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Review paper

Production, purification and properties of microbial phytases

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Abstract

Phytases (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyse the release of phosphate from phytate (myco-inositol hexakiphosphate). Several cereal grains, legumes and oilseeds, etc., store phosphorus as phytate. Environmental pollution due to the high-phosphate manure, resulting in the accumulation of P at various locations has raised serious concerns. Phytases appear of significant value in effectively controlling P pollution. They can be produced from a host of sources including plants, animals and micro-organisms. Microbial sources, however, are promising for their commercial exploitations. Strains of *Aspergillus* sp., chiefly *A. ficuum* and *A. niger* have most commonly been employed for industrial purposes. Phytases are considered as a monomeric protein, generally possessing a molecular weight between 40 and 100 kDa. They show broad substrate specificity and have generally pH and temperature optima around 4.5–6.0 and 45–60°C. The crystal structure of phytase has been determined at 2.5 Å resolution. Immobilization of phytase has been found to enhance its thermostability. This article reviews recent trends on the production, purification and properties of microbial phytases.

Keywords: Phytase; Micro-organisms; Solid state fermentation; Submerged fermentation; Gene expression; Purification; Properties

1. Introduction

Phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyses the release of phosphate from phytate (myco-inositol hexakiphosphate), which is the main form of phosphorus predominantly occurring in cereal grains, legumes and oilseeds. Hydrolysis of phytic acid (phytate) to myco-inositol and phosphoric acid is considered an important metabolic process in several bio-systems. Society's awareness and increasingly demanding recent regulations the world-over on controlling the agricultural pollution, particularly on phosphorus (P) pollution that limit the P content in manure, have intensified the phytase research. The focus has mainly been on its production and use as a means of reducing inorganic P supplementation in feed and consequent reduction in faecal P excretion. Environmental pollution due to the high-phosphate manure has resulted in the accumulation of P at various locations, especially in water bodies. Phytase supplementation can reduce the amount of P in manure up to approximately 30%.

Phytases are considered of significant value in upgrading the nutritional quality of phytate-rich feed. Phytase also improves the bio-availability of phytate P in plant foods for humans (for a review on phytic acid in human nutrition, see Martinez et al., 1996). Two important groups of animals, pigs and poultry lack the enzyme needed to digest efficiently phytate in their feed. As a result, they excrete large amounts of P into the environment. This results in pollution. However, for their proper skeletal growth, these animals need P at suitable concentration. This makes the situation complex. Supplementation of phytase to the feed provides an alternative to tackle both these conditions effectively.

One alternative approach to decrease the phytic acid content of the agricultural products could be the use of chemical methods, however, such methods are generally

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Table 1
Sources, techniques and substrates for the production of microbial phytases

Micro-organism	Expressed in	Production Technique	Substrate	References
Bacteria				
*Bacillus*sp.	–	SmF	Maltose	Choi et al. (1999)
*Bacillus*sp.	*Escherichia coli*	–	–	Kim et al. (1998)
B. amyloliquefaciens	–	–	–	Kim et al. (1999a), Ha et al. (1999)
B. amyloliquefaciens	*B. subtilis*	SmF	Complex medium[a]	Kim et al. (1999b)
B. subtilis	–	–	–	Shimizu (1992); Kerovuo et al. (1998); Kerovuo et al. (2000)
Enterobacter sp.	–	SmF	Complex medium[a]	Yoon et al. (1996)
Escherichia coli	–	SmF	Complex medium[a]	Sunitha et al. (1999)
E. coli		–	–	Greiner and Jany (1991); Jia et al. (1998); Kretz (1999); Wyss et al. (1999); Golovan et al. (2000); Lim et al. (2000)
Klebsiella sp.	–	–	–	Shah and Parekh (1990)
K. aerogenes	–	–	–	Tambe et al. (1994)
K. oxytoca	–	–	–	Jareonkitmongkol et al. (1997)
K. terrígena	–	–	–	Greiner et al. (1997)
Lactobacillus amylovorus	–	SmF	Glucose	Sreeramulu et al. (1996)
Megasphaera elsdenii[*]	–	–	–	Yanke et al. (1998), Cheng et al. (1999)
Mitsuokella multiacidus[*]	–	–	–	Yanke et al. (1998)
Pseudomonas sp.	–	–	–	Richardson and Hadobas (1997)
Prevotella ruminicola[*]	–	–	–	Yanke et al. (1998), Cheng et al. (1999)
Selenomonas ruminantium[*]	–	–	–	Yanke et al. (1998, 1999), Cheng et al. (1999)
Treponema sp.[*]	–	–	–	Yanke et al. (1998), Cheng et al. (1999)
Yeasts				
Arxula adeninivorans	–	SmF	Complex medium[b]	Sano et al. (1999)
Hansenula polymorpha	–	SmF	Glucose	Mayer et al. (1999)
Rhodotorula gracilis	–	–	–	Bindu et al. (1998)
Schwanniomyces castellii	–	SmF	Wheat bran, cotton flour	Segueilha et al. (1993)
S. castellii	–	SmF	–	Lambrechts et al. (1993)
S. occidentalis	*Candida boidinii*	–	–	Nakamura et al. (1999)
Fungi				
*Aspergillus*sp.	–	SmF	Complex medium[c]	Kim et al. (1999c)
A. carbonarius	–	SSF	Canola meal	Alasheh and Duvnjak (1994a,b, 1995)
A. carneus	–	–	–	Ghareib (1990)
A. ficuum	–	SSF	Canola meal	Ebune et al. (1995)
A. ficuum	–	SmF/SSF	Glucose, canola meal	Nair et al. (1991)
A. ficuum	–	–	–	Dischinger and Ullah (1992); Ullah and Dischinger (1993); Ullah (1988); Ullah and Sethumadhavan (1998); Kim et al. (1999b,c); Liu et al. (1999)

A. ficuum	*Nicotiana tabacum*	–	–	Ullah et al. (1999)
A. flavipes	–	–	–	Youssef et al. (1987)
A. fumigatus	*Pichia pastoris*	–	–	Rodriguez et al. (2000)
A. fumigatus	*A. niger*	–	–	Pasamontes et al. (1997a)
A. fumigatus	–	–	–	Wyss et al. (1998, 1999)
A. niger	–	SmF	Maize starch	Ahmad et al. (2000)
A. niger	–	SmF/SSF	Complex/wheat bran	Papagianni et al. (2000)
A. niger		–	–	Skowronski (1978); Kujawski and Zyla (1992); Vanhartingsveldt et al. (1993); Volfova et al. (1994); Dvorakova et al. (1997); Panchal and Wodzinski (1998); Kostrewa et al. (1999); Nagashima et al. (1999); Nasi et al. (1999); Wyss et al. (1999); Lehmann et al. (2000)
A. niger	*E. coli*	–	–	Phillippy and Mullaney (1997)
A. niger	*Saccharomyces cerevisiae*	–	–	Han et al. (1999)
A. niger	*Pichia pastoris*	–	–	Yao et al. (1998), Han and Lei (1999)
A. oryzae	–	–	–	Shimizu (1993)
A. terreus	–	–	–	Mitchell et al. (1997)
Emericella nidulans	–	–	–	Pasamontes et al. (1997b), Wyss et al. (1999)
Lentines edodes	–	–	–	Quaglia and diLena (1995)
Mycleiophthora thermophila	–	–	–	Mitchell et al. (1997), Wyss et al. (1999)
*Penicillium*sp.	–	–	–	Iwama and Sawada (1999)
Talaromyces thermophilus	–	–	–	Pasamontes et al. (1997b)
Thermomyces lanuginose	–	–	–	Berka et al. (1998, 1999)
Trichoderma reessei	–	–	–	Nasi et al. (1999)

[a] Glucose-based.
[b] Glucose or galactose-based.
[c] Sucrose-based.
[*] Anaerobic bacteria.

expensive and affect the nutritional quality of the product also.

2. Market trends and manufacture

Recent market trends have clearly shown that enzymes have emerged as big feed supplements. Feed enzymes (protease, xylanase, phytase, amylase, cellulase, lipase, β-glucanase) are the newest segment of the $5 billion animal nutrition market, which is increasing fast. Presently, only about 6% of manufactured animal feeds contain enzymes, against 80–90% for vitamins, which is considered as the largest animal nutrition category (Anons, 1998). Several major animal nutrition companies are getting involved in this area very actively and various products under different trade names are already available. For example, 'Cenzyme' is a product from Cenzone, which is a unique blend of concentrated digestive enzymes including phytase from a fungal source. It has application in animal feed (Cenzone, 1999). Gist–Brocades manufactures feed enzyme containing phytase under the trade name of 'Natu-phos'. The largest market share ($\sim$ 40%) in feed enzymes is held by Finnfeeds International, a unit of Finland's Cultor, followed by BASF (probably the number two feed enzyme producer). However, the latter, which has marketing agreement with Dutch enzyme producer company Gist–Brocades, is the leading marketer of phytase world-wide. Finnfeeds has recently developed a phytase. Novo industry too is marketing a phytase in Europe (Anons, 1998). Alltech has established a manufacturing facility in Mexico for the production of phytase (Anons, 1999).

3. Source of phytases

Phytases can be derived from a number of sources including plants, animals and micro-organisms. Recent research has shown that microbial sources are more promising for the production of phytases on a commercial level. Although several strains of bacteria, yeasts and fungi have been used for production under different conditions, two strains of *Aspergillus* sp., *A. niger* and *A. ficuum*, have most commonly been employed for its commercial production. Shieh and Ware (1968) made one of the earliest approaches on microbial sources of phytases in 1968. Table 1 cites several examples as the source of microbial phytases.

4. Production of microbial phytases

4.1. Screening and assay

Several screening programmes have been carried out aiming at the isolation of different groups of bacteria, yeast and fungi having extra-cellular phytase activity. Lissitskaya et al. (1999) screened micro-organisms producing phytase using museum and soil samples. It was found that moulds metabolised P more effectively than bacteria. Chen (1998) developed a bioassay method for the screening for extra-cellular phytase-producing micro-organisms. Washed cells of *Corynebacterium glutamicum* were used as indicator strain. About 71% soil isolates had phytase activity above 0.01 U/ml. Gargova et al. (1997) used a two-step procedure to screen some 200 fungi for phytase production. Volfova et al. (1994) screened 132 micro-organisms for phytase production. All isolates intensively producing active extra-cellular phytase were of fungal origin. Sano et al. (1999) screened about 1200 yeast strains from CBS Collection for their efficiency to grow on phytate as a sole source of carbon and phosphate. The vast majority of the strains did not grow. However, *Arxula adeninivorans* showed a particularly vigorous growth. This capacity was correlated with the presence of a high activity of secreted phytase. Of 21 yeast strains screened for ability to hydrolyse phytic acid salts, nine strains grew on sodium phytate as sole source of inorganic phosphate. Of the five most interesting strains for their growth parameters tested and for their phytase activity in batch-culture, *Schwanniomyces castellii* CBS 2863 showed the highest phytase activity in the presence of phytate (Lambrechts et al., 1992). Yanke et al. (1998) screened 334 strains of 22 species of anaerobic ruminal bacteria for phytase activity. Strains belonging to *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella ruminicola*, *Mitsuokella multiacidus* and *Treponema* sp. showed phytase activity. 438 soil bacteria that utilized inositol hexaphosphate (IHP) were isolated from a range of soils using defined selection media and screened by Richardson and Hadobas (1997). Four strains were able to utilize IHP and were identified as *Pseudomonas* sp. (fluorescent or non-fluorescent). The fluorescent *Pseudomonas* strains exhibited marked phytase activity.

A simple and rapid method has been described for determining the microbial phytase. The method consisted of determining the inorganic orthophosphate released on hydrolysis of sodium phytate at pH 5.5 (Engelen et al., 1994). Bae et al. (1999) developed a method for detecting phytase activity. Differential agar media were used for the detection of microbial phytase activity and the disappearance of precipitated calcium or sodium phytate was as an indication of enzyme activity. This technique, however, was unable to differentiate between phytase activity and acid production by ruminal bacteria.

4.2. Production techniques

Phytases can be produced from a host of micro-organisms including bacteria, yeasts and fungi (Table 1). During the past 40–50 years, the use of filamentous

fungi for the production of industrial enzymes has rapidly increased and phytases are no exception to this. Submerged fermentation (SmF) has largely been employed as the production technology. However, in recent years solid state fermentation (SSF) has gained much interest for the production of primary and secondary metabolites (Pandey, 1991, 1992, 1994). In a recent review, Pandey et al. (2000a) have discussed the potential of SSF emphasising that the current decade has witnessed an unprecedented spurt in SSF for the development of bioprocesses such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, biopulping, and production of value-added products such as biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, etc., enzymes, organic acids, biopesticides, including mycopesticides and bioherbicides, biosurfactants, biofuel, aroma compounds, etc. (Nampoothiri and Pandey, 1996; Pandey, 1998; Pandey et al., 1999a,b,c; Brand et al., 2000; Fan et al., 2000; Vandenberghe et al., 2000; Medeiros et al., 2000; Soares et al., 2000). SSF systems, which during the previous two decades were termed as 'low-technology' systems appear to be promising one for the production of value-added 'low volume-high cost' products such as biopharmaceuticals. Use of agro-industrial residues offers potential advantages in SSF processes (Pandey and Soccol, 1998, 2000; Pandey et al., 2000a,b,c,d).

Techniques of SmF as well as SSF have been employed for the production of phytases. Type of strain, culture conditions, nature of the substrate and availability of the nutrients are critical factors affecting the yield and should be taken into consideration for selecting a particular production technique. For example, a filamentous fungus in SmF is exposed to hydrodynamic forces but in SSF the surface of the solid particles acts as the matrix for the culture. Moo-Young et al. (1978) stated that microbial growth in such systems depends upon the availability of nutrients and the geometric configuration of the matrix. Papagianni et al. (2000) investigated qualitative relationships between medium composition, *A. niger* morphology, and phytase production in SmF and SSF. Media composition and fungal morphology greatly affected phytase production in SmF. Addition of wheat bran, and consequent addition of a slow releasing organic phosphate source, enhanced *A. niger* growth and phytase production in SmF as well as SSF. Han et al. (1987) used a semi-solid substrate fermentation system for the production of phytase.

4.3. Bacterial phytases

Several bacterial strains (wild or genetically modified) such as *Lactobacillus amylovorus*, *E. coli*, *B. subtilis*, *B. amyloliquefaciens*, *Klebsiella* sp., etc., have been employed for phytase synthesis. Sreeramulu et al. (1996) evaluated 19 strains of lactic acid-producing bacteria of the genera *Lactobacillus* and *Streptococcus* for the production of extra-cellular phytase. A number of them exhibited the enzyme activity in the fermentation medium but *Lactobacillus amylovorus* B4552 produced the maximum amounts of phytase, ranging from 125–146 units/ml in SmF using glucose and inorganic phosphate. The findings were claimed significant as *L. amylovorus* has potential in improving nutritional qualities of cereal and pulse-based food fermentations. A bacterial strain that produced extra-cellular phytase was isolated from soil near the roots of the leguminous plants and identified to be *Enterobacter* sp. 4. The optimum condition for phytase production in PSM medium was pH of 5.5 and 3 days of cultivation at 37°C (Yoon et al., 1996). Sunitha et al. (1999) optimized the medium for recombinant phytase production by *E. coli* BL21 using response surface methodology. A 2^3 central composite experimental design was used to study the combined effects of the medium components, tryptone, yeast extract and NaCl. The optimized medium with glucose showed a highest phytase activity of 2250 U/l. A genetically modified *B. subtilis* produced extra-cellular phytase (2.0 units/ml), which constituted over 90% of the total protein. The yield was 100-fold higher than the wild type *B. amyloliquefaciens* DS11 (Kim et al., 1999b). A bacterial strain, *Bacillus* sp. KHU-10, produced a high level of an extra-cellular phytase in a maltose, peptone and beef extract medium. Under the optimised conditions, the production of the phytase reached the highest level of 0.2 unit/ml after 4 days of fermentation (Choi et al., 1999).

4.4. Yeast phytases

Phytase production using yeast cultures has generally been carried out in SmF systems. The strains used include *Schwanniomyces castellii*, *S. occidentalis*, *Hansenula polymorph*, *Arxula adeninivorans*, *Rhodotorula gracilis*, etc. (Table 1). In a continuous culture using a strain of *S. castellii*, phytase production increased with pH and dilution rate. It decreased when the phytic acid or phosphate content increased. Mayer et al. (1999) developed an efficient process for the low-cost production of phytases using *Hansenula polymorpha*. Glucose or glucose syrups were used as main carbon sources during fermentation. Compared with the process using glycerol the use of glucose led to a reduction of more than 80% in the raw materials 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 exceed those reported so far for any yeast-based expression system.

4.5. Fungal phytases

Table 1 cites several examples of fungal cultures employed for the production of phytases in SmF or SSF. Ahmad et al. (2000) used maize starch-based medium for the production of phytase in SmF using *A. niger*. Activity of the enzyme was found to be 1.075 phytase units per min per ml of the crude culture filtrate at pH 5.5 and 40°C (10 days). Extra-cellular phytase produced by *Aspergillus* sp. 5990 showed a fivefold higher activity in liquid culture when compared with cultures of *A. ficuum* NRRL 3135. SmF was carried out at 35°C, pH 7 for four days. The phytase had a higher optimum temperature for its activity than the commercial enzyme, Natuphos, from *A. ficuum* NRRL 3135 (Kim et al., 1999c).

SSF was employed for phytase production using strains of *Aspergillus* sp. Ebune et al. (1995) used canola meal for phytase production by *A. ficuum*. Optimum substrate moisture was 64%. Age of the inoculum had a profound effect on enzyme synthesis by the culture. Using a strain of *A. carbonarius* on canola meal, Alasheh and Duvnjak (1995) found 53–60% moisture as the optimum. Addition of glucose at lower concentrations (6 g) and surfactants such as Na-oleate or Tween-80 in the medium increased biomass growth and enzyme synthesis (Alasheh and Duvnjak, 1994a,b).

5. Molecular biology and gene expression

Pasamontes et al. (1997a,b) cloned the phytases of the fungi *Emericella nidulans* and *Talaromyces thermophilus*. Phytase encoded by the *E. nidulans* sequence consisted of 463 amino acids and that of *T. thermophilus* 466 amino acids with a M–r of 51785 and 51450, respectively. Both predicted amino acid sequences exhibited high identity (48–67%) to known phytases. In view of the non-availability of naturally occurring phytases having the required level of thermostability for application in animal feeding, Lehmann et al. (2000) attempted to construct consensus phytases using primary protein sequence comparisons. A consensus enzyme based on 13 fungal phytase sequences had normal catalytic properties, but showed an unexpected 15–22°C increase in unfolding temperature compared with each of its parents. As a first step towards understanding the molecular basis of increased heat resistance, the crystal structure of consensus phytase was determined and compared with that of *A. niger* phytase. *A. niger* phytase unfolded at much lower temperatures. It was concluded that for fungal phytases apparently an unexpected direct link between protein sequence conservation and protein stability exists. Han et al. (1999) studied the expression of an *A. niger* phytase gene (phyA) in *Saccharomyces cerevisiae* and determined the effects of glycosylation on the activity of phytase and its thermostability. 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 extra-cellular phytase, The yield of total extra-cellular phytase activity was affected by the signal peptide and the medium composition. The expressed phytase had two pH optima (2.0–2.5 and 5.0–5.5), a temperature optimum between 55°C and 60°C, and a molecular size of approximately 120 kDa. De-glycosylation of the phytase resulted in losses of 9% of its activity and 40% of its thermostability. The gene (phyA) for the *A. niger* phytase with optima at pH 5.5 and 2.2 was expressed in *E. coli* under the control of the T7lac promoter (Phillippy and Mullaney, 1997).

Han and Lei (1999) studied the expression of a phytase gene (phyA) from *A. niger* in *Pichia pastoris*. The gene (1.4 kb) was inserted into an expression vector pPICZ alpha A with a signal peptide alpha-factor, under the control of AOX1 promoter. The resulting plasmid was transformed into two *P. pastoris* strains: KM71 (methanol utilization slow) and X33 (wild-type). Both host strains produced high levels of active phytase (25–65 units/ml of medium) that were largely secreted into the medium. Yao et al. (1998) modified the phytase gene phyA2, whose signal peptide encoding sequence and intron sequence had been removed. The Arg-encoding codons CGG and CAG in phyA2 were mutated into synonymous codon AGA. The modified phyA2 was fused behind the alpha-factor signal sequence under the control of AOX1 promoter in plasmid pPIC9, and then introduced into the host *P. pastoris* by electroporation. The results of Southern blotting analysis and Northern blotting analysis demonstrated that the phyA2 gene had integrated into the genome of *P. pastoris* and transcribed. The result of SDS-PAGE of the phytase expressed by *P. pastoris* showed that the modified phyA2 had been over-expressed and secreted. The concentration of the phytase expressed by *P. pastoris* with modified phyA2. exceeded 15,000 U/ml, which had a 3000-fold increase over that of original *A. niger* 963 and was 37 times higher than that of recombinant *P. pastoris* with non-modified phyA2.

Molecular characterization and expression of a phytase gene from the thermophilic fungus *T. lanuginsus* involved the cloning of the phyA gene encoding an extra-cellular phytase. The phyA gene encoded a primary translation product (PhyA) of 475 amino acids (aa), which included a putative signal peptide (23 aa) and propeptide (10 aa). The deduced amino acid sequence of PhyA showed limited sequence identity (ca. 47%) with *A. niger* phytase. The phyA gene was inserted into an expression vector under transcriptional control of the *Fusarium oxysporum* trypsin gene promoter and used to transform a *F. venenatum* recipient strain. The secreted recombinant phytase protein was enzymatically active

between pH 3 and 7.5. The *Thermomyces* phytase retained activity at 75°C and showed superior catalytic efficiency to any known fungal phytase at 65°C as optimum temperature (Berka et al., 1998).

An efficient expression system was developed in *B. subtilis* for the large-scale expression of phytase (Kim et al., 1999b). The phytase gene with a native promoter derived from *B. amyloliquefaciens* was cloned in the *Bacillus* expression vector pJH27 under a strong BJ27 promoter and its expression was optimized. Kim et al. (1998) cloned the gene encoding phytase from *Bacillus* sp. DS11 in *E. coli*. The phy cloned was encoded by a 2.2 kb fragment. This gene comprised 1152 nucleotides and encoded a polypeptide of 353 amino acids with a deduced molecular mass of 41,808 Da. Phytase was produced to 20% content of total soluble proteins in *E. coli*. This was claimed as the first nucleic sequence report on phytase from a bacterial strain.

6. Purification and properties

Purification studies on phytases were usually performed with an aim to study phytase properties. Both free and immobilized enzymes originating from various microbial sources have been characterized (Table 2). Nagashima et al. (1999) purified a phytase from *A. niger* SK-57 to homogeneity in four steps by using ion-exchange chromatography (two types), gel filtration, and chromatofocusing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme gave a single stained band at a molecular mass of approximately 60 kDa. However, The extra-cellular phytase from another strain of *A. niger* had a molecular weight approximately 100 kDa. It showed pH and temperature optima at 5.0 and 55°C, respectively. The *Km* value for dodecasodium phytate, calcium phytate and 4-nitrophenyl phosphate were 0.44, 0.45 and 1.38 mmol/l, respectively. The enzyme was inhibited by $Cu^{2+}, Zn^{2+}, Hg^{2+}, Sn^{2}+, Cd^{2+}$ ions and activated by Ca^{2+}, Mg^{2+} and Mn^{2+} ions (Dvorakova et al., 1997). Phytase from *A. carbonarius* produced in SSF had a *Km* value of 0.345 and pH and temperature optima were 4.7°C and 53°C, respectively. Pre-incubation of enzyme at higher temperatures activated the enzyme (Alasheh and Duvnjak, 1994a).

An extra-cellular phytase from *B. subtilis* (natto) N-77 was purified 322-fold to homogeneity by ultra-filtration and a combination of Sephadex G-100 and DEAE-Sepharose CL-6B column chromatography. The molecular weight of the purified enzyme was estimated to be 36 kDa on gel filtration and 38 kDa on SDS-polyacrylamide gel electrophoresis, suggesting that the native enzyme was a monomeric protein. The enzyme showed the isoelectric point at 6.25, *Km* value 0.5 mM and pH and temperature optima at 6.0–6.560°C and 60°C, respectively. The enzyme activity was greatly inhibited by EDTA, $Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+}$, and Al^{3+} (Shimizu, 1992). Kerovuo et al. (1998) purified a phytase from *B. subtilis* strain VTT E-68013, which showed pH and temperature optima at 7 and 55°C, respectively. The isolated enzyme required calcium for its activity and/or stability and was readily inhibited by EDTA. The phytase gene (phyC) was cloned from the *B. subtilis* VTT E-68013 genomic library. Due to the different homology sequences (when compared with known phytases or phosphatases). PhyC appeared not to be a member of the phytase subfamily of histidine acid phosphatases but a novel enzyme having phytase activity. Kerovuo et al. (2000) also studied the metal ion requirement of a *B. subtilis* phytase. Removal of metal ions from the enzyme by EDTA resulted in complete

Table 2
Properties of phytases

Micro-organism	Forms	pH		Opt. temperature (°C)	MW (KDa)	Reference
		Range	Optima			
Bacillus subtilis	1	–	7.0	55	–	Kerovuo et al. (1998)
B. subtilis (natto)	1	–	6.0–6.5	60	36–38	Shimizu (1992)
Enterobacter sp.	1	6.0–8.0	7.0–7.5	50	–	Yoon et al. (1996)
Escherichia coli	2	2.0–10.0	4.5	60	44.7	Golovan et al. (2000)
Klebsiella aerogenes	2	–	–	–	10–13	Tambe et al. (1994)
K. oxytoca	1	4.0–6.0	5.0–6.0	55	–	Jareonkitmongkol et al. (1997)
K. terrigena	1	–	5.0	58	40	Greiner et al. (1997)
Arxula adeninivorans	1	–	4.5	75	–	Sano et al. (1999)
Pichia pastoris	1	–	2.5, 5.5	60	95	Han and Lei (1999)
S. cerevisiae	1	–	2.0–2.5, 5.0–5.5	55–60	120	Han et al. (1999)
Schwanniomyces castellii	1	–	4.4	77	490	Segueilha et al. (1992)
A ficuum	1	–	5.0	60	–	Nair et al. (1991)
A. ficuum	1	–	5.0	50	–	Liu et al. (1999)
A niger	1	–	5.0	55	100	Dvorakova et al. (1997)

inactivation. The loss of enzymatic activity was 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 calcium. Only minor reactivation was detected with other divalent metal ions and their combinations. It was concluded that *B. subtilis* phytase required calcium for active conformation.

Golovan et al. (2000) purified a phytase from *E. coli*. The purified enzyme with a molecular mass of 44,708 Da was further separated by chromatofocusing into two isoforms of identical size with isoelectric points of 6.5 and 6.3. The isoforms had identical pH optima of 4.5 and were stable at pH values from 2–10. The temperature optimum for both phytase isoforms was 60°C. A phytase from *Klebsiella* sp. No. PG.-2 was purified 50-fold by ammonium sulphate fractionation, ion-exchange chromatography and gel filtration. The enzyme had pH and temperature optima at 6.0 and 37°C. It exhibited a *Km* of 2.0 mM and energy of activation 9120 cal/mole. The enzyme activity was inhibited by Ag^{2+}, Hg^{2+}, Cu^{2+}, fluoride and high substrate concentration (Shah and Parekh, 1990). Another phytase from *Klebsiella oxytoca* MO-3 was purified 400-fold and partially characterized. The enzyme was stable at pH 4–8 with optimum activity in the range of 5–6 and the optimum temperature at 55°C. It was strongly inhibited by NaF, Zn^{2+}, Fe^{2+}, and Cu^{2+}, but not inhibited by EDTA or *N*-ethylmaleimide (Jareonkitmongkol et al., 1997). Greiner et al. (1997) purified a phytase from one another strain of *Klebsiella* sp. (*K. terrigena*) to 410-fold with a recovery of 28%. The enzyme had a molecular mass of about 40 kDa. and optimal pH and temperature were 5.0 and 58°C, respectively. Tambe et al. (1994) reported two forms of phytases from *K. aerogenes* from the cell free extract on DE-52 by isoelectrofocusing technique and on Sephadex G-200. The two forms differed in their *Km*, pH optima, *pl* values and temperature sensitivity. This was claimed as one of the rare instances where such a small fragment of enzyme peptide retained a full complement of enzyme activity.

Phytase of an *Enterobacter* sp.4 was 81.7% extracellular nature; 4.4% activity was in the periplasmic space and the remaining activity was intra-cellular and cell-bound. It had pH optima at 7.0–7.5 and was most stable at pH 6.0–8.0. The optimum temperature was 50°C (Yoon et al., 1996).

Phytase from *Schwanniomyces castellii* was purified by anion exchange and gel filtration chromatography. The enzyme had a molecular weight of 490,000 with a glycosylation rate around 31%. The structure of the deglycosylated protein was tetrameric, with one large subunit (MW 125,000) and three identical small subunits (MW 70,000). The enzyme exhibited an uncommon preference for high temperature, with optimum activity at 77°C and thermostability up to 74°C. The optimum pH was 4.4. Phytate was completely dephosphorylated by the phytase and the *Km* value was 38.

6.1. Thermostability

Thermostability is considered an important and useful criterion for industrial application of phytase. The enzyme from *A. fumigatus* was found heat-stable with great potential (Rodriguez et al., 2000). Attempts were made to determine if a high level of functional expression of the *A. fumigatus* phytase gene could be produced in *Pichia pastoris* and how the recombinant phytase reacted to different substrates, heating conditions, and proteases. After 20 min of exposure to 65–90°C, the enzyme retained 20–39% higher residual activity in 10 and 200 mM sodium acetate than in sodium citrate. The enzyme seemed to be resistant to pepsin digestion, but was degraded by high levels of trypsin. Results showed that *P. pastoris* was a potential host to express high levels of *A. fumigatus* phytase. Phytases from *B. amyloliquefaciens* DS11 and *A. ficuum* were compared on the basis of thermal stabilities. The apparent half-life of the former enzyme at 80°C was 42 min (Kim et al., 1999a). Pasamontes et al. (1997a,b) reported a heat-stable phytase 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. This was claimed to be the first such highly thermostable phytase.

6.2. Crystal structure

Ullah (1988) and Ullah and Dischinger (1992, 1993) elucidated the primary structure of phytase from *A. ficuum*based on chemical sequencing. The crystal structure of *E. coli* phytase has been determined by a two wavelength anomalous diffraction method using the exceptionally strong anomalous scattering of tungsten. Despite a lack of sequence similarity, the structure closely resembles the overall fold of other histidine acid phosphatases (Lim et al., 2000). Jia et al. (1998) also studied the crystal structure of *E. coli* phytase. Crystals were obtained by the method of bulk crystallization in 10 mM sodium acetate buffer (pH 4.5) without using a conventional precipitant. A self-rotation function showed a clear twofold non-crystallographic symmetry relating two molecules of *E. coli* phytase in the asymmetric unit. Ha et al. (1999) carried out preliminary X-ray crystallographic analysis of a novel phytase from a *B. amyloliquefaciens* strain using the hanging-drop vapour-diffusion method. The amino-acid sequence of the enzyme did not show any homology to those of other known phytases or phosphatases, with the exception of a phytase from *B. subtilis*. The enzyme exhibited a thermal stability which was strongly dependent on calcium ions. High-quality single crystals of the enzyme in

the absence of calcium ions were obtained using a precipitant solution. Ha et al. (2000) also reported a new folding architecture of a six-bladed propeller for phosphatase activity revealed by the 2.1Åcrystal structures of a novel, thermostable phytase determined in both the partially and fully Ca^{2+}-loaded states. Binding of two calcium ions to high-affinity calcium binding sites resulted in a dramatic increase in thermostability by joining loop segments remote in the amino acid sequence. Crystal structure of phytase from *A. ficuum* was reported at 2.5 Åresolution by Kostrewa et al. (1997). The structure had an alpha/beta-domain and an alpha-domain with a new fold.

6.3. Immobilization

A few studies have been made on the application and properties of immobilized phytases (Dischinger and Ullah, 1992; Ullah and Cummins, 1987; Ullah and Phillippy, 1988; Greiner and Konietzny, 1996; Liu et al., 1999). Ullah and Phillippy (1988) described the immobilization of *A. ficuum* phytase and product characterization in a bioreactor. *E. coil* phytase was covalently immobilized on NHS-activated Sepharose(R) High Performance. The pH dependence of the phytase activity was not influenced by immobilization, whereas stability against heat treatment was enhanced as a consequence of immobilization. Compared to the free phytase the immobilized enzyme exhibited the same substrate specifity, but showed an increased *Km* value (Greiner and Konietzny, 1996). Mckelvie et al. (1995) developed a flow injection system, which utilised immobilized three phytase for the determination of phytase hydrolysable phosphorus (PHP) present in natural waters. The immobilized phytase exhibited good operational and storage stability over a period of several months. Liu et al. (1999) studied the effect of immobilization on pH and thermal stability of *A. ficuum* phytase. The pH and temperature optima were pH 5°C and 50°C. Under this condition, the k(cat) of 96 s(-1) was obtained, and the corresponding *Km* value for the catalysis of phytic acid was 2.34 mM. The optimum pH for the immobilized phytase was not much different from that of the intact enzyme. However, the optimum temperature was increased to 58°C, which was 8°C higher than that of the free enzyme.

7. Conclusions

The increasing economic pressures currently being placed upon animal producers demand more-efficient utilization of low-grade feedstuffs. In addition, consumer awareness and new legislation require that any increase in animal production cannot be achieved via growth-promoting drugs or other chemical substances. One increasingly popular approach to this problem is to supplement animal diets with hydrolytic enzymes in an attempt to aid the digestion and absorption of poorly available nutrients, or to remove anti-nutritional factors from the diet. Supplementation of phytases in such diets has desirable results. Phytases from microbial sources offer techno-economical feasibility for their production and application.

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