NO:9). The other primer was 24 bp long, with an EcoRI site at its 3' end: GGG AAT TCA TTA CAA ACT GCA GGC (SEQ ID NO:10). The PCR was run for 25 cycles, with 1 min denaturing at 95° C., 1 min annealing at 58° C., and 1 min chain extending at 72° C. A 1.3 kb fragment was

amplified, digested, and ligated into pYES2. After the insert was confirmed by restriction mapping, the construct (pYES2-SphyA-appA) was transformed into INVScI by lithium acetate method.

Expression. The selected transformants were inoculated into YEPD medium. The expression was induced by adding galactose into the culture after OD_{600} reached 2, as described previously. The cells were harvested 15 or 20 h after induction.

Activity Assay. Acid phosphatase activity was assayed at 37° C. in 25 mM glycine-HCI buffer (pH 2.5), using p-nitrophenyl phosphate as the substrate (stock 250 mM). Reaction buffer of 1.7 ml was added into 0.1 ml samples. After they were incubated for 5 min in a 37° C. waterbath, 0.2 ml of prewarmed substrate was added and mixed. The reaction solution was transferred into a prewarmed cuvette and incubated for 2 min in a 37° C. spectrophotometric compartment. The released p-nitrophenol was read continuously for 5 min at 405 nm for enzyme activity calculation.

In vitro study. Soybean meal (5.0 g) was suspended into 20 ml of 20 mM citrate buffer, pH 5.5, mixed with 200 mU of phytase, incubated at 37° C. for 4 h with continuous shaking. After chilling on ice for 10 min, the slurry was transferred into a centrifuge tube and spun for 15 min at 15,000×g. The supernatant was used to determine free phosphorus.

Example 11

Quantitation of Phytase Activity from Overexpression of *E. coli* appA Gene in *Saccharomyces cerevisiae*

The intracellular acid phosphatase activity in the appA overexpressed *E. coli* (pAPPA1) was 440 mU/mg protein. Unprecedently, an intracellular phytase activity greater than 4900 mU/mg protein was found in the transformed strain. But, there was only minimal phytase activity in the control (BL21). Thus, this acid phosphatase gene also codes for a phytase. The appA gene sequence was aligned with that of PhyA and found that these two genes shared 23% of identity.

Transforming INVScI with the construct of pYES2-Sphy-appA (led by the signal peptide of PhyA) produced extracellular phytase activity in the supernatant that was 2,000-fold greater than those of the wild type or of the transformant containing appA gene plus its own signal peptide (See Table 7).

TABLE 7

Extracellular phytase activity in transformants of appA gene with different signal peptides

Construct	Signal	Activity (mU/ml)	Activity (mU/mg protein)
PYES-appA	appA	Undetectable	Undetectable
pYES2-SphyA-appA	PhyA	1,158	445

The effects of medium (YEPD) inorganic phosphorus, phytate, pH, and temperature on the expression of phytase activity by pYES2-Sphy-A-appA are presented in Table 8. The highest phytase activity was 2,286 mU/ml (633 mU/mg protein) at the optimal condition.

TABLE 8

Effect of different conditions in the YEPD medium on phytase activity expression of pYES2-SphyA-appA in yeast.

Medium Conditions	Activity (mU/ml)
Phosphorus, mg/100 ml	
0	1402
1	714
5	722
10	456
Sodium phytate, g/100 ml	
0	870
0.1	1019
1.0	1748
Ph	
5.0	892
7.0	996
8.0	2286
Temperature, ° C.	
25	312
30	1036
37	996

The thermostability of the overexpressed extracellular phytase activity produced by the yeast transformant was greater than that of the intracellular phytase produced by *E. coli* transformed with pAPPA1 (See Table 9). Heating the extracellular phytase for 15 min at 80° C. resulted in 30% of loss of its phytase activity, while almost all the phytase activity from *E. coli* was lost under the same condition.

TABLE 9

Effect of heating different sources of phytases under 80° C. for 15 min on their activities

Phytase	Relative activity after heating, %
appA in *E. coli*	0.1
appA in *S. cerevisiae*	69
PhyA in *S. cerevisiae*	66
BASF phytase	50

Comparisons of the effect on releasing phosphorus from soybean meal by phytases (200 mU) of *E. coli,* overexpressed AppA in yeast, and BASF are presented in Table 10. The results indicate that all three sources of phytases released phytate-phosphorus effectively from soybean meal.

TABLE 10

Free phosphorus released from soybean meal by different sources of phytases

Phytase	Phosphorus (mg/g)
appA in *E. coli*	1.11
appA in *S. cerevisiae*	0.69
BASF	0.87

E. coli appA (acid phosphatase) gene when expressed in *Sacchacromyces cerevisiae* produces extracellular phytase activity in the media that was more than 2,000-fold greater than the control. The overexpressed phytase effectively releases phytate-phosphorus from soybean meal, and seems to be more thermostable than the presently available com-

mercial phytase or the intracellular phytase produced in *E. coli* by the same gene (appA).

Example 12

Methods and Materials for Overexpressing the *E. coli* appA Gene Encoding an Acid Phosphatase/Phytase in *Pichia pastoris*

Gene and Protein. The appA gene and the host *E. coli* strain CU1867 (No. 47092) were obtained from ATCC. The gene, an insert of 1.3 kb, was transformed into *E. coli* strain BL21 (No. 87441) using an expression vector pAPPA1 (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.*, 267:22830–36 (1992), which is hereby incorporated by reference).

Host and Vector. An EasySelect™ *Pichia* Expression Kit was obtained from Invitrogen (San Diego, Calif.). The kit provides hosts and vectors to express the gene either intracellularly or extracellularly in a wild-type strain (X-33). Two vectors were used, pPICZ B (3.3 kb) and pPICZαA (3.6 kb), both use AOX1 as the promoter.

Construction of the Expression Vector. Two primers were used to amplify the appA gene from pAPPA1 and two restriction sites EcoRI and KpnI were produced at the 5' and 3' ends, respectively.

(SEQ ID NO:11)
Upstream primer: GGA ATT CCA GAG TGA GCC GGA

(SEQ ID NO:12)
Downstream primer: GGG GTA CCT TAC AAA CTG CAC G

Template: pAPPA1 DNA isolated from ATCC 87441

PCR was run for 30 cycles, with 1 min denaturing at 94° C., 1 min annealing at 55° C., and 1 min chain extending at 72° C. A 1,245 base-pair fragment was amplified, digested with EcoRI and KpnI, and ligated (16° C. overnight) into pPICZ B (3.3 kb) and pPICZαA (3.6 kb). The ligation was confirmed by restriction mapping after transforming the constructs into DH5α.

Transformation of the construct into *Pichia* (X33). For each transformation, 100 μg of plasmid DNA was prepared and linearized by digesting with PmeI. After linearization, the DNA was purified and resuspended into 10 μL of sterile, deionized water. Half amount of the DNA was actually used for each transformation. Electroporation and the EasyComp chemical kit (Invitrogen) were both used to transform the DNA into X33. In the case of electroporation, an Electro Cell Manipulator (ECM 600, Gentromics, BTX Instrument Division, San Diego, Calif. 92121) and 2 mm cuvettes were used. The resistance was 186 Ohm, the charging voltage was 1.5 kilovolts, and the actual charging length was approximately 7 milliseconds. The electroporated cells were incubated on YPD agar plates containing 100 mg Zeocin/mL at 30° C. for 2–4 days for colony growth. In the case of chemical transformation, cells were grown on YPDS agar plates containing 100 mg Zeocin/mL. Compared with the electroporation, the chemical method had lower transformation efficiency.

Expression. Single colonies were inoculated into 10 ml of MGY medium (30 ml tube) and grown (16–18 h) to OD_{600} of 5–6 at 28–30° C. in a shaking incubator (200 rpm). The cells were collected by centrifugation (2,000 rpm) and resuspended into 10 ml of BMMY medium (containing 0.5% of methanol) to induce the expression. The samples (200 μL) were collected every 12 or 24 h after induction. Methanol (100%) was added at 100 μL every 24 to maintain a concentration of 0.5–1% in the media.

Assays. The cells were separated from the media (supernatant) and lysed with glass beads in breaking buffer. Extracellular phytase activity in the supernatant and intracellular phytase activity in the lysed cells were assayed as described previously (0.2 M citrate buffer, pH 5.5 under 37° C. using 10 mM sodium phytate). Acid phosphatase activity was assayed at 37° C. in 25 mM glycine-HCl buffer (pH 2.5), using p-nitrophenyl phosphate as the substrate (stock 250 mM). Reaction buffer of 1.7 ml was added into 0.1 ml samples. The released p-nitrophenol was read continuously for 5 min at 405 nm for enzyme activity calculation. SDS-PAGE (12%) was conducted to determine the size and relative amount of the expressed protein. The optimal pH and temperature of the expressed phytase were determined as described in the results.

In vitro study. Soybean meal (5.0 g) was suspended into 20 ml of 20 mM citrate buffer, pH 5.5, mixed with different levels of phytase, and incubated at 37° C. for 4 h with continuous shaking. After being chilled on ice for 10 min, the slurry was transferred into a centrifuge tube and spun for 15 min at 15,000×g. The supernatant was used to determine free phosphorus.

Example 13

Colony Phytase Activity Screening for *Phicia pastoris* Overexpressing the *E coli* appA Gene

Wild-type *Pichia* X33 produces minimal phytase activity intracellularly (<0.03 U/mg protein) or extracellularly (<0.05 U/mL). The X33 cells transformed with the appA gene inserted into pPICZB (without the α-factor and presumably produces intracellular phytase) did not show any increase in phytase activity (extracellular, 0.2 U/mL and intracellular, 0.05 U/mg protein).

Transforming X33 cells with the construct of pPIZαA-appA (led by the signal peptide of α-factor) produced extracellular phytase activity in the media. Initially, 72 colonies were screened. Only two colonies had activity <1 U/mL 40 hours after induction. Most of the colonies had activity ranging from 10 to 20 U/mL 40 hours after induction. All of the 70 colonies had phytase activity >80 U/mL 118 hours after induction. The highest phytase activity so far detected was 215 U/mL, 192 hours after the induction (See Table 11).

TABLE 11

Range of extracellular phytase activity in X33 colonies transformed with pPIZαA-appA 40 and 118 hours after induction.

Number of Colonies	40 hours after induction	118 hours after induction
2	<1 U/mL	
6	1 to 10 U/mL	
36	11 to 20 U/mL	
28	>20 U/mL	
70		>80 U/mL

Phytase and acid phosphatase activities in the transformant expressing 215U phytase activity/mL were compared with those of the wild-type of X33 (192 hours after induction) (See Table 12). Almost all of the expressed phytase protein was secreted from the cells, indicating that α-factor was a very effective signal peptide for phytase secretion.

TABLE 12

Phytase and acid phosphatase activities in the pPIZαA-appA transformant and the wild-type of X33 192 hours after induction.

	Wild-type X33		pPIZαA-appA transformant	
	Extra-cellular U/mL	Intra-cellular U/mg protein	Extra-cellular U/mL	Intra-cellular U/mg protein
Phytase	0.05	0.03	215	0.5
Acid phosphatase	0.01	0.002	5.88	0.9

Transformants of *E. coli* with the same acid phosphatase appA gene had intracellular phytase activity of 5 U/mg protein (Ostanin et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.*, 267:22830–36 (1992), which is hereby incorporated by reference). Transforming PhyA gene in *A. niger* produced an extracellular activity of 7.6 U/ml (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," *Gene* 127:87–94 (1993), which is hereby incorporated by reference). Compared with these results, the phytase expression system in *Pichia* is a very efficient expression system.

Example 14

Time-Course of Phytase Expression

There was a linear increase in extracellular phytase activity in the media almost in all of the selected colonies up to 192 hours after induction. FIG. **18** summarized the activity changes of five selected colonies from 24 to 163 hours after induction.

Example 15

Effects of Medium pH on the Expression of Phytase (Colony #23, Activity 136 U/mL at 186 h)

Using 0.1 M phosphate buffered media, the effects of different pH on the production of extracellular phytase in the transformants were studied against a control medium without buffer (pH 7.0). The medium buffered to pH 6 produced the highest phytase activity (See FIG. **19**).

Example 16

Size of the Expressed Extracellular Phytase

Using SDS-PAGE (12% gel) analysis, a clear band was noticed in the medium supernatant of culture inoculated with three different colonies (See FIG. **20**). The size was around 55 kDa, probably partially glycosylated. Because the expressed protein represented almost the only visible band in the supernatant, it would be convenient to collect the enzyme product without the need for a tedious purification.

Example 17

Optimum pH and Temperature of the Expressed Extracellular Phytase (Colony #23)

The optimum pH of the expressed phytase was 2.5 to 3.5 (See FIG. **21**). This is significantly different from that of phyA phytase either from *A. niger* (BASF) or our other expression systems. It is ideal for phytase function at the stomach pH.

The optimum temperature of the expressed enzyme was 60° C. (See FIG. **22**).

Example 18

Effect of the Expressed Phytase on Phytate-Phosphorus Hydrolysis from Soybean Meal

This overexpressed *E. coli* phytase (Colony #23) effectively hydrolyzed phytate-phosphorus from soybean meal (See FIG. **23**). The release of free phosphorus in the mixture was linear from 0 to 800 mU of phytase/g of feed.

Example 19

Effects of the Expressed *E. coli* AppA Phytase by *Pichia pastoris* on Phytate Phosphorus Bioavailability to Weanling Pigs

To determine the nutritional values of the expressed *E. coli* phytase by *Pichia* in swine diets, the efficacy of this new phytase was compared with those of inorganic phosphorus or the commercially available microbial phytase (Natuphos™, BASF Corp., Mt. Olive, N.J.). Forty-eight weanling pigs were selected from multiparous sows at Cornell Swine Research Farm. The pigs were weaned at 21 days of age and fed a commercial creep feed until day 28. They were then placed two per pen with six pens assigned randomly per treatment. The pigs were given two weeks to adjust to the corn-soybean meal basal diet (Table 13).

TABLE 13

Formulation of the Experiment Diets for Pigs.

Ingredient	+C % diet	−C % diet	YP % diet	MP % diet
Corn	60.5	61.57	61.07	61.07
Whey Protein Concentrate	3	3	3	3
SBM 44%	30	30	30	30
Corn Oil	3	3	3	3
Lime	0.8	0.93	0.93	0.93
Di-calcium phosphate	1.2	0	0	0
Vitamin and Mineral premix	0.5	0.5	0.5	0.5
ECAP premix	0	0	0.5	0
MP premix	0	0	0	0.5
Salt	0.5	0.5	0.5	0.5
CSP 250	0.5	0.5	0.5	0.5
Total	100	100	100	100
CP	20.6	20.6	20.6	20.6
Ca	0.73	0.47	0.47	0.47
P_{total}	0.6	0.39	0.39	0.39

Note:
All premixes use corn as the carrier
Vitamin and Mineral Premix supplies: 2,540 IU Vit. A, 660 IU Vit. D, 15 IU Vit. E, 2.2 mg Vit. K,
3.3 mg Riboflavin, 13.2 mg Pantothenic acid, 17.6 mg Niacin, 110.1 mg Choline, 1.98 ug B-12,
37.4 mg Mn, 0.6 mg I, 10 mg Cu, 0.3 mg Se, 100 mg Zn, and 100 mg Fe per Kg of diet

Then, each pen received one of the four treatment diets. The positive control group (+C) received the basal diet supplemented with dicalcium phosphate. The negative control group (−C) received just the basal diet. The yeast phytase group (YP) received the basal diet supplemented with the expressed *E. coli* phytase at 1,200 U/kg of feed. The microbial phytase group (MP) received the basal diet

supplemented with the BASF phytase at 1,200 U/kg of feed. Pigs were given free access to feed and water. Body weight gain of individual pigs was recorded weekly. Daily feed intake of individual pens was recorded daily. Blood samples from each of the individual pigs were taken weekly to assay plasma inorganic phosphorus concentrations. The results of body weight (BW), average daily gain (ADG), average feed intake (ADFI), and feed/gain ratio (F:G), and plasma inorganic phosphorus (PP) are presented in Table 14.

TABLE 14

Summary of PP, BW, ADG, ADFI, and F:G of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
		Initial		
PP	12.99	13.02	13.07	13.54
BW	11.54	11.63	12	11.5
		Week 1		
PP	10.83^{A}	6.48^{C}	8.59^{B}	8.35^{B}
BW	14	13.83	14.29	13.92
ADG	.351	.316	.327	.345
ADFI	.700	.684	.697	.697
F:G	2.04	2.20	2.18	2.13
		Week 2		
PP	9.76^{A}	5.64^{D}	8.72^{B}	7.84^{C}
BW	18.04	17.42	17.83	17.71
ADG	.578	.512	.506	.542
ADFI	.833	.855	.784	.837
F:G	1.46^{B}	1.67^{A}	1.56^{AB}	1.55^{AB}
		Week 3		
PP	11^{A}	6.26^{C}	8.64^{B}	8.13^{B}
BW	22.58	21.17	22	22.21
ADG	$.649^{A}$	$.536^{B}$	$.595^{AB}$	$.643^{AB}$
ADFI	1.166	1.02	1.001	1.003
F:G	1.8	1.92	1.71	1.36
		Week 4		
PP	10.94^{A}	6.31^{C}	9.65^{B}	9.2^{B}
BW	27.54	25.29	27.79	27.38
ADG	$.708^{AB}$	$.589^{B}$	$.827^{A}$	$.738^{AB}$
ADFI	1.395^{A}	1.049^{B}	1.309^{A}	1.273^{AB}
F:G	1.98	1.87	1.59	1.73

[1]Numbers in the same row without sharing a common letter are significantly different. Analysis of difference was conducted with the Bonferroni (Dunn) T-tests with alpha = 0.05 and df = 20

In addition, there was severe phosphorus deficiency in the negative control group in the end of the four-week experiment. But, there was no sign of phosphorus deficiency in the other three groups. Clearly, the expressed *E. coli* phytase by *Pichia* was at least, if not more, effective as the commercial microbial phytase in improving bioavailability of phytate-phosphorus from the corn-soybean meal diets for weanling pigs. It can be used to replace inorganic phosphorus supplementation to weanling pigs.

Example 20

Effects of the Expressed *E. coli* AppA Phytase by *Pichia pastoris* on Iron (Fe) and Phytate Phosphorus Bioavailability to Weanling Pigs

To determine the effect of the overexpressed *E. coli* phytase by *Pichia* on dietary phytate—bound Fe bioavailability to weanling pigs, 20 anemic pigs (21 days old and 7.3 g hemoglobin (Hb)/dL blood) were selected. The pigs were fed an Fe-deficient creep feed for 7 days and housed in metabolic cages at the age of 28 days old. The pigs were then fed the experimental diets at the age of 35 days old for 5 weeks. The treatment diets were as follows: Fe-deficient basal diet (−C, with added inorganic phosphorus), Fe-supplemented diet (+C), the Fe- and phosphorus-deficient diet supplemented with the expressed *E. coli* phytase (YP), or the commercial microbial phytase (BASF, MP) at 1,200 U/kg of feed. Body weight (BW), packed cell volume (PCV), Hb, and plasma inorganic phosphorus (PP) were determined weekly. The results are presented in Table 15.

TABLE 15

Summary of PCV, Hb, BW, and PP of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
		Initial		
PCV	25	25	26	24
Hb	7.73	7.22	7.85	7.08
BW	8.14	8.27	8.17	7.45
PP	7.92	7.76	7.21	7.36
		Week 1		
PCV	25	26	29	27
Hb	7.62	8.3	8.77	7.88
BW	9.44	8.84	9.63	8.57
PP	8.41	8.45	8.48	8.22
		Week 2		
PCV	29	26	30	28
Hb	8.6	7.34	8.93	8.27
BW	12.32	10.13	11.91	10.84
PP	10.28^{a}	9.05^{ab}	8.89^{ab}	8.22^{a}
		Week 3		
PCV	36^{a}	29^{b}	34^{a}	33^{a}
Hb	11.55^{a}	8.2^{b}	10.84^{a}	9.96^{ab}
BW	16.77^{a}	13^{b}	15.62^{ab}	14.62^{ab}
PP	12.14^{a}	11.37^{ab}	10.25^{bc}	9.71^{c}
		Week 4		
PCV	39	34	38	36
Hb	12.99^{a}	10.11^{b}	12.27^{a}	11.35^{ab}
BW	21.36^{a}	17.37^{b}	19.44^{ab}	18.56^{ab}
PP	10.19^{a}	9.34^{ab}	9.49^{ab}	8.8^{b}
		Week 5		
PCV	40	38	40	39
Hb	13.52^{a}	12.24^{b}	13.64^{a}	13.13^{ab}
BW	26.53	22.59	24.27	23.43
PP	9.27^{a}	8.95^{ab}	8.79^{ab}	8.02^{n}

[1]Values are means (n = 5). Means within the same row without sharing a common superscript ltter are significantly different (P < 0.10).

In conclusion, the overexpressed *E. coli* phytase by *Pichia* was at least as effective as the BASF phytase in improving phytate-phosphorus and Fe utilization in corn-soy diets for weanling pigs.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these therefore are considered within the scope of the invention as defined in the claims which follow.

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What is claimed:

1. A method of producing phytase in yeast comprising:
providing a heterologous polynucleotide from a non-yeast organism which encodes a protein or polypeptide comprising a phytase from *Escherichia coli;*
expressing the polynucleotide in a yeast; and
isolating the expressed protein or polypeptide, wherein said protein or polypeptide catalyzes the release of phosphate from phytate and has increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell.

2. The method according to claim 1, wherein the yeast is selected from the group consisting of *Saccharomyces* species, *Pichia* species, *Kluyveromyces* species, *Hansenula* species, *Candida* species, *Torulaspora* species, and *Schizosaccharomyces* species.

3. The method according to claim 2, wherein the yeast is *Pichia.*

4. The method according to claim 1, wherein the protein or polypeptide has an optimal phytase activity at a pH of less than about 4.

5. The method according to claim 1, wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted.

6. The method according to claim 5, wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.

7. The method according to claim 1, wherein the heterologous polynucleotide which encodes the protein or polypeptide is spliced in frame with a transcriptional enhancer element.

8. The method according to claim 1, wherein the heterologous polynucleotide is carried on a vector for stable transformation.

9. The method according to claim 1, wherein the heterologous polynucleotide is carried on an artificial chromosome.

10. The method according to claim 1, wherein the heterologous polynucleotide is integrated into a chromosome of the yeast.

11. A yeast strain comprising:
a heterologous polynucleotide from a non-yeast organism which encodes a phytase from *Escherichia coli* and is functionally linked to a promoter, wherein the phytase catalyzes the release of phosphate from phytate and has increased thermostability as compared to a phytase expressed in a non-yeast host cell.

12. The yeast strain according to claim 11, wherein the yeast is selected from the group consisting of *Saccharomyces* species, *Pichia* species, *Kluyveromyces* species, *Hansenula* species, *Candida* species, *Torulaspora* species, and *Schizosaccharomyces* species.

13. The yeast strain according to claim 12, wherein the yeast is *Pichia.*

14. The yeast strain according to claim 11, wherein the heterologous polynucleotide which encodes the protein or polypeptide is spliced in frame with a transcriptional enhancer element.

15. The yeast strain according to claim 11, wherein the heterologous polynucleotide is carried on a vector for stable transformation.

16. The yeast strain according to claim 11, wherein the heterologous polynucleotide is carried on an artificial chromosome.

17. The yeast strain according to claim 11, wherein the heterologous polynucleotide is integrated into a chromosome of the yeast.

18. The yeast strain according to claim 11, wherein the protein or polypeptide is preceded by a signal peptide.

19. A vector comprising:
a polynucleotide from a non-yeast organism which encodes a protein or polypeptide comprising a phytase from *Escherichia coli;*
a promoter functionally linked to the polynucleotide encoding the protein or polynucleotide; and
an origin of replication to direct replication of the vector in yeast.

20. The vector according to claim 19 further comprising:
a selectable marker.

21. The vector according to claim 20, wherein the selectable marker is selected from the group consisting of URA3, LEU2, TRP1, HIS3, HIS4, ARG4, and an antibiotic resistance gene.

22. The vector according to claim 19 further comprising:
an origin of replication to direct replication of the vector in a bacterial cell.

23. The vector according to claim 22, wherein the origin of replication is selected from the group consisting of ColE1, Ori, and oriT.

24. The vector according to claim 19, wherein the protein or polypeptide is preceded by a signal peptide.

25. The vector according to claim 19, wherein the polynucleotide which encodes the protein or polypeptide is spliced in frame with a transcriptional enhancer element.

26. Animal feed comprising the phytase made according to the method of claim 1.

27. The animal feed according to claim 26, wherein said phytase from *Escherichia coli* is an AppA phytase.

* * * * *

EXHIBIT 3

US008455232B2

(12) **United States Patent**
Lei

(10) **Patent No.: US 8,455,232 B2**
(45) **Date of Patent: *Jun. 4, 2013**

(54) **OVEREXPRESSION OF PHYTASE GENES IN YEAST SYSTEMS**

(75) Inventor: **Xingen Lei**, Ithaca, NY (US)

(73) Assignee: **Cornell Research Foundation, Inc.**, Ithaca, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 90 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/941,445**

(22) Filed: **Nov. 8, 2010**

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US 2011/0053246 A1 Mar. 3, 2011

Related U.S. Application Data

(63) Continuation of application No. 11/962,446, filed on Dec. 21, 2007, now Pat. No. 7,829,318, which is a continuation of application No. 11/372,851, filed on Mar. 10, 2006, now Pat. No. 7,312,063, which is a continuation of application No. 10/094,693, filed on Mar. 8, 2002, now Pat. No. 7,026,150, which is a continuation-in-part of application No. 09/104,769, filed on Jun. 25, 1998, now Pat. No. 6,451,572.

(51) **Int. Cl.**
C12N 9/16 (2006.01)

(52) **U.S. Cl.**
USPC **435/196**; 536/23.2

(58) **Field of Classification Search**
USPC 435/196, 252.3, 320.1; 536/23.2
See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to a method of producing a heterologous protein or polypeptide having phytase activity in a yeast system. The invention also provides proteins having phytase activity which have increased thermostability. Yeast strains which produce a heterologous phytase and the vectors used to produce the phytase are also provided.

4 Claims, 23 Drawing Sheets

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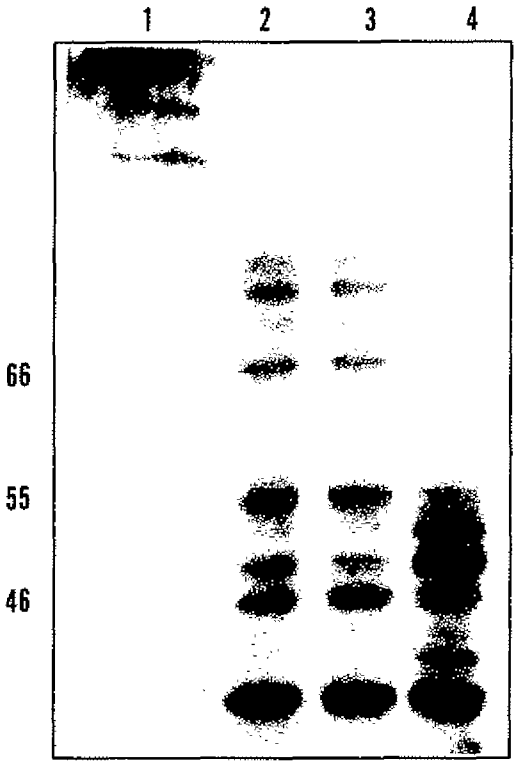

FIG. 1

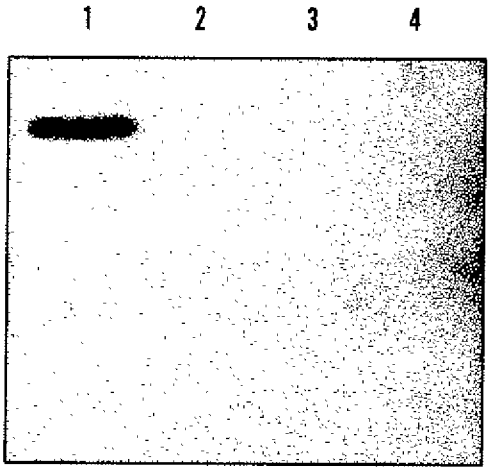

FIG. 2

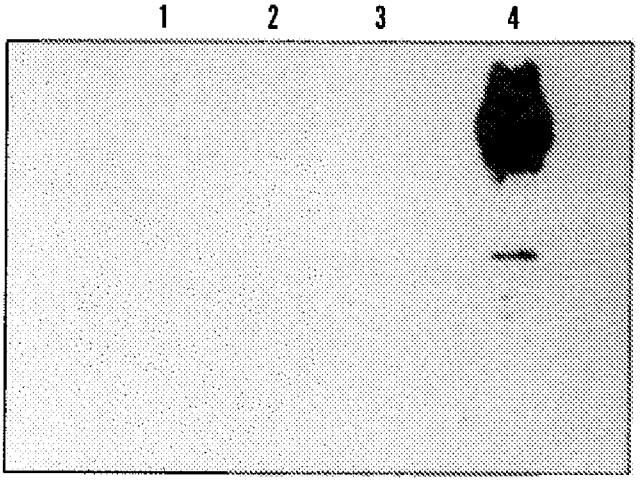

FIG. 3

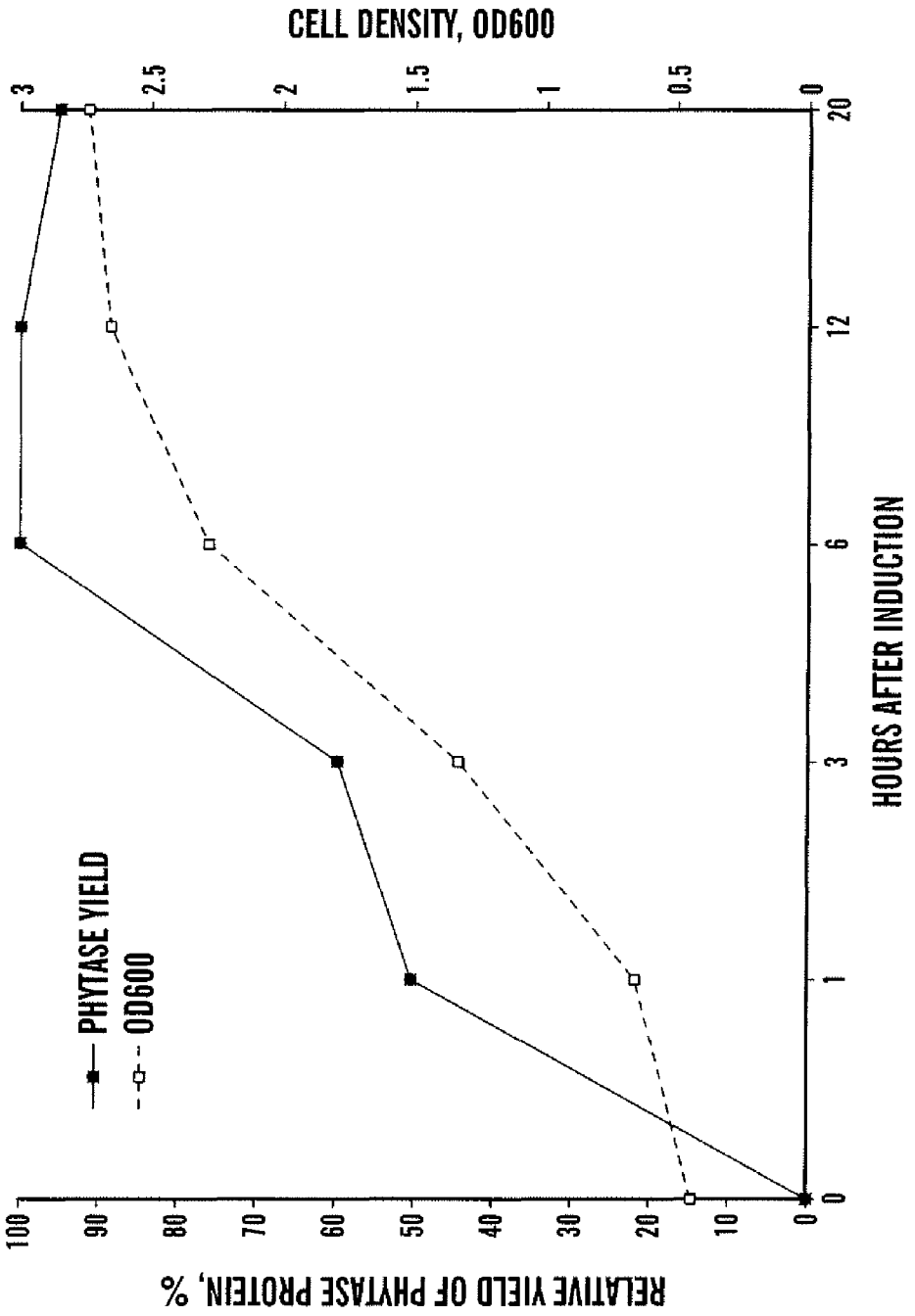
CELL DENSITY, OD600
RELATIVE YIELD OF PHYTASE PROTEIN, %
HOURS AFTER INDUCTION
PHYTASE YIELD
OD600
0
10
20
30
40
50
60
70
80
90
100
0
0.5
1
1.5
2
2.5
3
0
1
3
6
12
20

FIG. 4

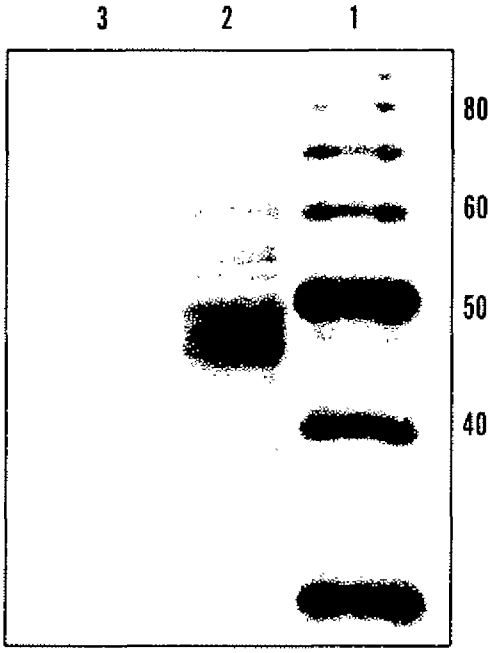

FIG. 5

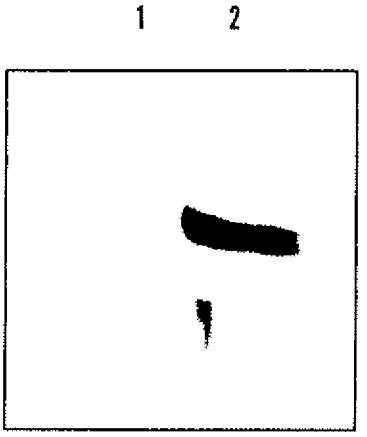

FIG. 6

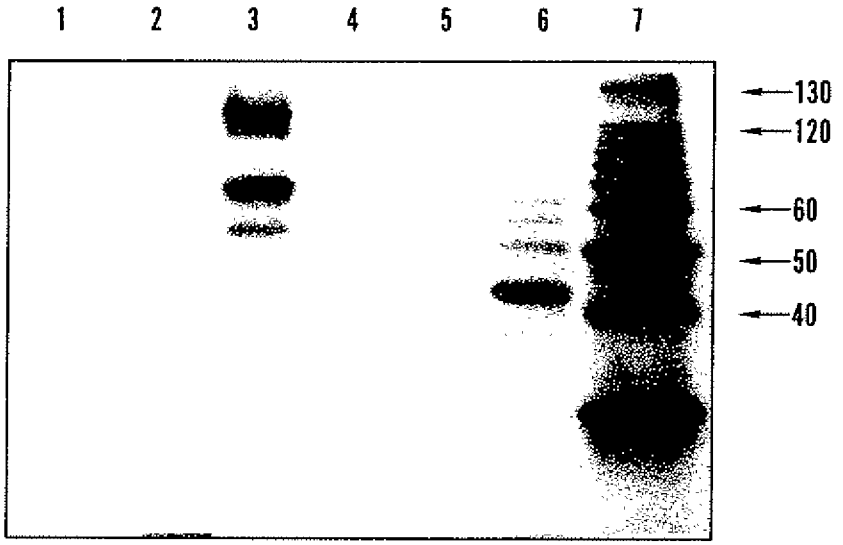

FIG. 7

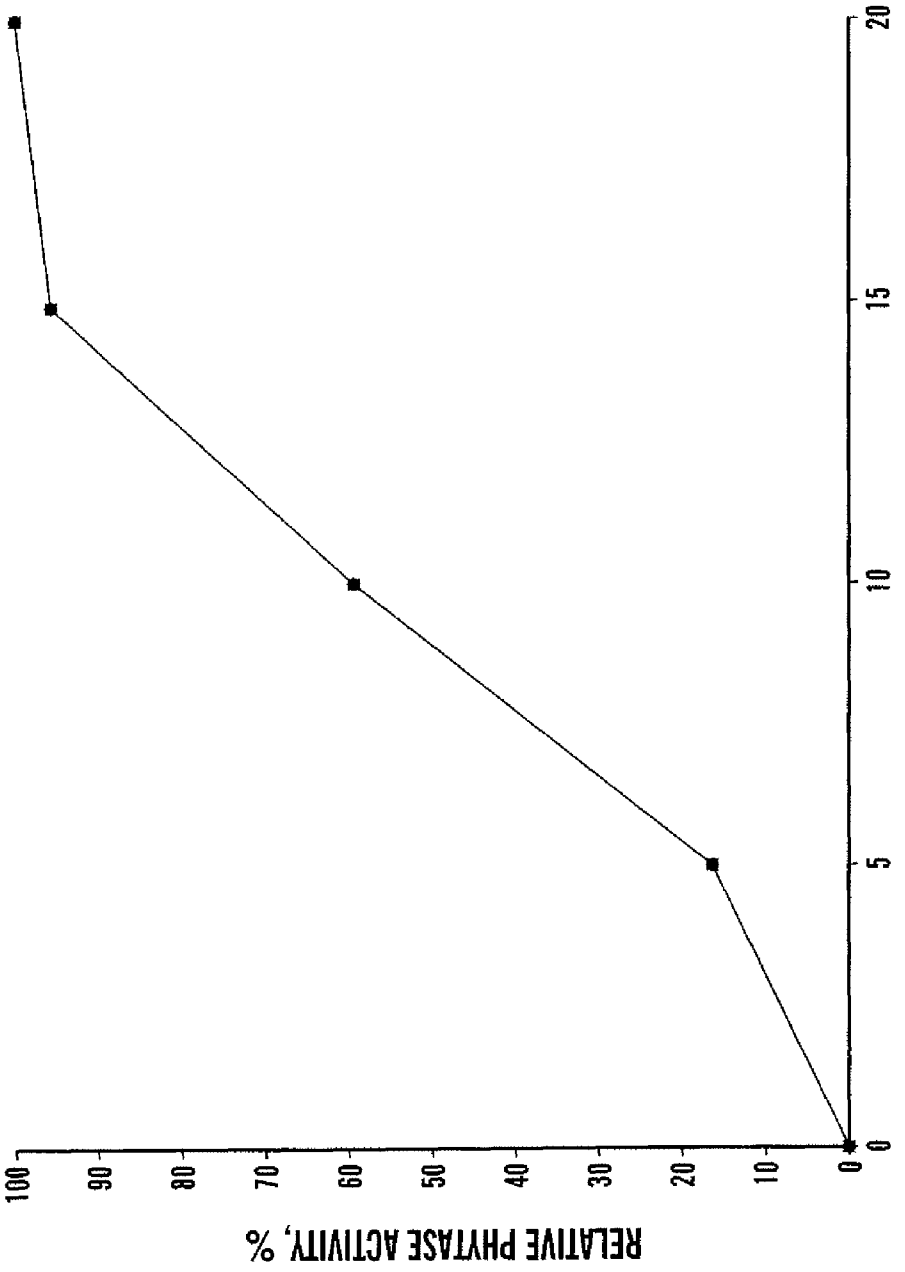
RELATIVE PHYTASE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
HOURS AFTER INDUCTION

FIG. 8

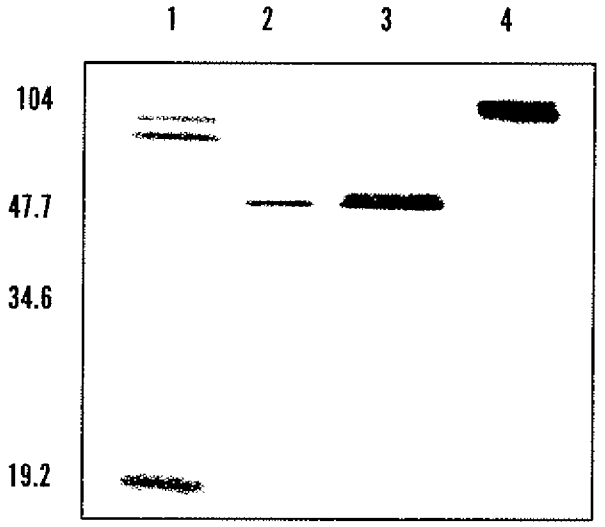

FIG. 9

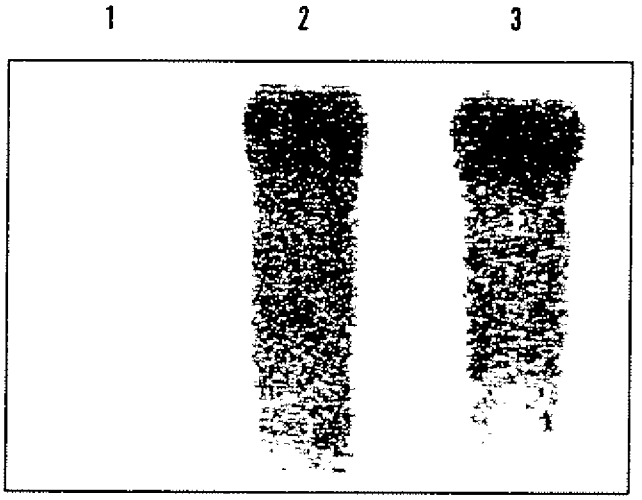

FIG. 10

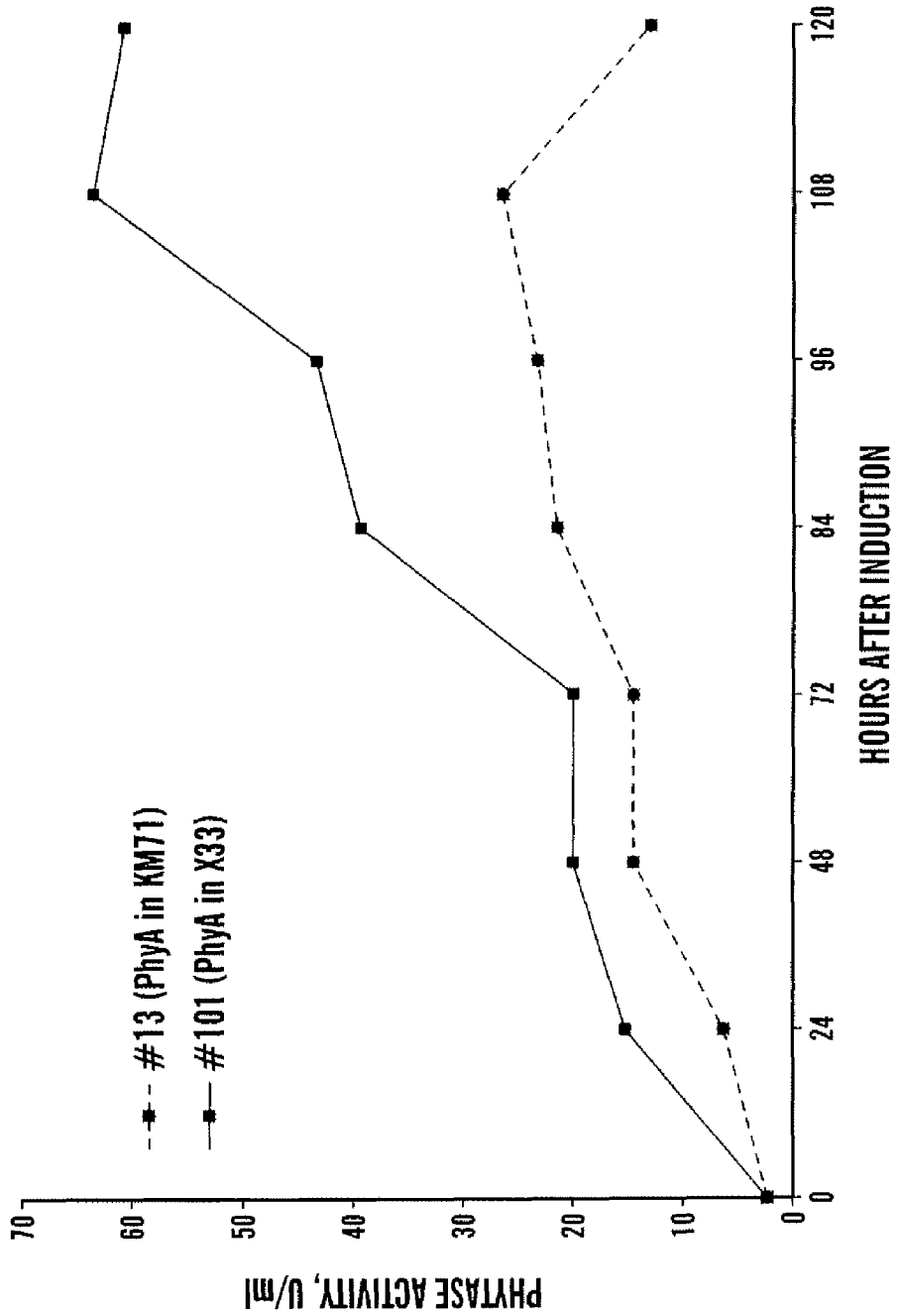
PHYTASE ACTIVITY, U/ml
70
60
50
40
30
20
10
0
0
24
48
72
84
96
108
120
HOURS AFTER INDUCTION
#13 (PhyA in KM71)
#101 (PhyA in X33)

FIG. 11

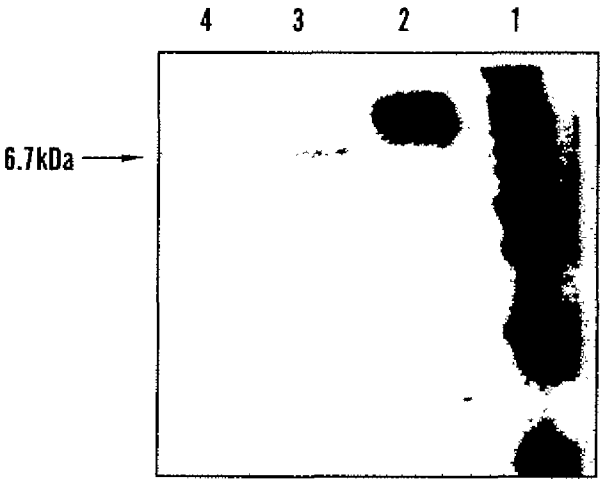

FIG. 12

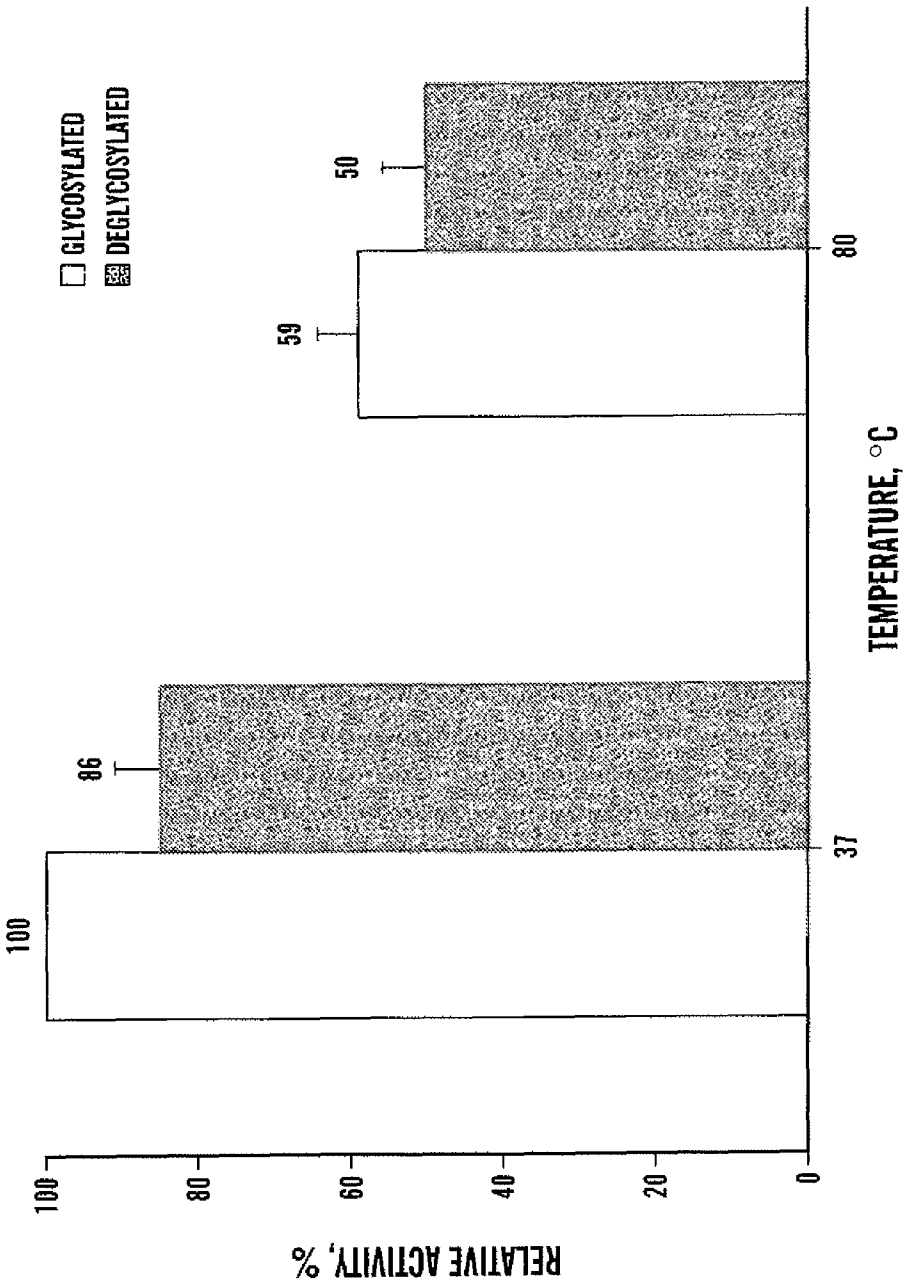
GLYCOSYLATED
DEGLYCOSYLATED
RELATIVE ACTIVITY, %
100
80
60
40
20
0
100
86
59
50
37
80
TEMPERATURE, °C

FIG. 13

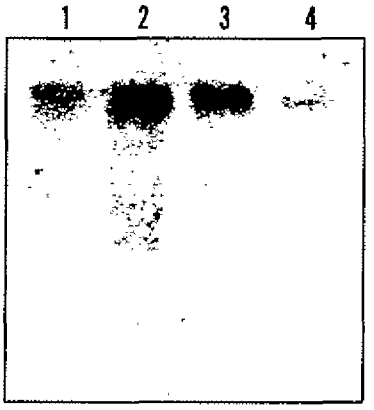

FIG. 14

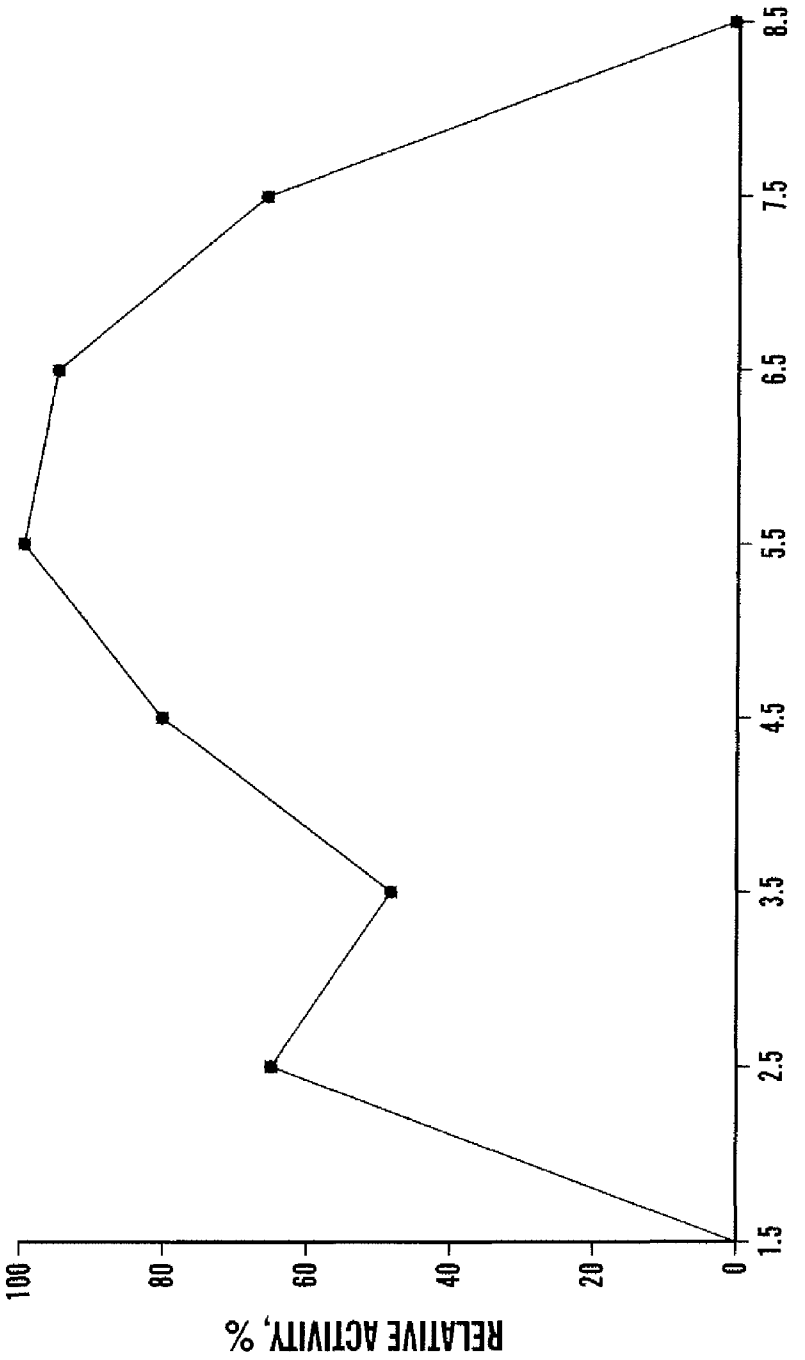

FIG. 15

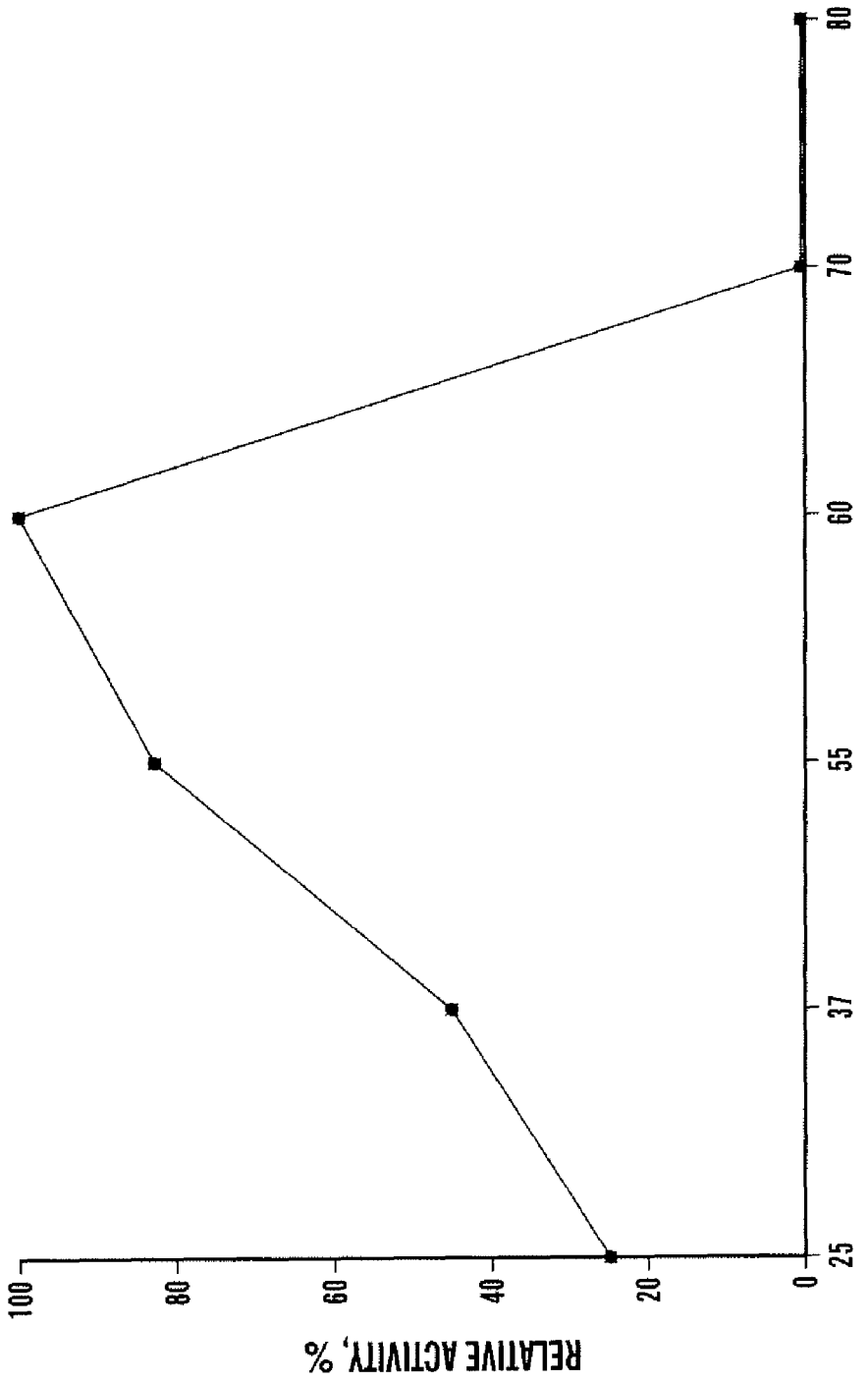
RELATIVE ACTIVITY, %
100
80
60
40
20
0
25
37
55
60
70
80
TEMPERATURE, °C

FIG. 16

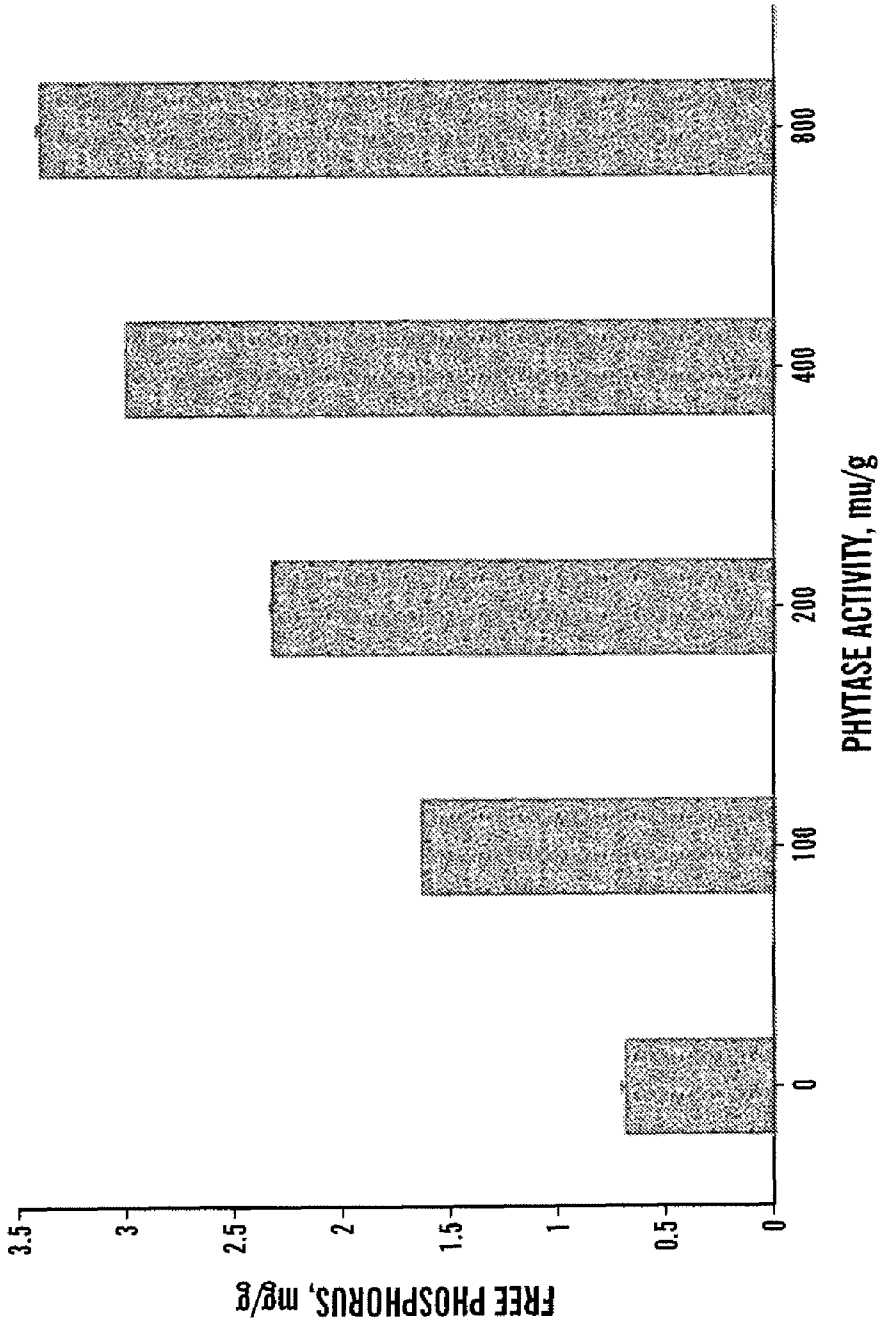
FREE PHOSPHORUS, mg/g
3.5
3
2.5
2
1.5
1
0.5
0
0
100
200
400
800
PHYTASE ACTIVITY, mu/g

FIG. 17

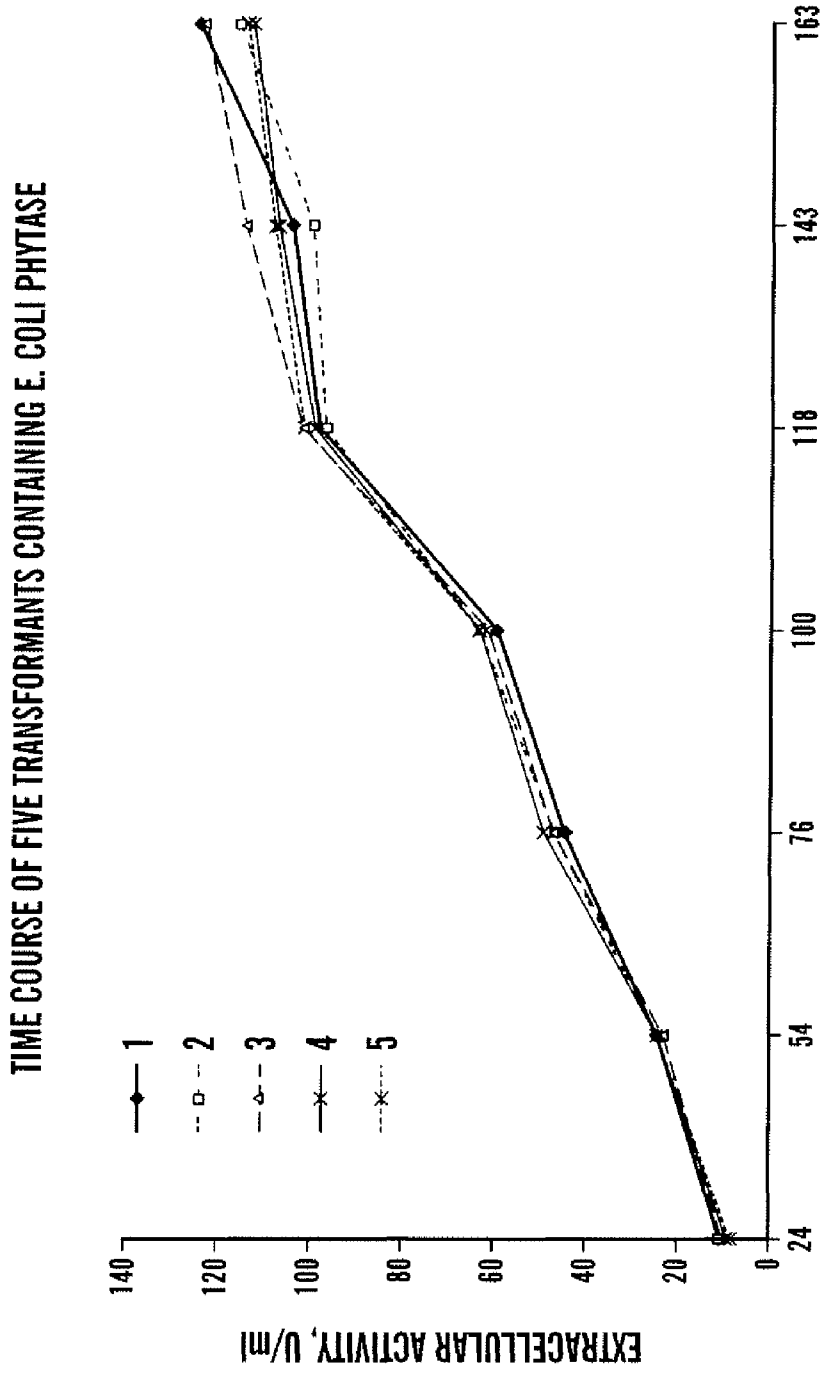
TIME COURSE OF FIVE TRANSFORMANTS CONTAINING E. COLI PHYTASE
EXTRACELLULAR ACTIVITY, U/ml
HOURS AFTER INDUCTION
1
2
3
4
5
0
20
40
60
80
100
120
140
24
54
76
100
118
143
163

FIG. 18

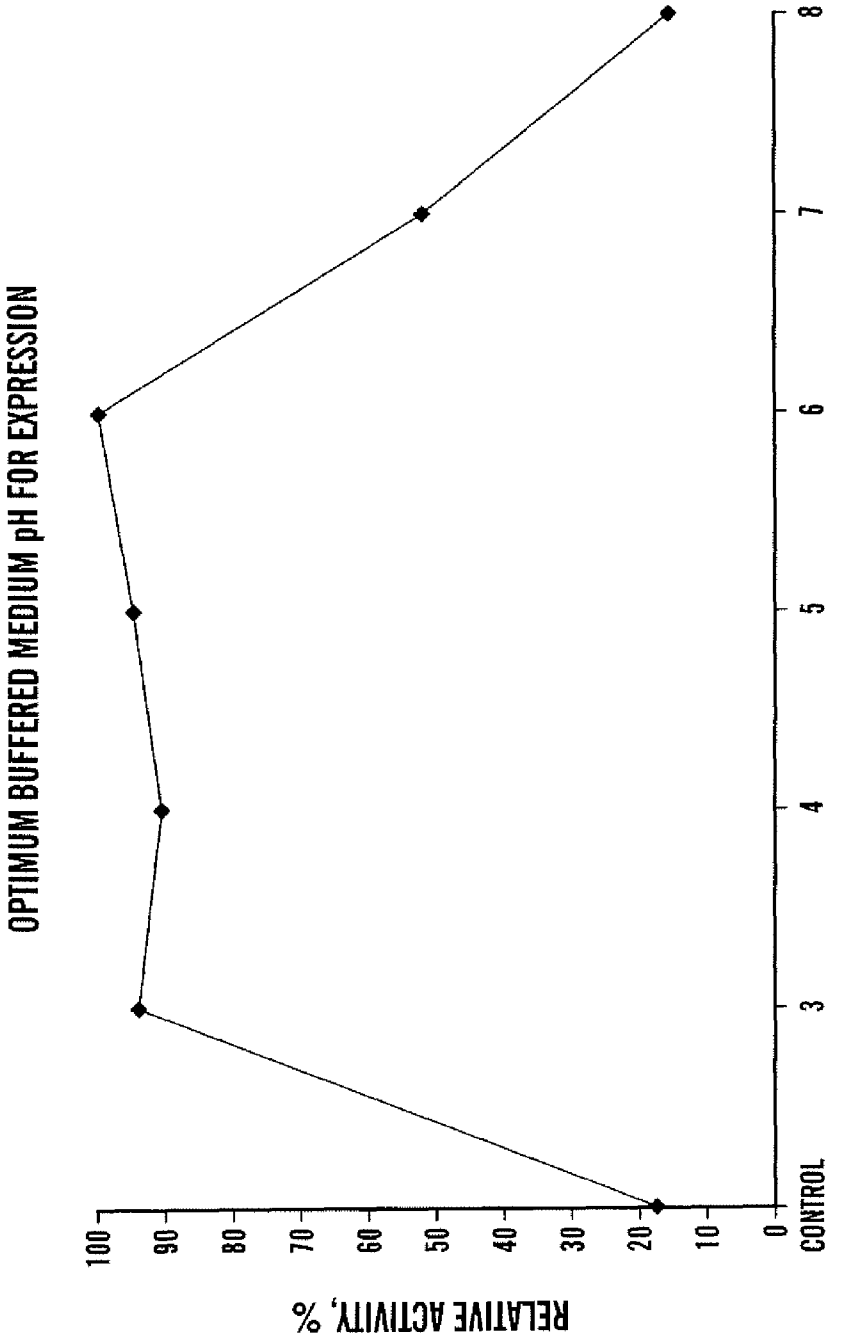
OPTIMUM BUFFERED MEDIUM pH FOR EXPRESSION
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
CONTROL
3
4
5
6
7
8
pH

FIG. 19

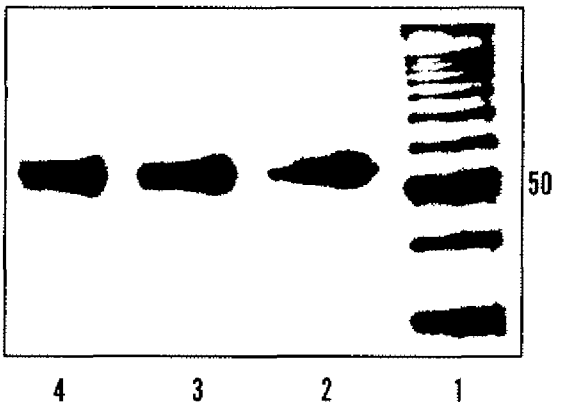

FIG. 20

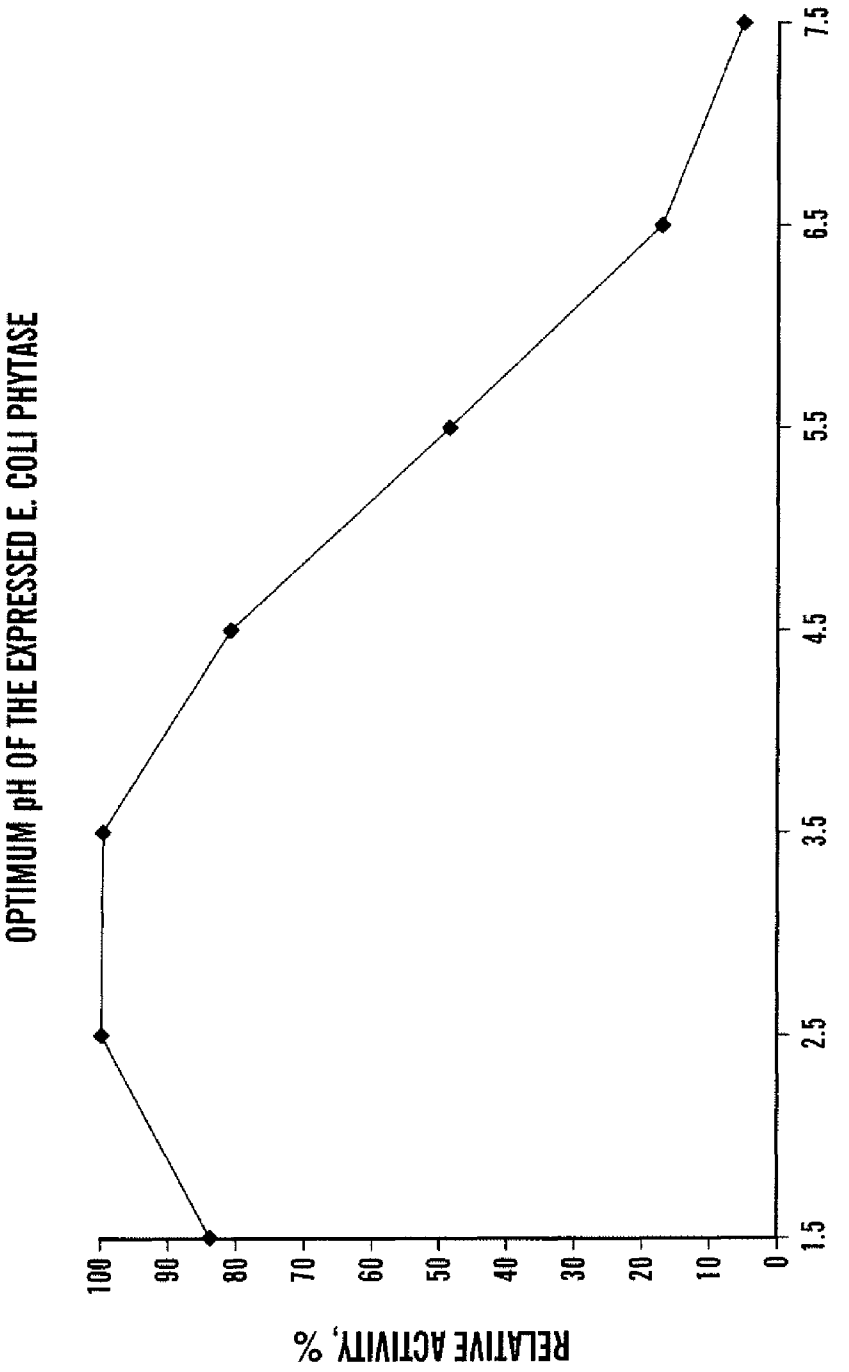
OPTIMUM pH OF THE EXPRESSED E. COLI PHYTASE
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
pH

FIG. 21

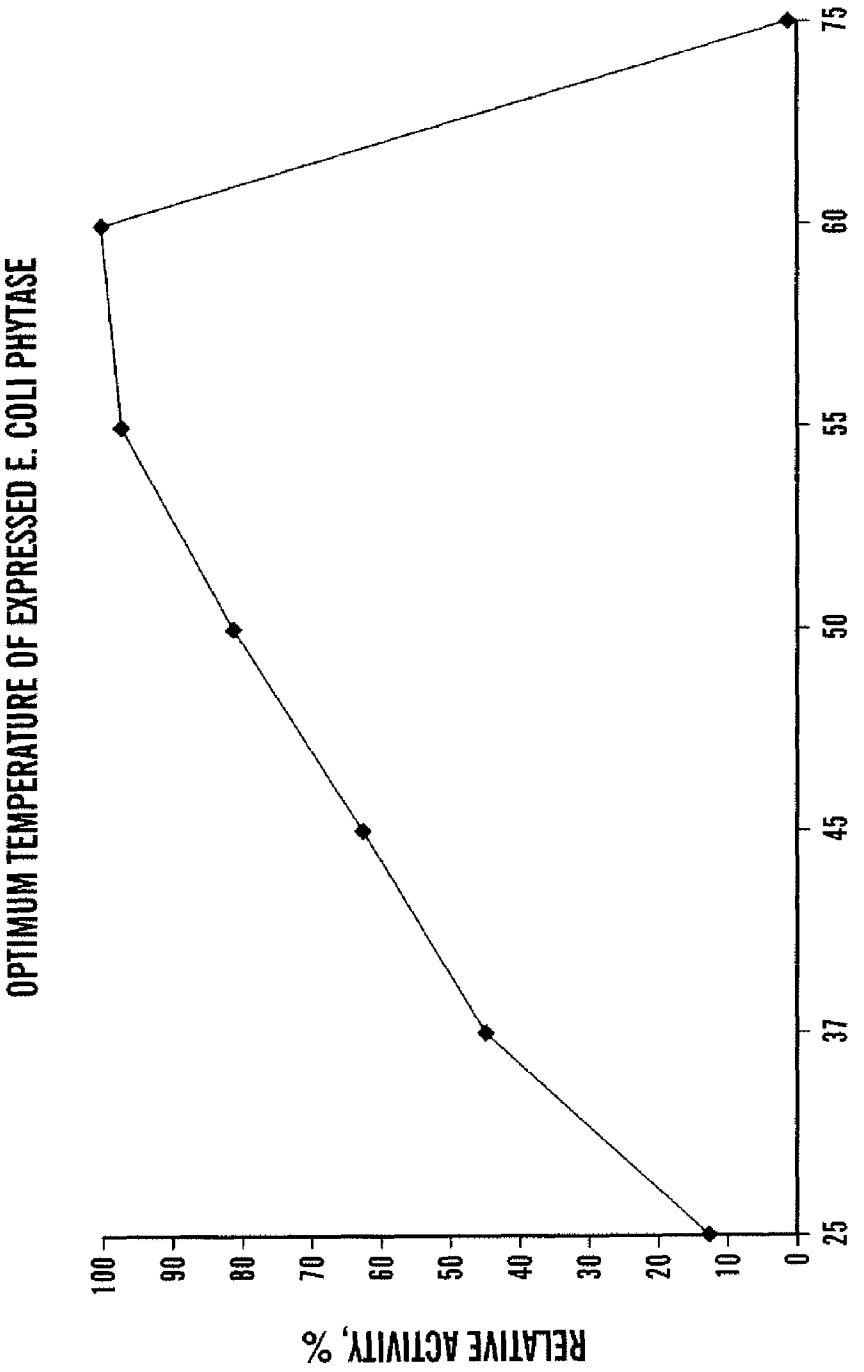
OPTIMUM TEMPERATURE OF EXPRESSED E. COLI PHYTASE
RELATIVE ACTIVITY, %
100
90
80
70
60
50
40
30
20
10
0
25
37
45
50
55
60
75
TEMPERATURE, C

FIG. 22

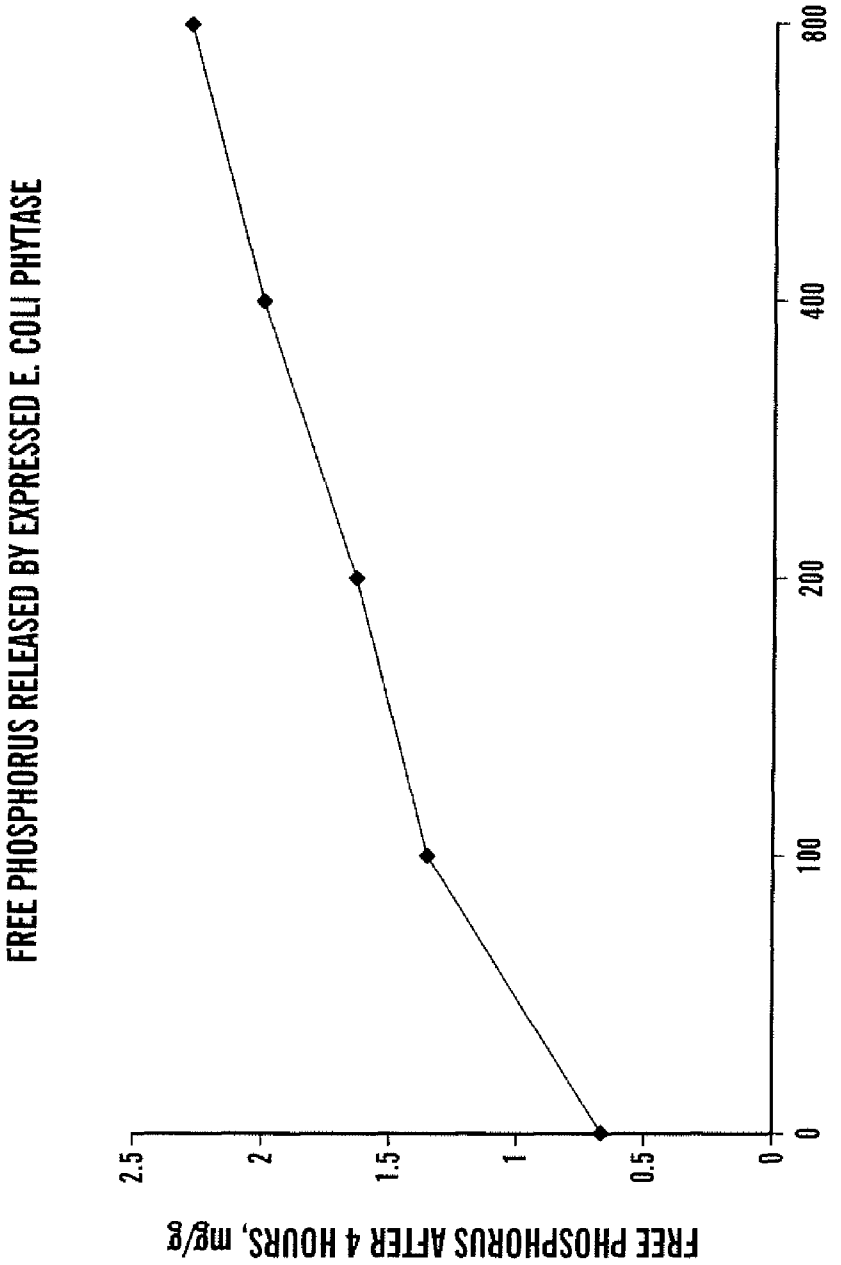
FREE PHOSPHORUS RELEASED BY EXPRESSED E. COLI PHYTASE
FREE PHOSPHORUS AFTER 4 HOURS, mg/g
2.5
2
1.5
1
0.5
0
0
100
200
400
800
PHYTASE, mU/g

FIG. 23

OVEREXPRESSION OF PHYTASE GENES IN YEAST SYSTEMS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation application of U.S. patent application Ser. No. 11/962,446 filed Dec. 21, 2007, which is a continuation application of U.S. patent application Ser. No. 11/372,851 filed Mar. 10, 2006 and issued as U.S. Pat. No. 7,312,063 on Dec. 25, 2007, which is a continuation application of U.S. patent application Ser. No. 10/094,693 filed Mar. 8, 2002 and issued as U.S. Pat. No. 7,026,150 on Apr. 11, 2006, which is a continuation-in-part of U.S. patent application Ser. No. 09/104,769 filed Jun. 25, 1998 and issued as U.S. Pat. No. 6,451,572 on Sep. 17, 2002, the contents of each of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to a method of producing phytase in yeast, yeast strains which express heterologous phytase, and the heterologous phytase produced by yeast.

BACKGROUND OF THE INVENTION

Phytases, a specific group of monoester phosphatases, are required to initiate the release of phosphate ("P") from phytate (myo-inositol hexophosphate), the major storage form of P in cereal foods or feeds (Reddy, N. R. et al., "Phytates in Legumes and Cereals," *Advances in Food Research,* 28:1 (1982)). Because simple-stomached animals like swine and poultry as well as humans have little phytase activity in their gastrointestinal tracts, nearly all of the ingested phytate P is indigestible. This results in the need for supplementation of inorganic P, an expensive and non-renewable nutrient, in diets for these animals. More undesirably, the unutilized phytate-P excreted through manure of these animals becomes P pollution of the environment (Cromwell, G. L. et al., "P-A Key Essential Nutrient, Yet a Possible Major Pollutant—Its Central Role in Animal Nutrition," *Biotechnology In the Feed Industry*; Proceedings Alltech 7th Annual Symposium, p. 133 (1991)). Furthermore, phytate chelates with essential trace elements like zinc and produces nutrient deficiencies such as growth and mental retardation in children ingesting mainly plant origin foods without removal of phytate.

Two phytases, phyA and phyB, from *Aspergillus niger* NRRL3135 have been cloned and sequenced (Ehrlich, K. C. et al., "Identification and Cloning of a Second Phytase Gene (phys) from *Aspergillus niger* (*ficuum*)," *Biochem. Biophys. Res. Commun.,* 195:53-57 (1993); Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori," Gene,* 133:56-62 (1993)). Recently, new phytase genes have been isolated from *Aspergillus terreus* and *Myceliophthora thermophila* (Mitchell et al., "The Phytase Subfamily of Histidine Acid Phosphatases: Isolation of Genes for Two Novel Phytases From the Fungi *Aspergillus terreus* and *Myceliophthora thermophila," Microbiology* 143:245-252, (1997)), *Aspergillus fumigatus* (Pasamontes et al., "Gene Cloning, Purification, and Characterization of a Heat-Stable Phytase from the Fungus *Aspergillus fumigatus" Appl. Environ. Microbiol.,* 63:1696-1700 (1997)), *Emericella nidulans* and *Talaromyces thermophilus* (Pasamontes et al., "Cloning of the Phytase

from *Emericella nidulans* and the Thermophilic Fungus *Talaromyces thermophilus," Biochim. Biophys. Acta.,* 1353: 217-223 (1997)), and maize (Maugenest et al., "Cloning and Characterization of a cDNA Encoding a Maize Seedling Phytase," *Biochem. J.* 322:511-517, 1997)).

Various types of phytase enzymes have been isolated and/or purified from *Enterobacter* sp. 4 (Yoon et al., "Isolation and Identification of Phytase-Producing Bacterium, *Enterobacter* sp. 4, and Enzymatic Properties of Phytase Enzyme," *Enzyme and Microbial Technology* 18:449-454 (1996)), *Klebsiella terrigena* (Greiner et al., "Purification and Characterization of a Phytase from *Klebsiella terrigena.," Arch. Biochem. Biophys.* 341:201-206 (1997)), and *Bacillus* sp. DS11 (Kim et al., "Purification and Properties of a Thermostable Phytase from *Bacillus* sp. DS11*," Enzyme and Microbial Technology* 22:2-7 (1998)). Properties of these enzyme have been studied. In addition, the crystal structure of phy A from *Aspergillus ficuum* has been reported (Kostrewa et al., "Crystal Structure of Phytase from *Aspergillus ficuum* at 2.5 A Resolution," *Nature Structure Biology* 4:185-190 (1997)).

Hartingsveldt et al. introduced phyA gene into *A. niger* and obtained a ten-fold increase of phytase activity compared to the wild type. ("Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87-94 (1993)). Supplemental microbial phytase of this source in the diets for pigs and poultry has been shown to be effective in improving utilization of phytate-P and zinc (Simons et al., "Improvement of Phosphorus Availability By Microbial Phytase in Broilers and Pigs," *Br. J. Nutr.,* 64:525 (1990); Lei, X. G. et al., "Supplementing Corn-Soybean Meal Diets With Microbial Phytase Linearly Improves Phytate P Utilization by Weaning Pigs," *J. Anim. Sci.,* 71:3359 (1993); Lei, X. G. et al., "Supplementing Corn-Soybean Meal Diets With Microbial Phytase Maximizes Phytate P Utilization by Weaning Pigs," *J. Anim. Sci.,* 71:3368 (1993); Cromwell, G. L. et al., "P-A Key Essential Nutrient, Yet a Possible Major Pollutant—Its Central Role in Animal Nutrition," *Biotechnology In the Feed Industry*; Proceedings Alltech 7th Annual Symposium, p. 133 (1991)). But, expenses of the limited available commercial phytase supply and the activity instability of the enzyme to heat of feed pelleting preclude its practical use in animal industry (Jongbloed, A. W. et al., "Effect of Pelleting Mixed Feeds on Phytase Activity and Apparent Absorbability of Phosphorus and Calcium in Pigs," *Animal Feed Science and Technology,* 28:233-242 (1990)). Moreover, phytase produced from *A. niger* is presumably not the safest source for human food manufacturing.

Yeast can be used to produce enzymes effectively while grown on simple and inexpensive media. With a proper signal sequence, the enzyme can be secreted into the media for convenient collection. Some yeast expression systems have the added advantage of being well accepted in the food industry and are safe and effective producers of food products.

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. This system is well-known for its ability to express high levels of heterologous proteins. Because it is an eukaryote, *Pichia* has many of the advantages of higher eukaryotic expression systems such as protein processing, folding, and post-transcriptional modification.

Thus, there is a need to develop an efficient and simple system to produce phytase economically for the application of food and feed industry.

SUMMARY OF THE INVENTION

The present invention relates to a method of producing phytase in yeast by introducing a heterologous gene which

encodes a protein or polypeptide with phytase/acid phosphatase activity into a yeast strain and expressing that gene.

The present invention also relates to a protein or polypeptide having phytase activity with optimum activity in a temperature range of 57-65° C. at pH of 2.5 to 3.5 or of 5.5. Optimal pH at 2.5 to 3.5 is particularly important for phytase, because that is the stomach pH of animals.

The invention further provides a yeast cell carrying a heterologous gene which encodes a protein or polypeptide with phytase activity and which is functionally linked to a promoter capable of expressing phytase in yeast.

Yet another aspect of the invention is a vector having a gene from a non-yeast organism which encodes a protein or polypeptide with phytase activity, a promoter which is capable of initiating transcription in yeast functionally linked to the gene encoding a peptide with phytase activity, and with an origin of replication capable of maintaining the vector in yeast or being capable of integrating into the host genome.

The invention also provides a method for producing a protein or polypeptide having phytase activity. An isolated appA gene, which encodes a protein or polypeptide with phytase activity, is expressed in a host cell.

The invention also includes a method of converting phytate to inositol and inorganic phosphate. The appA gene expresses a protein of polypeptide with phytase activity in a host cell. The protein or polypeptide is then contacted with phytate to catalyze the conversion of phytate to inositol and inorganic phosphate.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows an SDS-PAGE analysis of soluble protein prepared from the phytase gene transformed *E. coli* induced with IPTG. The cells were grown 4 hours before harvesting. Lane 1: Marker; Lanes 2 and 3: Transformants of pEP1 (the expressed protein was approximately 55 kDa); Lane 4: Transformant with only the expression vector pET25b(+).

FIG. **2** shows the Western blot analysis of the expressed phytase protein in *E. coli*. The antibody was raised against purified native phytase of *A. niger*. Each lane contained 50 μg total intracellular protein. Lanes 1 and 2: Recombinants after and before induction. Lanes 3 and 4: control (only the expressing vector) after and before induction.

FIG. **3** is a scan image of Northern blotting analysis of the mRNA of PhyA in *E. coli*. A 1.4 kb PhyA probe was used. Each lane contained 20 μg of total RNA. Lanes 1 and 2: RNA isolated from the control cells (only the expression vector) before and after induction. Lanes 3 and 4: RNA isolated from the recombinants containing PhyA before and after induction.

FIG. **4** is a time course of the induced expression of phytase (pEP1) in *E. coli* BL21(DE3). The cells were induced when the OD_{600} reached 0.5. The soluble protein, prepared at each time point, was quantified by SDS-PAGE analysis.

FIG. **5** shows an SDS-PAGE analysis of the expressed extracellular phytase protein by the phytase transformed *S. lividans* after growing for 72 hours. Cells were spun for 15 minutes at 8,000×g, and the supernatant was subjected to gel electrophoresis. Lane 1: Marker; Lane 2: Control with the only expression vector; Lane 3: Positive colony expressed phytase and the size was approximately 57 kDa.

FIG. **6** shows the Western blot analysis of the phytase expressed by *S. lividans*, using a phytase antibody raised against purified native phytase of *A. niger*. Each lane was loaded with 20 μg medium (supernatant) protein. Lane 1: Supernatant from the vector transformed control cell culture; Lane 2: Supernatant from the culture inoculated with the positive colony.

FIG. **7** depicts an SDS-PAGE analysis of the extracellular phytase expressed by *S. cerevisiae*. Each lane was loaded with 50 μg medium (supernatant) protein. Lanes 1 to 3: Supernatant from the culture inoculated with the positive colony harvested at 5, 10, and 25 hours after induction, respectively; Lanes 4 to 6: Supernatant from the vector-transformed control cell culture harvested at 5, 10, and 25 hours after induction, respectively; Lane 7: Marker (kDa). The expressed phytase was approximately 110 kDa (confirmed by Western blot).

FIG. **8** is a time course of the extracellular phytase activity expressed by the pYPP1 construct transformed *S. cerevisiae* after induced by galactose. The activity was analyzed in the supernatant of the collected medium.

FIG. **9** shows the Western blot analysis of the extracellular phytase expressed by *S. cerevisiae* before and after deglycosylation (Endo H), using a phytase antibody raised against purified native phytase in *A. niger*. Lane 1: Prestained SDS-PAGE standards (kDa) from Bio-Rad; Lanes 2 and 3: deglycosylated 10 and 20 μg phytase protein, respectively; Lane 4: glycosylated phytase (20 μg protein).

FIG. **10** is a scan image of Northern blot analysis for total RNA isolated from transformed *S. cerevisiae* cells. Lane 1: Control (with only the expression vector pYES2); Lanes 2 and 3: Transformants of pYPP1.

FIG. **11** is a time course of the extracellular phyA phytase activity produced by *Pichia pastoris* transformants of Mut^s (KM71) and Mut+ (X33) after induction.

FIG. **12** depicts an SDS-PAGE analysis of the overexpressed phytase in *Pichia*, with construct of pPICZαA-PhyA in KM71 (MUT^s). Lane 1: protein ladder. Lane 2: 40 μl of the supernatant of AK1 (a colony showed 21,700 mU/ml of extracellular phytase), collected 108 hours after induction. Lane 3: 40 μl of the supernatant of a control strain overexpressing human serum albumin (HAS, 6.7 kDa) at a level of 1 g/L. Lane 4: 40 μl of the supernatant of the KM71 control.

FIG. **13** depicts effects of deglycosylation by Endo H on the thermostability of the expressed phytase in *Pichia*. Phytase activity was measured after the enzymes were heated for 15 minutes under 37 or 80° C. in 0.2 M citrate buffer, pH 5.5.

FIG. **14** is a scan image of Northern analysis of the expressed phyA mRNA by the transformed *Pichia pastoris* strains (KM71 and X33). A 1.3 kb phyA probe was used for blotting. Lanes 1 and 2: the transformant of KM71 before and after induction; Lanes 3 and 4: the transformant of X33 after and before induction.

FIG. **15** shows the optimum pH of the expressed extracellular phytase by *Pichia* (X33). Buffers of 0.2 M glycine-HCl for pH 1.5, 2.5, 3.5; 0.2 M sodium citrate for pH 4.5, 5.5, 6.5, and 0.2 M Tris-HCl for pH 7.5 and 8.5 were used.

FIG. **16** shows the optimum temperature of the expressed extracellular phytase by *Pichia* (X33). The assays were conducted in 0.2 M citrate buffer, pH 5.5.

FIG. **17** depicts the release of free phosphorus from soybean by the expressed phytase in *Pichia* (X33). Five grams of soybean were suspended in 25 ml of 0.2 M citrate, pH 5.5, with different amounts of the enzyme. The incubation was conducted for 4 hours under 37° C. and the free phosphorus released in the supernatant was determined.

FIG. **18** shows a time course of the expression of the extracellular phytase activity from five transformants of *Pichia pastoris* containing the *E. coli* appA gene.

FIG. **19** graphically shows the relationship between medium pH and the expression of phytase activity by *Pichia pastoris*.

FIG. **20** is an SDS-PAGE analysis of the *E. coli* phytase overexpressed in *Pichia pastoris*. Lane 1: Protein ladder; Lanes 2 to 4: Supernatants collected from the cultures of positive colonies 23, 22, and 11, respectively, at 118 hours after induction.

FIG. **21** graphically shows the optimum pH of the overexpressed *E. coli* phytase by *Pichia pastoris*.

FIG. **22** graphically shows the optimum temperature of the overexpressed *E. coli* phytase by *Pichia pastoris*.

FIG. **23** shows the amount of free phosphorus released from soybean meal by the overexpressed *E. coli* phytase from *Pichia pastoris* after four hours treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing phytase in yeast. According to this method, a heterologous gene which encodes a protein or polypeptide with phytase activity is expressed in a yeast strain.

The enzymes which catalyze the conversion of phytate to inositol and inorganic phosphorus are broadly known as phytases. Phytase producing microorganisms comprise bacteria such as *Bacillus subtilis* (Paver et al., J. Bacteriol. 151, 1102 (1982), which is hereby incorporated by reference) and *Pseudomonas* (Cosgrove, *Austral. J. Biol. Sci.* 23:1207 (1970), which is hereby incorporated by reference); yeasts, such as *Saccharomyces cerevisiae* (Nayini et al., *Lebensmittel Wissenschaft and Technologie* 17:24 (1984), which is hereby incorporated by reference); and fungi, such as *Aspergillus terreus* (Yamada et al., *Agric. Biol. Chem.* 32:1275 (1986), which is hereby incorporated by reference), and *Aspergillus ficuum* (van Gorcom et al., European Patent Application 89/202,436, which is hereby incorporated by reference).

Phytases are also endogenously present in many plant species. Loewus, In: *Plant Biology* vol. 9: "Inositol Metabolism in Plants" (eds. D. J. Morre, W. F. Boss, F. A. Loewus) 13 (1990); and Gellatly, et al., *Plant Physiology* (supplement) 93:562 (1990), which are hereby incorporated by reference, mention the isolation and characterization of a phytase cDNA clone obtained from potato tubers. Gibson, et al., *J. Cell Biochem.*, 12C:L407 (1988) and Christen, et al., *J. Cell Biochem.*, 12C:L402 (1988), which are hereby incorporated by reference, mentions the synthesis of endogenous phytase during the germination of soybean seeds.

Preferably, the protein or polypeptide with phytase activity is secreted by the cell into growth media. This allows for higher expression levels and easier isolation of the product. The protein or polypeptide with phytase activity is coupled to a signal sequence capable of directing the protein out of the cell. Preferably, the signal sequence is cleaved from the protein.

In a preferred embodiment, the heterologous gene, which encodes a protein or polypeptide with phytase activity, is spliced in frame with a transcriptional enhancer element.

Preferred heterologous genes encoding proteins with phytase activity are isolated from a bacterial cell. A more preferred gene is the phyA gene of *Aspergillus niger*. A gene encoding phytase, phyA, from *Aspergillus niger* NRRL3135 has been cloned and sequenced (Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori*," *Gene*, 133:56-62 (1993), which are hereby incorporated by reference). Hartingsveldt et al. introduced phyA gene into *A. niger*, and obtained a tenfold increase of phytase activity compared to the wild type. (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," *Gene* 127:87-94 (1993), which is hereby incorporated by reference.)

Another preferred heterologous gene is the appA gene of *E. coli*. The gene, originally defined as *E. coli* periplasmic phosphoanhydride phosphohydrolase (appA) gene, contains 1,298 nucleotides (GeneBank accession number: M58708). The gene was first found to code for an acid phosphatase protein of optimal pH of 2.5 (EcAP) in *E. coli*. The acid phosphatase is a monomer with a molecular mass of 44,644 daltons. Mature EcAP contains 410 amino acids (Dassa, J. et al., "The Complete Nucleotide Sequence of the *Escherichia Coli* Gene AppA Reveals Significant Homology Between Ph 2.5 Acid Phosphatase and Glucose-1-Phosphatase," *J. Bacteriology*, 172:5497-5500 (1990), which is hereby incorporated by reference). Ostanin, et al. overexpressed appA in *E. coli* BL21 using a pT7 vector and increased its acid phosphatase activity by approximately 400-folds (440 mU/mg protein) (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia Coli* Acid Phosphatase," *J. Biol. Chem.*, 267:22830-36 (1992), which is hereby incorporated by reference). The product of the appA gene was not previously known to have phytase activity.

The appA or phyA gene can be expressed in any prokaryotic or eukaryotic expression system. A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Preferred vectors include a viral vector, plasmid, cosmid or an oligonucleotide. Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Preferred hosts for expressing appA or phyA include fungal cells, including species of yeast or filamentous fungi, may be used as host cells in accordance with the present invention. Preferred yeast host cells include different strains of *Saccharomyces cerevisiae*. Other yeasts like *Kluyveromyces, Torulaspora*, and *Schizosaccharomyces* can also be used. In a preferred embodiment, the yeast strain used to overexpress the protein is *Saccharomyces cerevisiae*. Preferred filamentous fungi host cells include *Aspergillus* and *Neurospora*. A more preferred strain of *Aspergillus* is *Aspergillus niger*.

In another preferred embodiment of the present invention, the yeast strain is a methylotrophic yeast strain. Methylotrophic yeast are those yeast genera capable of utilizing methanol as a carbon source for the production of the energy resources necessary to maintain cellular function and containing a gene for the expression of alcohol oxidase. Typical methylotrophic yeasts include members of the genera *Pichia, Hansenula, Torulopsis, Candida*, and *Karwinskia*. These yeast genera can use methanol as a sole carbon source. In a more preferred embodiment, the methylotrophic yeast strain is *Pichia pastoris*.

The present invention also provides a protein or polypeptide with phytase activity. PhyA is expressed in *Pichia* and the resulting protein produced has much higher extracellular activity (~65 mU/ml). The phytase activity yield was approximately 30-fold greater than that in phyA transformed *Saccha-*

romyces cerevisiae, 21-fold greater than that in wild type of *Aspergillus niger*, and 65,000-fold greater than that in the untransformed *Pichia*. The optimal pH of the expressed phytase was 2.5 and 5.5, and the optimal temperature was 60° C. Similarly, appA is expressed in *Pichia* and *Saccharomyces cerevisiae* with the resulting protein having much higher extracellular activity and a much preferred optimal pH of 2.5 to 3.5.

A preferred embodiment of the invention is a protein or polypeptide having phytase activity with optimum activity in a temperature range of 57 to 65° C. A more preferred embodiment is a protein or polypeptide having phytase activity, where its temperature range for optimum activity is from 58 to 62° C.

Yet another preferred embodiment is a protein or polypeptide having phytase activity where the protein retains at least 40% of its activity after heating the protein for 15 minutes at 80° C. More preferred is a protein or polypeptide having phytase activity where the protein retains at least 60% of its activity after heating the protein for 15 minutes at 60° C.

Purified protein may be obtained by several methods. The protein or polypeptide of the present invention is preferably produced in purified form (preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is secreted into the growth medium of recombinant host cells. Alternatively, the protein or polypeptide of the present invention is produced but not secreted into growth medium. In such cases, to isolate the protein, the host cell carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove cell debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the polypeptide or protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

The present invention also provides a yeast strain having a heterologous gene which encodes a protein or polypeptide with phytase activity. The heterologous gene should be functionally linked to a promoter capable of expressing phytase in yeast.

Yet another aspect of the invention is a vector for expressing phytase in yeast. The vector carries a gene from a non-yeast organism which encodes a protein or polypeptide with phytase activity. The phytase gene can be cloned into any vector which replicates autonomously or integrates into the genome of yeast. The copy number of autonomously replicating plasmids, e.g. YEp plasmids may be high, but their mitotic stability may be insufficient (Bitter et al., "Expression and Secretion Vectors for Yeast," *Meth. Enzymol.* 153:516-44 (1987), which is hereby incorporated by reference). They may contain the 2 mu-plasmid sequence responsible for autonomous replication, and an *E. coli* sequence responsible for replication in *E. coli*. The vectors preferably contain a genetic marker for selection of yeast transformants, and an antibiotic resistance gene for selection in *E. coli*. The episomal vectors containing the ARS and CEN sequences occur as a single copy per cell, and they are more stable than the YEp vectors. Integrative vectors are used when a DNA fragment is integrated as one or multiple copies into the yeast genome. In this case, the recombinant DNA is stable and no selection is needed (Struhl et al., "High-Frequency Transformation of Yeast: Autonomous Replication of Hybrid DNA Molecules," *Proc. Nat'l Acad. Sci. USA* 76:1035-39 (1979); Powels et al., *Cloning Vectors, I-IV, et seq*. Elsevier, (1985); Sakai et al., "Enhanced Secretion of Human Nerve Growth Factor from *Saccharomyces Cerevisiae* Using an Advanced δ-Integration System," *Biotechnology* 9:1382-85 (1991), which are hereby incorporated by reference). Some vectors have an origin of replication, which functions in the selected host cell. Suitable origins of replication include 2μ, ARS1, and 25 μM. The vectors have restriction endonuclease sites for insertion of the fusion gene and promoter sequences, and selection markers. The vectors may be modified by removal or addition of restriction sites, or removal of other unwanted nucleotides.

The phytase gene can be placed under the control of any promoter (Stetler et al., "Secretion of Active, Full- and Half-Length Human Secretory Leukocyte Protease Inhibitor by *Saccharomyces cerevisiae," Biotechnology* 7:55-60, (1989), which is hereby incorporated by reference). One can choose a constitutive or regulated yeast promoter. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073 (1980), which is hereby incorporated by reference) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149 (1968); and Holland et al., *Biochem.* 17:4900, (1978), which are hereby incorporated by reference), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in EP A-73,657 to Hitzeman, which is hereby incorporated by reference. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al., *J. Biol. Chem.* 258:2674 (1982) and Beier et al., *Nature* 300:724 (1982), which are hereby incorporated by reference.

One can choose a constitutive or regulated yeast promoter. The strong promoters of e.g., phosphoglycerate kinase (PGK) gene, other genes encoding glycolytic enzymes, and the alpha-factor gene, are constitutive. When a constitutive promoter is used, the product is synthesized during cell growth. The ADH2 promoter is regulated with ethanol and glucose, the GAL-1-10 and GAL7 promoters with galactose and glucose, the PHO5 promoter with phosphate, and the metallothionine promoter with copper. The heat shock promoters, to which the HSP150 promoter belongs, are regulated by temperature. Hybrid promoters can also be used. A regulated promoter is used when continuous expression of the desired product is harmful for the host cells. Instead of yeast promoters, a strong prokaryotic promoter such as the T7 promoter, can be used, but in this case the yeast strain has to be transformed with a gene encoding the respective polymerase. For transcription termination, the HSP150 terminator, or any other functional terminator is used. Here, promoters and terminators are called control elements. The present invention is not restricted to any specific vector, promoter, or terminator.

The vector may also carry a selectable marker. Selectable markers are often antibiotic resistance genes or genes capable of complementing strains of yeast having well characterized metabolic deficiencies, such as tryptophan or histidine deficient mutants. Preferred selectable markers include URA3, LEU2, HIS3, TRP1, HIS4, ARG4, or antibiotic resistance genes.

The vector may also have an origin of replication capable of replication in a bacterial cell. Manipulation of vectors is more efficient in bacterial strains. Preferred bacterial origin of replications are ColE1, Ori, or oriT.

A leader sequence either from the yeast or from phytase genes or other sources can be used to support the secretion of

expressed phytase enzyme into the medium. The present invention is not restricted to any specific type of leader sequence or signal peptide.

Suitable leader sequences include the yeast alpha factor leader sequence, which may be employed to direct secretion of the phytase. The alpha factor leader sequence is often inserted between the promoter sequence and the structural gene sequence (Kurjan et al., *Cell* 30:933, (1982); Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, (1984); U.S. Pat. No. 4,546,082; and European published patent application No. 324,274, which are hereby incorporated by reference). Another suitable leader sequence is the *S. cerevisiae* MF alpha 1 (alpha-factor) is synthesized as a prepro form of 165 amino acids comprising signal- or prepeptide of 19 amino acids followed by a "leader" or propeptide of 64 amino acids, encompassing three N-linked glycosylation sites followed by (LysArg(Asp/Glu, Ala)2-3 alpha-factor)4 (Kurjan, et al., *Cell* 30:933-43 (1982), which is hereby incorporated by reference). The signal-leader part of the preproMF alpha 1 has been widely employed to obtain synthesis and secretion of heterologous proteins in *S. cerivisiae*. Use of signal/leader peptides homologous to yeast is known from. U.S. Pat. No. 4,546,082, European Patent Applications Nos. 116,201; 123, 294; 123,544; 163,529; and 123,289 and DK Patent Application No. 3614/83, which are hereby incorporated by reference. In European Patent Application No. 123,289, which is hereby incorporated by reference, utilization of the *S. cerevisiae* a-factor precursor is described whereas WO 84/01153, which is hereby incorporated by reference, indicates utilization of the *Saccharomyces cerevisiae* invertase signal peptide, and German Patent Application DK 3614/83, which is hereby incorporated by reference, indicates utilization of the *Saccharomyces cerevisiae* PH05 signal peptide for secretion of foreign proteins.

The alpha-factor signal-leader from *Saccharomyces cerevisiae* (MF alpha 1 or MF alpha 2) may also be utilized in the secretion process of expressed heterologous proteins in yeast (U.S. Pat. No. 4,546,082, European Patent Applications Nos. 16,201; 123,294; 123 544; and 163,529, which are hereby incorporated by reference). By fusing a DNA sequence encoding the *S. cerevisiea* MF alpha 1 signal/leader sequence at the 5' end of the gene for the desired protein secretion and processing of the desired protein was demonstrated. The use of the mouse salivary amylase signal peptide (or a mutant thereof) to provide secretion of heterologous proteins expressed in yeast has been described in Published PCT Applications Nos. WO 89/02463 and WO 90/10075, which are hereby incorporated by reference.

U.S. Pat. No. 5,726,038 describes the use of the signal peptide of the yeast aspartic protease 3, which is capable of providing improved secretion of proteins expressed in yeast. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978), which is hereby incorporated by reference. The Hinnen et al. protocol selects for Trp transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

The gene may be maintained on stable expression vector, an artificial chromosome, or by integration into the yeast host cell chromosome. Integration into the chromosome may be accomplished by cloning the phytase gene into a vector which will recombine into a yeast chromosome. Suitable vectors may include nucleotide sequences which are homologous to nucleotide sequences in the yeast chromosome. Alternatively, the phytase gene may be located between recombination sites, such as transposable elements, which can mobilize the gene into the chromosome.

The present invention also provides a method of producing phytase by providing an isolated appA gene, which encodes a protein or polypeptide with phytase activity, and expressing the gene in host cell. Preferably the appA gene is isolated from *Escherichia coli*. Preferred host cells include yeast or filamentous fungi. The preferred filamentous fungi is *Aspergillus niger* and the preferred yeast are *Saccharomyces, Kluyveromyces, Torulaspora*, and *Schizosaccharomyces*, in particular, the yeast strain, *Saccromyces cerivesia*.

A method of converting phytate to inositol and inorganic phosphorus is also provided. An appA gene is isolated from an organism, using techniques well known in the art. A protein or polypeptide with phytase activity is then expressed from the gene in a host cell. The resulting protein or polypeptide is mixed or contacted with phyate. This technique is especially useful for treating phytate in food or animal feed. The preferred appA gene is isolated from *Escherichia coli*.

While the phytase enzyme produced in a yeast system released phytate-P from corn and soy as effectively as the currently commercial phytase, it appeared to be more thermostable. This phytase overexpression system in yeast can be used to provide thermostable phytase for use in the food and feed industries.

EXAMPLES

Example 1

Materials and Methods for Overexpressing PhyA in *E. Coli, S. Lividans*, and a *Saccharomyces* System

Phytase gene, host strains, and expression plasmids. Phytase gene, phyA, was kindly provided by Dr. E. J. Mullaney of the USDA. The gene (GenBank Accession number M94550) was contained in plasmid pMD4.21 in *E. coli* strain HB101. A 2.7 kb SphI fragment of *A. niger* DNA contained the coding region of the deglycosylated phytase and its 5' and 3' flanking sequences. A plasmid containing the signal peptide sequence, Spxy, of the active xylanase gene of *Aureobasidum pullulans* (GenBank Accession number U10298) was kindly provided by Dr. X. L. Li of the University of Georgia. The *E. coli* strain DH5α was used as an initial host for all the recombinant plasmids. In order to express phyA in *E. coli*, the expression vector, pET25b(+) (Novagen, Madison, Wis.) and the expression host, BL21 (DE3)pLysS, were used. In order to express phyA in *S. lividans* TK 24, plasmid pSES1 (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividansoglucanase* Gene and an Endoglucanase Gene from *Thermomonospora Fusca*," *Appl. Environ. Microbiol.*, 59:3032-43 (1993), which is hereby incorporated by reference), was used to construct the shuttle plasmid (from Dr. D. B. Wilson of Cornell University and he obtained it from Dr. D. A. Hopwood, John Innes Institute, Norwich, England). In order to express phyA in yeast, the expression vector pYES2 and the host *S. cerevisiae* strain, INVSc1 (Invitrogen, San Diego, Calif.) were used.

Plasmid cassette constructions and transformations. All the constructed plasmids and the correspondent hosts are listed in Table 1. A 1.4 kb PCR fragment of phyA gene was amplified

from the pMD4.21 by using two primers: upstream 5'-CGG AAT TCG TCA CCT CCG GAC T-3' (SEQ ID No. 1) and downstream 5'-CCC AAG CTT CTA AGC AAA ACA CTC-3' (SEQ ID No. 2). The resulting fragment contained the sequence coding for the deglycosylated phytase of *A. niger*, PhyA, and EcoRI and HindIII restriction site upstream and downstream, respectively. After purification with Geneclean II kit (Bio101, Inc., La Jolla, Calif.), the fragment was inserted into pET25b(+), and the resulting construct pEP1 (6893 bp) was transformed into BL21(DE3)pLysS after initial confirmation in DH5α cells. The expression was under the control of T7 promoter followed by the lead sequence (pel B) encoding 21 amino acids, and phyA. The host transformed with the pET25(+) vector only was used as the control.

TABLE 1

Expression vectors, constructs, and their host strains used in the study

Plasmid	Host	Description[1]	Reference[2]
pET25b(+)	*E. coli* DH5α and BL21 (DE3)pLysS	Expression vector	Novagen
pEP1	*E. coli* BL21 (DE3)pLysS	pET25bα(+) + phyA gene	This paper
PSES2	*E. coli* DH5α and *S. lividans* TK24	Expression vector	Jung et al.[2], 1993
PSPP1	*E. coli* DH5α and *S. lividans* TK24	pSES2 + Spe2 + phyA	This paper
PYES2	*E. coli* DH5α and *S. cerevisiae* INVSc1	Expression vector	Invitrogen
PYEP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Spe2 + phyA	This paper
PYXP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Spxy + phyA	This paper
PYPP1	*E. coli* DH5α and *S. cerevisiae* INVSc1	pYES2 + Sphy + phyA	This paper

[1]Spe2 is the signal peptide for endoglucanase E2 of *T. fusca* (Wilson, D. B., "Biochemistry and Genetics of Actinomycete Cellulases," Crit. Rev. Biotechnol., 12: 45-63 (1992), which is hereby incorporated by reference); Spxy is the signal peptide forxylanase of *A. pullulans* (Li and Ljungdahl, "Cloning, Sequencing, and Regulation of a Xylanase Gene from the Fungus *Aureobasidium pullulans* Y-2331-1," Appl. Environ. Microbiol., 60: 3160-66 (1994); Li and Ljungdahl, "Expression of *Aureobasidium pullulans* xynA in, and Secretion of the Xylanase from, *Saccharomyces cerevisiae*," Appl. Environ. Microbiol., 62: 209-13 (1996), which are hereby incorporated by reference); and Sphy is the signal peptide for phyA of *A. niger* (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," Gene 127: 87-94 (1993), which is hereby incorporated by reference).

[2]Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces* Lividansoglucanase Gene and an Endoglucanase Gene from *Thermomonospora Fusca*," Appl. Environ. Microbiol., 59: 3032-43 (1993), which is hereby incorporated by reference).

The construction of the plasmid for phyA expression in *S. lividans* started with the synthesis of a fragment containing pLT1 promoter and Spe2 signal peptide (Lao, G. et al., "DNA Sequences of Three Beta-1,4-endoglucanase Genes From *Thermomonospora Fusca*," *J. Bacteriol.*, 173:3397-407 (1991), which is hereby incorporated by reference) by PCR. An upstream primer, 5'-CAG CTA TGA CCA TGA TTA CGC C-3' (SEQ ID No. 3), and a downstream primer, 5'-CCT AGA ACG GGA ATT CAT TGG CCG CC-3' (SEQ ID No. 4), contained PstI and EcoRI restriction sites, respectively. The fragment was amplified from pBW2 (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene From *Thermomonospora Fusca*," *Appl. Environ. Microbiol.*, 59:3032-43 (1993), which is hereby incorporated by reference) and then digested with PstI and EcoRI, while the construct pEP1 and plasmid pBluescript SK$^+$ (Strategene, La Jolla, Calif.) were digested with EcoRI and HindIII, and PstI and HindIII, respectively. The three digested fragments were subsequently purified using Geneclean II kit and ligated into a single recombinant construct that contained the desired restriction sites of PstI and KpnI (from pBluescript SK*), pLT1 promoter and Spe2 leading peptide of endoglucanase E2 (551 bp, Lao, G. et al., "DNA Sequences of Three Beta-1,4-endoglucanase Genes From *Thermomonospora Fusca*," *J. Bacteriol.*, 173:3397-407 (1991), which is hereby incorporated by reference), and phyA gene (1365 bp). After the construct was digested with PstI and KpnI, the resulting fragment was inserted into the expression vector pSES1, and the formed shuttle plasmid (pSPP1, 9131 bp) was transformed into the host *S. lividans* protoplasts according to Hopwood et al. (Hopwood, D. A., et al., *Genetic Manipulation of Streptomyces-A Laboratory Manual*, The John Innes Foundation, Norwich, England (1985), which is hereby incorporated by reference). Likewise, a control was prepared by transforming *S. lividans* with expression vector pSES2.

Three shuttle plasmids with three different signal peptide sequences were constructed to express phyA in the yeast system (See Table 2). The first plasmid was originated from a HindIII digested fragment of pSPp1, including the promoter pLT1, lead sequence Spe2, and the coding region sequence of phyA. The fragment was ligated into the HindIII site of pYES2 treated with calf intestinal alkaline phosphatase and the plasmid was named pYEP1 (7783 bp) after its right orientation was confirmed. The second plasmid contained Spxy, a signal peptide sequence of xylanase gene from *A. pullulans* (Li, X. L. et al., "Cloning, Sequencing, and Regulation of a Xylanase Gene From the Fungus *Aureobasidium Pullulans*) Y-2311-1," *Appl. Environ. Microbiol.*, 60:3160-166 (1994); Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea*," *Appl. Environ. Microbiol.*, 62:209-13 (1996), which are hereby incorporated by reference), and phyA gene. Spxy was spliced with phyA by overlap extension (Horton, R. M., "In Vitro Recombination and Mutagenesis of DNA: SOEing Together Tailor-Made Genes," *PCR Protocols: Current Methods and Applications*, 251-61 (1993), which is hereby incorporated by reference) with two successive steps of PCR. One was to amplify Spxy sequence from pCE4 (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea*," *Appl. Environ. Microbiol.*, 62:209-13 (1996), which is hereby incorporated by reference) using upstream primer (5'-CCC AAG CTT GAT CAC ATC CAT TCA-3') (SEQ ID No. 5) with a HindIII restriction site (primer 1) and overlapping downstream primer (5'-CGG GGA CTG CTA GCG CAC GTT CGA T-3', primer 2) (SEQ ID No. 6). The other PCR was to amplify the coding region of phyA from pEP1 using overlapping upstream primer (5'-ATC GAA CGT GCG CTA GCA GCA GTC CCC G-3', primer 3) (SEQ ID No. 7) and downstream primer (5'-GCT CTA GAC TAA GCA AAA CAC TCC-3', primer 4) (SEQ ID No. 8) with a XbaI restriction site. The second step of PCR was conducted to merge the two fragments generated from the above two PCR by using the two purified fragments as the templates and primers 1 and 4. The resulting fragment contained HindIII and XbaI restriction sites and was cloned into pSES2. This plasmid was named pYXP1 (7219 bp). The third plasmid contained the signal peptide (Sphy) sequence of phyA and the coding region of phyA, excluding the intron between them (Hartingsveldt, W. van., et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," *Gene* 127:87-94 (1993), which is hereby incorporated by reference). Two primers, including a 70 bp of upstream primer contained the signal peptide with an engineered KpnI restriction site and a downstream primer that was the same one used for pYXP1 construction (primer 4) were used to amplify the desired fragment from pEP1. The PCR product was digested with KpnI and XbaI and cloned into pSES2, resulting in a plasmid named pYPP1 (7176 bp). All

the above three constructs were transformed into *S. cerevisiae* by the method of Ito et al., "Transformation of Intact Yeast Cells Treated with Alkali Cations," *J. Bacteriol.,* 153:163-68 (1983), which is hereby incorporated by reference.

TABLE 2

Signal peptides used for expression of phyA in *S. cerevisiae*

Construct Size (bp)	Peptide	Gene	Organism	Phytase activity[1] (mPU/ml)
pYEP1 7783	Spe2 (93 bp)	Cellulase E2	*T. fusca*	.80
pYXP1 7219	Spxy (102 bp)	Xylanase A	*A. pullulans*	Non-detectable
pYPP1 7176	Sphy (57 bp)	PhyA Phytase	*A. niger*	146
pSES1[2] 7224			*S. cerevisiae*	Non-detectable

[1]The phytase activity was detected in the supernatant of cell culture of Sabouraud-raffinose medium 15 hours after induced by adding galactose. See text for definition of phytase units.
[2]Expression vector for *S. cerevisiae*, used as a control.

Growth medium and induction of the gene expression. In the *E. coli* system, the transformants were grown in 50 ml of LB medium containing 50 μg/ml of ampicillin at 30° C. After the OD_{600} value of the medium reached 0.5 to 0.6, phytase gene expression was induced by adding IPTG (isopropyl b-D-thiogalactopyranoside) into the medium to a final concentration of 1 mM. Three hours after the induction, cells were collected by spinning down at 8000×g for 15 minutes, washed with 1×PBS, and lysed by lysozyme. Soluble and insoluble fractions of the cells were prepared, and a sample containing 500 μg of total protein (Lowry, O. H. et al., "Protein Measurement With the Folin Phenol Reagent," *J. Biol. Chem.,* 193:265-75 (1951), which is hereby incorporated by reference) was suspended in the same volume of 2×SDS buffer and analyzed by SDS-PAGE (Laemmli, U.K., "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4," *Nature* (*London*), 227:680-85 (1970), which is hereby incorporated by reference).

Recombinant *S. lividans* was grown in TSB broth with 5 μg/ml of *thiostrepton* at 30° C. (Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene from *Thermomonospora Fusca," Appl. Environ. Microbiol.,* 59:3032-43 (1993), which is hereby incorporated by reference). After 72 hours incubation, the cells and medium were harvested and prepared for SDS-PAGE (Wilson, D. B., "Biochemistry and Genetics of Actinomycete Cellulases," *Crit. Rev. Biotechnol.,* 12:45-63 (1992), which is hereby incorporated by reference).

Transformants of *S. cerevisiae* were initially grown in Sabouraud-raffinose (4%) medium (100 ml) without uracil for 48 hours, sterile galactose was then added into the medium (2%) to induce phytase expression. Samples of media and cells were collected at various time points, and extracellular and intracellular samples were prepared as described by Li and Ljungdahl, "Expression of *Aureobasidium pullulans* xynA in, and Secretion of the Xylanase from, *Saccharomyces cerevisiae," Appl. Environ. Microbiol.,* 62:209-13 (1996), which is hereby incorporated by reference. When needed, the supernatant of the expressed cell culture fractions was concentrated with Stirred Cells of Amicon (Beverly, Mass.) by using YM10 membranes (MW cutoff of 10,000). Other media were tested accordingly.

Enzyme protein and activity assay. Amounts of expressed phytase protein under various conditions were quantified by the relative densitometry of specific bands in SDS-PAGE, using IS-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, Calif.). Phytase activity in the samples of media and cells was determined as previously described (Piddington, C. S. et al., "The Cloning and Sequencing of the Genes Encoding Phytase (phy) and pH 2.5-optimum Acid Phosphatase (aph) from *Aspergillus niger* var. *awamori," Gene,* 133:56-62 (1993), which is hereby incorporated by reference) and the inorganic phosphate released was assayed by the method of Chen, P. S. et al., "Microdetermination of P," *Anal. Chem.,* 28:1756-58 (1956), which is hereby incorporated by reference. One phytase unit (PU) was defined as the amount of enzyme that releases one μmol of inorganic phosphate from sodium phytate per minute at 37° C.

Western blotting (immunoblot) analysis. The soluble fraction of the cell mass of the phytase transformed *E. coli* and the medium supernatant of *S. lividans* and *S. cerevisiae* transformants were collected as for SDS-PAGE. After electrophoresis, the proteins were then transferred onto Protran® nitrocellulose membrane (Schleicher & Schuell, Keene, N.H., USA) in 20 mM Tris-HCl (pH 8.3), 20% methanol, and 0.1% SDS, by using a Mini Trans-Blot cell (Bio-Rad Laboratories). Transfer was done overnight at a constant 50 V and the initial buffer temperature was 4° C. The membranes were then subjected to Western blot analysis. A rabbit polyclonal IgG (Kindly provided by Dr. A. H. J. Ullah of USDA. Dilution, 1:5,000) against purified native *A. niger* phytase was used as the first antibody. The blotting was finalized using Immuno-Blot Assay Kit (Bio-Rad Laboratories) containing a second antibody conjugated with horseradish peroxidase.

Total RNA isolation and analysis. Total RNA was isolated with TRIzol™ Reagent (GIBCO BRL, Gaithersburg, Md.) from *E. coli* and *S. cerevisiae* transformants 3 and 15 hours after induction, respectively. RNA samples (10 μg per lane) were then separated by formaldehyde agarose (1.5%, wt/vol) gel electrophoresis and transferred to Hyblot membranes (National Labnet, Woodbridge, N.J.) (Davis et al., *Basic Methods in Molecular Biology,* 2nd Ed., Appleton and Lange, Norwalle, Conn. (1994), which is hereby incorporated by reference). A 1.4 kb EcoRI-HindIII fragment in plasmid pEP1 was prepared and was random-primed labeled with ^{32}P using a DNA labeling kit followed by G-50 column purification (Pharmacia Biotech., Piscataway, N.J.) and then hybridized with the blotted RNA membranes in a hybridization oven (Hybaid, Middlesex, UK). The hybridized membranes were exposed to screens in Fuji Imaging Plate and analyzed by Bio-Imaging Analyzer (Kohshin Graphic Systems, Fuji, Japan).

Example 2

Expression of PhyA in *E. coli*

Four hours after the induction, a specific band (~55 kDa) was viewed in SDS-PAGE (12.5%) of the soluble cell fraction, compared to the only expression vector transformed control (See FIGS. **1** and **2**). This band represented 3.8% of the total soluble protein of this fraction. Correspondingly, northern analysis showed overexpression of phyA mRNA in these phytase gene transformants and no signal was viewed in the control cells (See FIG. **3**).

In order to optimize phytase protein expression, the time course and the effects of a series of factors on the expression were studied. These factors included incubation temperature (30 and 37° C.), medium pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), anaerobiosis (adding sterile mineral oil on the top of the growing cells), inorganic phosphate level in the medium (Dassa, E. et al., "The Acid Phosphatases with Optimum pH

of 2.5 of *Escherichia coli," J. Bio. Chem.,* 257:6669-76 (1982), which is hereby incorporated by reference), and sodium phytate (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM). Results indicated that expression of phytase protein was accumulated linearly with time for the first six hours after induction (See FIG. **4**). Thereafter, the expression remained relatively unchanged although bacterial cells continued to grow. Only medium pH and sodium phytate concentration significantly affected the phytase protein expression. Maximum protein was shown at pH 6.0 and 0.3 mM of sodium phytate, in which phytase protein was increased from 3.8 to 9.6% of the total soluble protein.

No phytase activity was detected extracellularly or intracellularly. This may not be completely unexpected, because the native phytase from *A. niger* is a glycoprotein with a size of 70-80 kDa (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87-94 (1993), which is hereby incorporated by reference). The protein expressed in the *E. coli* system of this study had a size of approximately 55 kDa. Presumably, the lack of glycosylation of the protein and other necessary post-translational modifications during secretion would preclude phytase activity.

Example 3

Expression of PhyA in *S. lividans*

Heterologous genes have been expressed in *S. lividans*, and the resulting products secreted into the medium with enzymatic activity (Ghangas, G. S. et al., "Cloning of the *Thermomonospora Fusca* Endoglucanase E2 Gene in *Streptomyces Lividans*: Affinity Purification and Functional Domains of the Cloned Gene Product," *Appl. Environ. Microbiol.,* 54:2521-26 (1988); Wilson, D. B., "Biochemistry and Genetics of Actinomycete Cellulases," *Crit. Rev. Biotechnol.,* 12:45-63 (1992); Jung, E. D. et al., "DNA Sequences and Expression in *Streptomyces Lividans* of an Exoglucanase Gene and an Endoglucanase Gene from *Thermomonospora Fusca," Environ. Microbiol.,* 59:3032-43 (1993), which are hereby incorporated by reference). Similarly, phyA gene was expressed in *S. lividans* and the protein was introduced into the medium, as shown in a specific band in the SDS-PAGE analyzed medium samples (See FIGS. **5** and **6**). This suggested that the signal peptide from endoglucanase E2 gene of *T. fusca* was able to lead phytase protein out of the cell. This protein was 57 kDa and represented 16.2% of the total protein in the medium. Changing medium pH to 6.0 and adding 0.3 mM of sodium phytate in the medium improved the protein yield to 20.3% of the total protein. Because phytase protein was secreted into the medium in such a high level, it should be easy to purify and used effectively for a variety of purposes such as producing phytase antibody. Once again, no increased phytase activity was found either in the medium or in the lysed cells. Although the protein size increased a little bit (2-3%) compared to the one expressed in *E. coli*, presumably due to glycosylation of phytase protein in this expression system, there was still no phytase activity.

Example 4

Expression of PhyA in *S. cerevisiae*

Three different signal peptides were used to compare the efficiency in leading the expressed protein out of the cells (See Table 2). The phytase activity was substantially increased in the Sabouraud-raffinose medium growing the transformants of pYEP1 and pYPP1, but not pYXP1. Visible phytase protein was shown by SDS-PAGE 20 hours after induction (FIG. **7**).

The expression of transformants of pYEP1 and pYPP1 were determined in three different types of medium: Sabouraud-raffinose (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea," Appl. Environ. Microbiol.,* 62:209-13 (1996), which is hereby incorporated by reference), Sabouraud-glycerol, and a modified general-purposed YEPD medium. As to transformants of pYEP1, similar phytase activity was expressed in the Sabouraud-raffinose and Sabouraud-medium, but there was no activity detected in the YEPD medium. In contrast, phytase activity in the medium cultured with transformants of pYPP1 varied greatly with the different types of medium. The activity was enhanced to 375 mU/ml when Sabouraud-glycerol medium was used. The activity was further increased to 1675 mU/ml, when the medium was changed to YEPD (See Table 3). While the YEPD medium was much cheaper than the Sabouraud-raffinose medium, the phytase yield was increased more than ten-folds. Thus, the putative signal peptide from the fungal phytase gene achieved the most efficient expression of the extracellular phytase activity. Nearly all the protein produced was secreted into the YEPD medium, because very little activity was detected in the yeast cells. The time course of the phytase expression in this system was shown in FIG. **8**.

TABLE 3

Phytase activity expressed from transformant with pYPP1 in different media
Hours after induction (mPU/ml)[1]

Medium	0	10	15
Sabouraud-raffinose	22	136	146
Sabouraud-glycerol	6	174	375
YEPD	18	1238	1675

[1]The phytase activity was detected in the supernatant of cell culture of the three media 0, 10, and 15 hours after induced by adding galactose. See text for definition of phytase units.

A variety of microorganisms including bacilli, yeasts, and filamentous fungi have phytase activity, while *A. niger* NRRL3135 strain produces the highest activity (340 mU/ml, Shieh, T. R. et al., "Survey of Microorganisms for the Production of Extracellular Phytase," *Appl. Environ. Microbiol.,* 16:1348-51 (1968), which is hereby incorporated by reference). *Schwanniomyces castellii* CBS 2863 has the highest phytase activity among 21 yeast strains (140 mU/ml, Lambrechts, C. et al., "Utilization of Phytate by Some Yeasts," *Biotechnology Letters,* 14:61-6 (1992), which is hereby incorporated by reference). Clearly, the recombinant yeast strain transformed with pYPP1 in the present study produced much higher phytase activity (1675 mU/ml) than *A. niger* (4-fold) and *S. castellii* CBS 2863 (11-fold). Maximum phytase production can be obtained in the system by optimizing the incubation conditions and modifying the plasmid cassettes (Demolder, J. W. et al., "Efficient Synthesis of Secreted Murine Interleukin-2 by *Saccharomyces Cerevisiae*: Influence of 3'-Untranslated Regions and Codon Usage," *Gene,* 111:207-13 (1992), which is hereby incorporated by reference).

The high level of phytase activity expression in *S. cerevisiae* was most likely due to the sufficient glycosylation of phytase protein and other post-translational modifications by yeast. After the medium supernatant was concentrated and subjected to SDS-PAGE analysis, there was a band with approximately 110 kDa (See FIGS. **7** and **9**), which was larger than the size of the native protein from *A. niger* (Hartingsveldt, W. van. et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger," Gene* 127:87-94 (1993), which is hereby incorporated by reference). Northern analysis confirmed the specific overexpression of phyA mRNA (See FIG. **10**). These results indicated that the yeast system was efficient to over-

express actively extracellular phytase enzyme. Yeast system has several advantages over bacteria or other systems such as *A. niger* (Hartingsveldt, W. van. et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," *Gene* 127:87-94 (1993), which is hereby incorporated by reference). It carries out post-translational modifications, including proper folding, glycosylation, disulfide bond formation, and proteolysis, during the translocation of proteins through the endoplasmic reticulum and the cell membrane. The secretion of proteins is facilitated by hydrophobic short signal peptides at the N-terminal regions of the protein precursors (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea*," *Appl. Environ. Microbiol.*, 62:209-13 (1996), which is hereby incorporated by reference). Proteins secreted by yeast cells are protected from aggregation and protease degradation. Most importantly, enzyme proteins produced by *S. cerevisiae* are easily purified, because it secretes only a few proteins. Considering the well-known safety of yeast products to both human beings and animals, this system is of great potential for human food and animal feed industry.

Example 5

Properties of the PhyA Phytase Overexpressed in *Saccharomyces cerevisiae*

The overexpressed phytase from transformants of pYPP1 plasmid was concentrated and used to study its property (See Table 4). The enzyme showed two optimum pH ranges: 2 to 2.5 and 5.0 to 5.5. However, enzyme activity at pH 2 to 2.5 was only 60% of the activity at pH 5 to 5.5. There was no activity detected at either pH 1 or 8. The optimum pH was virtually the same as the phytase from *A. niger* (Simons et al., "Improvement of Phosphorus Availability by Microbial Phytase in Broilers and Pigs," *Br. J. Nutr.*, 64:525 (1990), which is hereby incorporated by reference), thus active function in hydrolysis of phytate-P in the gastrointestinal tracts would certainly be expected. The optimum temperature of the enzyme was 60° C., while the current one on the market produced by Gist-Brocades is 55° C. (BASF, 1996). More than 80% of the activity remained at 50 to 55° C., but little activity was detected at 75 or 80° C. Heating the enzyme for 15 min at 37 and 80° C., the remaining activity for the expressed yeast phytase of the present invention was 100 and 63%, respectively, and for BASF Gist-Brocades phytase was 100 and 52%, respectively. The differences between the two enzyme sources at any given temperature were significant (See Table 5). Thus, the yeast phytase appeared to be more heat stable than the current commercial phytase product.

TABLE 4

Characteristics of the overexpressed phytase in yeast[1]

Optimum pH[2]									
	pH								
	1.0	2.0	2.5	3.0	4.0	5.0	5.5	6.0	8.0
Relative Activity (%)	$.5^{e} \pm .2$	$59.7^{c} \pm 3$	$64.8^{c} \pm 6$	$48.1^{d} \pm 4$	$81.0^{b} \pm 5$	$100.0^{a} \pm 1$	$95.0^{a} \pm 6$	$66.3^{c} \pm 1$	$.8^{e} \pm .4$
Optimum Temperature[3]									
	° C.								
	25	37	45	50	55	60	75	80	
Relative activity (%)	$24.2^{e} \pm .8$	$44.6^{d} \pm 3$	$63.9^{c} \pm 8$	$83.6^{b} \pm 2$	$89.8^{b} \pm 4$	$100.0^{a} \pm 4$	$.6^{f} \pm .1$	$.9^{f} \pm .2$	

[1]Data are means of relative activity ± standard deviation (n = 4). Means in a row with different superscript letters differ (P < 0.05). The general linear model of the statistical analysis system (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multiple treatment mean comparison. Significance level was set as P < 0.05.
[2]The activity was assayed at 37° C. (see context for phytase unit definition). Different buffers were used: 0.2 mM glycine-HCl buffer for pH 1.0 to 3.5; 0.2 mM sodium citrate buffer for pH 4.0 to 6.5; and 0.2 mM Tris-HCl buffer for pH over 7.
[3]Optimum temperature was determined at pH 5.5 (0.2 mM sodium citrate buffer).

TABLE 5

Comparison of the thermostability of overexpressed phytase in yeast and Gist-Brocades phytase produced by *A. niger*[1,2]

Relative activity, %	37° C.	80° C.
Yeast phytase	$100^{a} \pm 1$	$63^{b} \pm 1$
A. niger phytase	$100^{x} \pm 3$	$52^{y} \pm 2$
P[3] <		.03

[1]Data are means of relative activity ± standard deviation (n = 3). Means in a row with the different superscript letters differ (P < 0.05). The general linear model of the statistical analysis system (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multiple treatment mean comparison. Significance level was set as P < 0.05.
[2]The enzyme was heated for 15 minutes at different temperatures before reacting at 37° C. and pH 5.5.
[3]Significance (P values) of t-test between the activity of the two phytases at each temperature setting.

Although it is unclear how such improvement in thermostability is related to different post-translational modifications (folding, cleavage, glycosylation, etc.), (Li, X. L. et al., "Expression of *Aureobasidium Pullulans* XynA in, and Secretion of the Xylanase From, *Saccharomyces Cerevisiaea*," *Appl. Environ. Microbiol.*, 62:209-13 (1996), which is hereby incorporated by reference), it is certainly advantageous to have more thermostable phytase enzyme that can hopefully be resistant to the heat during feed pelleting, which is a problem with the current Gist-Brocades phytase.

Example 6

In Vitro Hydrolysis of Phytate-P from Corn, Soy, and Wheat Middlings by the Expressed Yeast Phytase

The expressed yeast phytase released phytate-P from corn and soybean meal as effectively as the Gist-Brocades phytase based on per unit activity (See Table 6). As expected, the

hydrolysis of phytate-P was a function of time and activity dosage. The expressed yeast phytase was also effective in releasing phytate-P from wheat middling, indicating its great potential in bread fermentation. Because the wheat middling used in this study contained much higher intrinsic phytase activity than commonly used wheat flour, much greater effect of the expressed yeast phytase on improving flour phytate-P hydrolysis and in trace element releasing would be expected, when it is used in a bakery (Hall, M. N. et al., "The Early Days of Yeast Genetics," *Cold Spring Harbor Laboratory Press* (1993), which is hereby incorporated by reference).

TABLE 6

Free phosphorus released from corn, soybean meal (SBM), and wheat middlings by overexpressed yeast phytase and fungus *A. niger* phytase in vitro[1]

Yeast phytase (PU/kg)		0	100	250	500	1000	250 (fungus phytase)
		Free phosphorus (mg/g)					
Corn:	1 hour	$.23^{d} \pm .03$	$.64^{c} \pm .08$	$1.14^{b} \pm .18$	$1.46^{a} \pm .04$	$1.54^{a} \pm .04$	$1.16^{b} \pm .15$
	4 hour	$.36^{c} \pm .02$	$1.26^{b} \pm .04$	$1.60^{a} \pm .03$	$1.66^{a} \pm .06$	$1.72^{a} \pm .04$	$1.68^{a} \pm .04$
SBM:	1 hour	$.68^{d} \pm .01$	$1.18^{cd} \pm .02$	$1.62^{c} \pm .18$	$2.48^{b} \pm .32$	$3.13^{a} \pm .19$	$1.68^{c} \pm .2$
	4 hour	$.73^{d}$	1.67^{c}	2.69^{b}	3.41^{a}	3.71^{a}	2.78^{b}
Wheat Middlings:	1 hour	$3.56 \pm .39$	$4.11 \pm .64$		$4.67^{2} \pm .05$		
	4 hour	$5.63 \pm .5$	$6.02 \pm .48$		$6.38^{2} \pm .07$		

[1]Each sample of 5 g was stirred in 20 ml of 0.2 mM sodium citrate buffer at 37° C. for 1 or 4 hours. The supernatant was obtained by spinning for 15 minutes at 8000 g. After going through Whatman 541 filter paper, the sample was subjected to free P assay by the method of Chen, P. S. et al., "Microdetermination of P," Anal. Chem., 28: 1756-58 (1956), which is hereby incorporated by reference. Data in the table are means of relative activity ± standard deviation(n = 4). The General Linear Model of the Statistical Analysis System (1988) was used to analyze the main treatment effects as randomized complete designs and Bonferroni t-test was used for multiple treatment mean comparison. Significance level was set as $P < 0.05$. A significant difference existed between 1 and 4 hour for every feed at each dose of enzyme as analyzed by t-test. Means in a row with different superscript letters differ ($P < 0.05$).
[2]n = 2

The overexpression of *Aspergillus niger* phytase (phyA) in *Escherichia coli, Streptomyces lividans*, and *Saccharomyces cerevisiae* were compared to develop an efficient and simple system to produce phytase economically. A 55 kDa soluble intracellular protein, representing 9.6% of the total soluble protein, was expressed in *E. coli* by using pET25b(+) system. A 57 kDa extracellular protein, representing 20.3% of the total protein in the medium, was expressed in *S. lividans* by using a shuttle plasmid containing the pLT1 promoter and Spell leading peptide of endoglucanase E2. No increase in phytase activity was shown in either expression system, presumably due to the lack of glycosylation and other necessary post-translational modification. In contrast, high extracellular phytase activity was produced in *S. cerevisiae* transformed with phyA gene. Three different signal peptides and three different types of medium were compared to identify the best expression vector and condition. Use of the signal peptide Sphy from phyA gene and YEPD medium produced the highest extracellular phytase activity. The overexpressed phytase in yeast was approximately 110 kDa, had two pH optima: 2.0 to 2.5 and 5.5 to 6.0, and the optimum temperature was at 60° C.

Example 7

Methods and Materials for Expression of phyA in *Pichia*

Host and vector. An EasySelect™ *Pichia* Expression Kit was purchased from Invitrogen (San Diego, Calif.). The kit provides hosts and vectors to express the gene either intracellularly or extracellularly, in strains of either Mut^{+} or Mut^{s} (Methanol utilization normal or slow). X33 was used as a Mut^{+} strain and KM71 as a Mut^{s} strain. Two vectors were used, pPICZ B (3.3 kb) and pPICZαA (3.6 kb), both use AOX1 as the promoter.

Construction of the Expressing Vectors. To compare the effect of different signal peptides on the expression of PhyA in *Pichia* system, two constructs were prepared. First, a 1.4 kb EcoRI-KpnI fragment, containing the PhyA sequence encoding the mature phytase protein, was ligated into pPICZαA. In this plasmid (pPICZα-phyA), PhyA was led by an alpha-factor, a very general-used signal peptide from *Saccharomyces cerevisiae*. Second, a 1.4 kb KpnI-XbaI fragment of pYPP1 was ligated into the vector (the coding region of phyA was led by its own signal peptide that was very effective in secreting the expressed phytase in *Saccharomyces cerevisiae*.)

Transformation and expression. The confirmed constructs were linearized by PmeI and transformed into GS115 and KM71, by EasyComp™ provided by the kit. Neocin™ was used to select the positive colonies. After a single colony was inoculated into 10 ml of MGY medium and grown to OD_{600} of 2-6 at 30° C., the cells were collected by centrifugation and resuspended into 10 ml of MMY medium (containing 0.5% of methanol). The samples were collected every 12 or 24 h after induction. The cells were separated from the supernatant and lysed with glass beads in breaking buffer. Phytase activity in the supernatant and cells was assayed as described previously. SDS-PAGE and Western blot were conducted to determine the size and relative amount of the expressed protein.

Example 8

PhyA Phytase Activity in *Pichia*

The expression construct using alpha-factor as the signal peptide for phyA was transformed into two *Pichia* strains. KM71 is a methanol utilization slow strain, while X33 is a *Pichia* wild-type utilizing methanol efficiently. The screening and incubation were conducted in 10 ml shake flasks under 29-30° C. For the transformants of KM71, 19 out of 20 picked colonies had extracellular phytase activity greater than 6 units/ml of culture supernatant after induction for 24 hours. Colony No. 13 showed the highest activity of 26 units/ml after incubated for 108 hours. For the transformants of X33, all colonies (20/20) had more than 10 units/ml after induced for 24 hours. One of the colonies (#101) produced phytase activ-

ity of 65 units/ml of supernatant. A time course study of the phytase expression in KM71 and X33 was summarized in FIG. **11**. Despite the difference of these two strains in utilizing methanol and, therefore, the ability in expressing phytase, it was found that alpha-factor was correctly processed by yeast cells. Besides, almost all of the expressed protein was secreted into the medium since not more than 5% of the total activity expressed was found intracellularly.

Effects of inorganic phosphorus and pH of media on the phytase expression were studied in the media (BMGY and BMMY) using a phyA recombinant of X33 (#101). The medium containing 50 mM phosphate produced the highest phytase activity, 66 units/ml at 168 hours after induction. By including 50 mM phosphate in the media, the effect of different pH of this buffer (3, 4, 5, 6, 7, and 8) on expression was also studied. When the pH was 6, this X33 transformant produced 75 units phytase/ml supernatant. Based on the protein concentration and SDS-PAGE analysis, the expression phytase protein yield was estimated to be between 3 to 4 mg/ml.

Example 9

Properties of the PhyA Phytase Expressed in *Pichia*

Molecular size and deglycosylation of the expressed phytase. After the supernatant of the medium inoculated with the phyA transformant was subjected to SDS-PAGE, a strong band around 95 kDa was seen (FIG. **12**). This was almost the only viewed protein in the supernatant. The expressed phytase reacted efficiently with the rabbit polyclonal antibody raised against purified native *A. niger* phytase. This indicated that the immunoreactivity of the expressed phytase was essentially the same as that of the native phytase from *A. niger*. The size was decreased to 50 kDa by deglycosylation using Endo H. The phyA antibody also reacted with the deglycosylated phytase. In addition, deglycosylation, conducted under native conditions, reduced the phytase activity about 15%, indicating that glycosylation was important for the activity of the phytases. Moreover, glycosylation affected the thermostability of the enzymes (FIG. **13**).

Northern analysis. As showed in FIG. **14**, a 1.3 kb phyA DNA probe hybridized with the mRNA of the induced transformants from both KM71 (#13) and X33 (#101). Response was also seen from the transformants prior to induction. Probably, the expression of phyA in this system was not controlled strictly at the level of transcription.

Optimal pH and temperature and phytate-phosphorus hydrolysis. Similar to *A. niger* phytase, the expressed phytase had two optimum pH, 2.5 and 5.5 (FIG. **15**). The optimum temperature of the expressed phytase was 60° C. (FIG. **16**). When the expressed phytase was incubated with soy samples at 100, 200, 400, 800 mU/g of sample at 37° C., phosphorus was released in a linear fashion with the phytase dose (FIG. **17**).

Example 10

Methods and Materials for Overexpression of *E. coli* appA Gene in *Saccharomyces cerevisiae*

Gene and Protein. This gene, originally defined as *E. coli* periplasmic phosphoanhydride phosphohydrolase (appA) gene, contains 1,298 nucleotides (GeneBank accession number: M58708). The gene was first found to code for an acid phosphatase protein of optimal pH of 2.5 (EcAP) in *E. coli*. The acid phosphatase is a monomer with a molecular mass of 44,644 daltons. Mature EcAP contains 410 amino acids (Dassa, J. et al., "The Complete Nucleotide Sequence of the *Escherichia coli* Gene appA Reveals Significant Homology Between pH 2.5 Acid Phosphatase and Glucose-1-Phosphatase," *J. Bacteriology,* 172:5497-5500 (1990), which is hereby incorporated by reference). Ostanin, K. et al. ("Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.,* 267: 22830-36 (1992), which is hereby incorporated by reference), overexpressed appA in *E. coli* BL21 using a pT7 vector and increased its acid phosphatase activity by approximately 400-fold (440 mU/mg protein).

The gene and a host *E. coli* strain CU 1869 (No. 47092) were purchased from ATCC. The gene, an insert of 1.3 kb, was transformed into *E. coli* strain BL21 (no. 87441) using an expression vector pAPPA1 (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.,* 267:22830-36 (1992), which is hereby incorporated by reference).

Host and Vector. The vector for overexpressing appA gene in *Saccharomyces cerevisiae* was pYES2 and the host was INVScI (Invitrogen, San Diego, Calif.).

Construction of the Expression Vector. Initially, a 1.3 kb XbaI fragment was isolated from pAPPA1. This fragment contained the appA gene with its own signal peptide. After being ligated into the XbaI site of pYES2, the construct (PYES2-appA) was transformed into *Saccharomyces cerevisiae*. But, no phytase activity was increased in either extra- or intra-cellular parts compared to the controls. pAPPA1 and pYPP1 (PhyA and its signal peptide in pYES2) were cotransformed into the yeast strain. Again, no increase in phytase activity due to pAPPA1 was detected in the media or the yeast cells.

Two primers were synthesized to construct the signal peptide of PhyA gene with the coding region of appA gene. One was 80 bp long containing the PhyA signal peptide and a KpnI site at 5' end: GGG GTA CCA TGG GCG TCT CTG CTG TTC TAC TTC CTT TGT ATC TCC TGT CTG GAG TCA CCT CCG GAC AGA GTG AGC CGG AG (SEQ ID No. 9). The other primer was 24 bp long, with an EcoRI site at its 3' end: GGG AAT TCA TTA CAA ACT GCA GGC (SEQ ID No. 10). The PCR was run for 25 cycles, with 1 min denaturing at 95° C., 1 min annealing at 58° C., and 1 min chain extending at 72° C. A 1.3 kb fragment was amplified, digested, and ligated into pYES2. After the insert was confirmed by restriction mapping, the construct (pYES2-SphyA-appA) was transformed into INVScI by lithium acetate method.

Expression. The selected transformants were inoculated into YEPD medium. The expression was induced by adding galactose into the culture after OD_{600} reached 2, as described previously. The cells were harvested 15 or 20 h after induction.

Activity Assay. Acid phosphatase activity was assayed at 37° C. in 25 mM glycine-HCl buffer (pH 2.5), using p-nitrophenyl phosphate as the substrate (stock 250 mM). Reaction buffer of 1.7 ml was added into 0.1 ml samples. After they were incubated for 5 min in a 37° C. waterbath, 0.2 ml of prewarmed substrate was added and mixed. The reaction solution was transferred into a prewarmed cuvette and incubated for 2 min in a 37° C. spectrophotometric compartment. The released p-nitrophenol was read continuously for 5 min at 405 nm for enzyme activity calculation.

In vitro study. Soybean meal (5.0 g) was suspended into 20 ml of 20 mM citrate buffer, pH 5.5, mixed with 200 mU of phytase, incubated at 37° C. for 4 h with continuous shaking. After chilling on ice for 10 min, the slurry was transferred into

a centrifuge tube and spun for 15 min at 15,000×g. The supernatant was used to determine free phosphorus.

Example 11

Quantitation of Phytase Activity from Overexpression of *E. coli* appA Gene in *Saccharomyces cerevisiae*

The intracellular acid phosphatase activity in the appA overexpressed *E. coli* (pAPPA1) was 440 mU/mg protein. Unprecedently, an intracellular phytase activity greater than 4900 mU/mg protein was found in the transformed strain. But, there was only minimal phytase activity in the control (BL21). Thus, this acid phosphatase gene also codes for a phytase. The appA gene sequence was aligned with that of PhyA and found that these two genes shared 23% of identity.

Transforming INVScI with the construct of pYES2-Sphy-appA (led by the signal peptide of PhyA) produced extracellular phytase activity in the supernatant that was 2,000-fold greater than those of the wild type or of the transformant containing appA gene plus its own signal peptide (See Table 7).

TABLE 7

Extracellular phytase activity in transformants of appA gene with different signal peptides

Construct	Signal	Activity (mU/ml)	Activity (mU/mg protein)
PYES-appA	appA	Undetectable	Undetectable
pYES2-SphyA-appA	PhyA	1,158	445

The effects of medium (YEPD) inorganic phosphorus, phytate, pH, and temperature on the expression of phytase activity by pYES2-Sphy-A-appA are presented in Table 8. The highest phytase activity was 2,286 mU/ml (633 mU/mg protein) at the optimal condition.

TABLE 8

Effect of different conditions in the YEPD medium on phytase activity expression of pYES2-SphyA-appA in yeast.

Medium Conditions	Activity (mU/ml)
Phosphorus, mg/100 ml	
0	1402
1	714
5	722
10	456
Sodium phytate, g/100 ml	
0	870
0.1	1019
1.0	1748
pH	
5.0	892
7.0	996
8.0	2286
Temperature, ° C.	
25	312
30	1036
37	996

The thermostability of the overexpressed extracellular phytase activity produced by the yeast transformant was greater than that of the intracellular phytase produced by *E. coli* transformed with pAPPA1 (See Table 9). Heating the extracellular phytase for 15 min at 80° C. resulted in 30% of loss of its phytase activity, while almost all the phytase activity from *E. coli* was lost under the same condition.

TABLE 9

Effect of heating different sources of phytases under 80° C. for 15 min on their activities

Phytase	Relative activity after heating, %
appA in *E. coli*	0.1
appA in *S. cerevisiae*	69
PhyA in *S. cerevisiae*	66
BASF phytase	50

Comparisons of the effect on releasing phosphorus from soybean meal by phytases (200 mU) of *E. coli*, overexpressed AppA in yeast, and BASF are presented in Table 10. The results indicate that all three sources of phytases released phytate-phosphorus effectively from soybean meal.

TABLE 10

Free phosphorus released from soybean meal by different sources of phytases

Phytase	Phosphorus (mg/g)
appA in *E. coli*	1.11
appA in *S. cerevisiae*	0.69
BASF	0.87

E. coli appA (acid phosphatase) gene when expressed in *Saccharomyces cerevisiae* produces extracellular phytase activity in the media that was more than 2,000-fold greater than the control. The overexpressed phytase effectively releases phytate-phosphorus from soybean meal, and seems to be more thermostable than the presently available commercial phytase or the intracellular phytase produced in *E. coli* by the same gene (appA).

Example 12

Methods and Materials for Overexpressing the *E. coli* appA Gene Encoding an Acid Phosphatase/Phytase in *Pichia pastoris*

Gene and Protein. The appA gene and the host *E. coli* strain CU1867 (No. 47092) were obtained from ATCC. The gene, an insert of 1.3 kb, was transformed into *E. coli* strain BL21 (No. 87441) using an expression vector pAPPA1 (Ostanin, K. et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.*, 267:22830-36 (1992), which is hereby incorporated by reference).

Host and Vector. An EasySelect™ *Pichia* Expression Kit was obtained from Invitrogen (San Diego, Calif.). The kit provides hosts and vectors to express the gene either intracellularly or extracellularly in a wild-type strain (X-33). Two vectors were used, pPICZ B (3.3 kb) and pPICZαA (3.6 kb), both use AOX1 as the promoter.

Construction of the Expression Vector. Two primers were used to amplify the appA gene from pAPPA1 and two restriction sites EcoRI and KpnI were produced at the 5' and 3' ends, respectively.

Upstream primer:
GGA ATT CCA GAG TGA GCC GGA (SEQ ID No. 11)

Downstream primer:
GGG GTA CCT TAC AAA CTG CAC G (SEQ ID No. 12)

Template:
pAPPA1 DNA isolated from ATCC 87441

PCR was run for 30 cycles, with 1 min denaturing at 94° C., 1 min annealing at 55° C., and 1 min chain extending at 72° C. A 1,245 base-pair fragment was amplified, digested with EcoRI and KpnI, and ligated (16° C. overnight) into pPICZ B (3.3 kb) and pPICZαA (3.6 kb). The ligation was confirmed by restriction mapping after transforming the constructs into DH5α.

Transformation of the construct into *Pichia* (X33). For each transformation, 100 μg of plasmid DNA was prepared and linearized by digesting with PmeI. After linearization, the DNA was purified and resuspended into 10 μL of sterile, deionized water. Half amount of the DNA was actually used for each transformation. Electroporation and the EasyComp chemical kit (Invitrogen) were both used to transform the DNA into X33. In the case of electroporation, an Electro Cell Manipulator (ECM 600, Gentromics, BTX Instrument Division, San Diego, Calif. 92121) and 2 mm cuvettes were used. The resistance was 186 Ohm, the charging voltage was 1.5 kilovolts, and the actual charging length was approximately 7 milliseconds. The electroporated cells were incubated on YPD agar plates containing 100 mg Zeocin/mL at 30° C. for 2-4 days for colony growth. In the case of chemical transformation, cells were grown on YPDS agar plates containing 100 mg Zeocin/mL. Compared with the electroporation, the chemical method had lower transformation efficiency.

Expression. Single colonies were inoculated into 10 ml of MGY medium (30 ml tube) and grown (16-18 h) to OD_{600} of 5-6 at 28-30° C. in a shaking incubator (200 rpm). The cells were collected by centrifugation (2,000 rpm) and resuspended into 10 ml of BMMY medium (containing 0.5% of methanol) to induce the expression. The samples (200 μL) were collected every 12 or 24 h after induction. Methanol (100%) was added at 100 μL every 24 to maintain a concentration of 0.5-1% in the media.

Assays. The cells were separated from the media (supernatant) and lysed with glass beads in breaking buffer. Extracellular phytase activity in the supernatant and intracellular phytase activity in the lysed cells were assayed as described previously (0.2 M citrate buffer, pH 5.5 under 37° C. using 10 mM sodium phytate). Acid phosphatase activity was assayed at 37° C. in 25 mM glycine-HCl buffer (pH 2.5), using p-nitrophenyl phosphate as the substrate (stock 250 mM). Reaction buffer of 1.7 ml was added into 0.1 ml samples. The released p-nitrophenol was read continuously for 5 min at 405 nm for enzyme activity calculation. SDS-PAGE (12%) was conducted to determine the size and relative amount of the expressed protein. The optimal pH and temperature of the expressed phytase were determined as described in the results.

In vitro study. Soybean meal (5.0 g) was suspended into 20 ml of 20 mM citrate buffer, pH 5.5, mixed with different levels of phytase, and incubated at 37° C. for 4 h with continuous shaking. After being chilled on ice for 10 min, the slurry was transferred into a centrifuge tube and spun for 15 min at 15,000×g. The supernatant was used to determine free phosphorus.

Example 13

Colony Phytase Activity Screening for *Phicia pastoris* Overexpressing the *E. coli* appA Gene

Wild-type *Pichia* X33 produces minimal phytase activity intracellularly (<0.03 U/mg protein) or extracellularly (<0.05 U/mL). The X33 cells transformed with the appA gene inserted into pPICZB (without the α-factor and presumably produces intracellular phytase) did not show any increase in phytase activity (extracellular, 0.2 U/mL and intracellular, 0.05 U/mg protein).

Transforming X33 cells with the construct of pPIZαA-appA (led by the signal peptide of α-factor) produced extracellular phytase activity in the media. Initially, 72 colonies were screened. Only two colonies had activity <1 U/mL 40 hours after induction. Most of the colonies had activity ranging from 10 to 20 U/mL 40 hours after induction. All of the 70 colonies had phytase activity >80 U/mL 118 hours after induction. The highest phytase activity so far detected was 215 U/mL, 192 hours after the induction (See Table 11).

TABLE 11

Range of extracellular phytase activity in X33 colonies transformed with pPIZαA-appA 40 and 118 hours after induction.

Number of Colonies	40 hours after induction	118 hours after induction
2	<1 U/mL	
6	1 to 10 U/mL	
36	11 to 20 U/mL	
28	>20 U/mL	
70		>80 U/mL

Phytase and acid phosphatase activities in the transformant expressing 215 U phytase activity/mL were compared with those of the wild-type of X33 (192 hours after induction) (See Table 12). Almost all of the expressed phytase protein was secreted from the cells, indicating that α-factor was a very effective signal peptide for phytase secretion.

TABLE 12

Phytase and acid phosphatase activities in the pPIZαA-appA transformant and the wild-type of X33 192 hours after induction.

	Wild-type X33		pPIZαA-appA transformant	
	Extracellular U/mL	Intracellular U/mg protein	Extracellular U/mL	Intracellular U/mg protein
Phytase	0.05	0.03	215	0.5
Acid phosphatase	0.01	0.002	5.88	0.9

Transformants of *E. coli* with the same acid phosphatase appA gene had intracellular phytase activity of 5 U/mg protein (Ostanin et al., "Overexpression, Site-Directed Mutagenesis, and Mechanism of *Escherichia coli* Acid Phosphatase," *J. Biol. Chem.*, 267:22830-36 (1992), which is hereby incorporated by reference). Transforming PhyA gene in *A. niger* produced an extracellular activity of 7.6 U/ml (Hartingsveldt et al., "Cloning, Characterization and Overexpression of the Phytase-Encoding Gene (phyA) of *Aspergillus Niger*," *Gene* 127:87-94 (1993), which is hereby incorporated by reference). Compared with these results, the phytase expression system in *Pichia* is a very efficient expression system.

Example 14

Time-Course of Phytase Expression

There was a linear increase in extracellular phytase activity in the media almost in all of the selected colonies up to 192 hours after induction. FIG. **18** summarized the activity changes of five selected colonies from 24 to 163 hours after induction.

Example 15

Effects of Medium pH on the Expression of Phytase (Colony #23, Activity 136 U/mL at 186 h)

Using 0.1 M phosphate buffered media, the effects of different pH on the production of extracellular phytase in the transformants were studied against a control medium without buffer (pH 7.0). The medium buffered to pH 6 produced the highest phytase activity (See FIG. **19**).

Example 16

Size of the Expressed Extracellular Phytase

Using SDS-PAGE (12% gel) analysis, a clear band was noticed in the medium supernatant of culture inoculated with three different colonies (See FIG. **20**). The size was around 55 kDa, probably partially glycosylated. Because the expressed protein represented almost the only visible band in the supernatant, it would be convenient to collect the enzyme product without the need for a tedious purification.

Example 17

Optimum pH and Temperature of the Expressed Extracellular Phytase (Colony #23)

The optimum pH of the expressed phytase was 2.5 to 3.5 (See FIG. **21**). This is significantly different from that of phyA phytase either from *A. niger* (BASF) or our other expression systems. It is ideal for phytase function at the stomach pH.

The optimum temperature of the expressed enzyme was 60° C. (See FIG. **22**).

Example 18

Effect of the Expressed Phytase on Phytate-Phosphorus Hydrolysis from Soybean Meal

This overexpressed *E. coli* phytase (Colony #23) effectively hydrolyzed phytate-phosphorus from soybean meal (See FIG. **23**). The release of free phosphorus in the mixture was linear from 0 to 800 mU of phytase/g of feed.

Example 19

Effects of the Expressed *E. coli* AppA Phytase by *Pichia pastoris* on Phytate Phosphorus Bioavailability to Weanling Pigs

To determine the nutritional values of the expressed *E. coli* phytase by *Pichia* in swine diets, the efficacy of this new phytase was compared with those of inorganic phosphorus or the commercially available microbial phytase (Natuphos™, BASF Corp., Mt. Olive, N.J.). Forty-eight weanling pigs were selected from multiparous sows at Cornell Swine Research Farm. The pigs were weaned at 21 days of age and fed a commercial creep feed until day 28. They were then placed two per pen with six pens assigned randomly per treatment. The pigs were given two weeks to adjust to the corn-soybean meal basal diet (Table 13).

TABLE 13

Formulation of the Experiment Diets for Pigs.

Ingredient	+C % diet	−C % diet	YP % diet	MP % diet
Corn	60.5	61.57	61.07	61.07
Whey Protein Concentrate	3	3	3	3
SBM 44%	30	30	30	30
Corn Oil	3	3	3	3
Lime	0.8	0.93	0.93	0.93
Di-calcium phosphate	1.2	0	0	0
Vitamin and Mineral premix	0.5	0.5	0.5	0.5
ECAP premix	0	0	0.5	0
MP premix	0	0	0	0.5
Salt	0.5	0.5	0.5	0.5
CSP 250	0.5	0.5	0.5	0.5
Total	100	100	100	100
CP	20.6	20.6	20.6	20.6
Ca	0.73	0.47	0.47	0.47
P_{total}	0.6	0.39	0.39	0.39

Note:

All premixes use corn as the carrier

Vitamin and Mineral Premix supplies: 2,540 IU Vit. A, 660 IU Vit. D, 15 IU Vit. E, 2.2 mg Vit. K, 3.3 mg Riboflavin, 13.2 mg Pantothenic acid, 17.6 mg Niacin, 110.1 mg Choline, 1.98 ug B-12, 37.4 mg Mn, 0.6 mg I, 10 mg Cu, 0.3 mg Se, 100 mg Zn, and 100 mg Fe per Kg of diet

Then, each pen received one of the four treatment diets. The positive control group (+C) received the basal diet supplemented with dicalcium phosphate. The negative control group (−C) received just the basal diet. The yeast phytase group (YP) received the basal diet supplemented with the expressed *E. coli* phytase at 1,200 U/kg of feed. The microbial phytase group (MP) received the basal diet supplemented with the BASF phytase at 1,200 U/kg of feed. Pigs were given free access to feed and water. Body weight gain of individual pigs was recorded weekly. Daily feed intake of individual pens was recorded daily. Blood samples from each of the individual pigs were taken weekly to assay plasma inorganic phosphorus concentrations. The results of body weight (BW), average daily gain (ADG), average feed intake (ADFI), and feed/gain ratio (F:G), and plasma inorganic phosphorus (PP) are presented in Table 14.

TABLE 14

Summary of PP, BW, ADG, ADFI, and F:G of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
Initial				
PP	12.99	13.02	13.07	13.54
BW	11.54	11.63	12	11.5
Week 1				
PP	10.83^{A}	6.48^{C}	8.59^{B}	8.35^{B}
BW	14	13.83	14.29	13.92
ADG	.351	.316	.327	.345
ADFI	.700	.684	.697	.697
F:G	2.04	2.20	2.18	2.13
Week 2				
PP	9.76^{A}	5.64^{D}	8.72^{B}	7.84^{C}
BW	18.04	17.42	17.83	17.71

TABLE 14-continued

Summary of PP, BW, ADG, ADFI, and F:G of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
ADG	.578	.512	.506	.542
ADFI	.833	.855	.784	.837
F:G	1.46^{B}	1.67^{A}	1.56^{AB}	1.55^{AB}
		Week 3		
PP	11^{A}	6.26^{C}	8.64^{B}	8.13^{B}
BW	22.58	21.17	22	22.21
ADG	$.649^{A}$	$.536^{B}$	$.595^{AB}$	$.643^{AB}$
ADFI	1.166	1.02	1.001	1.003
F:G	1.8	1.92	1.71	1.36
		Week 4		
PP	10.94^{A}	6.31^{C}	9.65^{B}	9.2^{B}
BW	27.54	25.29	27.79	27.38
ADG	$.708^{AB}$	$.589^{B}$	$.827^{A}$	$.738^{AB}$
ADFI	1.395^{A}	1.049^{B}	1.309^{A}	1.273^{AB}
F:G	1.98	1.87	1.59	1.73

[1]Numbers in the same row without sharing a common letter are significantly different. Analysis of difference was conducted with the Bonferroni (Dunn) T-tests with alpha = 0.05 and df = 20

In addition, there was severe phosphorus deficiency in the negative control group in the end of the four-week experiment. But, there was no sign of phosphorus deficiency in the other three groups. Clearly, the expressed *E. coli* phytase by *Pichia* was at least, if not more, effective as the commercial microbial phytase in improving bioavailability of phytate-phosphorus from the corn-soybean meal diets for weanling pigs. It can be used to replace inorganic phosphorus supplementation to weanling pigs.

Example 20

Effects of the Expressed *E. coli* AppA Phytase by *Pichia pastoris* on Iron (Fe) and Phytate Phosphorus Bioavailability to Weanling Pigs

To determine the effect of the overexpressed *E. coli* phytase by *Pichia* on dietary phytate-bound Fe bioavailability to weanling pigs, 20 anemic pigs (21 days old and 7.3 g hemoglobin (Hb)/dL blood) were selected. The pigs were fed an Fe-deficient creep feed for 7 days and housed in metabolic cages at the age of 28 days old. The pigs were then fed the experimental diets at the age of 35 days old for 5 weeks. The treatment diets were as follows: Fe-deficient basal diet (−C, with added inorganic phosphorus), Fe-supplemented diet (+C), the Fe- and phosphorus-deficient diet supplemented with the expressed *E. coli* phytase (YP), or the commercial microbial phytase (BASF, MP) at 1,200 U/kg of feed. Body weight (BW), packed cell volume (PCV), Hb, and plasma inorganic phosphorus (PP) were determined weekly. The results are presented in Table 15.

TABLE 15

Summary of PCV, Hb, BW, and PP of Pigs as Effected by Dietary Phytase.[1]

	+C	−C	YP	MP
		Initial		
PCV	25	25	26	24
Hb	7.73	7.22	7.85	7.08
BW	8.14	8.27	8.17	7.45
PP	7.92	7.76	7.21	7.36
		Week 1		
PCV	25	26	29	27
Hb	7.62	8.3	8.77	7.88
BW	9.44	8.84	9.63	8.57
PP	8.41	8.45	8.48	8.22
		Week 2		
PCV	29	26	30	28
Hb	8.6	7.34	8.93	8.27
BW	12.32	10.13	11.91	10.84
PP	10.28^{a}	9.05^{ab}	8.89^{ab}	8.22^{a}
		Week 3		
PCV	36^{a}	29^{b}	34^{a}	33^{a}
Hb	11.55^{a}	8.2^{b}	10.84^{a}	9.96^{ab}
BW	16.77^{a}	13^{b}	15.62^{ab}	14.62^{ab}
PP	12.14^{a}	11.37^{ab}	10.25^{bc}	9.71^{c}
		Week 4		
PCV	39	34	38	36
Hb	12.99^{a}	10.11^{b}	12.27^{a}	11.35^{ab}
BW	21.36^{a}	17.37^{b}	19.44^{ab}	18.56^{ab}
PP	10.19^{a}	9.34^{ab}	9.49^{ab}	8.8^{b}
		Week 5		
PCV	40	38	40	39
Hb	13.52^{a}	12.24^{b}	13.64^{a}	13.13^{ab}
BW	26.53	22.59	24.27	23.43
PP	9.27^{a}	8.95^{ab}	8.79^{ab}	8.02^{a}

[1]Values are means (n = 5). Means within the same row without sharing a common superscript ltter are significantly different (P < 0.10).

In conclusion, the overexpressed *E. coli* phytase by *Pichia* was at least as effective as the BASF phytase in improving phytate-phosphorus and Fe utilization in corn-soy diets for weanling pigs.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these therefore are considered within the scope of the invention as defined in the claims which follow.

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What is claimed:

1. A method of producing phytase in yeast, the method comprising:

providing a polynucleotide encoding a phytase from *Escherichia coli;*

expressing the polynucleotide in a yeast; and

isolating the expressed protein or polypeptide,

wherein said protein or polypeptide catalyzes the release of phosphate from phytate.

2. The method of claim **1** wherein said polynucleotide encodes *E. coli* AppA phytase.

3. The method of claim **1** wherein said yeast is a *Pichia* species.

4. The method of claim **1** wherein said polynucleotide is carried on a nucleic acid vector.

* * * * *

EXHIBIT 4

Exhibit 4

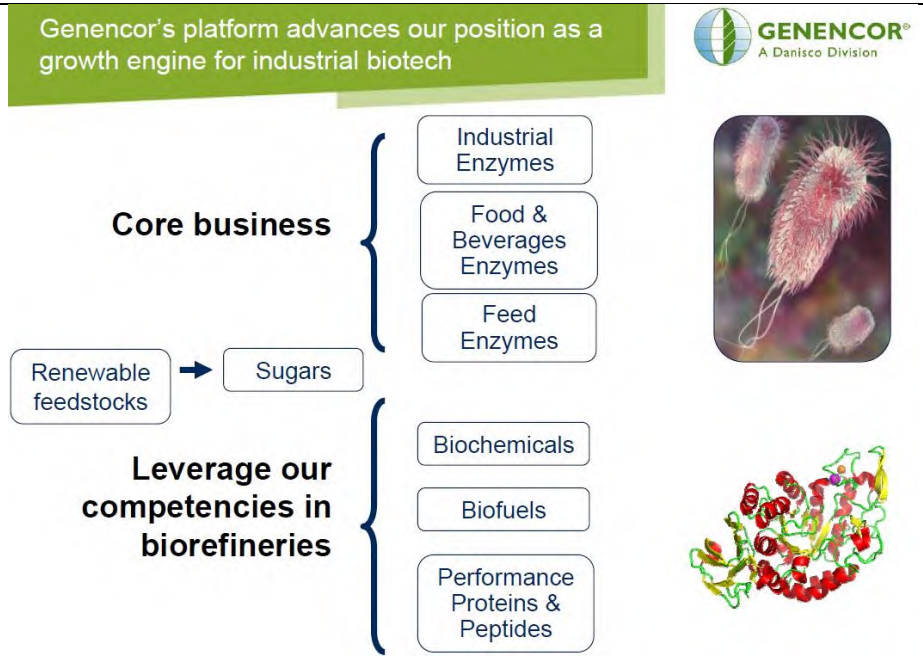
Huvepharma's Initial Claim Chart for Infringement of U.S. Patent No. 7,026,150 by Defendants' Phyzyme phytase products

On information and belief, the Defendants' Phyzyme phytase products infringe the claims of the '150 patent. Thus, the evidence and descriptions below describing the method of producing Phyzyme product lines is applicable to any one or more of the other Phyzyme phytase products including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT.

Claim 1	
Claim1. A method of producing phytase in yeast comprising:	Each use, offer for sale, and/or sale of Phyzyme phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase imported into the United States that was produced and/or is being produced in yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the photograph below of a packaging label of Phyzyme XP 10000 TPT acquired in the United States demonstrates that Phyzyme XP has been manufactured in Finland by Genencor International B.V. ("Genencor") and imported into the United States. Ex. 8 at 1. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, a packaging insert of Phyzyme XP 10000 TPT of a sample acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor for Danisco Animal Nutrition and contains Schizosaccharomyces pombe, which is a yeast. Ex. 14 at 1-7. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, Genencor operates biorefineries that manufacture Phyzyme XP as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme XP for animal feed. Ex. 9 at 1-6; Ex. 8 at 1; Ex. 18 at 2, 4-6.



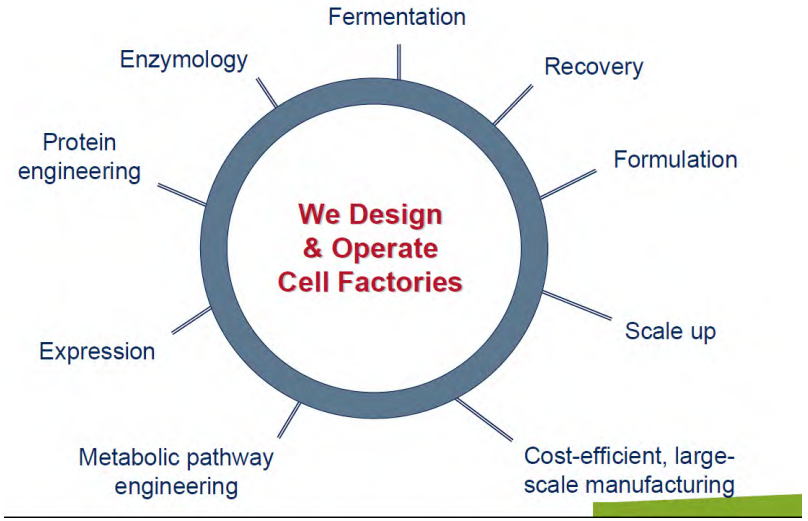
Ex. 9 at 3.

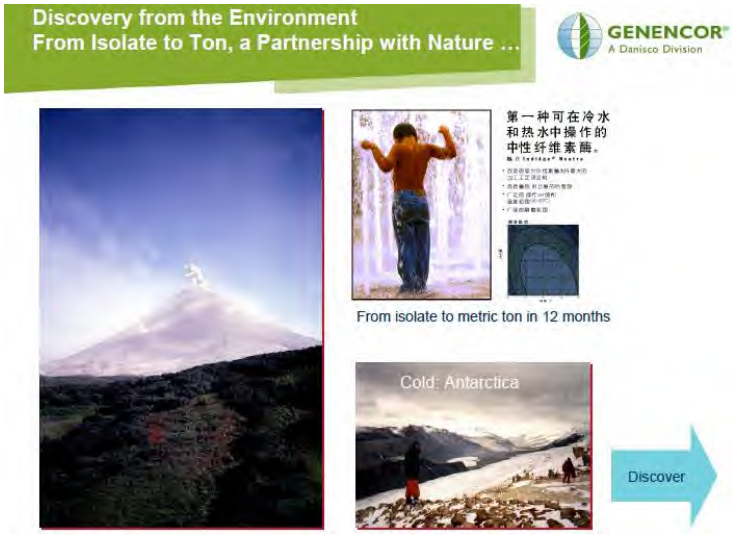
For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1.

For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G, and varies only in the final formulation. Ex. 11 at 5; Ex. 12 at 4.

providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase;	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase. For example, Phyzyme XP advertised and sold by DuPont Danisco Animal Nutrition was sourced from E.coli species bacterium and expressed in Saccharomyces pombe. Ex. 7 at 2, 4. For example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence that was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. In further examples, analytical results of the Phyzyme XP 10000 TPT

	sample of Exhibit 8 confirmed the presence of an AppA E.coli bacterium; and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA E.coli bacterium.
expressing the protein or polypeptide in a yeast; and	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by expressing the protein or polypeptide in a yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in the yeast Saccharomyces pombe. Ex. 7 at 2. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity, and the production strain for Phyzyme XP is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into

	S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a Schizosaccharomyces pombe yeast. Ex. 14 at 1-2. In an example, Genencor uses expression of engineered microorganisms to produce enzymes.  Ex. 9 at 5-6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast

	Schizosaccaromyces pombe.
isolating the expressed protein or polypeptide,	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced by isolating the expressed protein or polypeptide. For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides.  Ex. 9 at 8. For example, the production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e. >95%) are removed. In the clarification step, either conventional (depth) filtration or

	tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. Ex. 10 at 6.
wherein said protein or polypeptide catalyzes the release of phosphate from phytate and	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein said protein or polypeptide catalyzes the release of phosphate from phytate. For example, Phyzyme XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects. Ex. 7 at 2. In a further example, Phyzyme XP is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in the manure. Ex. 10 at 5. For example, the FEEDAP Panel considers that the change in the formulation (of Phyzyme XP 10000 L/TPT) will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion (relating to Phyzyme XP 5000 L/G). Ex. 13 at 8. For example, Phyzyme XP for Pigs and Chickens is a phytase designed specifically to improve the availability of phosphorus, calcium and amino acids contained in cereal grains, oil seed meals and their by-products.

	Ex. 15 at 1. For example, a Phyzyme XP 10000 TPT product insert states the product is phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig diets. Ex. 14 at 2.
has increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method that provides increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell. For example, Phyzyme XP TPT has “unrivalled heat stability, superior performance,” and “in a series of trials conducted at the Technological Institute, Kolding, Denmark, Phyzyme XP TPT is shown to be more heat stable than other leading phytase products.” Ex. 17 at 1-2. For example, significant loss of phytase activity during steam conditioning and subsequent pelleting of feed negatively impacts profitability. Phyzyme XP incorporates unique TPT coating technology, which offers heat stability up to 95C (203F). The TPT coating also allows rapid release of phytase in the upper part of the gut to achieve optimum bio-efficacy. Ex. 16 at 2. For example, Phyzyme XP 5000L is also stable after mixing with feedingstuffs with approximately 80% of activity remaining after six months storage in a feed mash at both 20°C and 35°C. Following pelleting of feeds at 80C, 82% of the initial phytase activity was still present, thus Phyzyme XP 5000G is considered to be stable when pelleted up to this temperature. Ex. 11 at 5. For example, Phyzyme XP 10000 TPT stability in pelleting, when tested

	with a maize-based diet and was conditioned for 30 seconds at 90C or 95C and pelleted, resulted in percentage of recovery of the initial enzymatic activity of 99% and 96%, respectively. Ex. 13 at 7. It is not expected that the double concentration of the Phyzyme XP 10000 L enzyme (compared to Phyzyme XP 5000L) will modify the stability of the product in feedingstuffs. Phyzyme XP 5000 showed an 80% of the activity remaining when added to feedingstuffs and stored for six months at 20°C and 35°C. Ex. 13 at 7. For example, Phyzyme XP is a phytase protected by unique Thermal Protection Technology coating from Danisco Animal Nutrition. Phyzyme XP TPT is heat stable to 95°C/203°F during pelleting. Ex. 7 at 3, 4. Phyzyme XP G is for mash feed only. A light brown, fine granular product on a wheat flour carrier. For optimum bioefficacy do no exceed conditioning and pelleting temperatures of 70°C/158°F. Ex. 7 at 4.
Claim 2	
The method according to claim 1, wherein the heterologous polynucleotide is an isolated appA polynucleotide.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein the heterologous polynucleotide is an isolated appA polynucleotide. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. The donor organism is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert

	was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.
Claim 3	
The method according to claim 1, wherein the yeast is selected from the group consisting of Saccharomyces species Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein the yeast is selected from the group consisting of Saccharomyces species Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1. For example, Phyzyme XP 5000L and Phyzyme XP 5000G is produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. Ex. 11 at 3. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3. For example, Phyzyme XP 10000 TPT product insert states the product contains dried Schizosaccharomyces pombe fermentation product. Ex.

	14 at 2. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
Claim 6	
The method according to claim 1, wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase imported into the United States that was produced by a method wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 10 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6.

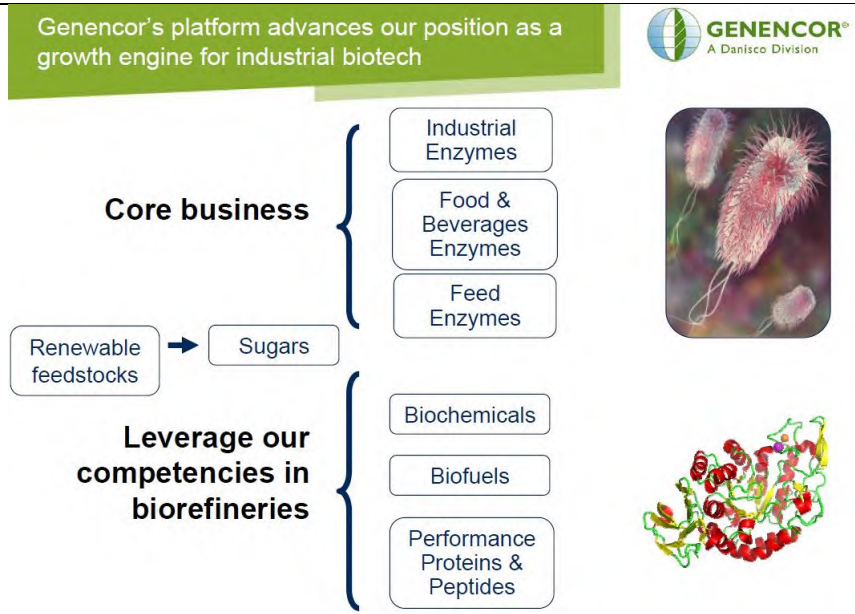
Claim 7	
The method according to claim 6, wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.	Each use, offer for sale, and/or sale of Phyzyme XP phytase is a use, offer for sale, and/or sale of Phyzyme XP imported into the United States that was produced by a method wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6. For example, Phyzyme XP is/has been sold in 5000 FTU and 10000 FTU concentrations. Ex. 11 at 3; Ex. 12 at 4; Ex. 13 at 4. For example, Phyzyme XP 5000L and Phyzyme XP 5000G are produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex.11 at 3. In another example, Phyzyme XP 10000L and Phyzyme XP 10000 TPT are produced by Schizosaccharomyces pombe with a minimum activity of 10000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex. 13 at 4. For example, the photograph below of a packaging label of Phyzyme XP acquired in the United States demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. and imported into the United States with a concentration of 10000 FTU/g. Ex. 8 at 1.

	  For example, the copy below of a packaging insert of Phyzyme XP acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. with a concentration of 10000 FTU/g. Ex. 14 at 2. DANISCO First you add knowledge... **Certificate of Conformance** Date: 20 Jul 2015 Our ref. no.: 41785009 10 Your ref. 1079908 Material: A140561083EU Phyzyme XP 10000 TPT Customer material no.: 10003912 Batch No.: 4882559945 Best before date: 19 Jun 2016 Production date: 20 Jun 2015
Claim 16	

The method according to claim 1, wherein the heterologous polynucleotide which encodes a protein or polypeptide with phytase activity is spliced in frame with a transcriptional enhancer element.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein the heterologous polynucleotide which encodes a protein or polypeptide with phytase activity is spliced in frame with a transcriptional enhancer element. For example, for example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.
Claim 28	
A method of producing a protein or polypeptide having phytase activity comprising:	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method using a protein or polypeptide having phytase activity. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the photograph below of a packaging label of Phyzyme XP 10000 TPT acquired in the United States demonstrates that Phyzyme XP is a protein having phytase activity that has been manufactured in Finland by Genencor and imported into the United States. Ex. 8 at 1.

For example, a packaging insert of Phyzyme XP 10000 TPT of a sample acquired in the Kingdom of Belgium demonstrates that Phyzyme XP has been manufactured in Finland by Genencor for Danisco Animal Nutrition and contains a protein with phytase activity. Ex. 14 at 1-3.

For example, Genencor operates biorefineries that manufacture Phyzyme XP as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme XP for animal feed. Ex. 9 at 6-12; Ex. 8 at 1; Ex. 18 at 2, 4-6.



Ex. 9 at 3.

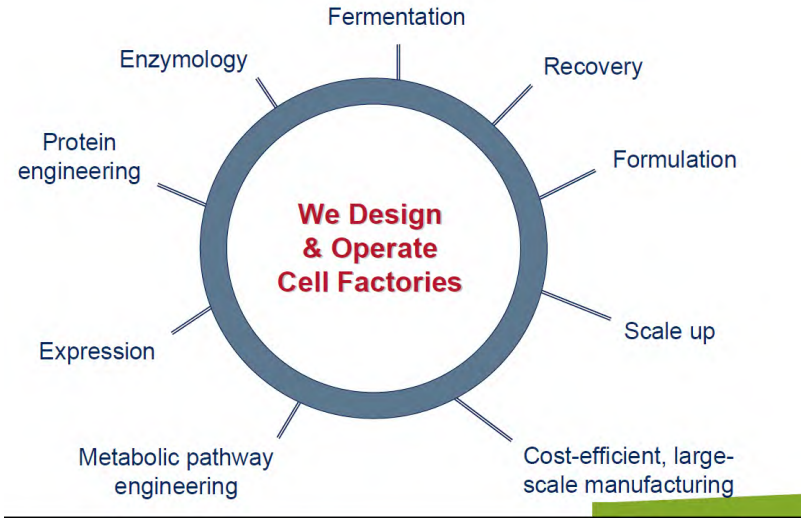
For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1.

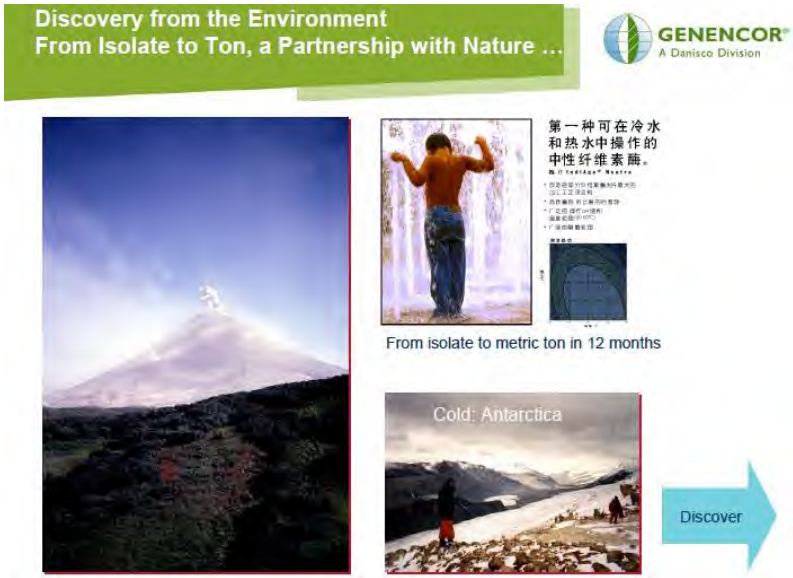
For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3; Ex. 12 at 4. The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G, and varies only in the final formulation. Ex. 12 at 4.

providing an isolated appA polynucleotide, which encodes a protein or polypeptide with phytase activity,	Each use, offer for sale, and/or sale of Phyzyme phytase XP imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by providing an isolated appA polynucleotide, which encodes a protein or polypeptide with phytase activity. For example, Phyzyme XP advertised and sold by DuPont and Danisco Animal Nutrition was sourced from E.coli species bacterium and expressed in Saccharomyces pombe. Ex. 7 at 2, 4. For example, for Phyzyme XP the final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex.10 at 9. For example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000.

	Ex. 13 at 3; Ex. 12 at 4. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of an AppA E.coli bacterium; and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA E.coli bacterium.

expressing said polynucleotide in a yeast hold cell under conditions effective to produce the protein or polypeptide having the phytase activity, and	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by expressing the polynucleotide in a yeast hold cell under conditions effective to produce the protein or polypeptide having the phytase activity. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in the yeast Saccharomyces pombe. Ex. 7 at 2. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3; Ex. 12 at 4. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using

	auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a Schizosaccharomyces pombe yeast. Ex. 14 at 1-2. In an example, Genencor uses expression of engineered microorganisms to produce enzymes.  Ex. 9 at 5-6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast

	Schizosaccaromyces pombe.
isolating the expressed protein or polypeptide.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by isolating the expressed protein or polypeptide. For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides.  Ex. 9 at 8. For example, the production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e. >95%) are removed. In the clarification step, either conventional (depth) filtration or

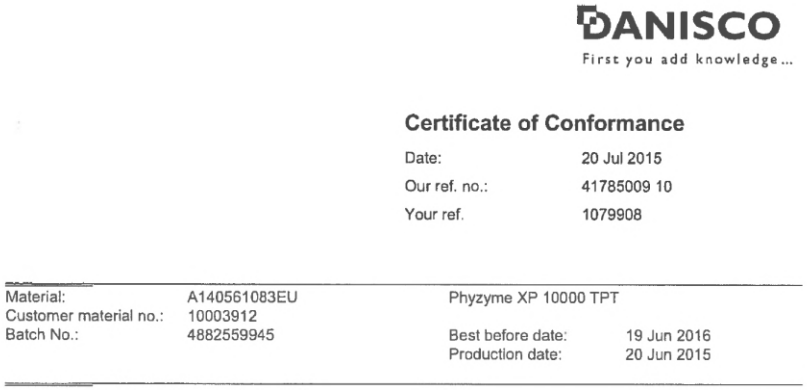
	tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. Ex. 10 at 6.
Claim 29	
The method according to claim 28, wherein the yeast host cell is selected from the group consisting of Saccaromyces species, Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein the yeast host cell is selected from the group consisting of Saccaromyces species, Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1. For example, Phyzyme XP 5000L and Phyzyme XP 5000G is produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. Ex. 11 at 3, 20. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3. For example, Phyzyme XP 10000 TPT product insert states the product

	contains dried Schizosaccharomyces pombe fermentation product. Ex. 14 at 2. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
Claim 31	
The method according to claim 28, wherein the protein or polypeptide, preceded by a signal peptide, is secreted by the cell into a growth medium or is not secreted.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced by a method wherein the protein or polypeptide, preceded by a signal peptide, is secreted by the cell into a growth medium or is not secreted. For example, a number of process steps serve to prevent the presence

	of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 10 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6.
Claim 32	
The method according to claim 31, wherein the protein or polypeptide is secreted by the yeast host cell into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced by a method wherein the protein or polypeptide is secreted by the yeast host cell into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6.

	For example, Phyzyme XP is/has been sold in 5000 FTU and 10000 FTU concentrations. Ex. 11 at 3; Ex. 12 at 4; Ex. 13 at 4. For example, Phyzyme XP 5000L and Phyzyme XP 5000G are produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex. 11 at 3. In another example, Phyzyme XP 10000L and Phyzyme XP 10000 TPT are produced by Schizosaccharomyces pombe with a minimum activity of 10000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex. 13 at 4. For example, the photograph below of a packaging label of Phyzyme acquired in the United States demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. and imported into the United States with a concentration of 10000 FTU/g. Ex. 8 at 1.

For example, the copy below of a packaging insert of Phyzyme XP acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor with a concentration of 10000 FTU/g. Ex. 14 at 2.



DANISCO

First you add knowledge...

Certificate of Conformance

Date:	20 Jul 2015
Our ref. no.:	41785009 10
Your ref.	1079908

Material:	A140561083EU	Phyzyme XP 10000 TPT	
Customer material no.:	10003912		
Batch No.:	4882559945	Best before date:	19 Jun 2016
		Production date:	20 Jun 2015

For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6.

Claim 33	
The method according to claim 28, wherein the appA polynucleotide is spliced in frame with a transcriptional enhancer element.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein the appA polynucleotide is spliced in frame with a transcriptional enhancer element. For example, for example, the donor organism is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6.

	The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.

EXHIBIT 5

Exhibit 5

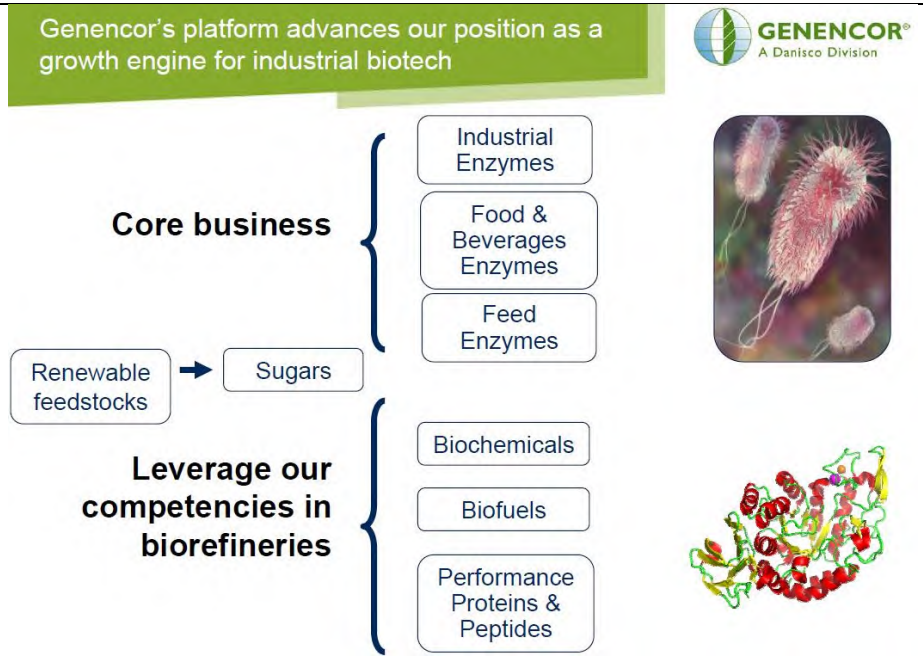
Huvepharma's Initial Claim Chart for Infringement of U.S. Patent No. 7,312,063 by Defendants' Phyzyme phytase products

On information and belief, the Defendants' Phyzyme phytase products infringe the claims of the '063 patent. Thus, the evidence and descriptions below describing the method of producing Phyzyme product lines is applicable to any one or more of the other Phyzyme phytase products including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT.

Claim 1	
Claim1. A method of producing phytase in yeast comprising:	Each use, offer for sale, and/or sale of Phyzyme phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase imported into the United States that was produced and/or is being produced in yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the photograph below of a packaging label of Phyzyme XP 10000 TPT acquired in the United States demonstrates that Phyzyme XP has been manufactured in Finland by Genencor International B.V. ("Genencor") and imported into the United States. Ex. 8 at 1. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, a packaging insert of Phyzyme XP 10000 TPT of a sample acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor for Danisco Animal Nutrition and contains Schizosaccharomyces pombe, which is a yeast. Ex. 14 at 1-7. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, Genencor operates biorefineries that manufacture Phyzyme XP as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme XP for animal feed. Ex. 9 at 1-6; Ex. 8 at 1; Ex. 18 at 2, 4-6.



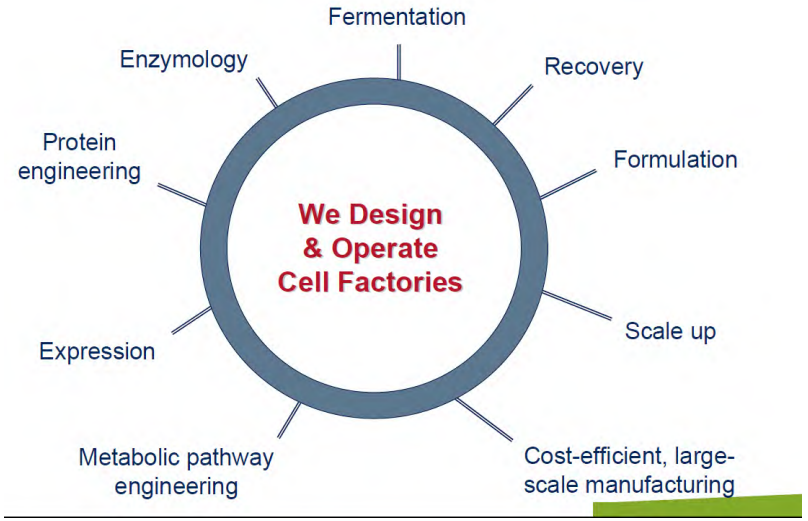
Ex. 9 at 3.

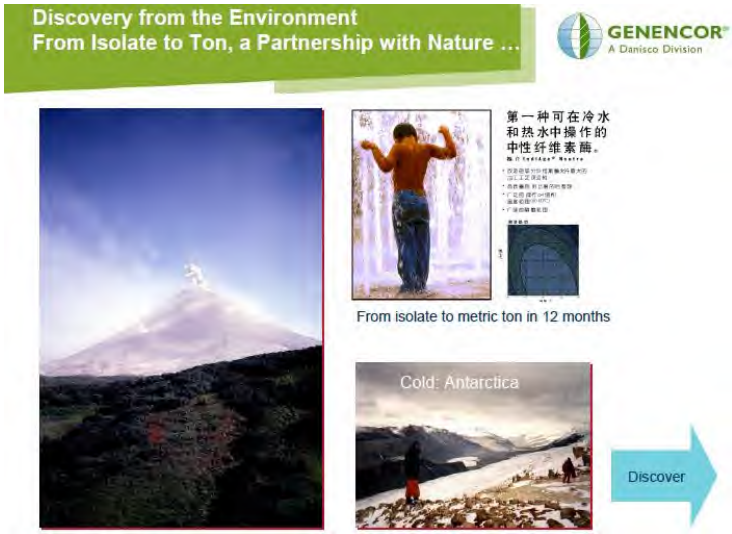
For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1.

For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3; Ex. 12 at 4. The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G, and varies only in the final formulation. Ex. 11 at 5; Ex. 12 at 4.

providing a heterologous polynucleotide from a non-yeast organism which encodes a protein or polypeptide comprising a phytase from Escherichia coli;	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase. For example, Phyzyme XP advertised and sold by DuPont Danisco Animal Nutrition was sourced from E.coli species bacterium and expressed in Saccharomyces pombe. Ex. 7 at 2, 4. For example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence that was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. In further examples, analytical results of the Phyzyme XP 10000 TPT

	sample of Exhibit 8 confirmed the presence of an AppA E.coli bacterium; and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA E.coli bacterium.
expressing the polynucleotide in a yeast; and	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by expressing the protein or polypeptide in a yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in the yeast Saccharomyces pombe. Ex. 7 at 2. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity, and the production strain for Phyzyme XP is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into

	S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a Schizosaccharomyces pombe yeast. Ex. 14 at 1-2. In an example, Genencor uses expression of engineered microorganisms to produce enzymes.  Ex. 9 at 5-6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast

	Schizosaccaromyces pombe.
isolating the expressed protein or polypeptide,	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced by isolating the expressed protein or polypeptide. For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides.  Ex. 9 at 8. For example, the production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e. >95%) are removed. In the clarification step, either conventional (depth) filtration or

	tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. Ex. 10 at 6.
wherein said protein or polypeptide catalyzes the release of phosphate from phytate and	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein said protein or polypeptide catalyzes the release of phosphate from phytate. For example, Phyzyme XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects. Ex. 7 at 2. In a further example, Phyzyme XP is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in the manure. Ex. 10 at 5. For example, the FEEDAP Panel considers that the change in the formulation (of Phyzyme XP 10000 L/TPT) will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion (relating to Phyzyme XP 5000 L/G). Ex. 13 at 8. For example, Phyzyme XP for Pigs and Chickens is a phytase designed specifically to improve the availability of phosphorus, calcium and amino acids contained in cereal grains, oil seed meals and their by-products.

	Ex. 15 at 1. For example, a Phyzyme XP 10000 TPT product insert states the product is phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig diets. Ex. 14 at 2.
has increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method that provides increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell. In another example, Phyzyme XP TPT has “unrivalled heat stability, superior performance,” and “in a series of trials conducted at the Technological Institute, Kolding, Denmark, Phyzyme XP TPT is shown to be more heat stable than other leading phytase products.” Ex. 17 at 1-2. For example, significant loss of phytase activity during steam conditioning and subsequent pelleting of feed negatively impacts profitability. Phyzyme XP incorporates unique TPT coating technology, which offers heat stability up to 95C (203F). The TPT coating also allows rapid release of phytase in the upper part of the gut to achieve optimum bio-efficacy. Ex. 16 at 2. For example, Phyzyme XP 5000L is also stable after mixing with feedingstuffs with approximately 80% of activity remaining after six months storage in a feed mash at both 20°C and 35°C. Following pelleting of feeds at 80C, 82% of the initial phytase activity was still present, thus Phyzyme XP 5000G is considered to be stable when pelleted up to this temperature. Ex. 11 at 5. For example, Phyzyme XP 10000 TPT stability in pelleting, when tested

	with a maize-based diet and was conditioned for 30 seconds at 90C or 95C and pelleted, resulted in percentage of recovery of the initial enzymatic activity of 99% and 96%, respectively. Ex. 13 at 7. It is not expected that the double concentration of the Phyzyme XP 10000 L enzyme (compared to Phyzyme XP 5000L) will modify the stability of the product in feedingstuffs. Ex. 13 at 7. Phyzyme XP 5000 showed an 80% of the activity remaining when added to feedingstuffs and stored for six months at 20°C and 35°C. Ex. 13 at 7. For example, Phyzyme XP is a phytase protected by unique Thermal Protection Technology coating from Danisco Animal Nutrition. Phyzyme XP TPT is heat stable to 95°C/203°F during pelleting. Ex. 7 at 3, 4. Phyzyme XP G is for mash feed only. A light brown, fine granular product on a wheat flour carrier. For optimum bioefficacy do no exceed conditioning and pelleting temperatures of 70°C/158°F. Ex. 7 at 4.
Claim 2	
The method according to claim 1, wherein the yeast is selected from the group consisting of Saccharomyces species, Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein the yeast is selected from the group consisting of Saccharomyces species Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1. For example, Phyzyme XP 5000L and Phyzyme XP 5000G is produced

	by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. Ex. 11 at 3, 20. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3. For example, Phyzyme XP 10000 TPT product insert states the product contains dried Schizosaccharomyces pombe fermentation product. Ex. 14 at 2. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
Claim 5	
The method according to claim 1, wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase imported into the United States that was produced by a method wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399. Ex. 10 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem

	repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6.
Claim 6	
The method according to claim 5, wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.	Each use, offer for sale, and/or sale of Phyzyme XP phytase is a use, offer for sale, and/or sale of Phyzyme XP imported into the United States that was produced by a method wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6. For example, Phyzyme XP is/has been sold in 5000 FTU and 10000 FTU concentrations. Ex. 11 at 3; Ex. 12 at 4; Ex. 13 at 4. For example, Phyzyme XP 5000L and Phyzyme XP 5000G are produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex.11 at 3. In another example, Phyzyme XP 10000L and Phyzyme XP 10000 TPT are produced by Schizosaccharomyces pombe with a minimum activity

	of 10000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex. 13 at 10. For example, the photograph below of a packaging label of Phyzyme XP acquired in the United States demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. and imported into the United States with a concentration of 10000 FTU/g. Ex. 8 at 1. SAMPLE NOT INTENDED FOR COMMERCIAL SALE Feed Grade PRODUCT Phyzyme XP 10000 TPT BATCH [redacted] QUANTITY 1 KG Man date 10/12 2017 MADE IN Finland Exp date 10/12 2018 Genencor International, B.V. Archimedesweg 30 2333 CN Leiden The Netherlands Tel +32 50 44 91 73 CAUTION: May Cause eye and skin irritation inhalation of dusts or mists may cause an allergic respiratory reaction. Avoid contact with eyes, skin and mucous membranes. Avoid breathing mist or dust. Use with adequate ventilation. FIRST AID: In Case of eye contact, flush eyes with water for at least 15 minutes and get prompt medical attention. In case of skin contact, wash thoroughly with soap and water. If irritation occurs and persists, consult a physician. If respiratory irritation or an allergic response occurs, consult a physician. GENENCOR® A Danisco Division For example, the copy below of a packaging insert of Phyzyme XP acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. with a concentration of 10000 FTU/g. Ex. 14 at 2.

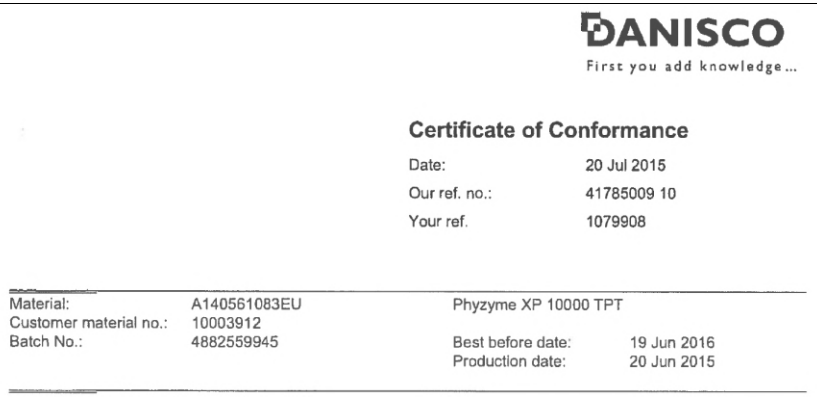
	
Claim 7	
The method according to claim 1, wherein the heterologous polynucleotide which encodes the protein or polypeptide is spliced in frame with a transcriptional enhancer element.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein the heterologous polynucleotide which encodes a protein or polypeptide with phytase activity is spliced in frame with a transcriptional enhancer element. For example, for example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.

EXHIBIT 6

Exhibit 6

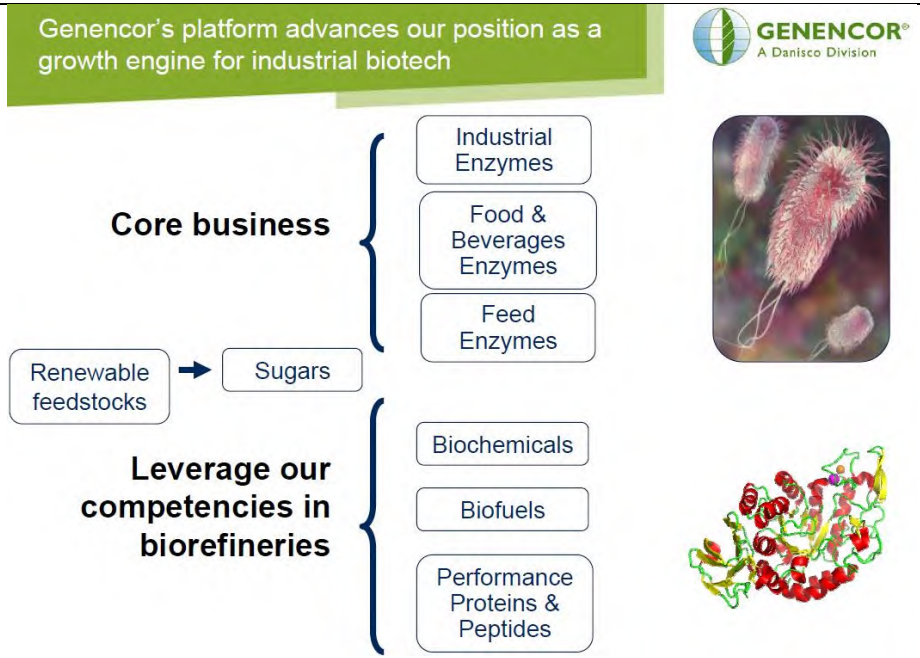
Huvepharma's Initial Claim Chart for Infringement of U.S. Patent No. 8,455,232 by Defendants' Phyzyme phytase products

On information and belief, the Defendants' Phyzyme phytase products infringe the claims of the '232 patent. Thus, the evidence and descriptions below describing the method of producing Phyzyme product lines is applicable to any one or more of the other Phyzyme phytase products including but not limited to Phyzyme XP, Phyzyme XP TPT, Phyzyme XP G, Phyzyme R, Phyzyme XP Liquid, Phyzyme XP 1500, Phyzyme XP 5000G, Phyzyme XP 5000L, Phyzyme XP 10000L, Phyzyme XP 150000L, and/or Phyzyme XP 10000TPT.

Claim 1	
A method of producing phytase in yeast, the method comprising:	Each use, offer for sale, and/or sale of Phyzyme phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme phytase imported into the United States that was produced and/or is being produced in yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the photograph below of a packaging label of Phyzyme XP 10000 TPT acquired in the United States demonstrates that Phyzyme XP has been manufactured in Finland by Genencor International B.V. ("Genencor") and imported into the United States. Ex. 8 at 1. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, a packaging insert of Phyzyme XP 10000 TPT of a sample acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor for Danisco Animal Nutrition and contains Schizosaccharomyces pombe, which is a yeast. Ex. 14 at 1-7. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, Genencor operates biorefineries that manufacture Phyzyme XP as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme XP for animal feed. Ex. 9 at 1-6; Ex. 8 at 1; Ex. 18 at 2, 4-6.



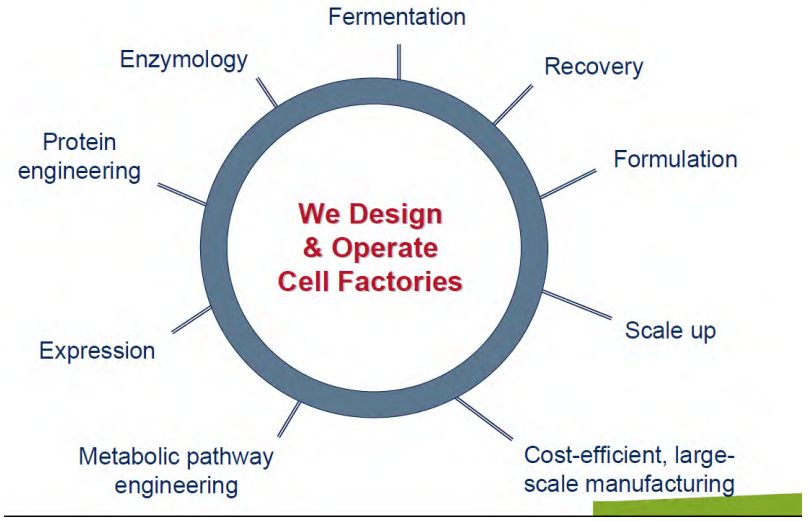
Ex. 9 at 3.

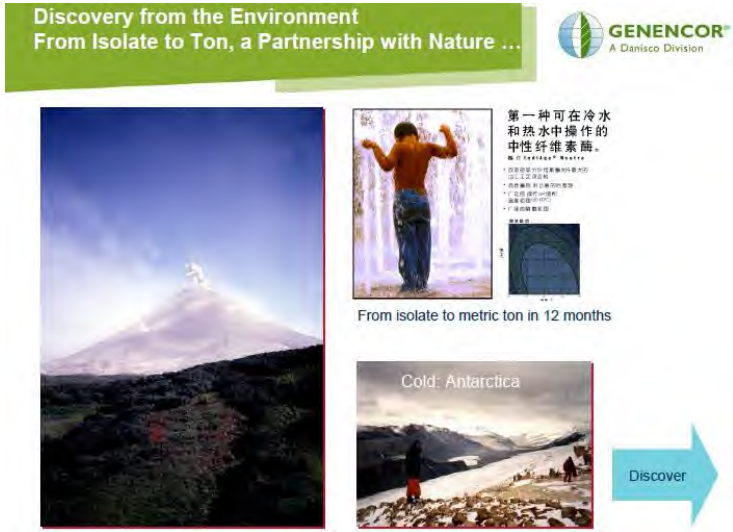
For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1.

For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G, and varies only in the final formulation. Ex. 11 at 5; Ex. 12 at 4.

providing a polynucleotide encoding a phytase from Escherichia coli;	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase. For example, Phyzyme XP advertised and sold by DuPont Danisco Animal Nutrition was sourced from E.coli species bacterium and expressed in Saccharomyces pombe. Ex. 7 at 2, 4. For example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence that was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of

	Exhibit 8 confirmed the presence of an AppA E.coli bacterium; and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA E.coli bacterium.
expressing the polynucleotide in a yeast; and	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by expressing the protein or polypeptide in a yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in the yeast Saccharomyces pombe. Ex. 7 at 2. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity, and the production strain for Phyzyme XP is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic

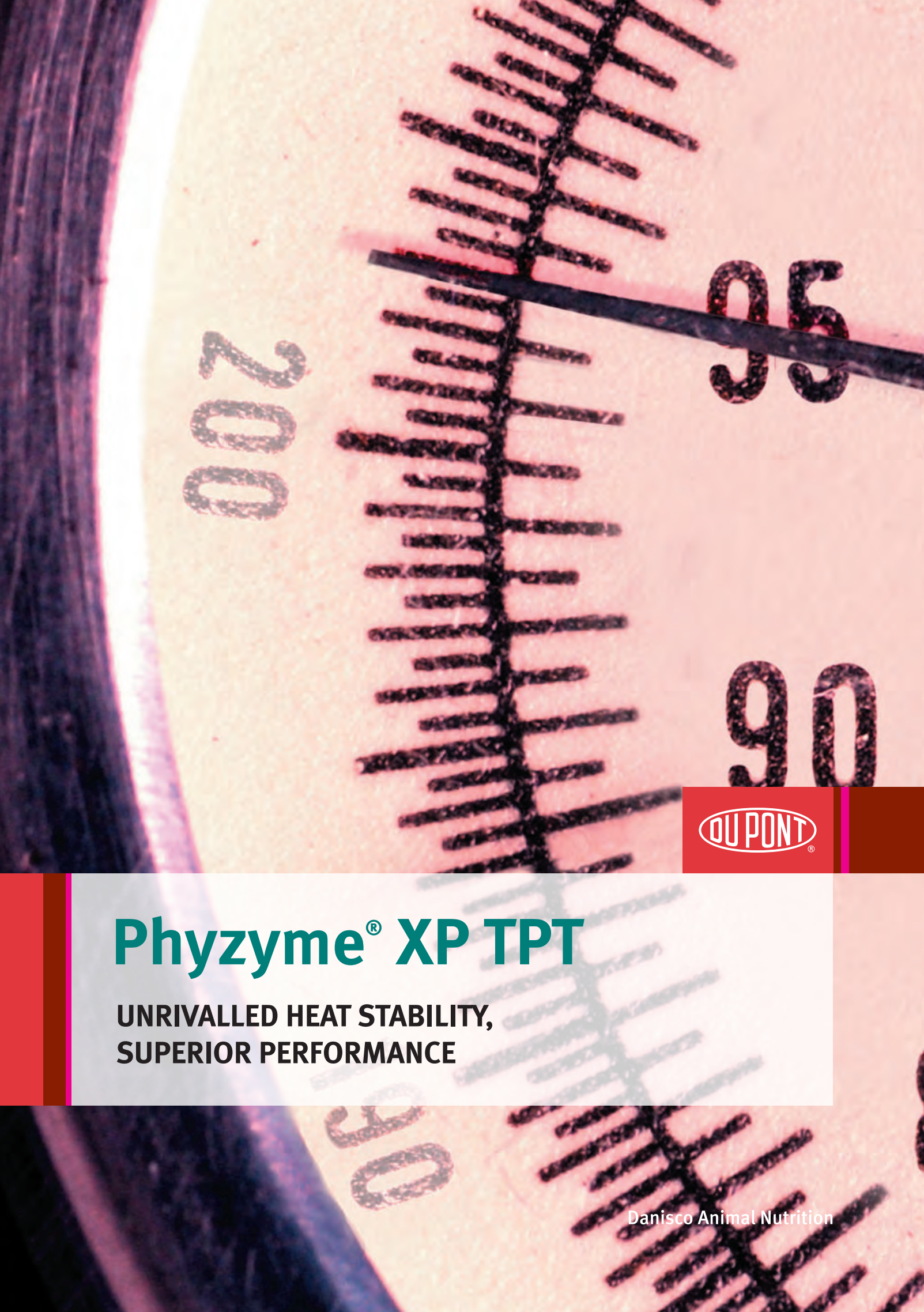
	mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a Schizosaccharomyces pombe yeast. Ex. 14 at 1-2. In an example, Genencor uses expression of engineered microorganisms to produce enzymes.  Ex. 9 at 5-6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
isolating the expressed protein or	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the

polypeptide,	United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced by isolating the expressed protein or polypeptide. For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides.  Ex. 9 at 8. For example, the production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e. >95%) are removed. In the clarification step, either conventional (depth) filtration or tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. Ex. 10 at 6.

wherein said protein or polypeptide catalyzes the release of phosphate from phytate.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein said protein or polypeptide catalyzes the release of phosphate from phytate. For example, Phyzyme XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects. Ex. 7 at 2. In a further example, Phyzyme XP is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in the manure. Ex. 10 at 5. For example, the FEEDAP Panel considers that the change in the formulation (of Phyzyme XP 10000 L/TPT) will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion (relating to Phyzyme XP 5000 L/G). Ex. 13 at 8. For example, Phyzyme XP for Pigs and Chickens is a phytase designed specifically to improve the availability of phosphorus, calcium and amino acids contained in cereal grains, oil seed meals and their by-products. Ex. 15 at 1. For example, a Phyzyme XP 10000 TPT product insert states the product is phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig diets. Ex. 14 at 2.
Claim 2	

The method of claim 1 wherein said polynucleotide encodes E. coli AppA phytase.	Each use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States is a use, offer for sale, and/or sale of Phyzyme XP phytase imported into the United States that was produced and/or is being produced by a method wherein the heterologous polynucleotide is an isolated appA polynucleotide. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. The donor organism is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.

EXHIBIT 7



Phyzyme® XP TPT

UNRIVALLED HEAT STABILITY, SUPERIOR PERFORMANCE

Danisco Animal Nutrition

Phyzyme® XP TPT

Market leading phytase: established and effective with unique TPT coating

Lower feed costs

- Increased nutrient and energy availability
- Reduced inorganic phosphate use
- Greater flexibility in ingredient use

Confidence in use

- Proven, superior, bioefficacy compared with fungal phytases
- Heat stability ideal for feed pelleting
- Reliable matrix values
- Optimize Feed™ Service and laboratory feed assay

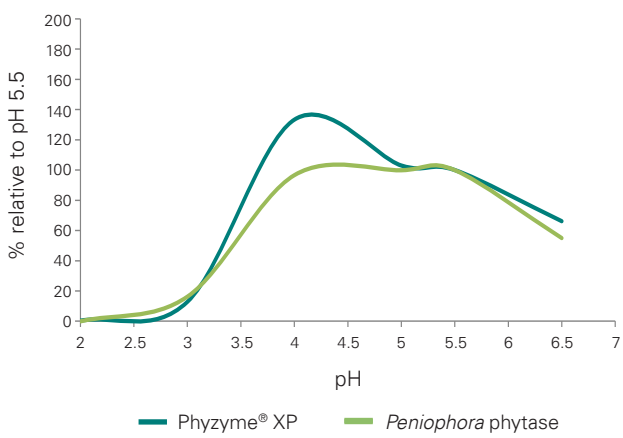
What is Phyzyme® XP?

Phyzyme® XP is a fully patented, unique bacterial phytase developed by Danisco Animal Nutrition.
Phyzyme® XP is sourced from an *E.coli* species bacterium and is expressed in a *Saccharomyces pombe*.

What is phytate?

Phytate, also known as phytic acid and inositol hexaphosphate (IP6), is the main natural store of phosphorus contained in variable amounts in plants. Phosphorus is an essential mineral but when bound in dietary phytate is inaccessible to monogastric animals as they lack the digestive enzyme phytase.

Phytase activity at varying pH - Phyzyme® XP versus competitor



Why is phytate a problem?

Phytate is both a potential phosphorus source and an 'anti-nutrient' because of its ability to bind to essential nutrients needed by the animal. In the upper digestive system, at low pH, phytate binds to proteins and amino acids. Further down the digestive system, at higher pH levels, phytate binds to minerals such as calcium and trace elements.

Phytate interferes with digestion and stimulates the animal to increase its production of digestive secretions which is an energy and nutrient consuming process. The net result is a reduction in animal performance.

Furthermore, unabsorbed phytate phosphorus is excreted by the animal, creating a major problem for the environment.

How does phytase help?

Phytase enzymes cleave phosphorus and the associated bound nutrients from the phytate molecule reducing its anti-nutrient effects. It is very important for phytase to start working rapidly at low pH levels which are found in the upper digestive system, e.g. pig's stomach and poultry gizzard. This minimizes the anti-nutrient effects of phytate throughout the digestive system and maximizes the time available for the animal to absorb the released nutrients.

Phyzyme® XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects.

Phyzyme® XP benefits

Phyzyme® XP rapidly degrades phytate, releasing more nutrients such as phosphorus, energy and amino acids compared to competitor phytase products. This offers many benefits in animal production.

Not all phytases are equal when countering the costly effect of phytate in the diet of animals. Phyzyme® XP has been consistently and successfully used by customers over many years. Recent studies show 500 FTU/kg feed of Phyzyme® XP is equivalent to >800 FTU/kg feed of competitor products (see graphs).

Feed cost savings

- Reductions in dietary inorganic phosphate, energy and amino acids
  - Fast and efficient breakdown of dietary phytate
  - Increased nutrient availability from phytate

Production benefit

- Body weight gain and FCR benefits
  - Efficient removal of phytate anti-nutrient effects at low pH
  - Release of nutrients from phytate improves energy and amino acid availability

- Improved environment
  - Reduced phosphorus excretion

Confidence in use

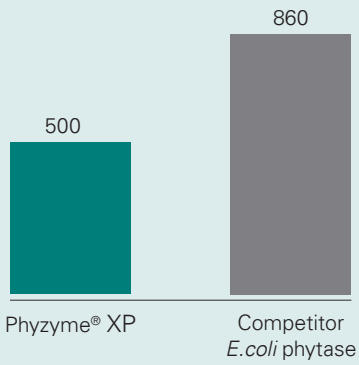
- Unrivalled heat stability
  - Phytase protected by unique Thermo Protection Technology (TPT) coating from Danisco Animal Nutrition
  - Phyzyme® XP TPT is heat stable to 95°C/203°F
  - Ideal for feed pelleting

- Reliable matrix values
  - Obtained from animal digestibility responses
  - Optimized according to the type of diet
  - Customer specific recommendations on Phyzyme® XP use
  - Over 10 years on the market

- Independent testing at external sites

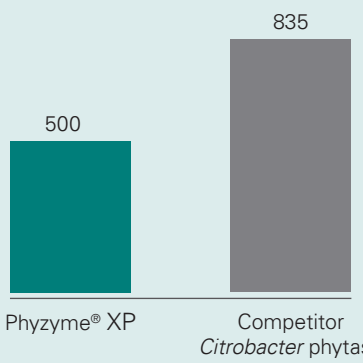
- Laboratory assay
  - Quickly and specifically detects active Phyzyme® XP in feed

Bioequivalence* Phyzyme® XP versus competitor E.coli phytase (FTU/kg feed)



TR: XPSPB.74

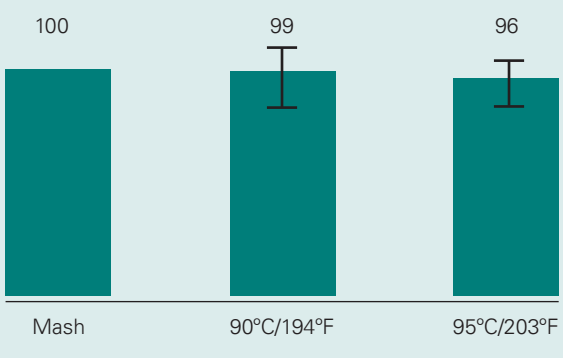
Bioequivalence* Phyzyme® XP versus competitor Citrobacter phytase (FTU/kg feed)



TR: XPSPB.75

*Based on tibia phosphorus measurements

Phyzyme® XP TPT heat stability during conditioning and pelleting



Relative recovery (% of mash activity)

Supporting services for Phyzyme® XP

- Fully quantitative traditional laboratory assay for phytase
- Applications expertise of our technical and business support teams
- Optimize Feed™ Service - provides accurate and specific matrices leading to customised recommendations on Phyzyme® XP use in poultry and swine diets, to maximize profit from phytase use

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Product form

Phyzyme® XP is a phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in animal diets.

Phyzyme® XP TPT
Thermostable up to 95°C / 203°F during pelleting.
An off-white to light tan, fine granular product.
Packed in 25 kg multi-wall polyethylene lined paper bags or carton with polyethylene inner bag and in 1000 kg bulk (tote) bags.

Phyzyme® XP is also available in two other forms

Phyzyme® XP G – for mash feed only
A light brown, fine granular product on a wheat flour carrier.
For optimum bioefficacy do not exceed conditioning and pelleting temperatures of 70°C / 158°F.
Packed in 25 kg multi-wall paper bags and in 1000 kg bulk (tote) bags.

Phyzyme® XP Liquid – for post pelleting applications
A brown liquid.
Packed in 200 kg and 1000 kg containers.

Contact your Danisco Animal Nutrition representative or distributor for recommendations about your specific application needs.

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To find your nearest local office or distributor visit www.animalnutrition.dupont.com

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PO Box 777, Marlborough, Wilts, SN8 1XN, UK
Tel +44 (0) 1672 517777
info.animalnutrition@dupont.com
www.animalnutrition.dupont.com

02/2014

EXHIBIT 8

SAMPLE NOT INTENDED FOR COMMERCIAL SALE

Feed Grade

PRODUCT Phyzyme XP 10000 TPT

BATCH [redacted]

QUANTITY 1 KG Man date 10/12 2017

MADE IN Finland Exp date 10/12 2018

Genencor International, B.V.
Archimedesweg 30
2333 CN Leiden
The Netherlands
Tel +32 50 44 91 73

GENENCOR®
A Danisco Division

CAUTION: May Cause eye and skin irritation Inhalation of dusts or mists may cause an allergic resiratory reaction. Avoid contact with eyes, skin and mucous membranes. Avoid breathing mist or dust. Use with adequate ventilation.

FIRST AID: In Case of eye contact, flush eyes with water for at least 15 minutes and get prompt medical attention. In case of skin contact, wash thoroughly with soap and water. If irritation occurs and persists, consult a physician. If respiratory irritation or an allergic response occurs, consult a physician.

EXHIBIT 9

Genencor's Industrial Biotech Platform

GENENCOR®
A Danisco Division

Concarneau

September 2009

Genencor - a Danisco Division

- Early pioneer of biotechnology – in 1982 Genentech & Corning Glassworks formed Genencor
- More than 250 commercial enzyme products
- 3500+ owned and licensed patents and applications
- Parent company is Danisco - more than $USD 3.5 billion in revenues and 9,700 employees
- Global sales and supply chain organization

Genencor's platform advances our position as a growth engine for industrial biotech

GENENCOR®
A Danisco Division

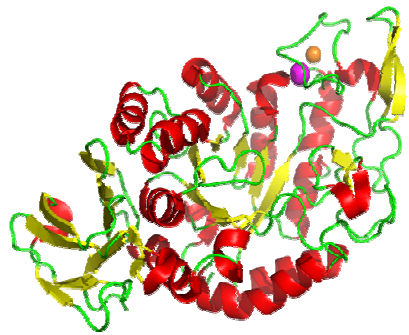
Core business

- Industrial Enzymes
- Food & Beverages Enzymes
- Feed Enzymes

Renewable feedstocks → Sugars

Leverage our competencies in biorefineries

- Biochemicals
- Biofuels
- Performance Proteins & Peptides



Our core business – **Enzymes** that empower industries to improve outcomes (environmental, performance, economic)

GENENCOR®
A Danisco Division

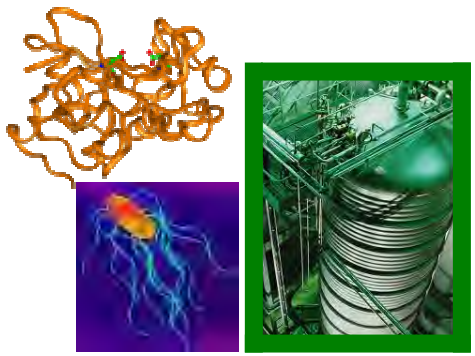
Brewing

Bio ethanol

Oil processing

Dairy

Animal feed



Baking

Wine & juice

Leather

Detergent

Starch

Pulp & Paper

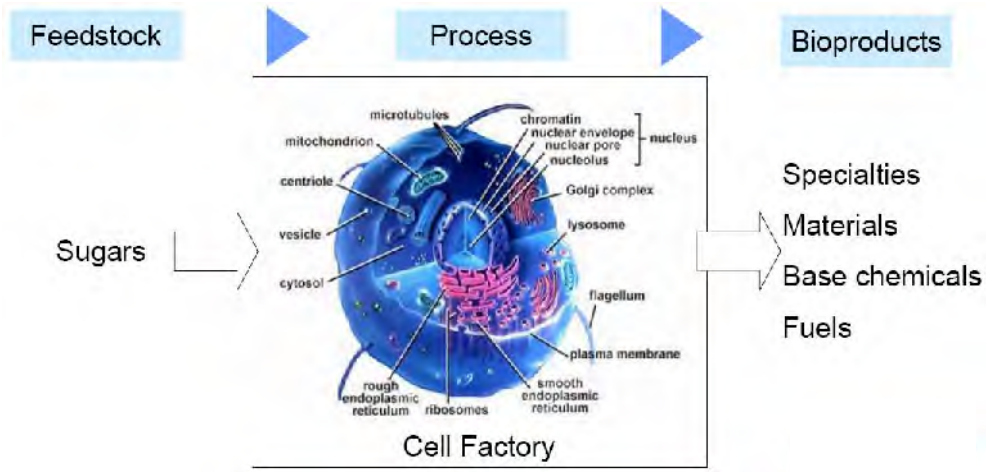
Waste processing

Textile

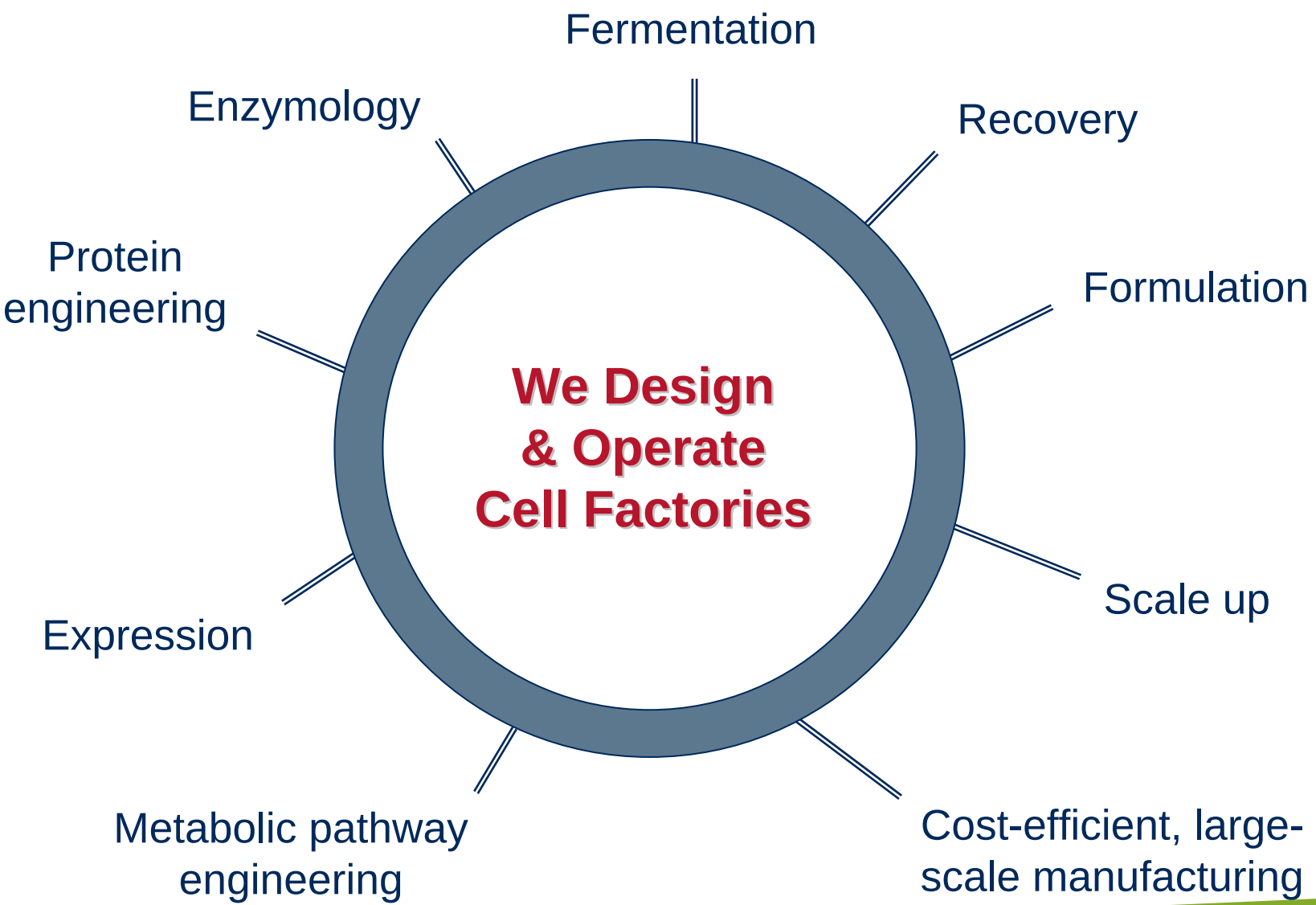
Our core competencies lie in discovery, enzyme manufacturing, and rapid scale-up

GENENCOR®
A Danisco Division

- Design and operate cell factories
- Engineer proteins that accomplish the previously impossible
- Engineer metabolic pathways of microorganisms to produce enzymes, proteins and other biomolecules at industrial scale and economics
- Fermentation, recovery, purification and formulation processing



What is Needed to Succeed

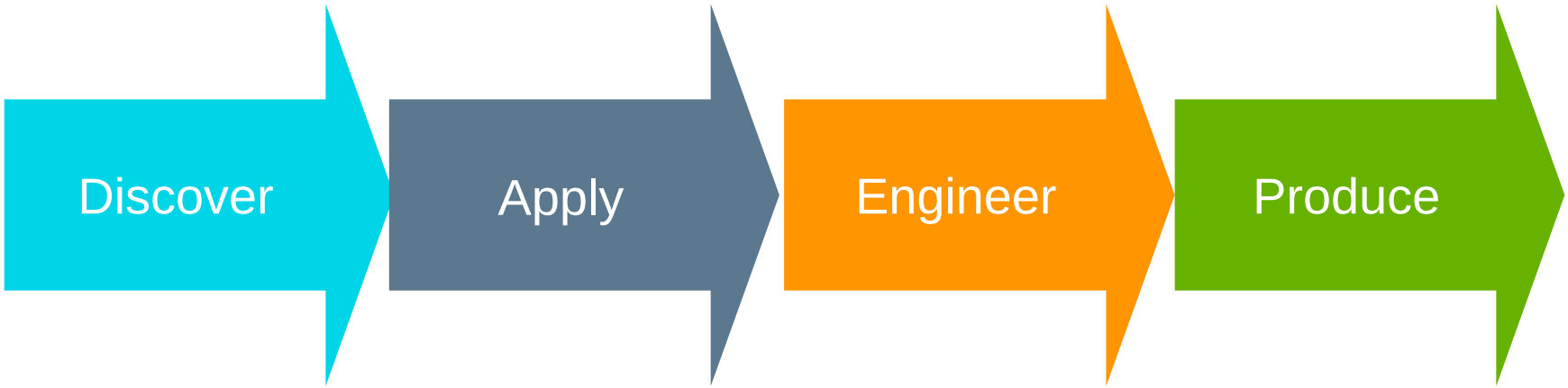


It Begins with Discovery

GENENCOR®
A Danisco Division

With an understanding of the customers' unmet needs, we

- Recruit from nature
- Build through applications research
- Drive through protein engineering
- Produce at metric ton scale



Discovery from the Environment
From Isolate to Ton, a Partnership with Nature …

GENENCOR®
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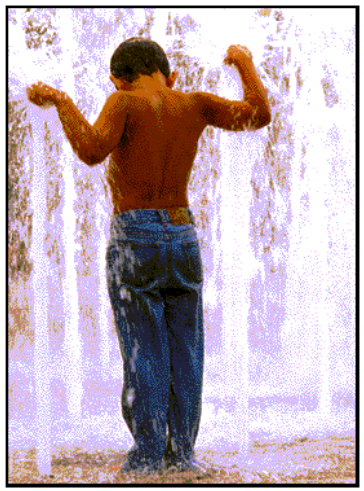
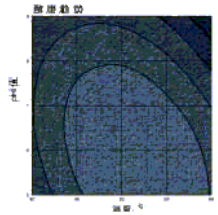


第一种可在冷水和热水中操作的中性纤维素酶。

推介 IndiAge® Neutra

- 改造的成分纤维素酶允许最大的加工工艺灵活性
- 高质量高 剂比量的后整理
- 广泛的 操作pH 值和温度范围(40-60°C)
- 广阔的酶磨范围



From isolate to metric ton in 12 months

Discover

We then put Discovery to the test - Laundry

GENENCOR®
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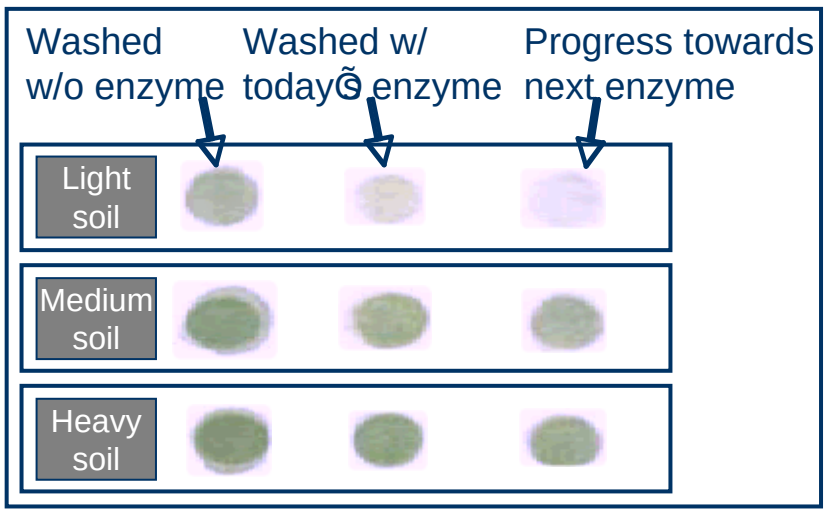

Tough soil removal

Apply

Next: Fermentation Processes Are Built from the Ground Up

GENENCOR®
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0.005 L

15 L

> 100,000 L

- Genencor produces proteins at chemical ingredient costs ($20 - $1000/Kg)
- Genencor rapidly creates engineered proteins that accomplish the previously impossible and deliver significant value to our customers.

Produce

Industrial (White) biotech is the 3rd wave of biotech – we are just at the beginning!

GENENCOR®
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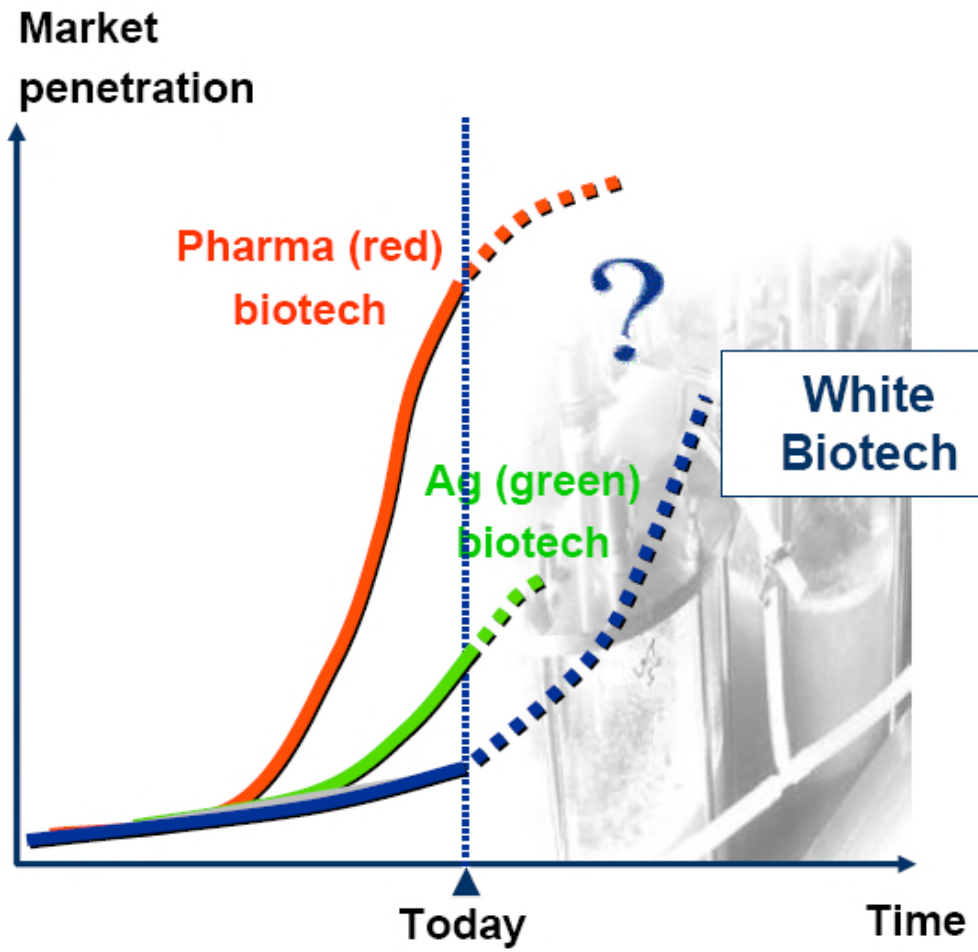

Industrial applications

Biofeedstock

Replacement of fossil feedstock by sugar and biomass

Bioprocesses

Use of natures toolbox for industrial processes (e.g., enzymes, bacteria)

Bioproducts

Current and new bio based products (e.g., fuel, materials)

Bioremediation

Use of biological processes for environ mental cleanup (e.g., waste water, oil pests)

Genencor

- We take customers' needs
- We find a solution - using all options
- …..and then we make it better

Marine world is full of opportunities to tap into, we are just at the beginning of seizing them!

Worldwide sales network & supply chain excellence

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- Production and distribution sites
- Regional distribution centers
- Regional customer relations locations
- R&D Center

Thank You!

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EXHIBIT 10

Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed and the Scientific Panel on Genetically Modified Organisms on the safety and efficacy of the enzymatic preparation Phyzyme XP (6- Phytase) for use as feed additive for chickens for fattening

(Question No EFSA-Q-2005-063)

Adopted by
the FEEDAP Panel on 20 April 2006
and by
the GMO Panel on 28 February 2006

SUMMARY

Phyzyme XP is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast *Schizosaccharomyces pombe,* designated ASP595-1 and deposited in the American Collection of Type Cultures as strain ATCC 5233. The final production strain is obtained after transformation with a linearised DNA fragment containing eight tandem repeats of an expression cassette which consists of the cytomegalovirus promoter, the 5' untranslated region of human lipocortin1 cDNA, the 6-phytase open reading frame, the terminator and the 3' untranslated region of human lipoprotein1 cDNA. No antibiotic resistance marker sequences were co-transformed as was confirmed by PCR. The expression cassettes are integrated in the *leu1* gene of the *S. pombe* chromosome. The applicant provided an acceptable risk analysis of the introduced viral sequences. Thus, no harmful sequences have been introduced, nor are there any antibiotic resistance marker sequences in the final production strain.

The safety of the donor and recipient organisms is based on the concept of familiarity.

After fermentation the enzyme is purified and the final enzyme product contains no culturable production organisms and recombinant DNA is below the level of detection.

Three pen trials conducted over the 42-day growing period showed significant effects on feed efficiency, daily weight gain and/or feed intake with the supplementation of 500 FTU Phyzyme XP kg^{-1} feed. P digestibility was also demonstrated to be increased with 500 FTU Phyzyme XP kg^{-1} feed. Only two studies were provided with supplementation of diets with 250 FTU Phyzyme XP kg^{-1} feed, both showing significant effects on weight gain, feed conversion and feed intake in two studies and on bone ash in one study. Therefore it is concluded that the efficacy of Phyzyme XP has been demonstrated at the dose of 500 FTU kg^{-1}, but insufficient data is provided to demonstrate efficacy at the minimum recommended dose of 250 FTU kg^{-1}.

A tolerance test carried out with chickens for fattening over a period of six weeks with the recommended dose (500 FTU kg^{-1}) and 7.5-fold overdose (7500 FTU kg^{-1}) of Phyzyme XP produced no evidence of any adverse effect on performance or mortality. Therefore it is concluded that the safety of Phyzyme XP has been demonstrated at the recommended dose and the 7.5-fold overdose.

On the basis of the results of three genotoxicity studies and one 90-day toxicity study there is no concern regarding the safety of this product for the consumer.

The product showed slight evidence of irritancy, low acute inhalation toxicity and no evidence of dermal sensitisation. By convention, the product, being an enzyme, is considered as a potential respiratory sensitizer.

It is concluded that there is no evidence for concern regarding safety for the environment.

Keywords: Enzyme, phytase, chickens for fattening, efficacy, safety, *Schizosaccharomyces pombe*, genetically modified micro-organism.

BACKGROUND

Regulation (EC) No 1831/2003 establishes rules governing the Community authorisation of additives for animal nutrition and in particular defines the conditions that a substance/product should meet to be granted authorisation. This Regulation replaces Council Directive 70/524/EEC. The Regulation foresees transition procedures for handling applications submitted under the earlier directive in its Article 25.

The applicant company Danisco Animal Nutrition is seeking permanent Community authorisation of its enzyme product "Phyzyme XP", which is a preparation of 6-Phytase EC 3.1.3.26, produced by *Schizosaccharomyces pombe* (ATCC 5233), as a feed additive intended for use in chickens for fattening under category "Enzymes" (Table 1).

Table 1. **Description of the preparation**

Product category:	Enzyme
Trade name:	Phyzyme XP
Description:	6-Phytase EC 3.1.3.26, produced by *Schizosaccharomyces pombe* ATCC 5233
Target animal category:	Chickens for fattening
Applicant:	Danisco Animal Nutrition
Member State Rapporteur:	UK
Type of request:	Permanent authorisation

In accordance with the above legislation, the Commission received a request from this company, through UK, the Member State *rapporteur* chosen by the company, to authorize its product under the conditions set out in Table 2. Supporting data produced by the company were compiled in a dossier and accompanied that application. All Member States received the dossier, which was introduced at the Standing Committee on the Food Chain and Animal Health. This dossier has been made available to the European Food Safety Authority.

TERMS OF REFERENCE

In view of the above, the Commission asks the European Food Safety Authority to deliver an opinion on the efficacy and on the safety of the enzyme preparation of trade name "Phyzyme XP", a preparation of 6-phytase produced by *Schizosaccharomyces pombe* (ATCC 5233), for the consumer, the user, the workers and for the target animal category specified and the environment, when used under the conditions set out in Table 2 below, taking into account the background and the information submitted by the applicant in the dossier.

Table 2: **Annex entry proposal**

Additive	Chemical formula, description	Species or category of animal	Maximum age	Minimum content	Maximum content	Other provisions
				Units of activity kg^{-1} of complete feedingstuff		
Phyzyme XP EC 3.1.3.26	Preparation of 6-Phytase produced by *Schizosaccharomyces pombe* (ATCC 5233) having a minimum activity of: Liquid form: 5000 FTU[11] g^{-1}	Chickens for fattening	-	250 FTU	-	1. In the directions for use of the additive and premixture, indicate the storage temperature, storage life and stability to pelleting. 2. Recommended dose per kg of complete feedingstuff: 250 - 1000 FTU. 3. For use in compound feed containing more than 0.23% phytin bound phosphorus

[1] FTU is the amount of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate substrate at pH 5.5 and at 37 °C

ASSESSMENT

1. Introduction

The subject of the present dossier is the permanent authorisation of Phyzyme™ XP liquid for chickens for fattening. This product is a preparation of 6-phytase produced by the genetically modified micro-organism (GMM) *Schizosaccharomyces pombe* (ATCC 5233). It is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in the manure.

2. Characterisation

2.1. Characterisation of the product

Phyzyme™ XP is an enzyme feed additive with 6-phytase as its main activity. The production strain, designated ASP595-1, is derived from the fission yeast *Schizosaccharomyces pombe* (ATCC 38399).

The product is a liquid enzyme concentrate complying with the following specifications:

Appearance	Homogeneous brown liquid
Specific gravity (g mL^{-1})	1.05 - 1.15
pH	4.4 – 4.8
Phytase activity (units g^{-1})	5000 guaranteed minimum
Sorbitol (% w v^{-1})	4.0%
Sodium Chloride (% w v^{-1})	13%

Units: µmoles phosphate per min at pH 5.5 and 37ºC

Routine quality control requires examination for microbiological (coliforms, *E. coli*, *Salmonella* spp., yeasts and molds), heavy metals (arsenic, cadmium, lead and mercury) and mycotoxin contamination. Appropriate action limits are set.

The product has been demonstrated to be stable on storage with 96% of the enzyme activity retained after 6 months at 5ºC, 92% after 6 months at 20ºC and 76% after 6 months at 35ºC. Phyzyme™ XP is also stable after mixing with feedingstuffs with approximately 80% of activity remaining after 6 months storage at 20ºC and 35ºC.

2.2. Characterisation of the production organism

2.2.1. Information relating to the genetically modified micro-organism (GMM)

2.2.1.1. Characteristics of the recipient or parental micro-organism

The recipient strain is *Schizosaccharomyces pombe* ATCC 38399, a teleomorph heterothallic$^-$ leucine auxotrophic yeast strain. The leucine auxotrophy results from a mutation at the leucine locus *leu1-32*.

S. pombe has a long history of safe use (OECD, 1992; IFBC, 1990). It is present in various traditionally fermented foods.

Schizosaccharomyces are members of the ascomycete subgroup Archaeascomycetes, a recently described but not yet formalized group designated primarily upon rDNA sequence analysis. The genus *Schizosaccharomyces* contains three recognized species: *S. japonicus*, *S. ocotsporus,* and *S. pombe. S. pombe* is taxonomically distinct from the most common recognized yeast pathogens. There are no reports of the existence of pathogenic, toxigenic or allergenic strains in the genus *Schizosaccharomyces* nor has any strain been described as being isolated from human faeces or from clinical samples derived from the human body.

2.2.1.2. Characteristics of the donor organism

The donor organism is *Escherichia coli* B ATCC 11303. The DNA was supplied by Sigma Co. The origin of the strain is not known. It was isolated in the late 1930's or early 1940's and since then it has been used as a common laboratory strain.

Escherichia coli is a well known species belonging to the family *Enterobacteriaceae*.

E. coli is found throughout the world in water, soil and as intestinal flora. Certain *E. coli* strains have pathogenic potential. Safety of the *E. coli* B strain is based on 1) the long-term use of this organism in numerous laboratories throughout the world, 2) the absence of genes encoding virulence factors as determined by PCR and other molecular methods, and 3) the lack of pathogenic potential in both mouse and chick models.

2.2.1.3. Description of the genetic modification process

The open reading frame (ORF) of the *appA* 6-phytase gene without its native *E. coli* leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. The expression cassette, which consists of the cytomegalovirus promoter, the 5' untranslated region of human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into *S. pombe* in two rounds of gene replacement recombination using auxotrophic mutants and selection for prototrophy. The strain producing the highest quantity of phytase was used as the final production strain. The copy number of the expression cassette was determined by quantitative PCR during the scaling up production process and varied from 4.35 ± 0.27 to 2.34 ± 0.22.

2.2.2. Information relating to the production process

Phyzyme™ XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain *S. pombe*. The 6-phytase enzyme is recovered from the fermentation broth by physical operation. The production process is controlled by process control parameters (media composition, sterilization, temperature, agitation, aeration, pressure, growth duration, pH, residual glucose level) and the fermentation process is followed by quality control parameters (culture purity, growth, phytase activity).

2.2.3. Information relating to the product purification process

2.2.3.1. Technique used to remove microbial cells from the product

A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (*i.e.*, > 95%) are removed. In the clarification step, either conventional (depth) filtration or tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. The absence of viable yeast cells is tested in the final product by plating reaching a detection level of 2 cfu ml^{-1}. Dot blot analysis was used to confirm that recombinant DNA was absent from the enzyme product. The limit of detection in this assay was 300 pg DNA kg^{-1} feed. The GMO Panel accepts the detection limit of this method as being adequate.

3. Efficacy for chickens for fattening

Four experiments have been carried out in order to demonstrate the efficacy of this enzyme preparation in chickens for fattening.

3.1. Trial 1

One pen trial with 1512 chickens for fattening was performed from 1 to 42 days (1 - 21 days = starter period, 21 – 42 days = finisher period). The chicks were allocated into 36 pens, in a randomised design, consisting of six dietary treatments with six replicates per treatment. The basal diet contained 5.16/4.50 g kg^{-1} total P (starter/finisher diet, respectively). Phyzyme XP was tested at 0, 250, 500, 750 and 1000 U kg^{-1}. For comparison, a positive control containing 6.35/5.69 g kg^{-1} total P was included. Enzyme activity in feed was measured and found appropriate.

The addition of Phyzyme XP at 250 FTU kg^{-1} resulted in a significant ($P \leq 0.05$) increase in body weight gain and feed efficiency during the study (Table 3). The highest improvement in feed efficiency was observed for the 500 FTU kg^{-1} group. Mortality was not affected by treatment.

Table 3. **Performance of chickens for fattening (1-42 days)**

Phyzyme XP (FTU kg^{-1})	Weight gain (g)	Feed intake (g $bird^{-1}$)	Feed conversion (gain/feed)	Mortality (%)
Positive control	2117[b]	3780	0.553[bc]	4.36
0	1939[a]	3688	0.516[a]	5.16
250	2126[b]	3847	0.544[b]	7.14
500	2160[b]	3827	0.558[c]	7.14
750	2195[b]	3862	0.559[c]	3.57
1000	2138[b]	3819	0.555[bc]	5.95

[a, b, c:] Means in a column not sharing a common superscript are significantly different ($P<0.05$)

3.2. Trial 2

One pen trial with 480 chickens for fattening from 1 to 42 days (1 - 21 days = starter period, 21 – 42 days = finisher period) was performed. The chicks were allocated into 40 pens, in a randomised design, consisting of 5 dietary treatments with 8 replicates per treatment.

The basal diet contained 5.1/4.4 g kg^{-1} total P (starter/finisher diet). Phyzyme XP was tested at 0, 500, 750 and 1000 FTU kg^{-1}. For comparison a positive control diet was used which contained 7.7/6.4 g kg^{-1} total P. Enzyme activity in feed was measured and found appropriate. At the end of the 3rd and 6th weeks, two chicks from each pen were killed and the left tibia and toe from each were excised for bone ash analysis.

Table 4. **Performance of chickens for fattening fed diets with graded levels of Phyzyme XP (1-42 days)**

Phyzyme XP (FTU kg^{-1})	Weight gain (g)	Feed intake (g $bird^{-1}$)	Feed conversion (gain/feed)	Mortality (%)	Ash (%)	
					Tibia	Toe
Positive control	2198[a]	3723[a]	0.591	4.16	54.3[a]	12.8[a]
0	2005[b]	3430[b]	0.585	6.25	48.5[b]	10.9[b]
500	2134[ab]	3674[a]	0.581	3.13	51.9[a]	12.2[ab]
750	2246[a]	3797[a]	0.591	4.17	52.7[a]	12.5[a]
1000	2209[a]	3740[a]	0.591	5.21	52.0[a]	11.7[ab]

[a, b:] Means in a column not sharing a common superscript are significantly different ($P<0.05$)

Supplementation of the diet with Phyzyme XP at 500 FTU kg^{-1} significantly increased feed intake (P≤0.05) without significantly affecting weight gain (Table 4), and increased tibia ash content (P≤0.05). Weight gain and toe ash content were only affected at Phyzyme XP levels of 750 FTU kg^{-1} or higher. Feed efficiency and mortality were not affected by treatment.

3.3. Trial 3

One pen trial with 1764 chickens for fattening at the age of 1 to 42 days (1 - 21 days = starter period, 21 – 42 days = finisher period) was performed. The chicks were allocated into 48 pens, in a randomised complete design, consisting of seven dietary treatments with six replicates per treatment. The basal diet contained 2.6/2.2 g kg^{-1} available P (starter/finisher diet). Phyzyme XP was tested at 0, 250, 500, 1000, 2000 FTU kg^{-1}. For comparison, a positive control containing 3.6/3.2 g kg^{-1} available P was included. At the end of the 6th week, the tibia of one bird from each pen was dissected.

Addition of Phyzyme XP at 250 FTU kg^{-1} resulted in a significant increase (P<0.05) of the final weight and feed intake of chickens (Table 5). Polynomial analysis of the phytase response revealed highly significant linear increase in feed intake and final body weight. Overall, feed efficiency was improved (P≤0.05) with 500 FTU kg^{-1} Phyzyme XP. Mortality to 42 days was not affected by treatment. Additions of phytase above 250 FTU kg^{-1} significantly increased (P≤0.05) bone ash percentage compared to the negative control diet.

Table 5. **Performance of chickens for fattening fed diets with graded levels of Phyzyme XP (1-42 days)**

Phyzyme XP (FTU kg^{-1})	Final weight (g)	Feed intake (g $bird^{-1}$)	Feed conversion (gain/feed)	Mortality (%)	Bone Ash (%)
Positive control	1982^{bc}	3544^{bc}	0.522^{a}	7.14	50.38^{de}
0	1791^{a}	3175^{a}	0.544^{ab}	3.97	44.83^{a}
250	1919^{b}	3371^{b}	0.560^{ab}	5.95	47.14^{b}
500	1982^{bc}	3424^{b}	0.573^{b}	4.76	47.43^{bc}
1000	2034^{cd}	3554^{bc}	0.565^{ab}	4.36	49.46^{cd}
2000	2109^{d}	3631^{c}	0.566^{ab}	2.78	48.43^{bcd}

$^{a, b, c, d, e:}$ Means in a column not sharing a common superscript are significantly different (P<0.05)

3.4. Trial 4

One digestibility trial with chickens for fattening from 8 to 22 days was performed. A total of 192 broilers (six chicks per pen and eight pens per treatment) were assigned to four diets in a way that the average weight of chicks was similar across dietary treatments. The basal diet contained 3.9 g kg^{-1} total P. Phyzyme XP was tested at 0, 500 and 1000 FTU kg^{-1}. For comparison, a positive control containing 7.7 g kg^{-1} total P was used. Enzyme activity in feed was measured and found appropriate.

When compared with the negative control diet, supplementation with 500 FTU Phyzyme XP kg^{-1} diet increased P retention and digestibility (54.5 *vs.* 66.4 and 53.1 *vs.* 68.8, P≤0.001, respectively). Ca retention or digestibility was not affected by the supplementation with Phyzyme XP.

3.5. Conclusions regarding efficacy

Three pen trials conducted over the 42-day growing period showed significant effects on feed efficiency, daily weight gain and/or feed intake with the supplementation of 500 FTU Phyzyme XP kg^{-1} feed. One digestibility trial demonstrated a significantly higher P retention and ileal digestibility in chickens given Phyzyme XP at 500 FTU Phyzyme XP

kg^{-1} feed. The increased bioavailability of P is demonstrated directly by increased ileal digestibility in one study, and indirectly by bone ash in three studies.

Only two studies were provided with supplementation of diets with 250 FTU Phyzyme XP kg^{-1} feed, both showing significant effects on weight gain, feed conversion and feed intake in two studies and on bone ash in one study.

The FEEDAP Panel thus concludes that the efficacy of Phyzyme XP has been demonstrated at the dose of 500 FTU kg^{-1}, but insufficient data is provided to demonstrate efficacy at the minimum recommended dose of 250 FTU kg^{-1}.

4. Safety

4.1. The safety aspects of the genetic modification

4.1.1. Information relating to the GMM and comparison of the GMM with its conventional counterpart

a) Description of the genetic trait(s) or phenotypic characteristics and any new trait which can be expressed or no longer expressed

The final production strain ATCC 5233 expresses the 6-phytase *appA* gene from *E. coli* B. It contains the expression cassette integrated into the chromosome. The final production strain does not contain any antibiotic resistance marker. This was verified by a PCR with a detection sensitivity of $\leq$ 3 pg DNA, which is sensitive enough to detect a single copy of the *bla* gene in the genome.

b) Structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified micro-organism

The applicant has provided a description of all the elements in the insertion cassette. It consists of the cytomegalovirus promoter, the 5' untranslated region of human lipocortin1 cDNA, the phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1. The applicant has provided an acceptable risk assessment of the virus sequences that are present in the final production strain.

4.1.2. Conclusions regarding the genetic modification

Phyzyme XP contains the 6-phytase enzyme produced after fermentation with the GM *Schizosaccharomyces pombe*. In the final production strain no harmful sequences have been introduced, nor are there any antibiotic resistance marker sequences.

The safety of the donor, *E. coli* B, and recipient organism *Schizosaccharomyces pombe,* is based on the concept of familiarity. For the donor organism, *E. coli* B, extra safety evidence is given by the absence of genes encoding virulence factors and the lack of pathogenic potential in both mouse and chick models.

After fermentation the enzyme product is purified so that no culturable production organisms are present and recombinant DNA is below the level of detection.

4.2. Safety for the target species

One tolerance trial with 756 chickens for fattening from 1 to 42 days (0 - 21 days = starter period, 21 – 42 days = finisher period) was performed. The chicks were allocated into 48 pens, in a randomised complete design, consisting of three dietary treatments with six replicates per treatment. The basal diet contained 2.6/2.2 g kg^{-1} available P (starter/finisher diet). Phyzyme XP was tested at 0, 500 (2X minimum recommended dose), 7500 (7.5X maximum recommended dose) FTU kg^{-1} (confirmed by recovery analysis).

No negative effects were observed with the supplementation of Phyzyme XP at 7.5 times the maximum recommended dose. In fact, feed intake and final weight were

significantly improved with the overdose of enzyme. No effects on feed conversion or mortality were observed.

Since this study was not performed at 10 times the maximum recommended dose, it does not fully satisfy the requirements for tolerance tests. Therefore, the safety for chickens for fattening has only been demonstrated at 7.5 times the maximum recommended dose.

4.3. Safety for the Consumer

4.3.1. Genotoxicity studies

The product tested in the genotoxicity assays was defined as RTA (refined test article) which was the production broth with cells removed but prior to adding stabilisers and preservatives. It was provided in lyophilised form and designated DV006R in study reports.

4.3.1.1. Bacterial Reverse Mutation Assay

The product as defined above was tested in compliance with GLP in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2*uvr*A (pKM101) both with and without microsomal enzyme activation systems. There was no evidence for genotoxicity of the test article in this study.

4.3.1.2. Mouse Lymphoma Assay

The product as defined above was tested in compliance with GLP for ability to induce forward mutations at the Thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line and was tested both with and without microsomal enzyme activation systems up to the highest non-cytotoxic dose. There was no evidence for any genotoxicity in the results of this assay.

4.3.1.3. Mammalian Micronucleus Assay

The product as defined above was tested in compliance with GLP for *in vivo* clastogenic activity by detecting micronuclei erythrocytes in mouse bone marrow following oral gavage administration of single doses of 500, 1000 and 2000 mg kg^{-1} bw. The study revealed no evidence of any toxicity to the bone marrow and showed no effect of the test article on the frequency of micronuclei.

4.3.2. Oral toxicity studies

The test article was provided in two forms for the oral toxicity studies: the Unrefined Test Article (UTA) which was the production broth with most of the cells removed and the RTA which was the same as that used for the genotoxicity assays.

4.3.2.1. 14-day range-finding study

Groups of 10 rats of each sex were administered test article daily by gavage for 14 days at doses which were multiples of the intended target species dose (50 units kg^{-1} bw day^{-1}). The administered doses were either 0, 100X, 600X, 1000X UTA or 2000X RTA. The study was conducted in compliance with GLP. Since no adverse effects were seen at the highest dose after 14 days these doses were selected for the 90-day study.

4.3.2.2. 90-day study

The dose levels used and the mode of administration were as described above for the 14-day study. Groups of 20 rats per sex per group were allocated for the low dose and intermediate group and 25 per sex per group for the control and highest dose of each test article. For each group which originally had 25 animals 20 were killed after 90 days

but the remaining five per group were kept for a further two weeks without treatment with test article before being brought to necropsy. The study was conducted in compliance with GLP and according to a current guideline protocol, and included a full range of clinical, haematological and pathological endpoints. There were no effects of treatment on clinical observations, body weight or food intake. Haematological and clinical chemistry results were similar for all groups although the females in both RTA and UTA highest dose group had globulin levels and albumin/globulin ratios which were statistically significantly higher than those of controls; a similar pattern was not seen in males. In the rats which were left for two weeks without treatment before necropsy the globulin levels of the same group were still higher than controls to a similar extent although the difference was not statistically significant, probably due to the smaller numbers of animals at this time-point.

Necropsy and histopathological examination showed no treatment-related changes apart from inflammatory changes in the lung which are identified as being consistent with the inhalation of a foreign substance. These effects are considered by the applicant and the contract laboratory to be due to the physiological effect of the high concentration of the test article leading to inhalation of particles after gavage. Although this is an unusual finding with such a product it is a known consequence of the use of gavage administration, and according to information provided by the applicant it has been seen with an enzyme product previously. It is likely that the differences seen in globulin levels are secondary to these pulmonary effects.

The FEEDAP Panel concludes that the effects seen are most likely to be a local effect of inhalation exposure, particularly to the unrefined product, and do not represent a relevant adverse effect for the purposes of safety evaluation. In the absence of any systemic effects of treatment the highest dose tested is concluded by the FEEDAP Panel to be the NOEL for this product.

4.3.3. Conclusions regarding consumer safety

On the basis of the results of three genotoxicity studies there is no concern regarding the genotoxicity of this product.

Although effects were seen in the 90-day study with this product they are considered by the FEEDAP Panel to be artefacts of the testing procedure used and as such not relevant to the safety evaluation. The FEEDAP Panel concludes that there is no evidence for concern regarding consumer safety.

4.4. Safety to the User

4.4.1. Skin irritation

When 0.5 mL of the UTA product was applied to the skin of three male and three female rabbits and left under semi-occluded dressing for four hours there was a slight erythema in five of the animals, which remained in one case for 24 hours. This is interpreted as evidence of slight irritancy. The study was conducted in compliance with GLP.

4.4.2. Eye irritation

When the UTA product was applied directly to the eye of three male and three female rabbits and apart from a slight redness in some animals immediately after treatment no irritant effects were observed. The study was conducted in compliance with GLP. The product is concluded to be non-irritant to the eye from the results obtained.

4.4.3. Sensitisation

A study was conducted in guinea pigs to the Buehler protocol and according to GLP. Groups of ten guinea pigs of each sex were tested against a control group of five of each

sex using the UTA product. There was no evidence of delayed contact hypersensitivity in this test.

4.4.4. Acute inhalation toxicity

Five male and five female rats were exposed to an aerosol of 2.2 mg L^{-1} of the UTA product for six hours and observed for 14 days. The study was conducted in compliance with GLP. A gross necropsy but no histological examination was performed. Dark material was reported around the nose and mouth of the treated group on the day of treatment but no mortality or other adverse effects were reported.

4.4.5. Conclusions regarding user safety

The product, even in its most unrefined form, shows little evidence of irritancy, low acute inhalation toxicity and shows no signs of dermal sensitisation potential. Although the pulmonary effects seen in the 90-day study are considered an artefact of the testing procedure they may indicate some inherent potential for the product to cause inflammation if inhaled. By convention the product is labelled as a potential sensitizer and thus protective clothing is recommended.

4.5. Environmental Safety

4.5.1. Potential environmental impact of the genetic modification

No environmental impact from the use of this product is expected on the basis of the sequences incorporated and the characteristics of the recipient micro-organism. The production micro-organism is removed from the product and the recombinant DNA is below the limit of detection.

4.5.2. Environmental impact of feed additive

The active ingredient is a 6-phytase enzyme which is a natural substance and is considered of no environmental concern. Thus, no further environmental assessment is required.

CONCLUSIONS

On the basis of the data submitted, the GMO Panel concluded that it is unlikely that the genetic modification of *S. pombe* to produce Phyzyme XP has any adverse effects on human and animal health or the environment.

Three pen trials conducted over the 42-day growing period showed significant effects on feed efficiency, daily weight gain and/or feed intake with the supplementation of 500 FTU Phyzyme XP kg^{-1} feed. P digestibility was also demonstrated to be increased with 500 FTU Phyzyme XP kg^{-1} feed. Only two studies were provided with supplementation of diets with 250 FTU Phyzyme XP kg^{-1} feed, both showing significant effects on weight gain, feed conversion and feed intake in two studies and on bone ash in one study. The FEEDAP Panel thus concludes that the efficacy of Phyzyme XP has been demonstrated at the dose of 500 FTU kg^{-1}, but insufficient data is provided to demonstrate efficacy at the minimum recommended dose of 250 FTU kg^{-1}.

A tolerance test carried out with chickens for fattening over a six-week period with a 2X minimum recommended dose (500 FTU kg^{-1}) and 7.5X maximum recommended dose (7500 FTU kg^{-1}) of Phyzyme XP produced no evidence of any adverse effect on performance or mortality. The FEEDAP Panel therefore concludes that the safety of Phyzyme XP has been demonstrated at 7.5X the maximum recommended dose (7500 FTU kg^{-1}). However, the tolerance of this product has not been demonstrated at 10X the maximum recommended dose.

On the basis of the results of three genotoxicity studies there is no concern regarding the genotoxicity of this product.

Although effects were seen in the 90-day study with this product they are considered by the FEEDAP Panel to be artefacts of the testing procedure used and as such not relevant to the safety evaluation. The FEEDAP Panel concludes that there is no evidence for concern regarding consumer safety.

The product, even in its most unrefined form, shows little evidence of irritancy, low acute inhalation toxicity and shows no signs of dermal sensitisation potential. Although the pulmonary effects seen in the 90-day study are considered an artefact of the testing procedure they may indicate some inherent potential for the product to cause inflammation if inhaled. By convention the product is labelled as a potential sensitizer and thus protective clothing for this purpose should be sufficient to minimise inhalation exposure, assuming that the clothing includes respiratory protection.

The FEEDAP Panel concludes that there is no evidence for concern regarding safety for the environment.

DOCUMENTATION PROVIDED TO EFSA

1. Dossier Phyzyme™ XP Chickens for fattening. December 2002. Submitted by Danisco Animal Nutrition.
2. Answers to Member State questions. October 2005. Submitted by Danisco Animal Nutrition.
3. Answers to EFSA questions. November 2005. Submitted by Danisco Animal Nutrition.
4. Answers to EFSA questions – Supplementary information. January 2006. Submitted by Danisco Animal Nutrition.
5. Supplementary information. March 2006. Submitted by Danisco Animal Nutrition.

REFERENCES

1. IFBC, International Food Biotechnology Council. 1990. Biotechnologies and Food: Assuring the Safety of Foods produced by Genetic Modification. National Academic Press, Washington DC. pp. S114-S128.
2. OECD. 1992. Safety Consideration for Biotechnology. Organization for Economic Cooperation and Development. OECD Council.

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EXHIBIT 11

Opinion the Scientific Panel on Additives and Products or Substances used in Animal Feed on the safety and efficacy of the enzyme preparation Phyzyme™ XP 5000L and Phyzyme™ XP 5000G (6-phytase) as a feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows in accordance with Regulation (EC) No 1831/2003

(Question No EFSA-Q-2005-080)

Adopted on 17 October 2006

SUMMARY

Phyzyme™ XP 5000L/G is an enzyme feed additive with phytase as its main activity. The additive exists in two forms, a liquid form (5000L) and a solid form (5000G). The production strain for Phyzyme™ XP 5000L/G is a genetically modified strain of the fission yeast *Schizosaccharomyces pombe*. Phyzyme™ XP 5000L has been previously assessed by EFSA and found to be safe for the consumer, user and the environment. The FEEDAP Panel considers the studies made with either one of the Phyzyme XP 5000 products (L and G) to be relevant for both.

The efficacy of Phyzyme™ XP 5000L/G has been demonstrated in chickens for fattening at 500 FTU kg^{-1}, laying hens at 150 FTU kg^{-1}, turkeys for fattening at 250 FTU kg^{-1}, ducks for fattening at 250 FTU kg^{-1} and in piglets, pigs for fattening and sows at 500 FTU kg^{-1}.

The safety of Phyzyme XP 5000L/G has been demonstrated at the maximum recommended dose for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets, pigs for fattening and sows.

Keywords: Phytase, efficacy, safety for target animals, chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets, pigs for fattening, sows

BACKGROUND

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lies down that any person seeking an authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from the company Danisco Animal Nutrition[2] for authorisation of the product Phyzyme™ XP 5000L and Phyzyme™ XP 5000G to be used as a feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows (category: zootechnical additives; functional groups: digestibility enhancers and substances which favourably affect the environment) under the conditions mentioned in Table 1.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4.1 (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application. According to Article 8 of the above mentioned Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 28 of September of 2005.

The additive Phyzyme™ XP 5000L and Phyzyme™ XP 5000G is a preparation of 6-phytase (EC 3.1.3.26) produced by the genetically modified micro-organism *Schizosaccharomyces pombe* (ATCC 5233). This product has not been authorised at Community level.

The FEEDAP and GMO Panels of EFSA delivered an opinion on the safety and efficacy of Phyzyme™ XP for chickens for fattening (adopted on 20 April 2006 by the FEEDAP Panel and on 28 February by the GMO Panel).[3]

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003 EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. Therefore, EFSA shall deliver an opinion on the efficacy and the safety for the target animals, user and consumer and the environment of the product Phyzyme™ XP 5000L and Phyzyme™ XP 5000G, which is a preparation of 6-phytase (EC 3.1.3.26) produced by the genetically modified micro-organism *Schizosaccharomyces pombe* (ATCC 5233) when used under the conditions described in Table 1.

[1] OJ L 268, 18.10.2003, p.29.
[2] Danisco Animal Nutrition, PO Box 777, Marlborough, Wiltshire, SN8 1XN, United Kingdom
[3] http://www.efsa.europa.eu/en/science/feedap/feedap_opinions/1468.html

Table 1. **Register entry as proposed by the applicant.**

Additive	Phyzyme™ XP
Registration number/EC No/No (if appropriate)	EC 3.1.3.26
Category of additive	Zootechnical additives
Functional group of additive	Digestibility enhancers. Substances which favourably affect the environment

Description			
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)
Preparation of 6-phytase (EC 3.1.3.26) produced by *Schizosaccharomyces pombe* (ATCC 5233) with a minimum activity of: Liquid form: 5000 FTU g^{-1} Solid form: 5000 FTU g^{-1}	-	Guaranteed minimum activity of 5000 FTU g^{-1}	1 FTU is the amount of enzyme which liberates 1 micromole of inorganic phosphate from sodium phytate at pH 5.5 and 37 °C in one minute

Trade name (if appropriate)	Phyzyme™ XP 5000L and Phyzyme™ XP 5000G
Name of the holder of authorisation (if appropriate)	Danisco Animal Nutrition

Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		Units of activity kg^{-1} of complete feedingstuffs		
Chickens for fattening	-	250	750	-
Turkeys for fattening	-	250	1000	-
Laying hens	-	150	900	-
Ducks for fattening	-	250	1000	-
Piglets (weaned)	-	250	1000	-
Pigs for fattening	-	250	1000	-
Sows	-	500	500	

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	In the directions for use of the additive, indicate the storage temperature, storage life and stability to pelleting. For use in compound feed containing more than 0.23% phytin bound phosphorous
Specific conditions or restrictions for handling (if appropriate)	Harmful. May cause sensitization by inhalation. Do not breathe dust (or spray). Avoid contact with skin. Wear

	suitable protective clothing and gloves
Post market monitoring (if appropriate)	All batches of Phyzyme™ XP are shipped in closed containers and the label information includes the name and address of the producer, a batch number and a bar code. All customers are supplied with copies of the Material Safety Datasheet for the product that includes an emergency contact number. This enables any user to contact the company and provide batch specific information on the use of a product. In addition batch traceability and complaints systems are in place so as to enable rapid investigation and resolution of any negative reports of usage or complaints. The traceability system ensures that any person to whom we have supplied the product can be rapidly identified.
Specific conditions for use in complementary feedingstuffs (if appropriate)	Not applicable

Maximum Residue Limit (MRL) (if appropriate)			
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues
-	-	-	-

ASSESSMENT

1. Introduction

Phyzyme™ XP has recently been assessed by the FEEDAP and GMO Panels of EFSA for use in chickens for fattening. The product Phyzyme™ XP 5000L which is the subject of the present application is identical to the Phyzyme™ XP previously reviewed and is derived from the same organism. The granulated product Phyzyme™ XP 5000G varies only in the final formulation as described below. Since the genetic modification characteristics and the safety for consumer and environment are identical to the Phyzyme™ XP product, recently reviewed, these are not considered further in this opinion.

2. Characterisation of the product

Phyzyme™ XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme™ XP, designated ASP595-1, is derived from a strain of the fission yeast *Schizosaccharomyces pombe* (ATCC 38399).

The product is available in two forms:

- Phyzyme™ XP 5000L: A formulated liquid enzyme concentrate complying with the following specifications:

Appearance	Homogeneous brown liquid
Specific gravity (g mL^{-1})	1.05 - 1.15
pH	4.4 – 4.8
Phytase activity (units g^{-1})	5000 guaranteed minimum
Heavy metals, as lead (mg kg^{-1})	30 max
Arsenic (mg kg^{-1})	3 max
Cadmium (mg kg^{-1})	1 max
Mercury (mg kg^{-1})	1 max
Sorbitol (% w v^{-1})	4.0%
Sodium Chloride (% w v^{-1})	13%

Units: µmoles phosphate liberated per min at pH 5.5 and 37°C

- Phyzyme™ XP 5000G: A formulated solid enzyme product containing the same enzyme product at the same enzyme activity but dispersed on a wheat flour carrier along with calcium propionate and citric acid as preservatives. A particle size study has demonstrated that approximately 9% of particles are <100 µm and 3.5% <10 µm.

2.1. Stability

Phyzyme™ XP 5000L has been demonstrated to be stable on storage at 20°C with 95% of the enzyme activity retained after six months, 92% after nine months and 88% after 12 months.

Phyzyme™ XP 5000G was stored for 12 months at 20°C with 87% of the enzyme activity remaining after this period.

Phyzyme™ XP 5000L is also stable after mixing with feedingstuffs with approximately 80% of activity remaining after six months storage in a feed mash at both 20°C and 35°C. Phyzyme™ XP 5000G had 72.4% of original activity after storage in feed mash for six months at 20°C.

Following pelleting of feeds at 80°C, 82% of the initial phytase activity was still present, thus Phyzyme™ XP 5000G is considered to be stable when pelleted up to this temperature. At higher temperatures significant loss of activity was seen.

2.2. Conditions of use

Phyzyme XP 5000L/G is intended to be used in animal feedingstuffs rich in phytates. The minimum and maximum recommended doses are the following: chickens for fattening, 250-750 FTU kg^{-1}; laying hens, 150-900 FTU kg^{-1}; turkeys for fattening, ducks for fattening, piglets and pigs for fattening, 250-1000 FTU kg^{-1}; sows, 500 FTU kg^{-1}.

2.3. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA has verified the CRL report as it relates to the methods used for the control of the active substance in animal feeds. The Executive Summary of the CRL report can be found in the Appendix.

3. Efficacy

3.1. Efficacy for chickens for fattening

The efficacy of Phyzyme™ XP 5000L in chickens for fattening has been evaluated by the FEEDAP Panel in its opinion adopted on 20 April 2006 (EFSA, 2006). In that opinion it was concluded that:

"Three pen trials conducted over the 42-day growing period showed significant effects on feed efficiency, daily weight gain and/or feed intake with the supplementation of 500 FTU Phyzyme™ XP kg^{-1} feed. P digestibility was also demonstrated to be increased with 500 FTU Phyzyme™ XP kg^{-1} feed. Only two studies were provided with supplementation of diets with 250 FTU Phyzyme™ XP kg^{-1} feed, both showing significant effects on weight gain, feed conversion and feed intake in two studies and on bone ash in one study. Therefore it is concluded that the efficacy of Phyzyme™ XP has been demonstrated at the dose of 500 FTU kg^{-1}, but insufficient data is provided to demonstrate efficacy at the minimum recommended dose of 250 FTU kg^{-1}."

The FEEDAP Panel considers that the solid form is very unlikely to differ in bioavailability from the liquid form, and therefore concludes that the efficacy data above are relevant to both formulations.

3.2. Efficacy for laying hens

Two trials were conducted in laying hens in two different locations. Each of these trials was subdivided in two different studies.

Trial 1[4]

This trial was subdivided into two separate studies. A total of 1056 laying hens (Hy-line) were randomly distributed between 12 dietary treatments, (22 replicates of four hens per treatment). Two types of basal diets were prepared, based on either a maize (Study 1) or wheat (Study 2). The dietary treatments included a positive control, which was designed to provide adequate levels of available P (0.25%), a negative control (0.13% available P) and the same diet supplemented with 150, 300, 600 or 900 FTU kg^{-1} of Phyzyme™ XP 5000G. Laying hens were 41 weeks of age at the start of the study and the diets in meal form were fed for a six week period. The intended enzyme activity in feed was confirmed by analysis. Titanium oxide was added to the diets as a marker and grab samples of excreta were collected on day 14 of the study for the assessment of digestibility of Ca, P, N, Mg, Na and K. At the end of the trial one hen from each cage was killed and sampled for toe ash assay. On the last day of the study, egg quality parameters were measured using one egg per cage (egg mass, Haugh units, egg yolk colour, and shell thickness).

[4] Technical Dossier/Section III/Ref. C10 & C11

Supplementation of the maize diets (study 1) with 600 FTU Phyzyme XP 5000G kg^{-1} significantly increased feed intake when compared to the negative control. No other differences were observed in any of the other production parameters or toe ash. In the wheat groups (study 2), supplementation with Phyzyme XP 5000G did not affect performance parameters or egg quality. Mortality was not affected by treatment in either of the studies. The apparent digestibility coefficient for P was significantly improved by phytase addition at 150 FTU kg^{-1} in the maize diets, and by the addition of 600 FTU kg^{-1} in the wheat diets. Ca apparent digestibility was not affected by phytase supplementation of maize diets, but was reduced in a dose related manner ($P<0.01$) by the inclusion of Phyzyme XP 5000G in the wheat diets (Table 2).

Trial 2[5]

This trial was also subdivided into two studies. One hundred laying hens (Brown Hy-Line) were distributed into individual balance cages. The ten dietary treatments were based on either maize (study 3) or wheat (study 4) and included a positive control (available P, 0.30%), a negative control (available P, 0.14%) and the negative control diet supplemented with 150, 300 or 600 FTU kg^{-1} Phyzyme™ XP 5000G. Laying hens were 27 weeks of age at the start of the study and the diets in meal form were fed for a six week period. The intended enzyme activity in feed was confirmed by analysis. Excreta were collected during a three-day period (day 14 to 17 of the study) for the assessment of digestibility of Ca, P, N and metabolisable energy (ME). At the end of the trial five hens from each group were killed and sampled for toe ash content.

Table 2. **Effect of Phyzyme™ XP 5000L/G on apparent P and Ca digestibility (%) of laying hens (Trials 1 and 2)**

	Trial 1				Trial 2			
	Maize diets (Study 1)		Wheat diets (Study 2)		Maize diets (Study 3)		Wheat diets (Study 4)	
	P	Ca	P	Ca	P	Ca	P	Ca
Positive control	54.5^{bc}	67.6	40.5^{bc}	57.0	27.1^{b}	47.0^{a}	28.5^{b}	54.4^{b}
0	52.2^{c}	68.4	33.5^{c}	62.3	15.1^{a}	46.9^{a}	17.1^{a}	48.8^{a}
150	63.3^{a}	71.8	34.7^{c}	63.6	21.8^{ab}	59.6^{c}	27.8^{b}	55.5^{b}
300	57.4^{abc}	70.2	37.0^{c}	58.4	20.3^{ab}	54.5^{b}	26.6^{b}	56.9^{b}
600	61.1^{ab}	68.2	48.2^{ab}	56.8	20.7^{ab}	52.2^{ab}	28.3^{b}	56.4^{b}
900	63.6^{a}	69.2	52.1^{a}	50.0				

$^{a, b, c}$: Values in a column not sharing a common superscript differ significantly ($P \leq 0.05$)

Supplementation of the negative maize control diet (study 3) with increasing levels of phytase did not influence feed intake, egg mass (g egg^{-1}) or toe ash. Egg production (%) and feed intake per egg was only improved by the supplementation with 600 FTU Phyzyme XP 5000G kg^{-1} compared with the negative control. Regarding the wheat diets (study 4), feed intake and toe ash were significantly improved by the supplementation with 150 FTU Phyzyme XP 5000G kg^{-1}. Apparent digestibility of Ca was significantly improved by the supplementation of both diets with 150 FTU kg^{-1}, while that of P was only improved at the same dose in the wheat diets (Table 2). No mortality was reported in either of the studies.

Conclusions on efficacy for laying hens

Four studies were conducted over a six week laying period with the addition of Phyzyme™ XP 5000L/G ranging between 150 and 900 FTU kg^{-1}.

[5] Technical Dossier/Section III/Ref. C12 & C13

The FEEDAP Panel considered the studies submitted as dose-response balance studies with sufficient duration and number of replicates per treatment group. Apparent digestibility of P was improved in two studies and Ca digestibility was improved in another study at a dose of Phyzyme™ XP of 150 FTU kg^{-1}. The FEEDAP Panel therefore concludes that efficacy of Phyzyme XP 5000L/G in laying hens is demonstrated at a dose of 150 FTU kg^{-1}.

3.3. Efficacy for turkeys for fattening

Trial 1[6]

A total of 650 male Nicholas turkey poults were distributed into 48 pens at one day of age and reared to 16 weeks of age. At four weeks of age, extra animals were removed to reduce stocking density to 12 birds per cage. Diets were formulated for the following age periods: 0-4 weeks, 4-8 weeks, 8-12 weeks and 12-16 weeks. Each diet was fed in mash form to six replicate pens of 12 birds. The diets comprised a positive control (available P: 0.60, 0.50, 0.43, 0.38% for the different periods), a negative control (available P: 0.40, 0.30, 0.21, 0.19% for the different periods), the negative control supplemented with 250, 500, 750 or 1000 FTU Phyzyme™ XP 5000G kg^{-1} diet and the negative control plus a commercial phytase at 500 or 1000 FTU kg^{-1} diet (data not shown). The intended enzyme activity in feed was confirmed by analysis. At 16 weeks of age, four turkeys per pen were euthanized and the right and left feet dissected.

Body weight, feed intake and toe ash content was significantly higher for all enzyme supplemented groups in comparison to the negative control (Table 3). Feed efficiency was improved with the addition of 750 FTU kg^{-1} Phyzyme™ XP 5000G over that of basal group. Mortality for the trial averaged 4.6% with the majority occurring during fist four weeks. Much of the mortality that occurred was unrelated to dietary treatment as compared to expected losses due to leg problems or deformities.

Table 3. **Efficacy of Phyzyme™ XP 5000G on performance and bone ash content of turkeys (0-16 weeks)**

	Positive Control	Phyzyme™ XP (FTU kg^{-1})				
		0	250	500	750	1000
Body weight, kg	13.83^{a}	11.79^{d}	12.52^{c}	13.24^{b}	13.34^{ab}	13.56^{ab}
Feed intake, g day^{-1}	299.6^{a}	266.1^{c}	286.3^{b}	295.6^{ab}	295.2^{ab}	302.9^{a}
Feed/gain, kg kg^{-1}	2.516^{c}	2.655^{a}	2.663^{a}	2.630^{ab}	2.557^{bc}	2.607^{abc}
Toe ash, %	13.79^{a}	12.51^{c}	12.99^{b}	13.56^{a}	13.48^{a}	13.63^{a}

$^{a, b, c}$: Values in a row not sharing a common superscript differ significantly ($P \leq 0.05$)

Trial 2[7]

A total of 768 male BUT 9 turkey poults were distributed into 96 pens at one day of age and reared to 16 weeks of age. Diets were formulated for the following age periods: 0-4 weeks, 4-8 weeks, 8-12 weeks and 12-16 weeks. Each diet was fed in crumbles/pellet form to six replicate pens. The experimental treatments consisted of a positive control (available P: 0.60, 0.50, 0.42, 0.38% for the different periods), a negative control (available P: 0.40, 0.30, 0.22, 0.18% for the different periods), and the negative control supplemented with 250, 500, 750 or 1000 FTU Phyzyme™ XP 5000L kg^{-1} diet or supplemented with a commercial phytase at 500 or 1000 FTU kg^{-1} diet (data not shown). The intended enzyme activity in feed was confirmed by analysis.

Body weight gain was significantly improved by the supplementation of the negative control with 250 FTU kg^{-1} ($P \leq 0.05$) (Table 4). Feed efficiency was improved when the

[6] Technical Dossier/Section III/Ref. C7

[7] Technical Dossier/Section III/Ref. C8

supplementation with Phyzyme XP was increased at 500 FTU kg^{-1}. The total mortality during the trial was 4.7% and it was unrelated to the dietary treatments. Of these just under half were culled mostly as a result of their inability to walk.

Table 4. **Efficacy of Phyzyme™ XP 5000L on performance of turkeys (0-112 days)**

	Positive control	Phyzyme™ XP (FTU kg^{-1})				
		0	250	500	750	1000
Body weight gain, kg $bird^{-1}$	14.2^{cd}	14.0^{d}	14.4^{bc}	14.5^{abc}	14.8^{a}	14.7^{ab}
Feed efficiency, kg kg^{-1}	2.45^{bc}	2.51^{c}	2.46^{bc}	2.42^{ab}	2.38^{ab}	2.35^{a}

$^{a, b, c}$: Values in a row not sharing a common superscript differ significantly ($P \leq 0.05$)

Trial 3[8]

A total of 576 male turkey poults were distributed into 64 pens at one day of age and reared to 16 weeks of age. Diets were formulated for the following age periods: 0-4 weeks, 4-8 weeks, 8-12 weeks and 12-16 weeks. Each diet was fed in crumble/pellet form to eight replicate pens (nine turkeys per pen). The diets comprised a positive control (P contents not available), a negative control, the negative control supplemented with 250, 500, 750 or 1000 FTU Phyzyme™ XP 5000L kg^{-1} diet or supplemented with an approved phytase at 500 or 1000 FTU kg^{-1} diet (data not shown). The intended enzyme activity in feed was confirmed by analysis. At six weeks of age, one turkey per pen was placed in a metabolism cage for three days to determine ME and digestibility of Ca, P and N. At the end of the trial, three birds per pen were euthanized and the middle toe of the right foot was collected.

The total mortality during the trial was 6.6% and it was unrelated to the dietary treatments.

Table 5. **Efficacy of Phyzyme™ XP 5000L on performance, bone ash content (0-16 weeks) and apparent digestibility of Ca and P (6 weeks) of turkeys**

	Positive Control	Phyzyme™ XP (FTU kg^{-1})				
		0	250	500	750	1000
Body weight gain, kg $bird^{-1}$	12.4^{a}	7.9^{d}	9.9^{c}	11.3^{b}	11.8^{ab}	11.7^{ab}
Feed efficiency, kg kg^{-1}	2.63^{b}	3.00^{a}	2.82^{ab}	2.78^{b}	2.69^{b}	2.67^{b}
Toe ash, %	8.42^{a}	6.23^{c}	7.19^{b}	7.71^{ab}	7.93^{ab}	8.18^{a}
Digestibility, %						
Ca	34.4^{c}	41.7^{abc}	54.6^{ab}	54.1^{ab}	40.4^{bc}	57.7^{a}
P	40.8^{c}	51.4^{bc}	62.8^{ab}	58.5^{ab}	55.9^{ab}	67.7^{a}

$^{a, b, c}$: Values in a row not sharing a common superscript differ significantly ($P \leq 0.05$)

Supplementation of the negative control with 250 FTU kg^{-1} of phytase significantly improved body weight gain (Table 5), being the weight gain of birds from the groups supplemented with 750 and 1000 FTU kg^{-1} similar to the positive control values. Feed efficiency was improved with the addition of 500, 750 and 1000 FTU kg^{-1} and was similar to the positive control group. Toe ash was increased by the supplementation with all enzyme levels. P digestibility was only improved by the supplementation with 1000 FTU kg^{-1}, while Ca digestibility was not affected by the enzyme supplementation.

Conclusions on efficacy for turkeys for fattening

Three trials conducted over the 16 weeks growing period with the addition of Phyzyme™ XP at 250 – 1000 FTU kg^{-1} showed positive effects on body weight gain. The increased bioavailability of P is demonstrated indirectly by bone ash in two studies.

The FEEDAP Panel thus concludes that the efficacy of Phyzyme™ XP 5000L/G has been demonstrated at the lowest recommended dose of 250 FTU kg^{-1}.

[8] Technical Dossier/Section III/Ref. C6

3.4. Efficacy for ducks for fattening

Trial 1[9]

A total of 768 one-day-old male Pekin ducklings were distributed into nine treatments (six replicates of 12 ducklings per pen). The diets comprised a positive control (available P: 0-21 days, 0.40%; 21-42 days, 0.30%), two negative controls (Diet 1: available P: 0-21 days, 0.30%; 21-42 days, 0.20%; Diet 2: available P: 0-21 days, 0.40%; 21-42 days, 0.30%), and the negative controls supplemented with 250, 500 or 1000 FTU Phyzyme™ XP 5000L kg^{-1} diet. Two further diets included the basal Diet 2 supplemented with 500 or 1000 FTU of a commercial phytase kg^{-1} (five replicate pens of 12 ducklings, data not shown).

All diets were provided as pellets, with the starter diets fed from 0-21 days and the corresponding finisher diets from 21-42 days. Diets were made into 3 mm pellets and the respective enzyme was sprayed onto the pellets at the required level.

The intended enzyme activity in feed was confirmed by analysis. Retentions of the dietary energy, N, Ca and P were calculated by carrying out analyses (titanium dioxide was used as an indigestible marker) on samples of droppings collected from the ducklings between 17 and 20 days. Ash contents of the tibias were determined on bones removed from a cross section of the birds (one duckling randomly selected from each pen) at the end of the trial.

Table 6. ***Efficacy of Phyzyme™ XP 5000L on performance of growing ducks***

	Positive Control	*Phyzyme™ XP (FTU kg^{-1})*							
		Diet 1				*Diet 2*			
		0	*250*	*500*	*1000*	*0*	*250*	*500*	*1000*
Feed intake, g $duck^{-1}$	*6157^{a}*	*6225^{ab}*	*6294^{ab}*	*6419^{b}*	*6260^{ab}*	*6283*	*6389*	*6308*	*6216*
Body weight gain, g	*3210^{ab}*	*3066^{c}*	*3104^{bc}*	*3226^{a}*	*3183^{ab}*	*3177*	*3187*	*3180*	*3200*
Feed conversion, kg kg^{-1}	*1.92^{a}*	*2.03^{c}*	*2.03^{c}*	*1.99^{bc}*	*1.97^{ab}*	*1.98^{bc}*	*2.01^{c}*	*1.99^{bc}*	*1.94^{ab}*
Tibia ash, %	*55.2^{ab}*	*51.6^{b}*	*56.0^{a}*	*54.1^{ab}*	*54.0^{ab}*	*54.8*	*57.1*	*54.4*	*54.2*
Retention, %									
Ca	*37.4^{b}*	*38.1^{b}*	*49.5^{a}*	*46.1^{a}*	*35.7^{b}*	*42.4*	*40.1*	*38.3*	*43.4*
P	*38.7^{c}*	*54.7^{ab}*	*55.9^{a}*	*56.1^{a}*	*49.1^{b}*	*46.1^{b}*	*54.5^{a}*	*53.4^{a}*	*55.6^{a}*

$^{a, b, c}$: Values in a row within a diet not sharing a common superscript differ significantly ($P \leq 0.05$)

For birds fed Diet 1, weight gain was significantly increased in comparison to the negative control by the supplementation with 500 and 1000 FTU Phyzyme™ XP 5000L kg^{-1}. Feed conversion was improved in birds receiving diets supplemented with 1000 FTU Phyzyme™ XP 5000L kg^{-1} compared to the negative control group. N and gross energy retention was not affected by the enzyme supplementation. Ducks fed on diets supplemented with 250 and 500 FTU Phyzyme™ XP 5000L kg^{-1} retained significantly more Ca than birds fed on the other diets. Tibia ash content was significantly increased by the supplementation with 250 FTU Phyzyme™ XP 5000L kg^{-1} compared to the negative control group. P retention was not affected by the supplementation with enzyme, when compared to the negative control (Table 6).

For Diet 2, supplementation of the negative control with increasing levels of phytase did not influence feed intake, body weight gain or tibia ash content over the entire experimental period. P retention was significantly higher in ducks fed diets containing 250 FTU Phyzyme™ XP 5000L kg^{-1} or higher compared to those fed the negative control diet (Table 6).

The total mortality was unrelated to the dietary treatments.

[9] Technical Dossier/Section III/Ref. C15 & C16

Trial 2[10]

A total of 480 White Pekin male ducks were assigned to 42 pens with 12 ducks per pen. The mash diets were based in corn and soybean meal and were provided in two phases, starter (0-14 days) and grower (15-42 days). The treatments comprised a positive control (available P: 0-14 days, 0.45%; 15-42 days, 0.35%), negative control (available P: 0-21 days, 0.30%; 21-42 days, 0.20%), and the negative control supplemented with 500, 1000 or 1500 FTU Phyzyme™ XP 5000G kg^{-1}. The intended enzyme activity in feed was confirmed by analysis.

Feed intake and weight gain were significantly improved with the supplementation with 500 FTU kg^{-1} (Table 7).

Table 7. **Efficacy of Phyzyme™ XP 5000G on performance of growing ducks (0-42 days)**

	Positive control	Phyzyme™ XP (FTU kg^{-1})			
		0	500	1000	1500
Feed intake, g $duck^{-1}$	7750[a]	6009[c]	6881[b]	7344[ab]	7613[a]
Body weight gain, g	2974[a]	2083[d]	2620[c]	2747[bc]	2858[ab]
Feed conversion, kg kg^{-1}	2.61[a]	2.89[b]	2.63[ab]	2.67[ab]	2.66[a]
Mortality, %	4.2	11.5	0	1.0	3.1

[a, b, c, d]: Values in a row not sharing a common superscript differ significantly ($P \leq 0.05$)

Conclusions on efficacy for ducks for fattening

Efficacy has been demonstrated in chickens and turkeys for fattening and laying hens. As the mode of action of phytase in ducks is the same as in the major species, no further demonstration of efficacy is necessary. In the first of two studies presented, which included two comparisons, efficacy was demonstrated at the lowest recommended dose (250 FTU kg^{-1}). In the second study, the lowest dose tested was 500 FTU kg^{-1}, also showing significant effects on feed intake and body weight gain. The FEEDAP Panel concludes that given that ducks are considered minor species, sufficient evidence has been provided to demonstrate efficacy at the lowest recommended dose (250 FTU kg^{-1}).

3.5. Efficacy for piglets (weaned)

Four experiments were carried out at three different locations.

First study (balance study)[11]

The four dietary treatments included a positive control (available P: 0.55%), a negative control (available P: 0.33%), the negative control supplemented with 500 or 1000 FTU Phyzyme™ XP 5000G kg^{-1} of feed. There were six individual piglets per treatment (total 24 piglets) with an initial body weight of 15 kg, housed in metabolic crates to allow collection of faeces and urine. The study consisted of a 5-day adaptation period followed by a 5-day collection period. This experiment allowed the determination of the apparent total tract digestibility of P (ATTD) and other nutrients such as dry matter (DM), N and gross energy. The intended enzyme activity in feed was confirmed by analysis.

Results from Table 8 show that supplementation with 500 FTU Phyzyme™ XP kg^{-1} significantly enhanced ($P<0.05$) the apparent digestibility of P with respect to the negative control.

[10] Technical Dossier/Section III/Ref. C17

[11] Technical Dossier/Section III/Ref. C19

Table 8. **Effect of Phyzyme™ XP 5000G on apparent total tract digestibility (%) of dry matter, P, N and gross energy of piglets**

	Positive control	Phyzyme™ XP (FTU kg^{-1})		
		0	500	1000
Dry matter^{y}	85.8^{c}	87.6^{ab}	87.2^{b}	88.3^{a}
N^{y}	80.3^{b}	84.0^{a}	82.6^{a}	84.2^{a}
P^{xy}	37.5^{b}	24.0^{d}	32.1^{c}	49.5^{a}
Gross energy^{y}	83.5^{c}	85.8^{ab}	84.9^{b}	86.2^{a}

$^{a, b, c}$: Values in a row not sharing a common superscript differ significantly (P≤0.05)
^{x}: Linear phytase effect (P<0.05)
^{y}: Quadratic phytase effect (P<0.05)

Second study[12]

The four dietary treatments included a positive control (available P: 0.57%), a negative control (available P: 0.40%), and the negative control supplemented with 500 or 1000 FTU Phyzyme™ XP 5000G kg^{-1} of feed. There were 12 individual piglets (six castrated males and six females) per treatment (total 48 piglets). The initial body weights were 10 kg and piglets were housed in individual metabolic crates. Piglets were weighed at the start of the study and at weekly intervals (days 7, 14 and 21) over the three weeks duration of the study. Feed intake and growth rate were calculated each week and feed conversion ratio was also calculated. The intended enzyme activity in feed was confirmed by analysis. Four animals were culled from the experiment from different treatments.

Table 9. **Efficacy of Phyzyme™ XP 5000G on P content in plasma of piglets (0-3 weeks)**

	Positive control	Phyzyme™ XP (FTU kg^{-1})		
		0	500	1000
Initial	4.21	4.02	4.05	3.95
Final ^{x}	3.45^{a}	1.43^{c}	2.04^{b}	2.34^{b}

$^{a, b, c}$: Values in a row not sharing a common superscript differ significantly (P ≤ 0.05)
^{x}: Linear phytase effect (P < 0.05)

The duration of the study was considered too short to conclude on performance characteristics. However, the P in blood plasma data was considered relevant for the assessment of efficacy. The level of P in blood plasma was reduced in the negative control and significantly increased by the phytase supplementation (Table 9).

Third study[13]

A total of 180 piglets were distributed to six dietary treatments (12 pens of three piglets per treatment). The diets comprised a positive control (available P: 0.40% 0-21 days, 0.32% 21-42 days), a negative control (available P: 0.30% 0-21 days, 0.22% 21-42 days) and the negative control supplemented with 250, 500, 750 or 1000 FTU Phyzyme™ XP 5000L kg^{-1} diet. The study lasted 42 days with two phases 0-21 days and 21-42 days. Diets were presented in pelleted form. The intended enzyme activity in feed was confirmed by analysis. Two piglets were removed from the study.

[12] Technical Dossier/Section III/Ref. C19
[13] Technical Dossier/Section III/Ref. C20

Table 10. **Efficacy of Phyzyme™ XP 5000L on performance parameters of piglets (0-6 weeks) (12 x 3 piglets per treatment)**

Overall (6 weeks)	Positive control	Phyzyme™ XP (FTU kg^{-1})				
		0	250	500	750	1000
Daily weight gain, g day^{-1} ^{x}	587^{a}	552^{c}	571^{bc}	578^{abc}	591^{ab}	591^{abc}
Feed intake, g day^{-1}	885^{a}	822^{bc}	807^{c}	827^{bc}	860^{ab}	845^{bc}
Feed/gain, kg kg^{-1}	1.506^{a}	1.49^{ab}	1.41^{d}	1.44^{cd}	1.46^{bc}	1.43^{cd}
Final weight, kg ^{x}	31.8^{ab}	30.3^{c}	31.1^{abc}	31.4^{abc}	31.9^{a}	31.9^{ab}

$^{a, b, c}$: Values in a row not sharing a common superscript differ significantly (P≤0.05)
^{x}: Linear phytase effect (P<0.05)

Piglets fed with 750-1000 FTU Phyzyme XP kg^{-1} of feed had higher final body weight and higher growth rate than the negative control and were similar to positive control (Table 10). Piglets fed diets containing 250 and 500 FTU Phyzyme™ XP had significantly better feed conversion (P<0.05) than those fed with the negative control.

Fourth study[14]

A total of 192 piglets were distributed to six dietary treatments (eight pens of four piglets per treatment (four pens of barrows and four pens of gilts)). The diets were presented in mash form and comprised a positive control (available P: 0.32% 0–14 days, 0.27% 15-28 days), a negative control (available P: 0.22% 0-21 days, 0.17% 21-42 days) and the negative control supplemented with 250, 500, 750 or 1000 FTU Phyzyme™ XP 5000G kg^{-1} feed. The study lasted 28 days with two phases, 0-14 days and 15-28 days. At the end of the experiment one piglet from each pen was slaughtered and the right fibula was removed in order to measure the ash content. The intended enzyme activity in feed was confirmed by analysis.

Animal performance and percentage of ash in fibula are presented in Table 11.

Table 11. **Performance parameters and ash content of right fibula of piglets fed diets with various levels of Phyzyme™ XP (0-4 weeks)**

Overall	Positive control	Phyzyme™ XP (FTU kg^{-1})				
		0	250	500	750	1000
Daily weight gain, g day^{-1} ^{xy}	419	371	389	401	415	403
Feed intake, g day^{-1}	723	710	737	727	748	725
Gain/feed, kg kg^{-1} ^{xz}	0.577	0.526	0.525	0.552	0.551	0.553
Fibula ash, % ^{z}	45.3^{ab}	41.5^{c}	43.0^{bc}	43.4^{abc}	45.2^{ab}	45.8^{a}

$^{a, b, c}$: Means within a row with different letters are different (P<0.05)
^{x}: Comparison of Positive control *vs.* Negative control (P<0.05)
^{y}: Comparison of Negative control *vs.* 750 FTU kg^{-1} (P<0.05)
^{z}: Linear phytase effect (P<0.05)

There was no significant effect (P>0.05) of dietary treatment on daily feed intake. The growth rate of piglets was significantly higher with the supplementation with 750 FTU kg^{-1}. The percentage of ash in the fibula was significantly increased in piglets fed with 750 and 1000 FTU phytase kg^{-1}, reaching levels similar percentage to that of the positive control.

Conclusion of efficacy in piglets

Phyzyme™ XP 5000L/G at 500 FTU kg^{-1} feed or more shows benefits in animal performance and P digestibility and P in blood plasma. However, the minimum recommended level claimed by the applicant (250 FTU kg^{-1}) cannot be supported by the

[14] Technical Dossier/Section III/Ref. C21

data provided since this dose was only tested in two experiments and shown effective only in one. Therefore, the FEEDAP Panel concludes that the efficacy of Phyzyme™ XP 5000 L/G is demonstrated in weaned piglets at a minimum dose of 500 FTU kg^{-1}.

3.6. Efficacy for pigs for fattening

Four studies were presented in the dossier. The experimental design was appropriate in order to test the efficacy of Phyzyme™ XP 5000L/G in pigs for fattening. The major criteria used in these studies were the digestibility of P and other additional parameters such as dry matter and gross energy. P digestibility for the different studies is summarized in Table 12.

Table 12. **Phosphorus digestibility (%) of pigs for fattening fed with different levels of Phyzyme™ XP 5000L/G**

Study[1,2]	Positive control	Phyzyme™ XP (FTU kg^{-1})				
		0	250	500	750	1000
1[15]	47.6[b]	40.0[c]		54.5[a]		53.8[a]
2[16x]	37.4[a]	24.5[b]		47.5[a]	46.1[a]	45.8[a]
3[17x]	39.1[c]	30.5[d]		49.6[ab]	52.4[a]	46.0[bc]
4[18x]	70.7[a]	65.0[b]	71.0[a]	72.0[a]		

[1]: The solid form of the additive was used in trials 1 and 4, and the liquid form in trials 2 and 3

[2]: Available P for the positive/negative control diets: Trial 1, 0.27/0.17%; Trial 2, 0.23/0.13%; Trial 3, 0.23/0.13%; Trial 4, 0.22/0.10%

[a, b, c]: Values in a row not sharing a common superscript differ significantly (P≤0.05)

[x]: Linear phytase effect (P<0.05)

Conclusions of efficacy in pigs for fattening

Phyzyme™ XP 5000 L/G at 500 FTU kg^{-1} feed or more showed increased P digestibility in four studies. However the minimum recommended level claimed by the applicant (250 FTU kg^{-1}) cannot be supported by the data provided since it was only tested in one experiment. Therefore, the FEEDAP Panel concludes that the efficacy of Phyzyme™ XP 5000 L/G has been demonstrated in pigs for fattening at 500 FTU kg^{-1}.

3.7. Efficacy for sows

Three studies were presented in the dossier. The experimental design was appropriate in order to test the efficacy of both forms of Phyzyme™ XP 5000L/G in sows (gestating and lactating sows). The major criteria used in these studies were the digestibility of P and other additional parameters such as dry matter, crude protein, Ca and gross energy. P digestibility from the different studies is summarized in Table 13.

Conclusion of efficacy in sows

The FEEDAP Panel concludes that P digestibility is improved by the addition of Phyzyme™ XP 5000L/G at the minimum recommended level claimed by the applicant (500 FTU kg^{-1}) in gestating and lactating sows.

[15] Technical Dossier/Section III/Ref. C23

[16] Technical Dossier/Section III/Ref. C24

[17] Technical Dossier/Section III/Ref. C25

[18] Technical Dossier/Section III/Ref. C26

Table 13. **Phosphorus digestibility (%) of sows fed with Phyzyme™ XP 5000 L/G**

Study[1,2]	Sow Status	Positive control	Phyzyme™ XP (FTU kg^{-1})		
			0	500	1000
1[19]	Gestating [x]	12.94^{b}	19.27^{ab}	26.76^{a}	21.73^{ab}
	Lactating [x]	25.92^{bc}	23.06^{c}	38.10^{ab}	40.33^{a}
2[20]	Gestating	26.31^{a}	16.89^{b}	13.14^{b}	24.52^{a}
	Lactating	38.73^{c}	37.97^{c}	55.25^{b}	59.31^{a}
3[21]	Gestating at 8 weeks	14.88^{b}	9.33^{b}	29.05^{a}	17.84^{ab}
	Gestating at 14 weeks	24.91^{b}	22.94^{b}	50.14^{a}	33.58^{b}
	Lactating	30.02^{b}	32.57^{b}	42.10^{ab}	46.56^{a}

[1]: Solid form of the additive used in trials 1 and 3, and liquid form in trial 2.

[2]: Available P for the positive/negative control diets: Trial 1, 0.32/0.20%; Trial 2, 0.35/0.14-0.34%; Trial 3, 0.32/0.20%

[a, b, c]: Values in a row not sharing a common letters differ significantly (P<0.05)

[x]: Linear phytase effect (P<0.05)

4. Safety for the target species

4.1. Safety for chickens for fattening[22]

A tolerance test with a total of 126 male broilers was performed. The chicks were allocated into 18 pens with seven chicks per pen. The chickens for fattening were fed starter diets from 0-3 weeks and grower diets from 3-6 weeks. The diets comprised negative control, the negative control plus phytase at 500 FTU kg^{-1} diet and at 7500 FTU Phyzyme™ XP 5000L kg^{-1} (10X). The analysed levels in the treatment groups indicate that the phytase (10X) was mixed into the feed at the expected level. Birds were monitored daily for visible signs of distress.

Supplementation of the diets with 10X the maximum recommended dose of Phyzyme™ XP 5000L did not result in any adverse effects in animal performance.

4.2. Safety for laying hens[23]

A total of 128 laying hens (ISA Brown) at 50 weeks of age were distributed to four treatments, which comprised a positive control (available P: 0.3%), a negative control P-deficient diet (available P: 0.15%) and the negative control supplemented with 900 (1X) or 15000 FTU Phyzyme™ XP 5000G kg^{-1} diet (16X) (analytical results: 814 and 11278 FTU kg^{-1} diet). The experiment lasted 61 days.

Treatments showed no significant effects on feed intake, laying intensity, egg weight, feed conversion, body weight and mortality in the study.

This trial therefore found no evidence of any adverse effect of added Phyzyme™ XP 5000G at more than 12X the recommended level in the diet.

4.3. Safety for turkeys for fattening[24]

A total of 216 commercial male turkey poults were distributed into 24 pens at one day of age and reared to 16 weeks of age. Diets were formulated for the following age periods: 0-4 weeks, 4-8 weeks, 8-12 weeks and 12-16 weeks. Each diet was fed in crumble/pellet form to eight replicate pens (nine turkeys per pen). The diets comprised a positive control, a negative control and the negative control supplemented with 10000 FTU Phyzyme™ XP

[19] Technical Dossier/Section III/Ref. C28
[20] Technical Dossier/Section III/Ref. C29
[21] Technical Dossier/Section III/Ref. C30
[22] Technical Dossier/Section IV/Ref. D10
[23] Supplementary information. July 2006
[24] Technical Dossier/Section IV/Ref. D11

5000G kg^{-1} diet (10X maximum recommended dose). At the end of the trial, three birds per pen were euthanized and the middle toe of the right foot was collected. The intended enzyme activity in feed was confirmed by analysis.

No adverse effects were observed in animal performance, bone mineralization or mortality.

4.4. Safety for ducks for fattening[25]

A total of 288 male White Pekin ducks were assigned to 24 pens with 12 ducks per pen. The diets were fed in mash form and the enzyme was added in solid form to eight replicate pens. The diets comprised positive control, negative control and the negative control supplemented with 15000 FTU Phyzyme XP 5000G kg^{-1} diet (15X maximum recommended dose, confirmed by analysis). Diets were provided in two phases – as starter and grower pellets, with the starter diets fed from 0-14 days and the corresponding finisher diets from 15-42 days.

Supplementation of the diets with 15X the maximum recommended dose did not adversely affect performance, health or mortality of ducks.

4.5. Safety for piglets (weaned)[26]

A total of 96 piglets were distributed to three dietary treatments (eight pens of four piglets per treatment (four pens of barrows and four pens of gilts)). The diets were presented in mash form and comprised a positive control (available P: 0.32% 0-21 days, 0.27% 21-42 days), a negative control (available P: 0.22% 0-14 days, 0.17% 15-28 days), and the negative control supplemented with 10000 FTU Phyzyme™ XP 5000G kg^{-1} feed (10X maximum recommended dose). The study lasted 28 days with two phases 0-14 days and 15-28 days. The health status of the piglets was monitored by veterinary inspection. The intended enzyme activity in feed was confirmed by analysis.

No adverse effects were observed in animal performance or health status of the piglets. No deaths were recorded.

4.6. Safety for pigs for fattening[27]

A tolerance test was carried out using a total of 96 pigs (eight pens of four pigs per treatment). The treatments included a positive control (available P: 0.23%), a negative control (available P: 0.13%) and the negative control supplemented with 10000 FTU Phyzyme™ XP 5000G kg^{-1} (10X maximum recommended dose). The study lasted 42 days. Diets were presented in mash form. The pigs were weighed at the start of the study, the end of study and every 14 days between, in order to determine the growth and the feed conversion rate. The mean weights at the start and the end of study were 24 kg and 59 kg, respectively. The health status of pigs was checked daily and any medical treatment recorded. The intended enzyme activity in feed was confirmed by analysis.

No adverse effects were seen in the production parameters or health status of pigs due to the overdose of the additive.

4.7. Safety for sows[28]

A tolerance test was carried out during the whole gestation and lactation of 24 confirmed pregnant sows, which were distributed into one of three dietary treatments (eight sows per treatment): a positive control (available P: 0.35%), a negative control (available P: 0.14%),

[25] Technical Dossier/Section IV/Ref. D13
[26] Technical Dossier/Section IV/Ref. D14
[27] Technical Dossier/Section IV/Ref. D15
[28] Technical Dossier/Section IV/Ref. D16

or the negative control supplemented with 7500 FTU Phyzyme™ XP 5000L kg^{-1} feed (15X maximum recommended dose). Diets were presented in mash form. Sows were weighed on day 60 and 70 of gestation, on day 1 and 10 of lactation and at weaning. Piglets were weighed at birth and at weaning. The health status of all sows and piglets was regularly checked by veterinary inspection. The intended enzyme activity in feed was confirmed by analysis. Four sows were removed from the experiment and 40 piglets died or were culled because of low viability. None of these problems were associated with dietary treatment.

No effects were observed on the reproductive parameters of the sows fed with a nominal 10X overdose of Phyzyme™ XP 5000L.

4.8. Conclusions on safety for target species

The safety of Phyzyme XP 5000L/G has been demonstrated at the maximum recommended dose for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets, pigs for fattening and sows.

5. Safety for the consumer, user and environment

The previous assessment of consumer and environmental safety of Phyzyme™ XP is fully relevant to the products Phyzyme™ XP 5000L and Phyzyme™ XP 5000G and is thus not reconsidered here. User safety data is also generally relevant, to both products. However since the previous assessment was for the liquid product and Phyzyme™ XP 5000G is a granular product the possibility of respirability also needs to be considered. Particle size studies suggest that up to 3.5% of particles may be respirable thus precautions are required to avoid such exposure. Since all enzyme products are considered potential respiratory sensitisers precautions appropriate to that risk are always advised. These would be sufficient to protect the user against any risk arising from inhalation of particles.

6. Post market monitoring

No specific requirements for post-market monitoring are foreseen as necessary.

CONCLUSIONS

Studies made with either one of the Phyzyme XP 5000 products (L and G) are assumed to be relevant for both.

The FEEDAP Panel concludes that the efficacy of Phyzyme™ XP 5000L/G has been demonstrated in chickens for fattening at 500 FTU kg^{-1}, laying hens at 150 FTU kg^{-1}, turkeys for fattening at 250 FTU kg^{-1}, ducks for fattening at 250 FTU kg^{-1} and in piglets, pigs for fattening and sows at 500 FTU kg^{-1}.

The safety of Phyzyme XP 5000L/G has been demonstrated at the maximum recommended dose for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets, pigs for fattening and sows.

The safety for the consumer, user and the environment of the Phyzyme™ XP 5000L product has previously been assessed. Although no additional data are provided on the user safety of the Phyzyme™ XP 5000G the precautions normally adopted for enzyme products are considered sufficient to protect against any risks that might derive from inhalation of particles.

REFERENCES

EFSA (European Food Safety Authority). 2006. Opinion of the Scientific Panel on Additives and Products or Substances used in Animal and the Scientific Panel on Genetically Modified Organisms on the safety and efficacy of the enzymatic preparation Phyzyme™ XP (6-phytase) for use as feed additive for chickens for fattening. http://www.efsa.europa.eu/en/science/feedap/feedap_opinions/1468.html

DOCUMENTATION PROVIDED TO EFSA

1. PHYZYME™ XP 5000L and PHYZYME™ XP 5000G. Application for authorisation for use in multiple species. March 2005. Submitted by Danisco Animal Nutrition.
2. Additional documents requested by EFSA upon completeness check of the dossier (letter dated 13th July 2005). September 2005. Submitted by Danisco Animal Nutrition.
3. Supplementary information. July 2006. Submitted by Danisco Animal Nutrition.
4. CRL Evaluation Report on the Analytical Methods of Phyzyme™ XP 5000L/5000G (December 2005).
5. Comments from the Member States submitted via the EFSAnet.

SCIENTIFIC PANEL MEMBERS

Georges Bories, Paul Brantom, Joaquim Brufau de Barberà, Andrew Chesson, Pier Sandro Cocconcelli, Bogdan Debski, Noël Dierick, Anders Franklin, Jürgen Gropp, Ingrid Halle, Christer Hogstrand, Joop de Knecht, Lubomir Leng, Anne-Katrine Lundebye Haldorsen, Alberto Mantovani, Miklós Mézes, Carlo Nebbia, Walter Rambeck, Guido Rychen, Atte von Wright and Pieter Wester

ACKNOWLEDGEMENT

The Scientific Panel on Additives and Products or Substances used in Animal Feed wishes to thank Arturo Anadón for his contribution to this opinion.

APPENDIX

Executive Summary of the Evaluation Report of the Community Reference Laboratory Feed Additives Authorisation on the Method(s) of Analysis for Phyzyme™ XP 5000L/G

In the current application authorisation is sought for Phyzyme™XP under the category zootechnical additives, groups 4.a and 4.c, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Phyzyme™XP as a digestibility enhancer for chickens, turkeys, laying hens, ducks, piglets, pigs and sows and as a substance that favourably affects the environment by reducing the release of phosphorus into the environment from manure when raising these animals. The feed additive is available in liquid or dry form. The active substance of this feed additive is the enzyme 6-phytase. Its content is expressed in terms of the enzyme activity FTU, where 1 FTU is the amount of enzyme which liberates one µmol of inorganic phosphate from sodium phytate at pH 5.5 and 37°C in one minute. The feed additive which has a target activity of 5000 FTU/g is intended to be mixed into compound feedingstuffs to a final phytase activity of 150–1000 FTU/kg.

For the determination of the phytase activity in the *feed additive* a photometric method is proposed. The method is based on the principle that phytase releases inorganic phosphate from a substrate, which in the presence of a molybdate/vanadate reagent forms a yellow complex. The yellow complex is measured with a spectrometer and the inorganic phosphate is quantified with a phosphate standard curve.

For the determination of the phytase activity in *premixtures* the applicant proposes a photometric method, which is based on the same principle as mentioned above. The protocol contains an additional clean-up step by ion-exchange, in order to separate endogenous phosphate in the sample. The relative standard deviation for repeatability (RSD_r) was 7.3 %, obtained on the measurement of a test sample with a phytase activity of 358 FTU/g.

For the determination of the phytase activity in *feedingstuffs* two very similar photometric methods are proposed which are based on the same principle as mentioned above. For the determination of phytase activity in feedingstuffs containing the *liquid* form of Phyzyme™XP the method is identical to the method used for premixtures. In contrast, the analytical protocol for the analysis of feed samples containing the *dry* form of Phyzyme™XP requires the determination of endogenous phosphate in the feed samples prior to the enzymatic reaction. The amount of endogenous phosphate is taken into account when measuring the phytase activity in the corresponding feed sample. For both methods the applicant reported an RSD_r of 5.3 %, obtained on samples with an appropriate phytase activity of 668 FTU/kg. Also the reported limits of quantification (LOQ) of about 11 FTU/kg were identical for both methods. Though the applicant's methods follow well known principles for the determination of phytase activity in various matrices, the suitability of these methods for official control purpose cannot be confirmed, given the limited validation data.

Several other analytical methods for the determination of the phytase activity in feedingstuffs exist and have been validated in interlaboratory studies. These include a method proposed by the Association of German Agricultural Analytical and Research Institutes (VDLUFA, Germany), which resulted in a relative between-laboratory standard deviation for reproducibility (RSD_R) of about 12 % for feedingstuffs and an AOAC method which obtained RSD_R values ranging from 14.0 to 27.6 % for feedingstuffs. However, neither of these methods allow for determination of phytase activity from all commercially available phytase products used in feed.

In order to allow for the measurement of the phytase activity in feedingstuffs, regardless of the specific phytase product used, the European Association of Feed Additive Manufacturers (FEFANA) developed a modified method based on the same principle as the applicant's method. The FEFANA method has been validated in an interlaboratory study which was performed on feedingstuffs containing different phytase products including Phyzyme™XP. The obtained values for the between-laboratory RSD_R ranging from 5 to 14 % are considered acceptable for the intended purpose. Therefore, this method which is currently under evaluation to become a standard of the European Committee for Standardisation (CEN), is recommended for official control purposes.

No further testing or validation is required.

EXHIBIT 12

SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of Phyzyme XP (6-phytase) as a feed additive for minor poultry species[1]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Phyzyme XP is a feed additive that contains 6-phytase, produced by a genetically modified strain of *Schizosaccharomyces pombe* (ATCC 5233) and is authorised for use in feed for chickens and turkeys for fattening, laying hens, ducks, piglets, pigs for fattening and sows. The applicant requested for an extension of use of the additive to all minor poultry species. Safety for chickens and turkeys for fattening and for laying hens was previously established at 750, 1000 and 1500 U/kg feed, respectively. Given the margin of safety established for the major poultry species, a maximum dose of 1000 U/kg feed was considered safe for all minor poultry species. Since the mode of action of phytases can be reasonably assumed to be the same in all avian species the minimum effective dose authorised for fattening birds (250 U/kg feed) and for laying birds (150 U/kg feed) was considered efficacious for minor poultry species.

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KEY WORDS

Enzyme, zootechnical additive, digestibility enhancers, minor poultry species, phytase, safety, efficacy

[1] On request from European Commission, Question No EFSA-Q-2011-00835, adopted on 07 March 2012.

[2] Panel members: Gabriele Aquilina, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Joop de Knecht, Noël Albert Dierick, Mikolaj Antoni Gralak, Jürgen Gropp, Ingrid Halle, Christer Hogstrand, Reinhard Kroker, Lubomir Leng, Secundino López Puente, Anne-Katrine Lundebye Haldorsen, Alberto Mantovani, Giovanna Martelli, Miklós Mézes, Derek Renshaw, Maria Saarela, Kristen Sejrsen and Johannes Westendorf. Correspondence: FEEDAP@efsa.europa.eu

[3] Acknowledgement: The Panel wishes to thank the members of the Working Group on Enzymes, including Nuria Canibe, Friedrich Schöne and Pieter Wester, for the preparatory work on this scientific opinion.

Suggested citation: EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific Opinion on the safety and efficacy of Phyzyme XP (6-phytase) as a feed additive for minor poultry species. EFSA Journal 2012;10(3):2619. [9 pp.] doi:10.2903/j.efsa.2012.2619. Available online: www.efsa.europa.eu/efsajournal

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SUMMARY

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of Phyzyme XP (6-phytase) as a feed additive for all poultry minor species. Phyzyme XP is a feed additive that contains 6-phytase, produced by a genetically modified strain of *Schizosaccharomyces pombe* (ATCC 5233).

Safety for chickens and turkeys for fattening and for laying hens was previously established at 750, 1000 and 1500 U/kg feed, respectively. Given the margin of safety established for the major poultry species, a maximum dose of 1000 U/kg feed was considered safe for all minor poultry species.

Since the mode of action of phytases can be reasonably assumed to be the same in all avian species the minimum effective dose authorised for fattening birds (250 U/kg feed) and for laying birds (150 U/kg feed) was considered efficacious for minor poultry species.

TABLE OF CONTENTS

Abstract 1
Summary 2
Table of contents 3
Background 4
Terms of reference 4
Assessment 7
1. Introduction 7
2. Conditions of use 7
3. Evaluation of the analytical methods by the European Union Reference Laboratory (EURL) 7
4. Safety 7
5. Efficacy 7
6. Post-market monitoring 8
Conclusions 8
Documentation provided to EFSA 8
References 8
Appendix 9

BACKGROUND

Regulation (EC) No 1831/2003[4] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Danisco Ltd[5] for authorisation of the product Phyzyme XP, 6-phytase, when used as a feed additive for all minor poultry species (category: zootechnical additives; functional group: digestibility enhancers) under the conditions mentioned in Table 1.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[6] According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 28 September 2011.

The additive Phyzyme XP is a preparation of 6-phytase produced by a genetically modified strain of *Schizosaccharomyces pombe* (ATCC 5233) available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). This product is authorised for use in chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows.[7, 8]

EFSA issued two opinions which considered the safety and efficacy of the enzyme preparation when used in chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows, including the assessment of the safety for the consumer, the user and the environment, as well as the safety aspects of the genetic modification (EFSA, 2006a and 2006b). A third opinion was adopted regarding the use of a two-times concentrated formulation of the product in 2008 (EFSA, 2008).

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animal(s), consumer, user and the environment and the efficacy of the product Phyzyme XP (6-phytase), when used under the conditions described in Table 1.

[4] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[5] Danisco Ltd, PO Box 777, SN8 1 XN, Marlborough, Wiltshire, United Kingdom.
[6] EFSA Dossier reference: FAD-2011-0015.
[7] Commission Regulation (EC) No 785/2007 of 4 July 2007 concerning the authorisation of 6-phytase EC 3.1.3.26 (Phyzyme XP 5000G Phyzyme XP 5000L) as a feed additive. OJ L 175, 25.07.2007, p.4.
[8] Commission Regulation (EC) No 379/2009 of 8 May 2009 concerning the authorisation of a new use of 6-phytase EC 3.1.3.26 as a feed additive for chickens for fattening, turkeys for fattening, laying hens, ducks for fattening, piglets (weaned), pigs for fattening and sows (holder of the authorisation Danisco Animal Nutrition, Legal Entity Danisco (UK) Limited. OJ L 116, 09.05.2009, p.6.

Table 1: Description and conditions of use of the additive as proposed by the applicant

Additive	6-phytase			
Registration number/EC No/No (if appropriate)	4a1640			
Category(-ies) of additive	Zootechnical additive			
Functional group(s) of additive	Digestibility enhancer			
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)	
Preparation of 6-phytase (EC 3.1.3.26) produced by Schizosaccharomyces pombe (ATCC SD 5233) PHYZYME XP 5000 L and PHYZYME XP 5000 G: 6-phytase: 5000 U/g PHYZYME XP 10000 L and PHYZYME XP 10000 TPT: 6-phytase: 10000 U/g	N/A	Guaranteed minimum activity of 6-phytase: 5000 U/g Guaranteed minimum activity of 6-phytase: 10000 U/g	1 unit is the amount of enzyme which liberates one micromole of inorganic phosphate per minute from a sodium phytate substrate at pH 5.5 and 37ºC	
Trade name (if appropriate)	PHYZYME XP 5000 L PHYZYME XP 5000 G PHYZYME XP 10000 L PHYZYME XP 10000 TPT			
Name of the holder of authorisation (if appropriate)	Dansico Animal Nutrition* *legal entity Danisco (UK) Limited			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg or Units of activity or CFU kg^{-1} of complete feedingstuffs (select what applicable)		
All other laying birds e.g ducks, goose pheasant	-	6-phytase: 150U	6-phytase: 1000U	-
All other fattening birds e.g ducks, goose, pheasant		6-phytase: 250 U	6-phytase: 1000U	
Other provisions and additional requirements for the labelling				

Specific conditions or restrictions for use (if appropriate)	In the directions for use of the additive, indicate the storage temperature, storage life and stability to pelleting. For use in compound feed containing more than 0.23 % phytin bound phosphorus.
Specific conditions or restrictions for handling (if appropriate)	Harmful: May cause sensitization by inhalation. Do not breathe spray. Avoid contact with skin. Wear suitable protective clothing and gloves. PHYZYME XP 5000 G and PHYZYME XP 10000 TPT Harmful: May cause sensitization by inhalation. Do not breathe dust. Avoid contact with skin. Wear suitable protective clothing and gloves.
Post-market monitoring (if appropriate)	All batches of PHYZYME XP 5000 L, PHYZYME XP 5000 G, PHYZYME XP 10000 L and PHYZYME XP 10000 TPT are shipped in closed containers and the label information includes the name and address of the producer, a batch number and a bar code. All customers are supplied with copies of the Material Safety Datasheet for the product that includes an emergency contact number. This enables any user to contact the company and provide batch specific information on the use of a product. In addition batch traceability and complaints systems are in place so as to enable rapid investigation and resolution of any negative reports of usage or complaints. The traceability system ensures that any person to whom we have supplied the product can be rapidly identified
Specific conditions for use in complementary feedingstuffs (if appropriate)	Not applicable

Maximum Residue Limit (MRL) (if appropriate)			
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues

ASSESSMENT

1. Introduction

The additive Phyzyme XP is a preparation of 6-phytase produced by a genetically modified strain of *Schizosaccharomyces pombe* (ATCC 5233) which is available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). This product is authorised for use in chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows.[9] The applicant is now asking for an extension of use of this product as a zootechnical additive for all minor poultry species.

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) considers that the safety aspects other than for the new target species are covered in the previous opinions and would not be affected by the requested extension of use. Therefore, the present opinion focuses on the safety and efficacy of this enzyme preparation for the new target species.

2. Conditions of use

The additive is intended for use in feed for minor poultry species for fattening at a minimum recommended dose of 250 U per kg feed and in feed for minor poultry species for laying at a minimum recommended dose of 150 U per kg feed. In all cases the maximum recommended dose is 1000 U per kg feed.

3. Evaluation of the analytical methods by the European Union Reference Laboratory (EURL)

EFSA has verified the EURL report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the EURL report can be found in the Appendix.

4. Safety

The safety of the product was previously established in chickens and turkeys for fattening and laying hens at a maximum dose of 750, 1000 and 1500 U/kg feed, respectively. The studies demonstrated the tolerance when the additive was added at 10-fold dose (EFSA, 2006b). Safety was also established in ducks at a dose of 1000 U/kg (EFSA, 2006b).

In the view of the FEEDAP Panel and given the margin of safety established for the major poultry species the conclusions can be extrapolated to all minor poultry species at the dose of 1000 U/kg.

5. Efficacy

The efficacy of Phyzyme XP was previously established in chickens and turkeys for fattening and laying hens (EFSA, 2006a and 2006b). The efficacy was demonstrated at 500, 250 and 150 U/kg feed in chickens for fattening, turkeys for fattening and laying hens, respectively.

Since the mode of action of phytases can be reasonably assumed to be the same in all avian species the conclusions on efficacy for major avian species can be extrapolated to include all minor avian species at the following corresponding doses:

- 250 U per kg feed for fattening birds
- 150 U per kg feed for laying birds

[9] OJ L 175, 25.07.2007, p.4 and OJ L 116, 09.05.2009, p.6.

6. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[10] and Good Manufacturing Practice.

CONCLUSIONS

Phyzyme XP is safe at the maximum dose of 1000 U/kg feed for all minor poultry species.

Since the mode of action of phytases can be reasonably assumed to be the same in all avian species the minimum effective dose authorised for fattening birds (250 U/kg feed) and for laying birds (150 U/kg feed) is considered efficacious for minor avian species.

DOCUMENTATION PROVIDED TO EFSA

1. Phyzyme XP. March 2011. Technical dossier submitted by Danisco Ltd.
2. Phyzyme XP. December 2011. Supplementary information submitted by Danisco Ltd.
3. Evaluation report of the European Union Reference Laboratory for Feed Additives on the methods(s) of analysis for Phyzyme XP.

REFERENCES

EFSA (European Food Safety Authority), 2006a. Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed and the Scientific Panel on Genetically Modified Organisms on the safety and efficacy of Phyzyme XP 5000 (G/L), 6-phytase) as a feed additive for chickens for fattening. The EFSA Journal (2006) 350, 1-14.

EFSA (European Food Safety Authority), 2006b. Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed and the Scientific Panel on Genetically Modified Organisms on the safety and efficacy of Phyzyme XP 5000 (G/L), 6-phytase) as a feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows. The EFSA Journal (2006) 404, 1-20.

EFSA (European Food Safety Authority), 2008. Safety and efficacy of Phyzyme XP 10000 (TPT/L), 6-phytase as a feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows. The EFSA Journal (2008) 915, 1-10.

[10] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

APPENDIX

Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Phyzyme XP[11]

In the current application authorisation is sought under article 4(1) for PHYZYME, under the category/functional group 4(a) "zootechnical additive"/"digestibility enhancers", according to the classification system of Annex 1 of Regulation (EC) No 1831/2003. According to the Applicant, the feed additive contains 6-phytase (EC 3.1.3.26) as active substance produced by the strain Schizosaccharomyces pombe (ATCC SD 5233). The product is intended to be marketed as different formulations: (i) liquid PHYZYME XP 5000 L and PHYZYME XP 10000 L; or (ii) solid PHYZYME XP 5000 G and PHYZYME XP 10000 TPT, with a guaranteed minimum activity of 5000 and 10000 U 6-phytase/g, respectively. The activity of 6-phytase is expressed in phytase units (U), where 1 U is defined as the amount of enzyme which hydrolyzes the phytate substrate producing 1 µmole of phosphate per minute at pH 5.5 and 37°C.

Specifically, authorisation is sought for the use of PHYZYME for all minor poultry species. The feed additive is intended to be incorporated into premixtures and/or complete feedingstuffs with an activity ranging from 150 to 1000 U 6-phytase/kg.

For the quantification of 6-phytase in the feed additive, the Applicant submitted a single-laboratory validated and further verified method - similar to the ring-trail validated EN ISO 30024 method for complete feedingstuffs - based on colour formation of phosphorous produced at pH 5.5 and 37 oC with a molybdate-vanadate reagent. The following performance characteristics were derived from the validation and the verification studies:

- a relative standard deviation for repeatability (RSDr) of 3.7 %;
- a relative standard deviation for intermediate precision (RSDip) ranging from 4.2 to 5.2 %; and
- a recovery rate (RRec) ranging from 101 to 102 %.

Based on the satisfactory performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric method submitted by the Applicant for the determination of 6-phytase in the feed additive.

For the quantification of 6-phytase in the premixtures and feedingstuffs, the Applicant proposed a single-laboratory validated and further verified method - similar to the above mentioned EN ISO 30024 method for feedingstuffs. For the premixtures, the sample is diluted with heat treated whole grain wheat flour. The performance characteristics derived from the validation and the verification studies are consistent with those published for the ring-trial validated EN ISO 30024 method, using the same analytical principle.

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated colorimetric method (EN ISO 30024) for the determination of 6-phytase in premixtures (after dilution with heat-treated whole grain flour) and feedingstuffs.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

[11] The full report is available on the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-2011-0015.pdf

EXHIBIT 13

European Food Safety Authority

The EFSA Journal (2008) 915, 1-10

Safety and efficacy of Phyzyme XP 10000 (TPT/L), 6-phytase, as feed additive for chickens for fattening, laying hens, ducks for fattening, turkeys for fattening, piglets (weaned), pigs for fattening and sows[1]

Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed

(Question No EFSA-Q-2008-272)

Adopted on 10 December 2008

FEEDAP PANEL MEMBERS

Georges Bories, Paul Brantom, Joaquim Brufau de Barberà, Andrew Chesson, Pier Sandro Cocconcelli, Bogdan Debski, Noël Dierick, Jürgen Gropp, Ingrid Halle, Christer Hogstrand, Joop de Knecht, Lubomir Leng, Sven Lindgren, Anne-Katrine Lundebye Haldorsen, Alberto Mantovani, Miklós Mézes, Carlo Nebbia, Walter Rambeck, Guido Rychen, Atte von Wright and Pieter Wester

SUMMARY

Following a request from the European Commission, the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the safety and efficacy of Phyzyme XP 10000 TPT/ L (6-phytase; EC 3.1.3.26) as feed additive for chickens for fattening, laying hens, ducks for fattening, turkeys for fattening, piglets (weaned), pigs for fattening and sows.

The product is a reformulation of an existing product (Phyzyme XP 5000) approved for all of the above species and containing double the concentration of phytase. It is intended to provide the same feed concentrations of phytase as the previous product by a lower rate of incorporation.

The FEEDAP Panel considers that the conclusions on safety and efficacy for Phyzyme XP 5000 apply fully to the new formulation given that the proposed doses are unchanged. The FEEDAP Panel therefore concludes that Phyzyme XP 10000 is effective in the target species at the phytase levels currently approved and is safe for the target species, consumer, user and the environment.

Key words: zootechnical additive, digestibility enhancer, substance favourably affecting the environment, phytase, chickens for fattening, turkeys for fattening, laying hens, ducks for fattening, piglets, pigs for fattening, sows, safety, efficacy

[1] For citation purposes: Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on a request from the European Commission on the safety and efficacy of Phyzyme XP 10000 (TPT/L), 6-phytase, as feed additive for chickens for fattening, laying hens, ducks for fattening, turkeys for fattening, piglets (weaned), pigs for fattening and sows. *The EFSA Journal* (2008) 915, 1-10.

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TABLE OF CONTENTS

FEEDAP Panel Members 1
Summary 1
Table of Contents 2
Background 3
Terms of reference 3
Acknowledgements 3
Assessment 6
1. Introduction 6
2. Characterisation of the product 6
2.1. Stability and homogeneity 7
2.2. Conditions of use 8
2.3. Evaluation of the analytical methods by the Community Reference Laboratory (CRL) 8
3. Efficacy 8
4. Safety 8
5. Post-market monitoring 9
Conclusions 9
Final remark 9
References 9
Documentation provided to EFSA 9

BACKGROUND

Regulation (EC) No 1831/2003[2] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from the company Danisco Animal Nutrition[3] for authorisation of the product Phyzyme XP 10000 (TPT/L) (6-phytase, EC 3.1.3.26), to be used as a feed additive for chickens for fattening, laying hens, ducks for fattening, turkeys for fattening, piglets (weaned), pigs for fattening and sows (category: Zootechnical additives; functional groups: digestibility enhancer, and substance which favourably affects the environment) under the conditions mentioned in Table 1.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[4] According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 1 August 2008.

The additive Phyzyme® XP 10000 (TPT/L) is a preparation of 6-phytase (EC 3.1.3.26) produced by the genetically modified micro-organism *Schizosaccharomyces pombe* (ATCC 5233). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme® XP 5000 (4a1640). The FEEDAP and GMO Panels of EFSA delivered an opinion on the safety and efficacy of Phyzyme® XP 5000 (L) for chickens for fattening (EFSA, 2006a). The FEEDAP Panel also issued an opinion on the safety and efficacy of Phyzyme® 5000 (L/G) as feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows (EFSA, 2006b). The product Phyzyme® 5000 XP (L/G), is authorised as a zootechnical additive under the functional group of digestibility enhancers for use in chickens and turkeys for fattening, laying hens, ducks for fattening, piglets (weaned), pigs for fattening, and sows (authorisation until 25 July 2017).

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the efficacy and the safety for the target animal(s), user and consumer and the environment of the product Phyzyme® XP 10000 (TPT/L), 6-phytase, when used under the conditions described in Table 1.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank Friedrich Schöne for his contribution to the preparation of this opinion.

[2] OJ L 268, 18.10.2003, p.29
[3] Danisco Animal Nutrition, Danisco (UK) Limited, PO Box 777, Marlborough, Wiltshire, SN8 1XN, United Kingdom.
[4] Dossier reference: FAD-2008-0003

Table 1. **Register entry as proposed by the applicant**

Additive	PHYZYME XP
Registration number/EC No/No (if appropriate)	EC 3.1.3.26
Category of additive	Zootechnical Additive
Functional group of additive	Digestibility enhancer, substance that favourably affects the environment

Description			
Composition, description	**Chemical formula**	**Purity criteria (if appropriate)**	**Method of analysis (if appropriate)**
Preparation of 6-phytase (EC 3.1.3.26) produced by *Schizosaccharomyces pombe* (ATCC 5233) with a minimum activity of: Liquid form: 10000 U g^{-1} Solid form: 10000 U g^{-1}	N/A	Guaranteed minimum activity of 10000 U g^{-1}	1 FTU is the amount of enzyme which liberates one micromole of inorganic phosphate per minute from a sodium phytate substrate at pH 5.5 and 37 º C

Trade name (if appropriate)	PHYZYME XP 10000 L and PHYZYME XP 10000 TPT
Name of the holder of authorisation (if appropriate)	Danisco Animal Nutrition* *legal entity Danisco (UK) Limited

Conditions of use				
Species or category of animal	**Maximum Age**	**Minimum content**	**Maximum content**	**Withdrawal period (if appropriate)**
		Units of activity kg^{-1} of complete feedingstuffs		
Chickens for fattening	-	250 FTU	750 FTU	-
Turkeys for fattening	-	250 FTU	1000 FTU	-
Laying hens	-	150 FTU	900 FTU	-
Ducks for fattening	-	250 FTU	1000 FTU	-
Piglets (weaned)	-	250 FTU	1000 FTU	-
Pigs for fattening	-	250 FTU	1000 FTU	-
Sows	-	500 FTU	500 FTU	-

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	In the directions for use of the additive, indicate the storage temperature, storage life and stability to pelleting. For use in compound feed containing more than 0.23% phytin bound phosphorus.
Specific conditions or restrictions for handling (if appropriate)	Harmful. May cause sensitization by inhalation. Do not breathe dust (or spray). Avoid contact with skin. Wear suitable protective clothing and globes.
Post market monitoring (if appropriate)	All batches of Phyzyme XP are shipped in closed containers and the label information includes the name and address of the producer, a batch number and a bar code. All customers are supplied with copies

	of the Material Safety Datasheet for the product that includes an emergency contact number. This enables any user to contact the company and provide batch specific information on the use of a product. In addition batch traceability and complaints systems are in place so as to enable rapid investigation and resolution of any negative reports of usage or complaints. The traceability system ensures that any person to whom we have supplied the product can be rapidly identified.
Specific conditions for use in complementary feedingstuffs (if appropriate)	Not applicable

Maximum Residue Limit (MRL) (if appropriate)			
Marker residue	**Species or category of animal**	**Target tissue(s) or food products**	**Maximum content in tissues**

ASSESSMENT

1. Introduction

The additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase (EC 3.1.3.26) produced by the genetically modified micro-organism *Schizosaccharomyces pombe* (ATCC 5233).

The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated form of the authorised product Phyzyme XP 5000L/G (4a1640). The FEEDAP and GMO Panels of EFSA delivered an opinion on the safety and efficacy of Phyzyme XP 5000 (L) for chickens for fattening (EFSA, 2006a). The FEEDAP Panel also issued an opinion on the safety and efficacy of the Phyzyme 5000 (L/G) as feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows (EFSA, 2006b); the product Phyzyme 5000 XP (L/G) is authorised for use in chickens and turkeys for fattening, laying hens, ducks for fattening, piglets (weaned), pigs for fattening, and sows (authorisation until 25 July 2017). The use levels of the enzyme in feed for each of the species remains the same as for the previous product.

2. Characterisation of the product

The product Phyzyme XP 10000 is presented in two different formulations, the liquid (L) form and the dry coated product (TPT), both formulations ensuring a minimum activity of 10000 FTU g^{-1}. Phyzyme XP 10000 liquid is a light brownish liquid preparation and the solid form is an off-white to light tan fine granular preparation. The composition of each form can be found in Tables 2 and 3, respectively. All the materials used in the preparation of the final products are of food grade quality.

Table 2. **Quantitative composition of Phyzyme XP 10000 L**

Component	Mean (%)	Range (%)
Water	78.9	-
Enzyme protein	1.0	0.6 – 2.0
Sorbitol	5.0	2.0 – 8.0
Sodium chloride	14.0	11.0 – 17.0
Sodium citrate	0.8	0.3 – 0.13
Sodium benzoate	0.2	0.15 – 0.25
Potassium sorbate	0.1	0.075 – 0.15

Phyzyme XP 10000 L has the same composition as Phyzyme 5000 L, the only difference being that the enzyme concentrate is doubled (1 % instead of 0.5 %) with regards to the 5000 L at the expense of water. The carriers/preservatives remain the same.

Table 3. **Quantitative composition of Phyzyme XP 10000 TPT**

Component	Mean (%)	Range (%)
Fermentation liquor	4	3 – 5
Enzyme protein	1.0	0.6 – 2.0
Sodium sulphate	80	64 – 96
Hydrated magnesium silicate	6	4.8 – 7.2
Starch	5	4 – 6
Polyvinyl alcohol	4	3.2 – 4.8

The composition of Phyzyme 10000 TPT is different from that of Phyzyme 5000 G in terms of carriers but all ingredients used are classified as food grade.

A study of the batch to batch variation (ten batches) of the enzymatic activity showed that the coefficient of variation is 3.3 % in the liquid form and 3.0 % in the solid form. The mean enzymatic activity found in the ten batches evaluated was 12564 and 12078 FTU g^{-1}, for the liquid and the solid formulations, respectively.

The liquid form of Phyzyme XP shows a specific gravity of 1.05 to 1.15 g mL^{-1} at 20 ºC, with a pH ranging from 4.4 to 4.8, and with a viscosity of 2.5 cp at 24º C. The solid form of Phyzyme XP has a bulk density of 1.35 kg L^{-1}, the 99.94 % of the particles have a particle size > 297 μm and it does not contain particles with a size below 150 μm.

The product is manufactured in accordance with chemical (heavy metals, Pb, Cd, Hg and As) and microbiological (total coliforms, *Salmonella, Escherichia coli*) purity criteria as specified by JECFA and FCC. The antimicrobial activity and mycotoxins content (sterigmatocystin, aflatoxin B1, ochratoxin A, zearalenone and T-2 toxin) meet JECFA specifications for food enzymes. Compliance with the limits was checked in ten batches of the enzyme concentrate.

2.1. Stability and homogeneity

The stability of the product, defined as phytase activity, at room temperature (20 ºC) was assessed in three batches of each form of Phyzyme XP 10000 for a period of 12 months. The recovery of the initial enzymatic activity obtained after 12 months for each formulation was 99 % for the liquid form and about 80 % for the solid form.

The stability of Phyzyme XP 10000 TPT was tested in a vitamin/mineral premix when added at a rate of 20 g kg^{-1} (200000 FTU kg^{-1}) and stored at room temperature (20 ºC) for six months. The recovery of the initial enzymatic activity after six months was of 76 %. The stability of Phyzyme XP 10000 TPT was also tested in mash feed (maize/soyabean meal) containing vitamin/mineral premix for six months. The enzyme was included at 100 mg kg^{-1} (1000 FTU kg^{-1}), and the mash feed was kept at room temperature (20 ºC). The recovery of the initial enzymatic activity after six months was 98 %. The stability of Phyzyme XP 10000 L when included in the feedingstuffs was not tested; however, it is not expected that the double concentration of the enzyme will modify the stability of the product in feedingstuffs. Phyzyme XP 5000 showed an 80 % of the activity remaining when added to feedingstuffs and stored for six months at 20 and 35 ºC.

Finally, the stability of Phyzyme XP 10000 TPT to pelleting was tested. A maize-based diet was supplemented with 500 mg Phyzyme XP 10000 kg^{-1} (5000 FTU kg^{-1}) and was conditioned for 30 seconds at 90 ºC or 95 ºC and pelleted. The percentage of recovery of the initial enzymatic activity was 99 % and 96 %, respectively. The stability of the enzyme after pelleting the feed (30 seconds at 90º C or 95º C) and storing it for six months at 20 ºC or 35 ºC was also tested when supplemented at 1000 FTU kg^{-1}. After six months at 20 ºC, the recovery of the enzyme activity was 123 %, and when stored at 35 ºC the recovery was 92 %.

The homogeneity of Phyzyme XP 10000 TPT was evaluated in a vitamin-mineral premixture when added at 20 g kg^{-1} (200000 FTU kg^{-1}), the coefficient of variation being 13 %. The coefficient of variation of the liquid form and the solid form when mixed in mash feed (maize/soybean meal) was tested (three batches) when added at 500 and 1000 FTU kg^{-1}, respectively. The values of the coefficient of variation were 7 % for the liquid and 18 % for the solid formulations; although they are high, they are indicated to be comparable to the expected CV of the assay method used.

2.2. Conditions of use

Phyzyme XP 10000 L/TPT is to be used in compound feed containing more than 0.23 % phytin bound P at the following inclusion rates (FTU kg^{-1}): at 250-750 in chickens for fattening; at 250-1000 in turkeys, ducks and pigs for fattening and weaned piglets; at 150-900 in laying hens and at 500 in sows. The use levels of the enzyme in feed for each of the species remains the same as for the previous product.

2.3. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA has verified the CRL report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the CRL report can be found in the Appendix.

3. Efficacy

The conditions of use of the product with a double concentration (Phyzyme XP 10000) have not been modified with respect to those proposed for Phyzyme XP 5000. Efficacy was previously assessed by the FEEDAP Panel (EFSA, 2006b) and it was concluded that the 'efficacy of Phyzyme XP 5000 L/G has been demonstrated in chickens for fattening at 500 FTU kg^{-1}, laying hens at 150 FTU kg^{-1}, turkeys for fattening at 250 FTU kg^{-1}, ducks for fattening at 250 FTU kg^{-1} and in piglets, pigs for fattening and sows at 500 FTU kg^{-1}.'

The FEEDAP Panel considers that the change in the formulation will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion.

Furthermore, the FEEDAP Panel would like to note that the data obtained in the efficacy trials showed that the use of Phyzyme XP permits an improvement in the digestibility of P when added to the diet, allowing the use of diets with a lower amount of inorganic P. This fact may in turn reduce the excretion of P by the animals to the benefit of the environment.

4. Safety

The conditions of use of the product when fed to the animals have not been modified with respect to those proposed for Phyzyme XP 5000. Therefore, the FEEDAP Panel does not see the need to reconsider the conclusions reached in the previous opinions with regards to safety for the target species and safety for the consumer.

In principle the differences between the original product and Phyzyme XP 10000 need to be considered regarding user safety. However, since the original assessment was based upon an unrefined product which is likely to maximise any adverse effects, the FEEDAP Panel considers that the change in formulation will not create any additional risk for the user which was not taken into account in the assessment of Phyzyme XP 5000.

The previous assessment of the environmental safety of Phyzyme XP (EFSA, 2006a) is fully relevant to the products being evaluated in the present opinion.

5. Post-market monitoring

No risks associated with the use of the product are foreseen. It is considered that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[5] and Good Manufacturing Practices.

CONCLUSIONS

The FEEDAP Panel considers that the conclusions on safety and efficacy for Phyzyme XP 5000 apply fully to the new formulation given that the proposed doses are unchanged. The FEEDAP Panel therefore concludes that Phyzyme XP 10000 is effective in the target species at the phytase levels currently approved and is safe for the target species, consumer, user and the environment.

FINAL REMARK

The current authorisation of Phyzyme XP 5000 L and G sets the minimum content of the product in chickens for fattening, piglets (weaned) and pigs for fattening at 250 FTU kg^{-1}; however, the efficacy was demonstrated in those species at a minimum dose of 500 FTU kg^{-1}.

REFERENCES

EFSA (European Food Safety Authority), 2006a. Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed and the Scientific Panel on Genetically Modified Organisms on the safety and efficacy of the enzymatic preparation Phyzyme XP (6-Phytase) for use as feed additive for chickens for fattening. <http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620769568.htm>

EFSA (European Food Safety Authority), 2006b. Opinion of the Panel on additives and products or substances used in animal feed (FEEDAP) on the safety and efficacy of the enzyme preparation Phyzyme™ XP 5000L and Phyzyme™ XP 5000G (6-phytase) as a feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening, piglets (weaned), pigs for fattening and sows in accordance with Regulation (EC) No 1831/2003. <http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620782208.htm>

DOCUMENTATION PROVIDED TO EFSA

1. Phyzyme XP Application for two-times concentrated liquid and dry product forms. January 2008. Submitted by Danisco Animal Nutrition.
2. Phyzyme XP Supplementary information. October 2008. Submitted by Danisco Animal Nutrition.
3. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods(s) of analysis for Phyzyme XP L/TPT.
4. Comments from Member States received through the ScienceNet.

[5] OJ L 35, 8.2.2005, p.1

APPENDIX

Executive Summary of the Evaluation Report of the Community Reference Laboratory for Feed Additives on the Method(s) of Analysis for Phyzyme XP L/TPT

In the current application authorisation is sought for Phyzyme XP 10000 TPT and Phyzyme XP 10000 L under the category zootechnical additives, groups 4.a and 4.c, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Phyzyme XP 10000 L and Phyzyme XP 10000 TPT as a digestibility enhancer and as a substance that favourably affects the environment for chickens for fattening, laying hens, ducks for fattening, turkeys for fattening, piglets, pigs for fattening and sows. The product is intended to be marketed as liquid (Phyzyme XP 10000 L) and as dry (Phyzyme XP 10000 TPT) form.

The active substance of Phyzyme XP 10000 TPT and Phyzyme XP 10000 L is 6-Phytase (EC 3.1.3.26) produced by S*chizosaccharomyces pombe* (ATCC 5233). The enzymatic activity is expressed in FTU, where 1 FTU is the amount of enzyme which liberates one µmol of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37°C. The liquid and the solid form of the product have an minimum enzymatic activity of 10000 FTU/g and the product is intended to be mixed into compound feedingstuffs to obtain a phytase activity range of 150 – 1000 FTU/kg depending on the target species or category of animal.

For the determination of the phytase activity in the *feed additive* a spectrophotometric method is proposed. The method is based on the principle that phytase releases inorganic phosphate from a sodium phytate substrate, which in the presence of a molybdate/vanadate reagent forms a yellow complex. The yellow complex is measured with a spectrophotometer and the inorganic phosphate is quantified against a phosphate standard curve. The applicant method was in-house validated, obtaining 7.7% for the percentage relative standard deviation for repeatability (RSD_r). The CRL considers this method suitable for the intended purpose.

For the determination of the phytase activity in *premixtures* the applicant proposes a method which is based on the dilution of the premixture sample into blank feed matrix and applying the corresponding method for the determination of the phytase activity in *feedingstuffs*. The CRL confirms the principle validity of such an approach. However, since a precise protocol of this method and corresponding validation data are missing, the suitability of the method for official control cannot be evaluated.

For the determination of the phytase in *feedingstuffs* a harmonised method is available, which is based on the same principle as the applicant's method for the determination of the phytase activity in the feed additive. The harmonised method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN) and has been validated in an interlaboratory study which was performed on *feedingstuffs* fortified with different phytase products including a feed additive that contained the specific enzyme of the present application (Gizzi et al., J. of AOAC International, 91, 259-267, (2008)). The obtained values of the precision for the various products could be pooled, obtaining 10% for the RSD_r and 12% for the relative standard deviation for reproducibility (RSD_R). Based on the acceptable method performance profile the CRL recommends this method for official control of 6-phytase in the *feedingstuffs*.

No further testing or validation is required.

EXHIBIT 14

DANISCO

First you add knowledge...

Certificate of Conformance

Date:	20 Jul 2015
Our ref. no.:	41785009 10
Your ref.	1079908

Material:	A140561083EU	Phyzyme XP 10000 TPT	
Customer material no.:	10003912		
Batch No.:	4882559945	Best before date:	19 Jun 2016
		Production date:	20 Jun 2015

We hereby declare that the above product conforms with the specifications stated in the Product Description PD 226386-3.

Production is based on controlled raw materials and the batch is manufactured on a fully automated production line, ensuring the right composition.

The product is produced by Danisco Animal Nutrition.

This certificate is generated automatically

Marjaana Veikkolainen

Quality Control Department

Genencor International B.V.
c/o Genencor International Oy
Orioninkatu 4
FI-10900 HANKO

DANISCO ANIMAL NUTRITION
info.animalnutrition@danisco.com
www.danisco.com

Valid from May 13, 2009

DANISCO
First you add knowledge

PRODUCT DESCRIPTION - PD 226386-1.0EN

Phyzyme® XP 10000 TPT

EC Registration No: 4a1640

Application

Phyzyme® XP 10000 TPT is a phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig diets.

Usage levels

Chickens for fattening: Use at a rate of 0.025 - 0.075 kg/tonne (0.0025-0.0075%) of complete feed as recommended by the Phycheck™ dose optimisation service, included either directly or via a premix

Laying hens: Use at a rate of 0.015 - 0.09 kg/tonne (0.0015-0.009%) of complete feed as recommended by the Phycheck™ dose optimisation service, included either directly or via a premix.

Turkeys for fattening: Use at a rate of 0.025 - 0 1 kg/tonne (0.0025-0 01%) of complete feed as recommended by the Phycheck™ dose optimisation service, included either directly or via a premix.

Ducks for fattening: Use at a rate of 0.025 - 0.1 kg/tonne (0.0025-0.01%) of complete feed as recommended by the Phycheck ™ dose optimisation service, included either directly or via a premix.

Piglets (weaned): Use at a rate of 0.025 - 0.1 kg/tonne (0.0025-0.01%) of complete feed as recommended by the Phycheck™ dose optimisation service, included either directly or via a premix.

Pigs for fattening: Use at a rate of 0.025 - 0.1 kg/tonne (0.0025-0.01%) of complete feed as recommended by the Phycheck™ dose optimisation service, included either directly or via a premix.

Sows: Use at a rate of 0.05 kg/tonne (0.005%) of complete feed included either directly or via a premix.

14 JAN 2015

Processing stability

For optimum bioefficacy, do not exceed conditioning and pelleting temperatures of 95°C (203°F)

Composition

Dried Schizosaccharomyces pombe fermentation product
Sodium sulphate
Poly-vinyl alcohol (PVA)
Starch
Hydrated magnesium silicate

Guaranteed minimum activity:

6-phytase (EC 3 1.3.26)	10000 FTU/g

Analysis methods detailing assay conditions are available upon request.

Properties

Phyzyme® XP 10000 TPT is supplied as an off-white to light tan, fine granular product as follows:

Typical particle size based on sieve fineness

Micrometers	Approx %
< 200	0 1
200 to 500	65
500 to 800	30
> 800	4.9

Particle size distribution studies by Method DIN 53734 indicate absence of particles less than 10 micrometers
Phyzyme® XP 10000 TPT is sieved to 1 2 mm prior to packaging

Bulk density	1350 kg/m³
Moisture content	Maximum 2 %

Physical/chemical specifications

Phyzyme® XP 10000 TPT is produced in accordance with FAO/WHO JECFA and FCC recommendations as follows.

Lead	< 5 ppm

Matters beyond the control of Danisco (UK) Limited (trading as Danisco Animal Nutrition) such as incorrect storage and use of the product, animal management, health and environment differences may cause variation in performance. Danisco (UK) Limited gives no expressed or implied warranty for individual results. Notwithstanding the disclaimer herein contained Danisco (UK) Limited's liability (if any) in respect of such matters shall under no circumstances exceed the purchase price of the product.

DANISCO

First you add knowledge...

PRODUCT DESCRIPTION - PD 226386-1.0EN

Phyzyme® XP 10000 TPT

EC Registration No: 4a1640

Microbiological specifications

Phyzyme® XP 10000 TPT is produced in accordance with FAO/WHO JECFA and FCC recommendations as follows:

Aflatoxins	Negative by test
Antibiotic activity	Negative by test
Salmonella/25g	Negative by test
E.coli/25g	Negative by test
Coliforms	< 30 CFUs/g

There are no producing micro-organisms in the final product None of the ingredients is of animal origin.

Storage

Store in dry conditions The constituent enzyme activities remain stable for at least 12 months when stored in original packaging at <20°C(<68°F) and 4 months when included in a vitamin/mineral premix and stored at <20°C(<68°F).

Packaging

Phyzyme® XP 10000 TPT is available in 25kg cartons with polyethylene inner bag and in 1000kg bulk bags.

Safety and handling

Provide for good ventilation and avoid dust formation It is recommended to use protective glasses, respiratory mask and gloves during handling. In case of accidental contact with skin or eyes, the only action needed is copious flushing with water. See the Material Safety Data Sheet for further information.

Additional information

For further information please contact:

Danisco Animal Nutrition
PO Box 777
Marlborough
Wiltshire
SN8 1XN
United Kingdom

Tel +44 (0) 1672 517777
Fax +44 (0) 1672 517778
www danisco.com/animalnutrition
info.animalnutrition@danisco.com

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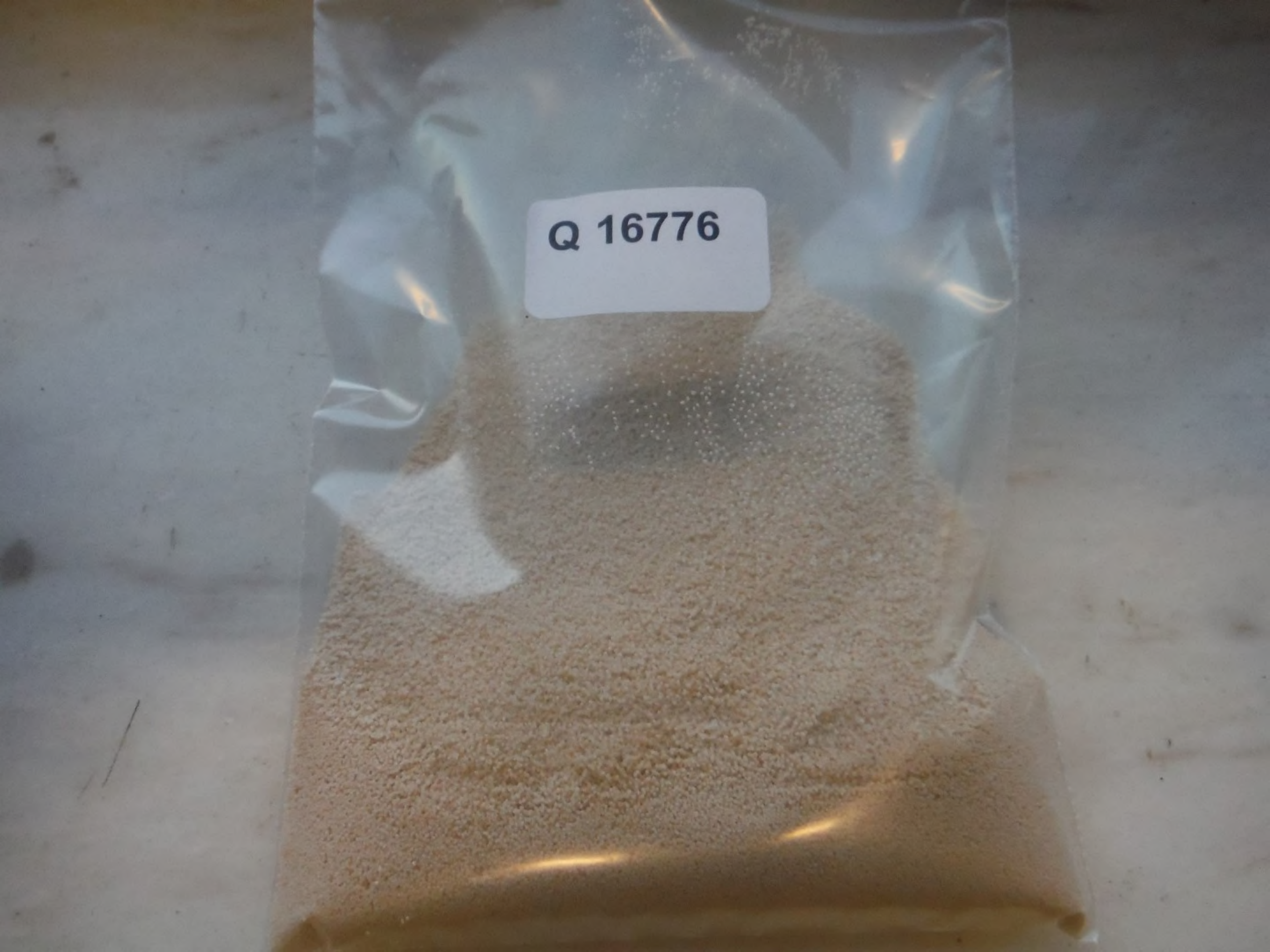
Q 16776

vitafor be.gium
Tel. +32 3 766 14 14
e-mail: vitafor@vitafor.com
www.vitafor.com
α BE2262

Product information
ARTICLE N°: 10-0277
MANUFACTURING DATE: 06/2015
NET WEIGHT: 20 KG
BATCH N°: 4882559945
EXPIRY DATE: 06/2016

DIGESTIBILITY ENHANCERS

COMPOSITION PER KG

4a1640 6-phytase (EC 3.1.3.26)	10 000 000
FTU	

Dosage in complete feedingstuff for:
- Chickens for fattening: 0.025-0.075 kg/ton
- Laying hens: 0.015-0.09 kg/ton
- Turkeys for fattening: 0.025-0.1 kg/ton
- Ducks for fattening: 0.025-0.1 kg/ton
- Piglets (weaned up to 35 kg): 0.025-0.1 kg/ton
- Pigs for fattening: 0.025-0.1 kg/ton
- Sows: 0.05 kg/ton
- For use in feed containing more than 0.23% phytin bound phosphorous
- Stability to pelleting 95 °C

Storage: cool, dark and dry

Hazard statements:
No classification

Precautionary statements:
No classification

Exclusively to be used in the preparation of animal feed.
Uniquement destiné pour la production d'aliments pour bétail.
Uitsluitend bestemd voor de bereiding van diervoeders.
Ausschließlich zur Herstellung von Mischfutter.

EXPORT

PHYZYME XP 10000 TPT

Productinformation

ARTICLE N°: 10-0277

MANUFACTURING DATE: 20/06/2015

BATCHN°: 4882559945

EXPIRY DATE: 19/06/2016

NET WEIGHT : 25 KG

ANCERS

10 000 000 FTU

EXHIBIT 15

Phyzyme XP for Poultry - Quick Reference Guide

phyzyme® XP

Application	A phytase feed enzyme designed specifically to improve the availability of phosphorus, calcium, energy and amino acids contained in cereal grains, oil seed meals and their by-products.
Economic benefits	• Lower feed costs • Improved environment
Recommendations for use	**Reformulation** For reduced feed costs and maintained bird growth and feed conversion. Reformulating the feed with Phyzyme XP provides opportunities to: • Reduce the phosphorus and calcium specification of the diet • Reduce the energy and protein/amino acid specification of the diet
Inclusion rate	Danisco's Phycheck™ optimisation service helps feed producers minimise feed costs when using Phyzyme XP. The service includes a unique software program to calculate customer-specific matrix values for phosphorus, calcium, amino acids and energy according to the phytate level of the diet, Phyzyme XP dose, bird age and economic inputs. For further advice on how to maximise your profit by reformulating your feeds with Phyzyme XP, please contact Danisco Animal Nutrition or your local distributor.
Storage stability	**Granular Phyzyme XP** 12 months when stored in original packaging at < 20°C (< 68°F). Store in dry conditions. 4 months when included in a vitamin/mineral premix and stored at < 20°C (< 68°F). Store in dry conditions. **Granular Phyzyme XP TPT** 12 months when stored in original packaging at < 20°C (< 68°F). Store in dry conditions. 4 months when included in a vitamin/mineral premix and stored at < 20°C (< 68°F). Store in dry conditions. **Liquid Phyzyme XP** 9 months when stored in original packaging at < 20°C (< 68°F). Do not freeze.
Processing stability	**Granular Phyzyme XP** For optimum bioefficacy, do not exceed conditioning and pelleting temperatures of 70°C (158°F). **Granular Phyzyme XP TPT** For optimum bioefficacy, do not exceed conditioning and pelleting temperatures of 95°C (203°F). **Liquid Phyzyme XP** For any processing conditions, particularly where conditioning and pelleting temperatures exceed 70°C (158°F). Apply after pelleting.
Packaging	**Granular Phyzyme XP** 25kg multi-wall paper bags or 1000kg bulk (tote) bags. **Granular Phyzyme XP TPT** 25kg box with heat sealed polyethylene liner. **Liquid Phyzyme XP** Containers of 200kg and 1000kg.

Related services	• Phycheck - provides customised recommendations for Phyzyme XP to maximise profit from phytase use • Engineering support - expert advice on the best choice of system for applying liquid Phyzyme XP products onto pelleted feed • Enzyme in-feed laboratory analysis - confirms that Phyzyme XP has been accurately included in the feed for maximum profit

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Phyzyme XP for Pigs - Quick Reference Guide

phyzyme® XP

Application	A phytase feed enzyme designed specifically to improve the availability of phosphorus, calcium, energy and amino acids contained in cereal grains, oil seed meals and their by-products.
Economic benefits	• Lower feed costs • Improved environment
Recommendations for use	**Reformulation** For reduced feed costs and maintained pig growth and feed conversion. Reformulating the feed with Phyzyme XP provides opportunities to: • Reduce the phosphorus and calcium specification of the diet • Reduce the energy and protein/amino acid specification of the diet
Inclusion rate	Danisco's Phycheck™ optimisation service helps feed producers minimise feed costs when using Phyzyme XP. The service includes a unique software program to calculate customer-specific matrix values for phosphorus, calcium, amino acids and energy according to Phyzyme XP dose and economic inputs. For further advice on how to maximise your profit by reformulating your feeds with Phyzyme XP, please contact Danisco Animal Nutrition or your local distributor.
Storage stability	**Granular Phyzyme XP** 12 months when stored in original packaging at < 20°C (< 68°F). Store in dry conditions. 4 months when included in a vitamin/mineral premix and stored at < 20°C (< 68°F). Store in dry conditions. **Granular Phyzyme XP TPT** 12 months when stored in original packaging at < 20°C (< 68°F). Store in dry conditions. 4 months when included in a vitamin/mineral premix and stored at < 20°C (< 68°F). Store in dry conditions. **Liquid Phyzyme XP** 9 months when stored in original packaging at < 20°C (< 68°F). Do not freeze.
Processing stability	**Granular Phyzyme XP** For optimum bioefficacy, do not exceed conditioning and pelleting temperatures of 70°C (158°F). **Granular Phyzyme XP TPT** For optimum bioefficacy, do not exceed conditioning and pelleting temperatures of 95°C (203°F). **Liquid Phyzyme XP** For any processing conditions, particularly where conditioning and pelleting temperatures exceed 70°C (158°F). Apply after pelleting.
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Related services	• Phycheck - provides customised recommendations for Phyzyme XP to maximise profit from phytase use • Engineering support - expert advice on the best choice of system for applying liquid Phyzyme XP products onto pelleted feed • Enzyme in-feed laboratory analysis - confirms that Phyzyme XP has been accurately included in the feed for maximum profit

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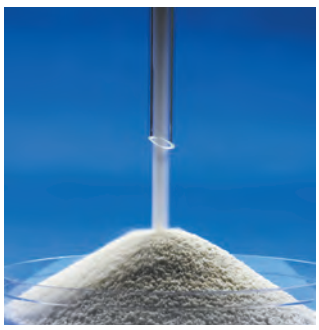
EXHIBIT 16

3 ways to achieve profitable phytase dosing

1. Select a highly bio-efficacious phytase



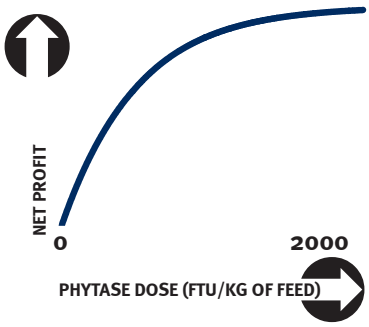
For over 20 years, the industry standard phytase dose has been 500 FTU/kg. Today, inorganic phosphorus costs more, phytase costs less and some phytases are more effective than others, yet the standard hasn't changed.

To achieve optimum phosphorus uptake, reduce the anti-nutrient effects of phytate and increase the availability of costly energy and amino acids today, you need to select:

- a phytase enzyme that is highly active at the low pH conditions prevailing in the animal's upper digestive tract
- the most appropriate dose for that phytase, supported by well–researched matrix values that vary according to animal species, diet variation and the age of the animal

Our highly bio-efficacious bacterial phytase products – Phyzyme® XP[1] and Axtra® PHY[1] – are supported by evidence-based matrix values that support flexible dosing and optimize profitability.

2. Use value-add services to optimize dose

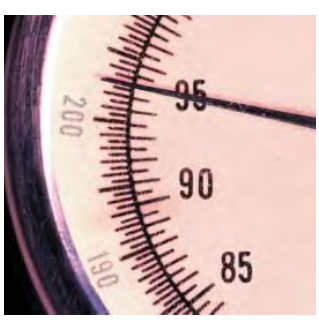


Our online Optimize Feed™ Service[2] is an easy-to-use tool enabling you to calculate the optimum dose of phytase needed to achieve the strongest performance benefits and cost savings. It uses accurate and well-researched matrix values based on substrate levels, animal species, diet variation and the age of the animal to maximize:

- uptake of phosphorus and reduce the need for costly inorganic phosphorus supplementation
- release of energy and amino acids by rapidly reducing the anti-nutritional effects of phytate, using extensive global data on raw material substrates

It also helps to establish the right dose of phytase to balance calcium and phosphorus levels, which is important for bone mineralization.

3. Make sure your phytase is heat stable

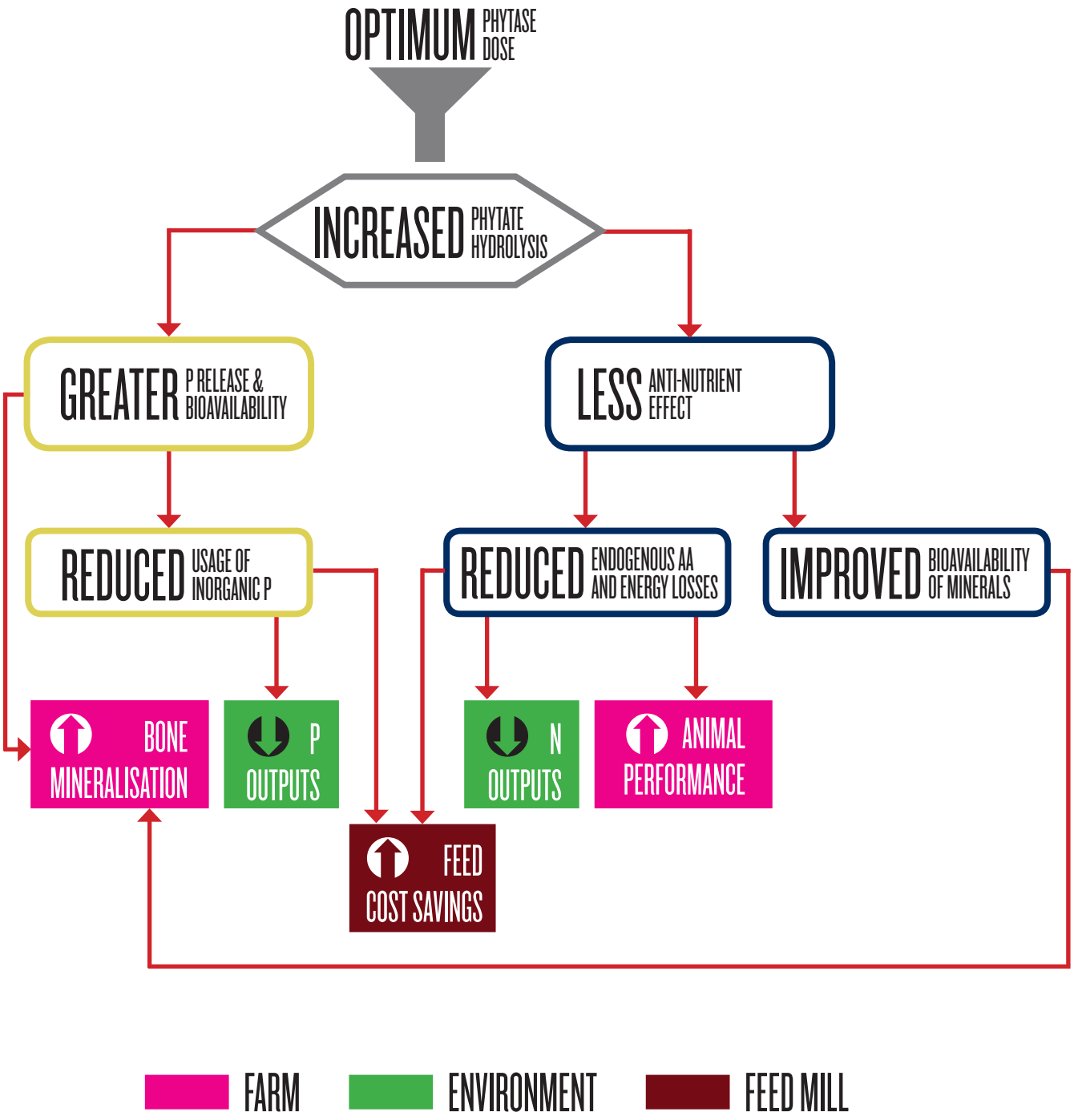


Significant loss of phytase activity during steam conditioning and subsequent pelleting of feed negatively impacts profitability. A phytase can be made more heat stable by using a well-researched coating to protect it.

Phyzyme® XP and Axtra® PHY incorporate unique TPT coating technology, which offers heat stability up to 95°C (203°F).[3] The TPT coating also allows rapid release of phytase in the upper part of the gut to achieve optimum bio-efficacy.

[1] Product availability is subject to national registrations [2] Available for customers only [3] For dry phytase pre-pelleting and liquid post-pelleting

Maximizing production value from phytase



Product form

Axtra® PHY and Phyzyme® XP are phytase feed enzymes specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in animal diets.

Axtra® PHY
Available as thermostable TPT and in a liquid.

Phyzyme® XP
Available as thermostable TPT, granular (on a wheat carrier) and in a liquid.

Packaging

Dry
Packed in 25 kg multi-wall polyethylene lined paper bags or carton with polyethylene inner bag and in 1000 kg bulk (tote) bags.

Liquid
Packed in 200 kg and 1000 kg containers.

More details on our Phyzyme® XP and Axtra® PHY phytase products are available at http://animalnutrition.dupont.com

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05/2014

EXHIBIT 17

Phyzyme XP TPT - unrivalled heat stability, superior performance

Phyzyme XP is a highly effective phytase feed enzyme. Trials have shown that Phyzyme XP offers superior value compared to leading competitor phytases. New Thermo Protection Technology (TPT) also enables Phyzyme XP TPT to be used in conditioning and pelleting processes which employ temperatures up to 95°C (203°F).

HEAT STABLE FOR ADDED CONFIDENCE

Animal feed is typically heat-treated and pelleted at temperatures up to 90°C (194°F). Enzymes are proteins, which are often inactivated if exposed to high temperatures. The Thermo Protection Technology used to produce Phyzyme XP TPT involves applying a specialised coating to the phytase. This coating protects the phytase from the high temperatures that occur during the feed manufacturing process and rapidly releases the phytase to deliver its effects in the bird's gut.

Benefit to the producer:

- Heat stable to 95°C (203°F) - allows feed producers to confidently use Phyzyme XP TPT in most pelleting and conditioning processes

In a large study at the Technological Institute, Kolding, Denmark, phytase activity was measured in feed which contained Phyzyme XP TPT and had been conditioned and pelleted at 90°C (194°F) or 95°C (203°F). The level of phytase activity retained in the pelleted feed was compared to the level of phytase activity in the corresponding mash feed before conditioning and pelleting.

PHYZYME XP TPT RETAINED VERY HIGH LEVELS OF PHYTASE ACTIVITY IN FEED CONDITIONED AND PELLETED AT 90°C (194°F) AND 95°C (203°F)

Phytase activity retained in heat treated pelleted feed compared to non heat treated mash feed

Pelleted feed

Processed at	90°C (194°F)	95°C (203°F)

Inlet steam pressure 2 bar (29 psi)
Conditioning time 30 seconds
6 tests

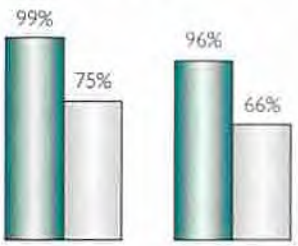
Trials conducted at the Technological Institute, Kolding, Denmark

17

UNRIVALLED HEAT STABILITY

In a series of trials conducted at the Technological Institute, Kolding, Denmark, Phyzyme XP TPT is shown to be more heat stable than other leading phytase products.

Phytase activity retained in heat treated pelleted feed compared to non heat treated mash feed



Processed at 90°C (194°F) 95°C (203°F)

Inlet steam pressure 2 bar (29 psi) Conditioning time 30 seconds
Phyzyme XP TPT: 6 tests Competitor R: 13 tests

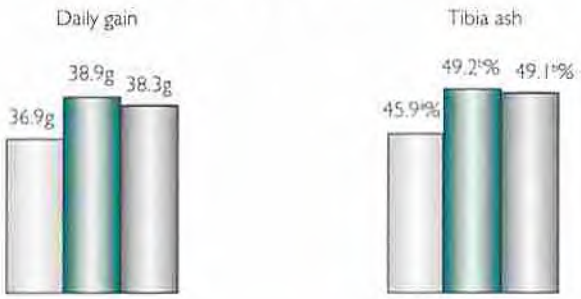
Phyzyme XP TPT Competitor R

Trials conducted at the Technological Institute, Kolding, Denmark

EFFECTIVE IN THE BIRD

The specialised coating protects Phyzyme XP TPT from the high temperatures experienced during the feed manufacturing process. The coating rapidly releases the phytase in the bird's gut to deliver unrivalled improvements in phosphorus, calcium, energy and amino acid digestibility.

Phyzyme XP TPT (coated) is as effective in the bird as Phyzyme XP (uncoated)



Average age 21 days
Reduced specification diet:
- 0.21% reduction in total phosphorus
- 0.11% reduction in total calcium

Trial conducted at the University of Manitoba, Canada *P<0.05

Reduced specification diet Reduced specification diet + Phyzyme XP (uncoated) Reduced specification diet + Phyzyme XP TPT (coated)

Related product card
Phyzyme XP inclusion rates, stability and packaging

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Biochemistry

“Enzymes are like tools designed by nature (or, Increasingly, by biotechnology) for a specific function. Our focus is on understanding enzyme performance and the industrial environments our products will be used in.

Our technical expertise is first to select the right enzymes to adapt for our customers’ needs. That could be improving the quality and shelf life of bread, for example, or producing biofuels from agricultural waste. We translate that required function into biochemical terms, i.e. what enzyme activity is needed.

Experience tells us which enzyme candidates might be robust enough to catalyse reaction in, for example, a high temperature oven or in a high or low pH environment.

Then we optimize the enzyme to perform its task, like a custom-designed tool, within the industrial systems of our customers.”

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Recovery

“The goal of enzyme recovery is to extract, concentrate, and purify as much enzyme as possible from the fermentation broth.

First, with fermentation and formulation, we develop and optimize the recovery process. We then scale up to production, making sure that stability, cost, yield and purity meet the formulation needs and the customer’s criteria.

Sometimes we have to develop a new recovery process. A good example is a new proprietary filtration technology for cell separation, which is now becoming a platform technology for our overall process.

The focus is always on the end product – that could be an enzyme that ‘stonewashes’ jeans, replacing high energy consuming processes. Or it might extend the shelf life of bread without additives, or save energy while doing the laundry or using the dishwasher.

Our goal is to develop enzymes that deliver significant environmental benefits.”

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Discovery

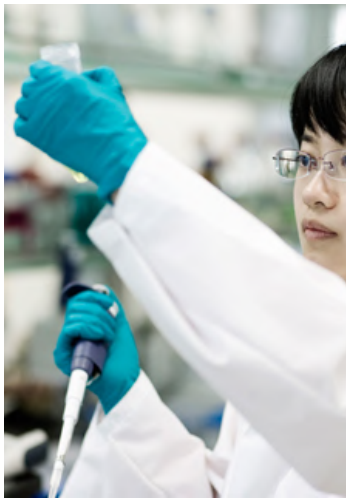
"New technologies are helping us harness the power of nature."

Discovery is the part of the continuous cycle where Genencor makes connections between the so-called ‘backbone molecules’ we discover and the ‘real-life’ usage we re-create in the lab.

If we find the results in our assays are encouraging, the enzyme would then be improved further in protein optimization.

Discovery is really about harnessing the power of nature. Every day, we find that we can sequence organisms that have never been sequenced before, thanks to new techniques of genome-mining we’ve developed.

Learn more about how our dedicated people contribute to this phase of our cycle, in their own words:



Genetics

Microbiology

Molecular Biology

Bioinfor-matics

Biochemical Characteriza-tion

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Formulation

“The purpose of formulation is to get the enzyme to the customer in a usable format.

We already know the enzyme performs well, based on application tests and molecule optimization. So we can now put it into a form that provides stability, improves the aesthetics or targets delivery.

Enzyme products may be exposed to harsh environments – such as high temperatures or pH values – in both storage and use. It is the formulation that helps protect the enzymes.

For example, enzymes are blended into powder detergents that contain a bleaching component. Both the enzyme and bleach are critical for the overall cleaning performance, but if the enzyme is unprotected the bleach can damage the enzyme during storage and its benefits may be lost.

By developing a solid granulated product that contains protective barriers, we can keep the enzyme and bleach separate during storage and optimize effective performance in the wash.

That’s pretty rewarding.”

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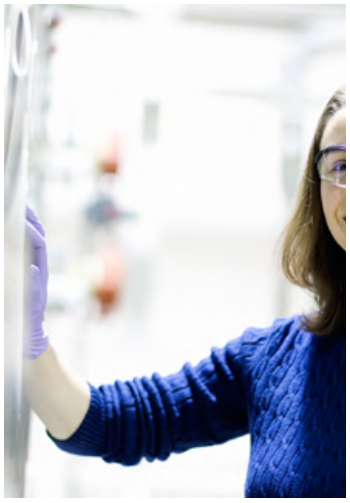
Production



"The closer we work with supply plants, the more smoothly the production transfer will go."

In this phase of the Innovation cycle, there is close collaboration with the production plant engineers to ensure that the transfer to large-scale production will be seamless – and as cost-effective as possible.

It starts with improving the organism that's going to express the enzyme – what we call the "cell factory". That includes predicting how it will behave during large scale production, to ensure optimum performance in our manufacturing plants. After strains are fermented and enzymes are produced, we then recover those and prepare them for formulation, ensuring stability in the customer's manufacturing process.

Insights gained throughout our Production processes are often fed back to Applications, and used to develop new game-changing biosolutions for our customers.

Learn more about how our dedicated people contribute to this phase of our cycle, in their own words:

Strain improvement

Cell Biology

Pathway Engineering

Bioanalytical chemistry

Fermentation

Recovery

Formulation

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Fermentation

“Fermentation really sits between R&D and production.

First, with both upstream (cell biology and strain improvement) and downstream (recovery and formulation) groups, we devise a strain and process where the molecule of interest is produced well. Our role is then to ensure we can produce huge quantities of stable, effective enzymes quickly and inexpensively enough to be commercially viable.

One of our core competencies is the ability to scale up from 10 millilitres to 3,000 litres right here in our laboratory. We then move to our fermentation plants, where the scale reaches as high as 360,000 litres.

It’s inspiring to see our re-engineered micro-organisms producing something that’s useful to people. A good example is the amylase we developed for the baking industry. This enzyme extends the shelf life of bread and other baking products. It also improves texture, reduces food waste, and is a far better solution than chemical additives.

It’s also very rewarding to see fresh bread containing our enzyme in the supermarket - only a year after we began working on it!”

“Process development ca
us from the petri dish to a
story fermentor.”

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Industry Applications Expertise

It's not enough to know how to build a better enzyme. Our Industry Applications experts use their deep knowledge of business to anticipate market needs and collaboratively develop solutions that will deliver the most value to our customers.

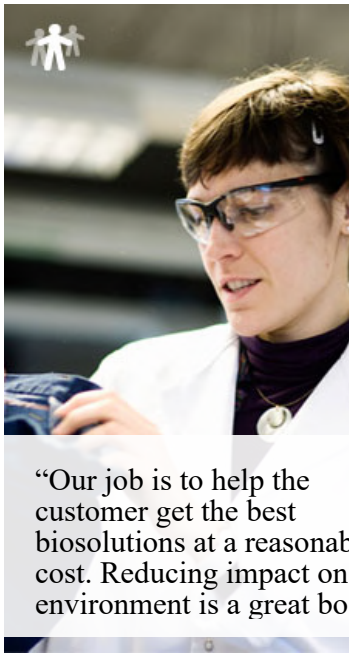
Focus on: Enzymatic Textile Processing

"We collaborate closely with the customer's technical team from the very beginning – translating their specific challenges into biochemical terms, to determine where and how we can add value.

In textile processing, that could be reducing operating costs. Or replacing the use of corrosive chemicals – or the use of silicon dust – to comply with safety legislation.

Often the need is to reduce consumption of water or energy. For example, we just launched a product for cotton 'de-fuzzing' that – combined with the dyeing step – enables a huge saving of precious water as well as production time.

The shorter production cycle and improved 'handle' of the end product offer our customer a marketable advantage to the time-sensitive fashion and retail markets. As a bonus, there's a significant reduction in environmental impact."



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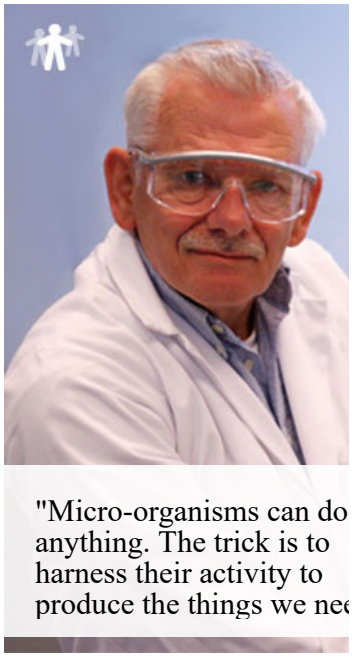
Microbiology

“Micro-organisms are endlessly fascinating; in nature, they can do anything. In fact, life on earth would be impossible without them.

The trick is to harness the capability of the highly efficient microbial proteins, and modify them to help produce the food, clothes, biofuels and animal feed we all rely on.

Genome mining can reveal a wide variety of interesting enzymes with industrial potential. We know how to pick the most interesting ones. Then we effectively design an artificial gene to place into a ‘screening host’ that will be very similar to the final production host.

We work very closely with our colleagues in production, so by the time the product is launched, the hosts are optimized to perform based on the needs of our customers’ production processes. Nature has no interest in washing machines. Fortunately, we know what it takes to optimize enzymes for cleaner clothes!”



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Model Systems

“The purpose of Model Systems is to enable us to assess product performance and substrate interaction in the lab.

We’re very close to the customer and their technical production requirements. So we aim to replicate their real world, large scale application – of, for example, Textile Processing or Animal Nutrition – in a small-scale test system.

That enables us to determine how the product would translate to the industrial environment, and the conditions our enzyme is going to have to perform in.

I like to think Model Systems is where ‘the rubber meets the road.’ It enables faster innovation, anchored in a customer-centric approach.

I’d say we’re innovating 5 times faster than a decade ago. Model Systems is an important contributor to that.”

“Determining product performance in the lab ge to market faster.”

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Protein Optimization

"Our specialist biotechnologists optimize the enzymes to get them to market faster and cheaper than ever before."

DuPont Industrial Biosciences has a long history of developing advanced biological techniques that enable us to leverage the diversity of enzymes found in nature.

Precise analysis of protein structure enables us to re-engineer enzymes to accelerate their effectiveness in our customers' applications.

Our biochemists can eliminate many candidates in the early stages following discovery. Gene selection and advanced screening technology then narrow down the immense numbers of potential enzymes.

That allows us to further reduce time to market, by focusing on only the fastest, most promising enzymes for application testing.

Learn more about how our dedicated people contribute to this phase of our cycle, in their own words:

Protein Engineering

High Throughput Screening

Protein Chemistry

Structural Biology

Enzymology

Related News

October 10, 2011

Genencor Scientist Wins Prestigious Enzyme Engineering Award

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Enzyme Substrate Interactions

“Industrial applications are a pretty tough environment for our enzyme systems. We have to make them very robust to perform and survive in such challenging conditions.

For example, our product might have to do its work inside a steam pipe in our customer's grain processing plant. It often has to survive wide temperature fluctuations during transportation and storage at commercial sites. If the substrate is a laundry stain, for example, our enzyme needs to bind with it effectively within detergent.

From experience, we know which part of the enzyme we can most effectively engineer to bind to the substrate. From our deep understanding of the biochemical nature of the substrate, we can accelerate that reaction.

But enzymes can still throw some curveballs. So we’re here to make sure we eliminate those before the product goes to market.”

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EXHIBIT 19

DUPONT MAJOR SUBSIDIARIES

Major Subsidiaries of E. I. du Pont de Nemours and Company	Operating Country
** Subsidiaries with >50 employees*	
Du Pont Luxembourg	Luxembourg
DuPont Nederland	Netherlands
DuPont Deutschland	Germany
DuPont Asturias, S.L.	Spain
DuPont De Nemours BVBA	Belgium
DU PONT DE NEMOURS SAS	France
DUP INTL OPERATIONS	Switzerland
DuPont Teijin Films UK	Great Britain
DuPont International SARL	Switzerland
DuPont Teijin Films	Luxembourg
DuPont (U.K.) Limited	Great Britain
DuPont Russia	Russia
DuPont UK Industrial	Great Britain
Du Pont Italiana	Italy
DUP SOLUTION (France)	France
DuPont Poland	Poland
DuPont Turkey Sanayi	Turkey
DuPont Iberica S.L.	Spain
DuPont Sverige AB	Sweden
DuPont Coord.Ctr. (Bel)	Belgium
DuPont Ukraine LLC	Ukraine
Solae Europe SA	Switzerland
Solae Belgium NV	Belgium
Solae do Brasil Industria e Comercio de Alimentos LTDA	Brazil
Danisco Argentina S.A.	Argentina
DuPont Argentina S.R.L.	Argentina
Solae do Brasil Holdings S/A	Brazil
DuPont Chile S.A.	Chile
DuPont de Colombia, S.A.	Colombia
DuPont S.A. de C.V.	Mexico
DuPont do Brasil S.A.	Brazil
DuPont Zheng Zhou Protein Company Limited	China
DuPont Shineway Luohe Food Company Limited	China
DuPont Shineway Luohe Protein Company Limited	China
Du Pont China Holding Company Ltd	China
E.I.Dupont India Private Limited	India
Du Pont Kabushiki Kaisha	Japan
DuPont Taiwan Ltd	Taiwan
E. I. DuPont Services Center India Private Limit	India
Du Pont Company (Singapore) Pte Ltd.	Singapore
DuPont (Korea) Inc.	Korea
DuPont Surface (Guangzhou) Co Ltd	China
PT DuPont Agricultural Products Indonesia	Indonesia
DuPont (Australia) Ltd.	Australia
Du Pont (Thailand) Ltd.	Thailand
Du Pont China Limited	Hong Kong
DuPont (Changshu) Fluoro Technology Co	China
DuPont Vietnam Ltd.	Vietnam
DuPont Malaysia Sdn Bhd	Malaysia
DuPont (China) Research Development Company Limited	China
Du Pont Far East Inc-Philippines	Philippines
Electronic Materials Du Pont Dongguan Ltd	China
Solae L.L.C.	USA
DuPont Electronics Microcircuits Industries, Ltd	USA

DuPont Agricultural Caribe Industries, Ltd.	USA
DuPont Authentication, Inc.	USA
DuPont Displays, Inc.	USA
DuPont Electronic Polymers LP	USA
EKC Technology, Inc.	USA
First Chemical Corporation	USA
Belco Technologies Corporation	USA
Coastal Training Technologies Corp.	USA
MECS Inc	USA
Sentinel Transportation, LLC	USA
E.I. du Pont Canada	Canada
DuPont Thetford (Granirex)	Canada
DuPont NHIB Denmark ApS	Denmark
Danisco USA Inc.	USA
Danisco US Inc.	USA
Danisco France SAS	France
Danisco Mexico S.A. de C.V.	Mexico
Danisco (China) Co. Ltd.	China
Danisco Deutschland GmbH	Germany
Genencor International OYGenen	Finland
Danisco Brasil Ltda	Brazil
Danisco Sweeteners Oy	Finland
Genencor (Wuxi)	China
Danisco (Zhangjiagang)	China
Danisco Malaysia Sdn.Bhd.	Malaysia
Danisco Sweeteners (Anyang) Co., LTD	China
Danisco Czech Republic	Czech Republic
Genencor International, B.V.	Netherlands
DuPont N&H Iberica SL	Spain
Danisco Chile S.A.	Chile
Danisco UK Ltd.	United Kingdon
Genencor International, BVBAGenen	Belgium
Danisco Argentina S.A.	Argentina
Danisco Slovakia s.r.o.	Slovakia
Danisco South Africa Pty	South Africa
Finnfeeds Finland Oy	Finland
Danisco (China) Holding	China
Danisco India Pvt. Ltd.	India
Danisco Landerneau S.A.S.	France
Danisco Singapore	Singapore
Danisco Austria GmbH	Austria
Beijing Lurou Berry Juice	China
Pioneer Hi-Bred International, Inc.	USA
Semillas Pioneer Chile	Chile
PHI Seeds Ltd.	India
Pioneer Hi-Bred Prod LP	Canada
Dunhuang Seed Pioneer	China
Shandong Denghai Pioneer	China
Pioneer Argentina S.R.L.	Argentina
PHI Servicios de C.V.	Mexico
Pioneer Hi-Bred Puerto Ri	USA
P.T. DuPont Indonesia	Indonesia
Pioneer Genetique S.A.R.L	France
Pioneer Hi-Bred Termelö	Hungary
Pioneer Hi-Bred RSA (PTY)	South Africa
Pioneer Hi-Bred Philippin	Philippines
Pioneer Hi-Bred NE-Servic	Germany
POC-India Branch	India
Pioneer Hi-Bred (Thailand	Thailand
Pioneer Hi-Bred Limited	Canada
Pioneer Hi-Bred Seeds Agr	Romania
Pioneer Pakistan Seed Ltd	Pakistan
Pioneer Semences SAS	France
Pioneer Hi-Bred Serv GmbH	Austria
Pioneer Hi-Bred Australia	Australia
MISR Pioneer Seed Co. SAE	Egypt
DuPont China Holdings	China
Tieling Pioneer Seed Res	China
Pioneer Hi-Bred NE-Sales	Germany
Pioneer Tohumculuk A.S.	Turkey

LLC Pioneer Hi-Bred UA	Ukraine
Pioneer Switzerland SA	Switzerland
Seed Consultants, Inc.	USA
Pioneer Hi-bred Hellas SA	Greece
Pannar Seed (Proprietary) Limited	South Africa
Pannar Seed (Pty) Ltd (South Africa)	South Africa
Pannar Seed (Pvt) Ltd. - Zimbabwe	Zimbabwe
Pannar Properties Zambia Limited	Zambia
Pannar Seed Ltda Mozambique	Mozambique
Pannar Seed BV (Netherlands)	Netherlands
Pannar Seed Kenya Ltd.	Kenya
Pannar Seed Malawi Ltd.	Malawi
Pannar Seed Tanzania Ltd.	Tanzania
Pannar Seed Lesotho (Pty) Ltd.	South Africa
Pannar Seed Zambia Ltd.	Zambia
Pannar Seed (Swaziland) Ltd.	Swaziland
Pannar Semillas SRL (Argentina)	Argentina

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EXHIBIT 20

Exhibit 20

Huvepharma's Initial Claim Chart for Infringement of U.S. Patent No. 7,026,150 by Defendants'

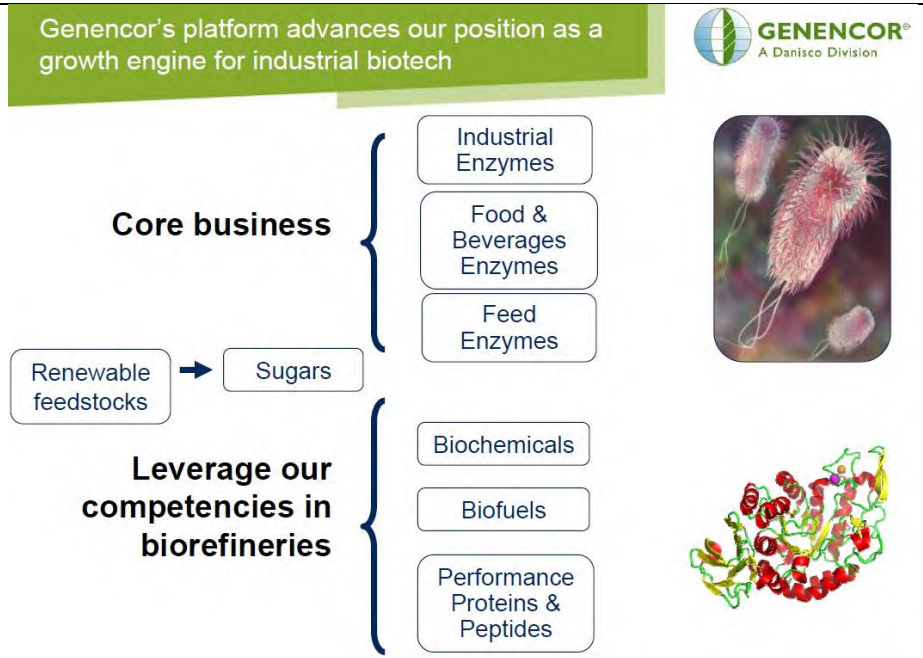
Optimash Phytase product

On information and belief, Defendants' Optimash Phytase product infringes the claims of the '150 patent identified below. On information and belief, Defendants' Optimash Phytase product is made according to production methods of the Defendants' Phyzyme XP phytase product lines. Thus, the evidence describing the method of producing the Phyzyme XP product lines is applicable to Defendants' Optimash Phytase product.

Claim 1	
Claim 1. A method of producing phytase in yeast comprising:	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced in yeast. For example, Defendants manufacture the Optimash Phytase product. Ex. 23. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the photograph below of a packaging label of Phyzyme XP 10000 TPT acquired in the United States demonstrates that Phyzyme XP has been manufactured in Finland by Genencor International B.V. ("Genencor") and imported into the United States. Ex. 8 at 1. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, a packaging insert of Phyzyme XP 10000 TPT of a sample acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor for Danisco Animal Nutrition and contains Schizosaccharomyces pombe, which is a yeast. Ex. 14 at 1-7. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, Genencor operates biorefineries that manufacture Phyzyme XP as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme XP for animal feed. Ex. 9 at 1-6; Ex. 8 at 1; Ex. 18 at 2, 4-6.



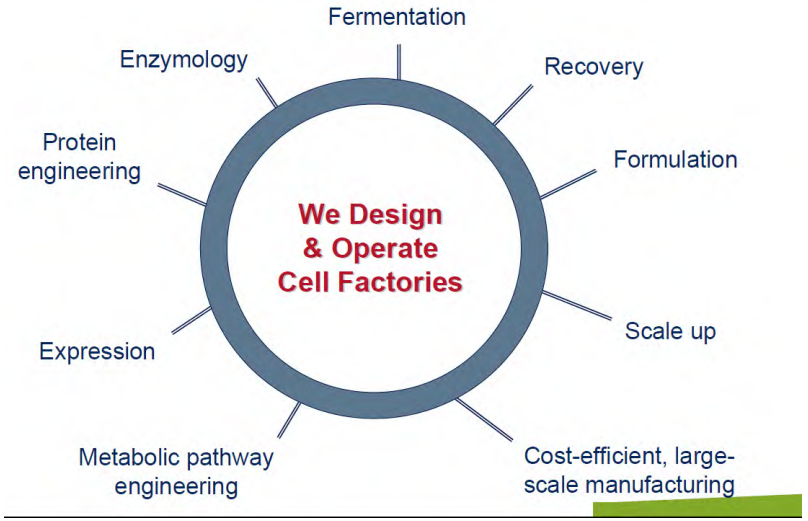
Ex. 9 at 3.

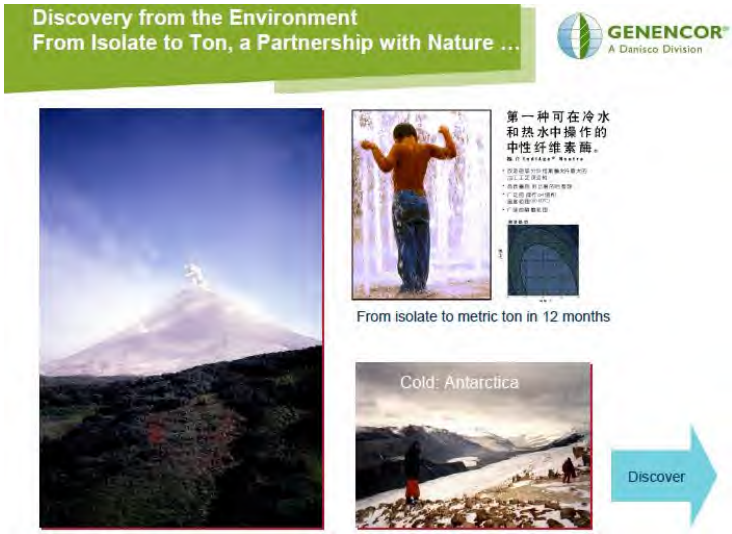
For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1.

For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G, and varies only in the final formulation. Ex. 11 at 5; Ex. 12 at 4.

providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase;	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase. For example, Phyzyme XP advertised and sold by DuPont Danisco Animal Nutrition was sourced from E.coli species bacterium and expressed in Saccharomyces pombe. Ex. 7 at 2, 4. For example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence that was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. In further examples, analytical results of the Phyzyme XP 10000 TPT

	sample of Exhibit 8 confirmed the presence of an AppA E.coli bacterium; and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA E.coli bacterium.
expressing the protein or polypeptide in a yeast; and	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or are being produced by expressing the protein or polypeptide in a yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in the yeast Saccharomyces pombe. Ex. 7 at 2. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity, and the production strain for Phyzyme XP is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into

	S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a Schizosaccharomyces pombe yeast. Ex. 14 at 1-2. In an example, Genencor uses expression of engineered microorganisms to produce enzymes.  Ex. 9 at 5-6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast

	Schizosaccaromyces pombe.
isolating the expressed protein or polypeptide,	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by isolating the expressed protein or polypeptide. For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides.  Ex. 9 at 8. For example, the production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e. >95%) are removed. In the clarification step, either conventional (depth) filtration or

	tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. Ex. 10 at 6.
wherein said protein or polypeptide catalyzes the release of phosphate from phytate and	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein said protein or polypeptide catalyzes the release of phosphate from phytate. For example, Optimash Phytase product releases inositol and increases the available free phosphorus in fermentation. Ex. 23 at 1. For example, Phyzyme XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects. Ex. 7 at 2. In a further example, Phyzyme XP is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in the manure. Ex. 10 at 5. For example, the FEEDAP Panel considers that the change in the formulation (of Phyzyme XP 10000 L/TPT) will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion (relating to Phyzyme XP 5000 L/G). Ex. 13 at 8. For example, Phyzyme XP for Pigs and Chickens is a phytase designed

	specifically to improve the availability of phosphorus, calcium and amino acids contained in cereal grains, oil seed meals and their by-products. Ex. 15 at 1. For example, a Phyzyme XP 10000 TPT product insert states the product is phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig diets. Ex. 14 at 2.
has increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method that provides increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell. For example, Optimash Phytase functions effectively during typical simultaneous saccharification and fermentation conditions (up to 70°C). Ex. 23 at 2. For example, Phyzyme XP TPT has “unrivalled heat stability, superior performance,” and “in a series of trials conducted at the Technological Institute, Kolding, Denmark, Phyzyme XP TPT is shown to be more heat stable than other leading phytase products.” Ex. 17 at 1-2. For example, significant loss of phytase activity during steam conditioning and subsequent pelleting of feed negatively impacts profitability. Phyzyme XP incorporates unique TPT coating technology, which offers heat stability up to 95C (203F). The TPT coating also allows rapid release of phytase in the upper part of the gut to achieve optimum bio-efficacy. Ex. 16 at 2. For example, Phyzyme XP 5000L is also stable after mixing with feedingstuffs with approximately 80% of activity remaining after six

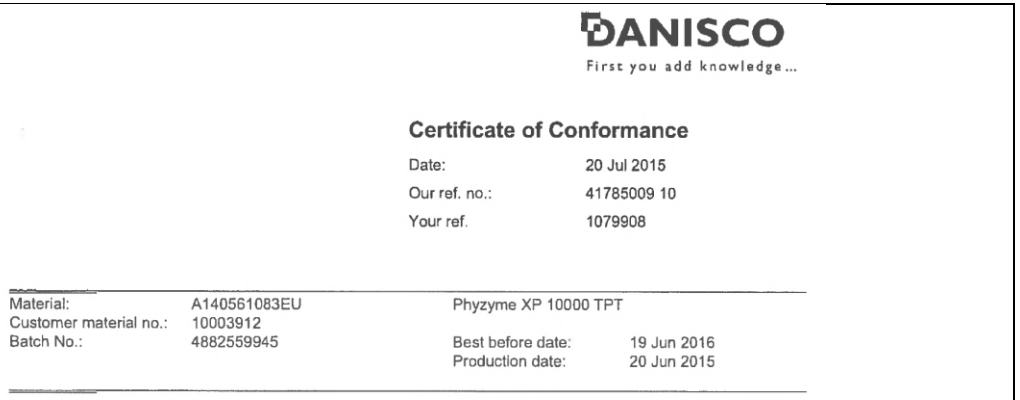
	months storage in a feed mash at both 20°C and 35°C. Following pelleting of feeds at 80C, 82% of the initial phytase activity was still present, thus Phyzyme XP 5000G is considered to be stable when pelleted up to this temperature. Ex. 11 at 5. For example, Phyzyme XP 10000 TPT stability in pelleting, when tested with a maize-based diet and was conditioned for 30 seconds at 90C or 95C and pelleted, resulted in percentage of recovery of the initial enzymatic activity of 99% and 96%, respectively. Ex. 13 at 7. It is not expected that the double concentration of the Phyzyme XP 10000 L enzyme (compared to Phyzyme XP 5000L) will modify the stability of the product in feedingstuffs. Phyzyme XP 5000 showed an 80% of the activity remaining when added to feedingstuffs and stored for six months at 20°C and 35°C. Ex. 13 at 7. For example, Phyzyme XP is a phytase protected by unique Thermal Protection Technology coating from Danisco Animal Nutrition. Phyzyme XP TPT is heat stable to 95°C/203°F during pelleting. Ex. 7 at 3, 4. Phyzyme XP G is for mash feed only. A light brown, fine granular product on a wheat flour carrier. For optimum bioefficacy do no exceed conditioning and pelleting temperatures of 70°C/158°F. Ex. 7 at 4.
Claim 2. The method according to claim 1, wherein the heterologous polynucleotide is an isolated appA polynucleotide.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the heterologous polynucleotide is an isolated appA polynucleotide. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the final production strain ATCC5233 expresses the 6-

	phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. The donor organism is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.
Claim 3. The method according to claim 1, wherein the yeast is selected from the group consisting of Saccharomyces species Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being by a method wherein the yeast is selected from the group consisting of Saccharomyces species Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1. For example, Phyzyme XP 5000L and Phyzyme XP 5000G is produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. Ex. 11 at 3. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism

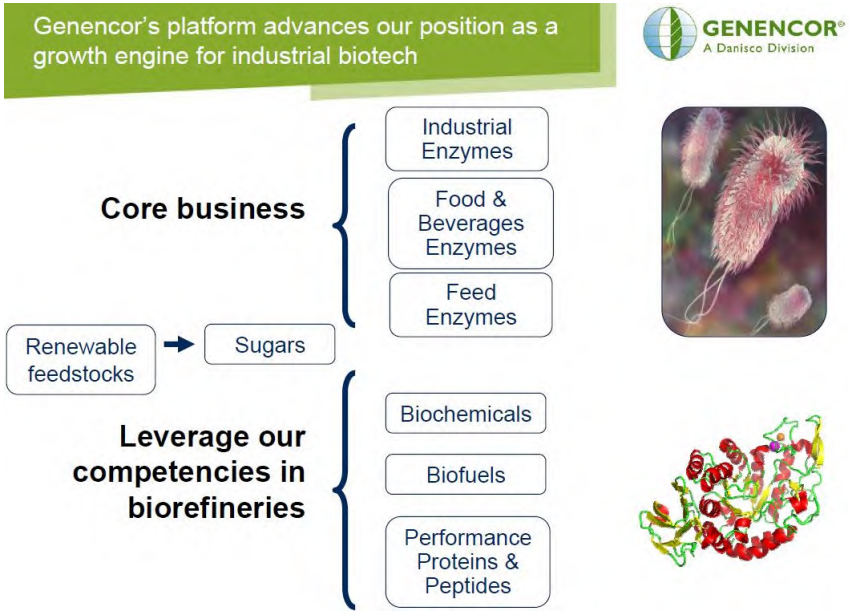
	Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3. For example, Phyzyme XP 10000 TPT product insert states the product contains dried Schizosaccharomyces pombe fermentation product. Ex. 14 at 2. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
Claim 6. The method according to claim 1, wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 10 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into

	S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6.
Claim 7. The method according to claim 6, wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.	Each use, offer for sale, and/or sale of Optimash Phytase is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6. For example, Phyzyme XP is/has been sold in 5000 FTU and 10000 FTU concentrations. Ex. 11 at 3; Ex. 12 at 4; Ex. 13 at 4. For example, Phyzyme XP 5000L and Phyzyme XP 5000G are produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex.11 at 3. In another example, Phyzyme XP 10000L and Phyzyme XP 10000 TPT are produced by Schizosaccharomyces pombe with a minimum activity of 10000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from

	sodium phytate. Ex. 13 at 4. For example, the photograph below of a packaging label of Phyzyme XP acquired in the United States demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. and imported into the United States with a concentration of 10000 FTU/g. Ex. 8 at 1. SAMPLE NOT INTENDED FOR COMMERCIAL SALE Feed Grade Phyzyme XP 10000 TPT PRODUCT BATCH [redacted] QUANTITY 1 KG Man date 10/12 2017 MADE IN Finland Exp date 10/12 2018 Genencor International, B.V. Archimedesweg 30 2333 CN Leiden The Netherlands Tel +32 50 44 91 73 CAUTION: May Cause eye and skin irritation. Inhalation of dusts or mists may cause an allergic respiratory reaction. Avoid contact with eyes, skin and mucous membranes. Avoid breathing mist or dust. Use with adequate ventilation. FIRST AID: In Case of eye contact, flush eyes with water for at least 15 minutes and get prompt medical attention. In case of skin contact, wash thoroughly with soap and water. If irritation occurs and persists, consult a physician. If respiratory irritation or an allergic response occurs, consult a physician. GENENCOR® A Danisco Division For example, the copy below of a packaging insert of Phyzyme XP acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. with a concentration of 10000 FTU/g. Ex. 14 at 2.

	
Claim 8. The method according to claim 1, wherein the heterologous polynucleotide which encodes a protein or polypeptide with phytase activity is spliced in frame with a transcriptional enhancer element.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the heterologous polynucleotide which encodes a protein or polypeptide with phytase activity is spliced in frame with a transcriptional enhancer element. For example, for example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.
Claim 28	
Claim 28. A method of producing a protein or polypeptide having phytase	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash

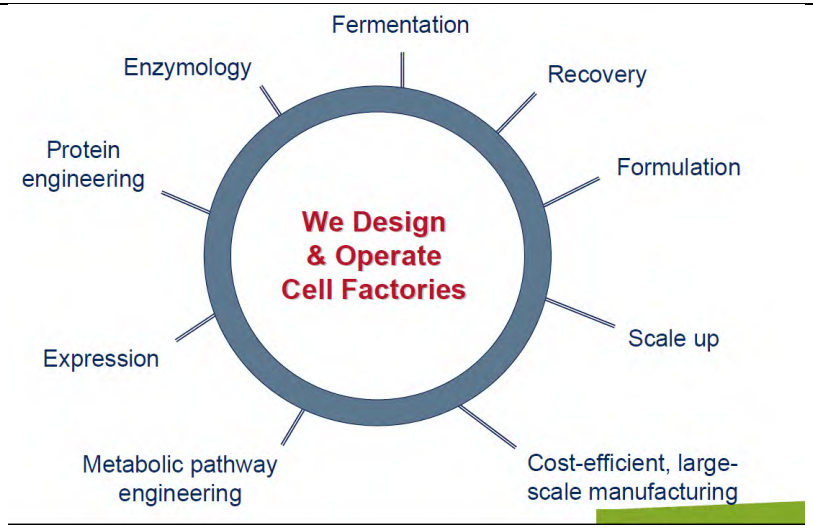
activity comprising:	Phytase imported into the United States that was produced and/or is being produced by a method using a protein or polypeptide having phytase activity. For example, Defendants manufacture the Optimash Phytase product. Ex. 23. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the photograph below of a packaging label of Phyzyme XP 10000 TPT acquired in the United States demonstrates that Phyzyme XP is a protein having phytase activity that has been manufactured in Finland by Genencor and imported into the United States. Ex. 8 at 1. For example, a packaging insert of Phyzyme XP 10000 TPT of a sample acquired in the Kingdom of Belgium demonstrates that Phyzyme XP has been manufactured in Finland by Genencor for Danisco Animal Nutrition

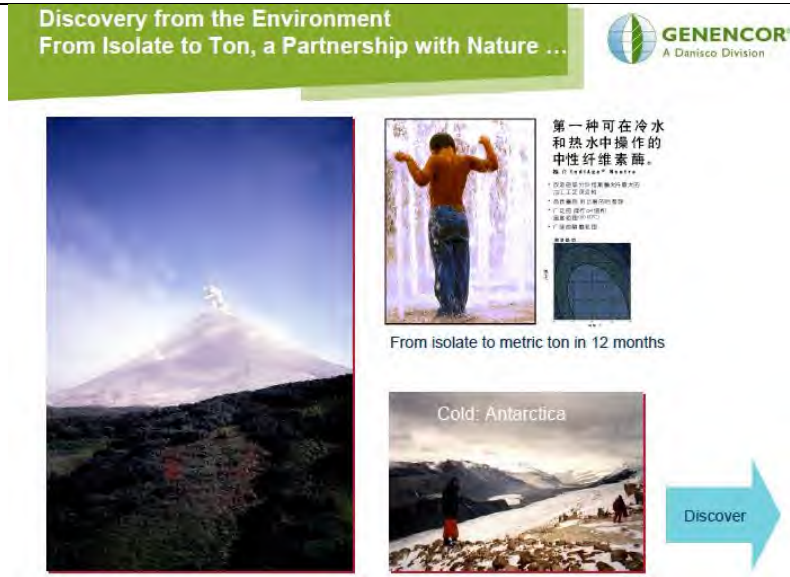
	and contains a protein with phytase activity. Ex. 14 at 1-3. For example, Genencor operates biorefineries that manufacture Phyzyme XP as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme XP for animal feed. Ex. 9 at 6-12; Ex. 8 at 1; Ex. 18 at 2, 4-6.  Ex. 9 at 3. For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and

	Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3; Ex. 12 at 4. The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G, and varies only in the final formulation. Ex. 12 at 4.
providing an isolated appA polynucleotide, which encodes a protein or polypeptide with phytase activity,	Each use, offer for sale, and/or sale of Optimash Phytase into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by providing an isolated appA polynucleotide, which encodes a protein or polypeptide with phytase activity. For example, Phyzyme XP advertised and sold by DuPont and Danisco Animal Nutrition was sourced from E.coli species bacterium and expressed in Saccharomyces pombe. Ex. 7 at 2, 4. For example, for Phyzyme XP the final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex.10 at 9. For example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP,

	designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3; Ex. 12 at 4. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of an AppA E.coli bacterium; and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA E.coli bacterium.
expressing said polynucleotide in a yeast hold cell under conditions effective to produce the protein or polypeptide having the phytase activity, and	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by expressing the polynucleotide in a yeast hold cell under conditions effective to produce the protein or polypeptide having the phytase activity. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in the yeast Saccharomyces pombe. Ex. 7 at 2. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000.

	Ex. 13 at 3; Ex. 12 at 4. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a Schizosaccharomyces pombe yeast. Ex. 14 at 1-2. In an example, Genencor uses expression of engineered microorganisms to produce enzymes.

	 Ex. 9 at 5-6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
isolating the expressed protein or polypeptide.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by isolating the expressed protein or polypeptide. For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides.

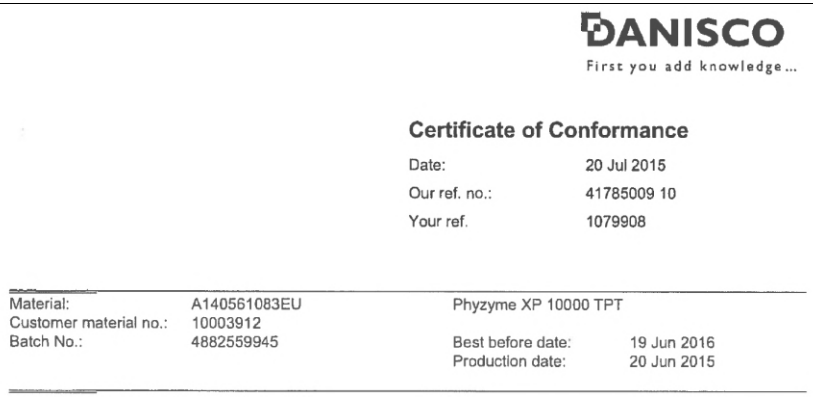
	 Ex. 9 at 8. For example, the production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e. >95%) are removed. In the clarification step, either conventional (depth) filtration or tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. Ex. 10 at 6.
Claim 29. The method according to claim 28, wherein the yeast host cell is selected from the group consisting of	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is

Saccaromyces species, Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species.	being produced by a method wherein the yeast host cell is selected from the group consisting of Saccaromyces species, Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1. For example, Phyzyme XP 5000L and Phyzyme XP 5000G is produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. Ex. 11 at 3, 20. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3. For example, Phyzyme XP 10000 TPT product insert states the product contains dried Schizosaccharomyces pombe fermentation product. Ex. 14 at 2. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was

	religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
Claim 31. The method according to claim 28, wherein the protein or polypeptide, preceded by a signal peptide, is secreted by the cell into a growth medium or is not secreted.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the protein or polypeptide, preceded by a signal peptide, is secreted by the cell into a growth medium or is not secreted. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 10 at 5. The expression cassette, which consists of the

	cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6.
Claim 32. The method according to claim 31, wherein the protein or polypeptide is secreted by the yeast host cell into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the protein or polypeptide is secreted by the yeast host cell into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6. For example, Phyzyme XP is/has been sold in 5000 FTU and 10000 FTU concentrations. Ex. 11 at 3; Ex. 12 at 4; Ex. 13 at 4. For example, Phyzyme XP 5000L and Phyzyme XP 5000G are produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex. 11 at 3.

	In another example, Phyzyme XP 10000L and Phyzyme XP 10000 TPT are produced by Schizosaccharomyces pombe with a minimum activity of 10000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex. 13 at 4. For example, the photograph below of a packaging label of Phyzyme acquired in the United States demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. and imported into the United States with a concentration of 10000 FTU/g. Ex. 8 at 1. For example, the copy below of a packaging insert of Phyzyme XP acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor with a concentration of 10000 FTU/g. Ex. 14 at 2.

	 DANISCO First you add knowledge... **Certificate of Conformance** Date: 20 Jul 2015 Our ref. no.: 41785009 10 Your ref. 1079908 Material: A140561083EU Phyzyme XP 10000 TPT Customer material no.: 10003912 Batch No.: 4882559945 Best before date: 19 Jun 2016 Production date: 20 Jun 2015 For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6.
Claim 33. The method according to claim 28, wherein the appA polynucleotide is spliced in frame with a transcriptional enhancer element.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the appA polynucleotide is spliced

	in frame with a transcriptional enhancer element. For example, for example, the donor organism is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399, a telemorph heterothallic leucine auxotrophic yeast strain. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.

EXHIBIT 21

Exhibit 21

Huvepharma's Initial Claim Chart for Infringement of U.S. Patent No. 7,312,063 by Defendants'

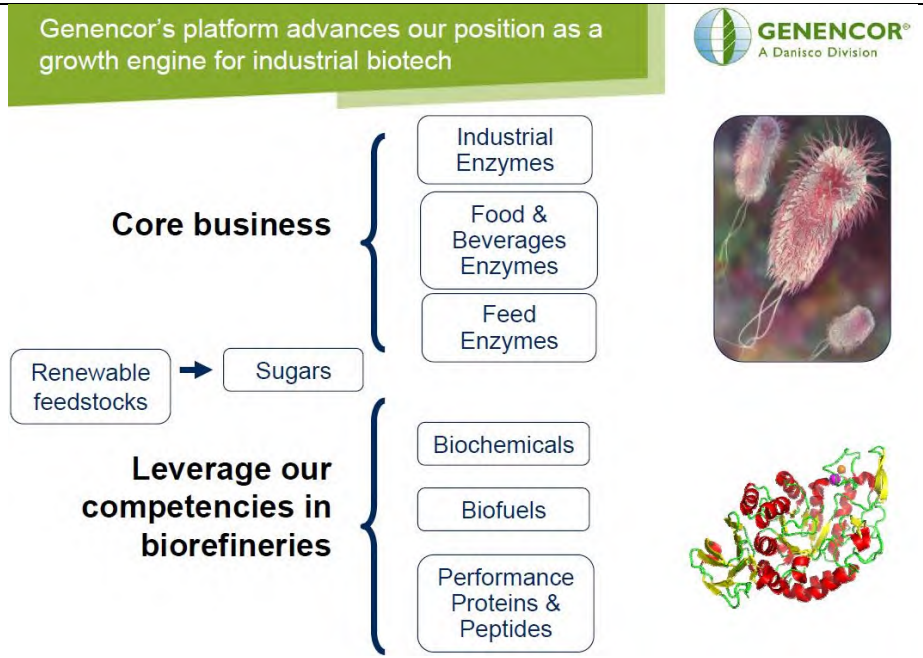
Optimash Phytase product

On information and belief, Defendants' Optimash Phytase product infringes the claims of the '063 patent. On information and belief, Defendants' Optimash Phytase product is made according to production methods of the Defendants' Phyzyme XP phytase product lines. Thus, the evidence describing the method of producing the Phyzyme XP product lines is applicable to Defendants' Optimash Phytase product.

Claim 1	
Claim 1. A method of producing phytase in yeast comprising:	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced in yeast. For example, Defendants manufacture the Optimash Phytase product. Ex. 23. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the photograph below of a packaging label of Phyzyme XP 10000 TPT acquired in the United States demonstrates that Phyzyme XP has been manufactured in Finland by Genencor International B.V. ("Genencor") and imported into the United States. Ex. 8 at 1. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, a packaging insert of Phyzyme XP 10000 TPT of a sample acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor for Danisco Animal Nutrition and contains Schizosaccharomyces pombe, which is a yeast. Ex. 14 at 1-7. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, Genencor operates biorefineries that manufacture Phyzyme XP as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme XP for animal feed. Ex. 9 at 1-6; Ex. 8 at 1; Ex. 18 at 2, 4-6.



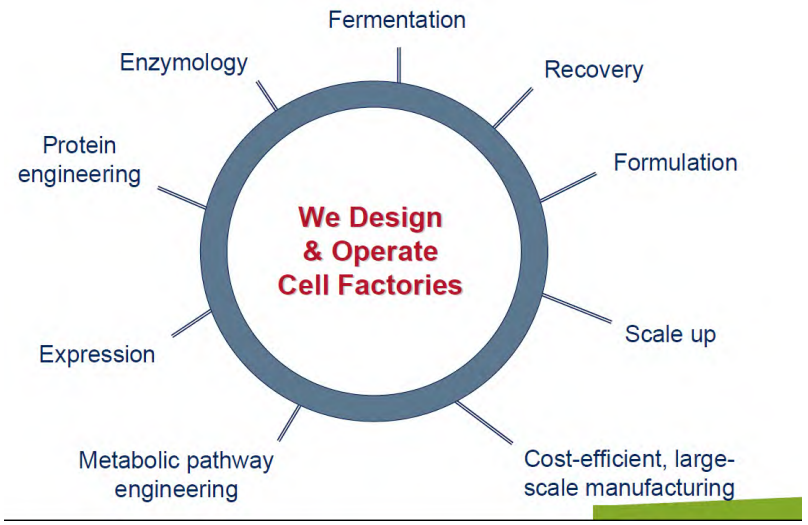
Ex. 9 at 3.

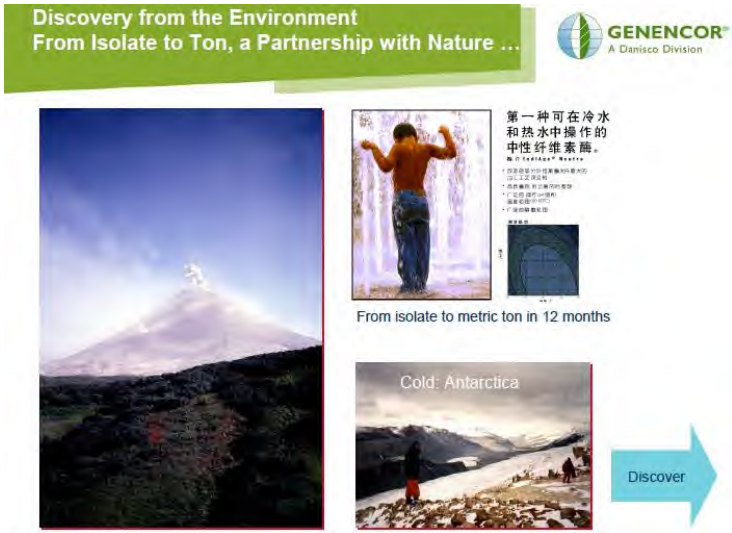
For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1.

For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3; Ex. 12 at 4. The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G, and varies only in the final formulation. Ex. 11 at 5; Ex. 12 at 4.

providing a heterologous polynucleotide from a non-yeast organism which encodes a protein or polypeptide comprising a phytase from Escherichia coli;	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase. For example, Phyzyme XP advertised and sold by DuPont Danisco Animal Nutrition was sourced from E.coli species bacterium and expressed in Saccharomyces pombe. Ex. 7 at 2, 4. For example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence that was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. In further examples, analytical results of the Phyzyme XP 10000 TPT

	sample of Exhibit 8 confirmed the presence of an AppA E.coli bacterium; and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA E.coli bacterium.
expressing the polynucleotide in a yeast; and	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase products imported into the United States that was produced and/or is being produced by expressing the protein or polypeptide in a yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in the yeast Saccharomyces pombe. Ex. 7 at 2. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity, and the production strain for Phyzyme XP is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid

	fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a Schizosaccharomyces pombe yeast. Ex. 14 at 1-2. In an example, Genencor uses expression of engineered microorganisms to produce enzymes.  Ex. 9 at 5-6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP

	10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
isolating the expressed protein or polypeptide,	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by isolating the expressed protein or polypeptide. For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides.  Ex. 9 at 8. For example, the production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e. >95%) are

	removed. In the clarification step, either conventional (depth) filtration or tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. Ex. 10 at 6.
wherein said protein or polypeptide catalyzes the release of phosphate from phytate and	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein said protein or polypeptide catalyzes the release of phosphate from phytate. For example, Optimash Phytase product releases inositol and increases the available free phosphorus in fermentation. Ex. 23 at 1. For example, Phyzyme XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects. Ex. 7 at 2. In a further example, Phyzyme XP is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in the manure. Ex. 10 at 5. For example, the FEEDAP Panel considers that the change in the formulation (of Phyzyme XP 10000 L/TPT) will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion (relating to Phyzyme XP

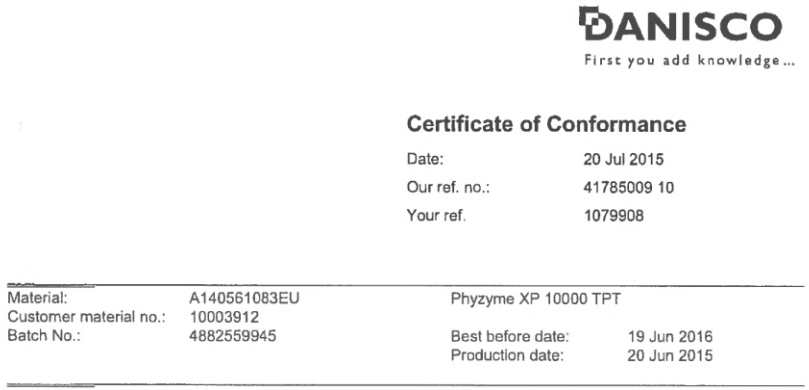
	5000 L/G). Ex. 13 at 8. For example, Phyzyme XP for Pigs and Chickens is a phytase designed specifically to improve the availability of phosphorus, calcium and amino acids contained in cereal grains, oil seed meals and their by-products. Ex. 15 at 1. For example, a Phyzyme XP 10000 TPT product insert states the product is phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig diets. Ex. 14 at 2.
has increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method that provides increased thermostability as compared to that of said protein or polypeptide expressed in a non-yeast host cell. For example, Optimash Phytase functions effectively during typical simultaneous saccharification and fermentation conditions (up to 70°C). Ex. 23 at 2. In another example, Phyzyme XP TPT has “unrivalled heat stability, superior performance,” and “in a series of trials conducted at the Technological Institute, Kolding, Denmark, Phyzyme XP TPT is shown to be more heat stable than other leading phytase products.” Ex. 17 at 1-2. For example, significant loss of phytase activity during steam conditioning and subsequent pelleting of feed negatively impacts profitability. Phyzyme XP incorporates unique TPT coating technology, which offers heat stability up to 95C (203F). The TPT coating also allows rapid release of phytase in the upper part of the gut to achieve optimum bio-efficacy. Ex. 16 at 2.

	For example, Phyzyme XP 5000L is also stable after mixing with feedingstuffs with approximately 80% of activity remaining after six months storage in a feed mash at both 20°C and 35°C. Following pelleting of feeds at 80C, 82% of the initial phytase activity was still present, thus Phyzyme XP 5000G is considered to be stable when pelleted up to this temperature. Ex. 11 at 5. For example, Phyzyme XP 10000 TPT stability in pelleting, when tested with a maize-based diet and was conditioned for 30 seconds at 90C or 95C and pelleted, resulted in percentage of recovery of the initial enzymatic activity of 99% and 96%, respectively. Ex. 13 at 7. It is not expected that the double concentration of the Phyzyme XP 10000 L enzyme (compared to Phyzyme XP 5000L) will modify the stability of the product in feedingstuffs. Ex. 13 at 7. Phyzyme XP 5000 showed an 80% of the activity remaining when added to feedingstuffs and stored for six months at 20°C and 35°C. Ex. 13 at 7. For example, Phyzyme XP is a phytase protected by unique Thermal Protection Technology coating from Danisco Animal Nutrition. Phyzyme XP TPT is heat stable to 95°C/203°F during pelleting. Ex. 7 at 3, 4. Phyzyme XP G is for mash feed only. A light brown, fine granular product on a wheat flour carrier. For optimum bioefficacy do no exceed conditioning and pelleting temperatures of 70°C/158°F. Ex. 7 at 4.
Claim 2. The method according to claim 1, wherein the yeast is selected from the group consisting of Saccharomyces species, Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the yeast is selected from the group consisting of Saccharomyces species Pichia species, Kluyveromyces species, Hansenula species, Candida species, Torulaspora species, and Schizosaccharomyces species.

Schizosaccharomyces species.	For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1. For example, Phyzyme XP 5000L and Phyzyme XP 5000G is produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. Ex. 11 at 3, 20. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 13 at 3. For example, Phyzyme XP 10000 TPT product insert states the product contains dried Schizosaccharomyces pombe fermentation product. Ex. 14 at 2. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
Claim 5. The method according to claim 1, wherein the protein or polypeptide preceded by a signal peptide is secreted by the yeast into a	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the protein or polypeptide

growth medium or is not secreted.	preceded by a signal peptide is secreted by the yeast into a growth medium or is not secreted. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399. Ex. 10 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6.
Claim 6. The method according to claim 5, wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium.	Each use, offer for sale, and/or sale of Optimash Phytase is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the protein or polypeptide is secreted by the yeast into the growth medium and has a concentration greater than 300 units per milliliter of the growth medium. For example, a number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e., > 95%) are removed. Ex. 10 at 6.

	For example, Phyzyme XP is/has been sold in 5000 FTU and 10000 FTU concentrations. Ex. 11 at 3; Ex. 12 at 4; Ex. 13 at 4. For example, Phyzyme XP 5000L and Phyzyme XP 5000G are produced by Schizosaccharomyces pombe with a minimum activity of 5000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex.11 at 3. In another example, Phyzyme XP 10000L and Phyzyme XP 10000 TPT are produced by Schizosaccharomyces pombe with a minimum activity of 10000 FTU/g in liquid and in solid form. 1 FTU is the amount of enzymes which liberates 1 micromole of inorganic phosphate from sodium phytate. Ex. 13 at 10. For example, the photograph below of a packaging label of Phyzyme XP acquired in the United States demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. and imported into the United States with a concentration of 10000 FTU/g. Ex. 8 at 1.

	For example, the copy below of a packaging insert of Phyzyme XP acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor International B.V. with a concentration of 10000 FTU/g. Ex. 14 at 2.  DANISCO First you add knowledge... **Certificate of Conformance** Date: 20 Jul 2015 Our ref. no.: 41785009 10 Your ref. 1079908 Material: A140561083EU Phyzyme XP 10000 TPT Customer material no.: 10003912 Batch No.: 4882559945 Best before date: 19 Jun 2016 Production date: 20 Jun 2015
Claim 7. The method according to claim 1, wherein the heterologous polynucleotide which encodes the protein or polypeptide is spliced in frame with a transcriptional enhancer element.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the heterologous polynucleotide which encodes a protein or polypeptide with phytase activity is spliced in frame with a transcriptional enhancer element. For example, for example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233

	expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.

EXHIBIT 22

Exhibit 22

Huvepharma's Initial Claim Chart for Infringement of U.S. Patent No. 8,455,232 by Defendants'

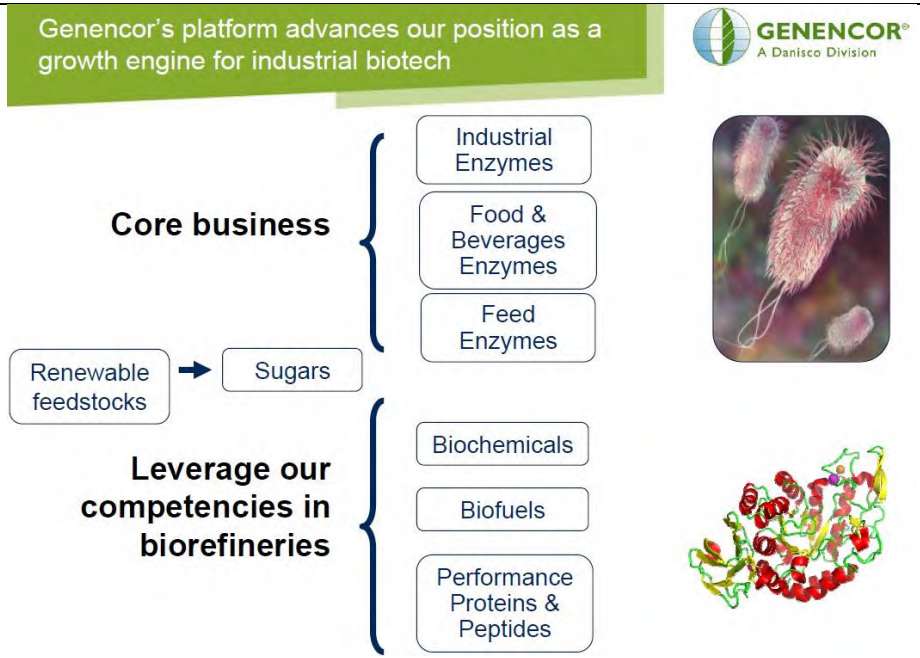
Optimash Phytase product

On information and belief, Defendants' Optimash Phytase product infringes the claims of the '232 patent. On information and belief, Defendants' Optimash Phytase product is made according to production methods of the Defendants' Phyzyme XP phytase product lines. Thus, the evidence describing the method of producing the Phyzyme XP product lines is applicable to Defendants' Optimash Phytase product.

Claim 1	
Claim 1. A method of producing phytase in yeast, the method comprising:	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced and/or are being produced in yeast. For example, Defendants manufacture the Optimash Phytase product. Ex. 23. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the photograph below of a packaging label of Phyzyme XP 10000 TPT acquired in the United States demonstrates that Phyzyme XP has been manufactured in Finland by Genencor International B.V. ("Genencor") and imported into the United States. Ex. 8 at 1. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, a packaging insert of Phyzyme XP 10000 TPT of a sample acquired in the Kingdom of Belgium demonstrates that Phyzyme XP is and/or has been manufactured in Finland by Genencor for Danisco Animal Nutrition and contains Schizosaccharomyces pombe, which is a yeast. Ex. 14 at 1-7. Analytical testing of this sample confirmed that the Phyzyme XP contained a yeast.

For example, Genencor operates biorefineries that manufacture Phyzyme XP as feed enzymes for animal feed. The manufacturing includes fermentation, recovery, purification, and formulation processing of Phyzyme XP for animal feed. Ex. 9 at 1-6; Ex. 8 at 1; Ex. 18 at 2, 4-6.



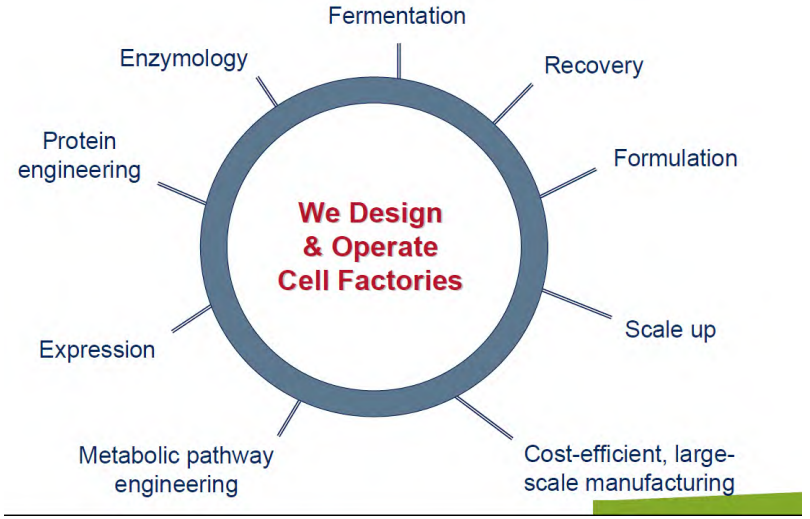
Ex. 9 at 3.

For example, the Phyzyme XP phytase is an enzyme feed additive with 6-phytase as its main activity, produced after fermentation with the genetically modified yeast Schizosacchoaromyces pombe. Ex. 10 at 1.

For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe available in solid (Phyzyme XP 5000G and Phyzyme XP 10000TPT) and liquid forms (Phyzyme XP 5000L and Phyzyme XP 10000L). The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. The product Phyzyme XP 5000L is derived from the same organism as the granulated Phyzyme XP 5000G, and varies only in the final formulation. Ex. 11 at 5; Ex. 12 at 4.

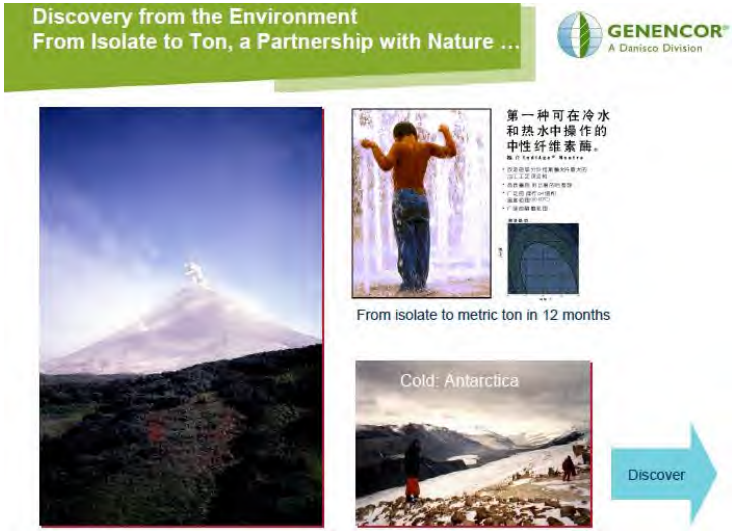
providing a polynucleotide encoding a phytase from Escherichia coli;	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by providing a heterologous polynucleotide from non-yeast organism which encodes a protein or polypeptide comprising either a PhyA phytase or an AppA phytase. For example, Phyzyme XP advertised and sold by DuPont Danisco Animal Nutrition was sourced from E.coli species bacterium and expressed in Saccharomyces pombe. Ex. 7 at 2, 4. For example, the donor organism for Phyzyme XP is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence that was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity. The production strain for Phyzyme XP, designated ASP595-1, is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of an AppA E.coli bacterium; and analytical

	results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of an AppA E.coli bacterium.
expressing the polynucleotide in a yeast; and	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by expressing the protein or polypeptide in a yeast. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in the yeast Saccharomyces pombe. Ex. 7 at 2. For example, Phyzyme XP 5000L/G is an enzyme feed additive with 6-phytase as its main activity, and the production strain for Phyzyme XP is derived from a strain of the fission yeast Schizosacchoaromyces pombe. Ex. 11 at 5; Ex. 12 at 4. For example, the additive Phyzyme XP 10000 (TPT/L) is a preparation of 6-phytase produced by the genetically modified micro-organism Schizosacchoaromyces pombe. The product Phyzyme XP 10000 (TPT/L) is a two-times concentrated version of the Phyzyme XP 5000. Ex. 12 at 4; Ex. 13 at 3. For example, the characteristics of the recipient or parental micro-organism are that the recipient strain is Schizosaccaromyces pombe ATCC 38399. Ex. 11 at 5. The expression cassette, which consists of the cytomegalovirus promotor, the 5' untranslated region of the human lipocortin1 cDNA, the 6-phytase ORF, the terminator and the 3' untranslated region of the gene encoding human lipoprotein1, was religated so that eight expression cassettes were constructed as tandem repeats. The eight tandem expression cassettes were digested from the plasmid and the linearised DNA fragment separated from the plasmid fragment encoding ampicillin resistance. They were then transferred into S. pombe in two round of gene replacement recombination using auxotrophic mutants and selection for prototrophy. Ex. 10 at 6. Phyzyme XP is produced

	by a contained system of submerged, fed-batch pure culture fermentation of the genetically modified strain S. pombe. Ex. 10 at 6. In another example, the Phyzyme XP 10000 TPT of Exhibit 14 was manufactured by Genencor in Finland in a Schizosaccharomyces pombe yeast. Ex. 14 at 1-2. In an example, Genencor uses expression of engineered microorganisms to produce enzymes.  Ex. 9 at 5-6. In further examples, analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 8 confirmed the presence of the yeast Schizosaccaromyces pombe, and analytical results of the Phyzyme XP 10000 TPT sample of Exhibit 14 confirmed the presence of the yeast Schizosaccaromyces pombe.
isolating the expressed protein or polypeptide,	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported

into the United States that was produced and/or is being produced by isolating the expressed protein or polypeptide.

For example, Genencor operates biorefineries that isolates expressed proteins or polypeptides.



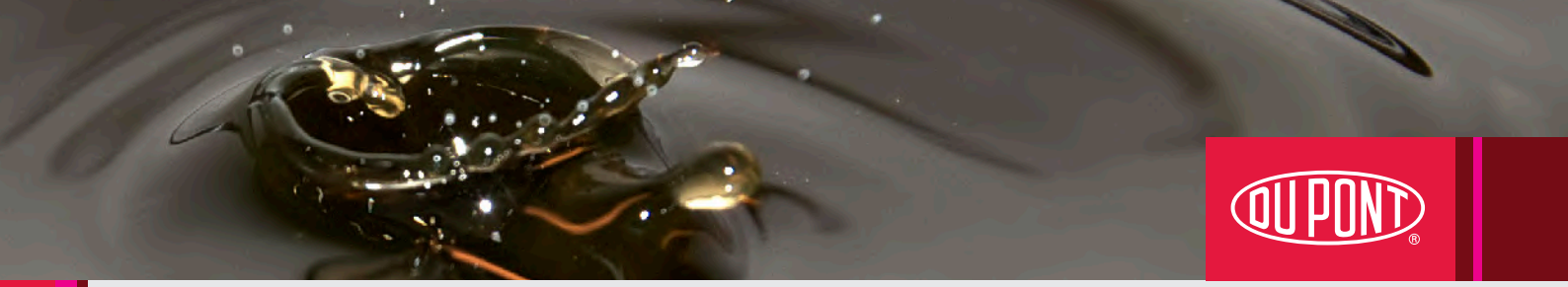
Ex. 9 at 8.

For example, the production process for Phyzyme XP includes the technique used to remove microbial cells from the product. A number of process steps serve to prevent the presence of the production organism in the final product. In the cell separation step, using centrifugation or rotary vacuum filtration, the majority of yeast cells (i.e. >95%) are removed. In the clarification step, either conventional (depth) filtration or tangential flow microfiltration ensures that a cell-free process stream is provided for the ultrafiltration step. The polish filtration step is an additional conventional (depth) filtration or tangential flow microfiltration step, which further ensures that no production organism is present in the final product. Ex. 10 at 6.

wherein said protein or polypeptide catalyzes the release of phosphate from phytate.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein said protein or polypeptide catalyzes the release of phosphate from phytate. For example, Optimash Phytase product releases inositol and increases the available free phosphorus in fermentation. Ex. 23 at 1. For example, Phyzyme XP has an exceptionally high relative activity at low pH levels compared to fungal phytases. It also has enhanced resistance to pepsin produced by the animal. This means it works quickly in the upper digestive tract to both release phosphorus and overcome phytate's anti-nutrient effects. Ex. 7 at 2. In a further example, Phyzyme XP is intended to be used as an additive to increase the bioavailability of phosphorus from the diet by hydrolysing the plant phytate. This would reduce the need to add inorganic phosphorus supplements to the animal diets and would decrease the excretion of total phosphorus in the manure. Ex. 10 at 5. For example, the FEEDAP Panel considers that the change in the formulation (of Phyzyme XP 10000 L/TPT) will not affect the phytase activity of the additive in the gastrointestinal tract and therefore the conclusions reached in the previous opinion (relating to Phyzyme XP 5000 L/G). Ex. 13 at 8. For example, Phyzyme XP for Pigs and Chickens is a phytase designed specifically to improve the availability of phosphorus, calcium and amino acids contained in cereal grains, oil seed meals and their by-products. Ex. 15 at 1. For example, a Phyzyme XP 10000 TPT product insert states the product is phytase feed enzyme specifically developed to increase the digestibility of phytin-bound phosphorus, calcium, energy and amino acids in poultry and pig

	diets. Ex. 14 at 2.
Claim 2. The method of claim 1 wherein said polynucleotide encodes E. coli AppA phytase.	Each use, offer for sale, and/or sale of Optimash Phytase imported into the United States is a use, offer for sale, and/or sale of Optimash Phytase imported into the United States that was produced and/or is being produced by a method wherein the heterologous polynucleotide is an isolated appA polynucleotide. For example, Phyzyme XP is sourced from an E.coli species bacterium and is expressed in Saccharomyces pombe. Ex. 7 at 2. For example, the final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9. The donor organism is Escherichia coli B ATCC 11303. Ex. 10 at 6. The genetic modification process includes the open reading frame (ORF) of the appA 6-phytase gene without its native E.coli leader peptide sequence was amplified and ligated in an expression vector. The nucleotide sequence of the insert was determined in its entirety to confirm that no mutations had been introduced. Ex. 10 at 6. The final production strain ATCC5233 expresses the 6-phytase appA gene from E.coli B. It contains the expression cassette integrated into the chromosome. Ex. 10 at 9.

EXHIBIT 23



OPTIMASH® PHYTASE

Phytase for Dry Grind Ethanol Production

DESCRIPTION

OPTIMASH® PHYTASE is a concentrated phytase solution characterized by its ability to efficiently hydrolyze phytate under ethanol fermentation conditions.

TYPICAL CHARACTERISTICS

Activity: 42,000 FTU/g (target)
Appearance: Brown Liquid
pH: 4.0 - 5.5
Specific gravity: 1.17 - 1.20 g/ml

The activity of OPTIMASH® PHYTASE enzyme is expressed in phytase units (FTU). One phytase unit (FTU) is defined as the amount of enzyme needed for the release of 1 µmol of inorganic orthophosphate from the substrate in one minute under the given reaction conditions of pH 5.5 and 37°C.

A detailed assay method is available upon request.

PERFORMANCE BENEFITS

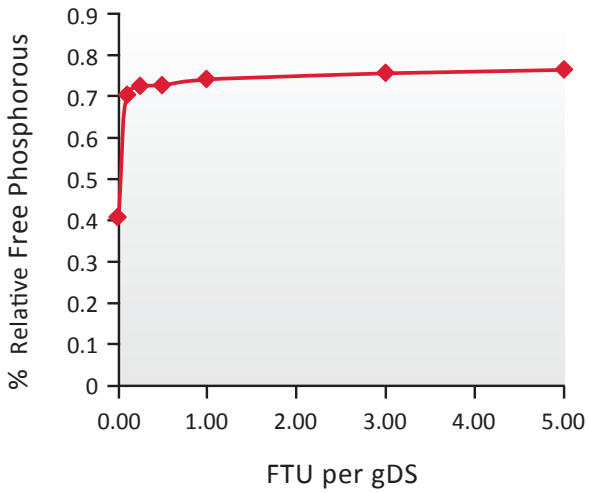
OPTIMASH® PHYTASE enzyme provides the following benefits to ethanol producers:

- Generates inositol*, an important yeast nutrient
- Maximizes the level of free phosphate in residuals (DDGS)
- Improves the heat transfer characteristics of plant heat exchangers

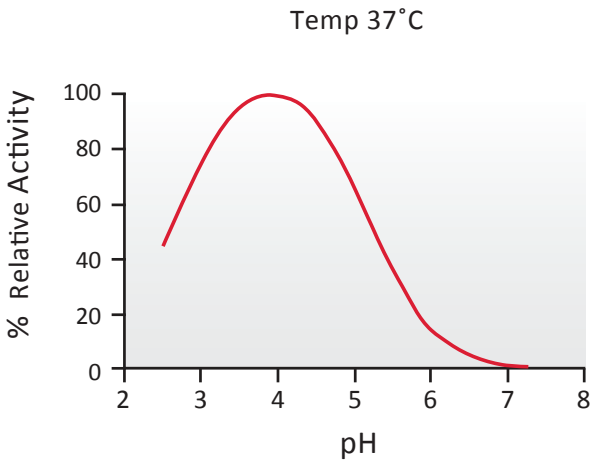
*"Determining the effects of inositol supplementation and the opi1 mutation on ethanol tolerance of *Saccharomyces cerevisiae,"* Erin L. Krause, Manuel J. Villa-García, Susan A. Henry, Larry P. Walker. Industrial Biotechnology. October 2007, 3(3): 260-268. doi:10.1089/ind.2007.3.260.

EFFECT OF OPTIMASH® PHYTASE DOSE ON FREE PHOSPHOROUS AT DROP

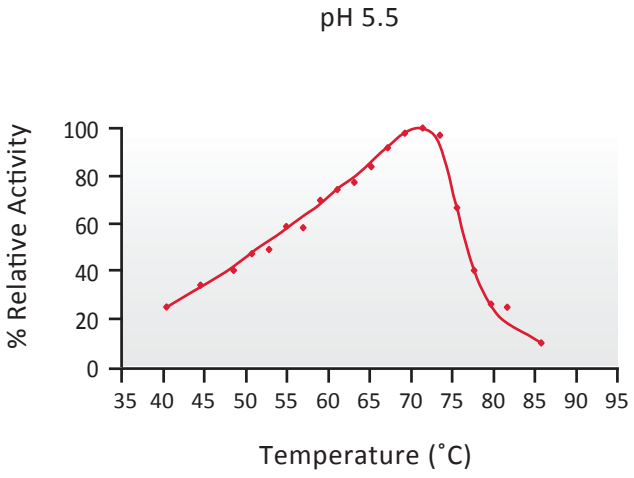
Fermentation conditions: 48 hours, 32°C, 4.8 initial pH



EFFECT OF PH ON OPTIMASH® PHYTASE ACTIVITY



EFFECT OF TEMPERATURE ON OPTIMASH® PHYTASE ACTIVITY



RECOMMENDED OPERATIONAL CONDITIONS	
Optimum pH range	3.5 - 5.0
Dry Substance	26 - 38%
Temperature	The enzyme is effective up to 70°C, but works well at fermentation temperatures of 28 - 35°C

OPTIMASH® PHYTASE enzyme functions effectively during typical simultaneous saccharification and fermentation(SSF) conditions: pH 3.5 to 5.0 and temperatures as low as 28° C. The product should be added in a single dose as early as possible to the propagation tank or the fermentor.

DOSAGE GUIDELINES

The optimum dosage levels of OPTIMASH® PHYTASE will vary with different substrates and operating conditions such as pH, temperature, and reaction time. An OPTIMASH™ PHYTASE dosage rate of 0.0024 kg per metric ton DS grain is recommended as a starting point for the optimizing of enzyme dosage.

REGULATORY STATUS

OPTIMASH® PHYTASE complies with the current recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

PACKAGING

OPTIMASH® PHYTASE is available in various package sizes. Please consult your DuPont representative for detailed information.

STORAGE

OPTIMASH® PHYTASE will meet the declared activity upon arrival at your plant. OPTIMASH® PHYTASE can be safely stored in sealed containers under cool conditions.

SAFETY & ENZYME HANDLING

Inhalation of enzyme dust and mists should be avoided. In case of contact with the skin or eyes, promptly rinse with water for at least 15 minutes.

For detailed handling information, please refer to the appropriate Material Safety Data Sheet, the **E**nzyme **T**echnical **A**ssociation (**ETA**) handbook Working Safely With Enzymes, and the **A**ssociation of **M**anufacturers and **F**ormulators of **E**nzyme **P**roducts (**AMFEP**) handbook *Guide to the Safe Handling of Microbial Enzyme Preparations*. All are available upon request from DuPont.

TECHNICAL SERVICE

DuPont is committed to working with customers to enhance processes and achieve their goals. More detailed information about the application of this product is available upon request. If you have any questions, please call us and let us know how we can be of assistance.

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REV0913 4077 OPTIMASH PHYTASE 01

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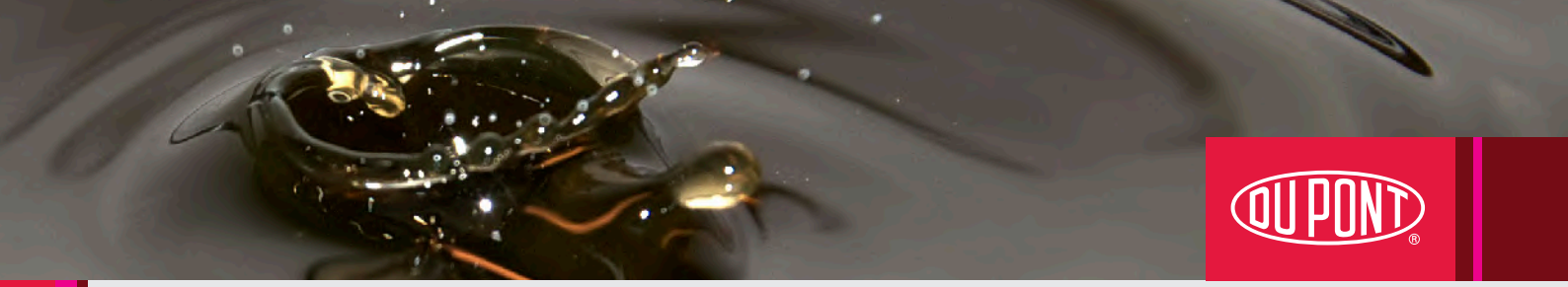
EXHIBIT 24



SPEZYME® RSL

High Performance Liquefaction Blend

DESCRIPTION

SPEZYME® RSL is a system of enzymes designed to liquefy dry grind corn or milo. The enzymes contained in the SPEZYME® RSL system are produced by genetically modified strains of Bacillus licheniformis and *Trichoderma reesei*.

TYPICAL CHARACTERISTICS

Activity: 20,100 NLC/g (target)
Appearance: Brown liquid
pH: 5.0 to 6.5
Specific gravity: 1.17

The activity of the SPEZYME® RSL system is expressed in NLC units per gram. Enzyme activity is determined by the rate of starch hydrolysis as reflected in the rate of decrease in iodine-staining capacity. A detailed assay method is available upon request.

PERFORMANCE BENEFITS

The SPEZYME® RSL system offers the following benefits to ethanol plant operators:

- Robust liquefaction at pH's ranging from 5.0 to 5.8
- Lower total liquefaction cost (enzyme, ammonia and sulfuric acid)
- Lower sulfur in distiller's grains
- Single enzyme dose

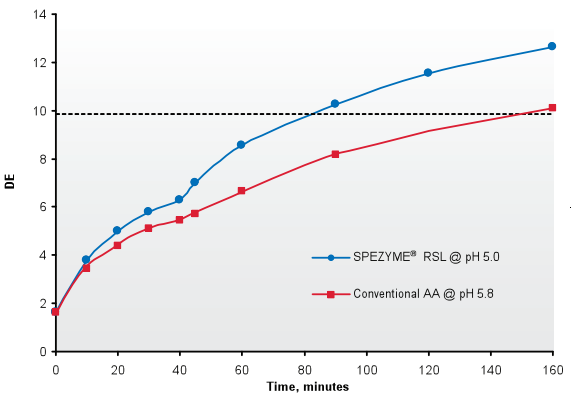
APPLICATION RECOMMENDATIONS

SPEZYME® RSL enzyme is suitable for use in a variety of liquefaction process designs.

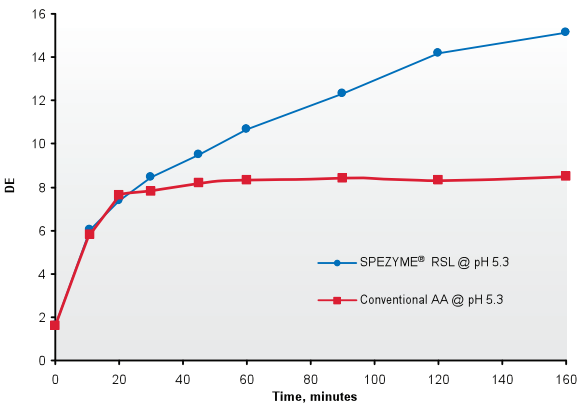
RECOMMENDED OPERATIONAL CONDITIONS	
Solids	30 to 36% weight/ weight
Optimal pH	5.0 to 5.3
pH Range	5.0 to 6.0
Temperature	
Slurry	83 - 86°C (182 - 187°F)
Liquefaction	83 - 88°C (182 - 191°F)
Time	90 to 140 minutes
Slurry	Preferably at least 15 minutes
Liquefaction	90 to 240 minutes

DuPont's technical sales professionals will provide specific process recommendations based on your objectives and plant characteristics.

Comparison of DE developemnt at equal weight / weight dosages (without jet)



Comparison of DE developemnt at equal weight / weight dosages (without jet)



Daily Concentrated H_2SO_4 Usage @ 52,000 bushels per day

To reduce liquefact pH by	kg
0.1	437
0.2	874
0.3	1,311
0.4	1,748
0.5	2,184

DOSAGE RECOMMENDATIONS

The SPEZYME® RSL system should be dosed at 0.020 to 0.024% weight enzyme / weight as is grain. To maximize performance, 100% of the dose should be added to the mix box or slurry.

REGULATORY STATUS

The enzymes and ingredients in SPEZYME® RSL are in compliance with TSCA and meet the standards for fuel ethanol production in the United States. In addition, the enzymes in the product have been dermined to be GRAS for distillers dried grains produced as a by-product in alcohol production. For other countries, please contact customer service.

PACKAGING

SPEZYME® RSL is available in various package sizes and bulk tankers. Please consult your DuPont representative for detailed information.

STORAGE

SPEZYME® RSL will meet the declared activity upon arrival at your plant. SPEZYME® RSL can be safely stored in sealed containers. Enzyme should be stored below 25°C (77°F) and sheltered from direct sunlight for maximum stability.

SAFETY & ENZYME HANDLING

Inhalation of enzyme dust and mists should be avoided. In case of contact with the skin or eyes, promptly rinse with water for at least 15 minutes.

For detailed handling information, please refer to the appropriate Material Safety Data Sheet, the **E**nzyme **T**echnical **A**ssociation (**ETA**) handbook Working Safely With Enzymes, and the **A**ssociation of **M**anufacturers and **F**ormulators of **E**nzyme **P**roducts (**AMFEP**) handbook *Guide to the Safe Handling of Microbial Enzyme Preparations*. All are available from DuPont.

TECHNICAL SERVICE

DuPont is committed to working with ethanol producers to achieve their goals. More detailed information about the application of this product is available upon request. If you have any questions, please call us and let us know how we can be of assistance.

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REV1012 3404 SPEZYME RSL 01

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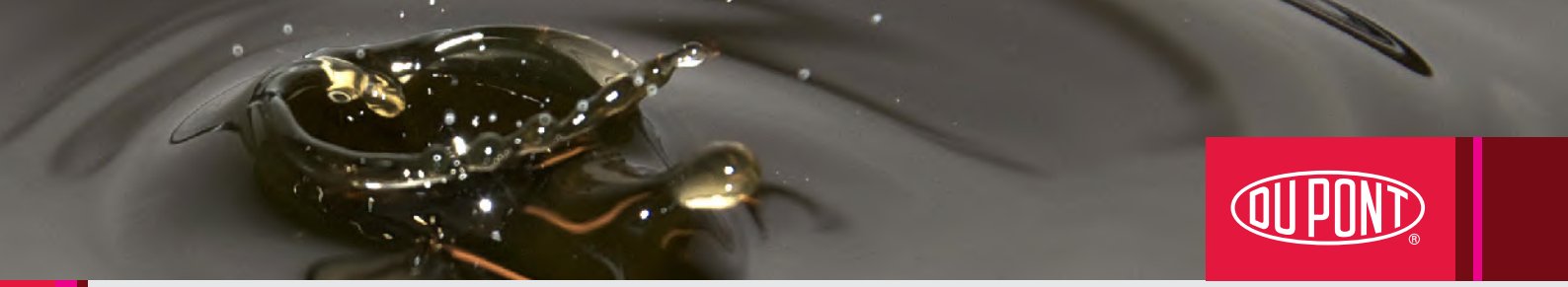
EXHIBIT 25



DISTILLASE® SSF+

Saccharifying System For Dry Grind Ethanol Production

DESCRIPTION

DISTILLASE® SSF+ is a system of enzymes designed to produce glucose from liquefied corn or milo mash during simultaneous saccharification and fermentation (SSF+) to ethanol. It contains: amylase (1,4-α-D-glucan glucanohydrolase - EC 3.2.1.1), glucoamylase (1,4-α-Dglucan hydrolyase E.C. 3.2.1.3), Aspergillopepsin 1 (EC 3.4.23.18) and phytase (EC 3.1.3.26).

The enzymes contained in DISTILLASE® SSF+ are produced by genetically modified strains of *Trichoderma reesei*.

TYPICAL CHARACTERISTICS

Activity: 380 GAU/g (minimum)
Appearance: Brown liquid
pH: 4.0 - 4.5
Specific gravity: 1.10 - 1.14 g/ml

UNIT DEFINITION

One **G**lucoamylase **U**nit (**GAU**) is the amount of enzyme that will liberate one gram of reducing sugars calculated as glucose per hour from soluble starch substrate under the conditions of the assay. A detailed assay method is available upon request.

PERFORMANCE BENEFITS

The DISTILLASE® SSF+ systems' unique composition improves fermentation yield and consistency. It does so by minimizing the fraction of starch that remains unfermented and enhancing yeast nutrition.

The glucoamylase in DISTILLASE® SSF+ system rapidly converts the dextrins to glucose across a broad range of starting pH (4.0 to 5.8). This results in 5 - 10% faster initial ethanol production.

The acid stable saccharifying amylase in the DISTILLASE® SSF+ effectively hydrolyzes starch that would not otherwise be converted to glucose during fermentation.

The protease in the DISTILLASE® SSF+ system hydrolyzes protein matrices in the corn kernel that bind the various fractions enabling the hydrolysis of "hard" to hydrolyze starch. It also increases the levels of essential yeast nutrients in the form of amino acids, peptides and free amino nitrogen.

The phytase in DISTILLASE® SSF+ generates inositol an important yeast nutrient and increases the available phosphorous in DDGS.

APPLICATION RECOMMENDATIONS

DISTILLASE® SSF+ is used to saccharify liquefied mashes of whole ground corn and milo. The resultant glucose is fermented by yeast to produce ethanol. All of the enzymes in DISTILLASE® SSF+ perform well within the temperature and pH ranges typical in whole ground corn and milo batch fermentations: 28 to 35°C (82 to 95°F) and 4.0 to 5.5 respectively. In high gravity (>34% solids) and/or milo fermentations performance can be further enhanced by the addition of FERMGEN® acid fungal protease.

DOSAGE RECOMMENDATIONS

DISTILLASE® SSF+ should be dosed at 0.06 to 0.08% weight enzyme / weight as is corn. The actual dose required will depend upon the conditions of your fermentation: time, initial pH and the level of solids.

REGULATORY STATUS

DISTILLASE® SSF+ is **GRAS** (**G**enerally **R**ecognized **A**s **S**afe) in the United States for use in alcohol production with the resultant distillers grains being used in animal feed.

The ingredients in DISTILLASE® SSF+ are in compliance with the Toxic Substances Control Act in the United States and Domestic Substances List in Canada.

PACKAGING

DISTILLASE® SSF+ is available in various package sizes and bulk tankers. Please consult your DuPont representative for detailed information.

STORAGE

DISTILLASE® SSF+ will meet the declared activity upon arrival at your plant. DISTILLASE® SSF+ can be safely stored in sealed containers. Enzymes should be stored below 25°C (77°F) and sheltered from direct sunlight for maximum stability.

SAFETY & ENZYME HANDLING

Inhalation of enzyme dust and mists should be avoided. In case of contact with the skin or eyes, promptly rinse with water for at least 15 minutes.

For detailed handling information, please refer to the appropriate Material Safety Data Sheet, the **E**nzyme **T**echnical **A**ssociation (**ETA**) handbook *Working Safely With Enzymes*, and the **A**ssociation of **M**anufacturers and **F**ormulators of **E**nzyme **P**roducts (**AMFEP**) handbook *Guide to the Safe Handling of Microbial Enzyme Preparations*. All are available from DuPont.

TECHNICAL SERVICE

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REV0712 3476 DISTILLASE SSF+ 01

EXHIBIT 26

How Ethanol Production Technology Impacts DDGS Value in Animal Nutrition

Don Cannon, Ph.D.; DuPont Industrial Biosciences
Biorefineries Applied Innovation Center in Cedar Rapids, IA
in collaboration with
DuPont Danisco Animal Nutrition (Milan Hruby, Ph.D.)

18th Annual Distillers Grains Symposium, Dallas, TX; May 14, 2014

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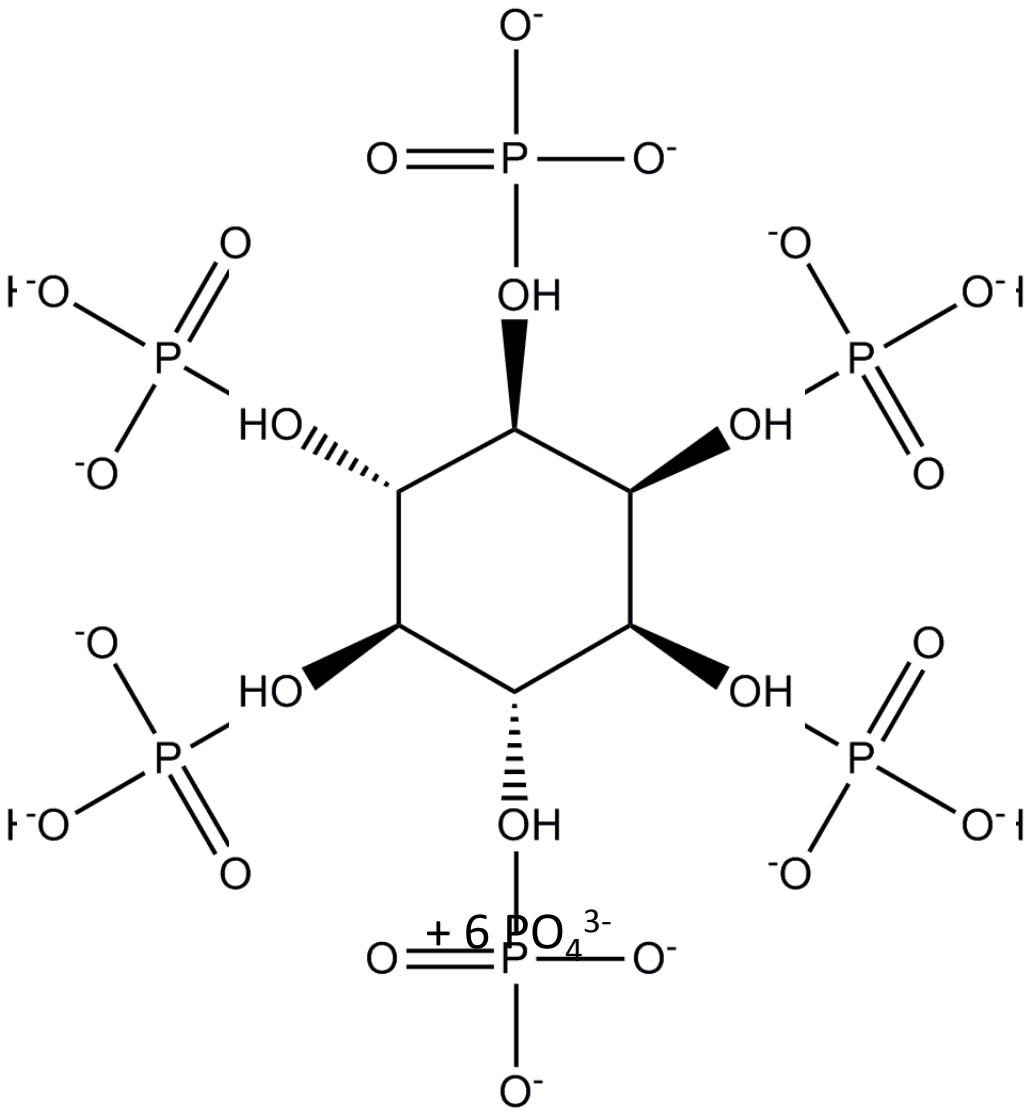
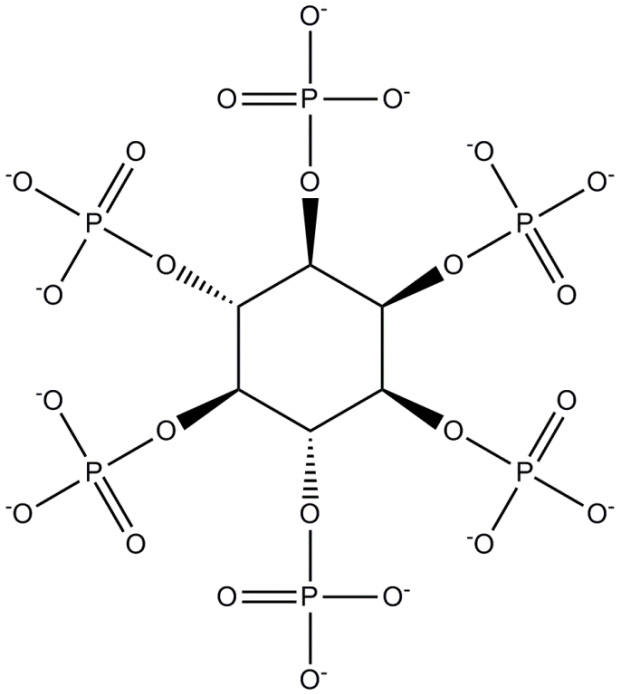
Phytic Acid Molecular Structures of Interest



Some Molecular Facts about Phytic Acid



***myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate**

- phytate (ionic form); phytin (mineral complexed); IP6
- reactive acidic compound that chelates cations
  - more strongly associates with divalent over monovalent
  - 12 protonation sites; 6 pKa's more acidic than the other 6 pKa's
- 1 to 3 %w/w in seeds, cereals, and oilseeds used in animal feed
  - different locations in different grains
  - associated with protein in oilseeds and grain legumes
  - various amounts of endogenous phytase (little to none in corn, high variability in wheat)
- phytin accounts for up to 80% of all P in animal feed
- phytin must be degraded for sufficient P availability (and reduced environmental impact)
- availability of energy and amino acids is reduced when IP6 is present in ingredients and animal feeds
- Phytate alters the secretion of endogenous compounds (animals digestive enzymes, HCl, mucins, etc.)
- DDGS with a reduced phytate content may have a superior nutritional value compared with conventional DDGS
- phytin levels in DDGS can vary greatly – phytase use in fuel ethanol process

Phytate Content and Endogenous Phytase Enzyme Activity in Grains

Grain/Cereal	Phytate [1] (% ds)	Endogenous Phytase [2] (units/kg)
Corn	0.8 -1.2	~20
Sorghum	0.8 -1.1	~20
Wheat	0.25 -1.37	1193
Barley	0.38 -1.16	582
Rye	0.54 -1.46	5130
Triticale	0.50 -1.89	n.d.

Reference: 1) Food Phytases,Edited by Reddy,N. R and Sathe,S. K (2002) CRC Press, New York

2) Ravidran V., W.L. Bryden and E. T. Kornegay. 1995. Phytates: Occurrence, bioavailability and implications in poultry nutrition. Poultry and Avian Biology Reviews. 6 (2): 125-143

DuPont Danisco Animal Nutrition Phytases

granular phytase enzyme for reduced feed costs and a better environment

Phyzyme® XP

Impact of Phytate on Fuel Ethanol Production

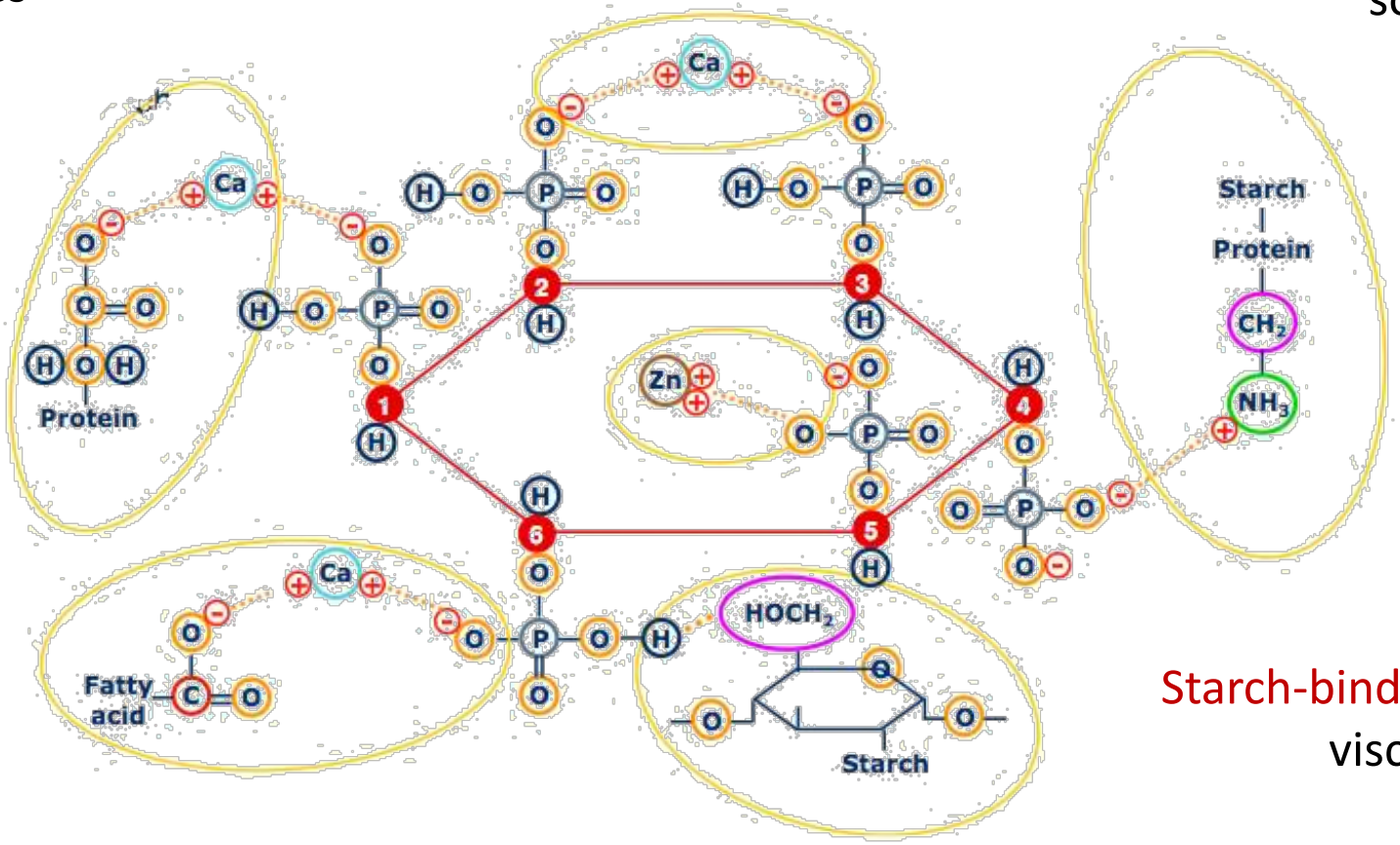
http://en.wikipedia.org/wiki/File:Ethanol_plant.jpg (photo by Steven Vaughn), May 2011

Why Do We Care About Phytate in Fuel Ethanol Production?

direct protein contact can reduce stability of enzymes

calcium is needed for α-amylase stability

starch-protein complexes are less soluble



Starch-binding increases viscosity

Zinc is an essential mineral for yeast

The Genencor Timeline for Phytase Application

March: Product Launch GC 491 a phytase product for ethanol production

Maximizing Ethanol Production from Grains

Paper presented at World Ethanol 2004 & Ethanol Production Workshop by George Virgil

INTERNATIONAL sugar

Article Phytase in fuel ethanol production published in the International Sugar Journal

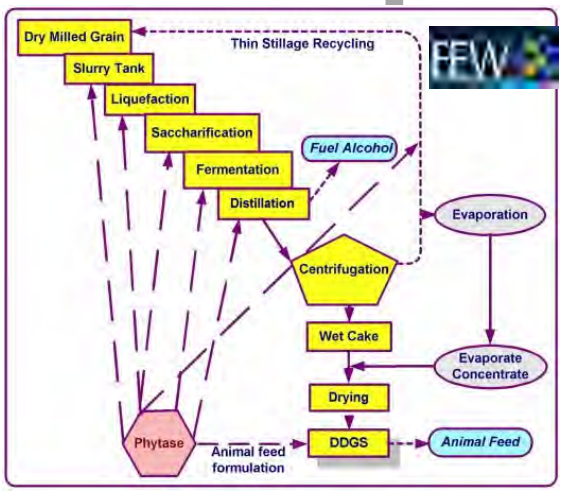
Product Launch MAXALIQ One

Product Launch SPEZYME® RSL

2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011

Application of Phytases in Fuel Ethanol Production

April: Paper presented at the 2001 Detmold Starch Conference by Gerhard Konieczny Janda

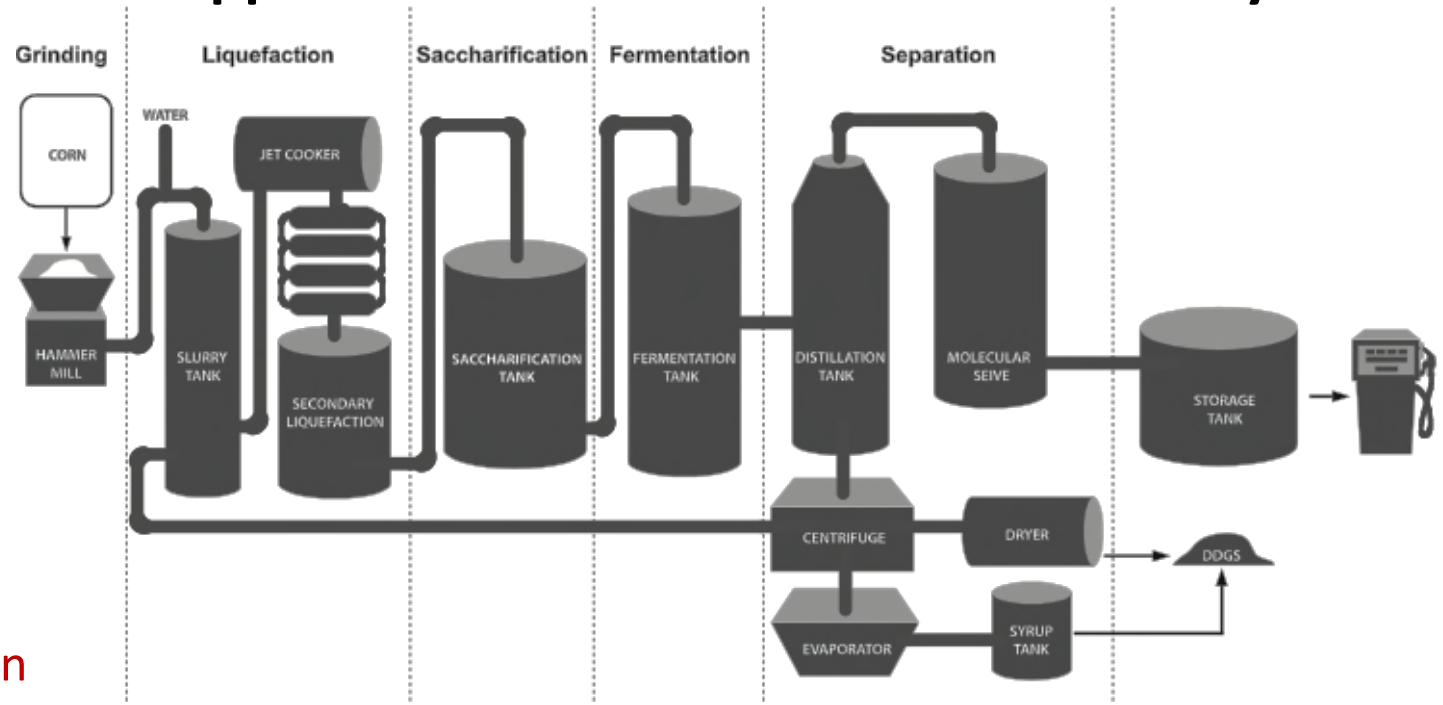


Fuel Ethanol Workshop presentation given by Dr. Jay Shetty

Product Launch DISTILLASE SSF+ a blend for SSF which contains phytase

Engineered Phytases *with Enhanced Thermostability*

Where Phytase is Applied in Fuel Ethanol Production Today



In Liquefaction

- To enhance the pH and temperature stability of alpha amylases
- Liquefaction at pH 5.0 to 5.3 reduces the amount of H_2SO_4 and anhydrous ammonia needed
  - Lower total cost of liquefaction
  - Decreasing the sulfur present in wet distillers grains and DDGS

In Fermentation

- To generate inositol*, an important yeast nutrient
- To maximize the level of free phosphate in residuals (DDGS)

Reference:

* Determining the effects of inositol supplementation and the *opi1* mutation on ethanol tolerance of *Saccharomyces cerevisiae Erin L. Krause, Manuel J. Villa-García, Susan A. Henry, Larry P. Walker. Industrial Biotechnology. October 2007, 3(3): 260-268. doi:10.1089/ind.2007.3.260.*

Dry Grind Liquefaction Has Some Issues

- Liquefaction has typically required two pH adjustments
  - From 5.1 to 5.8
  - Back to the chosen fermentation pH (5.8 to 4.5)
- It requires tight pH and temperature control
  - A pH variance of 0.2 can hurt performance
  - A temperature variance of +2° can hurt performance
- Adds significant sulfur to the system and eventually to DDGS (0.08 to 0.12% dmb)
- At many plants, two enzyme doses are necessary – adding complexity
- Poor liquefaction reduces ethanol yields and plant performance

Plant	Backset	Backset, Water and Corn
A	4.3	5.4
B	4.2	5.7
C	4.3	5.1
D	3.4	5.6
E	3.7	5.2
F	4.2	4.8
G	4.3	5.1
H	3.7	4.7
I	4.3	5.1
J	4.5	5.0
K	3.6	4.9
L	3.4	5.1
M	3.8	4.7
Average	4.0 ± 0.4	5.1 ± 0.3

Recommended Conditions for Liquefaction

Conventional α-amylase use:

Solids	30 to 36% wt/wt
pH Range	5.6 to 6.0
Temperature Range	170 to 190° F
Liquefaction Time	90 to 140 minutes

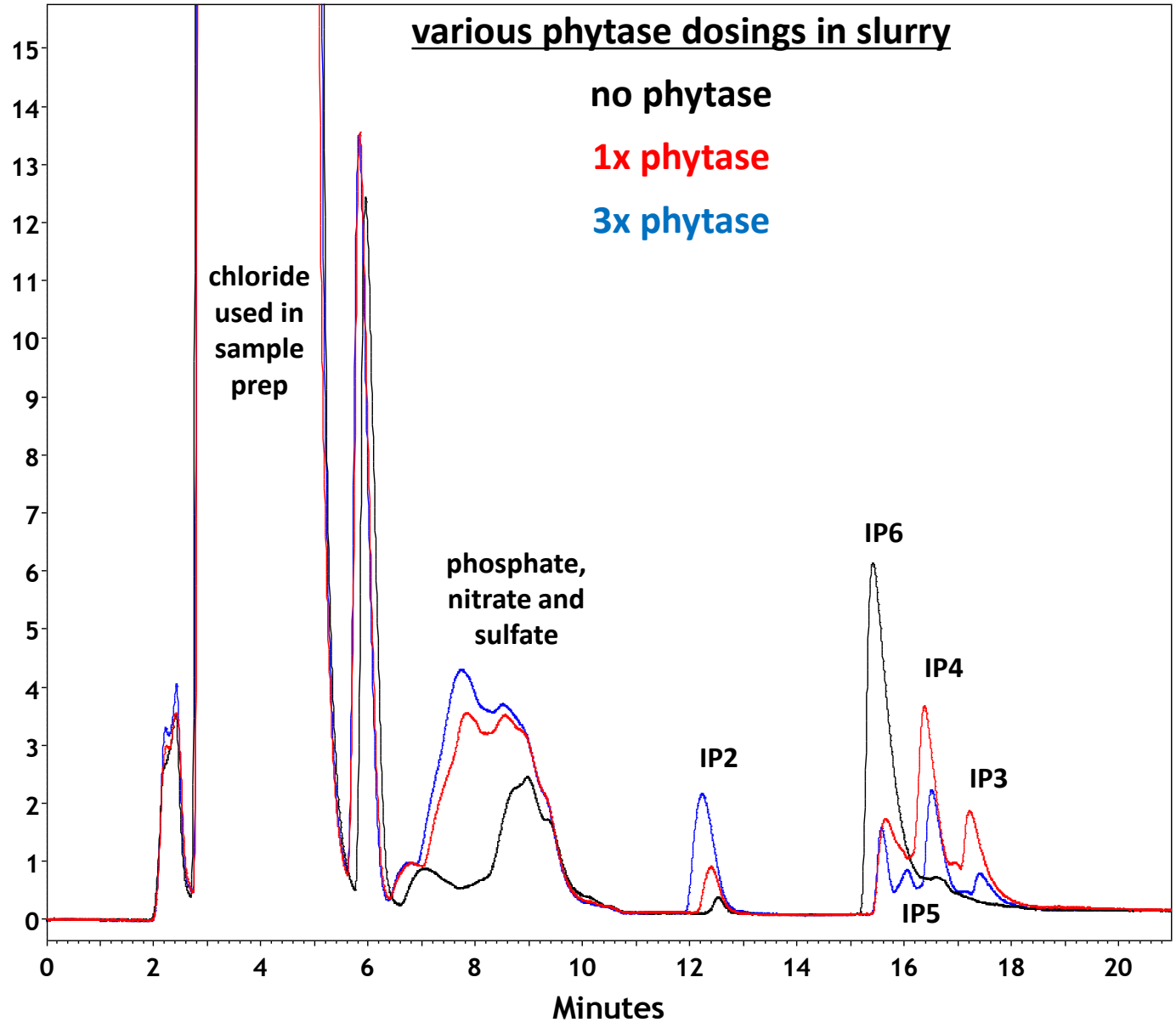
Recommended Dose: 0.020 to 0.024 %wt/wt as-is grain

SPEZYME® RSL (α-amylase + phytase) use:

Solids	30 to 36% wt/wt
pH Range	5.0 to 6.0
Temperature Range	170 to 195° F
Liquefaction Time	90 to 140 minutes

Recommended Dose: 0.020 to 0.024 %wt/wt as-is grain

Ion Chromatography for Inositol Phosphates



Dionex OmniPac PAX-100 column

0.20M NaOH gradient (2.5% IPA isocratic)

5%-25% in 8 min

25%-70% in 7 min

regen and equil steps

suppressed conductivity detection

supernatant dilution to 0.5M HCl

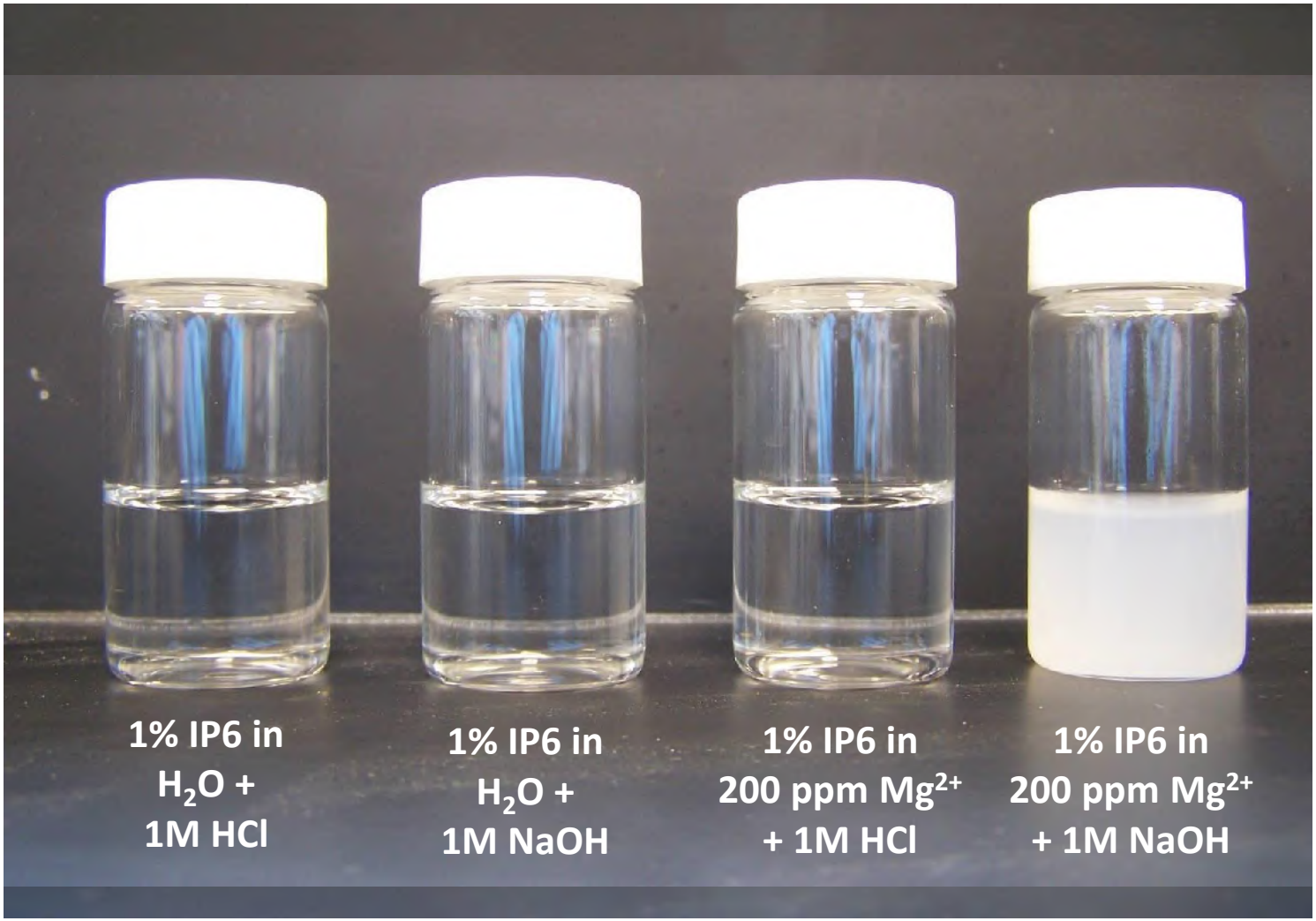
liquefaction supernatant: 0.25 %w/v supernatant, (31.6 DS% equates to 0.8 %w/w corn as is basis)

The Analytical Method: sample preparation

multiple chemical equilibriums to consider:

K_a's...K_{chel}'s...K_{sp}'s

$IP_6H_{12} \leftrightarrow IP_6^{12-}$; $IP_6^{12-} + M^{2+} \leftrightarrow IP_6M_6$; $\underline{IP_6}_{\ s} \leftrightarrow IP_{6\ aq}$



Phytate is Soluble in Acidic Environments

(i.e. phytate is NOT soluble in many conditions)

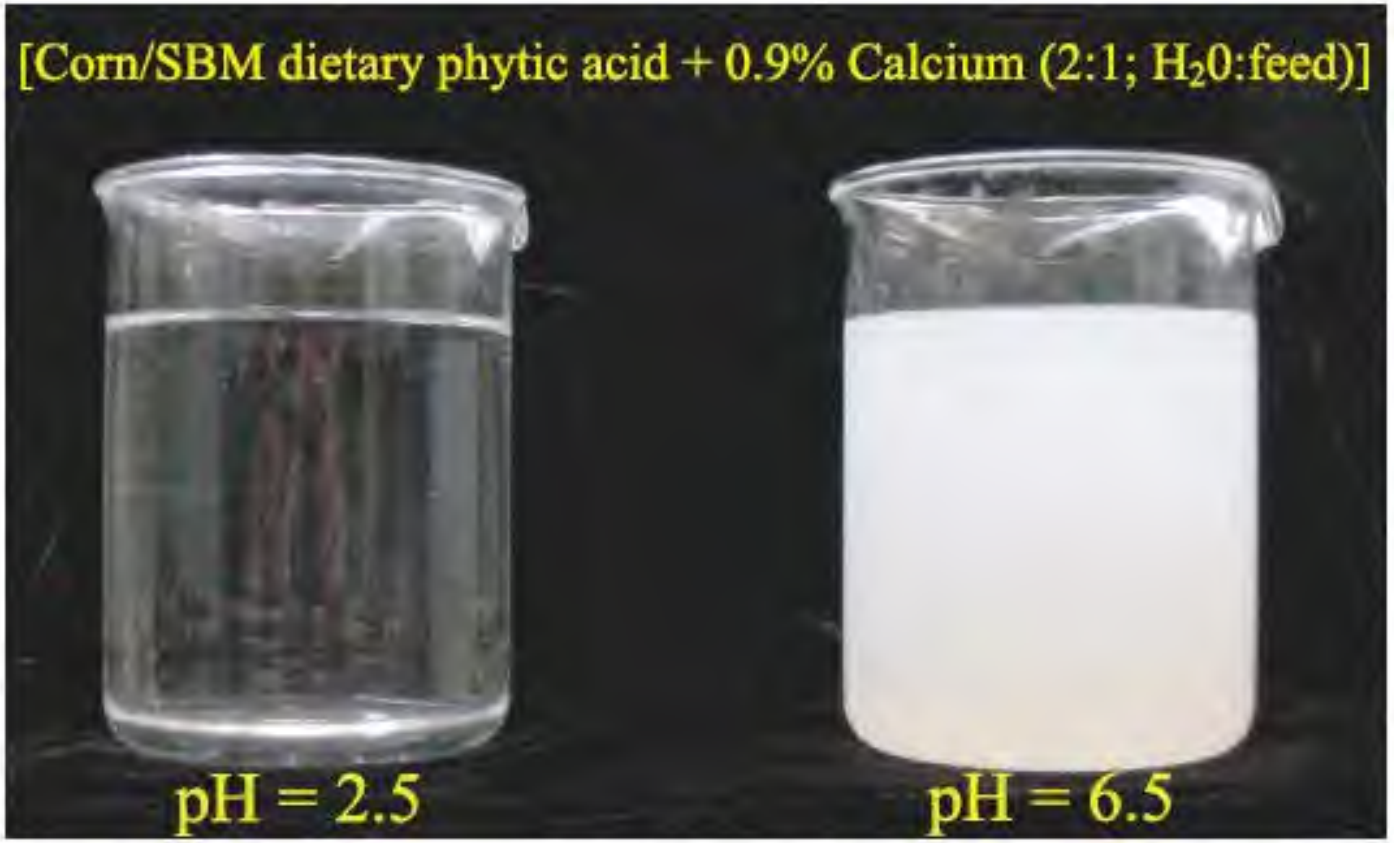
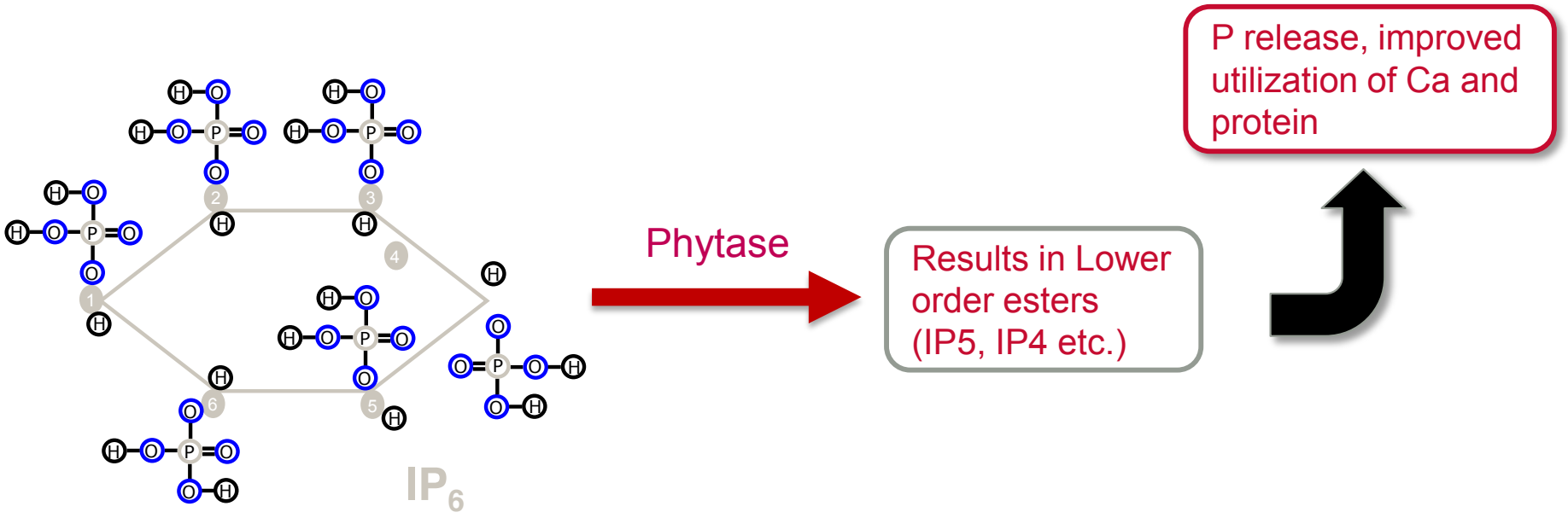


Figure 2: Demonstration of what occurs to phytin-Ca complex in the stomach (pH 2.5) and small intestine (pH 6.5). At the higher pH, phytase cannot work as easily on the substrate phytin because the subtstrate is precipitated.

Applegate, T.J. and Angel, R.; "Phytase: Basics of Enzyme Function," Purdue Univ. Extension, *Farm Animal Management @ Purdue*, **2004**, *Doc. AS-560-W*.

Phytase Use at Low pH Reduces the Antinutrient Effects of Phytate

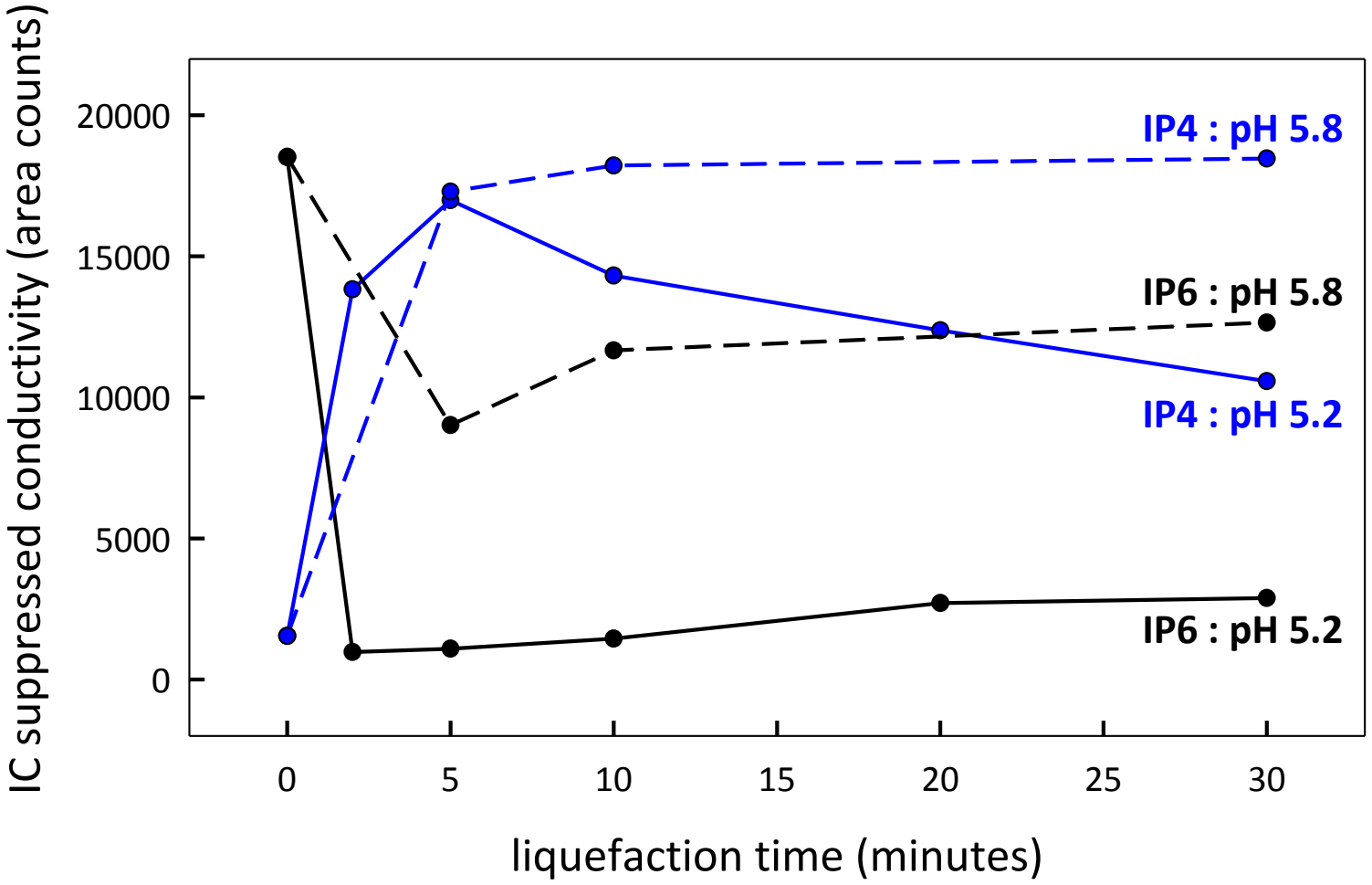
Phytate is more soluble at low pH and more susceptible to phytase attack



The most effective phytases will be those that lower **IP_6** concentrations **most rapidly at acidic pH**

Phytic Acid Reduction

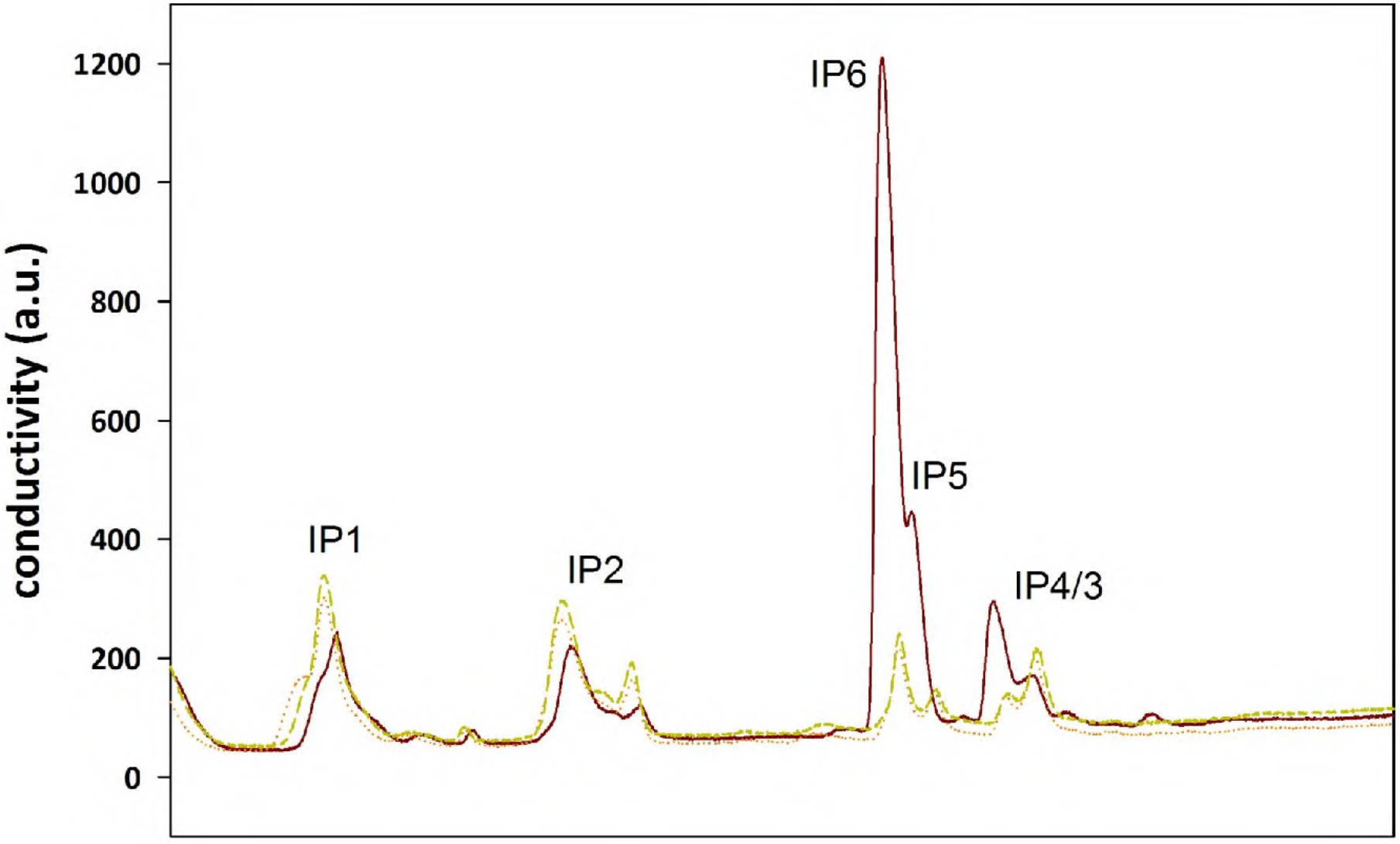
SPEZYME RSL Liquefaction; 32 %DS corn; 85 °C



Phytic Acid Monitoring in DDGS

DDGS w/ no phytase in process

DDGS w/ phytase in liquefaction



Phytate in DDGS using Spezyme® RSL

no phytase in liquefaction

	phytic acid (%w/w)
Plant 1	0.71
	0.71
Plant 1	0.75
	0.68
Plant 1	0.77
	0.80
Plant 2	0.62
	0.63
Plant 2	0.56
	0.60
Plant 2	0.58
	0.56
Plant 3	0.58
	0.62
Plant 3	0.70
	0.68
Plant 3	0.61
	0.62

each DDG was sampled twice,

same plants were sampled at later times

http://www.ethanolproducer.com/articles/8088/2011-ddgs-exports-to-japan-up-45-likely-to-set-records

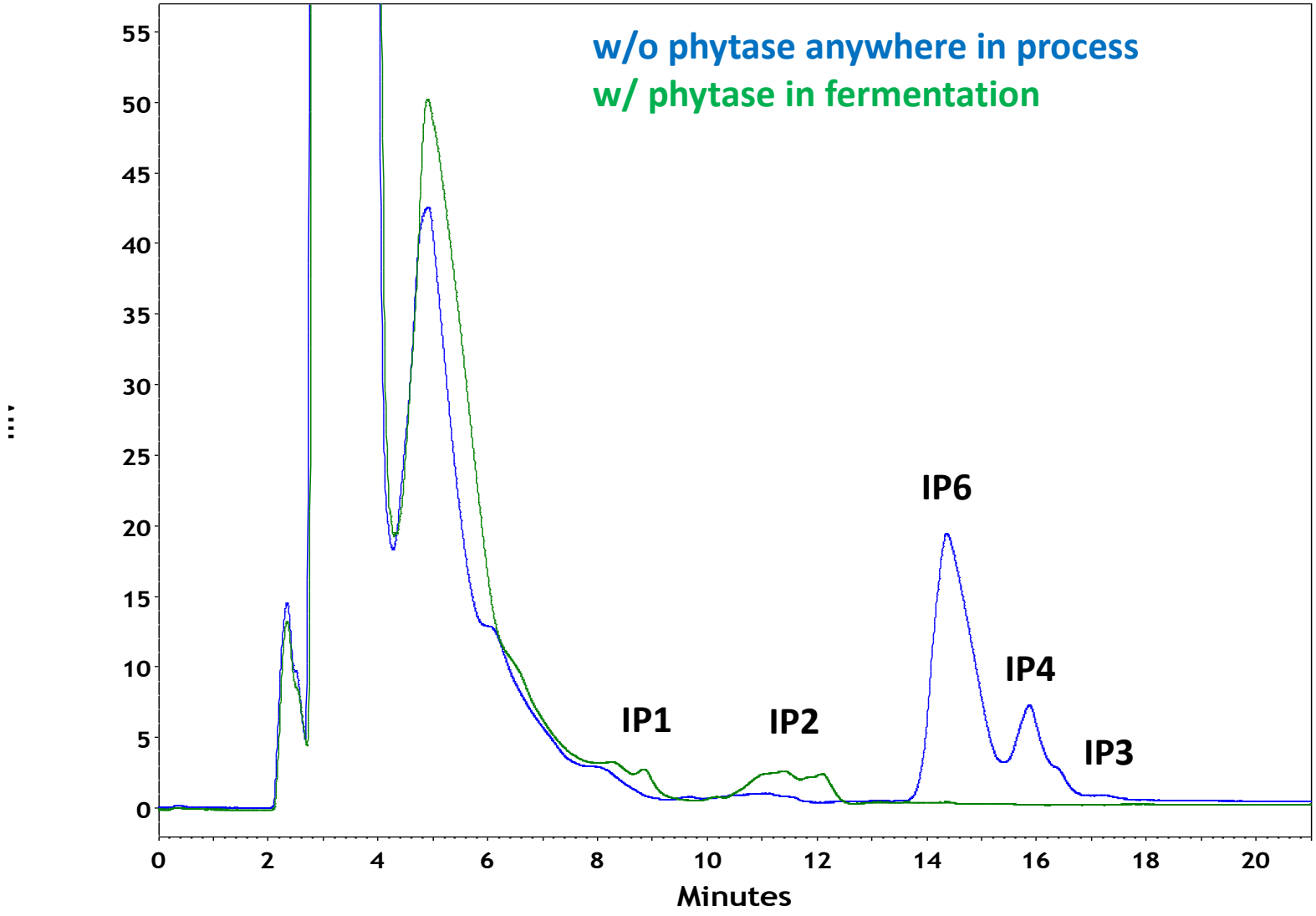
phytase in liquefaction

	phytic acid (%w/w)
Plant A	0.19
	0.21
Plant A	0.23
	0.23
Plant A	0.15
	0.16
Plant B	0.18
	0.17
Plant B	0.13
	0.13
Plant B	0.14
	0.15
Plant C	0.23
	0.24
Plant C	0.26
	0.28
Plant C	0.24
	0.25

DISTILLASE® SSF+ : phytase in fermentation

The DISTILLASE® SSF+ unique composition improves fermentation yield and consistency. It does so by minimizing the fraction of starch that remains unfermented and enhancing yeast nutrition.

It contains: glucoamylase, acid stable a-amylase, protease, and phytase



Fermentation is Better Environment for Phytase

Phytic Acid Monitoring in DDGS

DDGS Samples	phytic acid (%w/w)
no phytase added	*0.4 - 1.0*
SpezymeRSL	*0.1 - 0.3*
SSF and prop phytase	0.050 ± 0.006
SSF+	0.005 ± 0.002
SSF++	0.003 ± 0.001
SSF+ and prop phytase	0.001 ± 0.001

Estimating the Value of Improved DDGS

- Substantial Market
  - Swine: 2.8MM tons potential DDGS demand @ 10% inclusion
  - Poultry: 5.6MM tons
- Reduced phosphate supplementation by feed formulator
  - direct savings of added dicalcium phosphate
- Increased digestibility
  - sparing of more costly ingredients such as soybean oil or meal
- Increased DDGS consistency
  - More attractive ingredient, allows higher inclusion rates
- Less phosphorous runoff from feedlots

Estimating the Value of Improved DDGS: *reducing phosphorous waste*

You can't teach pigs to whistle, but you can reduce their impact on the environment

Environmental issues, including waste management, are the number one concern of livestock and poultry producers.

As the size and intensity of swine and poultry production operations have increased, so has the amount of manure produced, often with limited land area available for manure application, says E.T. Kornegay, professor of animal and poultry science. "This can lead to a build-up of nutrients in and on the soil with the potential for runoff into waterways."

Excess environmental phosphorus is suspected as a contributing factor in water-quality degradation and even in the bloom of *Pfiesteria piscicida*, which has caused fish kills in North Carolina and in tributaries to the Chesapeake Bay.

Virginia Tech animal and poultry science researchers, led by Kornegay, have developed feeding regimes for swine and poultry that result in massive reductions of phosphorus and other nutrients in manure. Kornegay's recommendations are being used nationwide, and he lectures about his research worldwide.

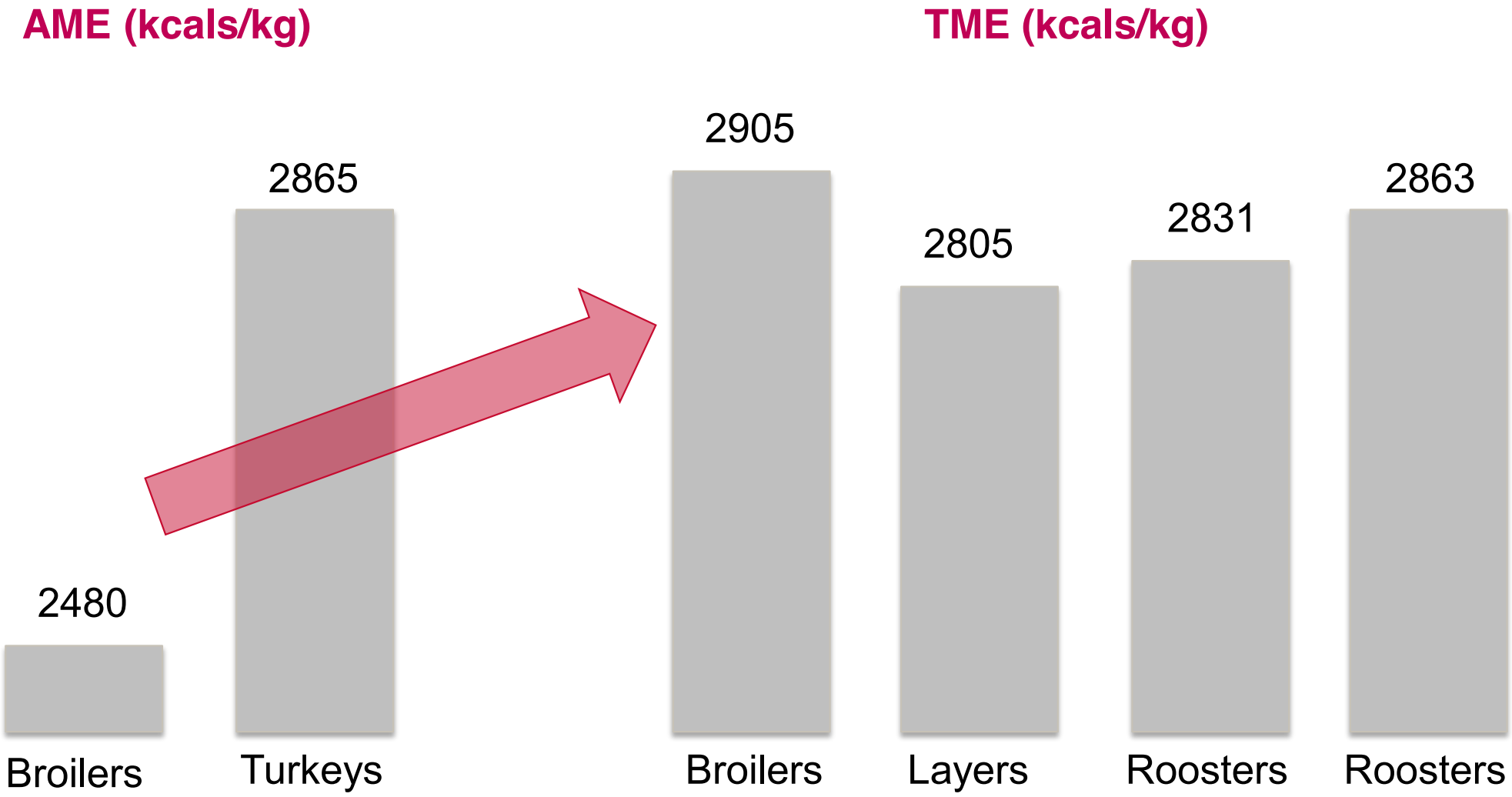
The key is what Kornegay calls "a miracle enzyme," microbial phytase.

Susan Trulove, in Virginia Tech Research magazine

Feed formulation savings example

- Each ton of DDGS saved up to $14
  - Broiler feed formulation
  - Reduced added phosphate, soybean meal and oil
- ~1 kg of Maxaliq® ONE enzyme consumed per ton DDGS
- **Each kg of Maxaliq® ONE added up to $14 / ton in premium DDGS value !!!**
- $1/ton of DDGS premium brings $146K to a 50 MGPY ethanol plant

DDGS can be a highly variable ingredient – difficult for nutritionists to predict its value



References: NRC 1994, Noll *et al* 2004, Lumpkins *et al* 2004, Lumpkins *et al* 2005, Batal & Dale 2004, Parsons *et al* 2006

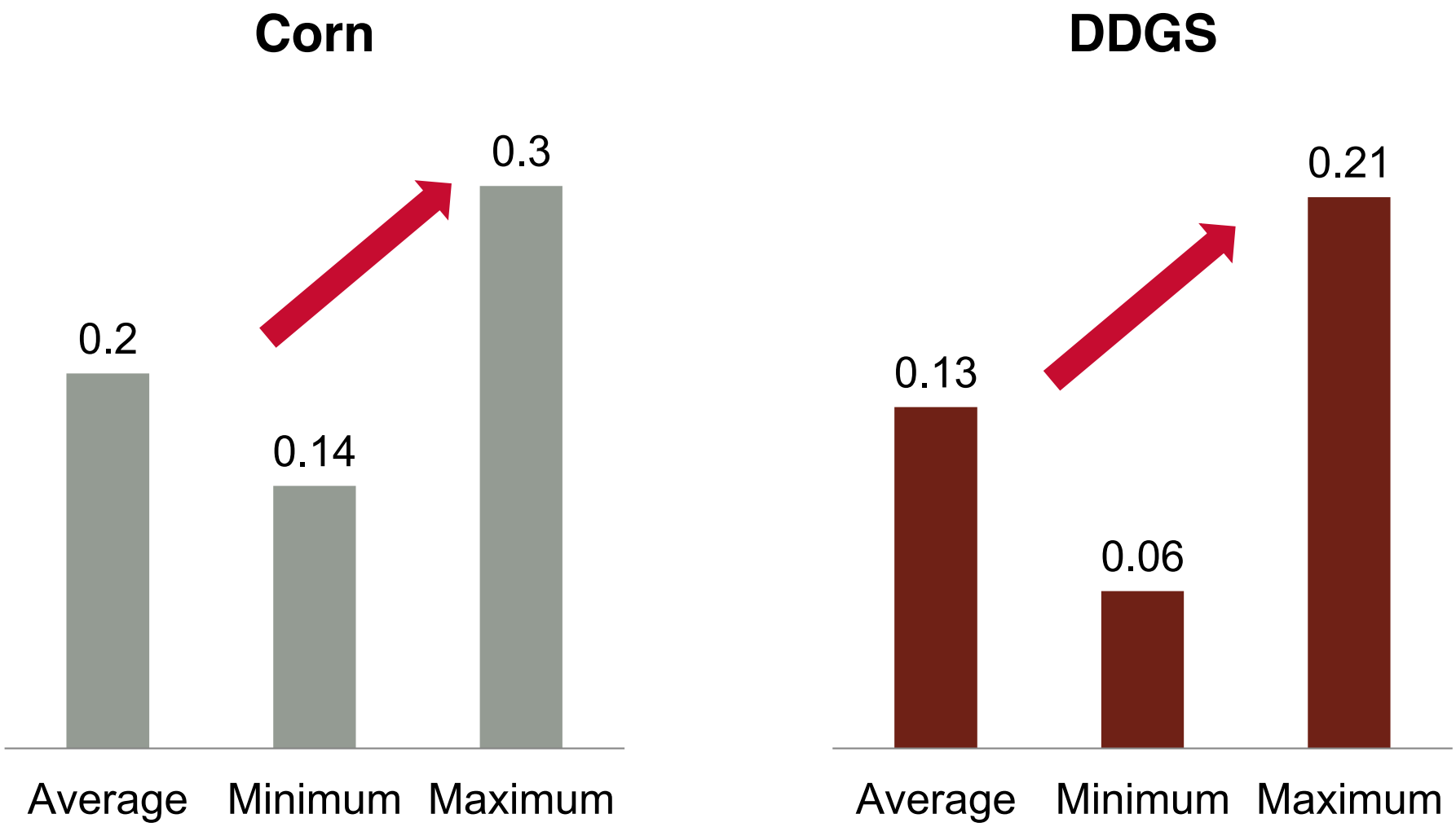
DDGS can be a highly variable ingredient – difficult for nutritionists to predict its value

Amino acid	Average	Range	CV (%)
Content (%)			
Lysine	0.73	0.59-0.89	11.6
Methionine	0.49	0.41-0.60	9.7
Threonine	0.98	0.85-1.14	6.0
Cystine	0.52	0.42-0.67	11.3
Digestibility (%)			
Lysine	72	59-84	11.2
Methionine	88	85-92	1.9
Threonine	76	69-83	4.8
Cystine	77	66-87	7.7

20 corn DDGS samples

Reference: Batal & Dale, 2006

Phytate P (%) levels in US corn and DDGS



DuPont database, harvest year 2012, corn (56 samples), DDGS (15 samples)

Nutrient Availability is Highly Variable - Phosphorus

DDGS Sample	Bioavailability (% relative to K_3PO_4)	Total P content (%)	Bioavailable P (%)
1	69	0.72	0.49
2	102	0.74	0.75
3	82	0.72	0.59
4	75	0.73	0.55
5	62	0.67	0.42
6	70	0.76	0.53
7	82	0.72	0.59
8	87	0.77	0.67
9	84	0.74	0.62
Average	79	0.73	0.58
Std deviation	11.8	0.03	0.10
CV%	15.0	3.9	16.8

The values are from a report presented at the 2006 Multistate Nutrition Conference. Ref: Parsons, C.M., C. Martinez, V. Singh, S. Radhakrishman, and S. Noll. 2006. Nutritional value of conventional and modified DDGS for poultry. Multi-State Poultry Nutrition and Feeding Conf., Indianapolis, IN. May 24-25, 2006.

Effect of Phytase in Ethanol Production on Nutritive Value of DDGS

Parameter	DDGS - phytase	DDGS + phytase	% impr.	P value
Dry matter digestibility, %	61.98	66.61	7.5	0.11
TME, kcal/kg DDGS (dmb)				
Total amino acid dig., %				
Essential AA dig., %				
Non-essential AA dig., %				

Essential amino acids: Arg, His, Iso, Leu, Lys, Met, Phe, Thr, Try, Val
Non-essential amino acids: Ala, Asp, Cys, Glu, Ser, Tyr

Same source of corn used to produce both types of DDGS: phytate P levels:
0.05% (phytase used) and 0.34% (no phytase)

Total P digestibility was increased by 27% (28.44 to 36.12%). However, the lower numbers probably reflect the fact that precision feeding method is not the best one to use to evaluate mineral digestibility.

Effect of Phytase in Ethanol Production on Nutritive Value of DDGS

Parameter	DDGS - phytase	DDGS + phytase	% impr.	P value
Lysine dig., %	71.87	75.94	5.7	0.24
Methionine dig., %				
Threonine dig., %				
Isoleucine dig., %				
Leucine dig., %				
Valine dig., %				

Same source of corn used to produce both types of DDGS: phytate P levels: 0.05% (phytase used) and 0.34% (no phytase)

Value of classic and phytase DDGS in a poultry diet

	w/ classic DDGS	w/ phytase DDGS
Corn	52.18	52.46
Classic DDGS	15.00	0.00
Phytase DDGS	**0.00**	**15.00**
SBM 48	24.20	24.16
L-LysHCL	0.21	0.21
DL-Met	0.18	0.18
NaCl	0.30	0.30
Limestone	1.09	1.25
Dical.Phosphate	0.73	0.44
Phytase	0.01	0.01
Vit/Min Premix	0.05	0.05
Fat/oil	6.06	5.96
Cost of diet, $	364.93	363.14

Diet cost difference **$1.8 per ton** of feed (with 15% DDGS) – value of 1 ton of phytase DDGS **~$12** higher than classic DDGS (based on higher P availability only)

Summary

- Phytate is a strong chelator of minerals and interacts with proteins and starch
- Ion chromatography with suppressed conductivity detection is used to resolve inositol phosphates.
- Sample preparation exemplifies the troublesome chemical nature of phytic acid
- Phytase use in dry-grind fuel-ethanol production provides various benefits
- SPEZYME® RSL provides enhanced liquefaction at lower pH's
- DISTILLASE® SSF+ provides yeast nutrients and is capable of reducing phytate levels in DDGs below detectable limits
- Phytate content in DDGS available to US animal feed industry is variable affecting P availability
- Almost no phytate P detected in DDGS samples from the ethanol processes, which are using phytase in fermentation
- DDGS derived from the phytase treatment can have significantly higher true metabolizable energy (TME) and digestibility of some amino acids, especially those present at high concentrations in endogenous protein
- DDGS with reduced phytate content has a superior nutritional value than classic DDGS

Thank You!

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EXHIBIT 27

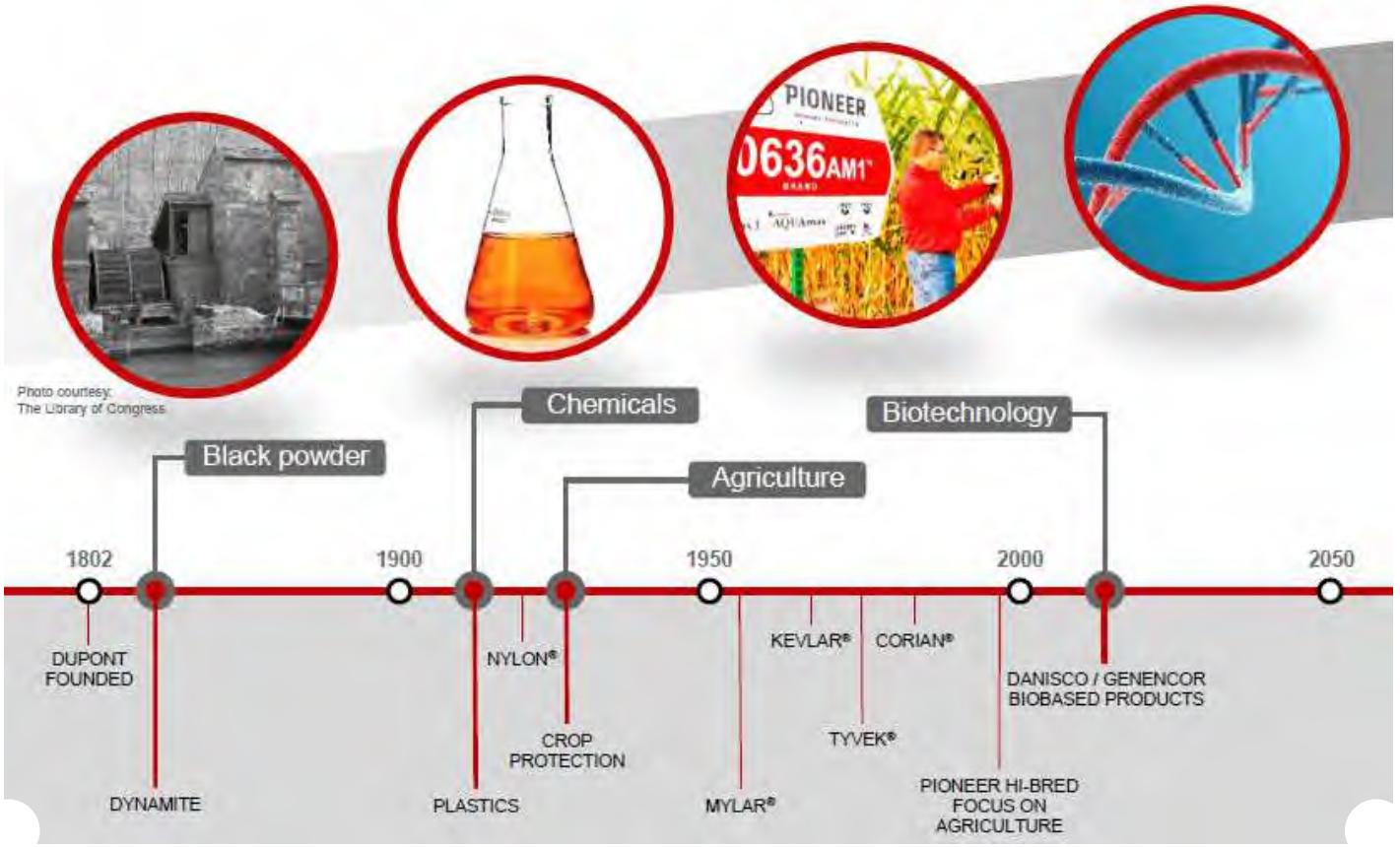
GREENWIN: White biotechnology: from research to industrial applications
Gembloux Espace Senghor, 13 May 2016

Development of microbial cell factories for lignocellulosic technologies

Arthur L. Kruckeberg

Industrial BioSciences Technology Team,
DuPont Corporation, Wilmington DE USA

- DuPont: two centuries of innovation



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- DuPont biotechnology in everyday life

©DuPont 2015

Bio-based Economy

- Biomass based products – benefits for a growing world

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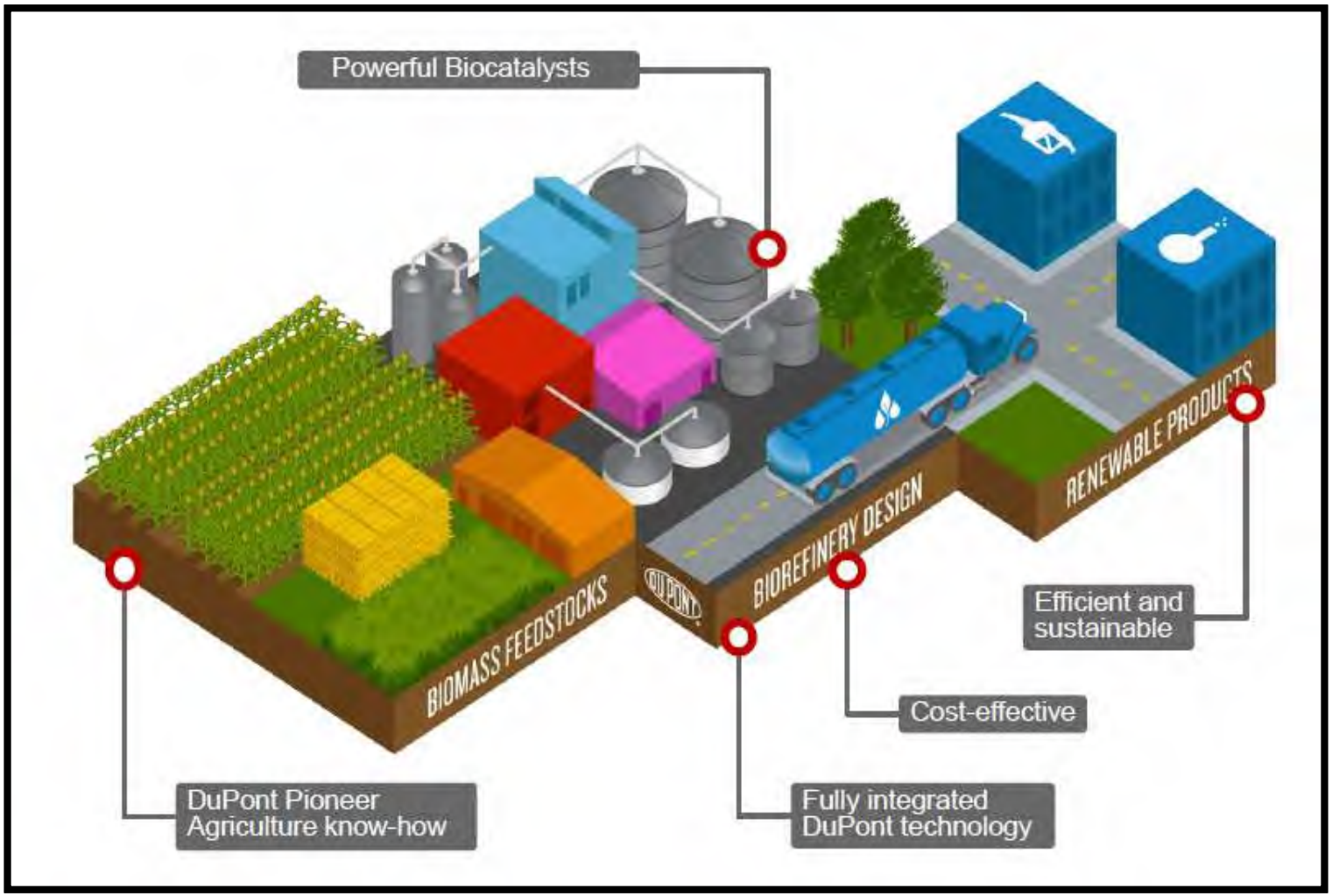
- DuPont value chain in cellulosic products



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Bio-based Economy

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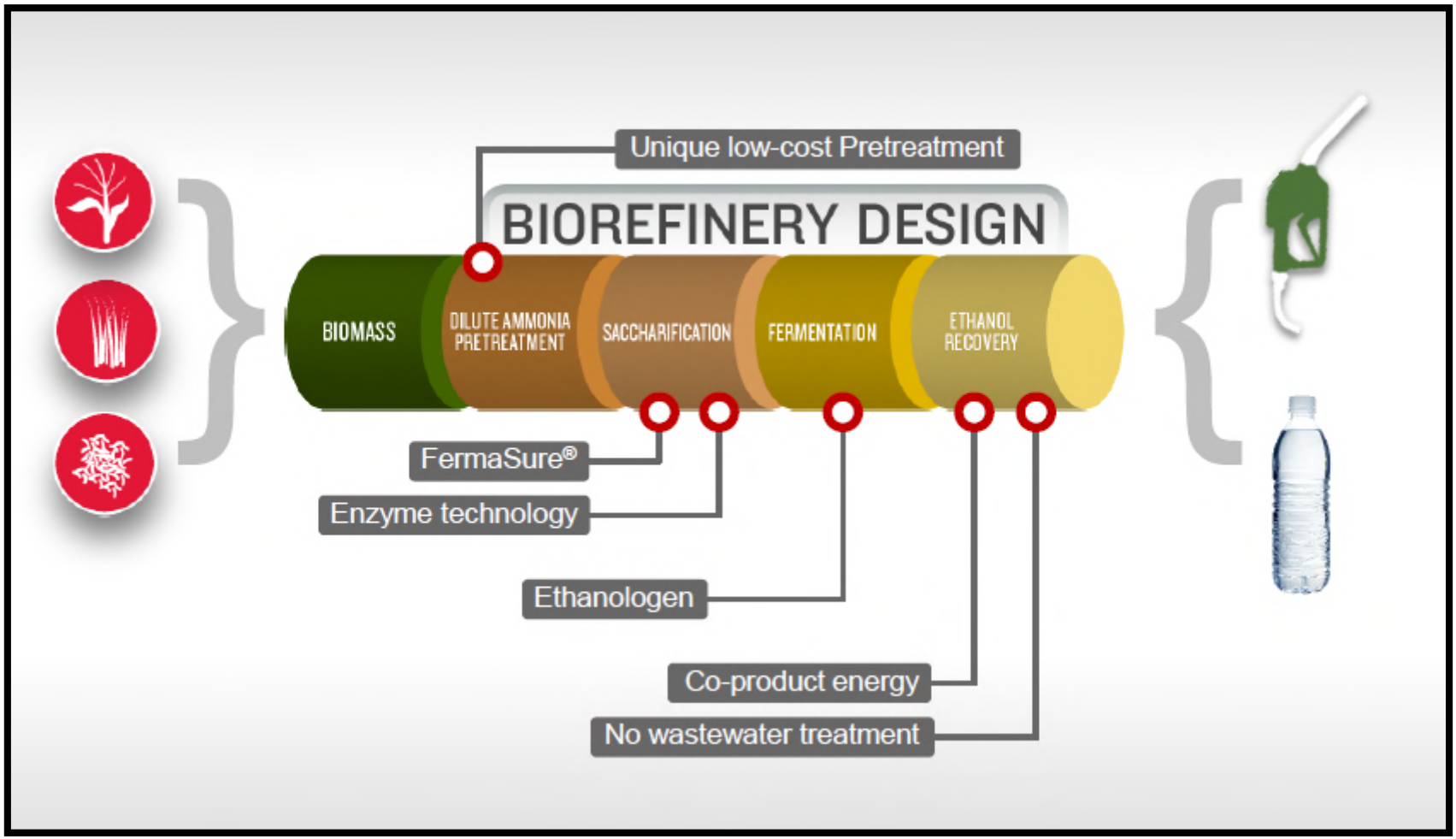
- DuPont Technologies for 2nd Gen Ethanol



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- Key benefits of DuPont Technology

FEEDSTOCK

Agronomic expertise

Multiple feedstocks

Supply chain development

CONVERSION

Integrated process

High yield

Low cost, low capital

SUSTAINABLE

~ Zero process water use

Low GHG footprint

Energy-efficient

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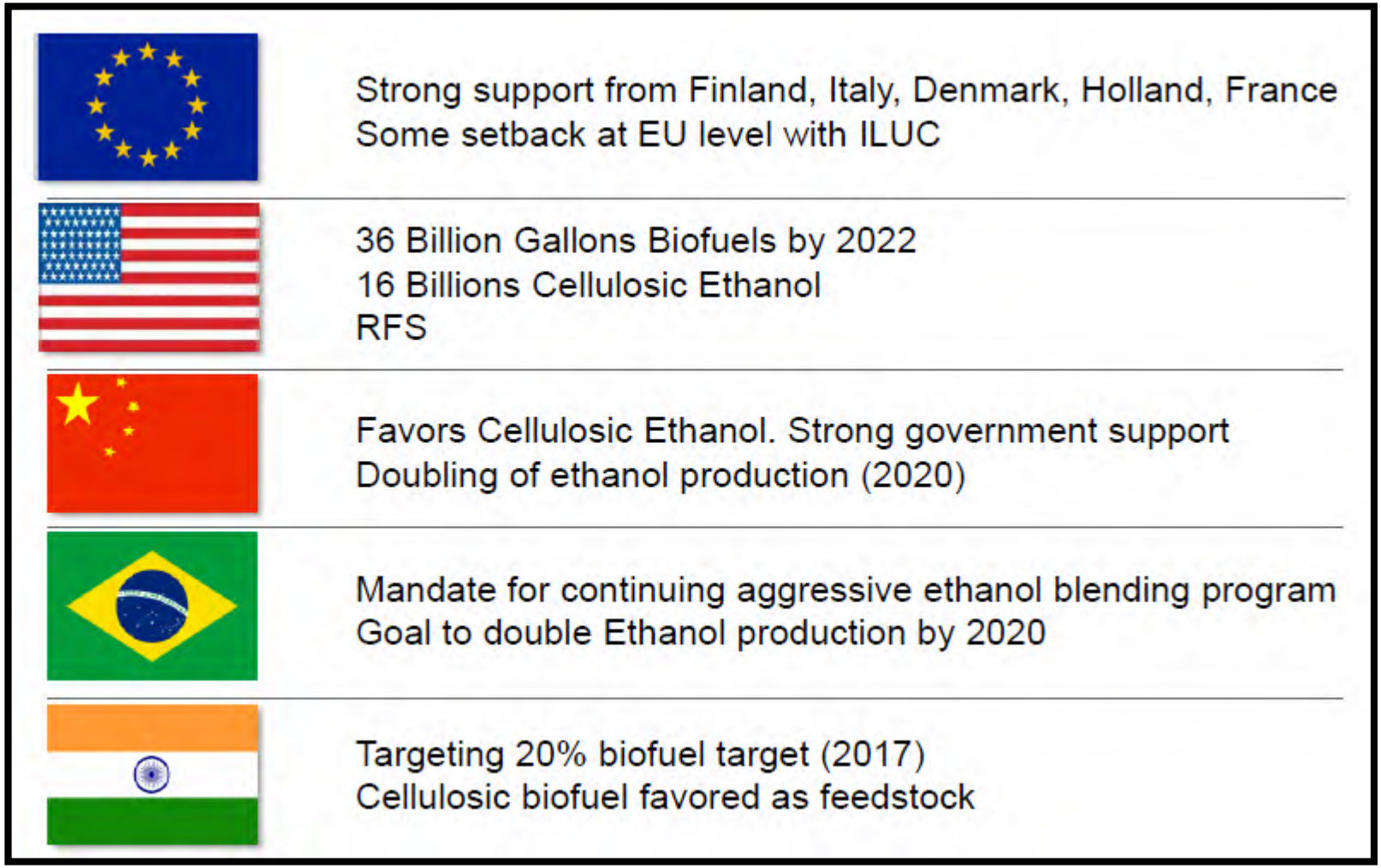
- International landscape



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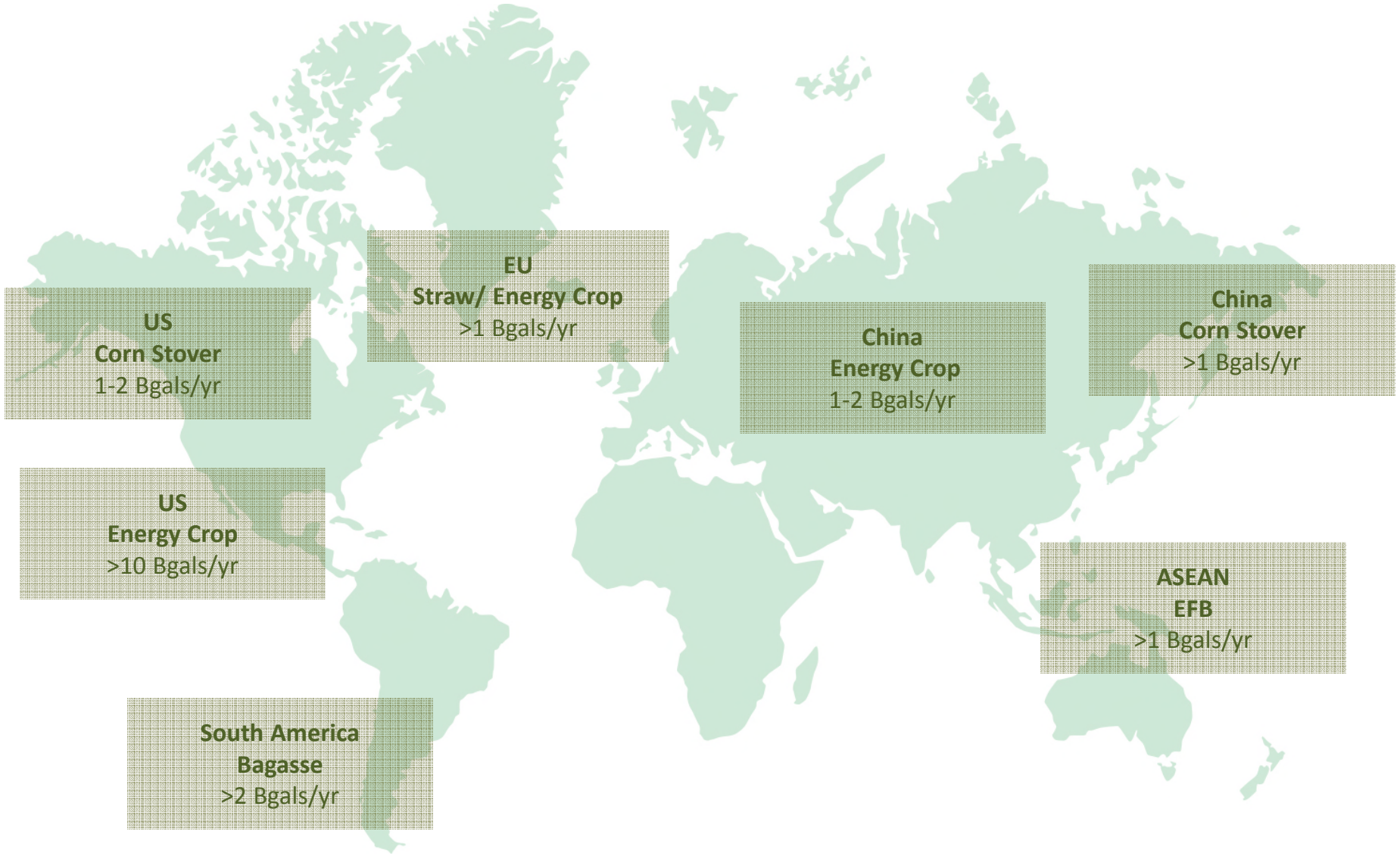
Global 2G Biofuels Feedstocks: Opportunity



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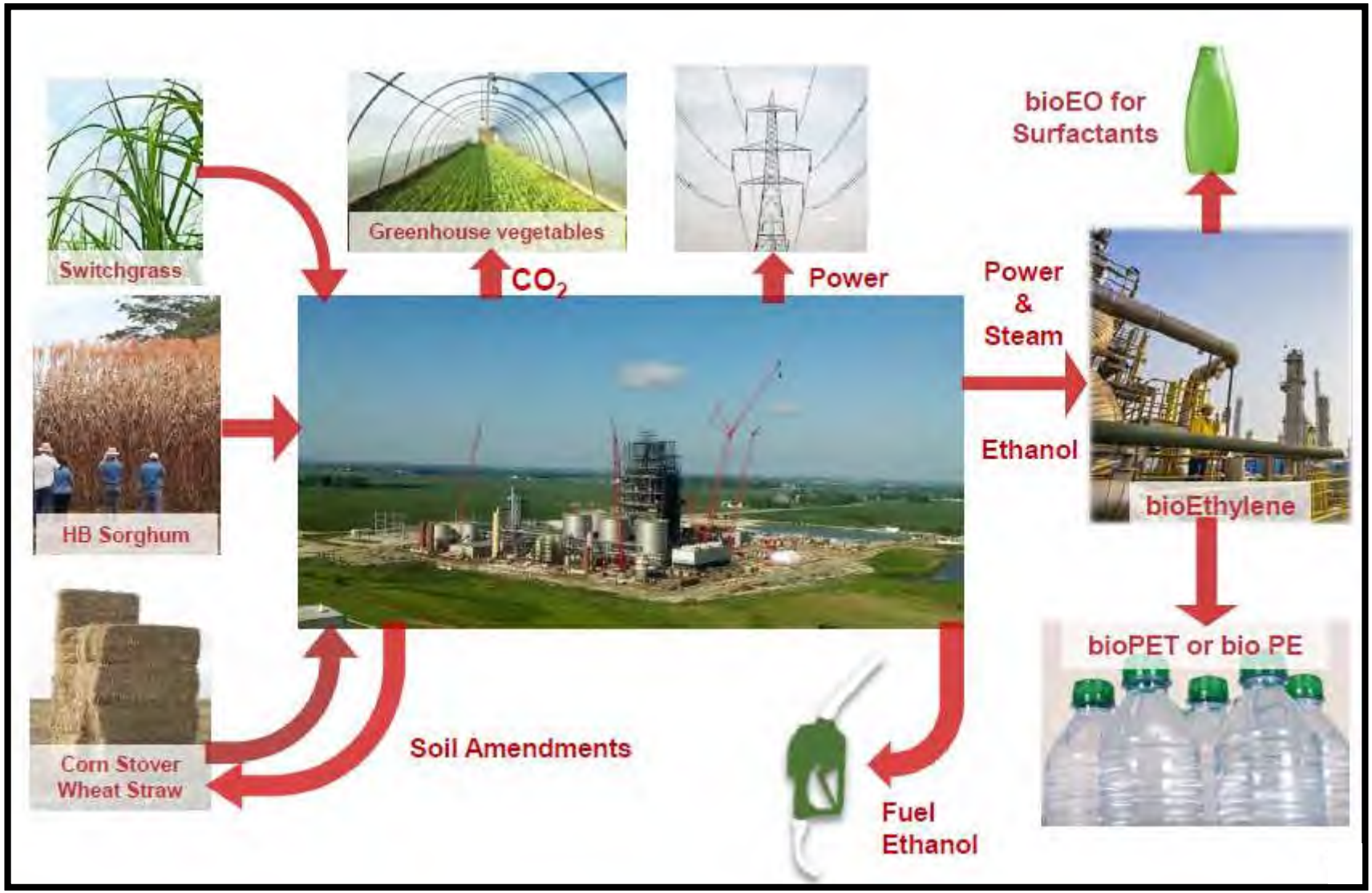
The cellulosic biorefinery



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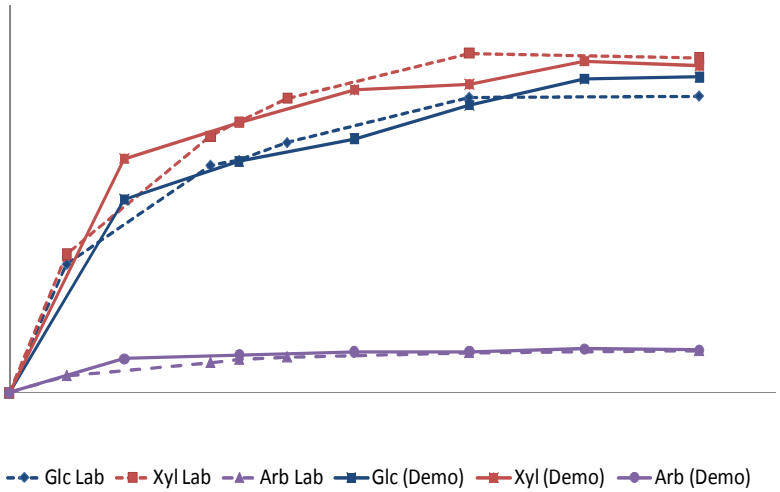
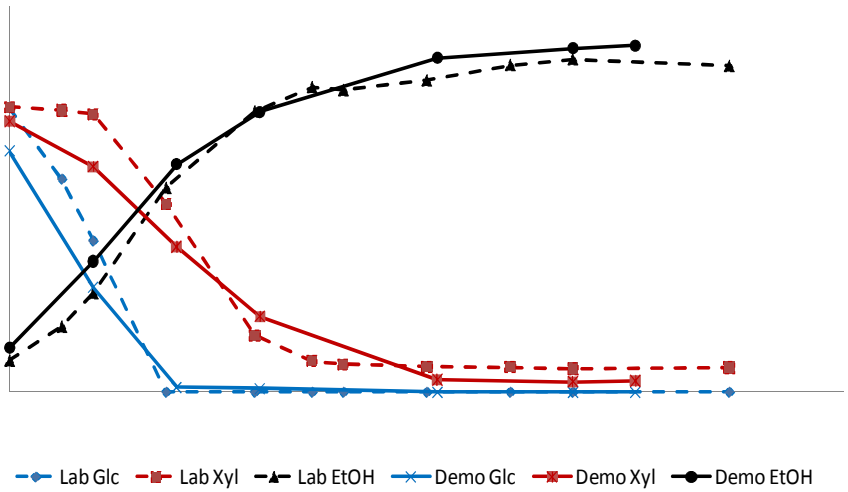
Scale-up: Lab → Pilot → …

Saccharification



Fermentation



Zymomonas mobilis: Performance comparison between 1 L and 75,000 L

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World's Largest Cellulosic Ethanol Plant
Grand opening October 30, 2015
"DuPont is leading the world in meeting our growing biofuel needs. We work with nature to create transformational new products, new business and even new industries that make a better world for us all to share," said Jim Collins, DuPont Executive Vice President leading the Bio-based Industrials strategic focus area.
"One of the largest challenges we faced was building an entirely new supply chain for plant-based raw materials. So we had to invent it," says Jan Koninckx, global business director at DuPont Advanced Biofuels.
© DuPont 2015
Photo: DuPont Cellulosic Ethanol Plant, Nevada, Iowa
16

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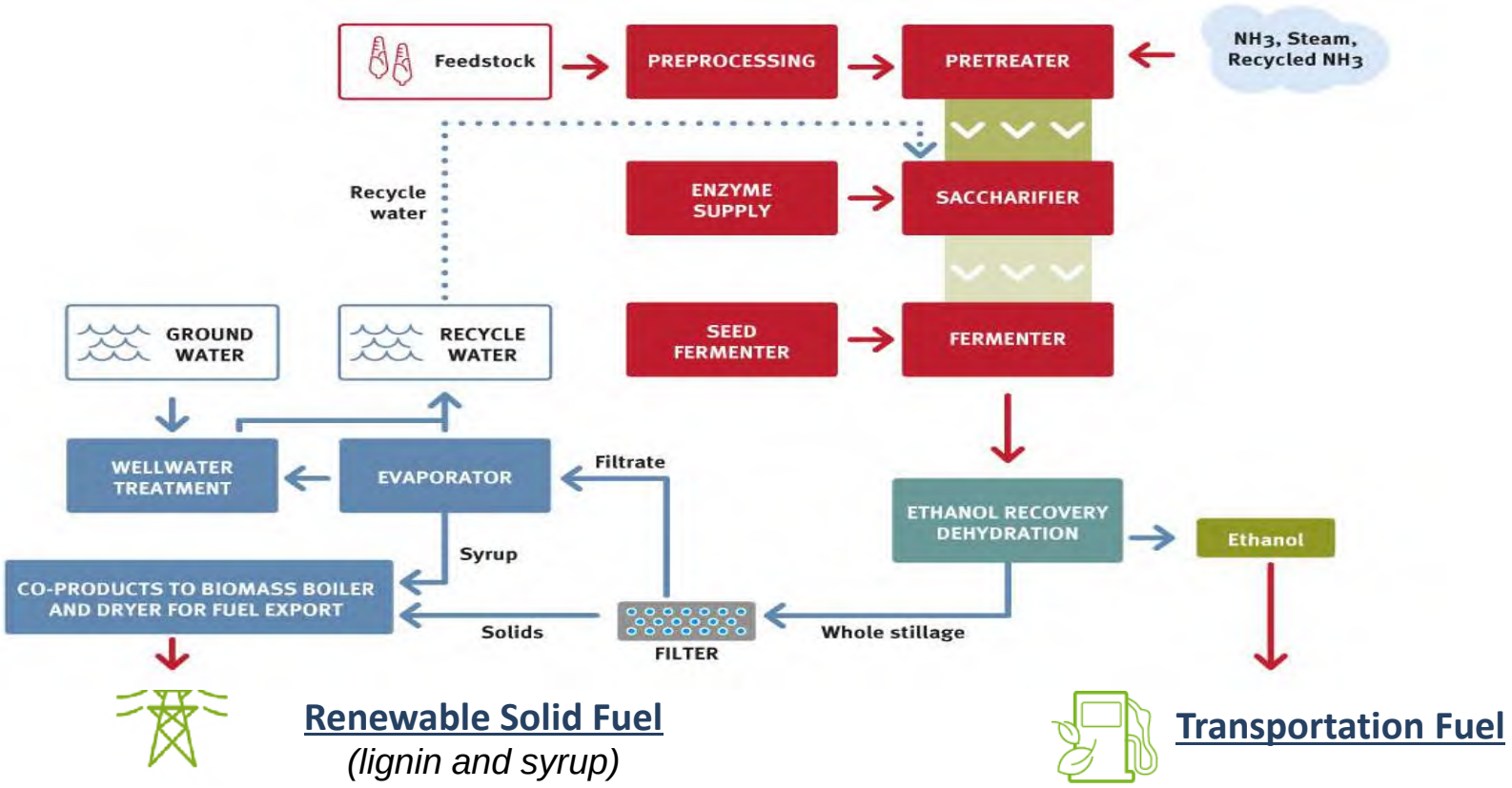
DCE Process Diagram: two renewable fuel products!



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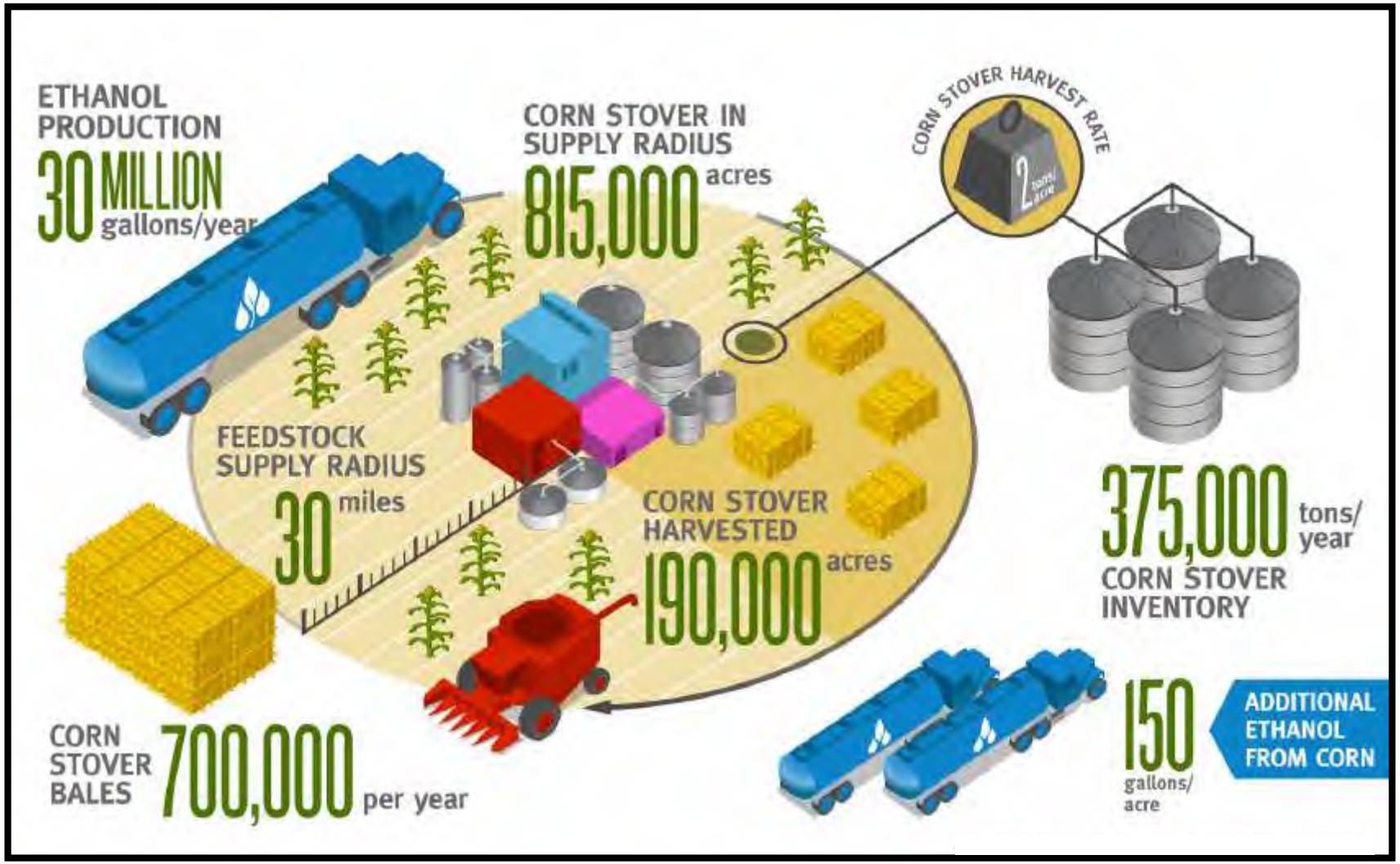
Cellulosic ethanol: At scale in Nevada Iowa



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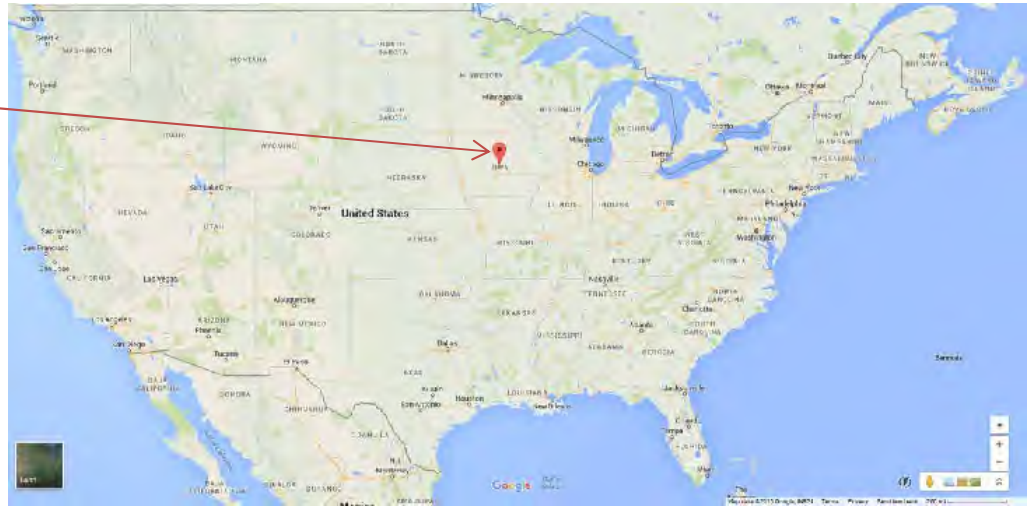
Nevada Iowa

- 114 million liters fuel-grade ethanol per year
- 500 farmers, 375 M dry tons corn stover (48 km radius)
- Clean; low water use
- Requires a half-ton bail of corn stover every 30 seconds



- Technology: Licensing model, world-wide
  - 1st license: Tianlong Industry, Jilin Province, China

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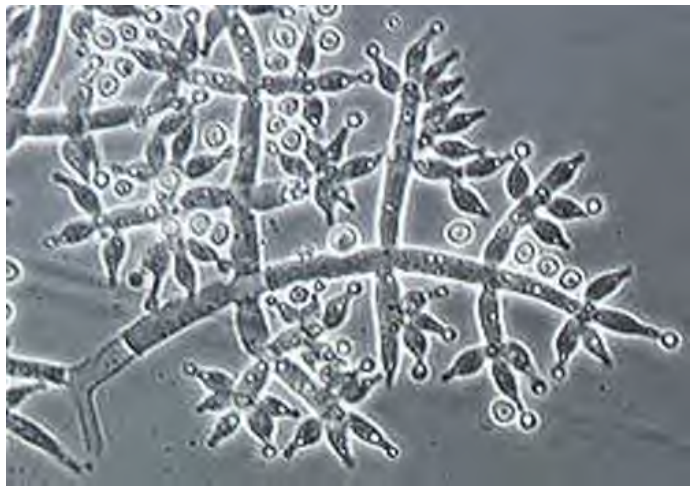
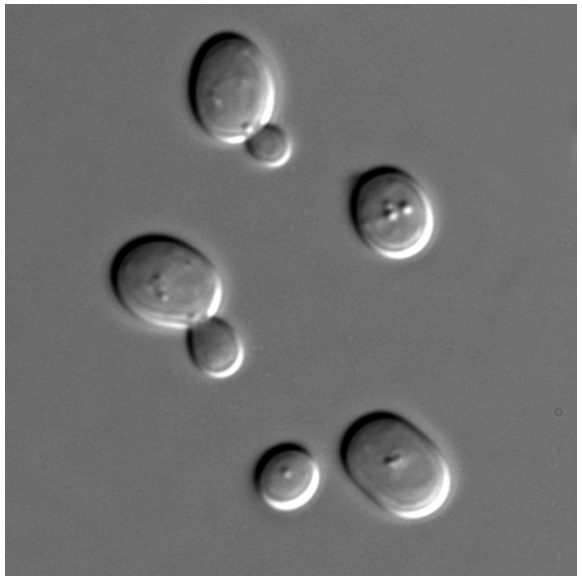
Cell Factories for DuPont Biotechnology

- Enzyme Production:
  - *Fungal species (Trichoderma reesei)*
  - *Bacillus species*



- Fermentation: 1st-Gen and Cellulosic Ethanol
  - *Zymomonas mobilis*
  - *Saccharomyces cerevisiae*



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Trichoderma

- Production host for cellulolytic enzyme package
  - Many enzymes in current enzyme package
    - Cellulases, xylanases, etc.
  - DuPont's *Trichoderma* platform
    - high yield on low cost carbon
    - high specific and volumetric productivity
    - versatility
    - thermotolerance
    - molecular biology toolbox
      - Same host for discovery, protein engineering and production
      - Rapid strain construction methods
    - low background proteins
    - predictable screen

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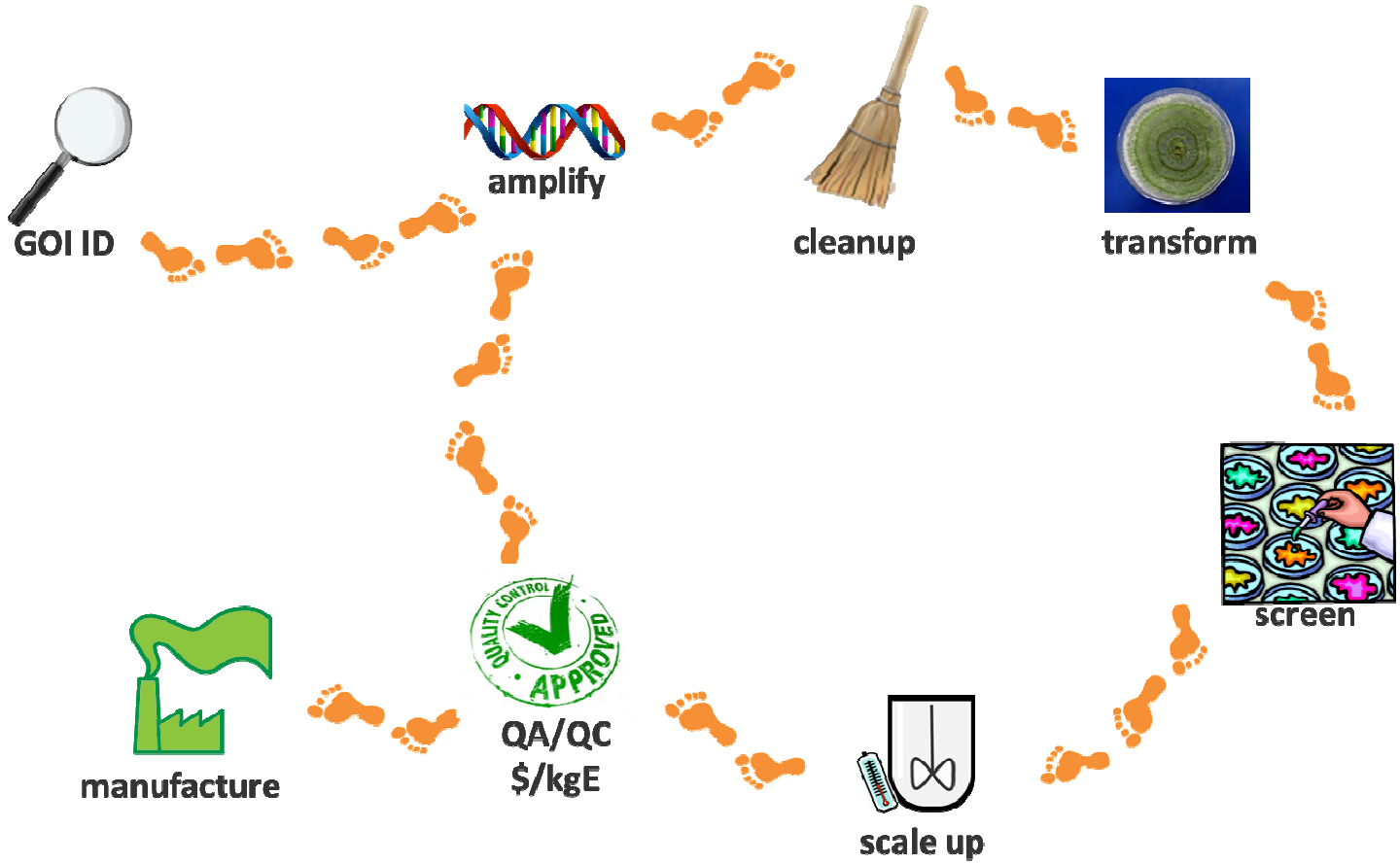
Trichoderma engineering

- Strain construction is laborious and time-intensive



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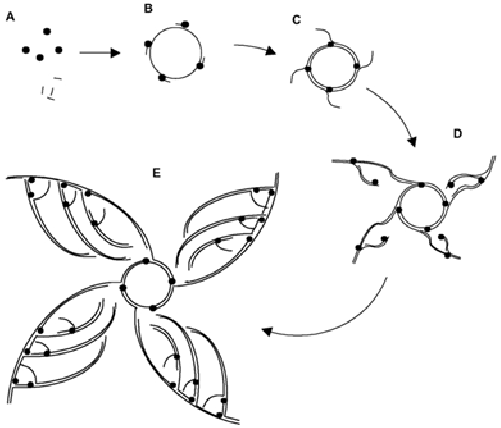
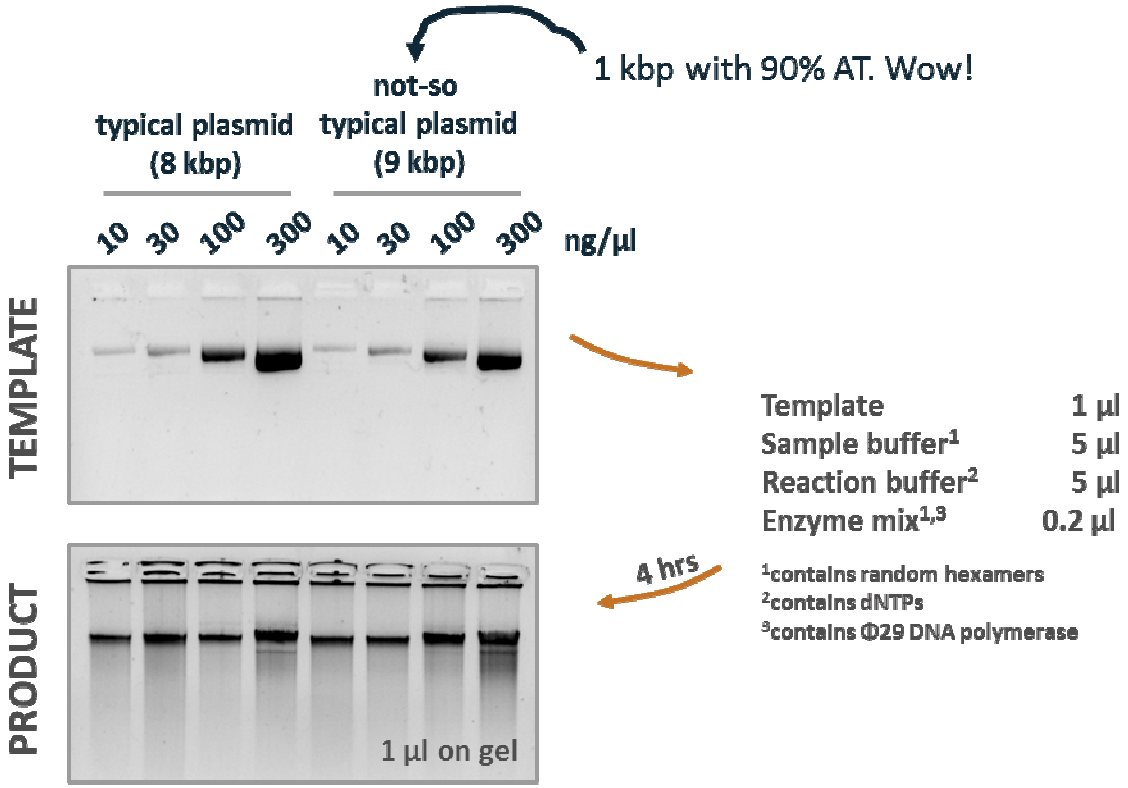
Trichoderma engineering

- DNA amplification
  - Conventional plasmid prep → rolling circle amplification (kit)

rolling circle amplification (RCA)



- Circular DNA as template
- Product concatemer dsDNA
- Error frequency ~10^{-7}



- very little starting material (e.g. ligation product without foreign DNA)
- very robust

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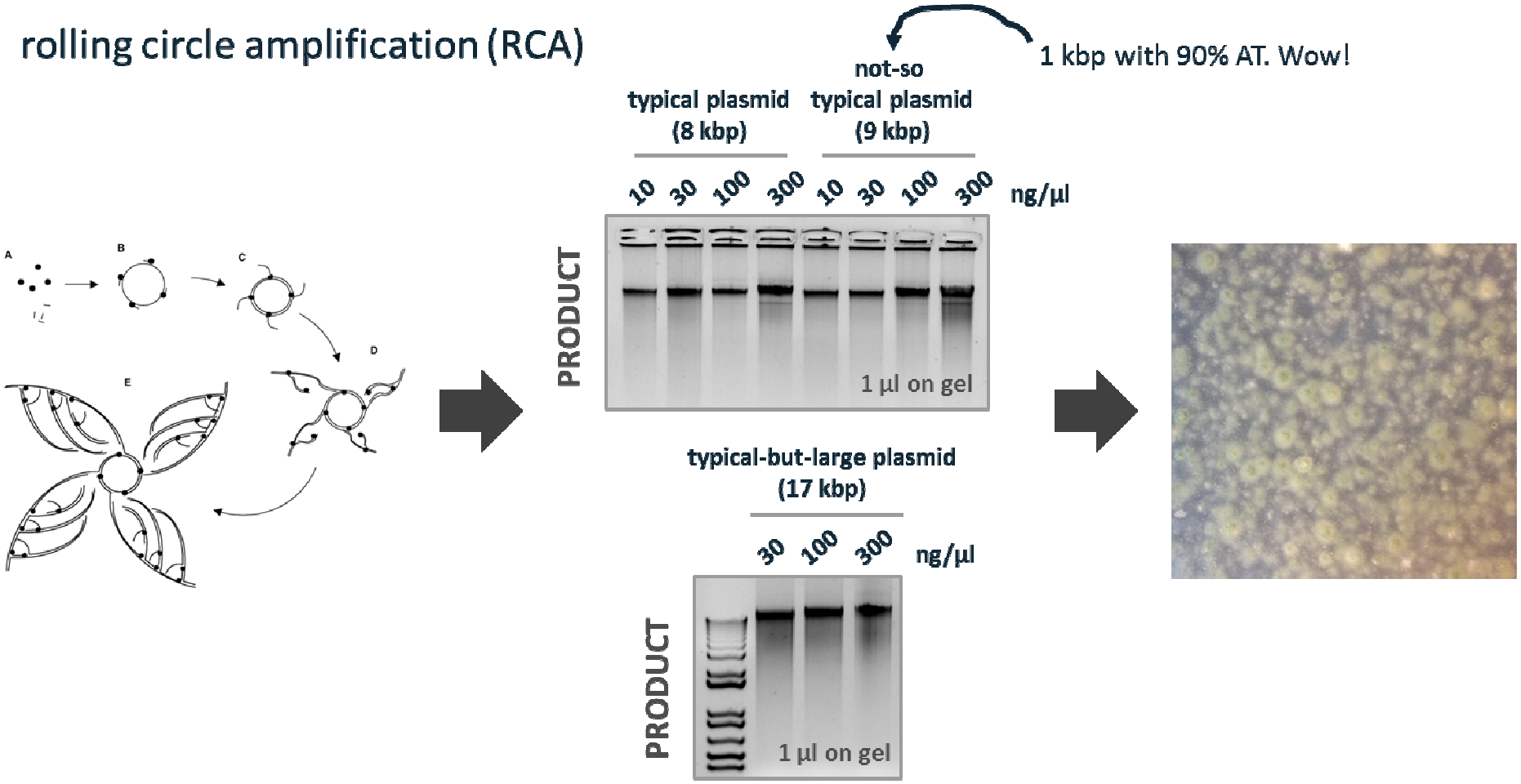
Trichoderma engineering

- Fungal transformation

rolling circle amplification (RCA)



- RCA gives functional products

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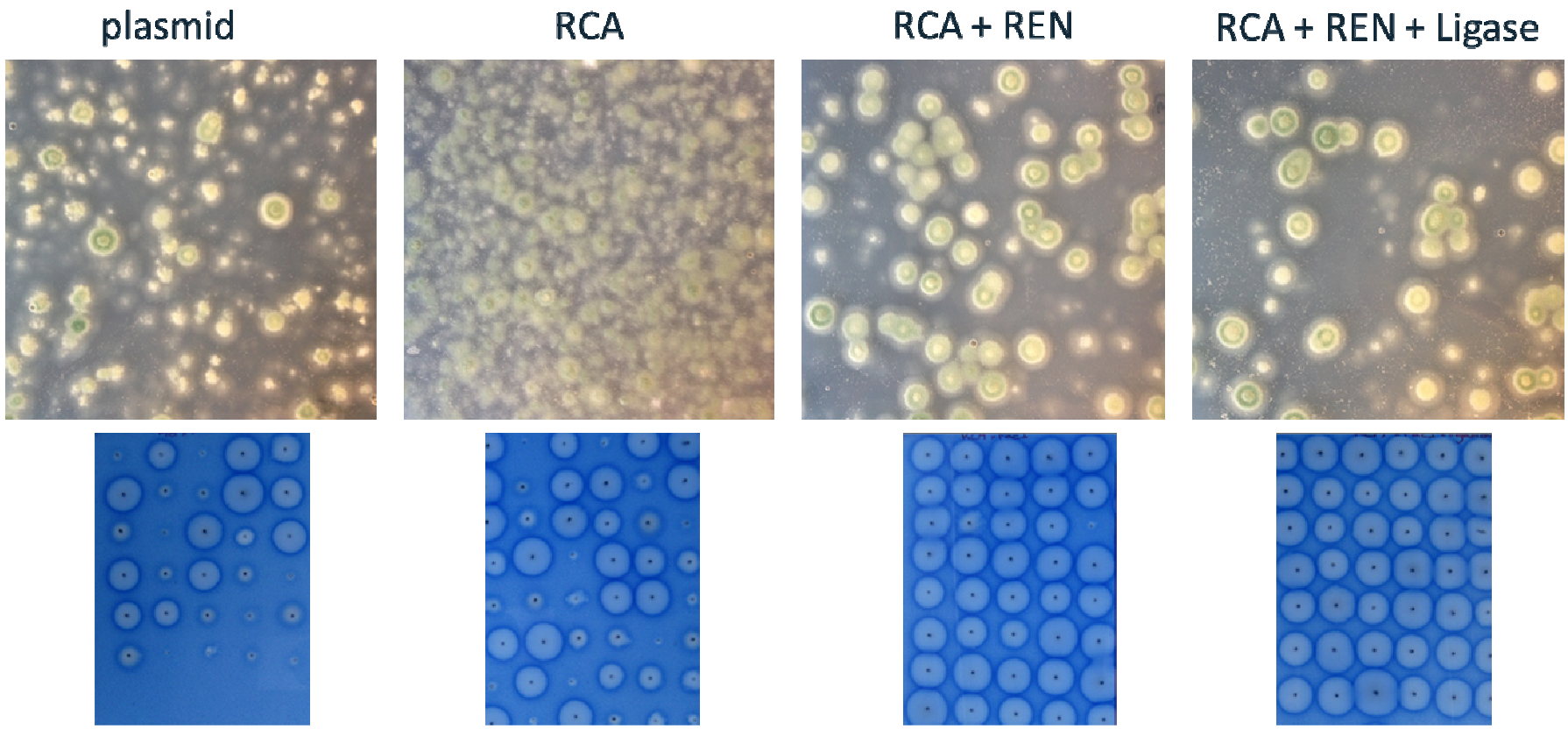
Transformation of RCA DNA without enzyme cleanup

Surprise! High yield of stable transformants, expressing desired protein at high levels



Apparently, DNA breaks function as landing sites for targeted genome integration

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Refinement of this serendipitous observation:

Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6829-1

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

I-*Sce*I-mediated double-strand DNA breaks stimulate efficient gene targeting in the industrial fungus *Trichoderma reesei*

Jean Paul Ouedraogo[1] · Mark Arentshorst[1] · Igor Nikolaev[2] · Sharief Barends[2] · Arthur F. J. Ram[1]

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Ethanologen Host Comparison

S. cerevisiae

- Eukaryote (budding yeast)
- 12 Mb genome on 16 chromosomes (diploid capable of sexual reproduction)
- Capable of respiratory or fermentative metabolism (Embden-Meyerhof, TCA cycle, pentose phosphate pathway). During fermentative growth, glycolytic enzymes comprise up to 25% soluble protein in the cell.
- Catabolizes a wide range of hexose sugars (and their oligosaccharides)
- Moderate specific rate of glucose consumption (2 g/g/h)
- Higher biomass yield (0.1 g/g anaerobically, up to 0.5 g/g aerobically)

Z. mobilis

- Prokaryote (alpha proteobacterium)
- 2.1 Mb genome on single chromosome
- Exclusively fermentative metabolism (Entner-Doudoroff only. ED pathway enzymes comprise up to 50% of total cell protein)
- Catabolizes only glucose, fructose and sucrose
- Very high specific rate of glucose consumption (10 g/g/h)
- Low biomass yield (0.03 g/g)

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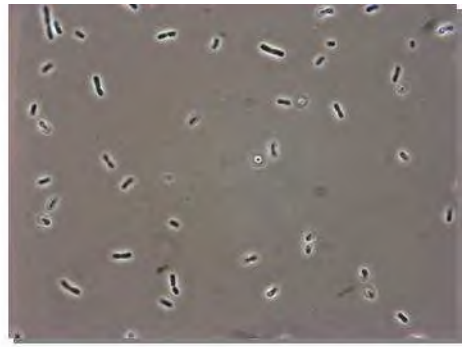
Zymomonas

Natural Substrates

Glucose
Fructose
Sucrose



Ethanol

- Natural fermentative microbe (GRAS)
- High ethanol yield (>0.48 g/g)
- High ethanol tolerance (>100 g/L)
- Tolerant to high sugar concentrations
- Higher yield & productivity than yeast

Lignocellulose Cell Factories

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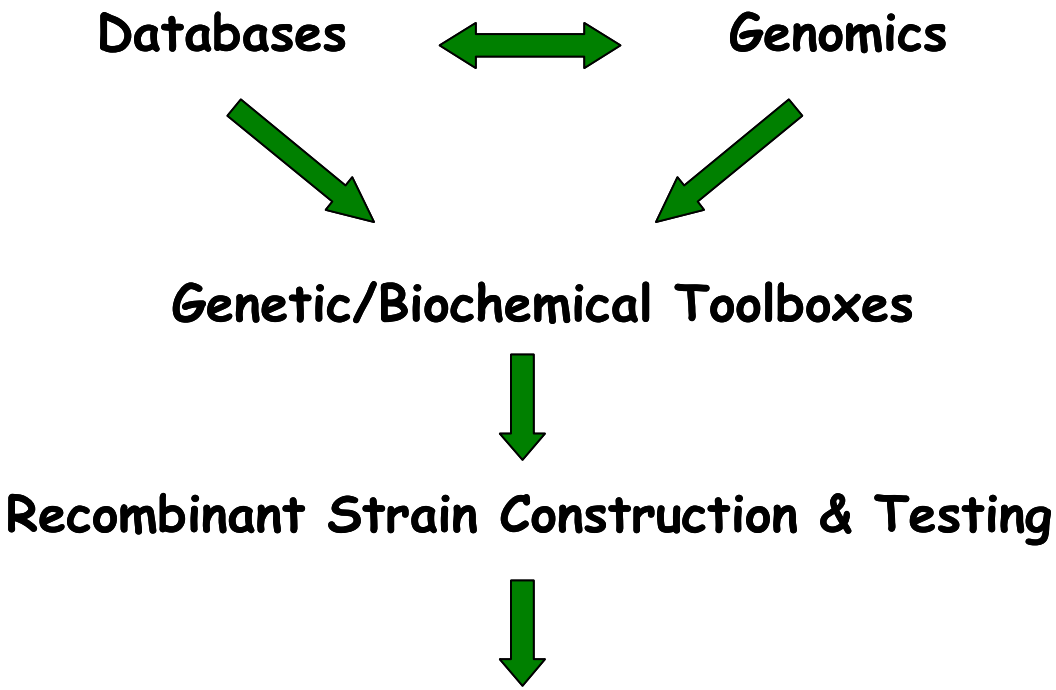
Path to Improved Ethanologen

Zymomonas mobilis



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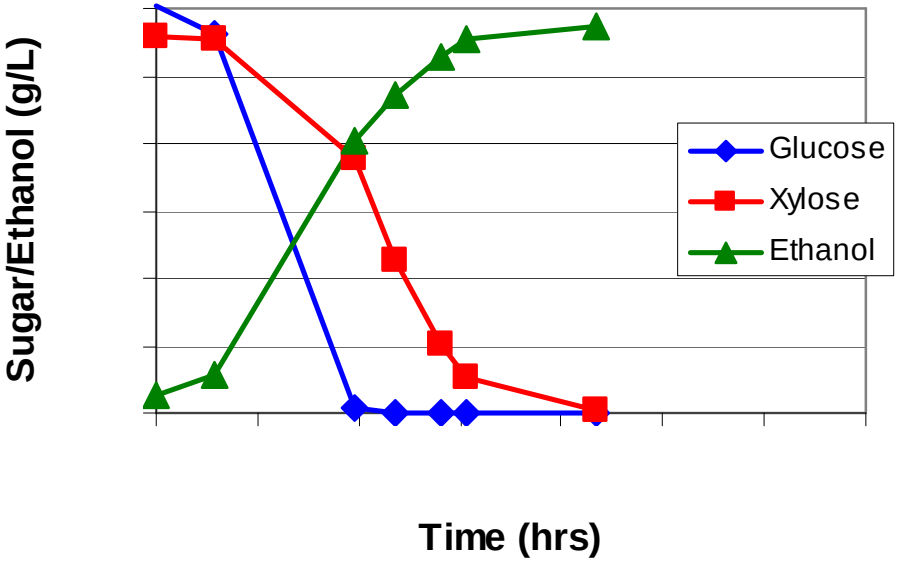
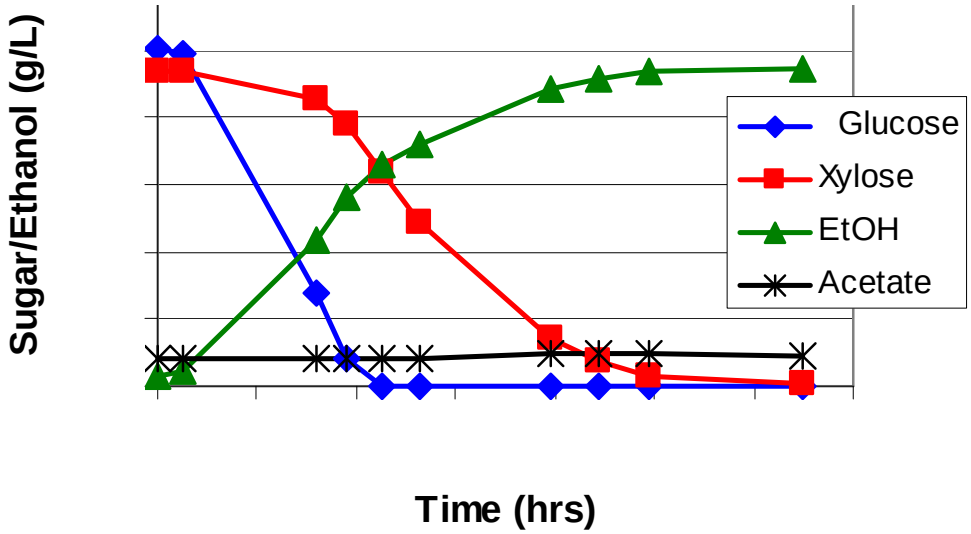
Fermentation of "Clean Sugars"





- Achieving full utilization of glucose and xylose with clean mixed sugars
- Achieved full sugar utilization with primary inhibitor from hydrolysate
  – acetic acid > 8 g/L

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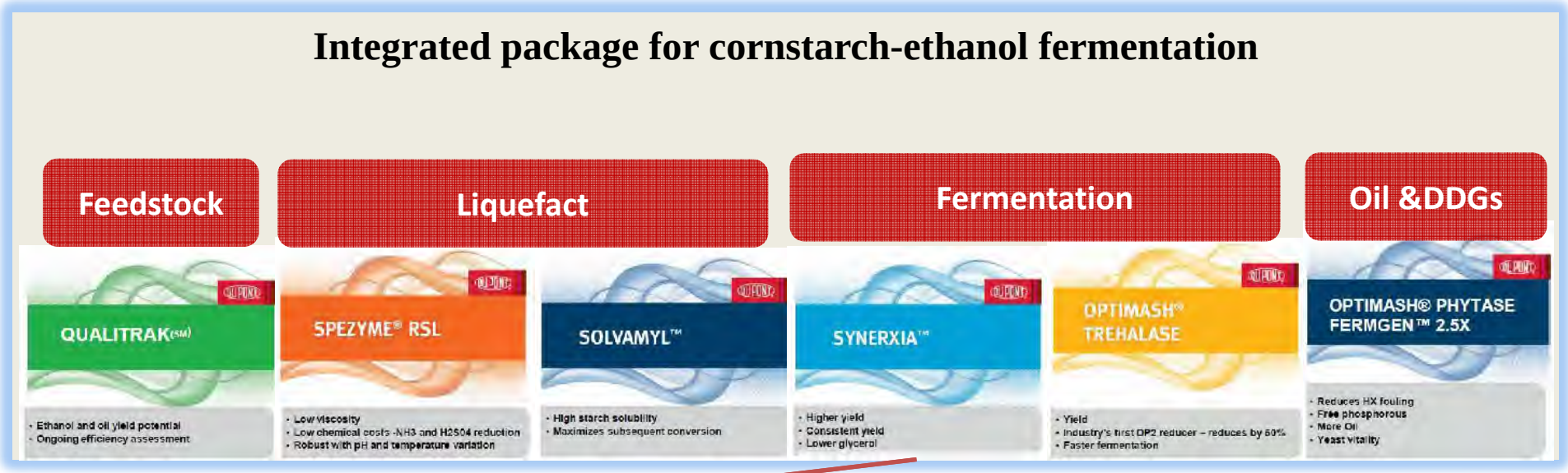
- DuPont Total Performance System (TPS) for 1st Gen Ethanol



Synerxia: “Bioengineered Active Dry Yeast Used In Fuel Alcohol Commercial Fermentations” Trademark, classified under “Industrial oils and greases.”

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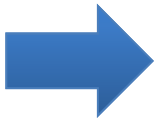
Typical Ethanol Plant Today

SPEZYME RSL	• Effective over broad pH and temp range • Decreased chemical costs • Drive Viscosity Break
DISTILLASE SSF+	• Hi-performance blend • Industrial leading GA • Phytase eliminates phytic acid • Cold Cook Alpha Amylase • Fermgen Protease



DuPont Total Performance System

SPEZYME RSL	•Effective over broad pH & temps •Decreased chemical costs •Drive Viscosity Break
SOLVAMYL	•Yield enhancing liquefaction AA •Drive Viscosity Reduction •Drive Solubility higher
SYNERXIA ADY	•Active Dry Yeast - Drop-in •Robust Performance •**Expresses Glucoamylase**
SYNERXIA Liquid Adjunct	• Industry leading GA •Add cold-cook alpha amylase •Control rate of fermentation
FERMGEN 2.5X	•Industry leading protease •Improved Oil recovery
OPTIMASH TREHALASE	•Industry's first DP2 reducer •Yield increase •Lower total sugar
OPTIMASH PHYTASE	•Designed for Fermentation •Improved heat exch. efficiency

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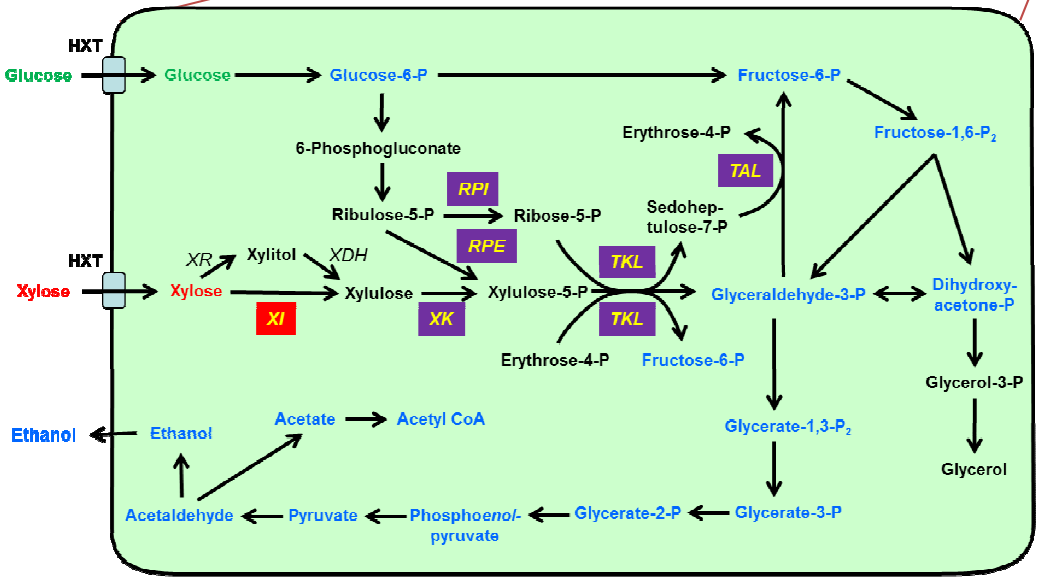
Grind → Hydrolyze → Saccharify → Ferment

Enzymes; Heat, 50C

Yeast; ~33C

Yeast



- Rational engineering
  - *Xylose isomerase*
  - *Lower pentose phosphate pathway*
  - *Pathway Balancing*
  - *Pentose transport*
- Non-directed approaches
  - *Adaptive evolution*
  - *Wide crosses: diversity*
  - *Genome shuffling*

→stress & inhibitor tolerance

→temperature tolerance

→process robustness

"Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels" Li et al. (2015) eLife 4.
"Evolved strains of Scheffersomyces stipitis achieving high ethanol productivity on acid- and base-pretreated biomass hydrolyzate at high solids loading" Slininger et al. (2015) Biotechnology for Biofuels 8:60.
"Enhanced xylose fermentation and ethanol production by engineered Saccharomyces cerevisiae strain" Vilela et al. (2015) AMB Express 5:16.
"Stress Tolerance Variations in Saccharomyces cerevisiae Strains from Diverse Ecological Sources and Geographical Locations" Zheng & Wang (2015) PLoS One 10:e0133889.

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- The end
  - Thanks for your attention!

- Thanks to the DuPont Cellulosic Ethanol team
  - Slides
    - Ken Hill
    - René Molina
    - Sharief Barends
    - Andrew Eliot
    - Brian Lefevbre
    - Perry Caimi

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