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