Identification and Characterisation of a *Cryptococcus laurentii* Abo 510 Phytase

Jason van Staden

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Science at the University of Stellenbosch

April 2004

Supervisor:
Dr. M. Bloom
Co-supervisor:
Prof. W.H. van Zyl

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Jason van Staden

Summary

Phosphorus is vital for growth of all life forms and is a fundamental component of nucleic acids, ATP and several other biological compounds. Oilseeds and cereal grains, two major constituents of the diet of animals, contain phytic acid, which is the main storage form of phosphorus in plant cells. Monogastric animals, such as poultry and pigs, are not capable of utilising the bound phosphorus in phytic acid since they do not produce phytase, the essential hydrolysing enzyme. Microbial phytase is therefore added to the animal feed to enhance the availability of phosphorus and thus minimise phosphorus pollution and phosphorus supplementation in diets. For a phytase to be effective in the poultry and swine industry, it needs to be able to release phytic acid phosphorus in the digestive tract, it must be thermo-stable to resist feed processing and must be inexpensive to produce. One approach for developing an efficient phytase for the animal feed industry is by identifying new phytases from microorganisms, plants and animals.

In this study, 11 strains of the genus *Cryptococcus* were screened for phytase activity. Initially, a differential agar plate screening method was employed to determine if any *Cryptococcus* species were able to express phytase, after which production was confirmed in different liquid media. *Cryptococcus laurentii* Abo 510 was identified as a strain with significant phytase activity. The *C. laurentii* Abo 510 strain showed clear zones on the differential media agar plates and the production of phytase at high levels was observed when using wet cells grown in liquid media. The *C. laurentii* Abo 510 strain produced maximal phytase activity at a relatively high temperature (62°C) and in an acidic pH range (pH 5.0). This phytase also showed a broad substrate specificity that may assist in the release of other phosphate compounds captured in feedstuff. Although the phytase did not require any metal ions for its activity, several metal ions caused inhibition of the phytase activity. The enzyme was stable when exposed to 70°C for up to 180 minutes with only 40% loss in activity. Phosphorus addition to the culture media and enzyme assay solution at concentrations exceeding 500 μM inhibited the phytase activity completely. Different carbon sources in the culture media also influenced the phytase activity. The enzyme was determined to be a cell wall-associated phytase with little intracellular activity.

Opsomming

Lewende organismes benodig fosfaat vir groei en oorlewing en fosfaat vorm 'n fundamentele komponent van nukleïensure, ATP en verskeie ander biologiese verbindings. Veevoer bestaan meestal uit twee groot bestanddele, naamlik oliesade en graansoorte wat fitiensuur bevat. Fitiensuur is die vernaamste vorm waarin fosfaat in veevoer gestoor word. Enkelmaagdiere soos pluimvee en varke is nie in staat om die fosfaat van die fitiensuur te benut nie, aangesien hierdie diere nie die geskikte hidrolitiese ensiem, fitase, vir die vrystelling van fosfaat besit nie. 'n Mikrobiese fitase-ensiem word derhalwe by veevoer gevoeg om die fosfaatbeskikbaarheid te verhoog. Sodoende word fosfaatbesoedeling en fosfaataanvullings tot die dieet van diere ook verminder. Vir 'n fitase om effektief in die pluimvee en vark-industrie te wees, moet dit fosfaat vanaf fitiensuur in die spysverteringskanaal vrystel, dit moet behandeling by hoë temperature tydens die veevoervervaardiging oorleef en die ensiem moet goedkoop geproduseer kan word. Een van die benaderings om 'n effektiewe fitase vir die dierevoer-industrie te ontwikkel, is om nuwe fitases in mikroörganismes, plante of diere te identifiseer.

In hierdie studie is die fitase-aktiwiteit van 11 stamme van die *Cryptococcus* genus bepaal. Die seleksie vir die produksie van fitase deur die verskillende *Cryptococcus* stamme was aanvanklik op differensiële agar plate gedoen en in verskillende vloeistofmedia bevestig . 'n *Cryptococcus laurentii* Abo 510 stam is geïdentifiseer as 'n goeie fitase produseerder. Die *C. laurentii* Abo 510 stam het helder sones op die differensiële media agar plate getoon en die produksie van hoë fitase-aktiwiteit is in nat selle waargeneem na opkweking in vloeistofmedia. Die *C. laurentii* Abo 510 ras produseer maksimum fitase-aktiwieit by 'n redelike hoë temperatuur (62°C) en in 'n suur pH reeks (pH 5.0). Die fitase het ook 'n wye substraatspesifisiteit wat tot die vrystelling van fosfaat vanaf ander komponente in die veevoer mag bydra. Die fitase het geen metaalione vir sy aktiwiteit benodig nie, maar sekere metaalione het die fitase-aktiwiteit onderdruk. Die ensiem was redelik stabiel by 70°C en het na 180 minute blootstelling slegs 'n 40% verlies in aktiwiteit getoon. Die byvoeging van fosfaat in die kultuurmedium en in die ensiem reaksiemengsel teen konsentrasies bo 500 µM, het die fitase aktiwiteit heeltemal onderdruk. Verskeie koolstofbronne het ook 'n effek op die optimale fitase-aktiwiteit getoon. Die fitase ensiem is met die selwand geassosieer en het baie min intrasellulêre aktiwiteit getoon.

Biographical Sketch

Jason van Staden was born on August 25, 1978 in Klerksdorp, South Africa and matriculated from Nelspruit High School in 1996. He enrolled at the University of Stellenbosch in 1997 and obtained a B.Sc. degree (Microbiology and Genetics) in 2000, as well as a Hons. B.Sc. degree (Microbiology) in 2001. In 2002, he enrolled for a M.Sc. degree (Microbiology) at Stellenbosch University under the supervision of Dr. M. Bloom and Prof. W.H. van Zyl.

Acknowledgements

Dr. Marinda Bloom, for her intellectual input, vision, loyalty and encouragement. Her support, guidance and financial support over the years are much appreciated.

Prof. W.H. van Zyl, for his advice and willingness to contribute to the success of this study.

Prof. A. Botha, for his creativity and enthusiasm throughout the study, as well as strains from his culture collection.

Dr. A.H.J. Ullah, for his intellectual input and technical assistance with the enzyme assays.

Dr. R. den Haan, for his support and intellectual contribution to this study.

My colleagues at the University of Stellenbosch, for their friendship, advice and support throughout this project.

The National Research Foundation (NRF) for financial support.

My parents and **friends**, for their love, support, understanding and belief in my abilities and to whom this thesis is dedicated.

Contents

1. General Introduction and Project Aims

1. General Introduction and Project Aims

Phytic acid (*myo*-inositol hexakisphosphate) is the main phosphorus-containing compound in cereal grains and oilseeds, which are generally used in feedstuff for the poultry and swine industries (Reddy *et al.*, 1982). These monogastric animals are not capable of utilising the phosphorus bound in phytate (Nelson *et al.*, 1971), the salt of phytic acid, because they either have a modest phytase activity in their digestive tract (Bitar and Reinhold, 1972) or lack a phytase enzyme to release the phosphorus bound to the phytate molecule (Reddy *et al.*, 1982). Furthermore, phytate has the ability to chelate calcium, zinc, iron, magnesium and some other minerals, which reduce the utilisation of these nutrients (Oberleas, 1973; O'Dell and Savage, 1960; Lei *et al.*, 1993a; Urbans *et al.*, 2000). To compensate for this problem, farmers regularly supplemented the diets of these animals with inorganic phosphorus, which was very expensive and resulted in phosphorus pollution due to the unutilised phytate-phosphorus being excreted (Sweeten, 1992). The utilisation of phytate-phosphorus by poultry and pigs has shown to be very successful with the addition of microbial phytases to the feedstuff (Lei and Stahl, 2000). Furthermore, the phosphate being excreted by these animals was reduced by up to 50% with the addition of phytases to their diet (Lei *et al.*, 1993b, Lei *et al.*, 1993c).

It is almost a century since Suzuki *et al.* (1907) discovered phytase while working on rice bran. They were also the first to prepare an extract that retained this enzyme's activity. Biotechnology companies, environmental protection groups and nutritionists have showed a significant interest in phytases since 1986 (Lei and Stahl, 2001). This interest led to the production of new phytases, isolated from a variety of sources (microorganisms, plants and animals) or via the genetic modification or expression of cloned phytases in a suitable host.

Phytase activity has been identified and characterised in cereals, legumes and oilseeds (Reddy *et al.*, 1982), as well as several bacteria, such as *Bacillus subtilis* (Shimizu, 1992) and *Escherichia coli* (Greiner *et al.*, 1993). Phytase has also been reported in yeast, i.e. *Saccharomyces cerevisiae* and *Schwanniomyces castellii* (Nakamura *et al.*, 2000), as well as filamentous fungi, with representatives of *Aspergillus* being the most active producers of extracellular phytase (Gibson, 1987).

An enzyme of commercial importance must fulfil certain criteria. In the case of the feed-additive phytase, these standards include high specific activity, broad substrate specificity, efficient release of phytate-phosphorus, a broad pH optimum, good stability

during storage and feed pelleting, and economical to produce (Lei and Stahl, 2001). At present, the current high cost to commercially produce this enzyme limits its application as a feed supplement in certain countries (Balander, 1998).

The specific aims of this study included:

1. The identification of a novel phytase;
2. Optimising the culture media for production of the enzyme; and
3. Characterising the functional properties of the enzyme.

An overview of the current literature on the production, characterisation and applications of phytases is given in Chapter 2, while the isolation and characterisation of the *Cryptococcus laurentii* Abo 510 phytase is discussed in Chapter 3.

Literature Cited

Balander, R. 1998. Egg production and egg specific gravity in laying hens fed reduced phosphorous diets supplemented with phytase. In: Proc. of the Alltech's 14th Ann. Symp, Passport to the year 2000, Biotechnology in the Feed Industry. Lyons, T.P., and Jaques, K.A. (Eds)., Nottingham University Press, UK, pp. 411-422.

Bitar, K., and Reinhold, J.G. 1972. Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf and man. Biochem. Biophys. Acta. **268:** 442-452.

Gibson, D.M. 1987. Production of extracellular phytase from *Aspergillus ficuum* on starch media. Biotechnol. Lett. **9** (5)**:** 305-310.

Greiner, R., Konietzny, U., and Jany, K.D. 1993. Purification and characterization of two phytases from *Escherichia coli*. Arch. Biochem. Biophys. **303:** 107-113.

Lei, X.G., Ku, P.K., Miller, E.R., Yokoyama, M.T., and Ullrey, D.E. 1993a. Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. J. Nutr. **123:** 1117-1123.

Lei, X.G., Ku, P.K., Miller, E.R., and Yokoyama, M.T. 1993b. Supplementing corn-soybean meal diets with microbial phytase linearly improves phytate phosphorus utilization by weanling pigs. J. Anim. Sci. **71:** 3359-3367.

Lei, X.G., Ku, P.K., Miller, E.R., Yokoyama, M.T., and Ullrey, D.E. 1993c. Supplementing corn-soybean meal diets with microbial phytase maximizes phytate phosphorus utilization by weanling pigs. J. Anim. Sci. **71:** 3368-3375.

Lei, X.G., and Stahl, C.H. 2000. Nutritional benefits of phytase and dietary determinants of its efficacy. J. Appl. Anim. Res. **17:** 97-112.

Lei, X.G., and Stahl, C.H. 2001. Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl. Microbiol. Biotechnol. **57:** 474-481.

Nakamura, Y., Fukuhara, H., and Sano, K. 2000. Secreted Phytase Activities of Yeasts. Biosci. Biotechnol. Biochem. **64** (4)**:** 841-844.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J., and Ware, J.H. 1971. Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J. Nutr. **101:** 1289-1294.

O'Dell, B.L., and Savage, J.E. 1960. Effects of phytic acid on zinc bioavailability. Proc. Soc. Exp. Biol. Med. **103:** 304-305.

Oberleas, D. 1973. Phytates. In: Toxicants occurring naturally in foods. National Academy of Sciences, Washington, DC, p. 363.

Reddy, N.R., Sathe, S.K., Salukhe, D.K. 1982. Phytates in legumes and cereals. Adv. Food. Res. (Academic Press, NY) **28:** 1-92.

Shimizu, M. 1992. Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77. Biosci. Biotech. Biochem. **56** (8)**:** 1266-1269.

Suzuki, U., Yoshimura, K. and Takaishi, M. 1907. Ueber ein Enzym "Phytase" das "Anhydro-oxymethylen diphosphorsaure" Spaltet. Tokyo Imper. Univ. Coll. Agric. Bull. **7:** 503-512.

Sweeten, J.M. 1992. Livestock and poultry waste management: a national overview. In: Blake, J.D., and Magette, W. (Eds)., National livestock, poultry and aquaculture waste management. Amer. Soc. Agric. Eng., St. Joseph, Minnesota, pp. 4-15.

Urbans, G., Lopez-Jurado, M., Aranda, P., Vidal-Valverde, C., Tenorio, E., and Porres, J. 2000. The role of phytic acid in legumes: antinutrient or beneficial function? A Review. J. Physiol. Biochem. **56:** 283-294.

2. Literature Review

The Production, Characterisation and Application of various Phytases

Table of Contents

1. Introduction

Cereals, legumes and oilseed crops serve as a major source of nutrients for the animal kingdom and constitute over 90% of the global harvested area. An imperative component of these crops is phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) (Reddy *et al.*, 1989), which is the major form of stored phosphorus. Posternak showed the presence of phytic acid for the first time in 1903 and the chemical structure of phytic acid was determined by Anderson in 1914. Phytate is a very effective chelating agent that chelates essential minerals such as calcium, sodium, magnesium, iron, zinc and sometimes proteins and starch (Pallauf and Rimbach, 1996). Phytate is thus considered as an antinutritional factor (ANF) for monogastric animals such as poultry and swine, that cannot degrade it and the enzymatic hydrolysis of phytate is considered advantageous for these animals (Pallauf and Rimbach, 1996). Given that the hydrolysis of phytic acid is of immense importance, a unique class of enzymes with the ability to hydrolyse phytic acid has evolved – the phytases. Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) hydrolyse phytic acid to form a less phosphorylated *myo*-inositol derivative and subsequently release inorganic phosphate (Wodzinski and Ullah, 1996). Phytases are prevalent in nature, occurring in plants, microorganisms, as well as in various animal tissues (Liu *et al.*, 1998).

2. Phytic Acid

Phytate is the salt form of phytic acid and is the primary source of inositol for animals, plants and microorganisms and serves as an important phosphorus reserve (Maga, 1982). The majority of animal feedstuff of plant origin contain 50 to 80% of their entire phosphorus as phytate (Harland and Morris, 1995).

2.1 Role in Plants and Nutrition

Numerous roles have been suggested for phytic acid in plant seeds and grains. These include a function as a phosphorus reserve, an energy reserve where phosphorus from phytic acid is liberated on germination and incorporated into ATP, a source of cations and a source of *myo*-inositol (a cell wall precursor) (Reddy *et al.*, 1989). Several applications of phytic acid

including rust removal, prevention of dental caries and its use as a hypocholesteromic agent were listed by Graf (1986), together with 56 other applications (Wodzinski and Ullah, 1996). Graf and co-workers also suggested that phytic acid functions as a natural antioxidant in seeds. The effectiveness of phytic acid in blocking iron-driven hydroxyl radical formation supported this assumption (Graf *et al.*, 1987). Furthermore, undigested phytic acid in the colon might protect against the development of colonic carcinoma in animals (Dvorakova, 1998).

2.2 The Chemical Structure of Phytic Acid

The conformation structure for phytic acid was obtained by means of X-ray analysis (Blank *et al.*, 1971) and ^{31}P-NMR techniques (Johnson and Tate, 1969). Johnson and Tate (1969) suggested that the phosphate at C_2 is in the axial position, with the other phosphates in an equatorial position. Data obtained from Costello and co-workers (1976) supported this conformation and the general approved conformation of phytic acid is shown in Figure 1.

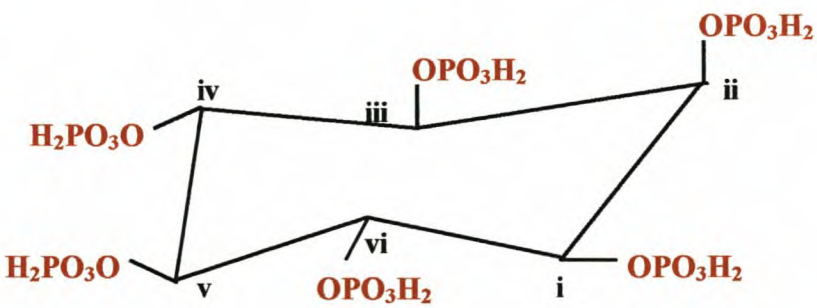

Figure 1. The generally approved conformation of phytic acid (*myo*-inositol hexakis dihydrogen phosphate). The numbering (i – vi) of carbon atoms is for the D-configuration (Liu *et al.*, 1998).

Phytic acid is a very strong negatively charged molecule with a broad pH range and therefore has a strong potential to form complexes with multivalent cations and positively charged proteins (Costello *et al.*, 1976). Using ^{31}P-NMR and pH titration methods, Costello and co-workers (1976) determined pKa values for the dissociating groups of phytic acid. Six

groups were in the strong acid range (pKa 1.1 to 2.1), one in the weak acid range (pKa 5.70), two had pKa values of 6.80 to 7.60, and three were in the alkaline range (pKa 10.0 to 12.0).

2.3 Antinutritional Effects of Phytic Acid

Phytic acid has a strong antinutritive effect (Pallauf and Rimbach, 1996) since, among other reasons, monogastric animals (humans, chickens and pigs) are not able to utilise the phytic acid-phosphorus (Nelson *et al.*, 1968). After complete dissociation of the phosphate groups, phytic acid carries twelve negative charges. This contributes enormously to the antinutritional effect since the negatively charged molecule will effectively bind to different mono-, di- and trivalent cations to form insoluble complexes (Reddy *et al.*, 1989). These phytate-mineral complexes that form in the intestinal tract reduce the bioavailability of essential minerals and will have a negative effect on the animal's health (Davies, 1982). This was supported by data that indicated that phytic acid influenced the bioavailability of zinc by growing rats when their diet were supplemented with phytic acid (Pallauf *et al.*, 1992).

Negatively charged phytic acid interacts with proteins to form phytate-protein complexes, which will render the proteins unavailable. Similar to the phytic acid-mineral complexes, this will also negatively influence the protein's bioavailability (De Rham and Jost, 1979; Fretzdorff *et al.*, 1995). The isoelectric points of plant proteins are generally around pH 4.0 to 5.0 and acidic conditions will therefore assist phytic acid in binding tightly to these plant proteins and decrease their bioavailability (Cheryan, 1980). Phytic acid was shown to interact with digestive enzymes such as trypsin, pepsin, α-amylase and β-galactosidase. This complex formation results in a decrease in the activity of these vital digestive enzymes and further contributes to the anti-nutritional effect of phytic acid (Deshpande and Cheryan, 1984; Inagawa *et al.*, 1987; Singh and Krikorian, 1982).

2.4 Degradation of Phytic Acid by Phytase

Throughout the hydration and germination periods of seeds, phytase present in the plant itself enzymatically dephosphorylates phytic acid (Harland and Harland, 1980). Depending upon the degree of dephosphorylation, phytase dephosphorylates phytic acid to produce phosphoric acid, free inositol and/or the intermediary compounds, including mono-, bis-, tris-, tetrakis-

and pentakisphosphate esters of inositol (Dasgupta *et al.*, 1996). The general dephosphorylation enzymatical reaction is shown in Figure 2.

Phosphatases represent a group of enzymes involved in the mobilisation of phosphate reserves, like those mentioned above (Andriotis and Ross, 2003). Phytases belong to the sub-class of acid phosphatases (Duff *et al.*, 1994) that, except for phytase, do not show an apparent preference for the substrate, phytic acid. The enzyme known as acid phosphatase also belongs to this sub-class of acid phosphatases and is distinguished from phytase by its non-specificity for phytic acid (Andriotis and Ross, 2003). The acid phosphatase might assist in the dephosphorylation of the intermediates of phytic acid during hydrolysis by phytase, but it is not capable of hydrolysing phytic acid in its native state (Zyla, 1993). For example, acid phosphatase isolated from garlic seedlings did not show any phytase activity, but the acid phosphatase was still efficient for the dephosphorylation of other phosphate substrates (Yenigun and Guvenilir, 2003). Phosphorus plays a very important role in the growth of plants and seeds and is an important part of the plant's metabolic functions. This may explain why several plant species enhance the expression of their extracellular acid phosphatases in their rhizosphere during phosphate shortages (Asmar *et al.*, 1995; Tarafdar and Jungk, 1987).

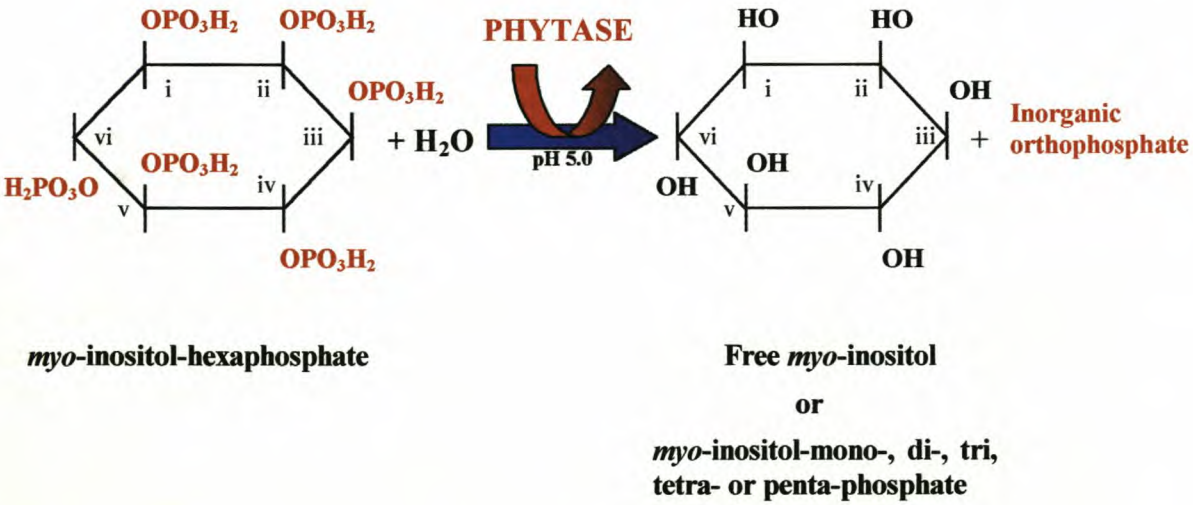

Figure 2. Enzymatic reaction of phytase (Liu *et al.*, 1998).

The Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) distinguishes between two types of phytase. The classification is based on the first phosphate group attacked by the phytase (numbering of these phosphate groups are shown in Figure 1 and Figure 2). The 3-phytase (EC 3.1.3.8) that first attacks phosphate at the third carbon position of the phytic acid, is characteristic for microorganisms (Johnson and Tate, 1969), except for the enzyme from *Escherichia coli* where the main pathway starts degradation at position 6 (Greiner *et al.*, 1993). The second phytase was classified as a 6-phytase (EC 3.1.3.26) that first attacks phosphate at the sixth carbon position of phytic acid (Cosgrove, 1969; 1970). This enzyme is usually found in plants (Maiti *et al.*, 1974).

Apart from *E. coli* that first degrade phytic acid at position 6, *Pichia rhodanensis* degrades phytic acid first at either position 3 or position 6 (Adelt *et al.*, 2003). The dephosphorylation pathway of this yeast in comparison with *Saccharomyces cerevisiae* is shown in Figure 3. The phytase of *S. cerevisiae*, similar to other microorganisms, attacks the phosphate group of phytic acid (*myo*-InositolP_6) first at position 3 and produces an end product of Inositol (1,2,6). Using *S. cerevisiae* to partially dephosphorylate phytic acid, resulted in the production of D-*myo*-inositol 1,2,6-triphosphate (Siren, 1986). Furthermore, immobilised phytase from *E. coli* was used to prepare inositol 1,2,3,4,5-pentakisphosphate, inositol 2,3,4,5-tetrakisphosphate, inositol 2,4,5-triphosphate, and inositol 2,5-biphosphate (Greiner and Konietzny, 1996).

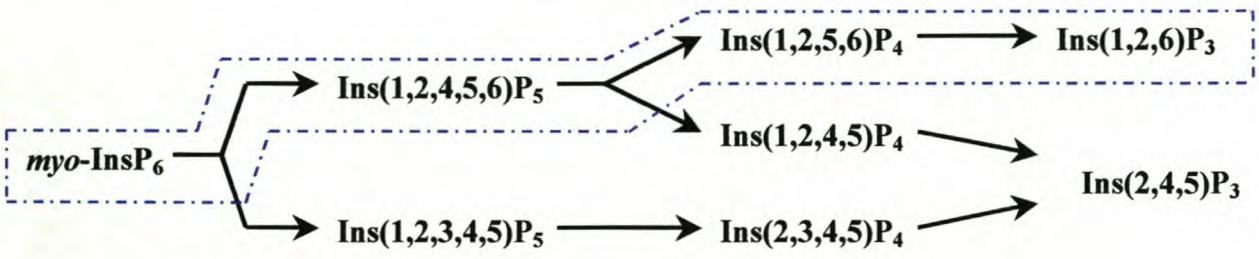

Figure 3: Major pathways for phytase from *P. rhodanensis* for the dephosphorylation of *myo*-InsP_6. The framed reaction shows the typical pathway for phytase from *S. cerevisiae* (Adelt *et al.*, 2003).

2.5 Metabolic Role of *Myo*-inositol-phosphate Derivatives

Transport of materials into the cell with the assistance of inositol phosphate intermediates that act as a secondary messenger and play a role in signal transduction in plant and animal cells, is a dynamic area of research (Wodzinski and Ullah, 1996). Inositol phosphates and phospholipids play essential roles in transmembrane signalling and mobilisation of calcium in the cell (Figure 4), which resulted in the requirement for a variety of inositol phosphate preparations (Billington, 1993). Moreover, particular inositol triphosphates have been recommended to avoid or ease diseases or circumstances associated with abnormal levels of Neuropeptide Y (NPY) (Siren *et al.*, 1992). These diseases account for various inflammatory and respiratory conditions (Siren *et al.*, 1992). Certain inositol triphosphates were also suggested to be used as pain killers (Siren, 1995).

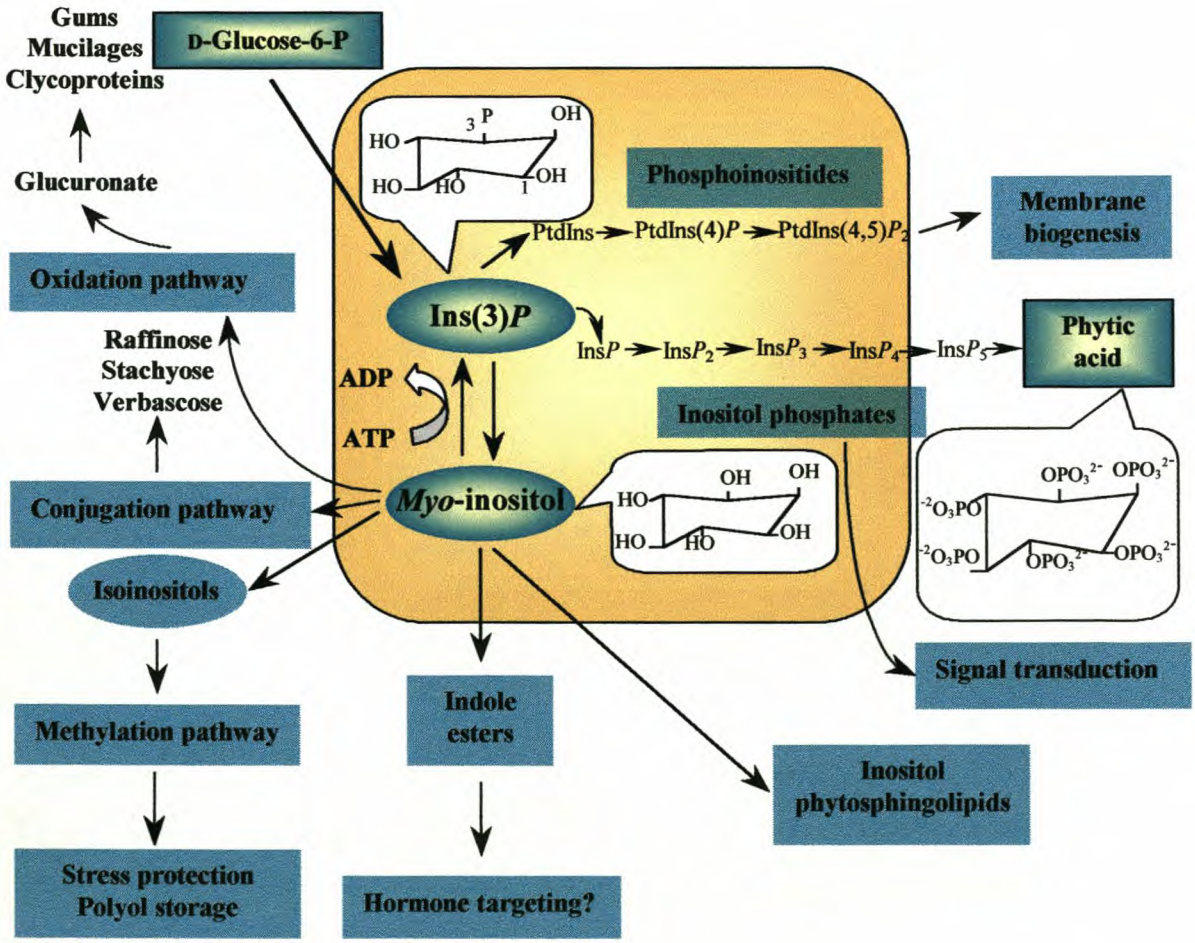

Figure 4. A representation of the role that *myo*-inositol and its phosphates play in the plant cell. Ins, inositol; PtdIns, phosphatidylinositol taken from (Brinch-Pedersen *et al.*, 2002).

These inositol intermediates and phytic acid have been shown to contribute to the enhancement of starch digestibility and blood glucose response (Thompson, 1986) in tumour formation inhibition (Shamsuddin *et al.*, 1988, 1989; Shamsuddin and Ullah, 1989; Ullah and Shamsuddin, 1990), in the treatment of Alzheimer's disease (Sabin, 1988, 1989) as well as treatment of Parkinson's disease (Sabin, 1992).

3. Isolation and Characterisation of Phytases

Phytase has a broad presence in plants, microorganisms and animal tissues. These various cells or tissues are capable of producing phytase either intracellularly or extracellularly; therefore, different purification techniques have to be employed for phytases from different sources. The production of phytase is also affected by many external properties, i.e. temperature, pH, growth media, etc. (Liu *et al.*, 1998)

3.1. Screening for Phytase Producers

Numerous screening programmes for the isolation of different groups of microorganisms with extracellular phytase activity have been investigated (Pandey *et al.*, 2001). Appropriate enzyme assays proved to be one of the fundamentals for screening large numbers of microbial isolates (Bae *et al.*, 1999). Liquid assays for measuring phytase activity are insufficient for the direct screening of numerous microbial isolates, since it is very time consuming (Shimizu, 1992; van Hartingsveldt *et al.*, 1993). Differential agar plating mediums proved to be appropriate for the screening of a large number of isolates (Gargova *et al.*, 1997; Sano *et al.*, 1999). Using a two-step procedure, 200 fungi were first tested for phytase production. First calcium phytate was used as main substrate. The second step was to verify if the fungi that produced extracellular phytase, also produced this enzyme in a medium containing cornstarch as the main substrate (Gargova *et al.*, 1997). A total of 132 microorganisms were screened for their phytase production and the majority of isolates producing phytase were of fungal origin (Volfova *et al.*, 1994).

About 1200 yeast strains were screened for their effectiveness to grow on phytate as a sole source of carbon and phosphate (Sano *et al.*, 1999). Many of the strains did not show any growth, but *Arxula adeninivorans* showed a good growth that was later linked to a high activity of secreted phytase. This same technique was used for 21 other yeast strains and only

one strain showed high phytase activity when used in a batch-culture (Lambrechts *et al.*, 1992). Defined selection media (phytic acid as main substrate) was used to screen 438 soil bacteria for phytase activity, but only four strains were capable of degrading the phytic acid (Richardson and Hadobas, 1997).

An alternative method employed non-specific chromogenic phosphatase substrates that are generally used for histochemical and molecular biology applications, including 5-bromo-4-chloro-3-indolyl phosphate (BCIP), naphthol AS phosphates (West *et al.*, 1990) and phenolphthalein diphosphate/methyl green (Riccio *et al.*, 1997). This approach, regrettably, lacked the required specificity.

Another indication of enzyme activity relied on the appearance of clearing zones on agar plates due to the disappearance of precipitated calcium phytate (Howson and Davis, 1983; Shieh and Ware, 1968). However, this solid medium assay yielded false positive reactions since some organisms produced acid that will precipitate the calcium phytate. It is therefore not possible to distinguish between phytase and acid production by these isolates on the selected differential medium (Bae *et al.*, 1999). A two-step counterstaining procedure was designed to overcome this predicament. The solid agar medium was first flooded with an aqueous cobalt chloride solution, followed by an ammonium molybdate/ammonium vanadate solution that reprecipitates acid-solubilised phytate (Bae *et al.*, 1999). This counterstaining technique can also be used to determine *in situ* phytase acivity in polyacrylamide gels (Bae *et al.*, 1999).

Determining the specific phytase activity for a single microbial isolate is based on the inorganic ortophosphate released through hydrolysis of sodium phytate at a pH of 5.5. This is a very straightforward method for obtaining the precise phytase activity (Engelen *et al.*, 1994). The enzyme activity is expressed in one of two ways. The first refers to the amount of inorganic phosphate (nmol) liberated per second from the substrate (sodium phytate) and is expressed in nKat (Ullah and Gibson, 1987). The second refers to the amount of inorganic phosphate (µmol) liberated per minute under the assay conditions, expressed in International Units (IU) (Bogar *et al.*, 2003).

3.2 Sources of Phytase and Purification Techniques

As mentioned earlier, phytase has a wide presence in plants, microorganisms and animal tissues (Table 1). Phytase in plants was found in cereals, legumes and various kinds of

Table 1. Sources of phytases, their cellular location and production techniques (Pandey *et al.*, 2001; Vohra and Satyanarayana, 2003).

Microorganisms	Location of enzyme	Production technique	Substrate	References
Bacteria				
Aerobacter aerogenes	Cell bound	SmF		Greaves *et al.* (1967)
Bacillus amyloliquefaciens	Extracellular	SmF		Ha *et al.* (1999)
Bacillus sp.	Extracellular	SmF	Maltose	Choi *et al.* (1999)
Bacillus subtilis	Extracellular	SmF, SSF	Steamed soybeans	Kerovuo *et al.* (1998); Shimizu (1992)
Bacillus sp. DS11	Extracellular			Kim *et al.* (1998)
Enterobacter sp. 4	Extracellular	SmF	Complex medium	Yoon *et al.* (1996)
E. coli	Cell bound	SmF	Complex medium	Greiner *et al.* (1993); Sunitha *et al.* (1999)
Klebsiella aerogenes	Cell bound	SmF		Tambe *et al.* (1994)
Klebsiella oxytoca MO-3	Cell bound	SmF		Jareonkitmongkol *et al.* (1997)
Lactobacillus amylovorus	Extracellular	SmF	Glucose	Sreeramulu *et al.* (1996)
Mitsuokella multiacidus	Cell bound			Yanke *et al.* (1998)
Pseudomonas sp.	Cell bound	SmF		Irving and Cosgrove (1971); Richardson and Hadobas (1997)
Selenomonas ruminantium	Cell bound	SmF		Yanke *et al.* (1999); Cheng *et al.* (1999)
Yeasts				
Arxula adeninivorans	Extracellular	SmF	Complex medium	Sano *et al.* (1999)
Candida spp.	Extracellular			Nakamura *et al.* (2000)
Clavispora lusitaniae	Extracellular			Nakamura *et al.* (2000)
Debaromyces 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	Cell bound	SmF	Glucose, beef extract	Vohra and Satyanarayana (2001)
Pichia spp.	Extracellular			Nakamura *et al.* (2000)
Rhodotorula gracilis	Cell bound	SmF	Glucose	Bindu *et al.* (1998)
Saccharomyces cerevisiae	Cell bound			Howson and Davis (1983)
S. cerevisiae	Extracellular			Nakamura *et al.* (2000)
Saccharomyces kluyveri	Extracellular			Nakamura *et al.* (2000)
Schwanniomyces castellii	Extracellular	SmF	Wheat bran, cotton flour	Segueilha *et al.* (1992)
Schwanniomyces occidentalis	Extracellular	SmF		Nakamura *et al.* (2000)
Torulaspora delbrueckii	Extracellular			Nakamura *et al.* (2000)
Torulaspora globosa	Extracellular			Nakamura *et al.* (2000)
Torulaspora pretoriensis	Extracellular			Nakamura *et al.* (2000)

Table 1. (continued) Sources of phytases, their cellular location and production techniques (Pandey *et al.*, 2001; Vohra and Satyanarayana, 2003).

Filamentous Fungi				
Aspergillus sp.	Extracellular	SmF	Complex medium	Kim *et al.* (1999b)
Aspergillus amstelodami	Extracellular			Howson and Davis (1983)
Aspergillus candidus	Extracellular			Howson and Davis (1983)
Aspergillus chevalieri	Extracellular			Howson and Davis (1983)
Aspergillus clavatus J239	Cell bound			Casida (1959)
Aspergillus ficuum	Extracellular	SSF	Canola meal	Ebune *et al.* (1995a)
Aspergillus ficuum	Extracellular	SmF/SSF	Glucose, canola meal	Nair *et al.* (1991)
Aspergillus ficuum	Extracellular	SmF	Glucose, corn starch	Ullah (1988)
Aspergillus flavipes Fla.A14	Cell bound			Casida (1959)
Aspergillus flavus	Extracellular			Shieh and Ware (1968)
A. flavus	Cell bound			Casida (1959)
Aspergillus niger	Extracellular	SmF	Maize starch	Ahmad *et al.* (2000)
Aspergillus niger	Extracellular	SmF/SSF	Complex/ wheat bran	Papagianni *et al.* (1999)
A. niger NRRL 67	Cell bound			Casida (1959)
A phoenicus QM 329	Cell bound			Casida (1959)
Aspergillus repens	Extracellular			Howson and Davis (1983)
Aspergillus syndowi	Extracellular			Howson and Davis (1983)
Aspergillus terreus	Extracellular			Yamada *et al.* (1968)
Aspergillus versicolor	Extracellular			Howson and Davis (1983)
Aspergillus wentii	Extracellular			Howson and Davis (1983)
Aspergillus fumigatus	Extracellular			Pasamontes *et al.* (1997b)
Aspergillus carbonarius	Extracellular	SSF	Canola meal	Alasheh and Duvnjak (1994, 1995)
Aspergillus carneus	Extracellular			Ghareib (1990)
A. nidulans QM-329	Cell bound			Casida (1959)
Aspergillus terreus 9A1	Extracellular			Mitchell *et al.* (1997)
Botrytis cinerea	Extracellular			Howson and Davis (1983)
Emericella nidulans	Extracellular			Pasamontes *et al.* (1997a)
Geotrichum candidum	Extracellular	SmF	Glucose	Howson and Davis (1983)
Mucor piriformis	Extracellular			Howson and Davis (1983)
Mucor recemosus	Extracellular	SSF	coconut oil cake	(Bogar *et al.*, 2003)
Myceliophthora thermophila	Extracellular			Mitchell *et al.* (1997); Wyss *et al.* (1999a); (Wyss *et al.* 1999b)
Penicillium sp.	Extracellular			Shieh and Ware (1968)
Penicillium spp. P-320	Cell bound			Casida (1959)
Rhizopus oryzae	Extracellular			Howson and Davis (1983)
Rhizopus oligosporus	Extracellular	SmF	Glucose	Howson and Davis (1983)
Rhizopus oligosporus	Intra- and Extracellular	SSF	Soybeans	Sutardi and Buckle (1988)
Rhizopus stolonifer	Extracellular			Howson and Davis (1983)
Sporotrichum thermophile	Extracellular			Ghosh (1997)
Talaromyces thermophilus	Extracellular			Pasamontes *et al.* (1997a)
Thermomyces lanuginosus	Extracellular			Berka *et al.* (1998)
Trichoderma reessei	Extracellular			Nasi *et al.* (1999)

SmF, Submerged Fermentation; SSF, Solid-state Fermentation

seeds and pollens. Numerous microorganisms including bacteria, yeast and fungal species, have a phytase-producing aptitude (Liu *et al.*, 1998). Phytase in animals is located in the erythrocytes and plasma of a variety of species and in the small intestine of some mammals (Cooper and Growing, 1983; Martin and Luque, 1985; Zyla, 1992). Various comprehensive procedures for phytase production are based on the source of phytase and the purity required (Liu *et al.*, 1998).

3.2.1 Plant Phytases

Approximately all plants have some phytase activity. However, the yield of phytase and the importance of this enzyme in hydrolysing phytic acid inside the seed vary noticeably among plants (Eeckhout and De Paepe, 1994). The first phytase activity in plants was found in rice and wheat bran (Suzuki *et al.*, 1907). Evaluation of 51 feedstuff used in feed mills for their ability to produce phytase, showed ample phytase activity in rye (85 $nkat.g^{-1}$), triticale (28 $nkat.g^{-1}$), barley (10 $nkat.g^{-1}$), wheat (20 $nkat.g^{-1}$) and soybean meal (Eeckhout and De Paepe, 1994). Phytase has also been found in maize, corn seeds, lettuces, dwarf beans, mung beans and other legumes or oilseeds (Eskin and Wiebe, 1983; Gibson and Ullah, 1990; Laboure *et al.*, 1993). Germinating seeds or pollen were shown to produce phytase during these developmental stages and a decrease in the phytic acid content in the seeds or pollen was noted (Reddy *et al.*, 1982; Gibson and Ullah, 1990). Mullaney *et al.* (2000) purified a phytase from scallion leaves, which indicated that phytase not only occurs in seeds and legumes.

For the purification of phytase from soybean seeds, an $(NH_4)_2SO_4$ precipitation and three column chromatographic steps were suggested (Gibson and Ullah, 1990). The separation of wheat bran phytase by DEAE cellulose resulted in two fractions that differed in their substrate degradation patterns (Lin *et al.*, 1987). Another plant to show phytase activity is *Arabidopsis thaliana*. This phytase shares several similar important features with the commercial phytase from *Aspergillus ficuum*, namely, size, active-site sequence, catalytic dipeptide and 10 cysteine residues situated in key positions of the molecule (Mullaney and Ullah, 1998). This phytase may therefore be just as successful as the commercially produced microbial phytases, but further research is needed to confirm such a suggestion. Purification of phytase from *Corylus avellana L.* seeds entailed the separation of phytase from the other acid phosphatases through consecutive $(NH_4)_2SO_4$ precipitations, gel filtrations and cation

exchange chromatography. This purification technique resulted in 300-fold purification and a reduction of phytase activity by 7.5% (Andriotis and Ross, 2003).

The plant's ability to produce phytase in the seeds during the germination period, allows the plant to release the phosphate captured in phytic acid during germination for its growth and to increase the nutritional quality of these seeds (Kyriakidis *et al.*, 1998). This process has been described in fababeans (Eskin and Wiebe, 1983), horse grams and mouth beans (Borade *et al.*, 1994). Maximal phytase activity varies during the germination stage where chickpeas and fababeans showed maximal phytase activity only on the 6 th day, whereas lentils, broad beans and runner beans showed maximal activity on day 8 of germination (Eskin and Wiebe, 1983; Kyriakidis *et al.*, 1998). Although these plant phytases do not show sufficient levels of phytase activity for its use in the industry, consumer acceptance may contribute to the feasibility of using endogenous plant phytases for commercial application (Kyriakidis *et al.*, 1998).

3.2.2 Bacterial Phytases

Phytases from bacterial sources are predominantly cell-associated, with the exception of *Bacillus subtilis*, *Lactobacillus amylovorus* and *Enterobacter* sp. 4 (Vohra and Satyanarayana, 2003). Generally, phytases from bacteria were purified by $(NH_4)_2SO_4$ precipitation, or ultrafiltration followed by ion-exchange and gel filtration (Liu *et al.*, 1998). A four-step chromatographic purification protocol was used for the purification of an *E. coli* phytase that was purified more than 16 000-fold (Greiner *et al.*, 1993). *B. subtilis* is able to grow and produce phytase using SSF (Solid-state Fermentation) on steamed soybean and SmF (Submerged Fermentation) media (Table 1) and a Sephadex G-100 column was used to purify this phytase (322-fold) (Shimizu, 1992). *Enterobacter* sp. 4 was isolated from soil near the roots of leguminous plants and maximal phytase activity was obtained when it was grown for three days in a minimal salt medium at pH 5.5 at 37°C (Yoon *et al.*, 1996). Localisation studies showed that the phytase activity of *Enterobacter* sp. 4 was mainly located in the extracellular fraction (82%) (Yoon *et al.*, 1996).

Lactic acid-producing bacteria (LAB) from the *Streptococcus* and *Lactobacillus* genera were evaluated for their extracellular phytase production (Sreeramulu *et al.*, 1996). The *L. amylovorus* B 4552 strain showed maximal phytase activity in comparison to the other 18 LAB screened for the enzyme in SmF. Among the 12 species of LAB from sourdough that

were screened for phytase activity, only intracellular phytase activity were shown for all of them, with *Lactobacillus sanfranciscensis* CB1 having the highest activity (De Angelis *et al.*, 2003). *Lactobacillus plantarum* NRRL B-4496 was shown to have a non-specific acid phosphatase that has a higher hydrolysis rate with monophosphorylated compounds which could explain the low level of phytase activity. The phytic acid structure could have degraded and would have led to the formation of phytic acid intermediates; which will make it possible for the acid phosphatase to hydrolyse these compounds and this might have led to the false positive activity of phytase (Zamudio *et al.*, 2001).

Under optimised conditions using a maltose, peptone and beef extract medium, *Bacillus* sp. KHU-10 produced a high level of an extracellular phytase after 4 days (0.2 U/ml) (Choi *et al.*, 1999). Intracellular phytase produced by *Citrobacter braakii* YH-15 was purified (12 800-fold) to homogeneity with $(NH_4)_2SO_4$, Phenyl Sepharose, DEAE Sepharose, CM Sepharose and Mono S HR 5/5 (Choi *et al.*, 1999). The phytase of the *Bacillus* sp. KHU-10 remarkably showed a specific activity of 3457 $U.mg^{-1}$ protein (Golovan *et al.* 2000), which was 1.9 times higher than the *E. coli* phytase (1800 $U.mg^{-1}$ protein) (Greiner *et al.*, 1993).

The extracellular phytase from *B. subtilis* VTT E-68013 grown in a wheat bran medium was very susceptible to routinely used chromatographic purification methods (ion exchange and gel filtration). $CaCl_2$ was required in the purification solutions to maintain the phytase activity and EDTA seemed to decrease the activity. A combination of ethanol and $(NH_4)_2SO_4$ precipitation was used for the purification of the phytase from *B. subtilis* VTT E-68013, which proved to be the most suitable purification technique (Kerovuo *et al.*, 1998).

Bacillus subtilis (natto) N-77, a strain that grows rapidly on steamed soybeans and produces an extracellular phytase, ferments soybeans to natto, a traditional soybean cheese produced in Japan. The enzyme was purified 322-fold through ultrafiltration and a combination of Sephadex G-100 and DEAE-Sepharose CL-6B columns to homogeneity. Maximal phytase activity was obtained with the addition of calcium (5 mM) and D-mannose (1% w/v) to the culture medium, with a specific activity of 8.7 U.mg $protein^{-1}$ (Shimizu, 1992).

Numerous strains of ruminal bacteria were shown to have phytase activity, including *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella* sp. and *Mitsuokella multiacidus* (Yanke *et al.*, 1998). These phytases are tightly associated with the outer membrane of the bacteria (D'Silva *et al.*, 2000; Yanke *et al.*, 1998).

3.2.3 Fungal Phytases

3.2.3.1 Filamentous Fungi

The majority of enzymes associated with the fungal cell wall are secreted proteins that are briefly retained in the cell wall before of their secretion into the environment (Klis, 1994). The majority of microbial phytases are repeatedly detected in fungal species, in particular species from the genus *Aspergillus* (Table 1). Over 2000 microorganisms isolated from 68 soil samples were screened for their production of phytase (Shieh and Ware, 1968); only 30 isolates produced an extracellular phytase activity, all of them being filamentous fungi. More than 90% of these extracellular phytase producers were from *Aspergillus*. A number of studies confirmed that strains from *A. niger* were the best extracellular producers of phytase (Howson and Davis, 1983; Volfova *et al.*, 1994).

SSF and semi-solid fermentation systems delivered much higher phytase activity than those produced in SmF systems (Krishna and Nokes, 2001; Papagianni *et al.*, 1999). At present, commercial phytase is produced by straightforward SmF systems by using strains of *A. niger* and *A. ficuum* (Igbasan *et al.*, 2000; Pandey *et al.*, 2001). Maximal activity was obtained with *A. niger* after 10 days of cultivation in a SmF system containing a maize starch based medium (Ahmad *et al.*, 2000). *A. ficuum* NRRL 3135 was thought to produce the highest extracellular phytase activity in SmF, until the discovery of an *Aspergillus* sp. 5990 that showed a five-fold increase in activity at 35°C after four days in SmF (Kim *et al.*, 1999b). Optimal phytase activity for *Aspergillus carbonarius* using canola meal as substrate showed that the moisture content had to be between 53 and 60% (Alasheh and Duvnjak, 1995). The addition of surfactants like Tween-80 in the culture medium increased the biomass growth and thus increased the phytase production by the culture (Alasheh and Duvnjak, 1994).

A. ficuum NRRL 3135 produced very high phytase activity (110 nkat/ml) in a cornstarch medium (Ebune *et al.*, 1995b; Nair *et al.*, 1991; Shieh and Ware, 1968; Ullah and Gibson, 1987; Ullah, 1988). In addition, an acid phosphatase from *A. ficuum* NRRL 3135 was obtained from a partly purified preparation (Irving and Cosgrove, 1974) and purified to homogeneity using ion exchange (SP-Trisacryl M and DEAE-Trisacryl M) and PBE 94 chromatofocusing. Only a 22-fold purification was required since this is one of the major secreted enzymes in *A. ficuum* NRRL 3135 and culturing in an 8% cornstarch medium simplified the filtration (Ullah and Gibson, 1987). This fungus also produced phytase in three alternative cultivation techniques, i.e. SSF (Ebune *et al.*, 1995a), semi-solid fermentation

(Han *et al.*, 1987) and SmF (Howson and Davis, 1983; Nair *et al.*, 1991; Shieh and Ware, 1968; Ullah and Gibson, 1987). Properties that had a profound negative influence on the phytase production by *A. ficuum* NRRL 3135, included the moisture content (64% for optimal activity) and the inoculum age (Ebune *et al.*, 1995b). Using a SmF batch culture technique, the addition of high concentrations of glucose and low levels of aeration negatively influenced the phytase production (Shieh and Ware, 1968). In SSF, both phytase and cell production were affected by the water quantity in the system (Ebune *et al.*, 1995a; Oriol *et al.*, 1988).

An *Aspergillus niger* var *teigham* hyper-producing strain was used in shake flasks as well as in fermenters for the production of phytase. Maximal production of phytase (184 nkat/ml) by this strain was obtained in minimal medium, with a specific activity of 21 367 $nkat.mg^{-1}$ protein. For optimal growth, as well as phytase production, the cultivation conditions for *A. niger* var *teigham* was 30°C with an initial pH of 6.5 and a combination of starch and glucose in the medium. In the case of an efficient nitrogen source, ammonium nitrate addition led to higher phytase activity than the addition of peptone to the medium (Vats and Banerjee, 2002). Extracellular phytase was purified from *A. niger* ATCC 9142 to homogeneity by using an initial ultrafiltration step, followed by chromatography (ion exchange), gel filtration and chromatofocusing. The specific activity for this strain was 89.6 $nkat.mg^{-1}$ protein (Casey and Walsh, 2003).

An *Aspergillus oryzae* K1 was isolated during the production of miso koji from steamed soybeans, a Japanese traditional soybean paste seasoning. This strain not only produced phytase, but also an acid phosphatase that assists in the hydrolysis of phytate intermediates (Shimizu, 1993). Sephadex G-100 and G-200, DEAE-Sepharose CL-6B column chromatography and chromatofocusing were used to purify the *A. oryzae* K1 phytase and maximal phytase activity (approximately 0.4 $U.ml^{-1}$) was obtained within 4 to 5 days of cultivation (Shimizu, 1993). Another strain of *A. oryzae* produced three types of acid phosphatases by using the SmF technique with phytic acid as the only phosphate source (Fujita *et al.*, 2003).

A thermophilic fungus, *Sporotrichum thermophile*, showed optimal phytase activity at 45°C with an initial pH of 6.0 (Ghosh, 1997). The optimal production of phytase from an *Aspergillus fumigatus* strain was at 37°C with the initial pH of the medium between pH 6.0 and 6.5 and the moisture content at 64% (Pasamontes *et al.*, 1997b). In addition, an acid phosphatase (glycosylphosphatidylinositol-anchored protein) was isolated from the cell wall

fraction (Bernard *et al.*, 2002). An *A. carbonarius* strain demonstrated maximum growth in a SSF system using canola meal as substrate and produced an extracellular phytase that eliminated phytic acid in the medium very rapidly. The moisture content had to be between 53% and 60% for optimal phytase production (Al-Asheh and Duvnjak, 1995). Phytase production from four Basidiomycete fungi, *Peniophora lycii*, *Agrocybe pediades*, a *Ceriporia* sp. and *Trametes pubescens*, was obtained (Lassen *et al.*, 2001). This indicated that phytases were more widely scattered in the fungal kingdom; previously reported cloned filamentous fungal phytases from the *Ascomycota* phylum were thought to be the only sources of phytase activity.

Rhizopus oligosporus CT11K2 isolated from tempeh, a product of soybean fermentation, produced an extra- and intracellular phytase. The phytase activity from the intracellular fraction was higher than the extracellular fraction obtained (Sutardi and Buckle, 1988). The phytase was partially purified by acetone fractionation, gel filtration with Sephadex G-100 and DEAE-cellulose chromatography (Sutardi and Buckle, 1988).

A food-grade fungus used in the fermentation of sufu in China, *Mucor racemosus* NRRL 1994, demonstrated high phytase activity in a SSF system. The substrate was coconut oil cake with a moisture level of 71%, initial pH of 5.5 and an incubation temperature of 25°C was required to produce optimal phytase activity (Bogar *et al.*, 2003).

3.2.3.2 Yeasts

The rationale for screening numerous yeasts for phytase production is due to the wide applications of yeasts in industrial processes and the fact that several yeasts have GRAS status, which should render it more favourable for market approval.

A small number of yeasts producing phytase have been reported (Table 1), the most important producers of yeast phytases being *S. cerevisiae* and *Schwanniomyces castellii* (Lambrechts *et al.*, 1992; Nayini and Markakis, 1984; Segueilha *et al.*, 1992). SmF systems were the only method being used thus far for the production of yeast phytases (Pandey *et al.*, 2001). A *S. castellii* CBS 2863 strain proved to be the strongest phytase producer from all other yeast phytases screened. The enzyme was purified by a three-stage purification technique using anion and gel filtration chromatography and showed a specific activity of 522 $U.mg^{-1}$ protein (Lambrechts *et al.*, 1992; Segueilha *et al.*, 1992). The phytase activity decreased in the presence of high phytic acid concentrations (Lambrechts *et al.*, 1992;

Segueilha *et al.*, 1992). *Torulopsis candida* CBS 940, *Debaryomyces castellii* CBS 2923 and *Candida tropicalis* CBS 5696 also have modest phytase activity (Lambrechts *et al.*, 1992). *Rhodotorula gracilis* was shown to produce phytase from whole cells and 15 cycles of freezing and thawing of the cells gave a phytase activity of 77.26 $U.g^{-1}$ cells in comparison to 40.84 $U.g^{-1}$ whole cells without permeabilisation (Bindu *et al.*, 1998). Phytase production was also reported for *Candida* spp. (Nakamura *et al.*, 2000) and a novel phytase was purified from *Candida krusei* WZ-001 (Quan *et al.*, 2001; Quan *et al.*, 2002). The phytase of the *C. krusei* strain was shown to be located in the cell wall of the yeast and the phytase activity was rather high relative to other commercial phytases. This high phytase producing strain might be looked at as a possible phytase to be used in industrial applications.

A total of 1200 yeast strains were screened based on their growth on agar plates that contain phytic acid as the sole carbon and phosphorus source. The only yeast that showed vigorous growth was *A. adeninivorans*, which has a very high cell mass yield on a broad range of substrates (Kunze and Kunze, 1996). The growth of this *A. adeninivorans* strain on the phytic acid agar screening plates suggested that this yeast might have a high phytase activity. This was confirmed by a solution assay that showed a phytase activity of 10 $U.mg^{-1}$ protein. This phytase was only partially purified by a 2ml-Sephadex G50 column filtration and/or further purified by one cycle of adsorption onto DEAE cellulose (Sano *et al.*, 1999).

A high intracellular phytase activity (68 $U.g^{-1}$ dry biomass) was produced by *Pichia anomala* when grown in a medium containing glucose and beef extract (Vohra and Satyanarayana, 2002). The optimal concentrations of these two substrates in the medium were obtained by the use of response surface methodology (statistical, computerised method for product or process improvement, including optimisation) to enhance the phytase production by this yeast. Using this method to optimise the media in contrast to doing it manually, decreased the cost of production of the phytase. A two-step method of acetone precipitation and anion exchange chromatography with DEAE-Sephadex was used to purify the phytase to near homogeneity (Vohra and Satyanarayana, 2002).

Nakamura *et al.* (2000) screened numerous yeast species for their extracellular phytase activity and two strains, *Pichia spartinae* and *P. rhodanensis*, showed high levels of phytase activity (Nakamura *et al.*, 2000).

3.2.4 Animal Phytases

In comparison to bacterial and fungal phytases, little research have been done on animal phytase producers. As mentioned earlier, phytate acts as an ANF for animals, therefore the gastrointestinal tract of various animals were screened for any phytase activity (Bitar and Reinhold, 1972). Although phytase activity has been reported in human tissue (Bitar and Reinhold, 1972), there is still doubt whether phytate may be hydrolysed in the human small intestine and stomach (Sandberg and Andersson, 1988). McCollum and Hart (1998) were the first researchers to report a phytase in calf liver and blood. In addition, phytase was noticed in the blood of birds, reptiles and certain types of fish (Rapoport *et al.*, 1941). An ancient protozoan pathogen of humans, *Leishmania donovani*, contains several specific members of the histidine-acid phosphatase enzyme family (Shakarian *et al.*, 2003) and the protozoan *Paramecium* was shown to have a phytase-like enzyme (Freund *et al.*, 1992).

In contrast, phytase has been found in the intestinal mucosa of the certain monogastric animals like rat, chicken and calves (Bitar and Reinhold, 1972; Cooper and Gowing, 1983; Yang *et al.*, 1991a). Since intestinal phytase does not appear to play an important role in phytate digestion, the addition of a dietary phytase might be an imperative factor for phytate hydrolysis and the health of these animals (Frolich, 1990).

3.3 Characterisation of Phytases

3.3.1 Temperature, pH Optima and Enzyme Stability

The temperature and pH optima for phytases from some plants and microorganisms are summarised in Table 2. Phytases vary in temperature optima from 40°C to 80°C (Vohra and Satyanarayana, 2003). Thermo-stability is important as the enzyme may be required to endure high temperatures of 60 to 90°C for a short period of time during the production of feed pellets (Pasamontes *et al.*, 1997b). The fungus *A. fumigatus* produced an exceptionally thermo-stable phytase that could endure temperatures of up to 100°C over a period of 20 minutes, while losing only 10% of its original activity (Pasamontes *et al.*, 1997b). The phytase from *A. carneus* lost 68% of its activity after a heating period of 60 minutes at 45°C (Ghareib, 1990). The same loss in activity was reported for the bacterial phytase from *E. coli*, which lost 24% of its activity when the enzyme was exposed to 60°C for 1 hour, and lost all activity when exposed to a temperature of 70°C (Yoon *et al.*, 1996).

Table 2. Characterisation of some plant and microbial phytases. (Liu *et al.*, 1998; Vohra and Satyanarayana, 2003)

Source	Optimal temp. (°C)	Optimal pH	Km (mM)	References
Plant				
Canola seed	50	5.2	0.36	Houde *et al.* (1990)
Maize	55	4.8	0.117	Laboure *et al.* (1993)
Soybean	55	4.5-4.8	0.048	Gibson and Ullah (1988); Sutardi and Buckle (1986)
Spelt	45	6.0	0.4	Konietzny *et al.* (1995)
Wheat bran	55	5.0-5.6, 7.0	0.022	Nagai and Funahashi (1962); Pears (1953); Lim and Tate (1973)
Bacteria				
Aerobacter aerogenes	25	4.0-5.0	0.135	Greaves *et al.* (1967)
Bacillus sp. DS11	70	7.0	0.55	Kim *et al.* (1998)
B. subtilis	55	7.0	0.04	Powar and Jagannathan (1982)
B. subtilis (natto) N77	60	6.0-6.5	0.50	Shimizu (1992)
E. coli	55	4.5	0.13	Greiner *et al.* (1993)
Klebsiella aerogenes	60	4.5-5.2	0.11	Tambe *et al.* (1994)
Pseudomonas sp.	40	5.5	0.016	Irving and Cosgrove (1971)
Yeasts				
A. adeninivorans	75-80	4.5	0.25	Sano *et al.* (1999)
P. anomala	60	4	0.20	Vohra and Satanarayana (2002)
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)
S. castellii	75-80	4.5	0.038	Segueilha *et al.* (1992)
Filamentous Fungi				
A. niger 92	55	5.0	0.44	Dvorokava *et al.* (1997)
A. ficuum NRRL 3135	58	2.5, 5.0-5.5	0.04	Ullah and Gibson (1987)
A. niger SK-57	50	2.5, 5.5	0.019	Nagashima *et al.* (1999)
A. niger ATCC 9142	65	5.0	0.10	Casey and Walsh (2003)
A. oryzae K1	50	5.5	0.33	Shimizu (1993)
R. oligosporus	55	4.5	0.15	Sutardi and Buckle (1988)

Phytase activity from *Enterobacter* sp. 4 was more stable (loss of 40% activity) when exposed to a temperature of 60°C for a period of 20 hours. The enzyme lost all its activity when the temperature was increased to 80°C for the same period (Yoon *et al.*, 1996). Phytase activity from *Bacillus* sp. DS11 showed an optimum temperature of 70°C, which is generally higher than temperature optima reported for most microorganisms. This phytase required the

inclusion of $CaCl_2$ in the assay solution to maintain a 100% activity during exposure to 70°C for 10 minutes. This suggested that the calcium ion had a protection effect on the stability of the phytase under high temperatures (Kim *et al.*, 1998a). Roughly, 50% of the original activity was lost when the enzyme was exposed to 90°C for 10 minutes (Kim *et al.*, 1998a).

Optimal phytase activity was reported for *S. castellii* at 77°C (Segueilha *et al.*, 1992), with an optimal temperature of 75°C for the yeast *A. adeninivorans* (Sano *et al.*, 1999). Phytases from two other yeast strains, *P. rhodanensis* and *P. spartinae*, demonstrated optimal phytase activity at temperatures of 70°C to 75°C, and 75°C to 80°C, respectively (Nakamura *et al.*, 2000), indicates that phytases from yeast may produce better phytase activity at high temperatures.

Optimal phytase activity between 50°C and 60°C was reported for bacterial phytases 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), *Klebsiella oxytoca* MO-3 (Jareonkitmongkol *et al.*, 1997), and *S. ruminantium* (Yanke *et al.*, 1999). Two exceptions were the phytase from *Aerobacter aerogenes* that had an optimum at 25°C (Greaves *et al.*, 1967), and the phytase from *Bacillus* sp. DS11 with an optimum at 70°C (Kim *et al.*, 1998a).

Maximal phytase activity was obtained for *A. fumigatus* when the enzyme was exposed to 37°C (Pasamontes *et al.*, 1997b), whereas the optimal activity for *A. ficuum* NRRL 3135 was reached at a temperature of 58°C (Howson and Davis, 1983). Furthermore, the thermophilic fungi, *Thermomyces lanuginosus*, displayed an optimum activity at 65°C (Berka *et al.*, 1998) and *S. thermophile* showed optimal activity at 45°C (Ghosh, 1997).

Phytases vary in their pH optimum from pH of 2.2 to pH 8 (Vohra and Satyanarayana, 2003). Bacterial phytases demonstrated maximal activity at a pH optimum between 4 and 5.5, including the phytases from *A. aerogenes* (Greaves *et al.*, 1967), *Pseudomonas* sp. (Irving and Cosgrove, 1971), *E. coli* (Greiner *et al.*, 1993), *S. ruminantium* (Yanke *et al.*, 1999) and *L. amylovorus* (Sreeramulu *et al.*, 1996). However, maximal phytase activity of *Enterobacter* sp. 4 (Yoon *et al.*, 1996) and *Bacillus* sp. DS11 (Kim *et al.*, 1998a) was shown to be in the neutral range of pH 7 to 7.6. Optimal phytase activity obtained from plant seeds was in the pH range from 4 to 7.5. Legume seed (Scott, 1991) and lily pollen (Hara *et al.*, 1985) showed optimal activity in the alkaline range of about pH 8.

The majority of microbial phytases have a pH optimum between 4.5 and 5.5, particularly the fungal sources. The *A. fumigatus* enzyme differ from most of the other fungal phytases in having a broad pH optimum range, where it showed 80% of its optimal activity between pH values of 4 and 7 (Pasamontes *et al.*, 1997b). *Citrobacter freundi*, as well as the phytase from *A. ficuum* NRRL 3135, showed two pH optima, *e.g.* pH 5.5 and pH 2.5 (Howson and Davis, 1983).

3.3.2 Substrate Specificity and Kinetic Parameters

The majority of phytases show a wide range of substrate specificity, with phytate being the substrate of choice (Vohra and Satyanarayana, 2003). The relative specificity of some phytases for a certain substrate is presented in Table 3. Only a small number of phytases demonstrated a very high specific activity for phytic acid. Although this allows one to distinguish between phytase and acid phosphatase activity, the substrate specificity of phytase may differ because of the different molecular characteristics of purified enzyme sources (Konietzny *et al.*, 1995). For example, the phytase from *Bacillus* sp. DS11 is extremely specific for phytate and had slight or no activity on *p*-nitrophenyl phosphate, ATP, ADP, AMP, β-glycerophosphate, sodium pyrophosphate and α-naphthylphosphate (Kim *et al.*, 1998a). Furthermore, the *Pseudomonas* sp. phytase also demonstrated no activity on inorganic pyrophosphate, β-glycerophosphate, ADP or AMP. Although it did show activity for *p*-nitrophenyl phosphate, this was only 14% in comparison to the activity for the phytate substrate (Irving and Cosgrove, 1971). The phytase from *E. coli, A. niger* and *Aspergillus terreus* seemed to be also fairly specific for phytic acid (Wyss *et al.*, 1999a).

In contrast to the examples mentioned above, phytases from *Emericella nidulans*, *Myceliophthora thermophila* and *A. fumigatus* proved to have a broad range of substrates (Wyss *et al.*, 1999a). Similarly, the phytase from *S. castellii* had the lowest Michaelis constant (K_m) of about 0.038 mM for phytate (Segueilha *et al.*, 1992). Higher K_m values for phytate were reported in *P. spartinae* (0.30 mM), *P. rhodanensis* (0.25) and *A. adeninivorans* (Vohra and Satyanarayana, 2003).

The phytase from mung beans had a K_m value of 0.65 mM for the hydrolysis of phytate (Majerus, 1992). This K_m value is comparable to some other phytase enzymes from canola seed (0.36 mM) (Houde *et al.*, 1990), cotton seed (0.37 mM) (Valikhanov *et al.*, 1981), maize (0.117 mM) (Laboure *et al.*, 1993) and spelt (0.4 mM) (Konietzny *et al.*, 1995).

Table 3. Substrate specificity for some sources of phytase (Liu *et al.*, 1998)

Substrate	Relative activity (%)					
	Spelt[a]	Canola seed[b]	*B. subtilis*[c]	*E. coli*[d]	*A. ficuum*[e]	*A. niger*[f]
Sodium phytate	100[i]	100	100	100	100	100
p-Nitrophenyl-phosphate	29	890	0	12.3	66	10
Sodium β-glycerophosphate	38	72	0	1.9	41	0
ATP	252	17 545	-	0	48	-
AMP	11	-	-	0	-	-
NADP	58	-	-	0	8	-
Fructose-1,6-diphosphate	103	-	-	8.5	27.3	-
Fructose-6-diphosphate	121	-	-	1.3	-	-
Sodium phosphate	-	1 860	21.1	-	-	-
Na_2H_2-pyrophosphate	517	23 190	4.2	0	19.4	-
Glucose-6-phosphate	0	-	-	0.4	13	-

[a] Konietzny *et al.*, 1995; [b] Houde *et al.*, 1990; [c] Shimizu, 1992; [d] Greiner *et al.*, 1993; [e] Irving and Cosgrove, 1974; Ullah, 1988; [f] Skowronski, 1978.
[i] Hydrolysis rate of sodium phytate was taken as 100% for comparison.
-: Not determined

Moderately lower K_m values were established for phytases from *A. ficuum* (0.040 mM) (Ullah and Gibson, 1987), *Typha latifolia L.* (0.017 mM) (Hara *et al.*, 1985), wheat bran (0.022 mM) (Lim and Tate, 1973) and soybean seed (0.048 mM) (Gibson and Ullah, 1988).

For most phytases, typical Michaelis-Menten kinetics were shown, where the liberation of inorganic phosphate by phytase is affected by the amount of substrate used (Feuillade and Dorizo, 1992; Shimizu, 1992). However, two phytase isolates from *M. thermophila* and *E. nidulans* displayed non-Michaelis-Menten behaviour (Wyss *et al.*, 1999a). As mentioned in a previous section, inorganic phosphorus may result in the inhibition of product formation by phytase (Greiner *et al.*, 1993; Howson and Davis, 1983; Konietzny *et al.*, 1995).

The K_m values of a purified phytase from *A. niger* SK-57 were determined for two different substrates, *i.e.* calcium phytate and 4-nitrophenyl phosphate that showed K_m values of 0.45 and 1.38 mM, respectively (Nagashima *et al.*, 1999). A K_m value of 0.345 mM was determined for the phytase from *A. carbonarius* produced in a SSF system (Alasheh and Duvnjak, 1994). *Klebsiella* sp. No. PG.-2 displayed a very high K_m of 2.0 mM (Shah and Parekh, 1990).

The kinetic effectiveness of an enzyme is confirmed by means of the K_{cat}/K_m values for a substrate, in this case phytate. The highest K_{cat}/K_m values for the substrate phytic acid were obtained from a phytase and acid phosphatase of *A. ficuum* NRRL 3135, these values were 1.29×10^7 and 6.10×10^6 $M.s^{-1}$, respectively (Ullah and Phillippy, 1994). Phytase from *E. coli* had a K_{cat}/K_m value of 4.78×10^7 $M.s^{-1}$ (Greiner *et al.*, 1993)

Specific activities for fungal phytases range between 23 $U.mg^{-1}$ protein for *A. fumigatus* to 198 $U.mg^{-1}$ protein for *A. terreus* when phytic acid was used as the substrate (Wyss *et al.*, 1999b). The specific phytase activities reported for bacteria differ approximately 100-fold, ranging from 8.7 $U.mg^{-1}$ protein for *B. subtilis* to 811 $U.mg^{-1}$ protein for *E. coli* (Greiner *et al.*, 1993; Shimizu, 1992).

3.3.3 Effect of Metal Ions and other Chemicals

Microbes seem to vary in their metal ion requirement to maintain or maximally produce a high phytase activity. A summary of the complete phytase inhibition by metal ions is shown in Table 4. The addition of 5 mM Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} inhibited the phytase from *S. castellii* to some extent. Zn^{2+} and Cu^{2+} at a concentration of 0.5 mM caused 50% inhibition of the phytase activity, and 5 mM Zn^{2+} and Cu^{2+} inhibited phytase activity completely (Segueilha *et al.*, 1992). EDTA, Cd^{2+} and Mn^{2+} inhibited the phytase activity at a concentration of 5 mM in *Bacillus* sp. DS11, while moderate inhibition was noted with the addition of 5 mM of Hg^{2+}, Mg^{2+}, Ba^{2+} and Cu^{2+} (Kim *et al.*, 1998). Phytase activity of *S. ruminantium* was inhibited by 5 mM Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+} (Yanke *et al.*, 1999). The sensitivity for these cations were very similar for the microbial phytases of *E. coli*, *Klebsiella terrigena* (Greiner *et al.*, 1993; Greiner *et al.*, 1997) and *A. ficuum* (Ullah and Cummins, 1988). Na^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} inhibited the partially purified phytase from *K. oxytoca* MO-3, but this phytase was not inhibited by N-ethylmaleimide or EDTA (Jareonkitmongkol *et al.*, 1997). Using EDTA to remove the metal ions from the phytase from *B. subtilis* resulted in total inactivation of the enzyme (Kerovuo *et al.*, 2000).

The cations Cu^{2+}, Zn^{2+}, Hg^{2+}, Sn^{2+} and Cd^{2+} inhibited the phytase activity of *A. niger* SK-57 (Dvorakova *et al.*, 1997). Phytase activity of *B. subtilis* (natto) N-77 proved to be inhibited by EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+} and Al^{3+} (Shimizu, 1992). Ag^{2+}, Hg^{2+}, Cu^{2+},

Table 4. Complete phytase inhibition by different metal ions.

Microorganisms	Metal Ions	References
Plant		
Spelt	Hg^{2+}, Cu^{+} and Cu^{2+}	(Konietzny *et al.*, 1995)
Bacteria		
Bacillus sp. Ds11	Cd^{2+} and Mn^{2+}	(Kim *et al.*, 1998)
B. subtilis (natto) N-77	Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+} and Al^{3+}	(Shimizu, 1992)
Klebsiella sp. No. PG.-2	Ag^{2+}, Hg^{2+}, Cu^{2+}	(Shah and Parekh, 1990)
S. ruminantium	5 mM Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+}	(Yanke *et al.*, 1999)
Yeasts		
S. castellii	5 mM Zn^{2+} and Cu^{2+}	(Segueilha *et al.*, 1992)
Filamentous Fungi		
A. niger SK-57	Cu^{2+}, Zn^{2+}, Hg^{2+}, Sn^{2+} and Cd^{2+}	(Dvorakova *et al.*, 1997)

fluoride and a high substrate concentration inhibited the phytase activity of *Klebsiella* sp. No. PG.-2 (Shah and Parekh, 1990). Konietzny *et al.* (1995) confirmed that Hg^{2+}, Cu^{+} and Cu^{2+} inhibited the phytase activity of spelt.

Ca^{2+} and Fe^{2+} did not have an effect on the purified phytase from *A. ficuum* NRRL 3135, however, Co^{2+} and Mn^{2+} increased the phytase activity by 13% and 30%, respectively (Ullah, 1988). Rat phytase activity was increased by 40% with the addition of 20~80 μM Zn^{2+} (Bitar and Reinhold, 1972). Phytase from pollen from *T. latifolia* and phytases from a number of other plants require Ca^{2+} for maximal phytase activity (Gibson and Ullah, 1988; Hara *et al.*, 1985; Laboure *et al.*, 1993; Scott and Loewus, 1986).

Fluoride and vanadate inhibited the phytase from cotyledons of germinating soybean seeds (Ullah and Gibson, 1988). Phytase from lily pollen (Barrientos *et al.*, 1994; Scott and Loewus, 1986) was also inhibited by fluoride. Furthermore, phytase from *K. terrigena* was competitively inhibited by fluoride, molybdate and vanadate (Greiner *et al.*, 1997)

3.3.4 Molecular Size

The majority of phytases are regarded as monomeric enzymes; including the fungal phytases (Dvorakova *et al.*, 1997; Ullah and Gibson, 1987; Wyss *et al.*, 1999a), *E. coli* and *K. terrigena* phytases (Greiner *et al.*, 1993; Greiner *et al.*, 1997), and the *B. subtilis* (natto) phytase (Shimizu, 1992). Nevertheless, there are phytases that consist of multiple subunits. A dimeric enzyme from maize seedlings during germination contained two subunits of 38 kDa each (Laboure *et al.*, 1993).

Phytases are high-molecular-weight proteins that vary between 40 to 700 kDa, depending on the phytase source (Vohra and Satayanarayana, 2003). The phytase from *S. ruminantium* was shown to be a single gene product (monomeric) with a molecular weight (Mr) of approximately 46 kDa (Yanke *et al.*, 1999). SDS-PAGE suggested a Mr of 44 kDa for the purified phytase from *Bacillus* sp. DS11 (Kim *et al.*, 1998) and 36 kDa for the phytase from *B. subtilis* (natto) N-77 (Shimizu, 1992).

Two protein bands of 70 and 90 kDa were obtained by SDS-PAGE for the purified rat intestinal phytase (Yang *et al.*, 1991b). Two dissimilar forms have been described for the phytase from *Klebsiella aerogenes*. The first one has a very large Mr of 700 kDa and is likely to be the native phytase. The second one (13 kDa) is possibly a subunit of the native 700-kDa phytase, although it did show 100% phytase activity and was the first small protein reported to have phytase activity (Tambe *et al.*, 1994).

Glycosylation may influence the catalytic, pI and expression properties of phytase. The native phytase of *A. ficuum* NRRL 3135 was 27% glycosylated. This glycosylation did not have any effect on the catalytic or refolding properties of the enzyme (Wyss *et al.*, 1999a). A Mr of 490 kDa was reported for the phytase from *S. castellii*, which had a 31% glycosylation. This glycosylated phytase was tetrameric, consisting of one large subunit of 125 kDa and three indistinguishable small subunits of 70 kDa (Segueilha *et al.*, 1992).

SDS-PAGE was used to determine the Mr of the purified phytase from *A. fumigatus*, revealing a 60 kDa protein (Pasamontes *et al.*, 1997b). The Mr of the purified phytase from *A. ficuum* NRRL 3135 was 85 kDa, the same size as the acid phosphatase (Ullah and Phillippy, 1988). The Mr for the purified phytase and acid phosphatase from *A. oryzae* K1 were 60 and 70 kDa, respectively (Vohra and Satayanarayana, 2003). For *A. terreus*, a Mr of 214 kDa was suggested for its native phytase; this protein was a homohexamer of 37 kDa subunits (Yamamoto *et al.*, 1972).

3.3.5 Amino acid Sequence Analyses of Phytases

Table 5 presents the amino acid composition of phytases from soybeans, *B. subtilis* and *A. ficuum*. The phytase from *A. ficuum* contained 594 amino acid residues, which are divided into 37% nonpolar, 42% polar, 11.5% acidic and 9.5% basic amino acids (Nayini and Markakis, 1984). The phytase contained more acidic residues than basic residues, which explained the acidic nature of the enzyme. The secondary structure of the *A. ficuum* phytase was projected to include 17.3% α-helixes, 29% β-sheet, 32.6% turns and 24.7% coils (Ullah *et al.*, 1994). Tryptophan was proposed to be important for the phytase activity of this fungus (Ullah and Dischinger, 1993), whilst disulfide bonds in the active center seem to be important in keeping its structural geometry. The phytase activity decreased when β-mercaptoethanol was used to reduce the disulfide bonds, which confirmed that the disulfide bridges are required for the phytase activity in *A. ficuum* (Ullah and Mullaney, 1996).

Table 5. The amino acid (aa) composition of some purified phytases (Liu *et al.*, 1998)

	Number of residues		
Amino acids	**Soybean[i]**	***B. subtilis*[ii]**	***A. ficuum*[iii]**
Alanine	29	29	45
Arginine	23	8	19
Asparagine	59	58	60
Cysteine	7	0	8
Glutamic acid	45	39	44
Glycine	47	36	56
Histidine	11	29	10
Isoleucine	25	18	22
Leucine	50	17	50
Lysine	24	20	11
Methionine	11	2	4
Phenylalanine	23	9	29
Proline	29	12	45
Serine	29	12	78
Threonine	18	3	56
Tyrosine	28	14	20
Valine	35	16	37
Total	**493 aa**	**322 aa**	**594 aa**

[i] Calculation on the molecular weight of 54 kDa (Gibson and Ullah, 1988)
[ii] Calculation on the molecular weight of 36.5 kDa (Powar and Jagannathan, 1982)
[iii] Calculation on the molecular weight of 61.7 kDa (Ullah, 1988)

The phytase amino acid sequence obtained from *Aspergillus niger* var. *awamorii* showed a 97.2% homology to the phytase sequence from *A. ficuum* NRRL 3135 phytase. It

was established that the phytase amino acid sequence of *A. ficuum* contained ten N-glycosylation sites (Piddington et *al.*, 1993; van Hartingsveldt *et al.*, 1993). The phytase from *P. lycii* appeared to have ten cysteine residues with three amino acid consensus regions when compared with other basidiomycete fungi (Figure 5) (Lassen *et al.*, 2001). The phytase from *E. nidulans* and *T. thermophilus* consisted of 463 and 466 amino acids, respectively, with 63% and 61% homology, with the amino acid sequence of the *A. niger* phytase (Pasamontes *et al.*, 1997a).

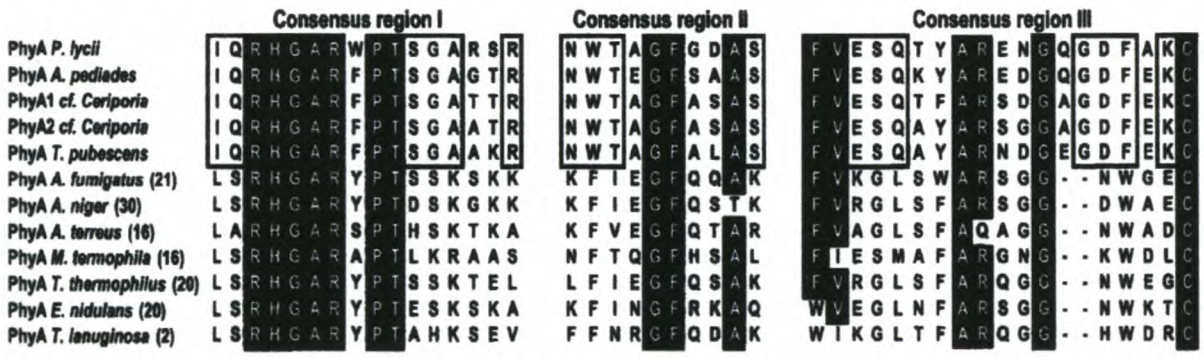

Figure 5. Amino acid sequence alignment to show the active site of phytase from some fungi strains and the high degree of amino acid conservation between four Basidiomycete strains in three regions (Lassen *et al.*, 2001).

By aligning all the amino acid sequences obtained from phytase sources, only two amino acids are preserved between all phytases published, i.e. Asn207 and Asn339. Using a 3D-modeling technique, it was determined that all phytases reported contained 21 identical amino acids that formed the substrate pouch of the protein (Figure 6) (Vohra and Satayanarayana, 2003).

3.3.6 Crystal Structure

Using a two wavelenght anomalous diffraction method with an exceptionally strong anomalous scattering of tungsten, the crystal structure of *E. coli* phytase was determined (Lim *et al.*, 2000) and compared to two other phytase crystal structures (Figure 7). The structure strongly resembled the overall fold of other histidine acid phosphatases regardless of a lack in sequence similarity.

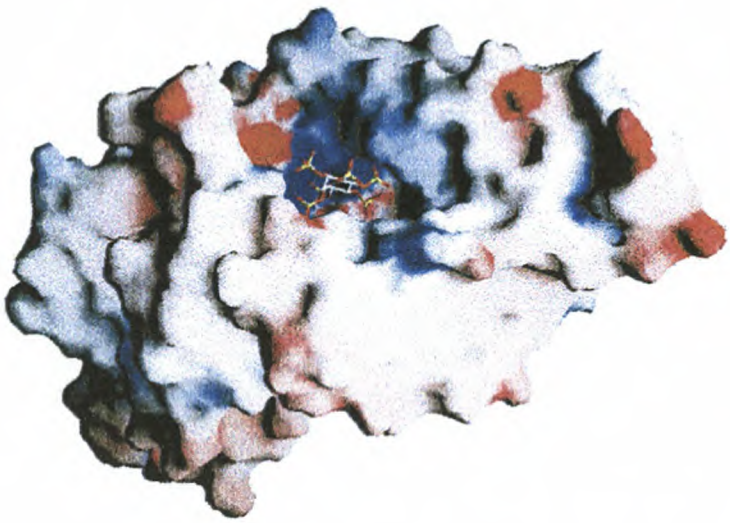

Figure 6. A GRASP electrostatic surface representation of the substrate binding pocket of *E. coli* phytase with potentials ranging from –20V (red) to +20V (blue). Phytate is shown as a stickmodel in the proteins binding pocket (Lim *et al.*, 2000).

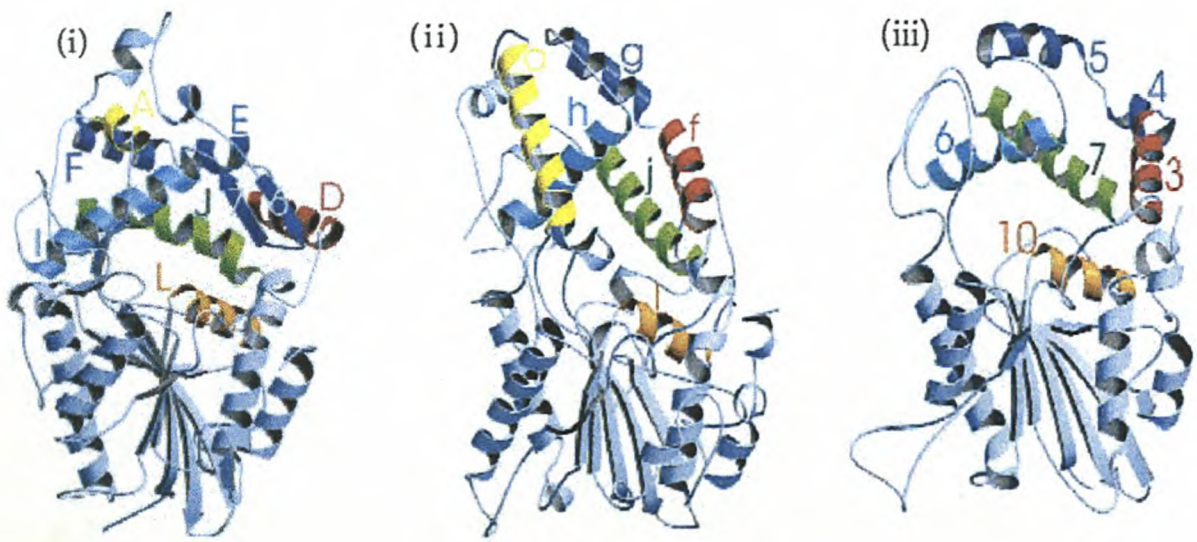

Figure 7. The similarities between three-dimensional crystal structures of (i) *E. coli* phytase, (ii) *A. niger* phytase and (iii) rat prostatic acid phosphatase. An outstanding central cavity separates the protein into an α-domain (red) and an α/β-domain (light blue). The α-helices surround the main β-sheet of the α/β-domain and this is well conserved among the three crystal structures. Capital letters are the labels for helices from *E. coli*; *A. niger* is labelled with small letters and numerals indicate rat prostatic acid phosphatase labels. The α-domain of *E. coli* has a unique feature of containing a β-hairpin structure (dark blue). The orange coloured helices represent the α/β-domain and are positioned in such a way that the helix dipole can assist in die binding of the scissile phosphate at the active site of the protein (Lim *et al.*, 2000).

The crystal structure of a phytase from *Bacillus amyloliquefaciens* was determined by using a X-ray crystallographic analysis that uses a hanging-drop vapour-diffusion method (Ha *et al.*, 1999). Premium single crystals of this phytase in the absence of calcium ions were achieved using a precipitant solution and the crystal structure was reported at 2.5 Å resolution (Kostrewa *et al.*, 1997). This crystal structure showed the α/β-domain and α-domain with an innovative fold. The advanced crystal structure of this phytase consisted of 434 amino acids, 115 H_2O molecules and one sulfide ion in the sulfide-binding site. This advance crystal structure provided important information regarding the substrate binding property of the enzyme (Figures 7 and 8) (Kostrewa *et al.*, 1997).

3.3.7 Active Site and Reaction Mechanism

Phosphate esters are optimally hydrolysed at low pH levels by acid phosphatases that are a heterologous group of enzymes. Several acid phosphatases from various sources share two similar sequence regions, each one reported to have a characteristic conserved histidine residue (van Etten *et al.*, 1991). There are 14 members of phytases that belong to this group

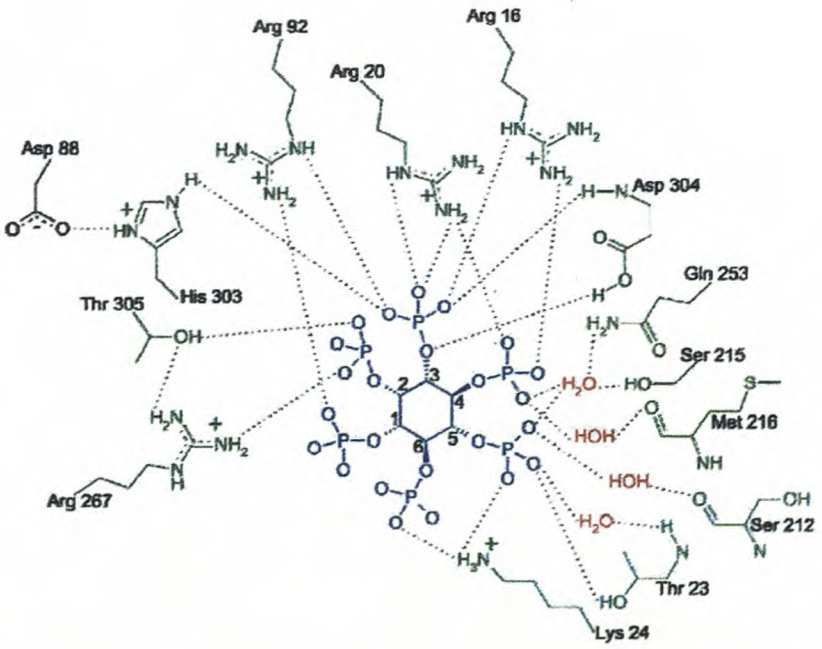

Figure 8. Schematic diagram of the interactions implicated in phytate binding with the phytase protein of *E. coli* (Lim *et al.*, 2000).

called "histidine acid phosphatases", each one sharing the sequence consensus pattern (histidine residues in bold) of [LIVM]-X(2)-[LIVMA]-X(2)-[LIVM]-X-R-**H**-[GN]-X-R-X-[PAS] near the N-terminus, and a conserved HD-motif [LIVMF]-X-[LIVMFFAG]-X(2)-[STAGI]-**H**-D-[STANQ]-X-[LIVM]-X(2)-[LIVMFY]-X(2)-[STA] near the C-terminus (Lassen *et al.*, 2001; van Etten *et al.*, 1991; Wodzinski and Ullah, 1996). This active site motif (R**H**GXRXP) is conserved in all the fungal phytase strains (Figure 5) and is present in the phytase from *E. coli* (Wodzinski and Ullah, 1996).

The fundamental role of the histidine and arginine residues in phytase activity were confirmed by probing active sites of fungal phytases by amino acid residue with specific modified reagents (Ullah *et al.*, 1991; Ullah and Dischinger, 1992). There were four tryptophan residues implicated in the phosphohydrolytic cleavage of phytic acid (Ullah and Dischinger, 1995). Only two were located in the hydrophilic region, Trp25 and Trp267; the other two residues were situated in the hydrophobic region and might not be able to play a role in the formation of the active site (Ullah and Dischinger, 1992).

A two-step reaction mechanism for phytases (histidine acid phosphatases) of fungi and *E. coli* has been suggested. Firstly, the arginine residue (because of its positively charged guanidine group in the tripeptide R**H**G) will interact directly with the phosphate group of the phytic acid. This function makes it vulnerable to nucleophilic attack, whereas the histidine residue serves as a nucleophile in the construction of covalent phosphohistidine intermediates. Secondly, the aspartic acid residue (from the **H**D-motif) protonates this group, which will subsequently leave the substrate (van Etten, 1982).

4. Commercial Production of Phytases

4.1 Enzyme Production Techniques and Directives

4.1.1 Submerged Fermentation vs. Solid-State Fermentation

Over the past half century, SmF technology has largely been employed for the production of commercially important products (Papagianni *et al.*, 1999). In recent years, SSF technology has expanded to include the production of value-added products (Pandey, 1991, 1992, 1994). This technology was regarded as low-technology systems for the inexpensive production of value-added low volume-high cost products such as biopharmaceuticals (Pandey and Soccol, 2000). Moreover, phytases have been produced with SmF as well as SSF techniques, as

shown in Table 1 (Papagianni *et al.*, 1999). It was found that phytases are produced more efficiently by SSF than with SmF (Howson and Davis 1983; Papagianni *et al.*, 1999). The reason for this might be that the SSF resembles a more natural way of life for some microorganisms (Krishna and Nokes, 2001). Han *et al.* (1987) also experimented with a semi-solid substrate fermentation technique to determine if a combination of SmF and SSF techniques could produce phytases more efficiently. They reported that the best technique for maximal phytase activity was actually SSF on its own, although, the mixture of SmF and SSF did produce higher phytase activity than in SmF alone.

There are many factors to be taken into consideration for the selecting of a certain production technique, which will have an effect on the yield. These factors include the type of strain, culture conditions and the nature and availability of nutrients for the efficient production of phytase (Moo-Young *et al.*, 1978; Papagianni *et al.*, 1999).

4.1.2 Physical and Nutritional Parameters

Temperature, pH, agitation, dissolved oxygen and pressure are the most significant physical parameters that will have a profound influence on the growth of organisms and the production of phytase. Most of the microorganisms produce phytase at a temperature optimum between 25 and 37°C. *Thermomyces lanuginosus* (Pasamontes *et al.*, 1997a), *T. thermophilus* (Pasamontes *et al.*, 1997a) and *S. thermophile* (Ghosh, 1997) are the only thermophilic microorganisms known to produce phytase; all the other known producers are mesophiles (Berka *et al.*, 1998).

The pH of the medium has an enormous effect on the microorganism's growth as well as production of the enzyme. The optimum pH for most phytase-producing microorganisms lies between pH 5.0 and 7.0 and there has been no report yet of a phytase that is produced in an alkaline environment (Vohra and Satayanarayana, 2003).

To maximise the production of phytases in SmF, it was found that the medium needed to be properly aerated and agitated to keep the medium constituents, microbial cells and oxygen equally suspended (Vohra and Satayanarayana, 2003). An aerated fermenter and agitation kept at 600 rpm was most efficient for the production of phytase from the yeast *S. castellii* (Sequeilha *et al.*, 1992). By just agitating the media (shaking at 270 rpm), maximum levels of phytase were produced by strains representing *A. niger, A. ficuum* NRRL

3135 and *A. terreus* (Shieh and Ware, 1968). Similar results were obtained with *Bacillus* sp. DS11 by shaking the culture at 230 rpm (Kim *et al.*, 1998).

Another very important factor in the production of phytase is the optimal concentration and source of carbon for the microorganism. Glucose is the prefered carbon source (substrate) for the production of phytase. Glucose at a concentration of 1% (w/v) was optimal for phytase production by two bacteria, *L. amylovorus* (Sreeramulu *et al.*, 1996) and *Enterobacter* sp. 4 (Yoon *et al.*, 1996). The production of phytase by *B. subtilis* was optimal with a glucose concentration of 2% (w/v) (Kerovuo *et al.*, 1998). For phytase production by *Bacillus* sp. DS11, wheat bran at a concentration of 6% (w/v) was found to be a good carbon source (Kim *et al.*, 1998). *Myo*-inositol, the product of total dephosphorylation of phytic acid, was used as the sole carbon source at a concentration of 0.2% (w/v) for the growth and production of phytase by the bacterium *Pseudomonas* sp. *4* (Irving and Cosgrove, 1971). Similar results were obtained for *K. aerogenes* when grown in a medium containing sodium phytate at a concentration of 2% (w/v) (Tambe *et al.*, 1994).

A several-fold improvement in phytase production was reported for *A. adeninivorans* by substituting the glucose with galactose in a medium consisting of yeast peptone and glucose (Sano *et al.*, 1999). The substitution of glucose with galactose also led to an improved production of phytase by *S. castellii* (Segueilha *et al.*, 1992). In the case of some fungi, a maltose yeast extract broth was used for optimal production (Shieh and Ware, 1968). However, when glucose or sucrose were used as sole carbon source, *A. ficuum* NRRL 3135 formed mycelial pellets that produced low yields of phytase (Wodzinski and Ullah, 1996). By using corn meal, the mycelial growth was dispersed and this led to an elevated enzyme yield (Shieh and Ware, 1968). This was also noted for the production of phytase by *A. ficuum* NRRL 3135 in a SSF technique (Ebune *et al.*, 1995).

Nitrogen is another very important parameter that influences the growth and production of enzymes in the medium. Using peptone (an organic form of nitrogen) at a concentration of 1% (m/v) improved the production of phytase by *A. aerogenes* (Greaves *et al.*, 1967). Similar results were obtained with *K. oxytoca* when the medium was supplemented with yeast extract (Jareonkitmongkol *et al.*, 1997). By including yeast extract and peptone in the medium at a concentration of 1% (m/v) each, the *A. adeninivorans* phytase yield improved several fold (Sano *et al.*, 1999). $(NH_4)_2SO_4$ (inorganic nitrogen source) used at a concentration of 1% (w/v) enhanced the phytase production in *Pseudomonas sp.* (Irving and Cosgrove, 1971), *Enterobacter sp.4* (Yoon *et al.*, 1996) and *S. castellii* (Lambrechts *et*

al., 1992). In the case of *B. subtilis*, the medium was supplemented with casein hydrolysate and $(NH_4)_2SO_4$ at 1% (w/v) each to improve the phytase yield in this microorganism (Powar and Jagannathan, 1967).

Apart from the necessity of carbon and nitrogen sources for growth and phytase production, some microorganisms require additional trace elements and vitamins (Galzy, 1964; Segueilha *et al.*, 1992). The majority of yeasts needed the inclusion of vitamins and trace elements in the medium to enhance their growth and phytase yield. In contrast, *B. 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), did not require trace element supplementation to produce efficient levels of phytase.

Inoculum size and age play a very important role in the phytase yields of the majority of microorganisms, in particular the fungal sources. If the inoculum size is too small, the fungi form pellets that resulted in very low extracellular enzyme yields. This phenomenon seems to be a universal characteristic of these microorganisms (Han *et al.*, 1987; Shieh and Ware, 1968; Wodzinski and Ullah, 1996). Inoculating a culture medium with 50-day-old spores of *A. niger* 307, resulted in maximal phytase production with an increase of phytase production by 3.29-fold (Gargova and Sariyska, 2003). Adding to this, it was shown that when younger inocula were used in SSF techniques, the phytase yields did not produce very high phytase activity (Nair and Duvnjak, 1990; Ebune, 1995).

The synthesis of phytase appears to be strongly regulated by the availability of inorganic phosphorus in the culture medium (Han *et al.*, 1987; Howson and Davis, 1983; Shieh and Ware, 1968; Ullah and Gibson, 1987). Inhibition of phytase synthesis is caused by a high concentration of phosphorus in the culture medium (Quan *et al.*, 2001). *B. amyloliquefaciens* DS11 sustained a good phytase activity with the addition of 5 mM phosphate to the medium. In contrast, the phytase from *A. ficuum* NRRL 3135 was inhibited by 50% with the addition of 3 mM phosphorus to the medium (Kim *et al.*, 1999b). The addition of up to 10 mM inorganic phosphorus did not show any inhibition of the phytase synthesis in *P. anomala* (Vohra and Satyanarayana, 2002). However, the addition of 0.05% (w/v) inorganic phosphate resulted in a drastic decline in phytase production by *A. niger* var *teigham*. The inclusion of 0.1% (w/v) or more inorganic phosphorus to the medium showed no phytase activity, probably due to product inhibition of phytase synthesis (Vats and Banerjee, 2002). When the culture medium of *A. niger* 307 included 0.1% (w/v)

inorganic phosphorus, a two-fold augmentation was noted in the phytase yield (Gargova and Sariyska, 2003). Inorganic phosphorus competitively inhibited phytase activity of cotyledons of dormant *C. avellana L.* seeds (Andriotis and Ross, 2003). Related inhibitions by inorganic phosphorus was reported for maize root and soybean phytases at concentrations of 300 μM and 20 mM, repectively (Hübel and Beck, 1996; Sutardi and Buckle, 1986). These results demonstrated that phytase activity would definitely be influenced by a number of parameters and that only one small adjustment to the temperature, pH, growth media, etc. will effect the level of phytase production and/or activity.

4.2 Expression Systems for the Efficient Production of Phytase

Attempts have been made for the cost-effective enhanced production of phytase, mostly due to its probable use in the animal feed industry. Different heterologous expression systems and hosts have been evaluated, including plants, bacteria, yeast, fungi and animals. Each system appeared to have some unique advantages along with certain limitations (Lei and Stahl, 2001). A few of these expression systems and hosts are discussed in the following sections.

4.2.1 Plant Expression Systems

Transgenic plants with an improved level of the 3-phytase were constructed by means of the transformation of the plant host with an expression vector containing fungal phytase DNA (Brocades, 1991). The phytase from *A. ficuum* NRRL 3135 was expressed in *Nicotiana tabacum* (tabacco) leaves and the recombinant phytase was compared with the phytase from its original host. It was found that the recombinant protein was catalytically identical to the native phytase, although the pH optimum of the recombinant phytase shifted from pH 5 to pH 4. The results were convincing enough to suggest that commercial production of this fungal phytase could be done by alternative means (Ullah *et al.*, 1999).

Alfalfa plants were also used to commercially produce phytase by this transgenic approach (Gutknecht, 1997). The expressed phytase in alfalfa plants maintained all the characteristics of the original *A. ficuum* phytase (Ullah *et al.*, 2002). The phytase from *A. ficuum* NRRL 3135 was expressed in soybean and showed similar properties to the native phytase, with the only exception being that the recombinant phytase had a lower Mr (Li *et al.*, 1997). High phytase activity levels of a foreign phytase gene, expressed in wheat (Brinch-

Pedersen *et al.*, 2000) and in seeds of canola and soybean (Denbow *et al.*, 1998; Zhang *et al.*, 2000), enhanced the phytate-phosphorus utilization by broilers and pigs.

4.2.2 Bacterial Expression Systems

The drawback of bacterial expression systems for the production of fungal phytases are their incapability to glycosylate the expressed proteins to the degree necessary for optimal activity. In contrast, phytases from *E. coli* and *Bacillus* sp. DS 11 expressed via the *E. coli* pET expression system (Golovan *et al.*, 2000; Kim *et al.*, 1998b) produced extremely high phytase activity in minimal media after a 30 hour growth period (Golovan *et al.*, 2000). The *phyC* gene from the *B. subtilis* strain was expressed extracellularly in *L. plantarum*, but the phytase activity was too low in this expression system to be used for industrial purposes (Kerovuo and Tynkkyen, 2000).

A *Streptomyces lividans* strain was used for the expression of two phytase-encoding genes, *A. niger phyA* and the *E. coli appA* (Lei and Stahl, 2001). Both of these recombinant phytases were expressed and secreted, but only the *appA* phytase from *E. coli* was active. The recombinant *appA* gene product had similar properties to the native phytase from *E. coli* (Golovan *et al.*, 2000). Another bacterial phytase, the *phyC* phytase from *B. subtilis* VTT E-68013, was expressed in *L. plantarum* strain 755 (Kerovuo and Tynkkynen, 2000). This strain was generally used to inoculate vegetable products due to its pH lowering effect (preservation of the products) and was thought to be a suitable expression host. It was concluded that the bacterium would withstand the acid levels in the stomach of monogastric animals and thus not affect the phytase activity.

4.2.3 Filamentous Fungi Expression Systems

Multiple cloned copies of the phytase encoding gene from *A. ficuum* NRRL 3135 (*phyA*) were inserted in a PluGBug® system (Gist-Brocades), which produced high phytase activity in the recombinant *A. niger* host after transformation (van Dijck, 1999; Vohra and Satyanarayana, 2003). The phytase gene from the basidiomycete, *P. lycii*, which showed very high phytase activity, was overexpressed in an *A. niger* expression system and this product is commercially available under the manufacturer's name of 'Biofeed' phytase (Vohra and Satyanarayana, 2003).

The phytase (*phyA*) from the thermophilic fungus, *T. lanuginosus*, was subcloned into an expression vector under transcriptional control of the *Fusarium oxysporum* trypsin gene promoter and used to transform a *Fusarium venenatum* recipient strain. This recombinant phytase retained its activity at 75°C and confirmed a superior catalytic efficiency to any recognized fungal phytase at 65°C (Berka *et al.*, 1998).

4.2.4 Yeast Expression Systems

The phytase from *A. fumigatus* was functionally expressed in *Pichia pastoris*. The 1.4-kb DNA fragment was subcloned into the expression vector pPICZalpha A and expressed as an active extracellular phytase (r-Afp). It compared well with expression of the gene in *A. niger* (Rodriguez *et al.*, 2000).

A 1.4-kb DNA fragment containing the phytase encoding gene of *A. niger* (*phyA*) was subcloned into the expression vector pYES2 and expressed in *S. cerevisiae* (Han *et al.*, 1999). The host has GRAS status and it has been widely used as a host organism to produce heterologous proteins. The properties of the recombinant and native phytase did not vary and the enzyme was capable of hydrolysing phytate phosphorus from soybean meal *in vitro*.

Low-cost phytase production was managed by using recombinant strains of *Hansenula polymorpha*. Changing the carbon source from glycerol to glucose or glucose syrup gave much higher productivity and conversion yields and showed optimal phytase productivity with enough aeration during growth (Mayer *et al.*, 1999).

4.2.5 Animal Expression Host

Phytase may be produced in the animal's own digestive tract by constructing transgenic poultry and hogs (Mullaney *et al.*, 2000). The *appA* phytase from *E. coli* has been expressed in transgenic mouse models where the phytase was engineered for expression in the salivary glands (Golovan *et al.*, 2001; Ward, 2001). A decrease of 11% in fecal phosphorus was reported when this phytase was expressed in the animals' salivary glands. Furthermore, this transgenic method was used to express this phytase gene in the pig's salivary glands. Almost no adding of inorganic phosphate was required for these transgenic pigs and they excreted up to 75% less fecal phosphorus than the control pigs. A number of efforts have already been

made to convert and express a fungal phytase in a monogastric animal (Mullaney *et al.*, 2000).

5. Applications for Commercial Phytases

5.1 Food industry

Addition of fungal phytases to commercial wholewheat bread production may reduce the level of phytate in the dough (Knorr *et al.*, 1981). Supplementing the dough with phytase will assist in rendering the nutrients and phosphate bound to the phytate more accessible for humans and could thus improve the health of humans by adding extra minerals to the diet. People consuming high amounts of cereals and babies eating soy-based formulas consume relatively high quantities of phytate (Simell *et al.*, 1989). A phytate-free soy protein was reported to include one of the commercial phytases, Finase® phytase. Futhermore, using a wheat phytase, soybean milk phytate was removed totally (Anno *et al.*, 1985). Iron absorption increased in humans who were fed a diet consisting of a flour enclosing wheat bran. The phytase present in the wheat bran together with an additional phytase from *A. niger* helped to release the phosphorus and other nutrients like iron from the flour and wheat (Sandberg *et al.*, 1996).

5.2 Animal Feed Additive

Since phytase hydrolyses phytate, phosphorus supplementation may be completely substituted by the addition of phytase to animal feed (250 to 1000 $U.kg^{-1}$) (Golovan *et al.*, 2001). This is one of the reasons why phytase manufacturing is booming, with annual sales of phytase as an animal feed additive reaching the $500 million mark at the end of the twentieth century (Abelson, 1999).

The Alko Co. (Finland), together with Altech (USA) and BASF (USA), initiated the industrial scale production of phytase, which were promoted under the names Finase™, Allzyme™ phytase and Natuphos™, respectively. Effective results were obtained from each of those commercial enzymes that improved the bioavailability of phytate phosphorus in poultry and swine (Baidoo *et al.*, 2003; Cromwell *et al.*, 1993; Cromwell *et al.*, 1995a, b; O'Quinn *et al.*, 1997; Yi *et al.*, 1996; Yi *et al.*, 1997).

The first addition of phytase to feedstuff was in a corn-soya diet; this phytase was a filtrate from an *A. niger* strain and was fed to 1-day-old chicks. An increase in bone ash was reported in the chicks, given that 90% of the phytate-phosphorus was reduced by this phytase filtrate (Nelson *et al.*, 1968). Broilers were fed a low-phosphorus diet with the addition of a microbial phytase and the availability of phosphorus increased to 60% and the total phosphorus in the droppings diminished by 50% (Nelson *et al.*, 1971). The microbial phytase supplementation in low-phosphorus diets fed to growing pigs improved the absorbability of phosphorus by 24% and decreased the phosphorus amount by 35% in the manure (Simons *et al.*, 1990).

The effectiveness of the microbial phytase Natuphos™ 1000 was evaluated in an experiment with 180-day-old broilers for a 3-week trial period. Body weight was increased in male (13.2%) and female (5.8%) chicks after 21 days with the addition of phytase. The retention of total phosphorus, calcium, copper and zinc was increased. The percentage ash (head and shaft) of dry, fat-free tibia bone was improved and a decrease of almost 10% was reported for the excretion of phosphorus when phytase was supplemented in the diet (Sebastian *et al.*, 1996).

The bioavailability of phytate phosphorus was increased when a novel phytase from *A. niger* was expressed in yeast and fed to weanling pigs (Stahl *et al.*, 2000). It was as efficient as the commercially used Natuphos at the supplementation level of 700 or 1200 $U.kg^{-1}$ in the diets of younger pigs.

5.3 Aquaculture

Fish, like poultry and swine, lack a sufficient phytase enzyme to successfully utilise the phytin phosphorus in their feedstuff, thus excreting this phosphate in the environment. Numerous fish feeding experiments have highlighted the probable value of phytase in diets containing plant feedstuffs like soybean meal (Mwachireya *et al.*, 1999; Robinson *et al.*, 1996). A considerable cost cutback might be obtained by substituting expensive fishmeal (protein source) with the addition of low-cost soybean meal proteins (Rumsey, 1993).

5.4 Phytase and Environmental Pollution

The major factor for surface water quality is the eutrophication of lakes and streams that

occurs when organic nutrients and minerals decrease dissolved oxygen to levels that support plants over animal life. The limiting nutrient for algae and some aquatic plant growth is phosphorus (Sharpley *et al.*, 1994). Plant over-growth, shifts in plant varieties and pH levels, interference with recreational and commercial navigation, clogging of water management plant filters, and a depletion of oxygen upon decomposition of plants are just some of the effects that a high level of phosphorus has on the environment (Wodzinski and Ullah, 1996). Harmful effects have been reported for a fish population when a decrease in dissolved oxygen levels in surface water were noticed. Furthermore, toxins are produced by specific blue-green algae and the over-growth of this specie may have potential health risks for animals and humans (Kotak *et al.*, 1993).

Up to 50% of total phosphorus in soil is from phytate, which is the most abundant identifiable phosphate composite in soil (Anderson, 1980). This is one of the reasons for the driving force behind the development of a phytase product, which will neutralise this environmental concern of releasing concentrated pollutants into the surroundings (Wodzinski and Ullah, 1996).

As mentioned before, monogastric animals are unable to metabolise phytic acid, because they do not have gastrointestinal phytases. For this reason, the diets of these animals are supplemented with inorganic phosphate to meet the phosphate requirement. The addition of this supplement raised the cost of production and it also contributed to the phosphate pollution problem (Mullaney *et al.*, 2000; Bali and Satyanarayana, 2001). The addition of phytase minimises cost and will have positive effects on the pollution problems. The significance of feeding phytase to poultry and pigs to minimise pollution has been quantitatively determined and is shown in Table 6. In the United States, a total of 8.23×10^4 tonnes of phosphate has been prevented to reach the environment per annum by the addition of phytase to feedstuffs for these monogastric animals (Wodzinski and Ullah, 1996). A 30 to 40% decrease in the phosphorus pollution in the Netherlands was reported when a phytase from *A. niger* was added to diets of pigs (Jongbloed *et al.*, 1992).

The animal feed experiments discussed earlier presented quantitative data on the quantity of supplied dietary phosphate digested by animals and the reduced amount of phosphorus excreted in the manure. The amount of phosphorus that is digested and not excreted when phytase was used in the diets of poultry and swine has been predictably estimated (Schöner *et al.*, 1993).

Table 6. The effect of phytase on the reduction of phosphorus excretion from certain animals (Wodzinski and Ullah, 1996).

Animal	Quantity in united states in 1992[i]	Average live weight. (kg)	Kg of feed per animal	g P excreted per animal if supplemented with P	g P excreted per animal if supplemented with phytase	Kg P per annum not excreted
Broilers	$6.14 X10^9$	2.01	3.8	14.5	8.4	$3.75 X10^7$
Layers	$3.64 X10^8$		36.4/annum	139/annum	80.5/annum	$2.20 X10^7$
Ducks	$1.8 X10^7$	2.95	7.08	27*	15.6*	$2.81 X10^5$
Turkeys	$2.89 X10^8$	9.91	26.4	101*	58.5*	$1.69 X10^7$
Pigs	$5.78 X10^7$	80.4	265	271*	177	$5.62 X10^6$
Total						**$8.23 X10^7$**

[i] National Agricultural Statistics 1993.
* Estimate; **P**: phosphorus

This data was implemented as a foundation for determining the reduced phosphate levels excreted in dung and are multiplied by the number of monogastric animals raised in the United States, i.e. 90 million ton of phosphorus would be prevented from entering the environment (Table 6) (Wodzinski and Ullah, 1996).

Throughout this review, there have been suggestions made and reports given on the application of phytase and its effectiveness in the poultry and swine industries. There have been numerous reports on improving and optimising the phytases from different sources such as, isolating new phytase sources, genetically engineering better phytases and developing cost-effective expression systems. However, our scientific knowledge of phytases has yet to yield a solution to meet its enormous nutritional and environmental demand. As mentioned earlier, supplementing feedstuff with commercial products containing microbial phytase activity has proved to be a practical and advantageous method for improving phytate-phosphorus utilisation by poultry and swine. Thus, one of the approaches would be to identify novel sources of phytase with high yields of phytase activity. This phytase must be thermo-stable (feed processing at 70-90°C) and have an acidic pH optimum to function in the digestive tract of monogastric animals and must be cost-effective to produce.

6. Literature Cited

Anderson, G. (1980) Assessing organic phosphorus in soils. In: Khasawneh, F., Sample, E. and Kamprath, E. (Eds), The Role of Phosphorus in Agriculture. Madison, USA: American Soc. of Agron. pp. 411–432.

Abelson, P.H. 1999. A Potential phosphate crisis. Science. **283:** 2015.

Adelt, S., Podeschwa, M., Dallmann, G., Altenbach, H. and Vogel, G. 2003. Stereo- and regiospecificity of yeast phytases – chemical synthesis and enzymatic conversion of the substrate analogues *neo*- and L-*chiro*-inositol hexakisphosphate. Bioorgan. Chem. **31:** 44-67.

Ahmad, T., Rasool, S., Sarwar, M., Haq, A. and Hasan, Z. 2000. Effect of microbial phytase produced from a fungus *Aspergillus niger* on bioavalability of phosphorous and calcium in broiler chickens. Anim. Feed Sci. Technol.. **83:** 103-114.

Alasheh, S. and Duvnjak, Z. 1994. Characteristics of phytase produced by *Aspergillus carbonarius* Nrc-401121 in canola meal. Acta Biotechnol. **14**(3)**:** 223-233.

Al-Asheh, S. and Duvnjak, Z. 1995. The effect of phosphate concentration on phytase production and the reduction of phytic acid content in canola meal by *Aspergillus carbonarius* during a solid-state fermentation process. Appl. Microbiol. Biotechnol. **43:** 25-30.

Andriotis, A.M.E. and Ross, J.D. 2003. Isolation and characterization of phytase from dormant *Corylus avellana* seeds. Phytochemistry. **64:** 689-699.

Anno, T., Nakanishi, K., Matsuno, R. and Kamikubo, T. 1985. Enzymatic elimination of phytate in soybean milk. J. Japan Soc. Food Sci. Technol. **32:** 174-180.

Asmar, F., Gahoonia, T.S. and Nielsen, N.E. 1995. Barley genotypes differ in extracellular phosphatase activity and depletion of organic phosphorous from rhizosphere. Plant and Soil. **172:** 117-122.

Bae, H.D., Yanke, L.J., Cheng, K.J. and Selinger, L.B. 1999. A novel staining method for detecting phytase activity. J. Microbiol. Methods. **39:** 17-22.

Baidoo, S.K., Yang, Q.M. and Walker, R.D. 2003. Effects of phytase on apparent digestibility of organic phosphorous and nutrients in maize-soya bean meal based diets for sows. Anim. Feed Sci. Technol. **104:** 133-141.

Bali, A. and Satyanarayana, T. 2001. Microbial Phytases in nutrition and combating phosphorous pollution. Everyman's Sci. **4:** 207-209.

Barrientos, L., Scott, J.J. and Murthy, P.P. 1994. Specificity of hydolysis of phytic acid by alkaline phytase from lily pollen. Plant Physiol. **106:** 1489-1495.

Bernard, M., Mouyna, I., Dubreucq, G., Debeaupuis, J., Fontaine, T., Vorgias, C., Fuglsang, C. and Latgé, J. 2002. Characterization of a cell-wall acid phosphatase (PhoAp) in *Aspergillus fumigatus.* Microbiology. **148:** 2819-2829.

Berka, R.M., Rey, M.W., Brown, K.M., Byun, T. and Klotz, A.V. 1998. Molecular characterization and expression of a phytase gene from the thermophilic fungus, *Thermomyces lanuginosus.* Appl. Environ. Microbiol. **64:** 4423-4427.

Billington, D.C. 1993. The Inositol Phosphates. Chemical Synthesis and Biological Significance. Verlag Chemie, Weinham.

Bindu, S., Somashekar, D. and Joseph, R. 1998. A comparative study on permeabilization treatments for in situ determination of phytase of *Rhodotolura gracilis.* Lett. Appl. Microbiol. **27** (6)**:** 336-340.

Bitar, K. and Reinhold, J.G. 1972. Phytases and alkaline phosphatase activities in the intestinal mucosa of rats, chicken, calf, and man. Biochim. Biophys. Acta. **268:** 442-452.

Blank, G.E., Pletcher, J. and Sax, M. 1971. The structure of *myo*-inositol hexaphosphate, dodecasodium salt octama-contahydrate: a single crystal x-ray analysis. Biochem. Biophys. Res. Commun. **44:** 319-325.

Bogar, B., Szakacs, G., Linden, J.C., Pandey, A. and Tengerdy, R.P. 2003. Optimization of phytase production by solid substrate fermentation. J. Ind. Microbiol. Biotechnol. **30:** 183-189.

Borade, V.P., Kadarn, S.S. and Salunke D.K. 1994. Changes in phytate, phosphorous and minerals during germination and cooking of horse gram and mouth bean. Qual. Plant Foods Hum. Nutr. **34:** 151.

Brinch-Pedersen, H., Olesen, A., Rasmussen, S.K. and Holm, P.B. 2000. Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. Molecular Breeding. **6:** 195-206.

Brinch-Pedersen, H., SØrensen, L.D. and Holm, P.B. 2002. Engineering crop plants: getting a handle on phosphate. Trends Plant Sci. **7:** 118-126.

Brocades, G. 1991. Production of phytase in transgenic plant or plant organ. Pat. EP 449 375.

Casey, A. and Walsh, G. 2003. Purification and characterization of extracellular phytase from *Aspergillus niger* ATCC 9142. Biores. Technol. **86:** 183-188.

Casida, L.E. Jr. 1959. Phosphatase activity of some common soil fungi. Soil Sci. **87:** 305-310.

Cheng, K.J., Selinger, L.B., Yanke, L.J., Bae, H.D., Zhou, L. and Forsberg, C.W. 1999. Phytases of rumen microorganisms, particularly of *Selenomonas ruminantium*, and uses therof in feed additives and in transgenic plants. US Patent 5, 985, 605, Nov. 16.

Cheryan, M. 1980. Phytic acid interactions in food systems. CRC Crit. Rev. Food Sci. Nutr. **13:** 297-335.

Choi, Y.M., Noh, D.O., Cho, S.H., Lee, H.K., Suh, H.J. and Chung, S.H. 1999. Isolation of a phytase-producing *Bacillus* sp. KHU-10 and its phytase production. J. Microbiol. Biotechnol. **9** (2)**:** 223-226.

Cooper, J.R. and Gowing, H.S. 1983. Mammalian small intestinal phytase (E.C. 3.1.3.8). Br. J. Nutr. **50:** 673-678.

Cosgrove, D.J. 1969. Ion-exchange chromatography of inositol polyphosphates. Ann. N.Y. Acad. Sci. **165:** 677-686.

Cosgrove, D.J., Irving, G.C.J. and Bromfield, S.M. 1970. Inositol Phosphate phosphatases of microbiological origin. The isolation of soil bacteria having inositol phosphate phosphatase activity. J. Biol. Sci. **23:** 339-343.

Costello, A.J.R., Glonek, T. and Myers, T.C. 1976. ^{31}P-nuclear magnetic resonance-pH titrations of *myo*-inositol hexaphosphate. Carbo. Res. **46:** 159-171.

Cromwell, G.L., Stahly, T.S., Coffey, R.D., Monegue, H.J. and Randolph, J.H. 1993. Efficacy of phytase in improving the bioavailability of phosphorous in soya bean meal and maize-soybean meal diets for pigs. J. Anim. Sci. **71:** 1831-1840.

Cromwell, G.L., Coffey, R.D., Parker, G.R., Monegue, H.J. and Randolph, J.H. 1995a. Efficacy of low activity microbial phytase in improving the bioavailability of phosphorous in corn-soybean meal diets for pigs. J. Anim. Sci. **73:** 449-456.

Cromwell, G.L., Coffey, R.D., Parker, G.R., Monegue, H.J. and Randolph, J.H. 1995b. Efficacy of a recombinant derived phytase in improving the bioavailability of phosphorous in corn-soybean meal diets for pigs. J. Anim. Sci. **73:** 2000-2008.

Dasgupta, S., Dasgupta, D., Sen M., Biswas, S. and Biswas, B.B. 1996. Interaction of myoinositoltriphosphate-phytase complex with the receptor for intracellular Ca^{2+} mobilization in plants. Biochemistry. **35:** 4994-5001.

Davies, N.T. 1982. Effects of phytic acid on mineral availability. In: Vahoung, G.V. and Kritchevsky, D. (Eds.), Dietary Fiber in Health and Disease. Plenum Press, New York.

De Angelis, M., Gallo, G., Corbo, M.R., McSweeney, P.L.H., Faccia, M., Giovine, M. and Gobbetti, M. 2003. Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CBI. Inter. J. Food Microbiol. **2723:** 1-12.

Denbow, D.M., Grabau, E.A., Lacy, G.H., Kornegay, E.T., Russel, D.R. and Umbeck, P.F. 1998. Soybeans transformed with a fungal phytase gene improve phosphorous availability for broilers. Poult. Sci. **77:** 878-881.

De Rham, O. and Jost, T. 1979. Phytate protein interactions in soybean extracts and low phytate soy protein products. J. Food Sci. **44:** 596-600.

Deshpande, S.S. and Cheryan, M. 1984. Effect of phytic acid, divalent cations, and their interactions on alpha-amylase activity. J. Food. Sci. **49:** 516-524.

D'Silva, G.C., Bae, H.D., Yanke, L.J., Cheng, K.J. and Selinger, L.B. 2000. Localization of phytase in *Selenomonas ruminantium* and *Mitsuokella multiacidus* by transmission electron microscopy. Can. J. Microbiol. **46:** 391-395.

Duff, S.M.G., Sarath, G. and Plaxton, W.C. 1994. The role of acid phosphatases in plant phosphorous metabolism. Physiol. Plant. **90:** 791-800.

Dvorokova, J. 1998. Phytase: Sources, preparation and exploitation. Folia Microbiol. **43**(4)**:** 323-338.

Dvorakova, J., Volfova, O. and Kopecky, J. 1997. Characterization of phytase produced by *Aspergillus niger*. Folia Microbiol. **42** (4)**:** 349-352.

Ebune, A., Al-Asheh, S. and Duvnjak, Z. 1995a. Effect of phosphate, surfactants and glucose on phytase production and hydrolysis of phytic acid in canola meal by *Aspergillus ficuum* during solid state fermentation. Biores. Technol. **54:** 241-247.

Ebune, A., Al-Asheh, S. amd Duvnjak, Z. 1995b. Production of phytase during solid state fermentation using *Aspergillus ficuum* NRRL 3135 in canola meal. Biores. Technol. 1995. **53:** 7-12.

Eeckhout, W. and De Paepe, M. 1994. Total phosphorous, phytate-phosphorous and phytase activity in plant feed stuffs. Anim. Feed Sci. Technol. **47:** 19-29.

Engelen, A.J., van der Heeft, F.C.P., Randsdorp, H. and Smit, E.L. 1994. Simple and rapid determination of phytase activity. J. AOAC Int. **77:** 760-764.

Eskin, N.A.M. and Wiebe, S. 1983. Changes in phytate activity and phytase during germination of two fababean cultivars. J. Food Sci. **48:** 270-271.

Feuillade, M. and Dorizo, J.M. 1992. Enzymatic release of phosphate in sediments of various origins. Wat. Res. **26:** 1195-1201.

Fretzdorff, B., Brummer, J.M., Rochen, W., Greiner, R., Konietzny, U. and Jany, K.D. 1995. Reduktion des Phytinsaure-Gehaites bei der Herstellung von Backwaren und Getreidenahrmitteln. Aid-Verhraucherdienst. **40:** 12-20.

Freund, W.D., Mayr, G.W., Tietz, C. and Schultz, J.E. 1992. Metabolism of inositol phosphates in the protozoan *Paramecium.* Eur. J. Biochem. **207:** 359-367.

Frolich, W. 1990. Chelating properties of dietary fibre and phytate. The role for mineral availability. Adv. Exp. Med. Biol. **270:** 83-93.

Fujita, J., Yamane, Y., Fukuda, H., Kizaki, Y., Wakabayashi, S., Shigeta, S., Suzuki, O. and Ono, K. 2003. Production and properties of phytase and acid phosphatase from a sake koji mold, *Aspergillus oryzae*. J. Biosci. Bioeng. **95** (4)**:** 348-353.

Galzy, P. 1964. Etude genetique et physiologique du metabolism de l'acide Lactique chez *Saccharomyces cerevisiae*. Hansen. Ann. Technol. Agric. **13:** 109-259.

Gargova, S. and Sariyska, M. 2003. Effect of culture conditions on the biosynthesis of *Aspergillus niger* phytase and acid phosphatase. Enz. Microbial Technol. **32:** 231-235.

Gargova. S., Roshkova, Z. and Vancheva, G. 1997. Screening of fungi for phytase production. Biotechnol Tech. **11** (4)**:** 221-224.

Ghareib, M. 1990. Biosynthesis, purification and some properties of extra-cellular phytase from *Aspergillus carneus.* Acta Microbiol. Hungarica. **37** (2)**:** 159-164.

Gibson, D.M. and Ullah, A.H.J. 1990. Phytase and their action in phytic acid. In: Inositol Metabolism in Plants. Wiley-Liss, Chichester, pp.77-92.

Ghosh, S. 1997. Phytase of a thermophilic mould *Sporotrichum thermophile* Apinis. M.Sc dissertation, Department of Microbiology, University of Delhi, Delhi.

Golovan, S.P., Hayes, M.A., Phillips, J.P. and Forsberg, C.W. 2001. Transgenic mice expressing bacterial phytase as a model for phosphorous pollution control. Nat. Biotechnol. **5:** 429-433.

Golovan, S.P. Wang, G., Zhang, J. and Forsberg, C.W. 2000. Characterization and overproduction of the *Escherichia coli appA* encoded bifunctional enzyme that exhibits both phytase and acid phophatase activities. Can. J. Microbiol. **46:** 59-71.

Graf, E. 1986. Chemistry and applications of phytic acid: an overview. In: Graf, E. (Ed.), Phytic Acid: chemistry and applications. Pilatus Press, Minneapolis, pp. 173-194.

Graf, E., Empson, K. and Eaton, J.W. 1987. Phytic acid. A natural antioxidant. J. Biol. Chem. **262:** 11647-11651.

Greiner, R. and Konitzny, U. 1996. Construction of a bioreactor to produce special breakdown products of phytate. J. Biotechnol. **48** (1-2)**:** 153-159.

Greiner, R., Konitzny, U. and Jany, K.D. 1993. Purification and characterization of two phytases from *Esherichia coli.* Arch. Biochem. Biophys. **303:** 107-113.

Greiner, R., Haller, E., Konietzny, U. and Jany, K.D. 1997. Purification and characterization of a phytase from *Klebsiella terrigena.* Arch. Biochem. Biophys. **341** (2)**:** 201-206.

Greaves, M., Anderson, G. and Wehley, D.M. 1967. The hydrolysis of inositol phosphatases by *Aerobacter aerogenes.* Biochim. Biophys. Acta. **132:** 412-418.

Gutnecht, K. 1997. Green genes: Alfalfa biofarming is about to take root. Wisconsin Agriculturist, Mid-March, pp. 8-10.

Ha, N.C., Kim, Y.O. and Oh, B.H. 1999. Preliminary X-ray crystallographic analysis of a novel phytase from a *Bacillus amiloliquefaciens* strain. Acta Crystallographics. Section D-Biological Crystallography. **55:** 691-693.

Han, Y.M., Wilson, D.B. and Lei, X.G. 1999. Expression of an *Aspergillus niger* phytase gene (*phyA*) in *Saccharomyces cerevisiae.* Appl. Environ. Microbiol. **65** (5)**:** 1915-1918.

Han, Y.W., Gallagher, D.J. and Wilfred, A.G. 1987. Phytase production by *Aspergillus ficuum* on semi-solid substrate. J. Ind. Microbiol. **2** (4)**:** 195-200.

Hara, A., Ebina, S. and Kondo, A. 1985. A new type of phytase from pollen of *Typha latifolia* L. Agric. Biol. Chem. **49:** 3539-3544.

Harland, B.F. and Harland, J. 1980. Fermentative reduction of phytate in rye, white and whole wheat breads. Cereal Chem. **57:** 226-229.

Harland, B.F. and Morris, E.R. 1995. Phytate: a good or a bad food component? Nutr. Res. **15** (5)**:** 733-754.

Houde, R.L., Alli, L. and Kermasha, S. 1990. Purification and characterisation of canola seed (*Brassica* sp.) phytase. J. Food Biochem. **14:** 331-351.

Howson, S.J. and Davies, R.P. 1983. Production of phytate hydrolyzing enzymes by some fungi. Enz. Microb. Technol. **5:** 377-389.

Hübel, F. and Beck, E. 1996. Maize root phytase: Purification, characterization, and localization of enzyme activity and its putative substrate. Plant Physiol. **112:** 1429-1436.

Igbasan, F.A., Manner, K., Miksch, G., Borriss, R., Farouk, A. and Simon, O. 2000. Comparative studies on the *in vitro* properties of phytases from various microbial origins. Arch. Fuer Tierernahrung. **53:** 353-373.

Inagawa, J., Kiyosawa, I. and Nagasawa, T. 1987. Effect of phytic acid on the hydrolyses of lactose woth beta-galactosidase. Agric. Biol. Chem. **51:** 3027-3032.

Irving, G.C.J and Cosgrove, D.J. 1971. Inositol phosphate phosphatase of microbial origin. Observations on the nature of the active center of a bacterial (*Pseudomonas* sp.) phytase. Austral. J. Biol. Sci. **24:** 1559-1564.

Irving, G.C.J. and Cosgrove, D.J. 1974. Inositol phosphate phosphatases of microbiological origin. Some properties of the partially purified phosphatases of *Aspergillus ficuum* NRRL 3135. Aust. J. Biol. Sci. **27:** 361-368.

Johnson, L.F. and Tate, M.E. 1969. The structure of *myo*-inositol pentaphosphates. Ann. N.Y. Acad. Sci. **165:** 526-532.

Jareonkitmongkol, S., Ohya, M., Watanabe, R., Takagi, H. and Nakamori, S. 1997. Partial purification of phytase from a soil isolate bacterium, *Klebsiella oxytoca* MO-3. J. Ferm. Bioeng. **83**(4)**:** 393-394.

Jongbloed, A.W., Mroz, M. and Kemme, P.A. 1992. The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorous, and phytic acid in different sections of the alimentary tract. J. Anim. Sci. **70:** 1159-1168.

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N. and Apajalahti, J. 1998. Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. Appl. Environ. Microbiol. **64:** 2079-2085.

Kerovuo, J. and Tynkkynen, S. 2000. Expression of *Bacillus subtilis* phytase in *Lactobacillus plantarum* 755. Lett. Appl. Microbiol. **30:** 325-329.

Kim, D.H., Oh, B.C., Choi, W.C., Lee, J.K. and Oh, T.K. 1999a. Enzymatic evaluation of *Bacillus amyloliquefaciens* phytase as a feed additive. Biotechnol. Lett. **21:** 925-927.

Kim, D.S., Godber, J.S. and Kim, H.R. 1999b. Culture conditions for a new phytase-producing fungus. Biotechnol. Lett. **21** (12)**:** 1077-1081.

Kim, Y.O., Lee, J.K., Kim, H.K., Yu, J.H. and Oh, T.K. 1998. Cloning of the thermostable phytase gene (*phy*) from *Bacillus* sp. DS11 and its overexpression in *Escherichia coli.* FEMS Microbiol Lett. **162:** 185-191.

Klis, F.M. 1994. Review: cell wall assembly in yeast. Yeast. **10:** 851-869.

Knorr, D., Watkins, T.R. and Carlson, B.L. 1981. Enzymatic reduction of phytate in whole wheat breads. J. Food Sci. **46:** 1866-1869.

Konietzny, U., Greiner, R. and Jany, K.D. 1995. Purification and characterization of a phytase from spelt. J. Food. Biochem. **18:** 165-183.

Kostrewa, D., Grüninger-Leitch, F., D'Archy, A., Broger, C., Mitchell, D. and Van Loon, A.P.G.M. 1997. Crystal structure of phytase from *Aspergillus ficuum* at 2.5 Å resolution. Nat. Struct. Biol. **4** (3)**:** 185-190.

Kotak, B. G., Kenefick, S. L., Fritz, D. L., Rousseaux, C. G., Prepas, E. E. and Hrudey, S. E. 1993. Occurrence and toxicological evaluation of cyanobacterial toxins in Alberta lakes and farm dugouts. Water Res. **27:** 495–506.

Krishna, C. and Nokes, S.E. 2001. Predicting vegetative inoculum performance to maximize phytase production in solid-state fermentation using response surface methodology. J. Ind. Microbiol. Biotechnol. **26:** 161-170.

Kunze, G. and Kunze, I. 1996. *Arxula adeninivorans*. In: Wolf, K. (Ed.), Nonconventional Yeasts in Biotechnology, a Handbook. Berlin, Springer-Verlag, pp. 389-409.

Kyriakidis, N.B., Galiotou-Panayotou, M., Stavropoulou, A. and Athanasopoulos, P. 1998. Increase in phytase activity and decrease in phytate during germination of four common legumes. Biotechnol. Lett. **20** (5)**:** 475-478.

Lambrechts, C., Boze, H., Moulin, G. and Galzy, P. 1992. Utilization of phytate by some yeasts. Biotechnol. Lett. **14:** 61-66.

Laboure, A.M., Gagnon, J. and Lescure, A.M. 1993. Purification and characterization of a phytase (*myo*-inositol hexakisphosphate phosphohydrolase) accumulated in maize (*zea mays*) seedlings during germination. Biochem. J. **295:** 413-419.

Lassen, S.F., Breinholt, J., Østergaard, P.R., Brugger, R., Bischoff, A., Wyss, M. and Fuglsang, C.C. 2001. Expression, Gene cloning and characterization of five novel phytases form four Basidiomycete Fungi: *Peniophora lycii, Agrocybe pediades,* a *Ceriporia* sp., and *Trametes pubescens*. Appl. Environ. Microbiol. **67:** 4701-4707.

Lei, X.G. and Stahl, C.H. 2000. Nutritional benefits of phytase and dietary determinants of its efficacy. J. Appl. Anim. Res. **17:** 97-112.

Lei, X.G. and Stahl, C.H. 2001. Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl. Microbiol. Biotechnol. **57:** 474-481.

Li, J., Hegeman, C.E., Hanlon, R.W., Lacy, G.H. Denbow, D.M. and Graban, E.A. 1997. Secretion of active recombinant phytase from soybean cell-suspension cultures. Plant Physiol. **114:** 1-9.

Lim, P.E. and Tate, M.E. 1973. The phytases. II. Properties of phytase fractions F1 and F2 from wheat bran and the myo-inositol phosphatases produced by fraction F2 . Biochim. Biophys. Acta. **302:** 316-328.

Lim, D., Golovan, S., Forsberg, C.W. and Jia, Z. 2000. Crystal structures *Escherichia coli* phytase and its complex wth phytate. Nat. Struc. Biol. **7** (2)**:** 108-113.

Lin, J.J., Dickinson, D.B. and Ho, T.H.D. (1987). Phytic acid metabolism in Lily (*Lilium longiflorum* Thunb.) pollen. Plant Physiol.. **83:** 408-413.

Liu, B.L., Rafiq, A., Tzeng, Y.M. and Rob, A. 1998. The induction and characterization of phytase and beyond. Enzyme Microbiol. Technol. **22:** 415-424.

Maga, J.A. 1982. Phytate: its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. J. Agric. Food Chem. **30:** 1-9.

Maiti, I.B., Majumber, A.L. and Biswas, B.B. (1974). Purification and mode of action of phytase from *Phaseolus aureus*. Phytochem. **13:** 1047-1051.

Majerus, P.W. 1992. Inositol phosphate biochemistry. Annu. Rev. Biochem. **61:** 225-250.

Martin, M. and Luque, J. 1985. Phytases activity in chicken erythrocytes and its control by organic phosphates (glycerate 2,3,-P_2 and inositol P_5) during avian development. Compar. Biochem. Physiol. **80** B: 557-561.

Mayer, A.F., Hellmuth, K., Schlieker, H., Lopez-Ulibarri, R., Oertel, S., Dahlems, U., Strasses, A.W.M. and van Loon, A.P.G.M. 1999. An expression system matures: a highly efficient and cost-effective process for phytase production by recombinant strains of *Hansensula polymorpha.* Biotechnol. Bioeng. **63** (3)**:** 373-381.

Mc collum, E.V. and Hart, E.B. 1998. On the occurrence of a phytin splitting enzyme in animal tissue. J. Biol. Chem. **4:** 497-500.

Mitchell, D.B., Vogel, K., Weimann, B.J., Pasamontes, L. and van Loon, A.P.G.M. 1997. The phytase subfamily of histidine acid phosphatases: Isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila.* Microbiology-UK. **143:** 245-252.

Moo-Young, M., Moreira, A.R. and Tengerdy, R.P. 1978. Principles of solid state fermentation. In: Smith, J.E., Berry, D.R. and Kristiansen, B. (Eds.), The Filamentous fungi. **3:** 116-143.

Mullaney, E.J., Daly, C.B., Sethumadhavan, K., Rodriquez, E., Gen-lei, X. and Ullah, A.H. 2000. Phytase activity in *Aspergillus fumigatus* isolates. Biochem. Biophys. Res. Commun. **275:** 759-763.

Mullaney, E.J. and Ullah, A.H.J. 1998. Identification of a histidine acid phosphatase (*phyA*)-like gene in *Arabidopsis thaliana.* Biochem. Biophys. Res. Commun. **251**(1)**:** 252-255.

Mwachireya, S.A., Beames, R.M., Higgs, D.A. and Dosanjh, B.S. 1999. Digestilbility of canola protein products derived from the physical, enzymatic and chemical processing of commercial canola meal in rainbow trout *Oncorhynchus mykiss* (Walbaum) held in fresh water. Aquacul. Nutr. **5:** 73-82.

Nagai, Y. and Funahashi, S. 1962. Phytase (*myo*-inositol-hexakisphosphate phosphohydrolase) from wheat bran. Part I. Purification and substrate specificity. Agric. Biol. Chem. **26:** 794-803.

Nagashima, T., Tange, T. and Anazawa, H. 1999. Dephosphorylation of phytate by using the *Aspergillus niger* phytase with a high affinity for phytate. Appl. Environ. Microbiol. **65** (10)**:** 4682-4684.

Nair, V.C. and Duvnjak, Z. 1990. Reduction of phytic acid content in canola meal by *Aspergillus ficuum* in solid state fermentation process. Appl. Microbiol. Biotechnol. **34:** 183-188.

Nair, V.C., Laflamme, J. and Uvnjak, Z. 1991. Production of phytase by *Aspergillus ficuum* and reduction of phytic acid content in canola meal. J. Sci. Food Agric. **54** (3)**:** 355-365.

Nakamuru, Y., Fukuhara, H. and Sano, K. 2000. Secreted phytase activities of yeasts. Biosci. Biotechnol. Biochem. **64:** 841-844.

Nasi, M., Partanen, K. and Piironen, J. 1999. Comparison of *Aspergillus niger* phytase and *Trichoderma reesei* phytase and acid phosphatase on phytate phosphorous availability in pigs fed on maize-soybean meal or barley-soybean meal diets. Archives of Animal Nutrition – Archiv fur Tierernahrung. **52** (1)**:** 15-27.

Nayini, N.R. and Markakis, P. 1984. The phytase of yeast. Lebensm. Wiss. Technol. **17:** 24-26.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J. and Ware, J.H. 1968. The availability of phytate phosphorous in soybean meal before and after treatment with a mold phytase. Poult. Sci. **47:** 1842-1848.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J. and Ware, J.H. 1971. Effect of supplement phytase on the utilization of phytate phosphorous by chiks. J. Nutr. **101:** 1289-1294.

O'Quinn, P.R., Knabe, D.A. and Gregg, E.J. 1997. Efficacy of Natuphos in sorghum-based diets of finishing swine. J. Anim. Sci. **75:** 1299-1307.

Oriol, E., Rainmbault, M., Roussos, S. and Vinegra-Gonzales, G. 1988. Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger* . Appl. Microbiol. Biotechnol. **27:** 498-503.

Pallauf, J. and Rimbach, G. 1996. Effect of supplemental phytase on mineral and trace element bioavailability and heavy accumulation in pigs with different type diets. In: Coehlbo, M.B. and Kornegay, E.T. (Eds.), Phytase in animal nutrition and waste management. BASF publication DC9601.

Pallauf, J., Höhler, D. and Rimbach, G. 1992. Effekt einer Zulage an mikrobieller Phytase zu einer Mais-Soja-Diät auf die scheinbare Absorption von Mg, Fe, Cu, Mn und Zn sowie auf Parameter des Zinkstataus beim Ferkel. J. Anim. Physiol. a. Anim. Nutr. **68:** 1-9.

Pandey, A. 1991. Aspects of design of fermenter in solid state fermentation. Process Biochem. **26:** 355-361.

Pandey, A. 1992. Recent developments in solid state fermentation. Process Biochem. **27:** 109-117.

Pandey, A. 1994. Solid state fermentation – an overview. In: Pandey, A. (Ed.). Solid State Fermentation. Wiley Eastern Limited, New Delhi, pp. 3-10.

Pandey, A. and Soccol, C.R. 2000. Economic utilization of crop residues for value addition – a futuristic approach. J. Sci. Ind. Res. **59** (1)**:** 12-22.

Pandey, A., Soccol, C.R., Rodriguez-Leon, J.A. and Nigam, P. 2001. Solid-State Fermentation in Biotechnology. Fundamentals and Applications. Asiatech Publishers, Inc., New Delhi.

Papagianni, M., Nokes, S.E. and Filer, K. 1999. Production of phytase by *Aspergillus niger* in submerged and solid-state fermentation. Process Biochem. **35** (3-4)**:** 397-402.

Pasamontes, L., Haiker, M., Henriquez-Huecas, M., Mitchell, D.B. and van Loon, A.P.G.M. 1997a. Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus.* Biochimica et Biophysica Acta. **1353:** 217-223.

Pasamontes, L., Haiker, M., Wyss, M., tessier, M. and van Loon, A.P.G.M. 1997b. Gene cloning, purification, and characterization of a heat-stable phytase from the fungus, *Aspergillus fumigatus*. Appl. Environ. Microbiol. **63** (5)**:** 1696-1700.

Pears, F.G. 1953. The phytase of wheat. Biochem. J. **53:** 102-109.

Piddington, C.S., Houston, C.S., Palobeimo, M., Cantrell, M., Micttinen-Oinonem, A., Nevalinen, H. and Ram Bosek, J. 1993. The cloning and sequencing of the genes encoding phytase (*phyA*) and pH 2.5 optimum acid phosphatase (*aph*) from *Aspergillus niger* var. *awamori*. Gene. **133:** 55-62.

Powar, V.K. and Jagannathan, V. 1967. Phytase from *Bacillus subtilis*. Indian. J. Biochem. **4:** 184-185.

Powar, V.K. and Jagannathan, V. 1982. Purification and properties of phytate specific phosphatase from *Bacillus subtilis*. J. Bacteriol. **151:** 1102-1108.

Quan, C., Fan, S. and Ohya, Y. 2002. Pathway of dephosphorylation of *myo*-inositol hexakisphosphate by a novel phytase from *Candida krusei* WZ-001. J. Biosci. Bioeng. **95:** 530-533.

Quan, C., Zhang, L., Wang, Y. and Ohta, Y. 2001. Production of phytase in a low phosphate medium by a novel yeast, *Candida krusei*. J. Biosci. Bioeng. **92:** 154-160.

Rayner, A.D.M. and Boddy, L. 1988. Fungal communities in the decay of wood. Adv. Microbial Ecol. **10:** 115-166.

Rapoport, S., Leva, E. and Guest, G.M. 1941. Phytase in plasma and crythrocytes of vertebrates. J. Biol. Chem. **139:** 621-632.

Reddy. N.R., Sathe, S.K. and Salukhe, D.K. 1982. Phytases in legumes and cereals. Adv. Food Res. Academic Press, N.Y. **28:** 1-92.

Reddy, N.R., Pierson, M.D., Sathe, S.K. and Salunkhe, D.K. 1989. Phytase in cereals and legumes. CRC Press, Inc., Boca Raton, Fla.

Riccio. M.L., Rossolini, G.M, Lombardi, G., Chiesurin, A. and Satta, G. 1997. Expression cloning of different bacterial phosphatase-encoding genes by histochemical screening of genomic libraries onto a indicator medium containing phenolphthalein diphosphate and methyl gree. J. Appl. Microbiol. **82:** 177-185.

Richardson, A.E. and Hadobas, P.A. 1997. Soil isolates of *Pseudomonas* spp, that utilize inositol phosphates. Can. J. Microbiol. **43** (6)**:** 509-516.

Rodriguez, E., Mullaney, E.J. and Lei, X.G. 2000. Expression of the *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. Biochem. Biophys. Commun. **268:** 373-378.

Robinson, E.H., Jackson, S. and Li, M.H. 1996. Supplemental phytase in catfish diets. Aquacul. Mag. **22:** 80-82.

Rumsey, G.L. 1993. Fish meal and alternate sources of protein in fish feeds: Update. Fisheries. **18:** 14-19.

Sabin, R. 1988. U.S. Patent No.4,758,430, July 19.

Sabin, R. 1989. U.S. Patent No.4,847,082, July 11.

Sabin, R. 1992. U.S. Patent No.5,112,814, May 12.

Sandberg, A.S. and Andersson, H. 1988. Effect of dietary phytase on the digestion of phytate in the stomach and small intestine of humans. J. Nutr. **118:** 469-476.

Sandberg, A.S., Hulthen, L.R. and Turk, M. 1996. Dietary *Aspergillus niger* phytase iron absorption in humans. J. Nutr. **126:** 476-480.

Sano, K., Fukuhara, H. and Nakamura, Y. 1999. Phytase of the yeast *Arxula adeninivorans.* Biotechnol. Lett. **21:** 33-38.

Segueilha, L., Lambrechts, C., Boze, H., Moulin, G. and Galzy, P. 1992. Purification and properties of the phytase from *Schwanniomyces castelii.* J. Ferment. Bioeng. **74:** 7-11.

Schöner, F.J., Hoppe, P.P., Schwarz., G. and Wiesdie, H. 1993. Effects of microbial phytase and inorganic phosphate in broiler chickens: performance and mineral retention at various calcium levels. J. Anim. Physiol. Anim. Nutr. **69**: 235–244.

Scott, J.J. 1991. Alkaline phytase activity in nonionic detergent extracts of legume seeds. Plant Physiol. **95:** 1298-1301.

Scott, J.J. and Loewus, F.A. 1986. A calcium-activated phytase from pollen of *Lilium longiflorum.* Plant Physiol. **82:** 333-335.

Sebastian, S., Touchburn, S.P., Chavez, E.R. and Lague, P.C. 1996. The effect of supplemental phytase on the performance and utilization of dietary calcium, phosphorous, copper and zinc in broiler chickens fed corn soybean diets. Poultr. Sci. **76:** 729-736.

Shah, V. and Parekh, L.J. 1990. Phytase from *Klebsiella* sp. no. pg-2 – purification and properties. Ind. J. Biochem. Biophys.. **27** (2)**:** 98-102.

Shakarian, M., Joshi, M. B., Ghedin, E. and Dwyer, D. M. 2002. Molecular Dissection of the Functional Domains of a Unique, Tartrate-resistant, Surface Membrane Acid Phosphatase in the Primitive Human Pathogen *Leishmania donovani.* J. Biol. Chem. **277** (20)**:** 17994 - 18001.

Shamsuddin, A.M. and Ullah, A. 1989. Inositol hexaphosphate inhibits large intestinal cancer in F344 rats 5 months after induction by azoxymethane. Carcinogenesis. **10:** 625-626.

Shamsuddin, A.M. Elsayed, A.M. and Ullah, A.H.J. 1988. Suppression of large intestinal cancer in F344 rats by inositol hexaphosphate. Carcinogenesis. **9:** 577-580.

Shamsuddin, A.M., Ullah, A.H.J. and Chakravarthy. A.K. 1989. Inositol and inositol hexaphosphate suppress cell proliferation and tumor formation in CD-1 mice. Carcinogenesis. **10:** 1461-1463.

Sharpley, A.N., Daniel, T.C. and Edwards, D.R. 1994. Phosphorus movement in the landscape. J. Production Agriculture. **6** (4)**:** 492-500.

Shieh, T.R. and Ware, J.H. 1968. Survey of microorganism for the production of extra-cellular phytase. Appl. Microbiol. **16:** 1348-1351.

Shimizu, M. 1992. Purification and characterization of phytase from *Bacillus subtilis* (natto) *n*-77. Biosci. Biotechnol. Biochem. **56** (8)**:** 1266-1269.

Shimizu, M. 1993. Purification and characterization of phytase and acid-phosphatase produced by *Aspergillus oryzae* K1. Biosci. Biotechnol. Biochem. **57 (8):** 1364-1365.

Skowronaski, T. 1978. Some properties of partially purified phytase from *Aspergillus niger*. Acta Micro. Pol. **27:** 41-48.

Simell, M. Turunen, M., Piironen, J. and Vaara, T. 1989. feed and food applications of phytase. Lecture at 3rd Meet. Industrial Applications of Enzymes, Barcelona (Spain).

Simons, P.C.M., Versteegh, H.A.J., Jongbloed, A.W., Kemme, P.A., Slump, P., Bos, K.D., Wolters, M.G.E., Beudeker, R.F. and Verschoor, G.J. 1990. Improvement of phosphorous availability by microbial phytase in broilers and pigs. Br. J. Nutr. **64:** 525-540.

Singh, M. and Krikorian, A.D. 1982. Inhibition of trypsin activity *in vivo* by phytate. J. Agric. Food Chem. **30:** 799-805.

Siren, M. 1986. Stabilized pharmaceutical and biological material composition. Pat. SE 003 165.

Siren, M. 1995. Method of treating pain using inosiltol triphosphate. U.S. patent 5407924.

Siren, M., Lofkvist, B. and Edvinsson, L. 1992. Method of treating cardiovascular diseases using inositoltriphsphate. U.S. Patent 5128332.

Sreeramulu, G., Srinivasa, D.S., Nand, K. and Joseph, R. 1996. *Lactobacillus amylovorus* as a phytase producer in submerged culture. Lett. Appl. Microbiol. **23:** 385-388.

Stahl, C.H., Roneker, K.R., Thornton, J.R. and Lei, X.G. 2000. A new phytase in yeast effectively improves the bioavailability of phytate phosphorous to weanling pigs. J. Anim. Sci. **78:** 668-674.

Stahl. C.H., Wilson, D.B. and Lei, X.G. 2003. Comparison of extracellular *Escherichia coli* AppA phytase expressed in *Streptomyces lividans* and *Pichia pastoris*. Biotechnol. Lett. **25:** 827-831.

Sunitha, K., Lee, J.K. and Oh, T.K. 1999. Optimization of medium components for phytase production by *E. coli* using response surface methodology. Bioprocess Engin. **21** (6): 477-481.

Sutardi and Buckle, K.A. 1986. The characteristics of soybean phytase. J. Food Biochem. **10:** 197-216.

Sutardi and Buckle, K.A. 1988. Characterization of extra- and intracellular phytases from *Rhizopus oligosporus* used in tempeh production. Int. J. Food Microbiol. **6** (1): 67-79.

Suzuki, U., Yoshimura, K. and Takaishi, M. 1907. Ueber ein Enzym "Phytase" das " Anhydro-oxy-methylen diphosphorsaure" Spallet. Tokyo. Imper. Univ. Coll. Agric. Bull. **7:** 503-512.

Tambe, S.M., Kaklij, G.S., Kelkar, S.M. and Parekh, L.J. 1994. Two distinct molecular forms of phytase from *Klebsiella aerogenes* – evidence for unusually small active enzyme peptide. J. Ferm. Bioeng. **77** (1)**:** 23-27.

Tarafdar, J. C. and A. Jungk (1987). Phosphatase activity in the rhizosphere and its relation to the depletion of soil organic phosphorus. Biology and Fertility of Soils **3** (4)**:** 199-204.

Thompson, L.U. 1986. Phytic acid: a factor influencing starch digestibility and blood glucose response. In: Graf, E.(Ed.), Phytic Acid: chemistry and applications. Pilatus Press, Minneapolis, p. 173-194.

Ullah, A.H.J. 1988. *Aspergillus ficuum* phytase – partial primary structure, substrate selectivity, and kinetic characterization. Prep. Biochem. **18** (4)**:** 459-471.

Ullah, A.H.J. and Cummins, B.J. 1988. *Aspergillus ficuum* extracellular phytase: Immobilization on gluteraldehyde-activated silicate. Ann. N.Y. Acad. Sci. **542:** 102-106.

Ullah, A.H.J. and Dischinger, H.C. 1992. Complete primary structure determination of *Aspergillus ficuum* phytase by chemical sequencing. FASEB Journal. **6** (1)**:** A63-A63.

Ullah, A.H.J. and Dischinger, H.C. 1993. *Aspergillus ficuum* phytase – complete primary structure elucidation by chemical sequencing. Biochem. Biophys. Res. Commun. **192** (2)**:** 747-753.

Ullah, A.H.J. and Dischinger, H.C. 1995. Aspergillus ficuum phytase active site: involvement of Arg and Trp residues**.** Ann. N.Y. Acad. Sci. **750:** 51-57.

Ullah, A.H.J. and Gibson, D.M. 1987. Extracellular phytase (EC 3.1.3.8) from *Aspergillus ficuum* NRRL 3135: purification and characterization. Prep. Biochem. **17:** 63-91.

Ullah, A.H.J. and Gibson, D.M. 1988. Purification and characterization of phytase from cotyledons of germinating soybeans seeds. Arch. Biochem. **260:** 503-513.

Ullah, A.H.J. and Mullaney, E.J. 1996. Disulfide bonds are necessary for structure and activity in *Aspergillus ficuum* phytase,. Biochem. Biophys. Res. Commun. **227:** 311-317.

Ullah, A.H.J. and Phillippy, B.Q. 1988. Immobilization of *Aspergillus ficuum* phytase – product characterization of the bioreactor. Prep. Biochem. **18** (4)**:** 483-489.

Ullah, A.H.J. and Phillippy, B.Q. 1994. Substrate selectivity in *Aspergillus ficuum* and acid phosphatases using *myo*-inositol phosphates. J. Agric. Food Chem. **42:** 423-425.

Ullah, A.H.J. and Shamsuddin, A.M. 1990. Dose-dependent inhibition of large intestinal cancer by inositol hexaphosphate in F344 rats. Carcinogenesis. **11:** 2219-2222.

Ullah, A.H.J., Cummins, B.J. and Dischinger, H.C. Jr. 1991. Cyclohexanedione modification of arginine at the active site of *Aspergillus ficuum* phytase. Biochem. Biophys. Res. Commun. **178:** 45-53.

Ullah, A.H.J, Mullaney, E.M. and Dischinger, H.C. 1994. The complete primary structure elucidation of *Aspergillus ficuum* (*niger*), pH 6.0, optimum acid phosphatase by Edman degradation. Biochem. Biophys. Res. Commun. **203:** 182-189.

Ullah, A.H.J., Sethumadhaven, K., Mullaney, E.J., Ziegelhoffer, T. and Austin-Phillips, S. 1999. Characterization of recombinant fungal phytase (*phyA*) expressed in tobacco leaves. Biochem. Biophys. Res. Commun. **264:** 201-206.

Ullah, A.H.J., Sethumadhaven, K., Mullaney, E.J., Ziegelhoffer, T. and Austin-Phillips, S. 2002. cloned and expressed fungal *phyA* gene in alfalfa produces a stable phytase. Biochem. Biophys. Res. Commun. **290:** 1343-1348.

Valikhanov, M.N., Abdullaeva, M.M. and Rakhimov, M.M. 1981. Some properties of cotton plant phytase. Biokhimiya. **46:** 100-102.

Van Dijk, P.W.M. 1999. Chymosin and phytate made by genetic engineering. J. Biotechnol. **67:** 77-80.

Van Etten, R.L. 1982. Human prostatic acid phosphatase: a histidine phosphatase. Ann. N.Y. Acad. Sci. **390:** 27-51.

Van Etten, R.L., Davidson, R., Stevis, P.E., MacArthur, H. and Moore, D.L. 1991. Covalent structure, disulfide bonding and identification of reactive surface and active site residues of humanprostatic acid phosphatase. J. Biol. Chem. **266:** 2313-2319.

Van Hartingsveld, W., Vanzeijl, C.M.J. Harteveld, G.M., Gouka, R.J., Suykerbuyk, M.E.G. Luiten, R.G.M., Vanparidon, P.A., Sellen, G.C.M., Veenstra, A.E., Vanorcom, R.F.M. and

vandenhondel, C.A.M.J.J. 1993. Cloning, characterization and over-expression of the phytase-encoding gene (phya) of *Aspergillus niger*. Gene. **127** (1)**:** 87-94.

Vats, P. and Banerjee, U.C. 2002. Studies on the production of phytase by a newly isolated strain of *Aspergillus niger* var teigham obtained from rotten wood-logs. Process Biochem. **38:** 211-217.

Vohra, A. and Satyanarayana, T. 2001. Phytase production by the yeast *Pichia anomala.* Biotechnol. Lett. **23** (7)**:** 551-554.

Vohra, A. and Satyanarayana, T. 2002. Purification and characterization of a thermostable and acid-stable phytase from *Pichia anomala.* World J. Microbiol. Biotechnol. **18:** 687-691.

Vohra, A. and Satyanarayana, T. 2003. Phytases: Microbial Sources, Production, Purification, and Potential Biotechnological Applications. Crit. Rev. Biotechnol. **23** (1)**:** 29-60.

Volfova, O., Dvorakova, J., Hanzlikova, A. and Jandera, A. 1994. Phytase from *Aspergillus niger*. Folia Microbiol. **39** (6)**:** 481-484.

Ward, K.A. 2001. Phosphorous-friendly transgenics. Nat. Biotechnol. **19:** 415-416.

West, S., Schroders, J and Kunz, W. 1990. A multiple-staining procedure for the detection of different DNA fragments on a single blot. Anal. Biochem. **190:** 254-258.

Wodzinski, R.J. and Ullah, A.H.J. 1996. Phytase. Adv. Appl. Microbiol. **42:** 263-302.

Wyss, M., Brugger, R., Kronenberger, A., Rémy, R., Fimbel, R., Oesterhelt, G., Lehmann, M. and van Loon, A.P.G.M. 1999a. Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl. Environ. Microbiol. **65:** 367-373.

Wyss, M., Pasamontes, L., Friedlein, A., Rémy, R., Tessier, M., Kronenberger, A., Middendorf, A., Lehmann, M., Schnoebelen, L., RØthlisberger, U., Kusznir, E., Wahl, G., Mòller, F., Lahm, H.W., Vogel, K. and van Loon, A.P.G.M. 1999b. Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): molecular size, glycosolation pattern, and engineering of proteolytic resistance. Appl. Environ. Microbiol. **65:** 359-366.

Yamada, K., Minoda, Y., Hidaka, Y., Matuo, H. and Kobayashi, M. 1968. Phytase from *Aspergillus terreus* I production, purification and some general properties of the enzyme. Agric. Biol. Chem. **32:** 1275-1282.

Yamamoto, S., Minoda, Y. and Yamada, K. 1972. Chemical and physiochemical properties of phytase from *Aspergillus terreus*. Agric. Biol. Chem. **36:** 2097-2103.

Yang, W.J., Matsuda, Y., Sano, S., Masutani, H and Nakagawa, H. 1991a. Purification and characterization of phytase from rat intestinal mucosa. Biochim. Biophys. Acta. **1075:** 75-82.

Yang, W.J., Matsuda, Y., Inomata, M and Nakagawa, H. 1991b. Development and dietary induction of 90 kDa subunit of rat intestinal phytase. Biochim. Biophys. Acta. **1075:** 83-87.

Yanke, L.J., Bae, H.D., Selinger, L.B. and Cheng, K.J. 1998. Phytase activity of anaerobic ruminal bacteria. Microbiology-UK. **144:** 1565-1573.

Yanke, L.J., Selinger, L.B. and Cheng, K.J. 1999. Phytase activity of *Selenomonas ruminantium:* a preliminary characterization. Lett. Appl. Microbiol. **29** (1)**:** 20-25.

Yenigun, B. and Guvenilir, Y. 2003. Partial purification and kinetic characterization of acid phosphatases from garlic seedling. Appl. Biochem. Biotechnol. **107** (1-3)**:** 677-688.

Yi, Z. Kornegay, E.T., Ravindran, V. and Denbow, D.M. 1996. Improving phytate phosphorous availability in corn and soybean meal for broilers using microbial phytase and calculation of phosphorous equivalency values for phytase. Poult. Sci. **75:** 240-249.

Yi, Z., Konegay. E.T. and Denbow, D.M. 1997. Supplemental microbial phytase improves zinc utilization in broilers. Poult. Sci. **75:** 540-546.

Yoon, S.J., Choi. Y.J., Min, H.K., Cho, K.K., Kim, J.W., Lee, S.C. and Jung, Y.H. 1996. Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. 4, and enzymatic properties of phytase enzyme. Enz. Microbial Technol. **18** (6)**:** 449-454.

Zamudio, M., González, A. and Medina, J.A. 2001. *Lactobacillus plantarum* phytase activity is due to non-specific acid phosphatase. Lett. Appl. Microbiol. **32** (3)**:** 181.

Zang, Z.B., Kornegay, E.T., Radcliffe, J.S., Wilson, J.H. and Veit, H.P. 2000. Comparison of phytase from genetically engineerd *Aspergillus* and canola in weanling pig diets. J. Anim. Sci. **78:** 2868-2878.

Zyla, K. 1992. Mold phytases and their application in the food industry. World. J. Microbiol. Biotechnol. **8:** 467-472.

Zyla, K. 1993. The role of acid phosphatase activity during enzymic dephosphorylation of phytates by *Aspergillus niger* phytase. World J. Microbiol. Biotechnol. **9:**117-119.

3. RESEARCH RESULTS

Identification and Characterisation of a Phytase from the Basidiomycete *Cryptococcus laurentii* Abo 510

Written in a style suitable for submission to
World Journal of Microbiology and Biotechnology

Identification and Characterisation of a Phytase from the Basidiomycete *Cryptococcus laurentii* Abo 510

Jason van Staden, W.H. van Zyl and M. Viljoen-Bloom

Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa

Summary

Phytase is a hydrolytic enzyme that dephosphorylates phytic acid (*myo*-inositol hexakisdihydrogen-phosphate), a major storage form of phosphorus in feedstuff of plant origin. Phytase thus improves the bioavailability of phytate phosphorus and reduces phosphorus pollution from animal waste. In this study, several *Cryptococcus* strains were screened for phytase activity. The *C. laurentii* Abo 510 strain showed the highest potential for phytase production and was selected for further characterisation. The cell wall-associated enzyme displayed an optimal temperature of 62°C and an optimal pH of 5.0. The phytase enzyme was thermo-stable at 70°C for up to 3 hours with a loss of only 40% of its original activity. The phytase displayed broad substrate specificity, including ATP, D-glucose-6-phosphate, D-fructose-1,6-diphosphate, *p*-nitrophenyl phosphate, but its preferred substrate was phytic acid (K_m of 21 μM). Inorganic phosphate at a concentration of 0.5 mM, or 5 mM phytic acid completely inhibited the phytase activity. Phytase activity was moderately inhibited in the presence of certain mineral ions, i.e. Hg^{2+}, Zn^{2+}, Cd^{2+} and Ca^{2+}. The enzymatic characteristics (especially the thermo-stability and pH range) of the phytase from *C. laurentii* Abo 510 suggest that the enzyme has the potential to be used as a feedstuff additive.

Introduction

The application of phytate-hydrolysing enzymes in the poultry and swine industry has strongly developed over the past few years (Mullaney *et al.*, 2000). The reason for this is that 60-90% of phosphorus from feedstuff of plant origin (cereal grains, oilseeds and legumes) is present in the form of phytic acid or phytate (Al-Asheh and Duvnjak, 1995; Mitchell *et al.*, 1997; Nelson, 1967; Reddy *et al.*, 1982; Ullah and Gibson, 1987). Phytate, the salt form of phytic acid, is indigestible to monogastric animals such as poultry, pigs and fish since these animals lack the required gastrointestinal tract enzyme(s) for the dephosphorylation of the phytate complex (Common, 1989; Cromwell *et al.*, 1995). To meet the dietary requirements

of these animals, producers therefore are required to add expensive supplementary phosphorus to the feedstuff. Moreover, phytic acid is capable of chelating multivalent cations, rendering these minerals biologically unavailable to the animal (Erdman, 1979; Harland and Morris, 1995) and making phytate an antinutritional factor (ANF).

Phytase was employed as a feed additive to overcome these problems and was shown to be a successful substitute for the supplementation of inorganic phosphorus (Cromwell *et al.*, 1995; Liu *et al.*, 1997; O'Quinn *et al.*, 1997). Phytase (*myo*-inositol hexakisphosphate phosphohydrolases) belongs to the family of histidine acid phosphatases and is effective in the hydrolysis of phytate to produce *myo*-inositol and inorganic phosphate (Jareonkitmongkol *et al.*, 1997; Kim *et al.*, 1998; Mitchell *et al.*, 1997; Ullah, 1988; Ullah and Phillippy, 1994; van Etten *et al.*, 1991). This pre-treatment of animal feed also decreased the phosphorus pollution caused by animal manure by up to 50% (Han *et al.*, 1997; Kornegay and Qian, 1996; Lei *et al.*, 1993) and was advantageous to the animal by increasing phosphorus and mineral uptake (Broz *et al.*, 1994; Mullaney *et al.*, 2000; Pen *et al.*, 1993; Walsh *et al.*, 1994). Some phytases are not capable of hydrolysing phytate completely and may need an alternative or additional enzyme to accelerate the process. One of these enzymes is acid phosphatase, which also belongs to the family of histidine acid phosphatases (Zyla, 1993). Although acid phosphatase is not capable of hydrolysing phytate, it assists the phytase in accelerating the release of the phosphate from lower *myo*-inositol phosphate structures.

Current available commercial phytases are expensive and there are some limitations (especially thermo-stability and acidic pH stability) that hamper their use as a feed additive. This resulted in the search for more attractive phytases (Kerovuo *et al.*, 1998; Kim *et al.*, 1998; Mitchell *et al.*, 1997; Wodzinski and Ullah, 1996). Phytase is produced by plants (Gibson and Ullah, 1988; Hübel and Beck, 1996), a few animal tissues (van der Kaay and van Haastert, 1995) and microorganisms (Greiner *et al.*, 1993; Kim *et al.*, 1999; Lambrechts *et al.*, 1993; Powar and Jagannathan, 1982; Ullah and Gibson, 1987; Wyss *et al.*, 1999). Phytase is broadly dispersed among fungi, in particular filamentous soil fungi such as *Aspergillus* spp. (Wodzinski and Ullah, 1996).

Various yeasts such as *Schwanniomyces castellii* (Segueilha *et al.*, 1992), *Arxula adeninivorans* (Sano *et al.*, 1999), *Pichia spartinae* and *Pichia rhodanensis* (Nakamura *et al.*, 2000) are also capable of producing extracellular phytases. Intracellular phytases were isolated from *Saccharomyces cerevisiae* (Nakamura *et al.*, 2000) and *Pichia anomala* (Vohra

and Satyanarayana, 2001), while *Rhodotorula gracilis* (Bindu *et al.*, 1998) and a *P. anomala* strain (Vohra and Satyanarayana, 2002) exhibited cell wall-associated phytase activity.

Cryptococcus is an anamorphic basidiomycetous, encapsulated yeast (Yarrow, 1998). A few *Cryptococcus* strains have been shown to produce other enzymes of economic importance, such as laccase (*C. laurentii*, *Cryptococcus curvatus* and *Cryptococcus albidus*) (Ikeda *et al.*, 2002), α-amylase (*Cryptococcus* sp. S-2) (Iefuji *et al.*, 1996a), β-xylosidase (*Cryptococcus podzolicus*) (Shubakov, 2000) and acid xylanase (*Cryptococcus* sp. S-2 and *Cryptococcus* sp. X-1) (Iefuji *et al.*, 1996b; Sripo *et al.*, 1997).

The aim of this study was to screen for phytase production among soil borne *cryptococci* and to evaluate the prospect of utilising a strain with potential for industrial applications. A novel cell wall-associated phytase from the yeast *Cryptococcus laurentii* Abo 510 was identified and characterised, the first description of a phytase from *Cryptococci*.

Materials and methods

Chemicals

Phytic acid (dodecasodium salt), *p*-nitrophenyl phosphate (*p*-NPP) and calcium phytate was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were standard or analytical grade unless otherwise specified.

Microorganisms and cultivation

The 11 *Cryptococcus* strains (nine *C. laurentii* strains and two *C. podzolicus* strains) were isolated from soil in the Western Cape region, South Africa, and strains were identified through their morphological characteristics and molecular sequences. The cultures were maintained by periodic transfer to Difco-Bacto malt extract agar plates and storage at 4°C.

Screening for phytase production

Two methods were used to screen for phytase activity. The first method was based on a differential agar media (Gargova *et al.*, 1997; Howson and Davis, 1983; Shieh and Ware, 1968) where phytase activity was indicated by the disappearance of precipitated calcium phytate, i.e. zones of clearing on the agar media indicated positive results for a microorganism

producing phytase. The *Cryptococcus* strains were plated onto phytase screening medium (PSM) agar containing 15 g/l D-glucose, 5g/l calcium phytate, 5 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 0.01 g/l $FeSO_4.7H_2O$, 0.01 g/l $MnSO_4.4H_2O$ and 15 g/l Difco-Bacto agar. The pH was adjusted to 5.5 with 1 M KOH before autoclaving at 121°C for 15 min. The inoculated PSM plates were incubated at 30°C for 2 to 7 days, depending on the growth and development of zones by the cultures. This was followed by a two-step counterstaining procedure that eliminated false positive results displayed by acid producing microorganisms on the agar media (Bae *et al.*, 1999). The colonies were washed from the agar surface with distilled water and the agar plate was then flooded with a 2% (w/v) aqueous cobalt chloride solution. This solution was replaced with a freshly prepared solution containing 6.25% (w/v) aqueous ammonium molybdate and 0.42% (w/v) ammonium vanadate. The solutions were removed after 5 min incubation at room temperature and the agar plates were examined for zones of clearing.

The strains that showed zones of clearing after the counterstaining method were subjected to a second screening to confirm the production of a phytase enzyme. The strains were inoculated in different media including PSM broth (substituting calcium phytate for equal amounts of sodium phytate), PSM broth without calcium phytate or sodium phytate, and a liquid starch medium that contained 20 g/l corn starch, 10 g/l soybean flour, 20 g/l glucose, 5 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 0.1 g/l $FeSO_4.7H_2O$ and the pH adjusted with 1 M HCl to 6.0 before autoclaving. Cultivation was done in 1-litre Erlenmeyer flasks containing 100 ml of the liquid starch medium or in 250 ml Erlenmeyer flasks containing 50 ml of the PSM broth. All the media were inoculated with 1×10^7 cells/ml and incubated at 30°C on an orbital shaker (150 rev/min) (Gargova *et al.*, 1997; Ullah and Gibson, 1987). Samples (2 ml) were taken daily and centrifuged at 5 000 x *g* for 10 min. The supernatant was removed and filtered through a 0.22-µm-syringe filter and the crude filtrate was used to determine extracellular phytase activity. The pellet was washed three times with distilled water and the wet cells were weighed. The wet cells were resuspended in 925 µl of 50 mM sodium acetate buffer (pH 5.0) and used to determine any cell-associated phytase activity.

Optimised media for phytase production

The growth media was optimised for maximal production of phytase by *C. laurentii* Abo 510. The strain was grown in a modified liquid glucose-yeast extract broth described by Shieh and

Ware (1968) containing 10 g/l glucose, 5 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 0.01 g/l $FeSO_4.7H_2O$, 0.01 g/l $MnSO_4.4H_2O$, 1.25 g/l yeast extract and pH adjusted to 6 with 1 M HCl. A pre-culture was made in Yeast Extract Peptone Dextrose (YEPD) broth and incubated on a rotary wheel (150 rev/min) at 30°C for 24 h. The culture was harvested by centrifuging at 5 000 x *g* for 10 min at 4°C and the pellet was washed twice with sterile distilled water. The pellet was resuspended in 1 ml of a 50 mM sodium acetate buffer and used to inoculate 50 ml glucose-yeast extract broth in a 100 ml Erlenmeyer flask to 1 x 10^7 cells/ml. The culture was incubated on an orbital shaker (150 rev/min) at 30°C for 7 days. After each day, 2 ml samples of the cell suspension were taken in duplicate and the cells were harvested by centrifuging at 5 000 x *g* for 10 min. The pellet was collected, weighed and assayed for phytase activity.

To evaluate the effect that various carbon sources might have on the phytase activity, the cells were cultivated in the modified PSM broth (without phytate) described above and supplemented with different carbon sources, i.e. 1% (w/v) glucose, maltose, fructose or sucrose (Gautam *et al.*, 2002). These carbon sources are soluble carbohydrates normally found in wheat flour (Gobbetti, 1998). Different culture media were also tested for growth and phytase production, i.e. 1% (w/v) YEPD broth, potato dextrose broth (PDB), malt extract and molasses (Bindu *et al.*, 1998).

Enzyme assays

Phytase activity was evaluated by examining the release of inorganic phosphorus from phytic acid colorimetrically using a modified method of Heinonen and Lahti (1981). Weighed wet cells were incubated at 62°C for varying time intervals (1, 2, 5, 10, 15, 20 min) to determine the reaction time with 10 mM sodium phytate and 50 mM sodium acetate buffer (pH 5) in a total volume of 1 ml (Ullah and Gibson, 1987). The liberated inorganic phosphorus was complexed by the addition of 2 ml of a freshly prepared AAM solution (acetone, 10 mM ammonium molybdate and 5 N H_2SO_4 mixed in a ratio of 2:1:1, v/v). The ammonium molybdate reaction with the phosphate was stopped through the addition of 100 µl 1 M citric acid and the phosphomolybdate concentration (yellow colour) was measured at 355 nm with a Spectronics (Genesis 5) spectrophotometer. One international unit (IU) of phytase activity was expressed as 1 µM of inorganic orthophosphate liberated per minute per millilitre or per gram wet cells (IU/ml or IU/g wet cells). To evaluate the *C. laurentii* Abo 510 strain's ability to produce an acid phosphatase, the sodium phytate substrate was replaced by 10 mM *p*-NNP

and the activity was determined at pH 2.5 and pH 4.5 at 82°C (Ullah and Cummins, 1988). The acid phosphatase activity was defined as 1 µM *p*-NPP released per min per millilitre or gram wet cells (IU/ml or IU/g wet cells).

The total protein concentration of the cell suspension was measured by using the Bradford method with bovine serum albumin as standard (Bradford, 1976).

Localisation of the phytase enzyme

Three different methods were evaluated to determine the extracellular and cellular phytase activity.

i) Permeabilisation: The cells were grown for 72 h in glucose-yeast extract medium, harvested by centrifugation at 5 000 x *g* for 5 min and washed twice with 1 ml 50 mM sodium acetate buffer (pH 5.0). The supernatant was used to determine the extracellular phytase activity. Fifty mg wet cells were suspended in 1 ml of 50 mM sodium acetate buffer (pH 5.0) containing a permeabilising agent, i.e. 1 or 10% (v/v) Triton X-100, 0.1 or 1% (v/v) Tween 80, 0.1 or 1% (w/v) SDS, 1 or 10% (w/v) EDTA, 50% (v/v) ethanol or 0.85% (w/v) NaCl, and incubated for 2 hours at room temperature (Bindu *et al.*, 1998). The cells were washed twice with the sodium acetate buffer and the pellet resuspended in the same buffer and used for measuring phytase activity.

ii) Freeze-thaw: 50 mg wet cells were resuspended in a 50 mM sodium acetate buffer, frozen in ethanol at -20°C for 15 min and then thawed for 10 min at room temperature (Decleirie *et al.*, 1987). The freezing and thawing were repeated for 1, 5, 10, 15 or 20 cycles after which the permeabilised cells were washed twice with the 50 mM sodium acetate buffer and the phytase activity measured as mentioned above.

iii) Homogenisation: 50 mg wet cells, suspended in 500 µl sodium acetate buffer with 1% Tween 80, were vortexed for 5 min with 300 mg glass beads (212-300 microns) in a 2 ml eppendorf (Bindu *et al.*, 1998). The homogenate was then centrifuged at 4°C for 5 min at 5 000 x *g* and the supernatant used for the reaction assay.

Effect of pH and temperature on phytase activity

The pH optimum of the phytase and acid phosphatase activity was determined at 52°C. A broad pH range of pH 2.0 to pH 9.0 was tested using different buffers at 50 mM to obtain the required pH: glycine-HCl (pH 2.0 – 3.0), sodium acetate (pH 3.0 – 5.0), 2-(N-Morpholino)-

ethanesulfonic acid (MES) (pH 5.0 – 7.0) and Tris-HCl (pH 7.0 – 9.0). The addition of the cell suspension only slightly effected the pH values (Wyss *et al.*, 1999).

To determine the optimum temperature for phytase and acid phosphatase activity, the enzyme reactions were carried out between 24°C to 90°C at a pH of 5.0 for 5 min. The reaction assay mixtures and enzyme extracts were equilibrated for 6 min at each temperature before the enzyme assay (Howson and Davis, 1983).

The thermal stability of the enzyme was determined by exposing the crude enzyme extract to various temperatures (4°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C) for 2 to 180 min. The enzyme extract was cooled to room temperature and the residual activity was assayed at 62°C and expressed as a percentage of the phytase activity of an untreated sample (Bogar *et al.*, 2003; Fujita *et al.*, 2003; Vohra and Satyanarayana, 2002).

Different solutions were evaluated for their ability to stabilise the enzyme during exposure to high temperatures (70°C, 80°C and 90°C). These included 1% and 5% (w/v) polyethylene glycol (PEG) 6000, $CaCl_2.2H_2O$, glycerol, sorbitol and mannitol (Vohra and Satyanarayana, 2002).

Substrate specificity and substrate affinity

The substrate specificity of the phytase was determined by replacing the phytic acid substrate in the reaction assay mixture with different phosphate compounds i.e. Adenosine triphosphate (ATP), 4-NPP, D-glucose-6-phosphate, α-D-glucose-1-phosphate, di-sodium phenyl phosphate dihydrate, *p*-NNP di (Tris) salt and D-fructose-1,6-diphosphate) at a concentration of 10 mM (Vohra and Satyanarayana, 2002). The activity of each phosphate compound was determined as described above. One unit of activity catalysed the liberation of 1 μM of inorganic phosphate per minute per gram wet cells.

The K_m values were calculated by using different concentrations of phytic acid (or other phosphate compounds). The assay mixture contained a series of concentrations (0.001, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 mM) in 50 mM sodium acetate buffer (pH 5.0) and each reaction was stopped after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or 30 min of incubation (Wyss *et al.*, 1999). The enzyme activity was determined as mentioned above.

Effect of phosphorus and phytic acid concentration on the phytase activity

To determine the effect that inorganic phosphate has on the phytase activity of the *C. laurentii* Abo 510 strain, KH_2PO_4 was added to the reaction mixture at final concentrations ranging from 0.005 mM to 10 mM (Vohra and Satyanarayana, 2002).

The inhibitory effect of phytic acid on the phytase activity was evaluated by adding phytic acid to a final concentration of 0.1 mM to 10 mM to the reaction mixture.

Effect of metal ions and inhibitors on phytase activity

Cells were harvested from 2 ml samples taken from glucose-yeast extract broth by centrifugation (4 000 x *g*, 5 min at 4°C) and washed twice with distilled water. The metal ions and inhibitors were pre-incubated with the enzyme extract for 15 min at room temperature before the addition of substrates. The different metal ions (concentrations of 1 mM or 5 mM) included: K^+, Ca^{2+}, Co^{2+}, Cd^{2+}, Li^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ba^{2+}, Zn^{2+}, Ni^{2+} and Hg^{2+} (added as chloride salts), Fe^{2+} (as $FeSO_4$) and Ag^+ (as $AgNO_3$). Potential inhibitors, EDTA, SDS and phenylmethanesulphonyl fluoride (PMSF), were added at a concentration of 1 mM to the reaction mixture to determine their effect on the phytase activity (Fujita *et al.*, 2003; Vohra and Satyanarayana, 2002).

Results

Screening for phytase production by *Cryptococcus strains*

Among the 11 *Cryptococcus* strains evaluated for phytase production, only *C. laurentii* Abo 510 showed phytase activity (4.55 IU/g wet cells) with a specific phytase activity of 29.74 IU/mg proteins. However, all the strains grew on the PSM agar plates and clear zones appeared on the differential plates. The counterstaining method was used to eliminate any false positive results due to acid production by these strains and only *C. laurentii* Abo 510 was identified as a positive phytase producer (Figure 1).

Medium optimisation for phytase production in *C. laurentii* Abo 510

Five different media were tested for optimal phytase production and the PSM (without phytate) was found to produce maximal phytase activity (Table 1). Although the cell growth in the PSM (without phytate) was not as high as in the other media, it produced the highest

phytase activity and was selected for further experimental purposes including temperature and pH optima and the effect of inhibitory agents on the phytase enzyme.

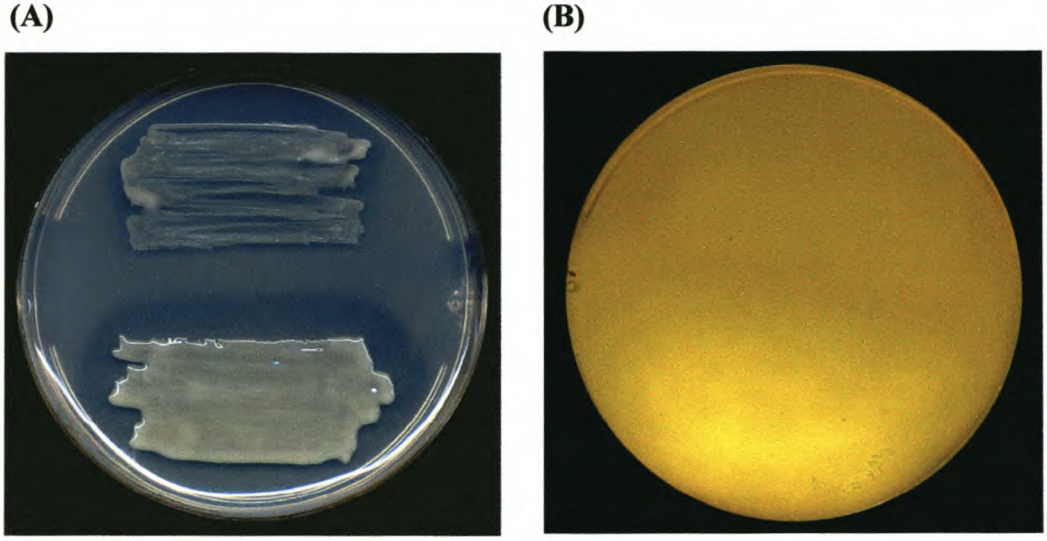

Figure 1. Screening for phytase production on a PSM agar plate. (A) PSM plate before counterstaining showing the growth and zones of clearing with *C. podzolicus* Abo 511 (top) and *C. laurentii* Abo 510 (bottom). (B) Counterstaining showed that *C. laurentii* Abo 510 is a positive phytase producer (zone of clearing stayed intact).

Table 1. Media for maximal phytase activity and growth, and the effect of different carbon sources on the phytase activity of *C. laurentii* Abo 510.

Culture Media	**[a]Growth (Cell count)**	**Phytase activity (IU/g wet cells)**
YEPD	$5.44 \times 10^7 \pm 0.14$ [b]	3.71 ± 0.10 [b]
Patato Dextrose Broth (PDB)	$8.50 \times 10^6 \pm 0.28$	4.05 ± 0.07
Malt Extract	$6.33 \times 10^7 \pm 0.25$	3.84 ± 0.21
Molasses	$4.55 \times 10^7 \pm 0.57$	2.00 ± 0.21
PSM (without phytate), 1% Glucose	$2.18 \times 10^7 \pm 0.32$	4.55 ± 0.02
[c]Carbon sources		
1% Maltose	$1.40 \times 10^7 \pm 0.35$	3.29 ± 0.19
1% Fructose	$1.69 \times 10^7 \pm 0.23$	3.07 ± 0.06
1% Sucrose	$2.32 \times 10^7 \pm 0.52$	2.75 ± 0.18

[a] Inoculated with $\pm 1 \times 10^6$ cells/ml; incubated for 7 days at 30°C

[b] The values are the mean and standard deviations of three repeats

[c] PSM broth (without phytate) with alternative carbon sources

Phytase production was evaluated by replacing D-glucose in the culture medium (PSM without phytate) with different carbon sources at 1% (w/v). Table 1 indicates that none of these carbon sources increased the production of phytase. Maximal phytase production was obtained after 72 hours (Figure 2) in PSM (without phytate) containing 1% glucose. This coincided with depletion of the glucose and the culture entering stationary phase.

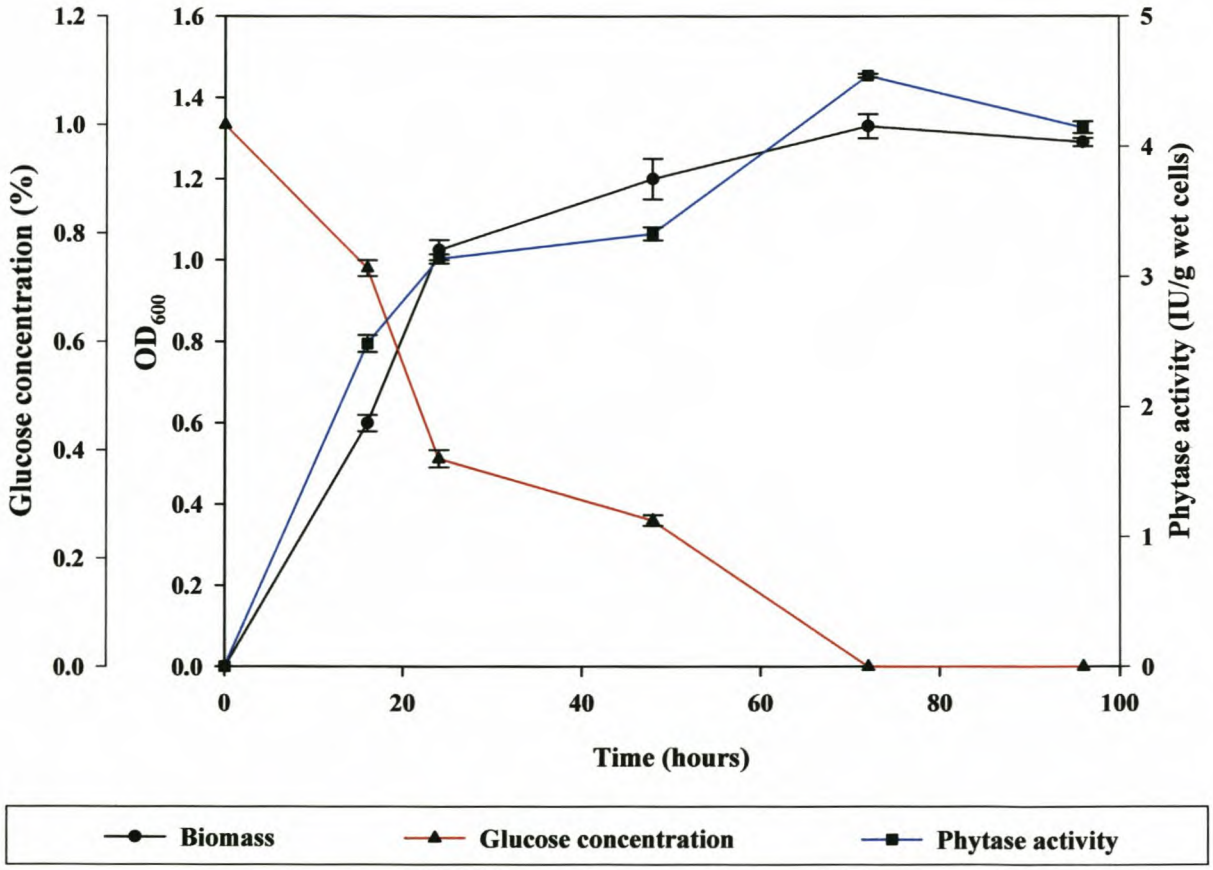

Figure 2. Evaluation of the interaction between glucose concentration, biomass and phytase activity in *C. laurentii* Abo 510 grown in PSM (without phytate) (error bars indicate standard deviation of three repeats).

Permeabilisation and localisation of the phytase enzyme

Phytase activity in *C. laurentii* Abo 510 was associated with the whole cells and there was no phytase activity found in the supernatant or the culture medium (Table 2). None of the permeabilisation agents resulted in higher phytase activity than found in the non-permeabilised cells; instead, these agents seem to inhibit the phytase activity. Tween 80 was the only detergent to yield an intracellular fraction with phytase activity, whereas SDS almost

completely inhibited the phytase activity associated with the cells. Homogenisation only produced a low level of intracellular phytase activity relative to the phytase activity obtained from whole cells.

The freeze-thaw method was successful in producing optimal phytase activity of whole cells from *R. gracilis* (almost double the phytase activity of non-permeabilised cells) (Bindu *et al.*, 1998). However, for *C. laurentii* Abo 510, the freeze-thaw method actually decreased the phytase activity from the whole cells. It is possible that he long incubation times could have destroyed the enzyme.

Table 2. Determining the localisation of the phytase enzyme from *C. laurentii* Abo 510 through permeabilisation of the cells with detergents and other permeabilising methods.

		Phytase activity	
Permeabilising agent	**Concentration (%)**	**Intracellular[a] (IU/ml)**	**Cell associated (IU/g wet cells)**
Non-permeabilised cells (control)		—	4.55 ±0.02[a]
Triton X-100	1	—	3.71 ±0.09
	10	—	3.68 ±0.04
Tween 80	0.1	0.06 ±0.03	2.94 ±0.26
	1	0.28 ±0.01	2.52 ±0.05
SDS	0.1	—	0.62 ±0.02
	1	—	0.08 ±0.01
EDTA	1	—	2.44 ±0.18
	10	—	2.11 ±0.06
ETOH	50	—	2.69 ±0.26
FSO		—	2.80 ±0.08
Freeze-thaw (5 Cycles)		—	2.58 ±0.21
Homogenisation		0.55 ±0.01	—

[a] The values are the mean and standard deviations of three repeats

Effect of pH and temperature on phytase activity

The *C. laurentii* Abo 510 phytase was active between pH 2.5 and pH 7.5 with an optimal phytase activity at pH 5.0 (Figure 3). The acid phosphatase enzyme showed the same optimal activity between pH 2.0 and pH 5.0 (data not shown).

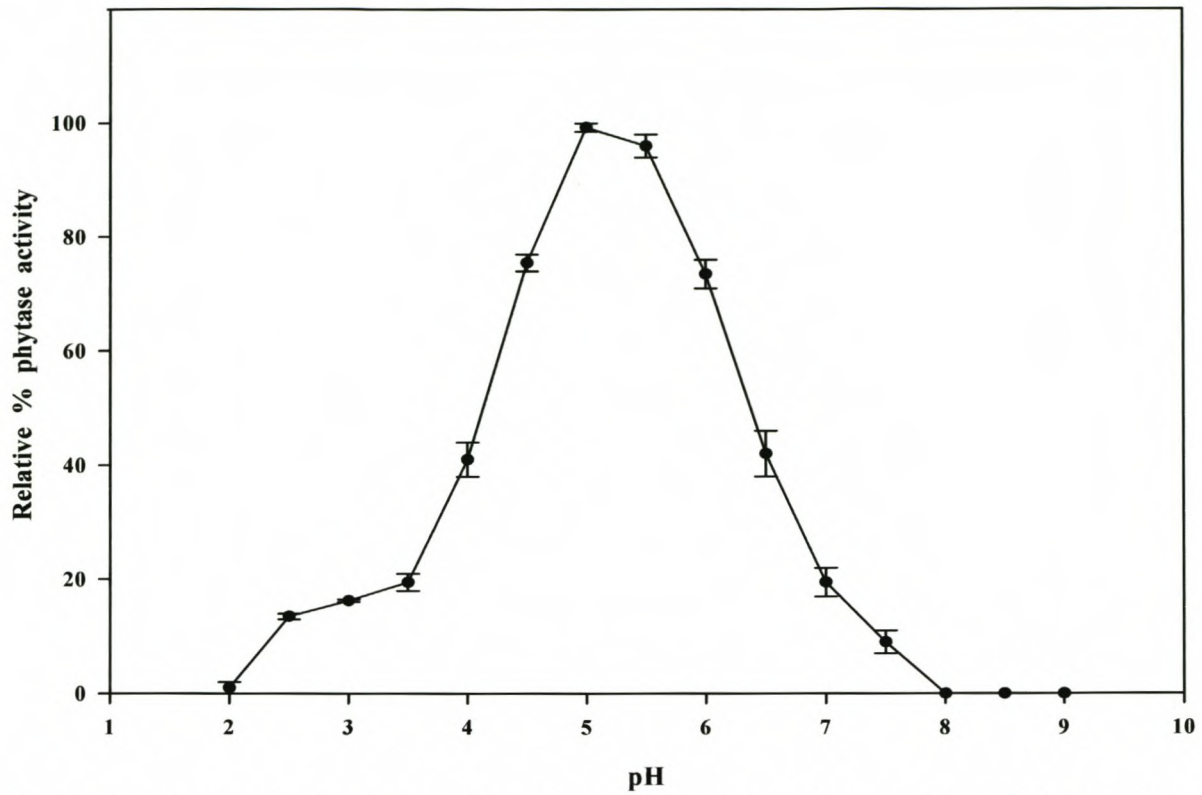

Figure 3. Effect of pH on the hydrolysis of sodium phytate by *C. laurentii* Abo 510 (error bars indicate standard deviation of three repeats).

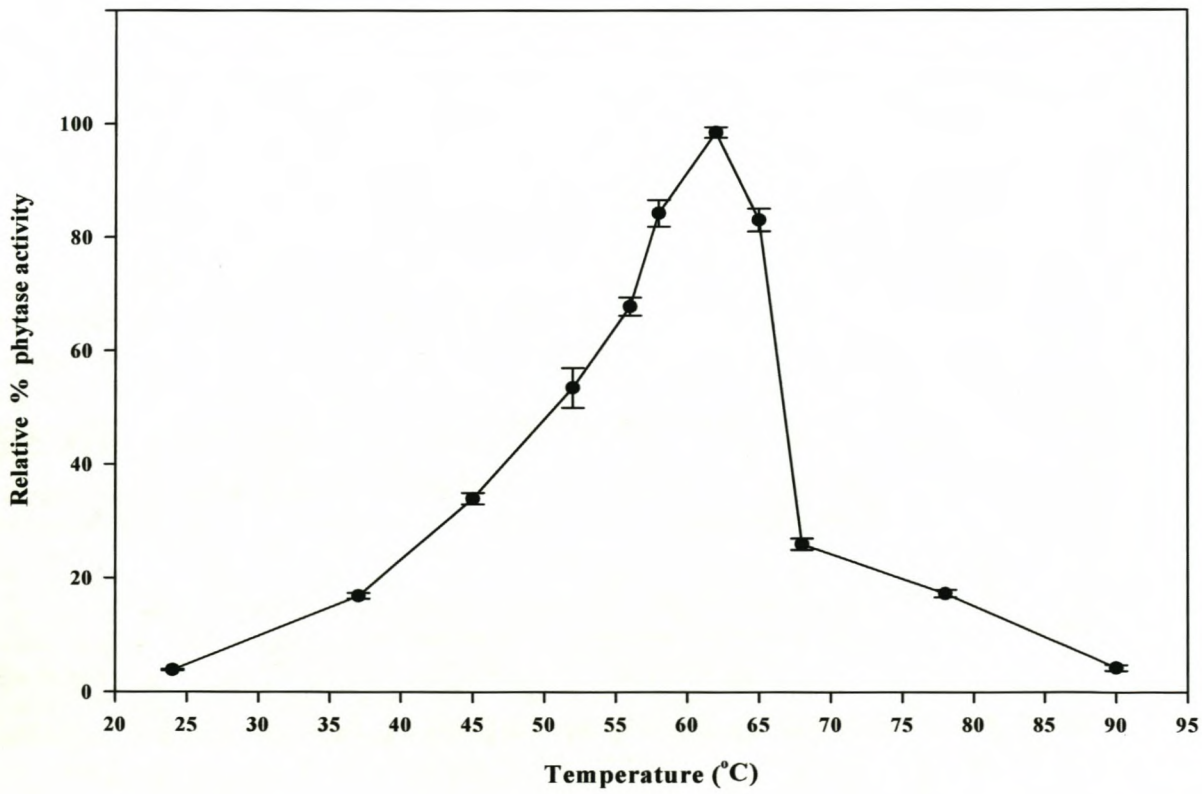

Figure 4. Effect of temperature on the hydrolysis of sodium phytate by *C. laurentii* Abo 510 (error bars indicate standard deviation of three repeats).

To determine its optimum temperature, phytase activity was assayed at various temperatures ranging from 24°C to 90°C. The optimum temperature for maximal phytase activity was found to be 62°C (Figure 4). The optimal temperature for acid phosphatase was 82°C (data not shown).

The residual phytase activity were stable at temperatures below 60°C (Figure 5). A loss of only 40% of the original activity was observed after exposure to 70°C for 180 min, but a complete loss in phytase activity was observed when the enzyme was exposed to 90°C for 5 min or to 80°C for 60 min.

The addition of different concentrations (1% or 5%) of solvents to the reaction assay mixture did not stabilise the phytase activity during the exposure to very high temperatures (above 70°C). However, the addition of 5% PEG 6000 helped to retain 22% of the phytase's original activity after exposure to 90°C for 15 min (data no shown).

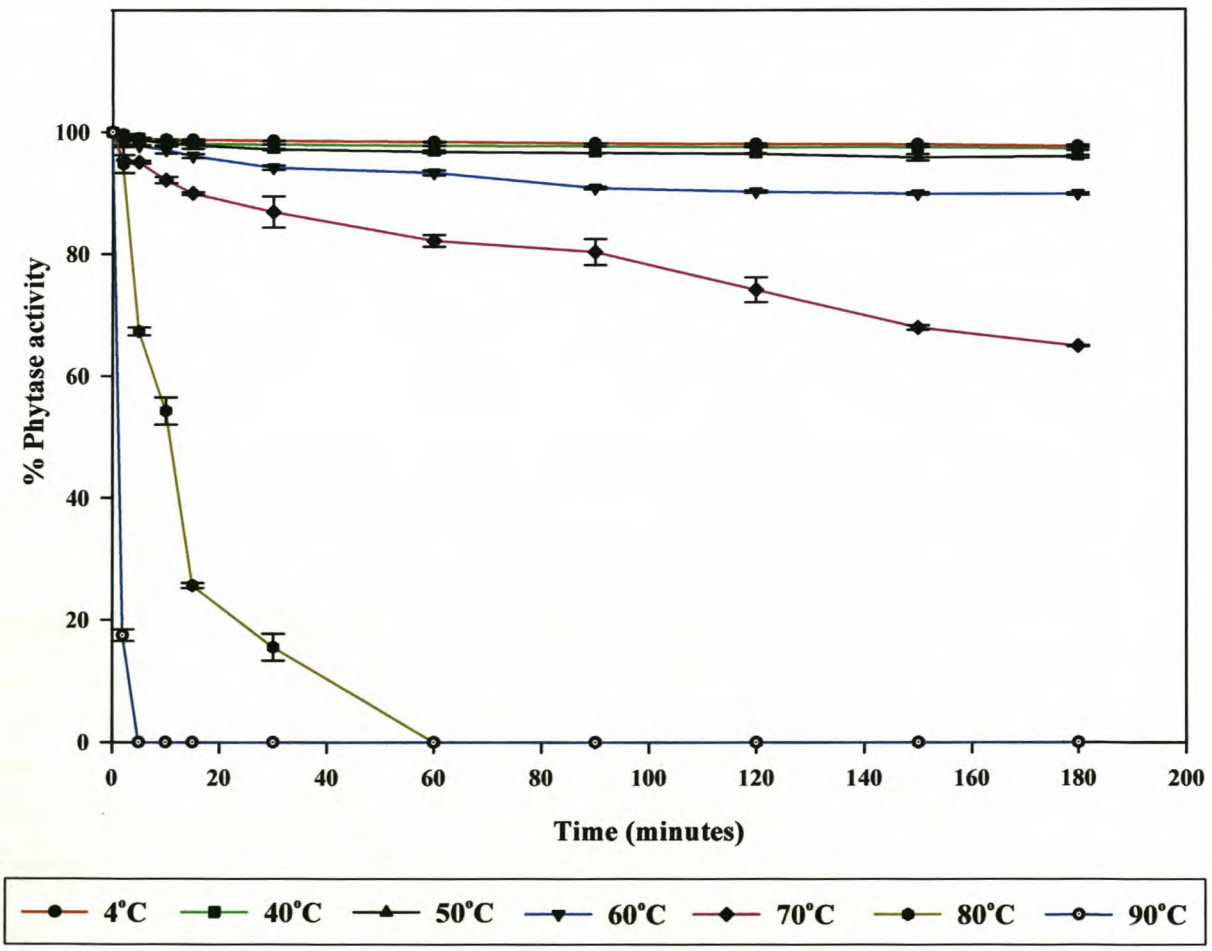

Figure 5. Thermo-stability of the *C. laurentii* Abo 510 phytase with exposure to different temperatures and time periods (error bars indicate standard deviation of three repeats).

Substrate specificity

Table 3 shows the phytase specificity for different substrates and the relative activity for each substrate. Although sodium phytate was not the compound with the highest relative rate of hydrolysis, the *C. laurentii* Abo 510 phytase had the lowest K_m value for sodium phytate.

Table 3. Substrate specificity of the *C. laurentii* Abo 510 phytase.

Substrates	Relative activity (%)	K_m (mM)
Sodium phytate	100[a]	0.02 ±0.01[b]
Calcium phytate	93.76 ±8.55[b]	0.03 ±0.01
4-Nitrophenyl phosphate	152.95 ±4.59	0.52 ±0.01
p-Nitrophenyl phosphate di (tris) salt	154.42 ±5.05	0.23 ±0.01
di-Sodium phenyl phosphate dihydrate	111.85 ±4.12	0.17 ±0.01
ATP	76.67 ±7.49	0.07 ±0.01
α-D-Glucose-1-phosphate	76.72 ±4.48	0.28 ±0.03
D-Glucose-6-phosphate	73.75 ±4.20	0.27 ±0.02
D-Fructose-1,6-diphosphate	202.19 ±6.34	0.14 ±0.02

[a] Hydrolysis rate of sodium phytate was taken as 100%

[b] The values are the mean and standard deviations of three repeats

Effect of phosphorus and phytic acid concentrations on the phytase activity

The addition of phosphate (KH_2PO_4) to the reaction assay mixture at different concentrations showed that the phytase activity was completely inhibited at a concentration of 0.5 mM or higher (Figure 6). Phytic acid concentrations of 5 mM or higher in the reaction assay mixture also completely inhibited the phytase activity from *C. laurentii* Abo 510 (Figure 7).

Effect of metal ions and inhibitors

Different metal ions were included in the standard phytase reaction assay in order to determine their effect on the *C. laurentii* Abo 510 phytase activity (Table 4). The cations Li^+, Na^+, K^+ and Mg^{2+} did not significantly affect the phytase activity when added at a concentration of 1 or 5 mM. Hg^{2+}, Zn^{2+} and Cd^{2+} showed a loss in phytase activity of more

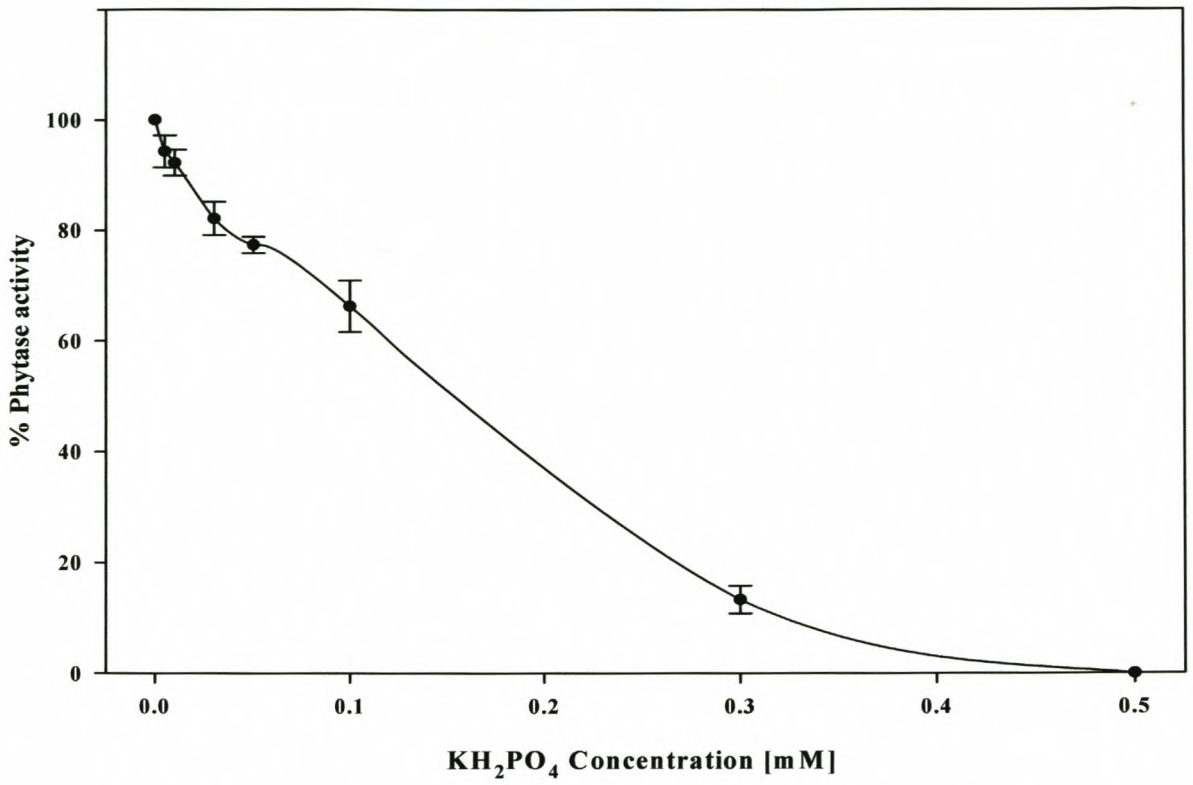

Figure 6. Effect of phosphorus concentration on the *C. laurentii* Abo 510 phytase activity (error bars indicate standard deviation of three repeats).

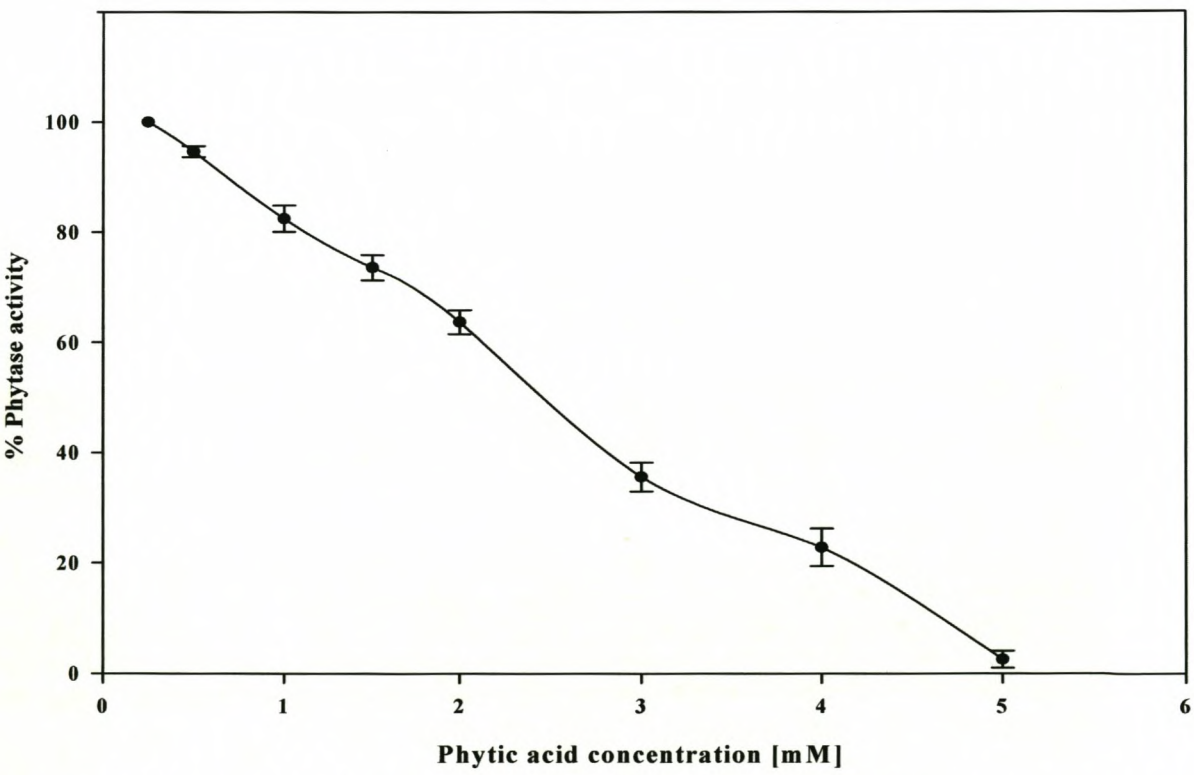

Figure 7. Effect of phytic acid concentration on the *C. laurentii* Abo 510 phytase activity (error bars indicate standard deviation of three repeats).

Table 4. Effect of metal ions and inhibitors on the *C. laurentii* Abo 510 phytase activity.

Effectors	Concentration (mM)	[a] Relative activity (%)
Mineral ions (salts)		
$HgCl_2$	1mM	36.46 ±1.56[b]
	5mM	31.17 ±2.36
$ZnCl_2$	1mM	35.46 ±2.14
	5mM	27.34 ±1.08
$CdCl_2$	1mM	40.54 ±0.59
	5mM	42.03 ±2.60
$CoCl_2$	1mM	83.17 ±1.09
	5mM	76.08 ±2.21
LiCl	1mM	99.33 ±0.29
	5mM	95.79 ±1.56
NaCl	1mM	94.36 ±0.37
	5mM	91.23 ±2.52
KCl	1mM	94.50 ±1.01
	5mM	96.03 ±0.57
$MnCl_2.4H_2O$	1mM	65.38 ±2.07
	5mM	36.01 ±0.21
$MgCl_2.6H_2O$	1mM	98.05 ±0.14
	5mM	95.01 ±0.86
$BaCl_2.2H_2O$	1mM	96.07 ±0.07
	5mM	71.95 ±1.33
$FeSO_4.7H_2O$	1mM	96.52 ±0.49
	5mM	41.30 ±2.45
$NiCl.6H_2O$	1mM	90.22 ±0.33
	5mM	79.25 ±0.20
$CaCl_2.2H_2O$	1mM	82.35 ±2.50
	5 mM	68.64 ±0.73
$CuCl_2.2H_2O$	1mM	29.89 ±0.88
	5 mM	14.03 ±1.67
$AgNO_3$	1 mM	98.09 ±1.46
	5 mM	77.12 ±0.12
Inhibitors		
PMSF	1 mM	50.61 ±1.25
SDS	1 mM	69.68 ±3.61
EDTA	1 mM	89.28 ±7.70

[a] The activity in the absence of a metal ion was defined as 100%
[b] The values are the mean and standard deviations of three repeats

than 50%. Cu^{2+} were shown to almost completely inhibit the phytase activity. Mn2+, Ba2+ and Fe2+ showed almost a 50% difference in their phytase inhibition when the concentration was increased to 5 mM. PMSF inhibited almost 50% of the phytase activity when added at 1 mM, whereas 1 mM EDTA showed almost no negative effect on the phytase activity. It is therefore recommended not to use an inhibitor (PMSF) in the assay mixture.

Discussion

The *Cryptococcus* genus was screened for phytase activity due to their presence in the droppings of poultry and other bird species (Mahmoud, 1999; Mattsson *et al.*, 1999). Species that were previously isolated from soil near feral pigeons were *Debaryomyces hansenii* var. *hansenii*, *C. laurentii*, *Cryptococcus uniguttulatus* and *Cryptococcus neoformans* var. *neoformans*. A few *Cryptococcus* strains were also shown to be associated with the trachea of chickens (Laubscher *et al.*, 2000). Since these monogastric animals are not able to digest the phytate in normal poultry feed, their manure will contain high concentrations of phytate (Common, 1989; Cromwell *et al.*, 1995). This would also lead to contamination of the surrounding soil with phytate. The presence of *Cryptococcus* strains in soil near the manure of bird species, therefore suggested that these organisms might be able to degrade phytate in the soil.

This study contains the first report of a *Cryptococcus* strain exhibiting phytase activity. The phytase was found to be associated with the cells with no extracellular activity observed. It seems that the phytase is cell wall-associated, since ionic and detergent solutions were unable to extract intracellular phytase activity (Table 2). Cell-associated phytase was also found in other yeasts, i.e. *R. gracilis* (Bindu *et al.*, 1998) and *Candida krusei* WZ-001 (Quan *et al.*, 2001). Some bacteria were also shown to have cell-associated phytase activity, i.e. *Selenomonas ruminantium* TY 35, *Mitsuokella multiacidus* 46/5 (Yanke *et al.*, 1998) and *E. coli* (D'Silva *et al.*, 2000; Greiner *et al.*, 1993).

Homogenisation did not only permeabilise the cells, but damaged the cell walls completely, thus releasing more intracellular phytase activity than the other methods investigated. The *C. laurentii* Abo 510 phytase activity of 4.55 IU/g cells compares well with the reported phytase activities of other fungi and bacteria, i.e. *Aspergillus carbonarius* (0.99 IU/ml) (Shieh and Ware, 1968), *Aspergillus niger* ATCC 9142 (1.09 IU/ml) (Casey and

Walsh, 2003), *Aspergillus ficuum* (6.62 IU/ml) (Shieh and Ware, 1968), *R. gracilis* (0.77 IU/g cells) (Bindu *et al.*, 1998), *Bacillus subtilis* (natto) (0.11 IU/ml) (Shimizu, 1992) and *Bacillus subtilis* (0.24 IU/ml) (Powar and Jagannathan, 1982).

Carbon sources need to be easily available to initiate growth and metabolism of yeast. Unfortunately not one of the alternative carbon sources investigated improved the production of phytase neither did they drastically increase the biomass. Maximal phytase activity was obtained only when the glucose concentration was depleted, possibly due to glucose repression.

Phytases usually have an acidic pH optima range (pH 4.5 to 6.0) with a rapid decrease in phytase activity at pH values above 6.0 (Ghareib, 1990; Irving and Cosgrove, 1971; Irving and Cosgrove, 1974; Ullah and Gibson, 1987). Yeast phytases have an optimal pH range of 4.0 to 5.0 (Nakamura *et al.*, 2000; Nayini and Markakis, 1984; Quan *et al.*, 2001; Sano *et al.*, 1999; Vohra and Satyanarayana, 2002). The *A. ficuum* NRRL 3135 phytase has an optimal pH of pH 2.5 and 5.5, enabling this filamentous fungus to be active at very high acidic levels. The phytase from *C. laurentii* Abo 510 has a pH optimum of 5.0, and the acid phosphatase has a broad pH optima range from pH 2.0 to pH 5.0. This acid phosphatase will assist the phytase in the hydrolysis of phytate intermediates under very high acidic levels.

The optimum temperature of yeast phytases is normally above 60°C (Nakamura *et al.*, 2000; Sano *et al.*, 1999; Segueilha *et al.*, 1992). The phytase from *C. laurentii* Abo 510 was active at temperatures between 24°C to 80°C with optimum phytase activity at 62°C, with acid phosphatase having an even higher optimum temperature of 82°C. The phytase was also thermostable for 3 hours when exposed to temperatures between 4°C and 70°C. The stability of the phytase at 4°C suggests that the enzyme will remain stable during the storage at this temperature. Phytase activity was completely lost after exposure to 80°C for 1 h or 90°C for 5 min.

The most thermo-stable phytase reported was that of *Aspergillus fumigatus*, which was able to withstand temperatures of up to 100°C for 20 min with only losing 10% of its initial phytase activity (Pasamontes *et al.*, 1997). It is crucial that the phytase enzyme must remain stable under these temperatures (70-90°C) due to the high temperature exposure during the feed manufacturing process (Lyons and Walsh, 1993). The addition of 5% PEG 6000 before the exposure to 90°C did help in maintaining 21% of the original phytase activity of *C. laurentii* Abo 510. Another solution might be to supplement the feedstuff with the culture

(cells) in a powdery form without any heat exposure except for the inactivation of pathogens in the animal feed prior to supplementation with the culture.

The phytase from *C. laurentii* Abo 510 exhibited broad substrate specificity, nevertheless, the preferred substrate for phytase was still phytate and the K_m value of 0.021 mM was the lowest reported so far for any yeast phytase (Nakamura *et al.*, 2000). The *S. castellii* phytase showed a broad substrate specificity by acting on glucose-1-phosphate, glucose-6-phosphate, *p*-NNP and ATP which also exhibited higher activity values than with phytate (Segueilha *et al.*, 1992), similar results were obtained with the *C. laurentii* Abo 510 phytase. Previous reported low K_m values for yeast phytases were from *C. krusei* WZ-001 (0.03 mM) (Quan *et al.*, 2001), *P. anomala* (0.2 mM) (Vohra and Satyanarayana, 2002) and *S. castellii* (0.038 mM) (Segueilha *et al.*, 1992). Low K_m values other than that from yeast phytases were, *A. ficuum* (0.027 mM) (Ullah and Gibson, 1987), *A. niger* SK-57 (0.019 mM) (Nagashima *et al.*, 1999), *B. subtilis* (0.04 mM) (Powar and Jagannathan, 1982), *Pseudomonas* sp. (0.016 mM) (Irving and Cosgrove, 1971) and wheat bran (0.022 mM) (Lim and Tate, 1973). Having a broad substrate specificity may be advantageous since the enzyme will more readily liberate intermediate phytates and will assist in hydrolysing all the other phosphate groups from phytic acid. The only drawback is that the broad substrate specificity is usually associated with low specific phytase activity (Wyss *et al.*, 1999).

Phosphate is known to repress the synthesis of various phosphatases in yeast (Ohta and Ohtani, 1979). A sharp decrease in phytase activity was observed with the addition of 0.1 mM phosphorus to the reaction assay mixture with no phytase activity observed at 0.5 mM. A final substrate concentration of 5 mM in the reaction assay mixture completely inhibited the phytase activity, this might indicate end product (phosphate) inhibition in phytase synthesis. In contrast, the *P. anomala* and *S. castellii* phytases were reported not to be inhibited by the presence of up to 10 mM and 40 mM inorganic phosphate in reaction mixture, respectively (Segueilha *et al.*, 1992).

Phytase activity of *C. laurentii* Abo 510 was moderately inhibited by Hg^{2+}, Zn^{2+} and Cd^{2+} (1 and 5 mM). Ca^{2+} almost completely inhibited the phytase activity. The inhibition of the *C. laurentii* Abo 510 phytase by Mn^{2+}, Ba^{2+}, Fe^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+} and Ag^{+} was concentration dependent, this meant that if the metal ion concentration of these cations were increased from 1 mM to 5 mM, the phytase activity inhibition increased. The inhibition of these mineral ions on the phytase activity might be ascribed to the strong chelating ability of phytate, which might form an insoluble metal-phytate structure and thus effectively reduce

the availability of phytate for the enzyme (Wang *et al.*, 1980). None of the mineral ions had a stimulatory effect on phytase activity. Similar levels of inhibition were observed with *P. anomala* (5 mM Fe^{3+} reduced phytase activity with 90%) (Vohra and Satyanarayana, 2002) and *S. castellii* (5 mM Zn^{2+} and Cu^{2+} strongly inhibited the reaction) (Segueilha *et al.*, 1992).

The phytase from *C. laurentii* Abo 510 is active in an acidic pH range, relatively thermo-stable, has a broad substrate specificity and high affinity for phytate and it does not need metal ions for its activity. These criteria are all in favour of the *C. laurentii* Abo 510 strain for the application in the animal feed industry. Supplementation with *C. laurentii* Abo 510 in the feedstuff of monogastric animals might improve the utilisation of phosphate bound to the phytate molecule (feedstuff) and eliminate the ANF of phytate.

References

Al-Asheh, S. and Duvnjak, Z. 1995. The effect of phosphate concentration on phytase production and the reduction of phytic acid content in canola meal by *Aspergillus carbonarius* during a solid-state fermentation process. *Appl. Microbiol Biotechnol.* **43**, 25-30.

Bae, H.D., Yanke, L.J., Cheng, K.J. and Selinger L.B. 1999 A novel staining method for detecting phytase activity. *J. Microbiol. Methods* **39**, 17-22.

Bindu, S., Somashekar, D. and Joseph, R. 1998. A comparative study on permeabilization treatments for *in situ* determination of phytase of *Rhodotorula gracilis*. *Lett. Appl. Microbiol.* **27**, 336-340.

Bogar, B., Szakacs, G., Pandey, A., Abdulhameed, S., Linden, J.C. and Tengerdy, R.P. 2003. Production of Phytase by *Mucor racemosus* in Solid-State Fermentation. *Biotechnol. Prog.* **19**, 312-319.

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochem.* **72**, 248-254.

Broz, J., Oldale, P., Perrin-Voltz, A.H., Rychen, G., Schulze, J. and Simoes Nunes, C. 1994. Effects of supplemental phytase on performance and phosphorous utilisation in broiler chickens fed a low phosphorous diet without addition of inorganic phosphates. *Br. J. Poultry Sci.* **35**, 273-280.

Casey, A. and Walsh, G. 2003. Purification and characterization of extracellular phytase from Aspergillus niger ATCC 9142. *Biores. Technol.* **86**, 183-188.

Common, F.H. 1989. Biological availability of phosphorous for pigs. *Nature.* **143**, 370-380.

Cromwell, G.L., Coffey, R.D., Parker, G.R., Monegue, H.J. and Randolph, J.H. 1995. Efficacy of a recombinant-derived phytase in improving the bioavailability of phosphorous in corn-soybean meal diets for pigs. *J. Anim.Sci.* **73**, 2000-2008.

Decleirie, M., De Cat, W. and Huynh, N.V. 1987. Comparison of various permeabilization treatments on *Kluyveromyces* by determining *in situ* β-galactosidase activity. *Enzyme Microb. Technol.* **9**, 300-302.

D'Silva, C.G., Bae, H.D. Yanke, L.J., Cheng, K.J. and Selinger, L.B. 2000. Localization of phytase in *Selenomonas ruminantium* and *Mitsuokella multiacidus* by transmission electron microscopy. *Can. J. Microbiol.* **46**, 391-395.

Erdman, J.W., Jr. 1979. Oilseed phytase: nutritional implications. *J. Am. Oil Chem. Soc.* **56**, 736-741.

Fuijita, J., Fukuda, H., Yamane, Y., Kizaki, Y., Shingeta, S., Ono, K., Suzuki, O. and Wakabayashi, S. 2003. Production and properties of phytase and acid phosphatase from a sake koji mold, *Aspergillus oryzae*. *J. Biosci. Bioeng.* **95**, 348-353.

Gargova, S., Roshkova, Z. and Vancheva, G. 1997. Screening of fungi for phytases production. *Biotechnol. Tech.*, **11** (4), 221-224.

Gautam, P., Sabu, A., Pandey, A., Szakacs, G. and Soccol, C.R. 2002. Microbial production of extra-cellular phatase using polystyrene as inert solid support. *Biores. Technol.* **83**, 229-233.

Ghareib, M. 1990. Biosynthesis, purification, and some properties of extracellular phytase from *Aspergillus carneus*. *Acta Microbiol. Hung.* **37**, 412-418.

Gibson, D.M. and Ullah, A.H.J. 1988. Purification and characterization of phytase from cotyledons of germinating soybean seeds. *Arch. Biochem. Biophys.* **260**, 503-513.

Gobbetti, M. 1998. The sourdough microflora: interactions of lactic acid bacteria and yeasts. *Trends Food Sci. Technol.* **9**, 267-274.

Greiner, R., Konietzny, U. and Jany, K.D. 1993. Purification and characterization of two phytases from *Escherichia coli*. *Arch. Biochem. Biophys*. **303**, 107-113.

Han, Y.M., Yang, F., Zhou, A.G., Miller, E.R., Ku, P.K., Hogberg, M.G. and Lei, X.G. 1997. Supplemental phytases of microbial and cereal sources improve dietary phytate phosphorous utilization by pigs from weaning through finishing. *J. Anim. Sci*. **75**, 1017-1025.

Harland, B.F. and Morris, E.R. 1995. Phytate: a good or bad food component. *Nutr. Res*. **15**, 733-754.

Heinonen, J.K. and Lahti, R.J. 1981. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Anal. Biochem*. **113**, 313-317.

Howson, S.J. and Davis, R.P. 1983. Production of phytate-hydrolysing enzyme by some fungi. *Enzyme Microb. Technol*. **5**, 377-382.

Hübel, F. and Beck, E. 1996. Maize root phytase: purification, characterization, and localization of enzyme activity and its putative substrate. *Plant Physiol.* **112**, 1429-1436.

Iefuji, H., Chino, M., Kato, M. and Iimura, Y. 1996a. Raw-starch-digesting and thermostable α-amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning and sequencing. *Biochem. J.* **318**, 989-996.

Iefuji, H., Chino, M., Kato, M. and Iimura, Y. 1996b. Acid xylanase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning, and sequencing. *Biochem. J.* **60**, 1331-1338.

Ikeda, R., Sugita, T., Jacobson, E.S. and Shinoda, T. 2002. Laccase and melanization in clinically important *Cryptococcus* species other than *Cryptococcus neoformans*. *J. Clin. Microbiol*. **40** (4), 1214-1218.

Irving, G.C.J. and Cosgrove, D.J. 1971. Inositol phosphate phosphatases of microbiological origin. Some properties of a partially purified bacterial (*Pseudomonas* sp.) phytase. *Aust. J. Biol. Sci*. **24**, 547-557.

Irving, G.C.J. and Cosgrove, D.J. 1974. Inositol phosphate phosphatases of microbiological origin. Some properties of the partially purified phosphatases of *Aspergillus ficuum* NRRL 3135. *Aust. J. Biol. Sci.* **24**, 547-557.

Jareonkitmongkol, S., Ohya, M. Watanbe, R., Takagi, H. and Nakmori, S. 1997. Partial purification of phytase from a soil isolate bacterium, *Klebsiella oxytoca* MO-3. *J. Ferment. Bioeng.* **83**, 393-394.

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N., Apajalahti, J. 1998. Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis*. *Appl. Environ. Microbiol.* **64**, 2079-2085.

Kim, D., Godbear, J.S. and Kim, H. 1999. Culture conditions for a new phytase-producing fungus. *Biotechnol. Lett.* **21**, 1077-1081.

Kim, Y.O., Kim, H.K., Bae, K.S., Yu, J.H. and Oh, T.K. 1998. Purification and properties of a thermostable phytase from *Bacillus sp.* DS11. *Enzyme Microbiol. Technol.* **22**, 2-7.

Kornegay, E.T. and Qian, H. 1996. Replacement of inorganic phosphorous by microbial phytase for young pigs fed on a maize-soybean-meal diet. *Br. J. Nutr.* **76**, 563-578.

Lambrechts, C., Boze, H., Segueilha, L., Moulin, G. and Galzy, P. 1993. Influence of culture conditions of *Schwanniomyces castellii* phytase. *Biotechnol. Lett.* **15**, 399-404.

Laubscher, W.D.F., Viljoen, B.C. and Albertyn, J. 2000. The yeast flora occurring in the trachea of broiler chicken. *Food Technol. Biotechnol.* **38**, 77-80.

Lei, X.G., Ku, P.K., Miller, E.R. and Yokoyama, M.T. 1993. Supplementing corn-soybean meal diets with microbial phytase lineary improves phytate phosphorous utilization by weanling pigs. *J. Anim. Sci.* **71**, 3359-3367.

Lim, P.E. and Tate, M.E. 1973. The Phytases. II. Properties of phytase fractions F_1 and F_2 from wheat bran and the *myo*-inositol phosphates produced by fraction F_2. *Biochim. Biophys. Acta.* **302**, 316-328.

Liu, J., Bollinger, D.W., Ledoux, D.R., Ellersieck, M.R. and Veum, T.L. 1997. Soaking increases the efficacy of supplemental microbial phytase in a low-phosphorous corn-soybean meal diet for growing pigs. *J. Anim. Sci.* **75**, 1292-1298.

Lyons, T., and Walsh, G. 1993. Applications of enzymes in feed manufacturing. In: Wenk, C., Boessinger, M. (Eds.), *Enzyme. Anim. Nutr.* ETH, Switzerland, pp. 241-254.

Mahmoud, Y.A.G. 1999. First environmental isolation of *Cryptococcus neoformans* var. *neoformans* and var. *gatti* from the Gharbia Governorate, Egypt. *Mycopathologia.* **148**, 83-86.

Mattsson, R., Haemig, P.D. and Olsen, B. 1999. Feral pigeons as carriers of *Cryptococcus uniguttulatus* and *Debaryomyces hansenii. Med. Mycol.* **37**, 367-369.

Mitchell, D.B., Vogel, K., Weimann, B.J. Pasamontes, L. and Van Loon, A.P.G.M. 1997. The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila. Microbiology.* **143**, 245-252.

Mullaney, E.J., Daly, C. and Ullah, A.J.H. 2000. Advances in phytase research. *Adv. Appl. Microbiol.* **47**, 157-199.

Nagashima, T., Tange, T. and Anazawa, H. 1999. Dephosphorylation of phytase by using the *Aspergillus niger* phytase with a high affinity for phytate. *Appl. Environ. Microbiol.* **65**, 4682-4684.

Nakamura, Y., Fukuhara, H. and Sano, K. 2000. Secreted Phytase Activities of Yeasts. *Biosci. Biotechnol. Biochem.* **64** (4), 841-844.

Nayini, N.R. and Markakis, P. 1984. The phytase of yeast. *Lebensm. Wiss. U. Technol.* **17**, 24-26.

Nelson, T.S. 1967. The utlilization of phytate phosphorous by poultry. *Poultry Sci.* **46**, 862-871.

Ohta, Y. and Ohtani, Y. 1979. Effect of phosphate concentration on growth of an alkane-degrading yeast *Candida lipolytica* ATCC 8661. *Microbiology.* **2**, 107-120.

O'Quinn, P.R., Knabe, D.A. and Gregg, E.J. 1997. Efficacy of Natuphos® in sorghum-based diets of finishing swine. *J. Anim. Sci.* **75**, 1299-1307.

Pasamontes, L., Halker, M., Wyss, M., Tessier, M. and Van Loon, A.P. 1997. Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus. Environ. Microbiol.* **63**, 1696-1700.

Pen, J., Verwoerd, T.C., Van Paridon, P.A., Beudeker, R.F., and 7 other authors. 1993. Phytase-containing transgenic seeds as a novel feed additive for improved phosphorous utilization. *Bio. Technology.* **11**, 811-814.

Powar, V.K. and Jagannathan, V. 1982. Purification and properties of phytate-specific phosphatase from *Bacillus subtilis. J. Bacteriol.* **151**, 1102-1108.

Quan, C., Zhang, L., Wang, Y. and Ohta, Y. 2001. Production af Phytase in a Low Phosphate Medium by a Novel Yeast *Candida krusei. J. Biosci. Bioeng.* **92** (2), 154-160.

Reddy, N.R., Sathe, S.K. and Salunkhe, D.K. 1982. Phytase in legumes and cereals. In: Chichester, C.O., Mrak, E.M., Stewart, G.F. (Eds.), *Adv. Food Chem.*, Academic Press, New York, pp. 1-92.

Sano, K., Fukuhara, H. and Nakamura, Y. 1999. Phytase of the yeast *Arxula adeninivorans. Biotechnol. Lett.* **21**, 33-38.

Segueilha, L., Lambrechts, C., Boze, H., Moulin, G. and Galzy, P. 1992. Purification and Properties of the Phytase form *Schwanniomyces castellii. J. Ferment. Bioeng.* **74** (1), 7-11.

Shieh, T.R. and Ware, J.H. 1968. Survey of microorganisms for the production of the extracellular phytase. *Appl. Microbiol.* **16**, 1348-1351.

Shimizu, M. 1992. Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77. *Biosci. Biotechnol. Biochem.* **56**, 1266-1269.

Shubakov, A.A. 2000. The β-xylosidase production by yeast *Cryptococcus podzolicus. Russian J. Bioorg. Chem.* **26**, 613-616.

Sripo, T., Ohara, T. and Phongdara, A. 1997. Purification and characterization of xylanases from *Cryptococcus* sp. X-1. *J. Sci. Soc. Thailand.* **23**, 307-322.

Ullah, A.H.J. 1988. *Aspergillus ficuum* phytase – partial primary structure, substrate selectivity and kinetic characterization. *Prep. Biochem.* **18**, 459-471.

Ullah, A.H.J. and Cummins, B.J. 1988. *Aspergillus ficuum* extracellular phytase: immobilization on glutaraldehyde activated silicate. *Ann. NY. Acad. Sci.* **542**, 102-106.

Ullah, A.H. J. and Gibson, D.M. 1987. Extracellular phytase (E.C. 3.1.3.8) From *Aspergillus ficuum* NRRL 3135: Purification and characterization *Prep. Biochem.* **17** (1), 63-91.

Ullah, A.H.J. and Phillippy, B.Q. 1994. Substrate selectivity in *Aspergillus ficuum* phytase and acid phosphateses using *myo*-inositol phosphates. *J. Agric. Food.* Chem. **42**, 423-425.

Van der Kaay, J. and van Haastert, P.J.M. 1995. Stereospecificity of inositol hexakisphosphate dephosphorylation by *Paramecium* phytase. *Biochem. J.* **312**, 907-910.

Van Etten, R.L., Davidson, R., Panayiotis, E.S., MacArthur, H. and Moore, D.L. 1991. Covalent structure, disulfide bonding, and identification of reactive surface and active site residues of human prostatic acid phosphatase. *J. Biol. Chem.* **266**, 2313-2319.

Vohra, A. and Satyanarayana, T. 2001. Phytase production by the yeast *Pichia anomala. Biotechnol. Lett.* **23**, 551-554.

Vohra, A. and Satyanarayana, T. 2002. Purification and characterization of a thermostable and acid-stable phytase from *Pichia anomala. World J. Microbiol. Biotechnol.* **18**, 687-691.

Walsh, G.A., Power, R.F. and Headon, D.R. 1994. Enzymes in the animal feed industry. *TIFS.* **5**, 81-87.

Wang, H.L., Savain, W. and Hesseltine, C.W. 1980. Phytase of molds used in oriental food fermentation. *J. Food Sci.* **45**, 1261-1266.

Wodzinski, R.J. and Ullah, A.H.J. 1996. Phytase. In: Neidleman, S.L. and Laskin, A.I. (Eds.), *Adv. in Appl. Microbiol.* , Academic Press, USA, pp. 263-302.

Wyss, M., Brugger, R., Kronenberger, A., Rémy, R., Fimbel, R., Oesterhelt, G., Lehmann, M. and van Loon, A.P.G. 1999. Biochemical Characterization of Fungal Phytases (*myo*-Inosital Hexakisphosphate Phasphohydrolases): catalytic Properties. *Appl. Environ. Microbiol.* **65**, 367-373.

Yanke, L.J., Bae, H.D., Selinger, L.B. and Cheng, K.J. 1998. Phytase activity of anaerobic ruminal bacteria. *Microbiology*. **144**, 1565-1573.

Yarrow, D. 1998. Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman, C.P. and Fell, J.W. (Eds.), *The Yeasts, A taxonomic study*, 4th Edition, Elsevier, Amsterdam, pp.77-100.

Zyla,-K. 1993. The role of acid phosphatases activity during enzymic dephosphorylation of phytates by *Aspergillus niger* phytase. *World J. Microbiol. Biotechnol.* **9** (1), 117-119.

4. Concluding Remarks

4. Concluding Remarks

We embarked on a study screening novel microbial taxa for phytase activity. The basidiomycete *Cryptococcus laurentii* Abo 510 showed the highest phytase activity of all the *Cryptococcus* strains screened.

For an enzyme to be employed as a commercial feed-additive phytase, it needs to fulfil certain criteria (Lei and Stahl, 2001). Firstly, the microorganism must produce a high phytase activity; the phytase activity for *C. laurentii* Abo 510 phytase was 4.55 IU/g wet cells. This high level of phytase activity compares well with other phytases such as bacterial phytases (0.1 IU/ml – 0.388 IU/ml) (Choi *et al.*, 1999; Yanke *et al.*, 1998), yeast phytases (0.02 IU/ml – 1.07 IU/ml) (Nakamura *et al.*, 2000; Sano *et al.*, 1999) and filamentous fungal phytases (0.6 IU/ml – 6.62 IU/ml) (van Hartingsveldt *et al.*, 1993). Secondly, the phytase must have broad substrate specificity. The *C. laurentii* Abo 510 strain showed broad substrate specificity that will assist the phytase in dephosphorylating the phytate molecule. Thirdly, the phytase must be active in a broad pH range, especially in the strong acidic pH range (gastrointestinal tract of monogastric animals) and be active and stable at high and low temperatures to survive thermal feed processing and storage of phytase. The phytase from *C. laurentii* Abo 510 is active at pH 2.5 to 7.5 with an optimum at pH 5.0 and has a temperature optimum of 62°C with a loss of only 40% of its activity at 70°C. At 4°C, the phytase from *C. laurentii* Abo 510 did not loose any of its phytase activity after 180 min. Lastly, the feed-additive phytase needs to be economical to produce since high cost to commercially produce feed-additive phytases limits its application as a feed supplement (Balander, 1998). Since *C. laurentii* Abo 510 produces a cell-associated phytase activity, it may be cost-effective to supplement the animal feedstuff with the cells, in a powdery form. This will assist the release of the phytate-phosphorus for utilisation by monogastric animals and thus minimise phosphorus pollution (Lei *et al.*, 1993). The phytase from *C. laurentii* Abo 510 thus fulfils the criteria of a commercially applicable phytase, suggesting that this enzyme may be suitable as an animal feed-additive.

Future research should be focussed on the molecular study of the *C. laurentii* Abo 510 phytase and to ensure the production of this phytase at a cost-effective level. One solution to this problem would be to express the gene heterologously in a yeast strain such as *Hansenula polymorpha* (Mayer *et al.*, 1999), which has already been shown to be a low-cost, high productivity production technique for phytase. Another alternative might be to express the

gene in an *A. niger* strain (Igbasan *et al.*, 2000; Pandey *et al.*, 2001), which is already used for the commercial production of the *A. ficuum* phytase at high yields.

In conclusion, we have shown that the phytase of *C. laurentii* Abo 510 shows high levels of phytase activity and its enzymatic properties are all in favour to use the native yeast or a recombinant strain expressing the gene for industrial feedstuff applications.

Literature Cited

Balander, R. 1998. Egg production and egg specific gravity in laying hens fed reduced phosphorous diets supplemented with phytase. In: Proc. of the Alltech's 14th Ann. Symp, Passport to the year 2000, Biotechnology in the Feed Industry. Lyons, T.P., and Jaques, K.A. (Eds)., Nottingham University Press, UK, pp. 411-422.

Choi, Y.M., Noh, D.O., Cho, S.H., Lee, H.K., Suh, H.J. and Chung, S.H. 1999. Isolation of a phytase-producing *Bacillus* sp. KHU-10 and its phytase production. J. Microbiol. Biotechnol. **9** (2)**:** 223-226.

Igbasan, F.A., Manner, K., Miksch, G., Borriss, R., Farouk, A. and Simon, O. 2000. Comparative studies on the *in vitro* properties of phytases from various microbial origins. Arch. Fuer Tierernahrung. **53:** 353-373.

Lei, X.G., Ku, P.K., Miller, E.R., Yokoyama, M.T., and Ullrey, D.E. 1993c. Supplementing corn-soybean meal diets with microbial phytase maximizes phytate phosphorus utilization by weanling pigs. J. Anim. Sci. **71:** 3368-3375.

Lei, X.G., and Stahl, C.H. 2001. Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl. Microbiol. Biotechnol. **57:** 474-481.

Mayer, A.F., Hellmuth, K., Schlieker, H., Lopez-Ulibarri, R., Oertel, S., Dahlems, U., Strasses, A.W.M. and van Loon, A.P.G.M. 1999. An expression system matures: a highly efficient and cost-effective process for phytase production by recombinant strains of *Hansensula polymorpha.* Biotechnol. Bioeng. **63** (3)**:** 373-381.

Nakamuru, Y., Fukuhara, H. and Sano, K. 2000. Secreted phytase activities of yeasts. Biosci. Biotechnol. Biochem. **64:** 841-844.

Pandey, A., Soccol, C.R., Rodriguez-Leon, J.A. and Nigam, P. 2001. Solid-State Fermentation in Biotechnology. Fundamentals and Applications. Asiatech Publishers, Inc., New Delhi.

Sano, K., Fukuhara, H. and Nakamura, Y. 1999. Phytase of the yeast *Arxula adeninivorans.* Biotechnol. Lett. **21:** 33-38.

Van Hartingsveld, W., Vanzeijl, C.M.J. Harteveld, G.M., Gouka, R.J., Suykerbuyk, M.E.G. Luiten, R.G.M., Vanparidon, P.A., Sellen, G.C.M., Veenstra, A.E., Vanorcom, R.F.M. and vandenhondel, C.A.M.J.J. 1993. Cloning, characterization and over-expression of the phytase-encoding gene (phya) of *Aspergillus niger*. Gene. **127** (1)**:** 87-94.

Yanke, L.J., Selinger, L.B. and Cheng, K.J. 1999. Phytase activity of *Selenomonas ruminantium:* a preliminary characterization. Lett. Appl. Microbiol. **29** (1)**:** 20-25.