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Phytases: Microbial Sources, Production, Purification, and Potential Biotechnological Applications

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ABSTRACT: The review deals with phytase-producing microorganisms along with optimum conditions for its production. Various methods used for purifying phytases and their characteristics are discussed. Heterologous gene expression, cost-effective large-scale phytase production, and various biotechnological applications of the enzyme in animal feed and food industries are also discussed.

KEY WORDS: phytase, phytic acid, animal feed, phosphorus pollution, antinutritional factor.

I. INTRODUCTION

Phytic acid is an abundant plant constituent comprising 1 to 5% by weight of edible legumes, cereals, oil seeds, pollens, and nuts. It is an organic form of phosphorus, which is chemically a myo-inositol hexakis-dihydrogenphosphate (IP6). In 1903 Posternak described phytic acid for the first time. It was discovered by Pfeffer as early as 1872. The molecular formula of phytic acid is $C_6H_{18}O_{24}P_6$ and its molecular weight is 659.86. Salts of phytic acid are called phytates. Phytin is the calcium/magnesium salt of phytic acid. The presence of phytates in plant foodstuffs is well documented. It is the primary source of inositol and storage form of phosphorus in plant seeds that are used as animal feed ingredients (oilseed meals, cereal grains and legumes) (Maga et al., 1982). The total phosphorus, phytate-P and phytic acid content of common poultry feedstuffs are presented in Table 1. Most foods of plant origin contain 50 to 80% of their total phosphorus as phytate (Harland and Morris, 1995).

The role of phytin-P in plant was earlier speculated as a storage product. It was believed that a large amount of phosphorus was stored in the seed, and it was liberated on germination and incorporated into ATP. Recent studies have established the role of inositol phosphate intermediates in the transport of materials into the cell. Their role in transport as secondary messengers and in signal transduction has been confirmed (Berridge and Irvine, 1989).

The reviews by Wodzinski and Ullah (1996), Bali and Satyanarayana (1997, 1999, 2001), and others have dealt with the production, characterization, and applications of phytases. In this review, we have focused our attention on production, purification, and characterization, the molecular characteristics such as the active site and amino acid sequence, the use of heterologous gene expression for enhanced phytase production, site-directed mutagenesis for improving the desired characteristics, and the potential applications of microbial phytases in the animal feed and other industries.

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TABLE 1
Total P and Phytate P of common poultry feedstuffs (Tyagi et al. 1998)

Ingredients	Total P (%)	Phytate P (%)	% of total P
Cereals/millets			
Maize	0.39	0.25	64
Rice	0.15	0.09	60
Wheat	0.44	0.27	61
Sorghum	0.30	0.22	73
Barley	0.33	0.20	61
Bajra	0.31	0.23	74
Oilseed meals			
Groundnut meal	0.60	0.46	77
Soybean meal	0.88	0.56	64
Cotton seed meal	0.93	0.786	82
Sunflower meal	0.90	0.45	51

II. INTERACTION OF PHYTATES WITH FOOD INGREDIENTS

Phytic acid interacts with other food ingredients due to which it acts as an antinutritional factor in several ways, as described below:

1. Six reactive groups in the molecules of IP6 make it a strong chelating agent that binds cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}. Under gastrointestinal pH conditions, insoluble metal phytate complexes are formed that make the metal unavailable for absorption in the intestinal tract of animals and humans (Maga, 1982). In the presence of phytate and added calcium interference with mineral absorption occurred as a result of the formation of insoluble complexes (Sandberg et al., 1993). Mellanby (1949) demonstrated reduced calcium absorption and subsequently induced rickets when phytate was added to dog diets. Iron represents the other nutritionally significant mineral that has been associated with phytate binding. Davies and Nightingale (1975) have shown phytate to be inhibitory to iron absorption. An inverse relationship exists between the level of phytic acid in the diet and zinc bioavailability (O'Dell and Savage, 1960).
2. Phytates reduce digestibility of proteins, starch, and lipids. Phytate complexes with proteins, making them less soluble so that they resist proteolysis (Dvorakova, 1998). Polyphenols and phytic acid may affect starch digestibility through interacion with amylase enzyme (Thompson and Yoon, 1984).
3. The action of certain enzymes such as amylase, trypsin, acid phosphatase, and tyrosinase has been shown to be inhibited by phytic acid and also by inositol pentaphosphate (Harland and Morris, 1995).

III. PHYTASE

It is an enzyme that hydrolyses phytic acid to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates. The research on phytase has expanded considerably in the last few years. The literature

search clearly demonstrates the recent surge of interest in the enzyme. The research on phytase spans 87 years from its discovery by Suzuki et al. (1907) until its commercialization in Europe in 1993 to 1994 by Gist-Brocades. The milestones in the discovery and commercialization of phytase have been described by Wodzinski and Ullah (1996) and Dvorakova (1998). At the close of the twentieth century, annual sales of phytase as an animal feed additive were estimated to be $500 million (Abelson, 1999). The upcoming of the market for this feed additive can be attributed to a chain of events that created the need for the enzyme and the means for its commercial development.

There are two phytases as classified by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) in consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN):

1. EC 3.1.3.8
Recommended name: 3-phytase
Systematic name: myo-inositol-hexakisphosphate 3-phosphohydrolase
Other name(s): phytase; phytate 3-phosphatase

2. EC 3.1.3.26
Recommended name: 6-phytase
Systematic name: myo-inositol-hexakisphosphate 6-phosphohydrolase
Other name(s): phytase; phytate 6-phosphatase

Phytases catalyze the reactions shown below:
A 3-phytase (EC 3.1.3.8) first attacks phytate at the 3-position (Johnson and Tate, 1969):
Myo-inositol hexakisphosphate + H_2O = D-myo-inositol 1,2,4,5,6-pentakisphosphate + orthophosphate.
While a 6-phytase (EC 3.1.3.26) first attacks phytate at the 6-position (Cosgrove, 1969; 1970):
Myo-inositol hexakisphosphate + H_2O = D-myo-inositol 1,2,3,4,5-pentakisphosphate + orthophosphate.

There are conflicting reports as to the final reaction product of phytase acting on phytate. In theory, the inositol pentakisphosphate can rebind to the enzyme, releasing a further phosphate group and the inositol tetrakisphosphate. The phosphate group released due to the action of phytase combines with molybdate reagent to form phosphomolybdenum blue that can be detected colorimetrically.

A. Sources of Phytase

1. Plant Sources

Phytase has been reported in rice, wheat, maize, soybeans, corn seeds, lettuces, dwarf beans, mung beans, fababean, rye, and other legumes or oil seeds (Chang, 1967; Eskin and Wiebe, 1983; Gibson and Ullah, 1990). In germinating seeds or pollen, the phytase seems responsible for phytin degradation (Greene et al., 1975). Suzuki et al. (1907) were the first investigators to make a preparation of phytase. They detected activity in rice and wheat bran and isolated inositol as a product of the reaction. A phytase has been purified from soybeans, and its amino acid sequence did not show homology with any known histidine acid phosphatase. Phytase from scallion leaves has also been purified, and it has maximum activity at pH 5.5 and 51°C (Mullaney et al., 2000). Laboure et al. (1993) purified and characterized phytase from germinating maize seedlings, and cDNA coding for this phytase was cloned (Maugenest et al., 1997). This would allow the isolation of corresponding genes and the study of their regulation during germination. Gibson and Ullah (1990) suggested ammonium sulfate precipitation and three-column chromatographic steps for the preparation of phytase from soybean seed. The phytase from wheat bran was separated by DEAE cellulose into two fractions, which had different substrate degradation patterns (Liu et al., 1998).

2. Animal Sources

The first report on animal phytase in calf liver and blood was that by Mc Collum and

Hart (1908). However, a further search for mammalian blood phytase was unsuccessful; phytase was detected in the blood of lower vertebrates such as birds, reptiles, fishes, sea turtle (Rapoport et al., 1941). Because phytate acts as an antinutritional factor for animals, the presence of phytase in the gastrointestinal tract of various animals was investigated. Patwardhan (1937) first noted phytate hydrolysis in the rat intestine. Phytase activity was also observed in the intestine of pig, sheep, and cow (Spitzer and Phillips, 1972). Bitar and Reinhold (1972) partially purified phytase from rat, chicken, calf, and human intestines. About 30 times lower phytase activity was found in the human intestine when compared with that of a rat. The normal human small intestine has a limited ability to digest undegraded phytates (Igbal et al., 1994). It does not seem to play a significant role in phytate digestion, but dietary phytase may be an important factor in phytate hydrolysis (Frolich, 1990).

The ruminants probably digest phytate through the action of phytase produced by microbial flora in the rumen. The inorganic phosphates produced by splitting of phytate is utilized by both the microbial flora and ruminant host.

3. Microorganisms Producing Phytases

Phytases have been reported in a number of bacteria. Bacterial phytases are mostly cell associated, with the exception of *Bacillus subtilis, Lactobacillus amylovorus,* and *Enterobacter* sp. 4.

Over 200 fungal isolates belonging to the genera *Aspergillus, Penicillium, Mucor,* and *Rhizopus* have been tested for phytase production (Liu et al., 1998). All isolates produced active extracellular phytase. *Aspergillus niger* was identified as the most active fungal phytase producer. *Aspergillus niger* NRRL 3135 produced two different phytases, one with pH optima at 5.5 and 2.5 and the other optimally active at pH 2.0 and temperature optima at 55°C. Later these enzymes were designated phyA and phyB, respectively. *Aspergillus ficuum* NRRL 3135 also produced phytase in solid state fermentation (SSF) using canola meal as the substrate. The thermophilic fungus *Thermomyces lanuginosus* exhibited optimum activity at 65°C and pH of 6.0 (Berka et al., 1998). Another thermophilic fungus *Sporotrichum thermophile* produced phytase optimally at 45°C and at a pH 6.0 (Ghosh, 1997). Phytase from *Aspergillus fumigatus* was optimally active at pH 6.0 to 6.5 and 37°C and moisture content of 64% (Pasamontes et al., 1997). In solid state fermentation (SSF) using canola meal as the substrate, *A. carbonarius* showed maximum growth associated phytase production after 72 h. The decrease in phytic acid content was high, with moisture content between 53% and 60% (Al-Asheh and Duvnjak, 1995). Phytase was detected in whole cells in the fungi such as *A. clavatus* J239, *A. flavipes* Fla. A-14, *A. flavus, A. nidulans* QM-329, *A. niger* NRRL 67, *A. niger* P330, *A. oryzae* QM228, *A. phoenicus* QM 329, *A. repens* QM-44C, *A. terreus* Fla.C-93, *A. tamarii* J1008, *Mucor* spp., *Penicillium* spp. P-320, and *Rhizopus* spp. (Casida, 1959).

Among yeasts, extracellular phytase has been reported in *Schwanniomyces castellii* (Segueilha et al., 1992) and *Arxula adeninivorans* (Sano et al., 1999), which have been characterized. The molecular weight of the native phytase of *S. castellii* was 490,000. It was a glycoprotein with an estimated glycosylation rate of 31%, which is similar to that reported for the phytase from *A. ficuum.* The optimum temperature for the activity of *S. castellii* phytase was 77°C, and the enzyme was stable for 1 h at 74°C. The enzyme had broad substrate specificity with phytate as its preferential substrate. Another yeast *Arxula adeninivorans* was reported to be one of the rare yeasts capable of assimilating phytate as a sole source of carbon and phosphate. Nakamura et al. (2000) screened several yeast species for extracellular phytase activity. *Pichia spartinae and P. rhodanensis* showed the highest levels of phytase activity. The enzyme of *P. spartinae*

exhibited the highest optimal reaction temperature at 75 to 80°C, whereas for *P. rhodanensis,* it was 70 to 75°C with optimum pH being 3.6 to 5.5 and 4.5 to 5, respectively. The phytase of *P. anomala* was found in the cellular fraction. The optimum temperature and pH were 60°C and 4.0, respectively. The enzyme exhibited a broad substrate specificity and was thermostable and acid stable (Vohra and Satyanarayana, 2002 b).

A list of phytase-producing microorganisms is presented in Table 2.

B. Production of Phytase

In view of the importance of this enzyme in the animal feed industry, the effects of nutritional and physical parameters have been studied after its production.

1. Physical Parameters

The most important physical parameters having a profound influence on growth of organisms and production of metabolites from them are pH, temperature, agitation, dissolved oxygen, and pressure. Microorganisms producing phytases are all mesophiles, with the exception of thermophilic fungi *Thermomyces lanuginosus* (Berka et al., 1998), *Talaromyces thermophilus* (Pasamontes *et al.,* 1997), and *Sporotrichum thermophile* (Ghosh, 1997). The optimum temperature for the production of phytases from most of the microorganisms lies in the range 25 to 37°C (Table 3).

The pH has a profound effect on the production of the enzyme. For phytase production the optimum pH of most bacteria and fungi is in the range between 5.0 and 7.0. There is no report of phytase production at alkaline pH (Table 3).

Proper aeration and agitation of the medium is important to keep the medium constituents, microbial cells, and oxygen uniformly suspended. In *Schwanniomyces castellii*, phytase production was carried out continuously in a fermenter aerated at 1 vvm and agitation kept at 600 rev min^{-1} (Segueilha et al., 1992). Phytase production by the fungi *A. niger, A. ficuum* NRRL 3135, and *A. terreus* was carried out by shaking at 270 rev/min (Shieh and Ware, 1968). Phytase was produced maximally by *Bacillus* sp. DS11 by shaking at 230 rpm (Kim et al., 1998). *Aspergillus carbonarius* produced phytase in solid state fermentation (SSF) using canola meal as the substrate (Al-Asheh and Duvnjak, 1994).

2. Nutritional Parameters

a. Carbon Source

The source and optimal concentration of carbon are important factors for the production of phytase. Glucose has been the most preferred substrate for phytase production. Glucose (1%) was optimal for phytase production by *Lactobacillus amylovorus* (Sreeramulu et al., 1996) and *Enterobacter* sp. 4 (Yoon et al., 1996). Glucose at 2% concentration was used for *Bacillus subtilis* (Kerovuo et al., 1998). Wheat bran (6%) was a good carbon source for phytase production by *Bacillus* sp. DS11 (Kim et al., 1998), whereas *Pseudomonas* sp. grew on myo-inositol (0.2%) as the sole carbon source and produced phytase (Irving and Cosgrove, 1971). The highest phytase production by *Klebsiella aerogenes* was reported in the medium containing sodium phytate (2%) as the sole carbon source (Tambe et al., 1994).

When glucose (2%) was substituted with galactose in yeast peptone glucose medium, phytase production by *Arxula adeninivorans* increased severalfold (Sano et al., 1999). Phytate had no inducible effect on phytase synthesis because there was no increase in the presence of 1 m*M* sodium phytate in the medium. Galactose (1%) was found to be the preferred carbon source for phytase production by *Schwanniomyces castellii* (Segueilha et al., 1992).

For fungi, malt yeast extract broth was used (Shieh and Ware, 1968). When simple sugars

TABLE 2
List of Microorganisms Producing Phytases

Bacteria	Location of the enzyme	Reference
Aerobacter aerogenes	Cell bound	Greaves et al., 1967
B. amyloliquefaciens	Extracellular	Ha et al., 1999
B. subtilis	Extracellular	Kerovuo et al., 1998
Bacillus sp., DS11	Extracellular	Kim et al., 1998
Bacillus subtilis	Extracellular	Powar and Jagannathan, 1982
Enterobacter sp.4	Extracellular	Yoon et al., 1996
Escherichia coli	Cell bound	Greiner et al., 1993
Klebsiella aerogenes	Cell bound	Tambe et al., 1994
Klebsiella oxytoca MO-3	Cell bound	Jareonkitmongkol et al., 1997
Lactobacillus amylovorus	Extracellular	Sreeramulu et al., 1996
Mitsuokella multiacidus	Cell bound	Yanke et al., 1998
Pseudomonas sp.	Cell bound	Irving and Cosgrove, 1971
Selenomonas ruminantium	Cell bound	Yanke et al., 1999
Fungi		
Aspergillus amstelodami	Extracellular	Howson and Davis, 1983
A. chevalieri	Extracellular	Howson and Davis, 1983
A. candidus	Extracellular	Howson and Davis, 1983
A. niger syn A. ficuum	Extracellular	Howson and Davis, 1983
A. flavus	Extracellular	Shieh and Ware, 1968
A. niger	Extracellular	Skowronski, 1978
A. niger	Extracellular	Shieh and Ware, 1968
A. repens	Extracellular	Howson and Davis, 1983
A. syndowi	Extracellular	Howson and Davis, 1983
A. terreus	Extracellular	Yamada et al., 1968
A. versicolor	Extracellular	Howson and Davis, 1983
A. wentii	Extracellular	Howson and Davis, 1983
A. fumigatus	Extracellular	Pasamontes et al., 1997
A. carbonarius	Extracellular	Al- Asheh and Duvnjak, 1994
A. carneus	Extracellular	Ghareib, 1990
Aspergillus terreus 9A1	Extracellular	Mitchell et al., 1997
Botrytis cinerea	Extracellular	Howson and Davis, 1983
Emericella nidulans	Extracellular	Pasamontes et al., 1997
Geotrichum candidum	Extracellular	Howson and Davis, 1983
Mucor piriformis	Extracellular	Howson and Davis, 1983
M. racemosus	Extracellular	Howson and Davis, 1983
Myceliophthora thermophila	Extracellular	Mitchell et al., 1997
Pencillium sp.	Extracellular	Shieh and Ware, 1968

TABLE 2 (continued)

Rhizopus oryzae	Extracellular	Howson and Davis, 1983
R. oligosporus	Extracellular	Howson and Davis, 1983
R. stolonifer	Extracellular	Howson and Davis, 1983
Sporotrichum thermophile	Extracellular	Ghosh, 1997
Talaromyces thermophilus	Extracellular	Pasamontes et al., 1997
Thermomyces lanuginosus	Extracellular	Berka et al., 1998
A. clavatus J239	Cell bound	Casida, 1959
A. flavipes Fla. A-14	Cell bound	Casida, 1959
A. flavus	Cell bound	Casida, 1959
A. nidulans QM-329	Cell bound	Casida, 1959
A. niger NRRL 67	Cell bound	Casida, 1959
A. niger P330	Cell bound	Casida, 1959
A. oryzae QM 228	Cell bound	Casida, 1959
A. phoenicus QM 329	Cell bound	Casida, 1959
A. repens QM-44C	Cell bound	Casida, 1959
A. terreus Fla C-93	Cell bound	Casida, 1959
A. tamarii J1008	Cell bound	Casida, 1959
Mucor spp.	Cell bound	Casida, 1959
Penicillium spp. P-320	Cell bound	Casida, 1959
Rhizopus spp.	Cell bound	Casida, 1959
Yeasts		
Arxula adeninivorans	Extracellular	Sano et al., 1999
Candida spp.	Extracellular	Nakamura et al., 2000
Clavispora lusitaniae	Extracellular	Nakamura et al., 2000
Debaryomyces yamadae	Extracellular	Nakamura et al., 2000
Hanseniaspora valbyensis	Extracellular	Nakamura et al., 2000
Kluyveromyces lactis	Extracellular	Nakamura et al., 2000
Metchnikowia pulcherrima	Extracellular	Nakamura et al., 2000
Pichia anomala	Extracellular	Nakamura et al., 2000
P. anomala	Cell bound	Vohra and Satyanarayana, 2001
Pichia spp.	Extracellular	Nakamura et al., 2000
Saccharomyces cerevisiae	Cell bound	Howson and Davis, 1983
S. cerevisiae	Extracellular	Nakamura et al., 2000
S. kluyveri	Extracellular	Nakamura et al., 2000
Schwanniomyces occidentalis	Extracellular	Segueilha et al., 1992
S. occidentalis	Extracellular	Nakamura et al., 2000
Torulaspora delbrueckii	Extracellular	Nakamura et al., 2000
T. globosa	Extracellular	Nakamura et al., 2000
T. pretoriensis	Extracellular	Nakamura et al., 2000

TABLE 3
Production of Phytases by Microorganisms

Bacteria	T opt (°C)	pH opt	Inducible/ constitutive	References
Aerobacter aerogenes	27	6.8	Constitutive	Greaves et al., 1967
Bacillus sp. DS11	37	6.5	Constitutive	Kim et al., 1998
Bacillus subtilis	30	6.5	Inducible	Powar and Jagannathan, 1982
Enterobacter sp.4	39	5.5	Inducible	Yoon et al., 1996
Escherichia coli	37	7.5	Constitutive	Greiner et al., 1993
Klebsiella aerogenes	30		Inducible	Tambe et al., 1994
Lactobacillus amylovorus	37	6	Inducible	Sreeramulu et al., 1996
Pseudomonas sp.	25		Constitutive	Irving and Cosgrove, 1971
Selenomonas ruminantium	39		Constitutive	Yanke et al., 1999
Yeast				
Arxula adeninivorans	28	5.5	Constitutive	Sano et al., 1999
Pichia rhodanensis	28	6	Constitutive	Nakamura et al., 2000
P. spartinae	28	6	Constitutive	Nakamura et al., 2000
Schwanniomyces castellii	30	6.5	Constitutive	Segueilha et al., 1992
Fungi				
Aspergillus carneus	30	6	Constitutive	Ghareib, 1990
A. carbonarius (SSF)	30		Constitutive	Al Asheh and Duvnjak, 1995
A. ficuum NRRL 3135	27	5	Constitutive	Shieh and Ware, 1968
A. niger	30	5	Constitutive	Shieh and Ware, 1968
Rhizopus oligosporus	25	5.5	Constitutive	Howson and Davis, 1983

such as glucose and sucrose were used as a sole source of carbon, mycelial pellets were formed and low yields of phytase were observed in *A. niger* NRRL 3135. Certain types of cornmeal, however, caused dispersed mycelial growth and higher enzyme yield (Shieh and Ware, 1968). Phytase production in SSF using canola meal as the substrate was reported in *Aspergillus ficuum* (Ebune et al., 1995). Supplementation of canola meal with glucose (5.2%) increased phytase production.

b. Nitrogen Source

The source of nitrogen in the culture medium is another important parameter that influences the growth and production of enzymes. The organic form of nitrogen such as peptone has been used extensively for the production of phytase. Peptone (1%) was used in *Aerobacter aerogenes* (Greaves et al., 1967), and in *K. oxytoca* it was supplemented with yeast extract (1%) (Jareonkitmongkol et al., 1997).

Inorganic nitrogen source such as ammonium sulfate (0.1%) was used for phytase production by *Pseudomonas* sp. (Irving and Cosgrove, 1971), *Enterobacter* sp. 4 (Yoon et al., 1996), and *S. castellii* (Lambrechts et al., 1992). Casein hydrolysate (1%) and $(NH_4)_2SO_4$ (0.1%) were used as N_2 sources for phytase production by *B. subtilis* (Powar and Jagannathan, 1967). For *A. adeninivorans,* yeast extract (1%) and peptone (1%) served as good nitrogen sources (Sano et al., 1999). Statistical optimization of the medium components (glucose and beef extract) by response surface methodology enhanced phytase production by *P. anomala* (Vohra and Satyanarayana, 2002a).

c. Requirement of Trace Elements and Vitamins

Besides carbon and nitrogen sources, some microorganisms require additional trace elements and vitamins for growth and enzyme production. For phytase production by yeasts, the screening medium contained vitamins and trace elements (Galzy, 1964; Segueilha et al., 1992). The addition of trace elements was not needed for phytase production by *Bacillus subtilis* (Powar and Jagannathan, 1967), *Bacillus* sp. DS11 (Kim et al., 1998), *E. coli* (Greiner et al., 1993), as well as fungi such as *A. niger* ATCC 9142 and *A. ficuum* NRRL 3135 (Shieh and Ware, 1968).

IV. REGULATION OF PHYTASE SYNTHESIS

A. Effect of Phosphorus Concentration

The available inorganic phosphorus content of the medium controlled the synthesis of the enzyme (Shieh and Ware, 1968). The most commonly used phosphorus sources for phytase production are KH_2PO_4 and K_2HPO_4. In *Bacillus* sp. DS11, the medium contained KH_2PO_4 (0.05%) and K_2HPO_4 (0.04%) (Kim et al., 1998). For *B. subtilis*, sodium phytate (0.06%) was used along with KH_2PO_4 (0.1%). For the plate assay, calcium phytate (0.5%) has been used as the insoluble substrate. In *S. castellii,* sodium phytate (0.06%) alone was used as the source of phosphorus (Segueilha et al., 1992). Phosphate present in different ingredients inhibited phytase synthesis in several species of yeasts and molds (Shieh and Ware, 1968; Nayini and Markakis, 1984; and Yamada et al., 1968). Phytase yield by fungi in corn meal medium depended on the phosphorus content of the corn meal. A high phosphorus content of corn grains suppressed phytase production by the mold (Shieh and Ware, 1968). After elimination of phosphate from the medium, the specific activity of phytase of *A. niger* AbZ4 in a molasses medium increased by 7.3-fold (Zyta and Gogol, 2002). The regulatory effect of high P on phytase synthesis was confirmed by Howson and Davis (1983), Han and Gallagher (1987), and Ullah and Gibson (1987). In the case of *Lactobacillus amylovorus,* the optimum phosphate level of 240 mg/L supported maximum phytase production (Sreeramulu et al., 1996). Phosphate concentration of canola meal influenced phytase production by *A. ficuum* in SSF process (Ebune et al., 1995). Phytase production by *Candida krusei* was controlled by phosphate concentration in the medium (Quan et al., 2001). Maximum production occurred in the medium containing 0.5 mg phosphorus per 100 ml. An increase in the concentration to more than 5 mg per 100 ml caused inhibition of phytase synthesis.

B. Effect of Surfactant

When a simple sugar such as glucose or fructose was used as a sole source of carbon for phytase production by *A. niger* NRRL 3135, mycelial pellets were formed, and the enzyme titres were low (Shieh and Ware, 1968; Han and Gallagher, 1987). In a medium containing surfactant (sodium oleate 0.5% v/v), growth

was, however, dispersed, and phyA yields were 4.7-fold higher than in controls.

During the growth of *A. carbonarius*, the rates of growth, phytase production, and reduction of phytic acid content in canola meal in SSF were higher in the presence of Na-oleate (Al Asheh and Duvnjak, 1995). Similarly, canola meal, Tween-80, and sodium oleate increased the rates of phytase production and hydrolysis of phytic acid when compared with the control, while Triton X-100 had a negative effect on these processes (Ebune et al., 1995).

C. Effect of Medium Ingredients and Inoculum Size

In glucose/fructose as a sole source of carbon, mycelial pellets were formed and the enzyme yields by *A. niger* NRRL 3135 were low (Shieh and Ware, 1968). Glucose concentration (up to 5.2%) increased secretion of phytase by *A. ficuum* in SSF (Ebune et al., 1995). High concentration of glucose (9.8 and 17.8%) had an adverse effect.

When the inoculum size is too small and in a relatively low viscosity medium, organisms tend to form pellets. Low extracellular enzyme yields, when pellets are formed, appear to be a general characteristic of the microorganisms (Wodzinski and Ullah, 1996).

V. PURIFICATION OF PHYTASES

Various methods have been used for purifying enzymes, including ammonium sulfate/acetone precipitation, ultrafiltration, ion exchange, and gel filtration chromatography. Very rarely proteins are purified by one method; therefore, usually a combination of these methods are used to purify.

An extracellular phytase from *Bacillus* sp. DS11 was purified to homogeneity by acetone precipitation and phenyl-sepharose, Resource S, and Superose 12 column chromatographies (Kim et al., 1998). Ultrafiltration was used for concentrating 10-fold enzyme sample from *Selenomonas ruminantium* JY35 with a molecular weight cut-off of 10,000 Da (Yanke et al., 1999). Phytase from *E. coli* was purified using ammonium sulfate precipitation (25 to 80% saturation) followed by CM-sepharose CL6B chromatography, DEAE-sepharose CL6B chromatography, phenyl sepharose CL4B chromatography, and Mono S HR, 5/5 chromatography (Greiner et al., 1993). Phytase from *K. aerogenes* was purified using ion exchange chromatography on DE-52 followed by gel filtration on Sephadex G-200 column (Tambe et al., 1994).

The phytase of *S. castellii* was purified by anion exchange followed by filtration chromatography (Segueilha et al., 1992). Partial purification of phytase from *A. adeninivorans* was achieved by Sephadex G50 filtration and/or DEAE chromatography (Sano et al., 1999).

Aspergillus fumigatus phytase overexpressed in *A. niger* NW205 was concentrated approximately 50-fold by ultrafiltration in Amicon 8400 cells (PM 30 membranes) and Ultrafree-15 centrifugal filter devices (Biomax-30K; Millipore, Bedford, MA) followed by ion exchange chromatography (Pasamontes et al., 1997). Phytase of *A. oryzae* NRRL 1988 was purified by acetone fractionation (60% v/v), gel filtration on G-100, followed by DEAE cellulose chromatography (Wang et al., 1980). Partial purification of phytase from *A. ficuum* NRRL 3135 was carried out by gel filtration and ion exchange dextran chromatography (Irving and Cosgrove, 1974). A phytase (EC 3.1.3.8) with a high affinity for phytic acid was found in *A. niger* SK-57 and purified to homogeneity in four steps using ion exchange chromatography (two types), gel filtration, and chromatofocusing (Ngashima et al., 1999). Phytase from *A. carneus* was purified 43-fold from the culture filtrate by acetone precipitation, gel filtration through Sephadex G-75, and ion exchange chromatography on DEAE-cellulose (Ghareib, 1990).

VI. CHARACTERIZATION OF PHYTASES

Properties of enzymes are important in determining their potential applications in different industries.

A. Temperature

Phytases with high-temperature optima are desirable in the animal feed industry because feed pelleting involves a step of 80 to 85°C for few seconds. Phytase from *B. subtilis* (Powar and Jagannathan, 1982), *E. coli* (Greiner et al., 1993), *Klebsiella aerogenes* (Tambe et al., 1994), *Enterobacter* sp. 4 (Yoon et al., 1996), *K. oxytoca* MO-3 (Jareonkitmongkol et al., 1997), and *Selenomonas ruminantium* (Yanke et al., 1999) were optimally active in the temperature range between 50 and 60°C, while phytase of *Aerobacter aerogenes* had an optima at 25°C (Greaves et al., 1967), and that of *Bacillus* sp. DS11 at 70°C (Kim et al., 1998).

Phytase of *S. castellii* was optimally active at 77°C (Segueilha et al., 1992) and that of *A. adeninivorans* showed maximum phytase activity at 75°C (Sano et al., 1999). Phytases from *Pichia rhodanensis* and *P. spartinae* showed optimal reaction temperature at 70 to 75°C and 75 to 80°C, respectively (Nakamura et al., 2000).

Among the thermophilic fungi, *Thermomyces lanuginosus* exhibited optimum activity at 65°C (Berka et al., 1998) and *Sporotrichum thermophile* at 45°C (Ghosh, 1997). Phytase of *A. fumigatus* and *A. niger* NRRL 3135 showed optimum activity at 37°C (Pasamontes et al., 1997) and at 55°C (Howson and Davis, 1983), respectively. These are interesting observations because all organisms except *T. lanuginosus* and *S. thermophile* are mesophiles, and the temperature optima for phytases are in the thermophilic range (Table 4).

B. pH

Most microbial phytases, especially those of fungal origin, show the main pH optimum between 4.5 and 5.5; some bacterial ones have a pH optimum at 6.5 to 7.5. For phytase of *A. aerogenes* (Greaves et al., 1967), *Pseudomonas* sp. (Irving and Cosgrove, 1971), *E. coli* (Greiner et al., 1993), *S. ruminantium* (Yanke et al., 1999), *L. amylovorus* (Sreeramulu et al., 1996), the optimum pH was in the range between 4 and 5.5. The pH optimum of *Enterobacter* sp. 4 (Yoon et al., 1996 and *Bacillus* sp. DS11 (Kim et al., 1998) was in the neutral range (7 to 7.5). *A. niger* NRRL 3135 produced two different phytases, one with pH optima at 5.5 and 2.5, and the other with 2.0; these enzymes were designated as phyA and phyB, respectively (Howson and Davis, 1983). Phytases of *T. lanuginosus* (Berka et al., 1998) and *A. fumigatus* (Pasamontes et al., 1997) were found to be optimally active at 6.0 to 6.5. The optimum pH for phytase of *S. castellii* was 4.4 (Segueilha et al., 1992) and that for *A. adeninivorans* was 4.5 (Sano et al., 1999) (Table 4).

Nakamura et al. (2000) studied a number of yeast strains for extracellular phytase activity. All the yeast phytases were found to have an optimal pH of 4 to 5 when measured at optimal temperature of 50 to 60°C. At 37°C, many strains, however, produced another phytase with the optimum pH of 3 to 4. This means either there is more than one phytase, or the same protein changes its optimal pH range depending on temperature.

C. Effect of Metal Ions

Phytases from different microbes differed in their requirement for metal ions for their activity. Phytase of *S. castellii* was slightly inhibited in the presence of 5 m*M* Ca^{2+} and Mg^{2+}, Mn^{2+} and Fe^{2+}. The cations Zn^{2+} and Cu^{2+} (0.5 m*M*) caused around 50% inhibition of activity; 5 m*M* Zn^{2+} and Cu^{2+} strongly inhibited the reaction (Segueilha et al., 1992).

Phytase of *Bacillus* sp. DS11 was strongly inhibited by EDTA, Cd^{2+}, and Mn^{2+} and moderately inhibited by Hg^{2+}, Mg^{2+}, Ba^{2+}, and Cu^{2+} at 5 m*M* (Kim et al., 1998). In the reaction mixtures containing 5 m*M* Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Hg^{2+}, phytase activity of *Selenomonas ruminantium* was strongly inhibited (Yanke et al., 1999). The sensitivity pattern was similar to that of *E. coli* and *K. terrigena* (Greiner et

TABLE 4
Physico-Chemical Properties of Purified Phytases

Bacteria	T opt (°C)	pH	*Km* (mM)	Mol. Wt. (kDa)	Reference
Aerobacter aerogenes	25	4-5	0.135	-	Greaves et al., 1967
Bacillus sp. DS11	70	7.0	0.55	-	Kim et al., 1998
Bacillus subtilis	55	7.0	0.04	36.5	Powar and Jagannathan, 1982
Enterobacter sp.4	50	7-7.5	-	-	Yoon et al., 1996
Escherichia coli	55	4.5	0.13	42	Greiner et al., 1993
Klebsiella aerogenes	60	4.5-5.2	0.11	700	Tambe et al., 1994
Klebsiella oxytoca MO-3	55	5-6	-	-	Jareonkitmongkol et al., 1997
Lactobacillus amylovorus	45	4.4	-	-	Sreeramulu et al., 1996
Pseudomonas sp.	40	5.5	0.016	-	Irving and Cosgrove, 1971
Selenomonas ruminantium	50-55	4-5.5	-	46	Yanke et al., 1999
Fungi					
Aspergillus carneus	40	5.6	-	-	Ghareib, 1990
A. carbonarius	53	4.7	-	-	Al Asheh and Duvnjak, 1994
A. niger 92	55	5.0	0.44	100	Dvorokava et al., 1997
A. terreus	70	4.5	-	214	Yamada et al., 1968
A. niger NRRL 3135	58	2.2; 5.0-5.5	0.04	85-100	Ullah and Gibson, 1987
Neurospora sp.	60	5.0-6.0	-	-	Ichibiki, 1995
Penicillium caseoicolum	45	3.0	-	60-81	Amano Pharmaceuticals, 1995
Rhizopus oligosporus	55	4.5	0.15	-	Sutardi and Buckle, 1988
Yeast					
Arxula adeninivorans	75-80	4.5	0.25		Sano et al., 1999
C. intermedia	65	4.5			Nakamura et al., 2000
C. tropicalis	65	4.5			Nakamura et al., 2000
Clavispora lusitaniae	70	4.0			Nakamura et al., 2000
Hanseniaspora valbyensis	60	4-5			Nakamura et al., 2000
Kluyveromyces thermotolerans	60-65	4-5			Nakamura et al., 2000
Pichia anomala	60	4	0.20	64	Vohra and Satyanarayana , 2002 b
P. rhodanensis	70-75	4.0-4.5	0.25		Nakamura et al., 2000
P. spartinae	75-80	4.5-5.5	0.33		Nakamura et al., 2000
Schwanniomyces occidentalis	75-80	4-5	0.038	490	Segueilha *et al.*, 1992

al., 1993; Greiner et al., 1997) and *A. ficuum* (Ullah and Cummins, 1988). The partially purified enzyme from *Klebsiella oxytoca* MO-3 was strongly inhibited by NaF, Zn^{2+}, Fe^{2+}, and Cu^{2+}, but it was not inhibited by EDTA or *N*-ethylmaleimide (Jareonkitmongkol et al., 1997).

In phytase of *B. subtilis,* the removal of metal ions from the enzyme by EDTA resulted in complete inactivation. The loss of enzymatic activity is most likely due to a conformational change, as the circular dichroism spectra of holoenzyme and metal-depleted enzyme were different. Metal-depleted enzyme was partially able to restore the active conformation when incubated in the presence of Ca^{2+}. Only minor reactivation was detected with other divalent metal ions and their combinations. Based on the data, it can be concluded that *B. subtilis* phytase required Ca^{2+} for its active conformation (Kerovuo et al., 2000).

D. Effect of Substrate

Phytases usually show broad substrate specificity, with the highest affinity for phytate. Only a few phytases have been described as highly specific for phytic acid. Phytase from *Bacillus* sp. DS11 was very specific for phytate and had little or no activity on phosphate esters such as *p*-nitrophenyl phosphate, ATP, ADP, AMP, β-glycerophosphate, sodium pyrophosphate, and α-naphthylphosphate (Kim et al., 1998). Similarly, phytase from *Pseudomonas* sp. did not show activity toward inorganic pyrophosphate, β-glycerophosphate, ADP or AMP, and activity against *p*-nitrophenyl phosphate was 14% of that on phytate (Irving and Cosgrove, 1971).

The phytases of *A. fumigatus*, *E. nidulans,* and *M. thermophila* exhibited broad substrate specificity, while phytases of *A. niger, A. terreus* CBS, and *E. coli* were rather specific for phytic acid (Wyss et al., 1999). A broad substrate specificity was reported for phytases of *S. castellii*. The *Km* value for phytate was the lowest, while glucose-1-phosphate, glucose-6-phosphate, *p*-nitrophenyl phosphate, and ATP exhibited higher values (Segueilha et al., 1992). The *Km* values of some published phytases are given in Table 4. Excess substrate caused the inhibition of the enzyme. Ullah and Cummins (1988) found that myo-inositol-P_6 concentration exceeding 2 mmol/L was inhibitory. The *Km* values of *Pichia spartinae* and *P. rhodanensis* phytases were 0.30 m*M* and 0.25 m*M*, respectively; these values are similar to that of *A. adeninivorans* but higher than that of *S. castellii* (0.038 m*M*).

It is generally observed that the presence of substrate along with the enzyme prevents thermal denaturation of the enzyme at temperatures above optimal temperature. The yeast phytases were not heat resistant in the absence of substrate phytate. Preincubation of the enzyme at 75°C for 20 min in the absence of substrate led to 90% inactivation (Sano et al., 1999).

E. Thermostability

Enzymes that are used as animal feed supplements should be able to withstand temperatures of 60 to 90°C, which may be reached during the feed pelleting process. Phytases are generally thermostable enzymes active in a broad pH range. Extremely thermostable phytase from *A. fumigatus* was able to withstand temperatures up to 100°C over a period of 20 min, with a loss of only 10% of the initial enzymatic activity (Pasamontes et al., 1997). The thermostability properties of three histidine acid phosphatases, *A. fumigatus* phytase, *A. niger* phytase and *A. niger* optimum pH 2.5 acid phosphatase were investigated by measuring circular dichroism, fluorescence, and enzymatic activity. The phytases of *A. fumigatus* and *A. niger* were both denatured at temperatures between 50 and 70°C. After heat denaturation at temperatures up to 90°C, *A. fumigatus* phytase refolded completely into a native-like, fully active conformation, while that of *A. niger*

on exposure to 55 to 90°C, an irreversible conformational change occurred, with losses in enzymatic activity up to 70 to 80% (Wyss et al., 1998). Phytase from *A. carneus* lost 68% of its activity on heating at 45°C for 60 min (Ghareib, 1990).

Phytase from *E. coli* retained only 24% activity when exposed to 60°C for 1 h, and completely lost the activity at 70°C. Similarly, a phytase from *Bacillus* sp. DS11 was incubated at various temperatures for 10 min. The enzyme stability was drastically reduced above 50°C in the absence of $CaCl_2$, while it was quite stable up to 90°C in the presence of 5 m*M* $CaCl_2$ (Kim et al., 1998). The residual activity of *Enterobacter* sp. 4 phytase was about 60% on exposure to 60°C for 20 h. A complete loss of activity was recorded when exposed to 70 to 80°C for 20 h (Yoon et al., 1996).

VII. MOLECULAR CHARACTERISTICS

A. Molecular Weight

Phytases are high-molecular-weight proteins ranging from 40 to 500 kDa. Zymogram analysis of phytase of *Selenomonas ruminantium* suggested that the activity was the result of a single gene product of a monomeric nature and approximately 46 kDa in size (Yanke et al., 1999). The molecular mass and the homogeneity of the purified enzyme from *Bacillus* sp. DS11 were estimated by gel filtration and SDS-PAGE. PAGE under denaturation conditions revealed a single protein band of 44 kDa whose size corresponded well with the molecular mass of 40 kDa obtained by Superose 12 column chromatography (Kim et al., 1998). An extracellular phytase from *B. subtilis* (natto) N-77 was purified 322-fold by gel filtration and DEAE chromatography (Shimizu, 1992). The molecular mass of the active monomeric form was determined as 36 kDa by SDS-PAGE.

Two periplasmic phytases, P_1 and P_2, were purified from *E. coli* to near homogeneity (Greiner et al., 1993). The active species were judged to be monomers with a molecular mass of 42 kDa. Both enzymes were very specific for phytate.

Phytase from *S. castellii* was purified by anion exchange and gel filtration chromatography. The enzyme had a molecular weight of 490 kDa with a glycosylation of around 31%. The glycosylated protein was tetrameric, with one large subunit (MW 125,000) and three identical small subunits (MW 70,000) (Segueilha et al., 1992).

Purified phytase from *A. fumigatus* revealed a protein with a molecular weight of 60 kDa by SDS-PAGE (Pasamontes et al., 1997). The determination of molecular mass of the *A. niger* NRRL 3135 phytase and acid phosphatases was determined by either gel filtration or SDS-PAGE. The enzymes were microheterogenous because they were differentially glycosylated (Ullah and Phillippy, 1988). The molecular masses of the monomeric form of phyA, phyB, and pH 6.0 optimum acid phosphatase were estimated by SDS-PAGE as 85, 65, and 85 kDa, respectively (Ullah and Phillippy, 1988).

An extracellular phytase and an extracellular acid phosphatase were purified from *A. oryzae* K1, and their molecular masses were found to be 60 and 70 kDa, respectively. A list of molar masses of phytases from various sources is given in Table 4.

B. Active Site

The active sites of phytases showed remarkable homology to the active site residues of the members of a particular class of acid phosphatase termed 'histidine phosphatase' (Van Etten et al., 1991; Ullah et al., 1991). Chemical probing at the active site of human prostatic acid phosphatase suggested that an arginine residue is involved in catalysis (Van Etten, 1982). A similar observation was also made in *A. niger* NRRL 3135 (Ullah et al., 1991). On close examination of the active site residues of phyA and phyB in *A. niger* NRRL 3135, pH 2.5 optimum acid phosphatase in

E. coli, pho3 and pho5 gene products in yeast, human prostatic, and lysosomal acid phosphatase, it was found that most conserved sequence is RHGXRXP. The acid phosphatases and phytases containing this active site motif in the N-terminal segment of the protein are grouped under 'histidine acid phosphatase' or HAP class of enzymes; a survey of the protein and DNA databases revealed 14 members belonging to this group of acid phosphatases.

Several bacterial, fungal, and plant phytases belong to this HAP class of phytases (Table 5). As one of the best characterized HAPs, the *A. niger* NRRL3135 phytase molecule is being employed as a model to better understand this class of enzymes (Mullaney et al., 2000). The positive charge of the guanidino groups of arginine is probably responsible for the recognition and anchoring of the negatively charged phosphate group to the proximity of a histidyl residue in the active site. The phosphate group is transiently transferred to the histidine group to form an unstable phosphoenzyme complex before hydrolytic cleavage to form orthophosphate (Van Etten, 1982). This mechanism is reasonable because it is known that a group attaches to the solitary histidine residue in phosphocarrier protein. In *A. niger* NRRL 3135 phyA, inactivation of tryptophan led to catalytic demise (Ullah and Dischinger, 1992). Of the four tryptophans, only Trp25 and Trp267 are in the hydrophilic region; the other two residues are in the hydrophobic region and may not play a role in active site formation.

C. Amino Acid Sequence and Structural Properties

The primary structure of phytase from *A. ficuum* was determined from both cloned DNA (GenBank Accession No. M94550) and chemical sequencing (Ullah and Dischinger, 1993). Both the sequences deduced are in full agreement with each other. Phytase sequence has also been deduced from the cloned DNA of *A. niger* strain van Tieghem (Van Hartingsveldt et al., 1993) and is identical. A third phytase sequence obtained from *A. niger* var. *awamorii* (GenBank Accession No. L02421) showed 97.2% homology to *A. niger* NRRL 3135 phytase. Table 6 shows the amino acid composition of phytase from *A. ficuum*, soybean, and *B. subtilis* obtained by a chemical sequencing method. *A. ficuum* phytase contained 594 amino acids that are composed of 37% nonpolar, 42% polar, 11.5% acidic, and 9.5% basic amino acids. The enzyme contains more acidic than basic residues. The secondary structure of *A. ficuum* has been proposed to contain 17.3% α-helixes, 29% β-sheet, 32.6% turns, and 24.7% coils. Ullah and Dischinger (1993) suggested that tryptophan is essential for phytase activity of *A. ficuum*. One of the reasons for higher thermostability of phytase from *A. niger* NRRL 3135 could be the presence of 10 cysteine residues that allow the protein to have five disulfide bridges (Ullah and Mullaney, 1996). A complete loss of activity occurred after the reduction of disulfide bonds by β-mercaptoethanol, thus implying that these disulfide bonds play an important role for maintaining the structural integrity and catalytic properties of the enzyme (Ullah and Mullaney, 1996). The peptide sequence contained 10 potential glycosylation sites. Also, the enzyme has a very high content of aspartic acid, partly in the amidated form and possibly participating in the formation of N-glycosidic bonds with N-acetyl glucosamine. The N-linked mannose and galactose of intact protein of phytase from *A. ficuum* were estimated to be 27.3% of the molecular weight, demonstrating that the enzyme is a glycoprotein (Ullah and Gibson, 1987). Crystallographic data suggested that the structure contains a larger α/β domain similar to that of rat acid phosphatase and a smaller α domain. A large, deep indentation is centered at the interface of the two domains. The refined crystal structure of this enzyme consisted of 434 amino acids, 115 water molecules, and 1 sulfide ion in the sulfide binding site (Kostrewa et al., 1997).

TABLE 5
The Accession Numbers of Fungal Histidine Acid Phosphatase (HAP) phytases

Phy A	Accession Number
A. niger NRRL 3135	JNO656
A. niger var *awamorii*ATCC 38854	P34753
A. niger SK57	BAA74433
A. fumigatus ATCC 130703	AAB96872
Emericella nidulans	O00093
A. terreus CBS	AAB52507
Myceliophthora thermophila ATCC 48102	AAB52508
Talaromyces thermophilus ATCC 20186	AAB96873

TABLE 6
The Amino Acid Composition of Purified Phytase Determined Using the Chemical Sequencing Method

	Number of residues			
Amino acid	*B. subtilis*	*Bacillus sp. DS11*	Soybean	*A. ficuum*
Ala	29	34	29	45
Asp	58	48	59	60
Glu	39	35	45	44
Phe	9	13	23	29
Gly	36	33	47	56
His	29	9	1	10
Ile	18	31	25	22
Lys	20	31	24	11
Leu	17	26	50	50
Met	2	4	11	4
Pro	12	18	29	45
Arg	8	10	23	19
Ser	12	22	29	78
Thr	3	22	18	56
Cys	0	0	7	8
Val	16	18	35	37
Tyr	14	15	28	20
Total	**322**	**369**	**493**	**594**

The composition is given as number of residues per molecule of protein.

A phytase from *Peniophora lycii,* a basidiomycete, has been reported recently and is awaiting approval for marketing as Bio-Feed[â] Phytase by Novo Nordisk (Lassen et al., 1997). It appears to be a histidine acid phosphatase with 10 cysteine residues, but, unlike *A. niger* NRRL 3135 phy A, it is a 6-phytase.

Phytase of *Emericella nidulans* comprised 463 amino acids, while that from *T. thermophilus* contained 466 amino acids. Both proteins encoded by the cloned phyA genes of *E. nidulans* and *T. thermophilus* showed a high percentage of amino acid identity to the phytases of *A. niger* (63% and 61%, respectively), *A. terreus* 9A1 (59%, 58%), *M. thermophila* (50%, 48%), and *A. fumigatus* (67%, 61%) (Pasamontes et al., 1997). The *A. niger* strain NRRL 3135 phytase was highly glycosylated, with an approximate molecular mass of 80 to 100 kDa. The function of the carbohydrate moiety of glycoproteins remains still unclear. Based on Edman degradation analysis performed on phytase fragments of *A. niger* obtained by trypsin digestion, free asparagine residues 27, 105, 207, 230, 339, and 376 were not present, suggesting that these potential glycosylation sites were utilized. Alignment of the phytase amino acid sequence showed that two of the six Asn207 and Asn339 are conserved among all phytases reported. The Asn376 site could also be defined as a conserved potential glycosylation site, except for the *M. thermophila* phytase. According to the structure models of the *E. nidulans* and *T. thermophila* phytases, all three residues are located at the surface of the enzymes, and therefore accessible for glycosylation. All phytases contain, in addition to the aforementioned conserved three sites, additional potential glycosylation sites of the type NXS/NXT (Pasamontes et al., 1997).

By the 3D-modeling approach, 21 amino acids were identified to be conserved in all phyA phytases and these belong to the residues forming the substrate pocket of the enzyme. Some of these conserved residues belong to motif I (RHGARXP) considered to be the phosphate acceptor site (Ullah et al., 1991) and motif II (HD) where the aspartate is proposed to be the proton donor for the substrate-leaving group (Ostanin et al., 1992). Both motifs are characteristic for histidine acid phosphatases (Pasamontes et al., 1997).

Amino acid sequence of a phytase from *B. subtilis* consisted of 383 residues. It showed neither homology to the sequences of other phytases nor to those of any known phosphatases. PhyC did not have the conserved RHGXRXP sequence found in the active site of known phytases, and therefore phyC did not appear to be a member of the phytase subfamily of histidine acid phosphatase but a novel enzyme having phytase activity (Kerovuo et al., 1998). The phytase gene cloned from *B. amyloliquefaciens* comprised 1152 nucleotides and encoded a polypeptide of 383 amino acids, including a signal sequence, in contrast to the fungal phytases, which comprised about 450 amino acids. The deduced amino acid sequence did not align with those of any other known phytases, or did this enzyme contain a RHGXRXP motif (Ha et al., 1999). These two bacterial enzymes may represent novel phytases whose active sites and possibly protein folds are different from those of all other phosphatases.

D. Heterologous Gene Expression

Due to the potential use of the enzyme in the animal feed industry, attempts have been made for its cost-effective enhanced production. A high level of functional expression of *A. fumigatus* phytase gene was achieved in *Pichia pastoris*. A 1.4-kbp DNA fragment containing the coding region of the gene was inserted into the expression vector pPICZalpha A and expressed in *P. pastoris* as an active, extracellular phytase (r-Afp). The enzyme in r-Afp shared similar pH and temperature optima, molecular size, extent of glycosylation, and specificity for *p*-nitrophenyl phosphate and sodium phytate to those of the same enzyme expressed in *A. niger*. In conclusion, *P. pastoris* is a potential host to express high levels of *A. fumigatus* phytase (Rodriguez et al., 2000).

A. niger phytase gene (phyA) was expressed in *S. cerevisiae*. A 1.4-kb DNA fragment containing the coding region of the phyA gene was inserted into the expression vector pYES2 and was expressed in *S. cerevisiae* as an active extracellular phytase. The expressed phytase had two pH optima (2 to 2.5 and 5 to 5.5) and a temperature optimum between 55 and 60°C. Due to the heavy glycosylation, the expressed phytase had a molecular size of 120 kDa and appeared to be more thermostable than the commercial enzyme. Deglycosylation of the phytase resulted in losses of 9% of its activity and 40% of its thermostability. In conclusion, the phyA gene was expressed as an active extracellular phytase in *S. cerevisiae,* and its thermostability was affected by glycosylation (Han et al., 1999).

With the aim of improving cost-effective phytase production, the gene phy A from *A. niger* var. *awamorii* ALKO243 encoding phytase was cloned and sequenced; the reintroduction of the cloned *phy* A gene resulted in severalfold overproduction of phytase (Piddington et al., 1993).

One company, Gist-Brocades, has cloned multiple copies of the *A. niger* NRRL 3135 phyA gene into their PluGBug® system that yielded high levels of phytase in their *A. niger* host. This product is now being marketed as Natuphos (Van Dijck, 1999). Another enzyme producer, Novo Nordisk, is replacing their current phytase product, Phytase Novo™, with another phytase cloned from *Peniophora lycii*, a basidiomycete (Novo Nordisk A/S, 1999). This *P. lycii* phytase will be overexpressed in *A. niger* expression system and sold under the product name Biofeed phytase (Ronozyme P). A list of commercial preparations of phytase is presented in Table 7.

Recombinant strains of *Hansenula polymorpha* have been used for low-cost phytase production. Compared with the process using glycerol, the standard carbon source used for this process until now, the use of glucose led to a reduction of more than 80% in the raw material costs. In addition, exceptionally high concentrations of active enzyme (up to 13.5 g/L) were obtained in the medium, with phytase representing over 97% of the total accumulated protein. These levels greatly exceeded those reported so far for any yeast-based expression system (Mayer et al., 1999).

A two- to sixfold increase in phytase titre was observed by Moore et al. (1995) after *S. cerevisiae* and *A. niger* acid phosphatase expression in *A. oryzae*. They used the enzyme for phytic acid removal from a feedstuff.

Patents were issued describing an expression system to enhance the secretion of exoproteins, including phytase in Gram-positive bacteria, for example, *Bacillus subtilis, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, and B. thuringiensis* (Finnish Nat. Publ. Health Inst., 1994). Vectors comprising a DNA sequence encoding 3-phytase derived from *A. niger* var. *ficuum* or *A. niger* were expressed in *A. niger* var. *ficuum, A. niger, A. awamorii, A. oryzae, Trichoderma reesei, Rhizomucor meihei, Kluyveromyces lactis, S. cerevisiae, B. subtilis,* and *B. licheniformis,* and the enzyme was secreted from the host cells (Brocades, 1991a)

The phyA gene encoding an extracellular phytase from the thermophilic fungus *Thermomyces lanuginosus* was cloned and heterologously expressed and the recombinant gene product was biochemically characterized. The phyA gene was inserted into an expression vector under transcriptional control of the *Fusarium oxysporum* trypsin gene promoter and used to transform a *Fusarium venenatum* recipient strain. The *Thermomyces* phytase retained activity at 75°C and demonstrated a superior catalytic efficiency to any known fungal phytase at 65°C (Berka et al., 1998). Phytases of the fungi *E. nidulans* and *Talaromyces thermophilus* have also been cloned. The putative enzyme encoded by *E. nidulans* sequence consisted of 463 amino acids with a molecular weight of 51.78 kDa. The protein deduced from *T. thermophilus* sequence comprised of 466 amino acids corresponding to a molecular weight of 51.44 kDa. Both predicted amino acid sequences exhibited high identity (48 to

TABLE 7
List of Commercial Preparations of Phytase

Company	Trade name	pH	Temp (°C)	Unit	Unit definition
BASF / DSM	Natuphos	5.5	37.0 ± 0.1	FTU (IU)	The amount of enzyme which liberates 1mmole inorganic phosphorus per minute from sodium phytate (0.0051 mol/L)
Novo Nordisk	Biofeed ®	5.5	37.0	FYT (IU)	The amount of enzyme that liberates 1 mmole of inorganic phosphate per minute from sodium phytate (0.005 mol/L)
AB Enzymes GmbH		5.0	37.0 ± 0.5	PPU (IU)	The amount of enzyme which liberates 1 mmole of inorganic phosphate from sodium phytate in one minute
Alko Co.	Finase				
Alltech	Allzyme phytase				

67%) to the known phytases (Pasamontes et al., 1997). The phyA gene encoding heat-stable phytase was cloned from *A. fumigatus* and overexpressed in *A. niger* (Pasamontes et al., 1997). The phyA gene from *A. niger* NRRL 3135 was partially cloned in a λgt11 expression library as identified by immunoprobe and sequence verification (Mullaney et al., 1991). The full-length gene was cloned subsequently, and the sequence submitted to the Gene Bank (Accession No. M94550). A second phytase gene (phyB) from *A. niger* NRRL 3135 was cloned; this DNA fragment coded for a 479 amino acid enzyme and was found to contain four exons (Ehrlich et al., 1993).

The phytase gene (phyC) was cloned from *B. subtilis* VTT E-68013 genome library. Its amino acid sequence (383 residues) showed no homology to the sequence of other phytases or to those of any known phosphatases. It appeared to be a novel enzyme having phytase activity (Kerovuo et al., 1998). The gene en-

coding phytase from *Bacillus* sp. DS11 was cloned in *E. coli,* and its sequence was determined. A 560-bp DNA fragment was used as a probe to screen the genomic library. The phy gene cloned was encoded by a 2.2-kbp fragment. This gene comprised 1152 nucleotides and encoded a polypeptide of 383 amino acids with a deduced molecular mass of 41.80 kDa (Kim et al., 1998).

An *E. coli* strain exhibiting high phytase activity was isolated from pig colon. Using primers derived from the *E. coli* (optimum pH 2.5) acid phosphatase appA sequence (Dassa et al., 1990), a 1482-bp DNA sequence was cloned from the isolate. Despite 95% homology between the sequenced gene and the appA, seven amino acids were different in their deduced polypeptide. To characterize the properties and functions of the encoded protein, the coding region of the isolated DNA fragment and appA was expressed in *Pichia pastoris*, as r-appA2 and r-appA, respectively. The recombinant protein r-appA2, like r-appA and the r-phyA phytase expressed in *A. niger*, was able to hydrolyze phosphorus from sodium phytate and *p*-nitrophenyl phosphate (Rodriguez et al., 1999).

Phytase production is also included in the new concept in plant biotechnology using transgenic plant engineering for pharmaceutical protein and industrial enzyme production (Pen et al., 1994; White-lam, 1995; Day, 1996). The method was developed to produce transgenic plants with an enhanced level of 3-phytase using transformation of plant host with an expression vector containing fungal phytase DNA, preferably obtained from *A. niger, A. awamorii,* and *A. nidulans,* operably linked to regulatory sequences (Brocades, 1991b). The phyA gene from *A. ficuum* coding for a 441 amino acid full-length phytase was expressed in *Nicotiana tabacum* (tobacco) leaves. The expressed phytase was purified, and when the recombinant phytase was compared with its counterpart from *A. ficuum* for physical and enzymatic properties it was found that catalytically the recombinant protein was indistinguishable from the native phytase. While the temperature optima of the recombinant protein remained unchanged, the pH optima shifted from pH 5 to 4. The results are encouraging enough to open up the possibility of overexpressing the phyA gene from *A. ficuum* in other crop plants as an alternative means of commercial production of this important enzyme (Ullah et al., 1999). Phytase was expressed as 1% of the soluble protein in mature seeds of transgenic plants (Pen et al., 1993, 1994).

University of Wisconsin researchers have also developed alfalfa plants to commercially produce phytase. They performed cloning and expression of the fungal phytase gene, so that most of its product was contained in the juice collected after the alfalfa was processed (Gutknecht, 1997). The expressed enzyme from alfalfa leaves was purified to homogeneity and biochemically characterized. The expressed phytase retained all the properties of the *A. ficuum* phytase (Ullah et al., 2002). The equipment investment for this biofarming process is minimal and potentially turns out a byproduct into a source of additional income for the farmer. Other enzymes have also been expressed in these plant 'bioreactors'. However, the results achieved with phytase enhance feasibility of future development of this technology to produce the enzyme commercially.

Another application of biotechnology in plants is to reduce the need for phytase by lowering phytate levels in the plant cereal or meals. Maize cultivars with reduced levels of phytic acid have already been produced (Raboy and Gerbasi, 1996). Transgenic soybean isolates that overexpress fungal phytases and thus eliminate or reduce the need to supplement meal with phytase are also being pursued. Li et al. (1997) have expressed the *A. niger* NRRL 3135 phytase gene in soybean. The recombinant phytase had a lower molecular weight than the native fungal enzyme, but its temperature and pH optima were almost identical.

In the future, transgenic poultry and hogs may produce phytase in their own digestive tract. Several attempts have already been made

to transform and express a fungal phytase in an animal (Mullaney et al., 2000). Transgenic mouse models have been developed expressing app A phytase gene from *E*. coli regulated for expression in salivary glands. The expression of salivary phytase reduced fecal phosphorus by 11% (Golovan et al., 2001).

VIII. APPLICATION OF PHYTASES

There are two main areas where phytase can be used. The first one is phytate elimination in the feed and food industries, and the second is preparation of myo-inositol phosphates as tools for biochemical investigation. Besides this, an emphasis has also been laid on its use in aquaculture as a soil amendment and in the semisynthesis of peroxidase.

A. Animal Feed Industry

Plant feedstuffs are the major constituents of poultry diets. About two-thirds of phosphorus of feedstuffs of plant origin is present as phytic acid in the form of phytate. Under most dietary conditions, phytate P is unavailable to poultry (Nelson, 1967). In addition, phytate P chelates several important minerals and thereby reduces their availability.

Ruminant animals sustain the microflora that enzymatically release inorganic phosphorus from phytic acid. However, monogastric animals such as humans, chickens, and pigs produce little or no phytase in the intestine. This requirement of phosphorus is met by supplementing soybean and other meals with relatively inexpensive rock phosphate, which provide the animal with this necessary nutrient. The excess phytin phosphorus is disposed of in the animal's manure (Mullaney et al., 2000). Phytic acid present in the manure of these animals is enzymatically cleaved by soil and water-borne microorganisms. The phosphorus thus released is transported into the water bodies causing eutrophication (Bali and Satyanarayana, 2001). This results in oxygen depletion due to excessive algal growth. The availability of phosphorus can be improved by adding microbial phytase to the feed or by using phytase-rich cereal diet (Nelson, 1967). The enzyme minimizes the need for supplementation with inorganic phosphorus due to improvement in the utilization of organic phosphorus in poultry, and thus markedly reducing the excretion of phosphorus in manure (Mohanna and Nys, 1999). Phytase hydrolyzes phytate, and the addition of phytase to feed (250 to 1000 U/kg) can fully replace phosphorus supplementation (Golovan et al., 2001)

However, the use of phytase as a feed additive is limited by cost, by inactivation at the high temperatures required for pelleting feed (plus 80°C), and by the loss of activity during storage. These problems can be overcome if phytase is produced endogenously by swine and poultry. This enzyme could increase the bioavailability of plant phytate and in turn lead to reduced phosphorus excretion. For this a transgenic mouse has been developed that secretes phytase in its saliva (Golovan et al., 2001). The cost of using phytase as an animal feed additive can also be reduced if the heat tolerance of the enzyme is increased since during the processing of the plant meal most of the mash is heated briefly to a high temperature (65 to 95°C). The *A. niger* phytase that is being marketed would be denatured at these temperatures. This means an additional step of adding phytase after pelletization is required, thus adding to the cost. Thus, the desirable phytase would be the one that has a high heat tolerance for animal feed applications (Mullaney et al., 2000). This has led to the cloning of phytase from thermophilic fungi such as *M. thermophila* and *T. thermophilus*. The use of compounds to enhance thermostability of phytase has also been investigated. Salts and polyols have been used to increase the thermostability of enzymes (Lamosa et al., 2000). Sorghum liquor wastes supplemented with starch has also been used to increase phytase thermostability (Chen et al., 2001). Phytate is reported to enhance the activ-

ity of *A. adeninivorans* phytase (Sano et al., 1999), and calcium to the heat tolerance of *Bacillus* sp. DS11 (Kim et al., 1998). Immobilization of *E. coli* phytase (Greiner and Konietzny, 1996) and glycosylation of *A. niger* phytase expressed in *S. cerevisiae* (Han et al., 1999) contribute to the thermostability of the enzyme produced. Protein engineering has also been used to increase phytase thermostability such as the replacement of proline (Watanbe and Suzuki, 1998) and introducing disulfide bonds (Scott and Steven, 2000).

For industrial applications in animal feed, a phytase of interest must be optimally active in the pH range prevalent in the digestive tract. Site-directed mutagenesis confirmed that a replacement of Gly-277 and Tyr-282 of *A. fumigatus* wild-type phytase with the corresponding residues of *A. niger* phytase (Lys and His) gave rise to a phytase with a pH optimum at 2.8 to 3.4 (Tomschy et al., 2002). Further, Tomschy et al. (2000 a,b) have shown that the mutagenesis of Arg-274 to Gln and of Gln 27 to Leu/Thr/Ile of *A. niger* T213 and *A. fumigatus* phytases, respectively, increased the specific activity significantly. This was thought to be due to improvement in product (myoinositol pentakis phosphate) release.

On a number of occasions, workers exposed to microbial enzymes have suffered allergic responses. There is evidence that phytase is an occupational allergen that can cause specific IgE immune responses. Bio-Feed® Phytase from Novo Nordisk is available in a coated granulated form; this is a dust-free product that offers several advantages over the powdered enzyme (Mullaney et al., 2000).

The Alko Co. (Finland) as well as Altech (USA) and *BASF* (USA) started the industrial scale production of phytase marketed under the names Finase, Allzyme phytase, and Natuphos, respectively, and successfully utilized it in feed applications. Finase added to a corn–soybean pig diet converted one-third of the unavailable phosphorus to an available form (Cromwell et al., 1993). In a similar way, experiments with addition of the Allzyme and Natuphos to a pig and chicken diet also indicated that phytase is efficacious in improving the bioavailability of phytate phosphorus for pigs (Cromwell et al., 1995a,b; Yi et al., 1996; O'Quinn et al., 1997) and broilers (Yi et al., 1997).

Nelson and his co-workers (1968) were the first to pretreat a corn–soya diet with culture filtrate containing phytases of *A. niger* and fed it to 1-day-old chicks. The chicks showed an increase in bone ash since 90% of the phytin-P was hydrolyzed by phytase. When microbial phytase was fed to low-P diets for broilers, the availability of P increased to 60%, and the amount of P in the droppings decreased by 50%. Phytase activity occurred in the alimentary tract of the chick and not in the feed prior to ingestion (Nelson et al., 1971).

A 3-week feeding trial with 180-day-old broiler chickens was conducted to study the efficacy of microbial phytase (Natuphos 1000) on growth performance, relative retention of P, Ca, Cu, and Zn and mineral contents of plasma and bone. Phytase supplementation increased body weight in male and female chicks by 13.2 and 5.8%, respectively, in 21 days. The supplementation of the low-P diet with phytase increased the relative retention of total P, Ca, Cu, and Zn by 12.5, 12.2., 19.3, and 62.3 percentage units, respectively. Microbial phytase increased the plasma P by 15.7% and reduced the Ca^{2+} concentration by 34.1%. It increased the percentage ash in both head and shaft portions of dry, fat-free tibia bone to a level comparable to that of the normal-P diet. The excretion of P was also reduced from 42 to 51% when the feed was supplemented with phytase (Sebastian et al., 1996).

The growth rate and feed conversion ratio on the low-P diets containing microbial phytase were comparable to or even better than those obtained on control diets. The addition of microbial phytase to diets for growing pigs increased the apparent absorbability of P by 24%. The amount of P in the feces was 35% lower (Simons et al., 1990).

Canola meal, used as a feedstuff for livestock and fowl, was successfully dephytinized

by *A. niger* NRRL 3135 in a solid state fermentation (Nair and Duvnjak, 1990; Nair et al., 1991; Ebune et al., 1995). Segueilha et al. (1993) removed phytic acid in wheat bran and glandless cotton flour using phytase from the yeast *S. castellii*.

Among several species of yeast, *Saccharomyces cerevisiae* is the ideal yeast for yeast culture production based on its growth and metabolic characteristics (Panda et al., 1998).

A new phytase was expressed in yeast, and this, when fed to weanling pigs, improved the bioavailability of phytate phosphorus. This phytase was found to be as effective as Natuphos at the inclusion level of 700 or 1200 U/kg of a P-deficient, corn–soybean meal diet in improving phytate-P utilization of young pigs (Stahl et al., 2000).

At the close of the twentieth century, annual sales of phytase as an animal feed additive were estimated to be $500 million and growing (Abelson, 1999). The evolution of the market for this feed additive can be attributed to a chain of events during the late twentieth that both created the need for this enzyme and provided a means for its commercial development.

B. Food Industry

The presence of phytates in plant foodstuffs (De Boland et al., 1975) is well known. Molds commonly used in oriental food fermentation have been examined for their ability to produce phytase. Tempeh is a popular oriental fermented food made from soyabeans inoculated by molds (*Rhizopus oligosporus*) in the koji process. The digestibility, vitamin content, and flavor of soyabean improved with the mold fermentation (Fardiaz and Markakis, 1981).

Since phytate binds to proteins, protein isolates from soybeans are rich in phytate. Simell et al. (1989) used Finase S phytase for the preparation of phytate-free soy protein isolates being more soluble at low pH (pH 3) than the control protein. The Finase phytase can also be used in the production of phytate-free soybean milk. Anno et al. (1985) eliminated phytate from soybean milk before its consumption using wheat phytase from Sigma, and Khare et al. (1994) successfully hydrolyzed soy milk phytate using immobilized wheat phytase. Extracellular phytase from *A. ficuum* NRRL3135 brought about dephosphorylation in legume products (Shieh et al., 1969). A 78% phytate loss occurred when the enzyme preparation from *A. niger* was mixed with soyabean meal and incubated for 15 h (Han, 1988). A crude preparation *of* intracellular acid phosphatase rich in phytase obtained from *A. niger* caused nearly complete dephosphorylation of protein isolates from soybean in few hours (Zyta et al., 1989)

Dietary phytase is inactivated during cooking so the phytate digestion is very poor, thereby affecting mineral absorption. The addition of *A. niger* phytase to the flour containing wheat bran increased iron absorption in humans (Sandberg et al., 1996). The use of phytase was suggested for producing low phytin bread.

In commercial whole wheat bread, phytic acid is present at levels of 0.29 to 1.05% (w/w). By adding mold phytases during bread-making, dough phytates could be almost completely eliminated. The desirable properties of phytase would have to be Ca^{2+} independent, pH optimum of 4.5 to 5.0 and have a high reaction rate at 30°C (Knorr et al., 1981).

Caransa et al. (1988) reported that microbial phytase could accelerate the process of steeping required in the wet milling of corn, thereby improving the properties of corn steep liquor.

Phytic acid has, however, also some positive effects. It exerts an antineoplastic effect in animal models of both colon and breast carcinomas. It has been observed that adding phytate to a 2% level in drinking water reduced the incidence of tumor in rats (Harland and Morris, 1995). The presence of undigested phytate in the colon may protect it against the development of colonic carcinoma (Igbal et al., 1994).

C. Preparation of Myo-Inositol Phosphates

The increasing interest in inositol phosphates and phospholipids that play a role in transmembrane signaling and mobilization of calcium from intracellular reserves resulted in the need for various inositol phosphate preparations (Billington, 1993). Enzymic hydrolysis of phytic acid using *S. cerevisiae* resulted in the production of D-myo-inositol 1,2,6-triphosphate, D-myo-inositol 1,2,5-triphosphate, L-myo-inositol 1,3,4-triphosphate and myo-inositol 1,2,3-triphosphate (Siren, 1986a). Greiner and Konietzny (1996) prepared inositol 1,2,3,4,5-pentakisphosphate, inositol 2,3,4,5-tetrakisphosphate, inositol 2,4,5-triphosphate, and inositol 2,5-biphosphate using immobilized phytase from *E. coli*. Inositol phosphate derivatives can be used as enzyme stabilizers (Siren, 1986b), enzyme substrates for metabolic investigation, as enzyme inhibitors and therefore potential drugs, and as chiral building blocks (Laumen and Ghisalba, 1994).

Phytase for converting phytic acid to lower myo-inositol phosphate derivatives or free myo-inositol and inorganic phosphate was suggested for the industrial production of inositol or inositol phosphates (Brocades, 1991a). Among the advantages of enzymic hydrolysis is its stereo specificity and mild reaction conditions.

D. Potential in Aquaculture

Several studies have been conducted on the use of soybean meal or other plant meals in aquaculture (Mwachireya et al., 1999). By substituting lower-cost plant protein for a more expensive protein source, such as menhaden fish meal, a significant cost reduction could be achieved. Feed costs constitute up to 70% of total fish production costs (Rumsey, 1993). As in poultry and hogs, fish lack an adequate digestive enzyme to effectively utilize the phytin phosphorus in this feed, thereby excreting it in the water. Therefore, phytase has been evaluated as a means to both increase the use of low-cost plant meals in the aquaculture industry and to maintain acceptable phosphorus levels in the water. Several fish feeding studies have documented the potential value of phytase in diets containing high levels of plant feedstuffs (Robinson et al., 1996; Mwachireya et al., 1999).

E. As a Soil Amendment

In certain locations, phytic acid and its derivatives may represent up to 50% of the total organic phosphorus in the soil (Dalal, 1978). Findenegg and Nelemans (1993) studied the effect of phytase on the availability of phosphorus from phytic acid in the soil for maize plants. Growth stimulation was reported as the result of an increased rate of phytin degradation when phytase was added to the soil. This study also suggests that the expression of phytase in the roots of transgenic plants may increase the availability of phosphorus to plant roots (Day, 1996).

F. Semisynthesis of Peroxidase

A semisynthetic peroxidase was designed by taking advantage of the structural similarity of the active site of vanadium-dependent haloperoxidases and fungal phytases and acid phosphatases (van de Velde et al., 2000). The Delft group incorporated vanadate ion into the active site of *A. niger (ficuum)* NRRL 3135 phytase. This resulted in a transformation of native phosphohydrolase activity of phytase into semisynthetic peroxidase, which catalyzed enantioselective oxidation of prochiral sulfides. It was found that only histidine acid phosphatase with the active site sequence RHGXRXP could function as a peroxidase when vanadate ion was incorporated into the active site. The vanadium chloroperoxidase shares structural similarities with some membrane bound acid phosphates (Hemrika et al., 1997)

IX. CONCLUSIONS AND FUTURE PERSPECTIVES

Phosphorus is essential for all life forms. It is a basic component in nucleic acids, ATP, and numerous other biological compounds. However, it has no cycle that replenishes the Earth's supply. Phytases are now being recognized for their beneficial environmental role in reducing the phosphorus levels in manure and minimizing the need to supplement phosphorus in diets. Their use as an animal feed additive is growing because it is ecofriendly. They have an immense importance in the feed and food industry because they also improve the nutritional status by degrading phytic acid, which acts as an antinutritional factor. Also, because lower inositol phosphates and phospholipids play important roles in transmembrane cell signaling and calcium mobilization from intracellular shock, an investigation into the potential role of phytase in this mechanism would be interesting. One area that offers tremendous opportunity is increasing the use of phytase in aquaculture in order to allow the use of low-cost plant meals. Other areas for expanded use range from the use of phytase as a soil amendment and its transformation into a peroxidase. Phytase research efforts are now focussed on the engineering of an enzyme with improved thermostability, pH optimum, and substrate specificity. Thus, a program aiming at improving the industrial production of phytase at a cost–effective level should continue.

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