"Studies on phytase from Aspergillus niger NCIM 563 under solid state fermentation and its correlation with submerged phytase I and II"

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By

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Dedicated to

MY FATHER AND MOTHER

Thank you, Mom and Dada.

For all your love and your support

a million words would be too short.

Three simple words, "I love you" seem too few

to express the love and respect I have for you.

CONTENTS

CERTIFICATE

This is to certify that the work entitled **"Studies on phytase from *Aspergillus niger* NCIM 563 under solid state fermentation and its correlation with submerged phytase I and II"** is the result of investigations carried out by **Ms. Kavita P. Bhavsar** at the NCIM RESOURCE CENTRE, National Chemical Laboratory, Pune, India under my supervision and the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

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DECLARATION

I hereby declare that the research work reported in the thesis entitled "**Studies on phytase from *Aspergillus niger* NCIM 563 under solid state fermentation and its correlation with submerged phytase I and II "** is entirely original and was carried out by me under the supervision of Dr. J. M. Khire at the NCIM Resource Centre, National Chemical Laboratory, Pune, India.

I further declare that the scientific contents of this thesis have not been the basis for award of any degree, fellowship, associate ship or any other similar title of any University or Institution.

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ABSTRACT

On the global level, there is a clear trend of steadily increasing extraction and use of both renewable and non-renewable natural resources for the production of goods and services. The larger fraction are non-renewable resources, i.e. resources which cannot be produced, re-grown or regenerated on a time scale which can sustain their consumption rate (e.g. strategic resources such as fossil fuels, phosphorus, uranium, lithium, neodymium, platinum, rare earths). Unremarked and unregulated by the United Nations and other high-level assemblies, the world's supply of phosphate rock, the dominant source of phosphorus for fertilizer, is being rapidly — and wastefully — drawn down. Phosphate rock, like oil, takes 10-15 million years to cycle naturally. While all farmers need access to phosphorus, just 5 countries control around 90% of the world's remaining phosphate rock reserves, including China, the US and Morocco (which also controls Western Sahara's reserves). Studies suggest current high-grade reserves will be depleted within 50-100 years. Further, peak phosphorus could occur by 2030. While the exact timing might be disputed, it is widely accepted that the quality of phosphate rock is decreasing and costs increasing. In mid 2008 the price of phosphate rock reached a peak 800% higher than early 2007.

The phosphorus situation has many similarities with oil, yet unlike oil, there is no substitute for phosphorus in food production. Phosphorus cannot be manufactured, though fortunately it can be recovered and reused over and over again. Phosphorus is one of Mother Nature's paradoxes as it is Life's bottleneck for existence on earth, but at the same time destructive in excess quantities in an aquatic environment. The only way to avert a supply crisis, researchers say, is to adopt the "3 R's" of sustainability: "Reduce, Reuse and Recycle."

Hence dephosphorylation of antinutritional and indigestible phytate, a phosphorus locking molecule, to digestible phosphorus, calcium and other mineral nutrients by phytases is an important metabolic process. The existing commercial microbial phytases produced by submerged

fermentation (SmF) conditions are expensive because of diluted product, production using recombinant strains, conventional purification techniques and high product recovery costs. Although, a limited number of phytases have been reported and studied, our scientific knowledge of phytases has yet to yield a solution to meet the nutritional and environmental requirements that a real-world solution demands.

So development of a viable process for phytase production, recovery and purification with techno-economic feasibility is necessary as the available methods such as SmF and column chromatography have several limitations. The available processes are also expensive, time consuming and difficult to scale-up. These traditional approaches are currently employed due to lack of alternative methods. For the above reasons, we chose to study the application of statistical methods to increase the phytase activity under SSF and suitability of ATPE system for phytase purification.

The same fungus produces two dissimilar phytase Phy I and Phy II under (SmF). Many studies on SSF and SmF for phytase have focused on process and fermenter design while the organism has been considered as a black box. The role of the physiological and genetic properties of the microorganisms producing phytase used during growth on solid substrates compared with aqueous solutions has so far been all but neglected. Hence we have tried to correlate different protein secretion in Smf and SSF and these studies can provide new insights to the existing “black box” of SSf/SmF biotechnology for phytase production.

Chapter I- Introduction

This chapter covers the literature on scope of the study, phytate and phytase enzyme, diversity of phytases, production, different sources, applications and recent advance in phytase research. It also covers the production and purification for phytase.

Chapter 2-Production of phytase by *Aspergillus niger* NCIM 563 under solid state fermentation

SSF provides a more economic alternative for enzyme production and application as compared to SmF. So phytase production by *Aspergillus niger* NCIM 563 was optimized using wheat bran in SSF. The present work demonstrates that using response surface optimization employing PBD and BBD gave a high level of phytase production of 154 IU/g DMB along with accessory enzymes in SSF. Phytase production improved from 50 IU/g dry moldy bran (DMB) to 154 IU/g DMB indicating 3.08 fold increase after optimization. A simultaneous reduction in fermentation time from 7days to 4days shows a high productivity of 38500 IU/kg/day. Scaling up the process in trays gave reproducible phytase production overcoming industrial constraints of practicability and economics. Some fungi are known to produce phytase and accessory enzymes by SSF but their low productivities are not comparable with the highest phytase productivity of 38500 IU/kg/day by *A. niger* NCIM 563 as shown by studies here. This demonstrates the potential applicability of SSF enzyme as a source of phytase supplement for phosphorus nutrition and environmental protection in animal feed industry.

Chapter 3 Downstream processing of solid state phytase from *Aspergillus niger* NCIM 563

There is a clear need for efficient, scalable and economical process for phytase bioseparation as available methods have several limitations. Hence the application of single step aqueous two-phase extraction (ATPE) for the downstream processing of phytase from *Aspergillus niger* NCIM 563, produced under SSF, has been studied and compared with the traditional multi-step procedure involving salt precipitation and column chromatography. High phytase recovery (98.5%) within a short time (3hrs) and improved thermostability was attained by ATPE in comparison to 20% recovery in 96hrs by chromatography process. The ATPE method, therefore, seems to be an interesting alternative for simultaneous partitioning and purification of phytase. This is the first report to show phytase extraction in a single step from fermentation broth by a liquid–liquid extraction process using ATPE The results presented in this work show that the ATPE technique

has considerable potential for the commercial development of an efficient process for separation and purification of SSF phytase.

Chapter 4-Biochemical characterization and application of solid state phytase from *Aspergillus niger* NCIM 563

The purified enzyme has been extensively characterized for its biochemical, molecular properties and synthesis of hollow silica nanocontainers in ionic liquids. The purified SSF phytase (Phy III) possessed an optimal pH of 5.6 and an optimal temperature of 60^0C. The protein is a monomer and exhibited a molecular mass of 85kDa in gel filtration and SDS–PAGE. Phy III exhibited broad substrate specificity but had high affinity for sodium phytate. It was markedly inhibited by N-bromosuccinimide suggesting a possible role of tryptophan in its catalysis. Based on MalDI-LC-MS/MS identification amino acid sequences of the peptides, the enzyme did not show homology with any other known phytases from the literature suggesting its unique nature. Importantly, the phytase released more inorganic phosphorus from soybean meal in a broad pH range from 1.5-6.5 under emulated gastric conditions and facilitates its use for the bioremediation of phytic acid in poultry feed.

Chapter 5- Correlation studies of solid state Phy III with submerged (Phy I and II) produced by *Aspergillus niger* NCIM 563

One of the differences between SSF and SmF cultures is that in the former, the moisture content of the substrate is low, resulting in a limitation of growth and metabolism of the microorganism. Moisture content being related to many factors can greatly influence the path of enrichment, leading to products that differ both quantitatively and qualitatively. *A. niger* NCIM 563 produces dissimilar phytases, Phy I and II in SmF and Phy II under SSF. From our studies, it can be seen that wheat bran and rice bran supported maximum phytase production under SSF and SmF. This may due to the reason that the fungus is confronted with gradients in concentration of

substrates and enzymes, the presence of a substrate-air interface, and gradients in water content and temperature. Production of accessory enzymes was higher in SSF when compared with SmF. This may be due to the conditions of SSF being more similar to fungal growth conditions in nature. This is the first report that aims at elucidating the mechanisms behind the differences in SSF and SmF for dissimilar phytases by comparison of their culture conditions and biochemical properties.

Chapter 6 Conclusions

This chapter details the salient feature of the work presented in the thesis and emphasized on possible future potential developments in the area.

Abbreviations

[BMIM][BF4]	1-butyl-3-methylimidazolium tetrafluoroborate
°C	degree centigrade
Å	Angstrom
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of VAriance
ATP	Adenosine triphosphate
ATPE	Aqueous twp phase extraction
BBD	Box Behnken Design
BPP	β propeller phytase
CCD	Central Composite design
CP	Cysteine Phosphatase
DCP	Dicalcium phosphate
DDA	Data dependent acquisition
DMB	Dry Mouldy Bran
DTT	Diethriotol
EDTA	Ethylene diamine tetraacetic acid
g, mg, µg, ng	gram, milligram, microgram, nanogram
GRAS	Generally regarded as safe
HAP	Histidine acid phosphatase
HCl	hydrochloric acid
IL	Ionic Liquid
IP1, IP2, IP3,IP4,IP5	Inositol mono-, bis-, tris-, tetrakis-, pentakis-phosphate
IP6	Phytic acid
IU	International Unit
kDa	Kilodalton
L, mL, µL	liter, milliliter, microliter

M, mM, µM	molar, millimolar, micromolar
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time Of Flight
MCP	Mono calcium phosphate
MW	Molecular Weight
NADP	Nicotinamide adenine dinucleotide phosphate
NBS	N-bromosuccinimide
NCIM	National Collection of Industrial Microorganisms
OVAT	One Variable Approach
P	Phosphorus
PAGE	Polarcylamide gel electrophoresis
PAP	purple acid phosphatases
PBD	Placket Burman Design
PDA	Potato Dextrose Agar
Phy I	SmF Phytase from *A. niger*
Phy II	SmF Phytase from *A. niger*
Phy III	SSF Phytase from *A. niger*
pI	Isoelectric point
PMSF	Phenylmethyl-sulphonilfluoride
R^2	Coefficient of Determination
RB	Rice Bran
RSM	Response surface methodology
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SmF	Submerged fermentation
SSF	Solid state fermentation
TEM	Transmission Electron Microscopy
TEOS	Tetraethyl orthosilicate
WB	Wheat Bran

Chapter 1

Introduction

This chapter is an introduction to the thesis and starts with the rationale and significance behind the work, carried out in this thesis followed by an overview of phytase production and purification. Although P is a basic component and energy conduit of life, it does not have a cycle to constantly replenish its supply. So dephosphorylation of phytate, a P locking molecule, by phytase is a crucial process. This chapter describes recent findings concerning the production, biochemical properties, molecular characteristics, and expression of phytases. Several potential applications of the phytases in animal nutrition, human health, and synthesis of lower myo-inositol phosphates are also summarized.

1. General Introduction

The cycling of P, a biocritical element in short supply, in nature is an important but slow biogeochemical process. P is a vital mineral important for bone and tissue growth in poultry. The massive growth of livestock production has made it the third most expensive nutrient in poultry production after energy and protein. Plants store P in the form of phytate (inositol 6-phosphate) carrying 6 phosphate groups. But this P present in seed grain as phytate is not readily available to mono-gastric animals, as they lack phytase activity. Phytate also acts as an antinutrient by chelating metal ions and reducing energy uptake.

To meet the P requirement, animal diets are generally supplemented with excess of commercial synthetic fertilizers. Human influences on the phosphate cycle come mainly from the introduction and use of these fertilizers. Strict norms for the excretion of large quantities of P effluents, human interference, decomposition of underutilized phytate cause phosphate pollution and price hike in synthetic fertilizers have currently led to the use of microbial phytase in animal feed. P is non-renewable and at the current extraction and usage rate, the existing phosphate reserves will be exhausted in next 80 years.

Use of phytase in animal feed will seize the anti-nutritional effects of phytate, decrease environmental pollution, increase availability of starch, protein, amino acids, calcium and P and abolish the addition of inorganic phosphate in animal feed. They are also imminent candidates for production of special isomers of different lower phosphate esters of myo-inositol, some of which are considered to be pharmacoactive and important intracellular secondary messengers. The FDA has approved “generally recognized as safe (GRAS)” petition for use of phytase in food, and it has been marketed as an animal feed enzyme in US since 1996. All these factors have concurrently made it as the third largest feed enzyme.

1.1 P

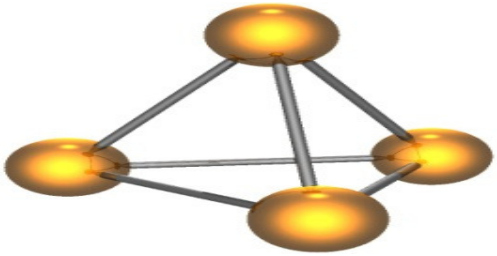

Fig. 1 P_4 molecule

Elemental P (Fig. 1) exists in two major forms- white and red P but due to its high reactivity, P is never found as a free element on Earth. The P cycle is the biogeochemical cycle that describes the movement of P through the lithosphere, hydrosphere, and biosphere. It is widely distributed in many minerals, mainly phosphates. Phosphate rock is a non-renewable natural resource, mainly found in sedimentary and igneous deposits. Its sustainable production and management is of critical importance. Most of the world phosphate production is used in agriculture.

P is essential to all known life forms. It is the second most abundant mineral in the human body, surpassed only by calcium. It makes about 1% of today body weight and is largely confined to the skeleton in addition to an important part of the active structure of the muscles, central nervous systems and the energy circuits. P compounds are important ingredients in high-grade detergents, cleaning agents, dental creams, toothpastes, flame retardants, stabilizer of plastics, corrosion inhibitors, dispersion agents in paints and primers and metal surface treatment.

Living cells also use phosphate to transport cellular energy in the form of adenosine triphosphate (ATP). Nearly every cellular process that uses energy obtains it in the form of ATP. ATP is also important for phosphorylation, a key regulatory event in cells (Fig. 2)

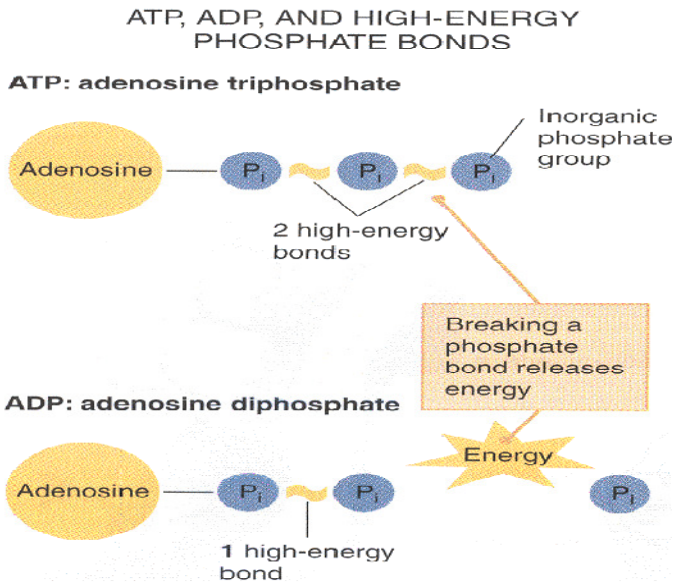

Fig. 2 High energy P bonds

Effects of P –

- In ecosystem an excess of P can be problematic, especially in aquatic systems, resulting in eutrophication which sometimes lead to algal blooms.
- Excess phosphate can lead to diarrhoea and calcification (hardening) of organs and soft tissue, and can interfere with the body's ability to use Fe^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+}.
- Hypophosphatemia is an electrolyte disturbance in which there is an abnormally low level of phosphate in the blood
- Osteomalacia (deficient calcification of bones; rickets)
- Anorexia (lack of appetite)
- Reduced performance (growth, milk yield or egg production)
- Pica is an appetite for objects not fit as food. Cattle develop an appetite for bones, which often contain *Clostridium botulism*.
- Lethargy, muscle weakness, seizures, erythrocyte deformity, hemolysis.

Despite its importance, P production, utilization and recycling is a slow process due to absence of gaseous phase and thus is therefore well-known as an imperfect cycle. Existing

phosphate reserves will be exhausted in next 80 years. All animal diets must contain adequate amounts of this element. So to meet their P requirements, inorganic P especially dicalcium phosphate is supplemented in diet of livestock and poultry animals. This has made it as the third most expensive nutrient in poultry production after energy and protein.

1.2 Phytate

Phytate (myo-inositol-1,2,3,4,5,6-hexakisphosphate; IP6) is the principal storage form of P, inositol, and variety of minerals in plants, representing approximately 75–80% of the total P in plant seeds [28, 52].

Structure of IP6:

IP6 bears six phosphate groups on one six-carbon molecule with low molecular weight of 660 and molecular formula $C_6H_{18}O_{24}P_6$ (Fig. 3). On the basis of Andersons structure [5], the systematic name for IP6 is myo-inositol-1,2,3,4,5,6-hexakisphosphate.

Occurrence

IP6 is a primary storage of inositol and storage form of P in plant seeds that are used as animal feed ingredients (oilseed meal, cereal grains and legumes) [93]**.** The total P, Phytate-P and IP6 content of common poultry feed are presented in Table 1. Most foods of plant origin contain 50-80% of their total P as phytate [53].

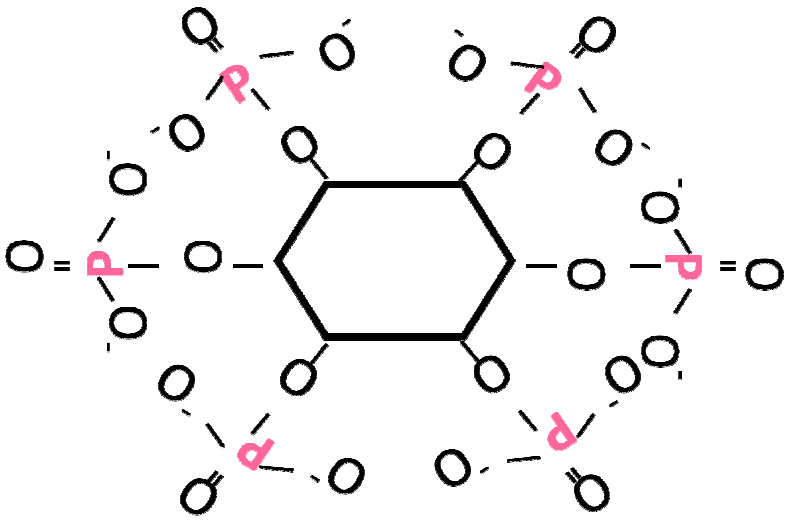

Fig. 3 Primary structure for myo-inositol hexakisphosphate

Table 1 Total P and Phytate P of common poultry feedstuffs [138]

Ingredients	Total P (%)	Phytate P (%)	% of total P
Cereals/ Millets			
Maize	0.39	0.25	64
Rice	0.15	0.09	60
Wheat	0.44	0.27	61
Sorghum	0.3	0.22	73
Barley	0.33	0.20	61
Bajra	0.31	0.23	74
Oilseed meals			
Groundnut meal	0.60	0.46	74
Soyabean meal	0.88	0.56	64
Cotton seed meal	0.93	0.786	82
Sunflower meal	0.90	0.45	51

Physiological nature of phytase

Phytate can exist in a metal-free form or in metal–phytate complex, depending on the pH of the solution and the concentration of metal cations (Fig. 4A). At acidic pH, protonation of the phosphate groups of phytate generates the metal-free form. At neutral pH, in contrast, deprotonation of the phosphate groups of phytate enhances the affinity for divalent metal cations and thus phytate forms metal–phytate complexes with divalent metal cations, mostly Mg^{2+} and Ca^{2+} [23, 91].

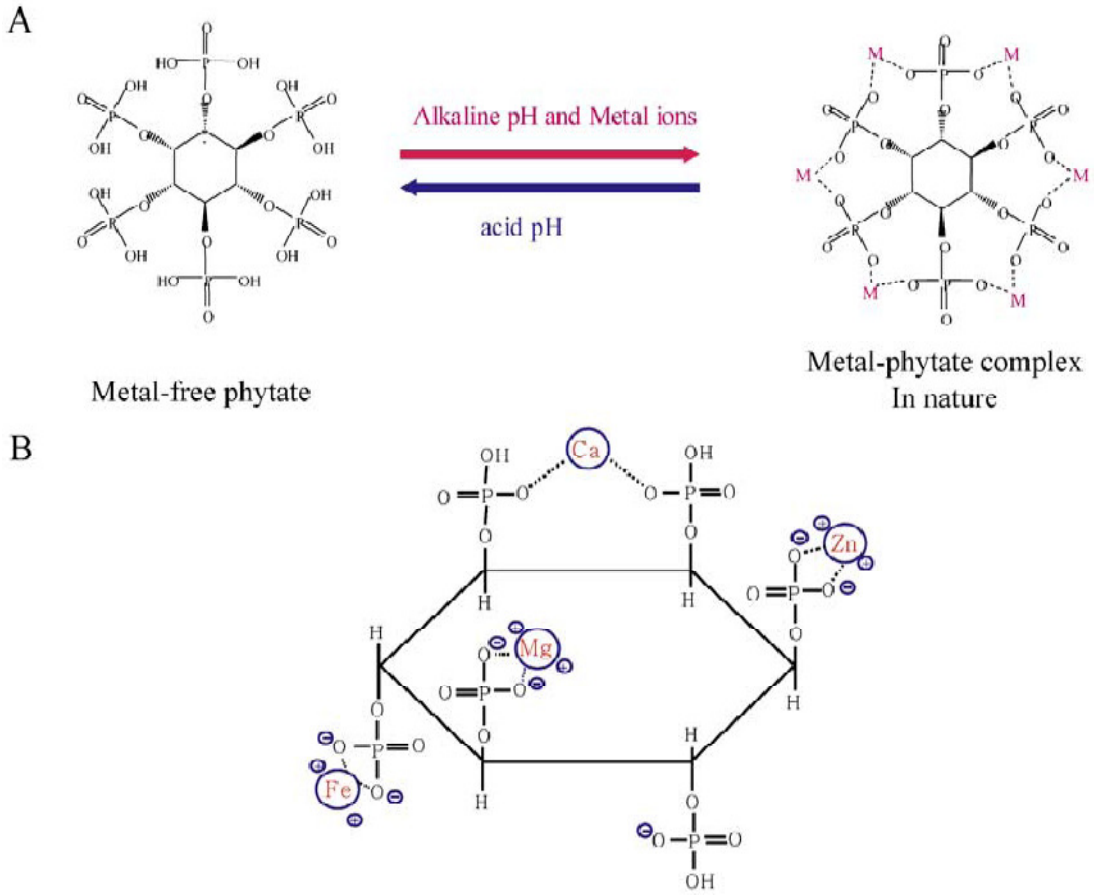

Fig. 4 Effects of pH and divalent metal cations on physiological nature of phytate

A Phytate exists as a metal-free phytate or a metal–phytate complex, depending on the pH and divalent metal cations. The extent of binding is dependent upon both pH and divalent metal cations to phytate ratios. In addition, at acidic pH and high cation concentration, a metal–phytate complex is formed due to direct electrostatic interaction.
B Divalent metal cations specifically bind to the phosphate groups of phytate, depending on the ionic radii of the metal cations. The formation of the bidentate metal complex prefers metal cations with large ionic radii.

In the metal–phytate complex, divalent metal cations with large ionic radii, such as Ca^{2+} (0.99 Å) and Sr^{2+}(1.12 Å), bind two oxianions from the phosphate groups of phytate in a bidentate fashion [95]**.** However, divalent metal cations with small radii, such as Mg^{2+} (0.65 Å), Fe^{2+} (0.74 Å), and Zn^{2+} (0.71 Å), bind in a monodentate fashion within two oxygen atoms from the phosphate groups of phytate (Fig. 4B). Therefore, bidentate metal-complex formation prefers divalent metal cations with large ionic radii [66]**.**

Negative aspects of phytate

Table 2 presents an overview of the negative interactions of phytate with nutrients and the mode of actions for the negative effects of phytate [76].

Table 2 Negative interaction of phytate and nutrients in food

Nutrients	Mode of action
Mineral ions (zinc, iron, calcium, magnesium, manganese and copper)	Formation of insoluble phytate-mineral complexes leads to decrease in mineral availability
Protein	Formation of nonspecific phytate-protein complex, not readily hydrolysed by proteolytic enzymes
Carbohydrate	Formation of phytate carbohydrate complexes making carbohydrate less degradable. Inhibition of amylase activity by complexing with Ca^{2+} ion and decrease of carbohydrate degradation
Lipid	Formation of 'lipophytin' complexes, may lead to metallic soaps in gut lumen, resulting in lower lipid availability

Effect of mineral uptake

Six reactive groups in the molecules of IP6 make it a strong chelating agent that binds cations Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}. The order of the ability of the mineral cations to form complexes with phytate in vitro has been found to be: $Cu^{2+} > Zn^{2+} > Cd^{2+}$ at pH 3–7 [108]. Dietary phytate forms an insoluble phytate-mineral complex. These complexes are not readily absorbed by the human gastrointestinal tract, which reduces the bioavailability of minerals. Moreover, the small intestine of the human is devoid of phytate degrading enzyme and also the microbial population in the upper part of the digestive tract is limited [62]**.**

Effect on protein digestibility

Phytate forms a strong complex with some proteins and resists their proteolysis. In general, the interaction of phytate with protein is dependent on pH. At a pH value lower than the isoelectric point of proteins [68]**,** phosphoric acid groups of phytate bind with the cationic group of basic amino acid, e.g., arginine, histidine, lysine, and form binary protein–phytate complexes. They are insoluble complexes that dissolve only below pH 3.5. Such complex formations may affect the protein structures that can hamper enzymatic activity, protein solubility and protein digestibility.

Effect on carbohydrate utilisation

Phytate may bind with starch either directly, via hydrogen bonds, or indirectly via proteins associated with starch [120].

Effect on lipid utilization

Phytate forms 'lipophytins' (complexes with lipid and its derivatives), along with other nutrients. Lipid and Ca phytate may be involved in the formation of metallic soaps in gut lumen of poultry, which is a major restraint for energy utilisation derived from lipid sources [77].

Environemtal perspective

Bound P (18-88% of total P content) in fact exists as phytate which is already present in animal feed. But this phytate P is not utilized by monogastric animals like poultry and pigs due to lack of intrinsic phytase in their gastrointestinal tracts. Phytate in addition acts as an antinutrient by chelating various cations such as Ca^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+} and thereby reducing their bioavailability. This unutilized phytate is the origin of P pollution as it builds up in areas of livestock production leading to eutrophication and algal blooms [103].

Degradation of phytate

The dephosphorylation of phytate is a prerequisite for improving nutritional value because removal of phosphate groups from the inositol ring decreases the mineral binding strength of phytate. This results in increased bioavailability of essential dietary minerals [126].

Non-enzymatic hydrolysis of phytates normally happens under high temperature conditions. By autoclaving at 121°C for 1h, Phillippy et al [112] studied the hydrolysis of IP6 (InsP6) and found that at pH 1.0, 2.0, 4.0, 6.0, 8.0, and 10.8, the percentages of InsP6 decomposed were 67.7, 76.8, 89.6, 81.9, 65.8, and 45.1%, respectively. The hydrolysis products were a variety of isomers of InsP1 to InsP5 (Myoinositol mono-, bis-, tris-, tetrakis-, and pentakis-phosphate). They also found that in the pH ranges of 1.0–10.8, the lower the pH, the more even distribution of inositol phosphate isomers. Based on this property, Chen et al [22] prepared reference standards of myoinositol phosphates by heating InsP6 solution which contains 2M HCl at 140°C for 1h, obtaining a total of 27 peaks representing InsP2–InsP6 isomers. During the process, the decomposition percentage of InsP6 is 95.3%.

Enzymatic hydrolysis of phytate

Phytases are chemically known as myo-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolase, and catalyze the sequential release of phosphate from phytate. Phytase sequesters orthophosphate groups from the inositol ring of IP6 to produce free inorganic P, along with a chain of intermediate myo-inositol phosphates (inositol pentaphosphate to inositol monophosphate) [34]**.** Phytase not only releases the P from plant-based diets but also makes available calcium, magnesium, protein and lipid. Thus, by releasing bound P in feed ingredients of vegetable origin, phytase makes more P available for bone growth and protects the environment against P pollution [11].

1.3 Phytase

In recent years, considerable efforts have been made to improve nutritive value of animal feedstuff through supplementation with exogenous enzyme. Currently used feed enzymes are divided into two main groups, the hemicellulases and phytases. Phytases *myo*-inositol hexaphosphate phosphorhydrolase) hydrolyze IP6 to *myo*-inositol and inorganic phosphates through a series of *myo*-inositol phosphate intermediates, and eliminate its anti-nutritional characteristics.

Source

In general, there are four possible sources: plant phytase, microbial phytase (fungal and bacterial phytase), phytase generated by the small intestinal mucosa and gut-associated micro floral phytase. Generally, phytase activity of animals is negligible compared to their plant and microbial counterparts [146]**.** Most of the scientific work has been done on microbial phytases, especially on those originating from filamentous fungi such as *Aspergillus ficuum*, *Mucor piriformis* and *Cladosporium* species [132]. Although some plants such as wheat and barley are rich in intrinsic

phytase, because of a narrower pH spectrum of activity and low heat stability their phytase activity is less effective than microbial phytases. Additionally, the bio-efficacy of plant phytases was only 40% compared to microbial phytases [156].

Diversity of phytases

Since the first phytase was found by Suzuki et al [134] in 1907, many different phytases from a variety of sources have been discovered and described. The International Union of Biochemists [63] currently distinguishes between three classes of phytase enzymes depending on the position (3, 6 or 5) on the inositol ring where the dephosphorylation is initiated as shown in Fig. 5.

1. **3-phytases** - (EC 3.1.3.8) yield 1, 2, 4, 5, 6-pentakisphosphate, does not always completely dephosphorylate IP6 and are normally produced by microorganism.
2. **6-phytases -** (EC 3.1.3.26) give 1,2,3,4,5- pentakisphosphate as the first product along with Pi and always completely dephosphorylate IP6 and are present in plants [100, 119].
3. **5-phytases-** (EC 3.1.3.72) from *Medicago sativa*, *Phaseolus vulgaris*, and *Pisum sativum* initiate phytate hydrolysis at the fifth phosphate group.

However, there are some exceptions: soybean phytase is a 3-phytase [109] and *Escherichia coli* phytase is a 6-phytase [48].

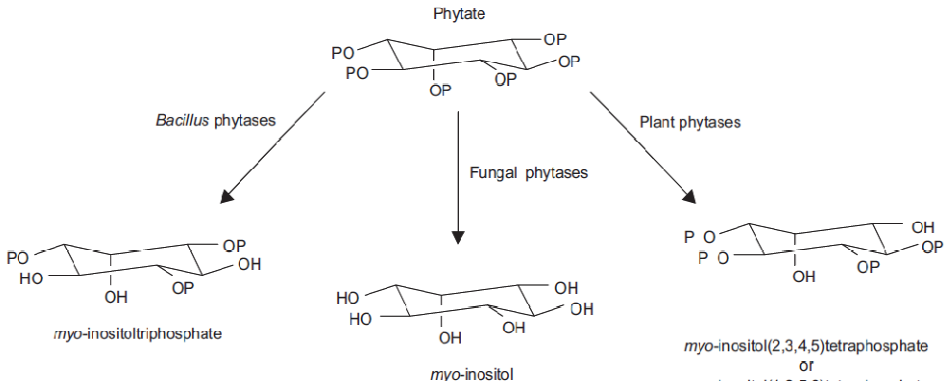

Fig. 5 Schematic diagram showing end products resulting from phytate hydrolysis mediated by different phytases

Based on biochemical properties and amino acid sequence alignment, phytases can be categorized into two major classes [104] (Fig. 7)

1. **Histidine acid phosphatase** – This class shows broad substrate specificity and hydrolyzes metal-free phytate at the acidic pH range and produces myo-inositol monophosphate as the final product.

2. **Alkaline phytase** – This class exhibits strict substrate specificity for the calcium–phytate complex and produces myo-inositol triphosphate as the final product. Phylogenic analysis clearly shows that alkaline phytases are not a subfamily of HAPs but are indeed novel phytases (Fig 5). Despite considerable differences between alkaline phytases and HAPs, only limited knowledge on the biochemical and catalytic properties of alkaline phytases is currently available. More focus has been on acidic phytases because of their applicability in animal feed and broader substrate specificity than those of alkaline phytases. On the basis of their catalytic properties, phytases are classified as histidine acid phosphatases (HAP), β propeller phytase (BPP), and purple acid phosphatases (PAP) [99].

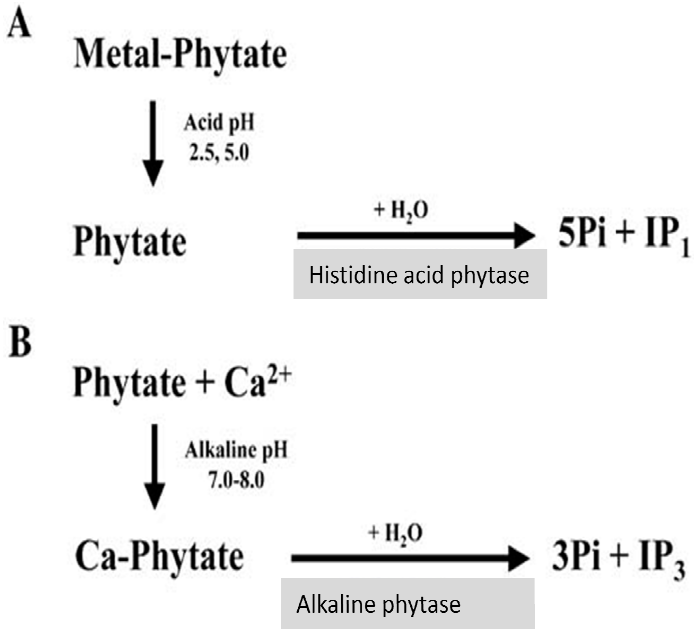

Fig. 6 Schematic illustrations of substrate hydrolysis by histidine acid phosphatases (HAPs) and alkaline phytases [128]

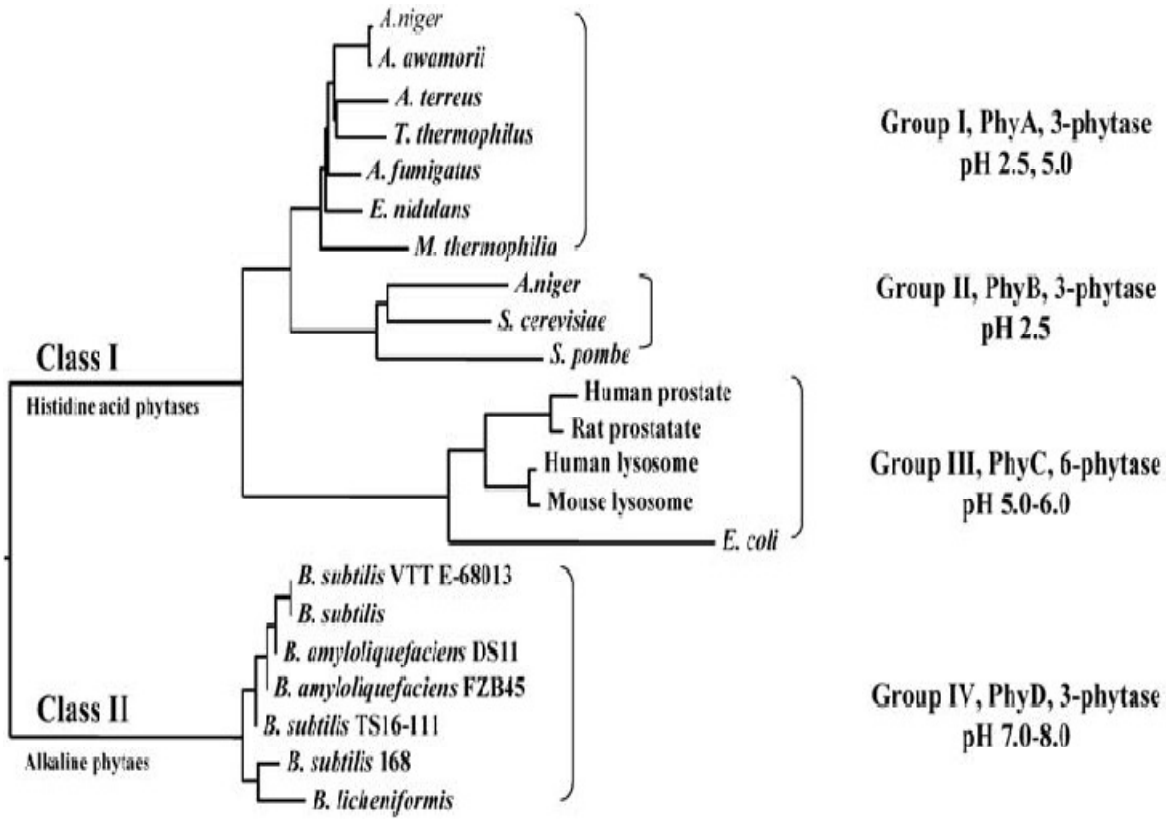

Fig. 7 Phylogenetic analysis of various HAPs and alkaline phytases [102]

Market trend and manufacture

Recent market trends have clearly shown that enzymes have emerged as big feed supplements. Feed enzymes (protease, xylanase, phytase, amylase, cellulase, lipase, β-glucanase) are the newest segment of the $5 billion animal nutrition market, which is increasing fast. Presently, only about 6% of manufactured animal feeds contain enzymes, against 80±90% for vitamins, which is considered as the largest animal nutrition category. The first phytase product, which entered the feed market in 1991, was manufactured by Gist Brocades (now DSM) and sold by BASF under the trade name Natuphos. Natuphos is available as powder, granulate, or liquid formulation.

Table 3 Commercial phytase preparations

Company	Country	Phytase source	Production strain	Trademark
AB Enzymes	Germany	*A. awamori*	T. reesi	Finase
Alko Biotechnology	Finland	*A. oryzae*	*A. oryzae*	SP,TP,SF
Alltech	USA	*A. niger*	*A. niger*	Allzyme phytase
BASF	Germany	*A. niger*	*A. niger*	Natuphos
BioZyme	USA	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	USA	*P. lycii*	*A. oryzae*	Bio-Feed phytase
Fermic	Mexico	*A. oryzae*	*A. oryzae*	Phyzyme
Finnfeeds International	Finland	*A. awamori*	T. reesi	Avizyme
Genecour International	USA	*P. simplicissimum*	P. funiculosu m	ROVABIO
Roal	Finland	*A. awamori*	T. reesi	Finase
Novozymes	Denmark	*A. oryzae*	*A. oryzae*	Ronozyme Roxazyme

Later, other products from different companies appeared, but only a limited number of commercial phytase products are currently available. These first phytases produced on commercial scale were either derived from fungal strains mutated via standard means or by using recombinant DNA technology. Several major animal nutrition companies are getting involved in this area very actively and various products under different trade names are already available as shown in Table 3 [16]**.** The FDA has approved "generally recognized as safe (GRAS)" petition for use of phytase in food, and it has been marketed as an animal feed enzyme in US since 1996. The expressed phytase genes are of fungal origin and originate in most cases from the genus *Aspergillus*. At present, all phytase preparations authorized in the EU as feed additives are produced by recombinant strains of filamentous fungi.

Ideal phytase and its designing

The phytase that has the desirable characteristics for application in animal feed industry can be called an 'ideal phytase', which should be active in the stomach, stable during animal feed processing and storage, and easily processed by the feed manufacturer for its suitability as an animal feed additive. It should satisfy the following points

1. Phytase should not be detected at the end of the small intestine. This is necessary because in this way the phytase produced by genetically modified organisms should not enter the environment [65]**.**
2. It should be effective in releasing phytate-P in the digestive tract.
3. It should be stable to resist proteases (trypsin and pepsin)
4. It should be able resist inactivation by heat during feed pelleting and storage
5. Low cost of production.

Finally, a phytase produced in high yield and purity by a relatively inexpensive system is attracting for food industries worldwide. It is now realized that any single phytase may never be

'ideal' for all feeds and foods. For example, the stomach pH in finishing pigs is much more acidic than that of weanling pigs [115]. Thus, phytase with optimum pH close to 3.0 will perform better in the former than in the latter. For poultry, an enzyme would be beneficial if it is active over broad pH range, that is, acidic (stomach) to neutral pH (crop) [121]. Phytases used for aquaculture application require a lower temperature that is optimum than the swine or poultry [116]. The choice of an organism for phytase production and development is, therefore, dependent upon the target application using directed evolution and protein engineering. All these features are not present within a single phytase, and therefore, based on the sequence of the available phytases, a consensus phytase could be designed [78, 79, 80].

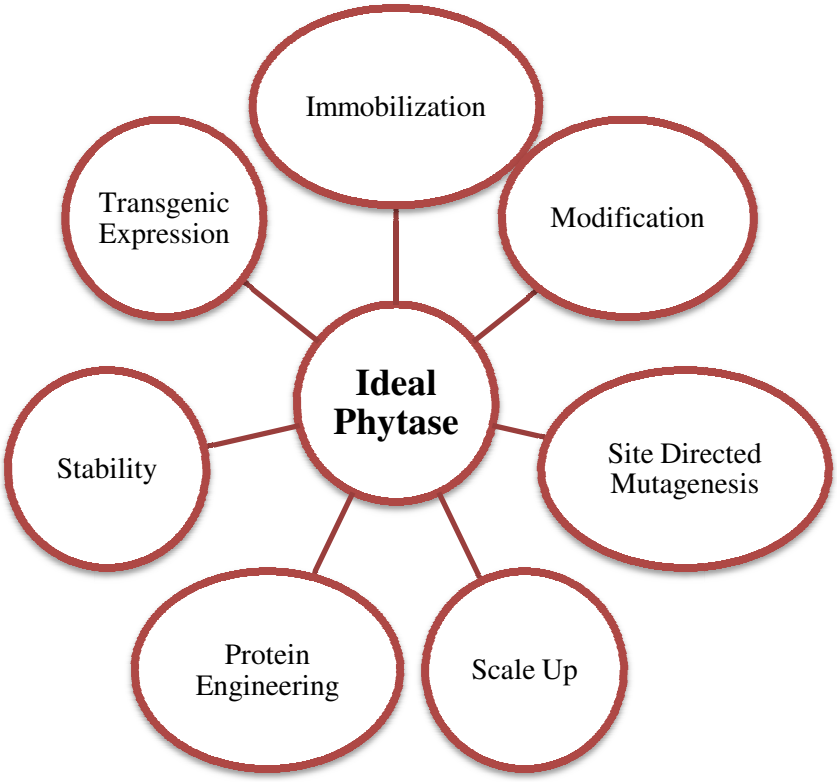

Fig. 8 Designing of ideal phytase

Genetic engineering techniques such as site directed mutagenesis could be employed for further ameliorating the properties. The strategies used for the designing and developing of an ideal phytase are presented in Figure. 8.

1. **Immobilization**- Immobilizing phytase for application in food, feed and pharmaceutical industry and biosensor.
2. **Modification-** Active site modification for enhanced thermostability and efficient catalysis of phytase by incorporating vanadium in active site for peroxidase activity.
3. **Site directed mutagenesis-** Modification for enhanced phytase thermostability and protease resistance
4. **Transgenic expression-** Expression in plants and animal for improving their nutrition and growth.
5. **Protein engineering-** Engineering phytase for enhanced thermostability and pH stability
6. **Scale up-** Economical and large scale phytase production
7. **Stability-** Understanding the role of glycosylation in phytase stability

The available phytase preparations used as feed additives are of fungal origin, produced by recombinant strains under submerged fermentation condition and have shortcomings especially with regard to obtaining diluted product, sensitivity to heat, inactivation under low pH conditions present in the stomach of animals [3] and high product recovery costs. These disadvantages need to be alleviated while at the same time producing phytase with high yield and purity

1.4 Microbial Production of phytase

Screening and assay

Several screening programmes have been carried out aiming at the isolation of different groups of bacteria yeast and fungi having extra-cellular phytase activity. Lissitskaya et al [87] screened micro-organisms producing phytase using museum and soil samples. It was found that moulds metabolized P more effectively than bacteria. Chen **[21]** developed a bioassay method for the screening for extra-cellular phytase-producing micro-organisms. Washed cells of *Corynebacterium glutamicum* were used as indicator strain. About 71% soil isolates had phytase activity above 0.01 U/ml. Gargova et al [42] used a two-step procedure to screen some 200 fungi for phytase production. A simple and rapid method has been described for determining the microbial phytase. The method consisted of determining the inorganic orthophosphate released on hydrolysis of sodium phytate at pH 5.5 [36]. Bae et al [8] developed a method for detecting phytase activity. Differential agar media were used for the detection of microbial phytase activity and the disappearance of precipitated calcium or sodium phytate was as an indication of enzyme activity. This technique, however, was unable to differentiate between phytase activity and acid production by ruminal bacteria.

Production technique

Phytases can be produced from a host of micro-organisms including bacteria, yeasts and fungi (Table 4). Submerged fermentation (SmF) has largely been employed as the production technology. However, in recent years solid state fermentation (SSF) has gained much interest for the production of phytase. Techniques of SmF as well as SSF have been employed for the production of phytases. Type of strain, culture conditions, nature of the substrate and availability of the nutrients are critical factors affecting the yield and should be taken into consideration for selecting a particular production technique. For example, a filamentous fungus in SmF is exposed

to hydrodynamic forces but in SSF the surface of the solid particles acts as the matrix for the culture.

Several bacterial strains (wild or genetically modified) such as *Lactobacillus amylovorus*, *E. coli*, *B. subtilis B. amyloliquefaciens*, *Klebsiella sp*., etc., have been employed for phytase production. The fermentation technique employed is SmF with glucose and yeast extract as main carbon and nitrogen source widely used. Sreemula et al [131] evaluated 19 strains of lactic acid-producing bacteria of the genera *Lactobacillus* and *Streptococcus* for the production of extra-cellular phytase. A number of them exhibited the enzyme activity in the fermentation medium but *Lactobacillus amylovorus* B4552 produced the maximum amounts of phytase, ranging from 125±146 units/ml in SmF using glucose and inorganic phosphate. Sunitha et al [133] optimized the medium for recombinant phytase production by *E. coli* BL21 using response surface methodology. A 23 central composite experimental design was used to study the combined effects of the medium components, tryptone, yeast extract and NaCl. The optimized medium with glucose showed a highest phytase activity of 2250 U/l. Phytase production using yeast cultures has generally been carried out in SmF systems. The strains used include *Schwanniomyces castellii*, *Pichia*, *Arxula adeninivorans* and *Candida kruzei*. Galactose and glucose were the preferred carbon sources. Phytase production from *P. anomala* has been extensively studied using RSM.

The available phytase preparations used as feed additives are of fungal origin, produced by recombinant strains under submerged fermentation condition. There is no defined medium for optimum production of phytase from different microbial sources especially fungi because each fungus has its own special conditions and specific substrates for maximum enzyme production especially in SSF. But the reports are few because of the low productivities and difficulties associated with operating and up scaling SSF conditions as seen in Table 4.

Table 4 Culture conditions for phytase production by various microorganisms [128]

Microbial Strain	pH_{opt}	$Temp_{opt}$	Fermentation	Carbon source	Nitrogen source
Filamentous fungi					
A. fumigatus SRRC 322	5	37	SmF	Hylon Starch	$NaNo_3$
A. niger	5.5	30	SmF	Glucose starch	-
A. ficuum	5	30	SmF	Corn starch,glucose	$NaNo_3$
A. oryzae	6.4	37	SmF	Glucose	$(NH_4)_2SO_4$
Rhizopus oligosporus	5.5	27	SmF	Corn starch,glucose	$NaNo_3$
R. oryzae	5.5	30	SSF	Glucose	NH_4NO_3
Mucor racemosus	5.5	30	SSF	Starch	$NaNo_3$
Peniophora lycii	5.5	26	SmF	Maltodextrin, soya flour	Peptone
Thermoascus aurantiacus	5.5	45	SmF	Starch,glucose,Wheat bran	Peptone
Rhizomucor pusillus	8	50	SSF	Wheat bran	Asparagine
Myceliopthora thermophila	5.5	45	SmF	Glucose	$NaNo_3$
Sporotrichum thermophile	5	45	SmF	Starch,Glucose	Peptone
S. thermophile	5	45	SSF	Sesame oil cake,glucose	$(NH_4)_2SO_4$
Yeasts					
Pichia anomala	6	25	SmF	Glucose	Beef Extract
Schwanniomyces castellii	4.4	77	SmF	Galactose	$(NH_4)SO_4$
Arxula adeninivorans	5.5	28	SmF	Galactose	Yeast extract
P. rhodanensis	4.5	70	SmF	Glucose	-
P. spartinae	4.5	75	SmF	Glucose	-
Candida krusei	4.6	40	SmF	Glucose	Polypeptone
Bacteria					
B. subtilis	7	37	SmF	Glucose	NH_4NO_3
B. amyloliquefaciens	6.8	37	SmF	Glucose	Caesin,Peptone
Escherichia coli	7	37	SmF	-	Tryptone
Klebsiella aerogenes	7	30	SmF	Sodium phytate	Yeast Extract
Lactobacillus sanfranciscensis	5.5	37	SmF	Maltose, Glucose	Yeast Extract
L. fructivorans	5.5	37	SmF	Maltose, Glucose	Yeast Extract
L. lactis subsp lactis	5.5	37	SmF	Maltose, Glucose	Yeast Extract
L. rhamnosus	6.5	37	SmF	Glucose	Yeast Extract
L. amylovorus	6.5	37	SmF	Glucose	Yeast Extract
Pediococcus pentosaceus	6.5	37	SmF	Glucose	Yeast Extract

Mutagenesis

Comparatively few reports are published regarding the improvement of phytase production via mutagenesis. Chelius and Wodzinski [20] during the strain improvement studies of *A. niger* NRRL 3135 by UV radiation, isolated a phytase catalytic mutant producing 3.3-fold higher phytase (phyA) than the wild type strain. The production of mutant phyA was highly repressed 60% by the inorganic phosphate (0.006%, w/v), however, their approach was limited by lack of specificity and sensitivity to discriminate between phytase and acid-phosphatase activity during primary screening process.

Transgenic studies

Although phytases are widely distributed in nature, the production in wild-type organisms is far from an economically viable level. Hence, cloning and expression of phytase genes in suitable host organisms is necessary in order to reach higher productivities. As the cost effectiveness of phytase production is a major limiting factor for its application, different heterologous expression systems and hosts have been evaluated. These are plants, bacteria, and fungi including yeast. As expected, each system bears some unique advantages, along with certain limitations.

1. **Plants-** The *A. niger* phyA gene has been successfully expressed in tobacco seeds [107] or leaves [141] and soybean cells [83]. But the difficulties of thermostability and phytase performance under adverse conditions are still a major concern.

2. Fu**ngi-** Phytase genes from *A. niger*, *A. terreus*, *A. fumigatus*, *E. nidulans*, and *M. thermophila* have all been expressed and secreted as active enzymes by *A. niger*. While fungal systems produce and secrete active phytases, other proteins, including proteases, are often produced at relatively high levels. Thus, there may be a need for further purification or inhibition of proteolysis that adds to the production cost.

3. **Yeast-** Though there are several reports of heterologous gene expression of bacterial and mold phytases in yeast expression systems, there are very few on homologous or heterologous expression of yeast phytase in microbial systems. *P. pastorisis* a potential host to express high levels of *A. fumigatus* phytase [122]**.**

4. **Bacteria-** The obstacle in using bacterial systems to produce fungal phytases is their inability to sufficiently glycosylate the expressed proteins to the extent necessary for activity. Thus, inactive *A. niger* PhyA protein was expressed intracellularly in *E. coli* [110] and extracellularly in *Streptomyces lividans*. The glycosylation of this protein or the expression host affect the properties of the expressed enzyme in terms of yield or non active state.

1.5 Biochemical characterization of phytase

Phytase is an ester-hydrolyzing enzyme with an estimated molecular weight of 35–700 kDa depending upon the source of origin and are usually active within pH range of 4.5-6.0.

Purification

Purification studies on phytases were usually performed with an aim to study properties of phytase originating from various microbial sources. They possess distinct aspects in molecular features as well as catalytic properties depending on the source of origin. Only a few phytate-degrading enzymes have been purified to homogeneity or near homogeneity (Table 5).

Purification of phytate-degrading enzymes includes common biochemical techniques such as ammonium sulphate fractionation, acetone precipitation, gel filtration, ion-exchange chromatography, affinity chromatography and hydrophobic interaction. One major problem in the purification of phytate-degrading enzymes especially from plants is the separation of phytate-degrading enzymes from contaminating nonspecific acid phosphatases [72]**.**

The recovery and purification of phytase has been achieved through several steps using different techniques. Boyce and Walsh [1], purified phytase from *Mucor hiemalis*, utilizing five steps (ultrafiltration, diafiltration, ion exchange, gel filtration and hydrophobic interaction), achieving 51% recovery and purification factor of 14.1; Spier et al [130] purified from *Aspergillus niger* phytase in three steps (SP Sepharose, Mono Q and single pass), obtained recovery (6.35%) and purification factor (10.1), whereas Azeke et al [7] obtained two phytases from *Rhizopus oligosporus* in five steps (Acetone Fractionation, Mono-S HR 5/50 Cationic-Exchange Chromatography, 16/60 Sephacryl S-200 HR chromatography, Mono-S HR 5/50 Cationic-Exchange Chromatography, Mono-Q HR 5/5 Anionic-Exchange Chromatography) with recovery: phytase 1 (1.3%) and phytase 2 (1.6%) and purification factor (75, 46), respectively.

Molecular and biochemical characteristics of phytase

Phytases are high-molecular-weight proteins ranging from 40-500 kDa. They are monomeric proteins, except for phytase B from *A.niger*, which is a tetramer. Phytases from eukaryotic organisms (yeasts, fungi, plants and animals) are often glycosylated and have higher molecular weights: 85-150 kDa for fungal phytases, around 500 kDa for yeast phytases, and 50-150 kDa for phytases from plants and animal tissues. Average molecular masses of bacterial phytases are smaller than those of fungal phytases (40–55 vs. 85–150 kDa), mainly due to glycosylation differences. Glycosylation has no effect on the specific activity and thermostability of phytases.

Most phytases have an optimal temperature of 44–60°C. In contrast, phytases from *A. fumigatus* and *B. amyloliquefaciens* have an optimum temperature of about 70°C. The phytate-degrading enzymes most resistant to high temperatures reported so far have been isolated from *A. fumigatus* and *Schwanniomyces castellii* [127]. In general, phytase enzymes of microbial origin are more pH and thermostable than their plant counterparts. The stability of most of the plant enzymes decreased dramatically at pH values below 4 and above 7.5, whereas the majority of the

corresponding microbial enzymes are rather stable even at pH values above 8.0 and below 3.0. Most phytases belong to either the acid phytases or the alkaline phytases, depending on their optimal pH for catalytic activity. Generally, the phytases from bacteria have an optimum pH in neutral to alkaline range while in fungi the optimum pH range is 2.5-6.0. All of the fungal, bacterial and plant phytate degrading enzymes investigated so far have acidic pI values with the exception of the *A. fumigates* enzyme, which has a pI of about 8.6. Bacterial phytases seem to be less acidic than fungal phytases: their pI is generally above 6, whereas fungal enzymes have pI values below 5.5.

Phytases usually show broad substrate spectrum with the highest affinity for phytate. The *A. fumigatus*, *Emericella nidulans* and *M. thermophila* phytases exhibited broad substrate specificity, while phytases of *A. niger, A. terreus* CBS and *E. coli* were rather specific for IP6. Broad substrate specificity was reported for phytases of *S. castellii* and *S. thermophile*, while cell-bound phytase from *P. anomala* exhibited broad substrate specificity. Only a few phytases have been described as highly specific for phytate such as the alkaline phytases from *B. subtilis.* The *Km* values of the phytases ranged between 10 and 650 μM.

Metal ions have been shown to modulate phytase activity. However, it is difficult to determine whether the inhibitory effect of various metals is due to direct binding to the enzyme, or whether the metal ions form poorly soluble complexes with IP6 and therefore decrease the active substrate concentration. Phytase activities of *Bacillus* sp were found to be Ca^{2+} dependent. Metal depletion caused by EDTA in them resulted in complete enzyme inactivation owing to a conformational change, as evidenced by the differences observed in the circular dichroism spectra of the holozyme versus metal-depleted enzyme [69]. Both of these enzymes, as well as two other *Bacillus* phytases were greatly inhibited by EDTA, indicating that a metal ion (Ca^{2+}) is needed for the activity the molecular weight and the properties, such as optimum pH, temperature, specificity and *Km* of phytase previously reported in microbes are summarized in Table 5.

An effective phytase needs to have a strong resistance to hydrolytic breakdown by digestive proteinases in the digestive tract. Fungal and bacterial phytases show different sensitivities to pepsin and trypsin [123]**,** and the latter seem to have a higher resistance to proteolytic degradation than the former [61]. The protease-sensitive sites of phytases, normally in the exposed loops at the surface of the molecules, may be blocked or modified using site-directed mutagenesis. Fluoride, a well known inhibitor of different acid phosphatase, was also found to be a strong competitive inhibitor of several acid bacterial, fungal and plant phytate-degrading enzymes. The reported inhibitor constants range from 0.1 to 0.5 mm. In contrast, the alkaline phytases from *B. subtilis*, *B. amyloliquefaciens* and lily pollen [9] show no reduction in activity in the presence of fluoride. Furthermore, the hydrolysis product orthophosphate was recognized as a competitive inhibitor of enzymatic phytate degradation. Molybdate, wolframate and vanadate are also known to inhibit phytate-degrading enzymes. It has been suggested that these transition metal oxoanions exert their inhibitory effects by forming complexes that resemble the trigonal bipyramidal geometry of the transition state [152].

Table 5 Source and properties of phytase

Phytase source	Mol wt (kDa)	Temp $_{opt}$	pH $_{opt}$	Km (mM)	pI	Specificity
F**ungi**						
A. fumigatus	75	58	5.0	-	8.6	-
A. niger	85	58	2.5,5.0	0.040	4.5	P
A. niger SK-57	60	50	5.5,2.5	0.0187	-	P
A. niger	-	55	5.5	0.200	4.9	-
A. niger	353	55	2.5	0.606		P
A. oryzae	120-140	50	5.5	0.33	4.2	B
A nidulans	77.8	55	5.5	-	-	-
R oligosporous	-	55	4.5	0.150	-	-
A. niger ATCC 9142	84	65	5.0	0.10	-	B
R oligosporous	124	65	5.0	0.01	-	B
Peniophora lyci	72	50-55	4.0-4.5	-	3.6	-
Ceriporia sp	59	55-60	5.5-6.0	-	7.36-8.01	-
Agrobyce pediades	59	50	5.0-6.0	-	4.15-4.86	-
Trametes pubescens	62	50	5.0-5.5	-	3.6	-
Thermomyces lanuginosus	60	65	7.0	0.11	4.7-5.2	B
Theroascus aurantiacus	-	55	-	-	-	-
Rhizomucor pusilis	-	70	5.4	-	-	B
Myceliopthora thermophile	-	37	6.0	-	-	B
Sporotrichum thermophile	456	60	5.5	0.15	4.9	B
Yeast	-	-	-	-	-	-
Saccharomyces cerevisiae	-	45	4.6	-	-	-
Schwanomyces castelli	490	77	4.4	0.038	-	B
Arxula adeninivorans	-	75	4.5	0.25	-	P
Candida krusei WZ OO1	330	40	4.6	-	-	-
Pichia anomala	64	60	4.0	0.20	-	B
P rhodanesis	-	70-75	4.0-4.5	0.25	-	-
P. spartinae	-	75-80	4.5-5.0	0.33	-	-
Bacteria						
Aerobacter aeerogenes	-	25	4.0-5.0	0.135	-	-
Bacillus sp DS 11	-	70	7.0	0.55	5.3	P
Bacillus subtilis	37	60	7.5	0.04	-	-
B. subtilis	38	60	6.0-6.5	-	-	-
B. subtilis	43	55	7.0-7.5	-	6.5	P
B. subtilis	44	55	6.0-7.0	-	5.0	P
B. icheniformis	47	65	6.0-7.0	-	5.1	-
B. amyloliquefaciens	44	70	7.0-7.5	-	-	-
Escherichia coli	42	55	4.5	0.13	6.3-6.5	P
Klebsiella oxytoca	40	55	5.0-6.0	-	-	-
K aerogenes	700	65	4.5	-	3.7	P
Psuedomonas syringe	47	40	5.5	0.38	-	P
L sanfranciscensis	50	45	4.0	-	5.0	B

B-broad spectrum, P- phytate specific

1.6 Kinetics and End Products of IP6 degradation

Phytate-degrading enzymes catalyze the stepwise hydrolysis of myo-inositol hexakisphosphate to orthophosphate and lower myo-inositol phosphates. The reaction intermediates are released from the enzymes and serve as substrates for further hydrolysis. Wyss et al [149] investigated the kinetics of phosphate release and the kinetics of accumulation of reaction intermediates, as well as the end products of IP6 degradation by various phytases. They concluded that all fungal phytases studied released five of the six phosphate groups, the end product being *myo*-inositol 2-monophosphate when excess enzyme is used. This indicates that all of these phytases have a pronounced stereo specificity and a strong preference for equatorial phosphate groups, whereas they are virtually unable to cleave the axial phosphate group.

Only in rare cases were traces of free *myo*-inositol or *myo*-inositol 1-monophosphate detected. *A. fumigatus* phytase readily degraded IP6 to *myo*-inositol 2-monophosphate, and only *myo*-inositol bisphophate (stereoisomer not known) accumulated to some extent. In contrast, *A. niger* and *A. terrus* phytases had to be used at much higher initial activities in order to obtain degradation down to *myo*-inositol 2-monophoshate, and considerable amounts of *myo*-inositol tris- and bisphosphates accumulated during the degradation. When *E. coli* phytase was used at an even higher initial activity, there was a pronounced accumulation of *myo*-inositol tetrakisphosphate during IP6 degradation. *Myo*-inositol bis- and triphosphates comprised more than 90% of the end products after a 90-min incubation period (with excess enzyme) and almost no *myo*-inositol monophosphate were detected. Therefore, lower *myo*-inositol phosphates appear to be less suitable substrates for *A. niger*, *A. terrus* and especially *E. coli* phytases than IP6. The stereoisomer assignment of the reaction intermediates and degradation pathway was not determined for these enzymes. The fact that the end products of IP6 hydrolysis for most phytases is identical do not necessarily means that the degradation pathways for IP6 are identical. 3-Phytase starts hydrolyzing the phosphate esters at the D-3 position, giving rise to D-Ins (1,2,4,5,6)P5 as the first intermediate [29 , 45]. 6-Phytase

starts the hydrolysis at the L-6 (or D-4) position, yielding L-Ins(1,2,3,4,5)P5 as the first intermediate. An alkaline phytase from lily pollen (Scott and Loewus, 1986) was shown to start the hydrolysis of IP6 at position D-5, with two subsequent dephosphorylation steps to yield Ins(1,2,3)P3 as the final product [10]. Inositol triphosphate is also the end product of IP6 hydrolysis for the phytase from *Typha latifolia* pollen [51]**.** Rat hepatic multiple inositol polyphosphate phosphatase (MIPP) catabolizes inositol hexakisphosphate without specificity towards a particular phosphate group. However, it hydrolyzed Ins(1,3,4,5,6)P5 via Ins(1,4,5,6)P4 to Ins(1,4,5)P3 by consecutive 3- and 6-phytase activities [31]. A detailed characterization of the phytase from the protozoan *Paramecium* by Freund et al [38] revealed that this enzyme degrades IP6 by stepwise dephosphorylation via D/L-Ins(1,2,3,4,5)P5, D/L-Ins(1,2,3,4)P4 and Ins(1,2,3)P3 finally to D/L-Ins(1,2)P2. Appearance of D/L-Ins(1,2,3,4)P4 clearly precedes that of Ins(1,2,3)P3. The slow conversion of inositol triphosphate to inositol bisphosphate indicates that Ins (1,2,3)P3 is the main end product. Powar and Jagannathan [113] showed that *myo*-inositol monophosphate (phosphate position not determined) is the end product for *B. subtilis* phytase. Kinetics, reaction intermediates and degradation pathways of IP6 degradation have not been reported for *Bacillus* phytases, neither is it known whether these enzymes are 3- or 6-phytases. The strong stereo specificity for the equatorial phosphate groups over the axial phosphate appears to be common to all phytases. This might indicate that only the phosphate groups protruding equatorially from the inositol ring can access the catalytic sites of these enzymes.

The pathway of hydrolysis by IP6-degrading enzymes seems to be unique for each species, and these enzymes, IP6, and its derivatives may play a variety of roles in biological systems [2]. To date, there is little knowledge of the sequence in which phytases hydrolyze phosphate groups from phytate and the IP6 derivatives. However, it is known that most characterized phytases hydrolyze IP6 in a stepwise manner, yielding myo-inositol pentakis-, tetrakis-, tris-, bis-and mono-phosphate products [71]**.** Biochemical mechanisms in the phytase involved still need to be further explored.

Table 6 Structural classification of phytases

Phytae	Enzyme family	Unique structural feature	Names	Mol mass	Natute of phytate	Inhibition	Stimulation	Glycos-ylation	Opt pH	Opt Temp	NCBI structure no	Example
Acidic	Histidine acid phosphatase	N-terminal RHGXRXP C-Terminal HD consensus motif	Phy A	62-128	Metal free	Ca^{2+}	EDTA	Yes	2.5-5.0	55-60	IIHP	*A. niger*, *A. terreus*, *M. thermophila*
			Phy B	270	Metal free	Ca^{2+}	EDTA	Yes	2.5	55-60	IQFX	*A. niger*, *S. cerevisiaae*, *S. pombe*
			Phy C	42-45	Metal free	Ca^{2+}	EDTA	No	5.0-6.0	40-60	IDKP	*E. coli*, Lyososomal phosphatase, Prostatic phosphatase
Acidic	Cysteine phosphatase	P loop structure contains HCXXGXXR(T/S) consensus motif	CP	46	-	Pb^{2+}	Cu^{2+}, Zn^{2+}, Hg^{2+}	-	4.0-5.5	50-55	-	*S. ruminatium*
Alkaline	β- propeller phytase	Six blade β-propeller shaped molecule	Phy D	38-45	Ca-phytate	EDTA	Ca^{2+}	No	7.0-8.0	-	-	*B. subtilis*, *B. amyloliquefaciens*
Acidic	Purple acid phosphatase	consensus motif DXG/GDXXY/GNH (ED)/VXXH/GHXH	Plant PAP	55	Fe-Zn center in active site		-	Yes	-	-	-	Glycine max, M truncatola
			Animal PAP	35	Fe-Fe center in active site		-	Yes	-	-	-	bovine spleen PAP, porcine uterus PAP (uteroferrin)

1.7 Structural classification of phytases

The basic structural features of several phytate degrading enzymes have been established and X-ray crystallographic studies have confirmed that they belong to a class with a novel catalytic mechanism [49]. In both instances, the elucidation of the 3-D molecular structure of different phytate degrading enzymes has enhanced our understanding of the linkage between the molecular structure of the molecule and its catalytic function.

It is now evident that different phytases have evolved to supply the unique nutritional requirements found in various forms of plant, animal and microbial life. At this time, four classes of phosphatase enzymes are known to have representatives that can degrade IP6: (1) HAP, (2) BPP, (3)CP and (4) PAP. Each one of these has unique structural features due to their distinct catalytic apparatus that allows them to utilize IP6 as a substrate in various environments (Table 6).

Several fungal, bacterial and plant phytases belong to the HAPs class of enzymes. All of these phytases share a conserved active site hepta-peptide motif RHGXRXP and the catalytically active dipeptide HD, unique to this class of enzymes [37]**.** This groups of enzymes catalyses the IP6 hydrolysis in a two-step process: a nucleophilic attack on the phosphorous atom by the histidine in the active site, followed by hydrolysis of the resulting phospho-histidine intermediate [144]**.** Phytases from *Bacillus* species constitute an exception: These enzymes share a sequence identity of 90–98% each other but are unrelated to HAPs and other phosphatases. Unlike other HAPs, they require Ca^{2+} for activity and show a different pH optimum of 7.0–8.0 [70].

Meanwhile, a phytase isolated from soybean was found to be unrelated to previously characterized microbial or maize (Zea mays) phytases, classified as HAPs. This soybean phytase is a PAP, characterized by seven conserved residues (bold) in the five conserved motifs – DXG, GDXXY, GNH(D⁄ E), VXXH and GHXH – involved in the coordination of the dimetal nuclear centre [56, 82]. In contrast, *S. ruminantium* phytase neither contains the conserved RHGXRXP

motif nor is affected by divalent metal ions. The active site is located near a conserved cysteine-containing (Cys241) P loop.

1.8 Crystal Structure of phytases

Crystal structure analyses of a number of phytases have revealed a range of distinct folds for these enzymes and have allowed their biophysical properties to be rationalized in terms of their structure. The crystal structure of *A. ficuum* phytase at 2.5 A° resolution revealed three distinct domains, including a large α-helical domain and β-sheet domain, and a small α-helical domain. The large α-helical domain and small α-helical domain contain five α-helixes and four α-helixes, respectively, and the β-sheet domain contains eight β-sheets [74]**.** Crystal structure analysis of *Escherichia coli* phytase with a resolution of 2.5 A° also showed two domains. One contains five α-helixes and two β-sheets, and the other includes six α-helixes and nine β-sheets [86]. A three-dimensional model of *A. ficuum* phytase (1IHP) from the National Center for Biotechnology Information's (NCBI) website is shown in Fig 9a.

The crystal structure of *B. amyloliquefaciens* phytase (TsPhy) at 2.1 A resolution revealed a six-bladed β-propeller in which each blade consists of a four- or five-stranded antiparallel β-sheet (Fig. 9b, PDB code 1H6L). The enzyme binds seven $Ca^{2+:}$ two near the periphery, one in the central channel and four near the 'top' of the molecule. Unlike other β-propeller structures, it does not show any conserved sequence repeats in the β-sheet. The crystal structures of TsPhy at 2.1 A° resolution in both the partially and the fully Ca^{2+}-loaded states were determined. And the dependence of thermostability of TsPhy on Ca^{2+} was assessed by differential scanning calorimetry.

The binding of two Ca^{2+} to high-affinity Ca^{2+}-binding sites results in a dramatic increase in thermostability (with an increase of as much as c. 30^{o}C in the melting temperature), because of the joining of loop segments remote in the amino acid sequence. Three Ca^{2+} bind to the active Ca^{2+}-binding sites and create an ideal conformation and charge distribution for the substrate. Substrate

binding to the active site would appear to be followed by occupation of the fourth Ca^{2+} site to offset the negative charge of the substrate phosphate group already coordinated by lysine and arginine.

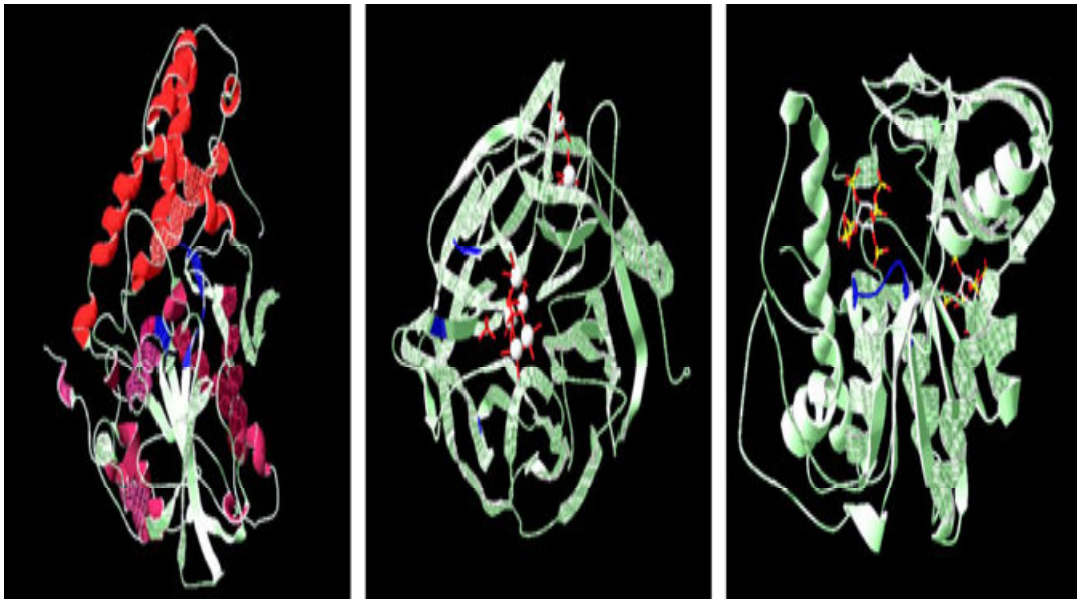

Fig 9 Swiss-Pdb viewer-prepared molecular models from the National Center for Biotechnology Information (NCBI)'s website (http://www.ncbi.nlm.nih.gov), representing three types of phytases: (a) 1IHP, PhyA, a histidine acid phosphatase; (b) 1H6L Ts-Phy, a b propeller phytase; (c) 1U26, SrPhy, a cysteine phytase [151]

Selenomonas ruminantium phosphatase (SrPhy) represents a third, dual-specificity phosphatase type with a conserved cysteine (C241) in its so-called P loop. Two distinct crystal packing arrangements have been observed of the complex of SrPhy with the inhibitor myo-inositol hexasulfate. The inhibitor is bound to both 'standby' and 'inhibited' conformations. In a pocket slightly away from the conserved P loop Cys241 and at the substrate binding site, the phosphate group to be hydrolysed is held close to the -SH group of Cys241. Further, mutagenesis studies verify that the P loop-containing phytase attracts and hydrolyses the substrate (phytate) sequentially via a complicated mechanism [25]. Figure 9c shows 1U26 to underscore the structural differences in these three classes of enzyme.

1.9 Development of effective phytases

Public awareness of the environmental impact of animal agriculture has led to legislation that limits the quantity of P in animal excreta in some parts of the world, and will likely extend to other parts of the world in the near future. Under these conditions, phytase will be widely used in animal diets to improve phytate-P bioavailability and reduce P excretion. Significant progress has been made in phytase research during the last 15 years. However, a limited number of phytases have been reported and studied, and our scientific knowledge of phytases has yet to yield a solution to meet the nutritional and environmental requirements that a real-world solution demands. Further research into identifying new phytases, engineering better phytases and developing more cost-effective expression systems should be continued. Two approaches have been taken to develop effective phytases: identifying new native phytase proteins from microorganisms or plants, and genetically modifying these cloned phytases.

Identification of new native phytase

Phytases are produced in a wide range of plant, bacterial, fungal and animal tissues. Most scientific work has, however, been performed on microbial phytases, particularly those from filamentous fungi such as *A. ficuum* [43], *a fumigatus* [105] or *Mucor piriformis* [57], *Rhizopus oligosporous* [18] and *Cladosporium* species [114]. The search for phytases with higher thermostability resulted in the cloning of the phytase gene from *A. fumigatus*, the purified enzyme of which retains 90% of its initial activity after being maintained at 100^{o}C for 20min. recently, a novel phytase gene from *A. niger* N-3 was cloned and expressed in *Pichia pastoris*. The purified enzyme of which retains 45% of its initial activity after being maintained at 90^{o}C for 5min. It showed a greater affinity for sodium phytate than for *p*-nitrophenyl phosphate. Dual optimum pH values were obtained at 2.0 and 5.5. The activity at pH 2.0 was about 30% higher than that at pH 5.5, which is more similar to conditions in the stomachs of monogastric animals. Two novel

thermostable genes were identified in *A. japonicus* BCC18313 (TR86) and BCC18081 (TR170), respectively. The thermostable nature of this phytases gives it valuable potential for applications [111].

Apart from the phytase genes identified in fungi, others have been cloned and identified in other microbes, motivated by their potential for applications. To find a phytase with high activity at low temperature and neutral pH, two phytases have been isolated from *Pedobacter nyackensis* MJ11 CGMCC 2503 and *Erwinia carotovora var. carotovota* ACCC 10276. The *Pedobacter* phytase belongs to the BPP family and shares very low identity (approximately 28.5%) with *Bacillus subtilis* phytase. Compared with the major commercial phytases and *B. subtilis* phytase, the purified recombinant enzyme from *E. coli* displayed higher activity and hydrolysed phytate from soybean meal with better efficiency at neutral pH and 25^{o}C. These characteristics suggest that this phytase has a great potential as an aquatic feed additive in the rapidly developing aquaculture industry. The *Erwinia* phytase contains a conserved active site hepta-peptide motif RHGXRXP and the catalytically active dipeptide HD that is typical of HAPs and shares a 50% amino acid identity to the *Klebsiella pneumoniae* phytase [58, 59]. And except for potential application in aquaculture, the latter is also attractive for food processing by avoiding damage to the food in gradients at low temperatures [47]. Moreover, owing to its typical properties as a low-temperature-active enzyme, it could be a good model protein to study the relationship between structure and function. The gene appA, encoding a phytase from *Yersinia kristeensenii*, was cloned and heterologously expressed in *P. pastoris*. The data show that the *Y. kristeensenii* phytase is highly pH stable at pH 1.5–11.0 and thermostable, providing significant advantages for processing, transportation, storage and application. Comparison of r-APPA with other well known phytases suggested that the *Y. kristeensenii* phytase would be an attractive enzyme for feed industry use [41]. In addition, phytases from yeast have also been identified and characterized (motivated by their potential as a feed additive for improving the phytate-P digestibility in monogastric animals), such as the marine

yeast *Kodamaea ohmeri* BG3 [85], *Pichia anomala* [67] and wastewater treatment yeast *Hansenula fabianii* J640 [145].

Protein engineering of phytase

Although properties of phytases vary, there is no single wild-type enzyme that is perfect or ideal for field applications. Theoretically, an 'ideal' phytase should be catalytically efficient, proteolysis-resistant, thermostable and cheap [81]**.** In reality, phytases possessing all of these qualities may never be found or generated. To obtain enzymes with modified and desired properties, two different strategies are used: rational protein design and directed (molecular) evolution, which are increasingly, applied in a synergistic manner to tailor-design the enzyme for a given process [14, 24].

1. **Thermostability-** Because commercial feeds are often pelleted, a process sing high temperature (60–80^{o}C) and steam, all feed enzymes need to be heat stable to avoid substantial activity loss during this process. The thermostability of an enzyme can be enhanced by multiple amino acid exchanges, each of which slightly increases the unfolding temperature of the protein. The rational approaches for thermostability engineering involve the comparison of the amino acid sequence of the protein of interest with a more thermostable, homologous counterpart, followed by replacement of selected amino acids. The thermostabilization concepts include the introduction of additional disulfide bridges, improvements in the packing of the hydrophobic core, engineering of surface salt bridge networks or helix dipole interactions, changes in helix propensity and changes in entropy [50, 136, 137].

2. **Catalytic activity**- Site directed mutagenesis of amino acid residue 300 was resulted in a high phytase activity by *A. niger* NRRL 3135 at pH 3.0 to 5.0, while a single mutation (K300E) resulted in an enhanced hydrolysis of IP6 at pH 4.0 and 5.0. In this study, the

basic amino acid residue lysine (K) was replaced by acidic residue. However, this replacement with another basic residue, or an uncharged but polar residue, did not significantly alter the activity at pH 4.0; but a replacement with basic residue arginine (R) lowered the activity over the pH range from 2.0 to 6.0 [98].

3. **Proteolysis resistance-**An effective phytase needs to have a strong resistance to hydrolytic breakdown by digestive proteinases in the digestive tract. Fungal and bacterial phytases show different sensitivities to pepsin and trypsin, and the latter seem to have a higher resistance to proteolytic degradation than the former. The protease-sensitive sites of phytases, normally in the exposed loops at the surface of the molecules, may be blocked or modified using site-directed mutagenesis [150]. Site-directed mutagenesis, based on crystal structure of phytases [86], has been used to improve pH profile of *A. niger* PhyA phytase. When expressed in *A. niger*, several fungal phytases were susceptible to proteases. N terminal sequences of the fragments revealed that cleavage invariably occurred at exposed loops on the surfaces of the molecules. Site directed mutagenesis at the protease-sensitive sites of *Aspergillus fumigatus* (S151N and R151L/ R152N) and *Emericella nidulans* phytase (K186G and R187R) yielded mutants with reduced susceptibility to proteases, without affecting the specific activity.

The rapidly growing number of successful phytase engineering studies using rational protein design and directed (molecular) evolution is conducted to improve the desirable enzyme characteristics. Tian et al [135] improved *A. niger* 113 (PhyI1s) activity by studying the effect of amino acid residues near the catalytic active centre or substrate specificity site – as well as some residues far from this site using site directed mutagenesis. Zhu et al [155] improved the thermostability of *Escherichia coli* (AppA) by 23.3% as compared to wild type using error-prone PCR and high-throughput screening. This mutant I408L could be used for the large-scale commercial production of phytases. Phytase from *Penicillium sp*. was studied by Zhao et al [154]

for high thermal stability, low optimal temperature and pH using Mn^{2+}-dNTP random mutation method. Two mutants were obtained with improved thermal stability and optimal temperature and pH that retained their high resistance to pepsin. Structure-guided consensus approach was used by Viader-Salvado et al [142] for broadening the pH profiles (2.5-9.0) of β-propeller phytase and it was proposed that P257, D336 play an important role for forming a larger number of hydrogen bonds and this resulted in new properties at pH 5.5 and 7.5. Site directed mutagenesis was employed to obtain mutant (A58E P65S Q191R T271R) of *Aspergillus niger* phytase (PhyA) with higher thermostability. This study shows that hydrogen bond network and ionic interactions play an important role [153].

1.10 Immobilization

Phytases act sequentially on *myo*-inositolhexakisphosphate to liberate various lower isomers. Thus, an efficient immobilized bioreactor could be used to produce various isomers of IP6 besides rendering the molecule non-chelator of metal-ions, proteins, etc Fungal phytase are hydrolytic enzymes with a high catalytic turnover number-typically the range being from 220-1000 per second. Thus these categories of enymes are ideal for immobilization and construction of packed bed reactors. A few studies have been made on the application and properties of immobilized phytate-degrading enzymes [46, 139]. The pH dependence of the phytate-degrading activity was not influenced by immobilization, whereas stability against heat treatment was enhanced as a consequence of immobilization. In addition, the immobilized phytate-degrading enzymes exhibited good optional and storage stability over a period of several months. Liu et al [88] increased the temperature optima of *A. ficuum* phytase to 58^{o}C, which was 8^{o}C higher than that of free enzyme. This was done by immobilization in gelatin gels and further hardening with formaldehyde. Apparent *K*m increased to 3.28mM (*K*m = 2.34mM for free enzyme) and the residual enzyme activity was 34.6% only.

1.11 Biotechnological applications of phytase

Since the first commercial phytase product Natuphos® was launched in 1991, the market volume has reached ca. 150 million Euros and will likely expand with new applications. The main application is still as a feed supplement to improve P bioavailability in plant feed-stuffs via the enzyme-mediated hydrolysis of phytate. Most importantly, the improved utilization of the phosphate deposits in the feed results in a substantial reduction in the phosphate content in animal manure and hence decreases of phosphate load on the environment in areas of intensive animal agriculture. High dietary P bioavailability reduces the need for supplemental inorganic P such as mono- and dicalcium-phosphate (MCP, DCP).

Because of the strong economic growth in China and India along with the oil price hike, the supply and cost of MCP and DCP has become a practical issue. Furthermore, inorganic phosphate is non-renewable resource, and it has been estimated that the easily-accessible phosphate on earth will be depleted in 50 years. Thus, phytase is an effective tool for natural resource management of P on a global scale.

The ban of dietary supplementation of meat and bone meal, as a cheap source of feed P, in Europe to prevent possible cross-species transfer of diseases such as BSE, has led to a profound change in the feed P management. This has given phytase a new socio-economic impact as a cost effective alternative to ensure animals to obtain adequate available P from the plant-based diets. Being the major storage form of P in seeds, plant phytate was produced in 2000 at a global yield >51 million metric tons. This amount accounts for approximately 65% of the elemental P sold worldwide as fertilizers [89]**.** Apparently, phytase can turn the plant phytate into a very valuable resource of P by improving its bioavailability for animal nutrition. Denmark and the Netherlands have imposed regulations to promote the use of microbial phytases.

Because of the potential value of phytases for improving the efficiency of P use, biotechnology has led the rapid development of the field to its current stage. With the development of heterologous gene expression, large amounts enzymes could be produced at relatively low cost. The importance of phytases as potential biotechnological tools has been recognized in various fields (Table 7). However, only a limited number of phytases have been reported and studied, and our knowledge of the mechanisms and factors regulating phytase activity is limited. Further research into developing new technologies and identifying the most efficient phytases must continue.

Phytases in animal nutrition:

Monogastric animals such as swine, fish, and poultry show negligible or no phytase activity in their digestive tracts. Consequently, phytates cannot be metabolized by the animals, thus creating a need to enhance phosphate and mineral bioavailability via phytase supplementation of animal feed. Of late, phytases are also viewed as environment friendly products, which can reduce the level of phosphate pollution in intensive livestock management areas by avoiding the addition of exogenous phosphate [140]. Undigested phytate of monogastric manure is washed off the farmland that imperils adjacent waterways by eutrophication [27]. The effect of feeding phytase to animals on pollution has been quantitatively determined. If phytase were used in the feed of all of the monogastric animals reared in the U.S., it would release P with a value of 168 million U.S dollars and would preclude 8.23 × 104 tonnes of phosphate from entering the environment per annum. The use of phytase as a feed additive has been approved in 22 countries.

Table 7 Potential applications of phytases

Application	Role and Effect	Properties	Challenges
Feed industry	Increased P utilization, metal bioavailability, decreased P conc. in excrement, Substitutes expensive Di-calcium phosphate	Resistance to low pH, active in the stomach, stable during animal feed processing and storage, low cost of production and easily processed by the feed manufacturer	Lack of desirable properties, High cost of production
Food industry	Increased P utilization, metal bioavailability, technical improvement of food processing	-	It will be a challenge to minimize the negative effect of phytate on iron and zinc nutrition without losing its potential health benefits
Myoinositol phosphate	Myoinositol phosphate intermediates used as enzyme stabilizers, enzyme inhibitors, potential drugs, chiral building blocks	-	Further intensive investigations, using diverse phytases, need to be undertaken for designing and producing pharmacologically important lower *myo*-inositol phosphates
Aquaculture	Substitute for expensive protein source such as menhaden fish meal and maintains the acceptable levels of P in water	Phytase active at low temperature and broad pH optima is required	Effects of phytase supplementation on various physiological and endocrine parameters like secretion of other enzymes, bile salts, on the immune response, hormone levels including growth hormone, thyroid hormone, insulin etc needs to be studied
Soil Amendment	Plant growth stimulation by mobilization of soil phytate into inorganic P	Phytase with broad pH optima and catalytic activity	Needs more research on phytase supplementation for boosting the productivity in agriculture and horticulture

The FDA (The Food and Drug Administration) has approved the phytase preparation as GRAS [147]. During the past two decades, there has been significant increase in the use of phytases as feed additive in pig, poultry, and fish diets. In numerous studies, the efficacy of microbial phytases to release phytate-bound P has been demonstrated in various animals [32, 15, 104, 39, 96, 117, 118, 157]. Phytases were also found to enhance the utilization of different minerals. Phytases from different sources have been evaluated individually and in combination for their efficacy as feed additives in poultry [19, 35, 106, 148]. Use of both bacterial and fungal phytases together as feed additive would be another promising alternative in improving the P utilization and alleviation of mineral deficiency, owing to their synergistic activities throughout the gastrointestinal tract of the animals. The use of phytase as a feed enzyme sets certain demands on the properties of the enzyme. Particularly, the enzyme should withstand high temperatures. This is because poultry and pig feed is commonly pelleted, which ensure that the animals have a balanced diet and facilitates the preservation of enzyme-containing product in the feed industry**.** During the pelleting process the temperatures may temporarily reach 90^{o}C**.** The first commercial phytase product, which became commercially available 10 years ago, offered animal nutritionists the tool to drastically reduce P excretion of monogastric animals by replacing inorganic phosphates with microbial phytase. Depending on diet, species, and level of phytase supplementation, P excretion can be reduced between 25 and 50% [73]**.**

Phytases in human nutrition:

Mineral deficiency of diets, caused by radical changes in food habits, is a major concern for developing countries. Processing and manufacturing of human food is also a possible application field for phytase. Up to now, no phytase product for a relevant food application is on the market. Research in this field focuses on better mineral absorption or technical improvement of food processing. Phytate present in cereal-based and legume-based complementary foods has been found to inhibit mineral absorption [60]**.** The human small intestine has limited ability to digest

undegraded phytates, resulting in adverse nutritional consequences with respect to metabolic cation imbalances**.** IP6 containing 12 dissociable protons with pKa values ranging from ~1.5 to 10—is a highly reactive and potent chelator of many mineral ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}. IP6 forms insoluble salts, at normal acidity (pH 3.0–6.8), in the human digestive tract, thereby reducing the bioavailability of these critical mineral nutrients for absorption [30]**.** Mucosal phytase and alkaline phosphatases, even if present in the human small intestine, do not seem to play a significant role in the phytate digestion, while dietary phytase serves as an important factor in phytate hydrolysis [125]**.** Haros et al [54] investigated the possible use of phytase in the process of bread making. Different amounts of fungal phytase were added in whole wheat breads, and it was shown that phytase is an excellent bread-making improver. The main achievement of this activity was the shortened fermentation period without affecting the bread dough pH. An increase in bread volume and an improvement in crumb texture were also observed.

Phytases in aquaculture:

A major concern in aquaculture is the utilization of dietary phosphates which critically affects fish growth as well as the aquatic environment. An efficient utilization of feed leading to optimum fish growth serves as a benchmark of successful aquaculture worldwide. Studies using phytase as feed additive in aquaculture amply establish that phytase supplementation could enhance the bioavailability of P, nitrogen, and other minerals, thereby decreasing P-load in the aquatic environment [101, 143]**.**

Role of phytases in soil amendment:

P is an essential plant nutrient that limits agricultural production on a global scale. Approximately 30–80% of the total P in soils is bound in organic form [55]**.** Phytate constitutes ~50% of the total organic P pool in the soil and is poorly utilized by plants [4]**.** Extracellular phytase activities have been reported under phosphate stress conditions, in diverse plant species,

namely, tobacco [90], barley [6], tomato, alfalfa [84], and so on. The ability of plants to use P from low phosphate or phytate containing media and/or from soil is improved when soil/media are inoculated with microorganisms that possess the ability to exude phytase, or when a purified phytase is added.

Phytases for the production of lower myo-inositol phosphates:

Lower phosphoric esters of myo-inositol (mono, bis, tris, and tetrakisphosphates) play a crucial role in transmembrane signaling processes and in calcium mobilization from intracellular store in animal as well as in plant tissues [12, 97, 33, 75, 124]. Research interest in this field prompted the need for various inositol phosphate preparations. However, chemical synthesis [13] is difficult. In contrast, an enzymatic synthesis has the advantage of high stereospecifity and mild reaction conditions. The use of phytase has been shown to be very effective in producing different inositol phosphate species.

Different isomers of *myo*-inositol phosphates have shown pharmacological effects for the prevention of diabetic complications, anti-inflammatory effects [17, 26], and antiangiogenic and antitumor effects [92]. *Myo*-inositol phosphates are also known to ameliorate heart disease conditions by controlling hypercholesterolemia and atherosclerosis [64], and also prevent renal stone formation [44].

1.12 Future prospects

P: Both a curse and necessity

Peak P is the point in time at which the maximum global P production rate is reached. P is a scarce finite resource on earth and due to its non-gaseous environmental cycle has resulted in alternative means other than mining being unavailable. According to some researchers, Earth's P reserves are expected to be completely depleted in 50–100 years and peak P to be reached in

approximately 2030. It is this nutrient which is necessary to our well-being -- in fact our food security -- that we don't have in plentiful supply. P is an indispensable resource that has been mismanaged to the point that we are jeopardizing our long-term food and water security. The depletion of P is more relevant to our world today than the depletion of oil is. It is a major component in fertilizer, without which fertilizer will be rendered useless. Without fertilizer, two thirds of the world's population will starve because the Earth cannot support our demands for food. There are no alternatives to P and no synthetic ways of creating it. Without new sources for high quality mineable P agriculture will face major problems within the next 50-100 years. P is an essential ingredient in animal and plant production; however, too much or too little P can be a problem both for animal production and the environment. Phosphate recycling is essential for the sustainable future of our society as it is inconceivable to continue to simply throw away a non-renewable resource which is essential for life. As the need to conserve the world's phosphate reserves increases the role of phytase will broaden.

Role of phytase

Phytases are now being recognized for their beneficial environmental role in reducing the P levels in manure and minimizing the need to supplement P in diets. Their use as an animal feed additive is growing because it is ecofriendly. They have an immense importance in the feed and food industry because they also improve the nutritional status by degrading IP6, which acts as an antinutritional factor. Also, because lower inositol phosphates and phospholipids play important roles in transmembrane cell signaling and calcium mobilization from intracellular shock, an investigation into the potential role of phytase in this mechanism would be interesting. One area that offers tremendous opportunity is increasing the use of phytase in aquaculture in order to allow the use of low-cost plant meals. Other areas for expanded use range from the use of phytase as a soil amendment and its transformation into a peroxidase.

The growing demand for phytase is amply reflected by the multitude of sources screened for phytases with desired attributes. Identification of various phytases from diverse sources and their expression in heterologous systems need to be worked out not only to enhance the enzyme production but also to decrease the cost of production. Physicochemical properties of phytases, namely, broad pH range to survive under varied pH conditions in animal digestive tract, resistance to proteolytic degradation, thermal stability to resist higher temperatures during feed pelleting and substrate specificity, and so on need thorough evaluations to design versatile "second-generation" phytases with wider applicability. Modification and upgradation of enzymatic properties can be achieved through adoption of genetic and protein engineering methods. Combination of fungal and bacterial phytases as feed additives might improve the bioavailability of P and minerals owing to their synergistic activity in animal digestive system.

1.13 Lacunae and need for more research

There are various reports on phytase production by bacteria, yeast and fungi among which fungal phytases are widely employed in animal feed due to their acid tolerance and higher yield. All available phytase preparations used as feed additives today are of fungal origin and produced by recombinant strains in SmF and are active at pH 5. These preparations are expensive because of diluted product and high product recovery costs. In addition, they have important shortcomings, especially with regard to their sensitivity to heat and inactivation under low pH conditions generally present in the stomach.

Likewise production, downstream processing also is an integral part of any product development as the final cost of the product largely depends on the cost incurred in extraction and purification. Hence development of a viable process for phytase recovery and purification with techno-economic feasibility is necessary as the available chromatographic methods have several limitations.

Hence efforts are needed to alleviate these disadvantages while at the same time producing produce cost effective phytase with fast and economic downstream processing. SSF provides a more economic alternative for enzyme production and application as compared to SmF. But the reports are few because of the low productivities and difficulties associated with operating and up scaling SSF conditions. Earlier, we have reported phytase production by *Aspergillus niger* NCIM 563 under SSF using wheat bran with no additional nutrients [94]**.** Micro-organisms produce low levels of phytase and it would be beneficial if these production rates be improved with desirable process features by employing statistical techniques.

In this work, our objective is to evaluate application of statistical methods to increase the phytase activity under SSF and up- scaling to tray fermenter. The present work also reports the use of ATPE for separation and purification of phytase and compares it with the conventional chromatography process.

The same fungus produces two dissimilar phytase Phy I and Phy II under SmF [129]**.** Many studies on SSF and SmF for phytase have focused on process and fermenter design while the organism has been considered as a black box. The role of the physiological and genetic properties of the microorganisms producing phytase used during growth on solid substrates compared with aqueous solutions has so far been all but neglected. Hence we have tried to correlate different protein secretion in Smf and SSF and these studies can provide new insights to the existing "black box" of SSf/SmF biotechnology for phytase production.

1.14 **Objectives of the study**

"Studies on phytase from *Aspergillus niger* NCIM 563 under solid state fermentation and its correlation with submerged phytase I and II" was taken up with the following objectives:

- Production of phytase by *Aspergillus niger* NCIM 563 under solid state fermentation
- Downstream processing of solid state phytase from *A niger* NCIM 563
- Characterization and application of solid state Phy III from *A. niger* NCIM 563
- Correlation studies of solid state Phy III with submerged (Phy I and II) produced by *A. niger* NCIM 563

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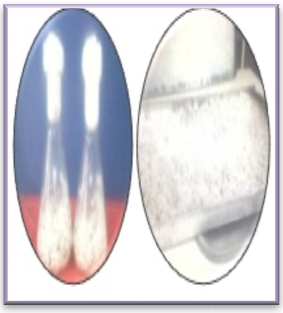

Chapter 2

Production of phytase by *Aspergillus niger* NCIM 563 under solid state fermentation

This chapter discusses high level phytase production by *Aspergillus niger* NCIM 563 in solid state fermentation (SSF) using an integrated statistical optimization approach involving the combination of Placket Burman design (PBD) and Box-Behnken design (BBD) was employed. PBD was used to evaluate the effect of eleven variables related to phytase production, and five statistically significant variables, namely, glucose, dextrin, $NaNO_3$, distilled water and $MgSO_4.7H_2O$ were selected for further optimization studies. The levels of five variables for maximum phytase production were determined by a BBD. Phytase production improved from 50 IU/g dry moldy bran (DMB) to 154 IU/g DMB indicating 3.08 fold increase after optimization. A simultaneous reduction in fermentation time from 7days to 4days shows a high productivity of 38500 IU/kg/day. Scaling up the process in trays gave reproducible phytase production overcoming industrial constraints of practicability and economics.

Part of the work presented in this chapter is published

1. Bhavsar KP, Ravi Kumar V, Khire JM (2011) High level phytase production by *A. niger* NCIM 563 in solid state culture: response surface optimization, up scaling and its partial characterization. J Ind Microbiol Biotech 38:1407-1417.

1. Introduction

P is an essential constituent of life like nitrogen, but, unlike nitrogen, P does not have a cycle to constantly replenish its supply. All animal diets must contain adequate amounts of this element. So to meet their P requirements, inorganic P especially dicalcium phosphate is supplemented in diet of livestock and poultry animals. This has made it as the third most expensive nutrient in poultry production after energy and protein. At the current extraction and usage rate, the existing phosphate reserves will be exhausted in next 80 years [30].

Bound P (18-88% of total P content) in fact exists as phytate which is already present in animal feed. But this phytate P is not utilized by monogastric animals like poultry and pigs due to lack of intrinsic phytase in their gastrointestinal tracts. Phytate in addition acts as an antinutrient by chelating various cations such as Ca^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+} and thereby reducing their bioavailability. This unutilized phytate is the origin of P pollution as it builds up in areas of livestock production leading to eutrophication and algal blooms [17].

So use of phytase in animal feed will seize the anti-nutritional effects of phytate, decrease environmental pollution, increase availability of starch, protein, amino acids, calcium and P and abolish the addition of inorganic phosphate in animal feed. They are also imminent candidates for production of special isomers of different lower phosphate esters of myo-inositol, some of which are considered to be pharmacoactive and important intracellular secondary messengers [28].The FDA has approved “generally recognized as safe (GRAS)” petition for use of phytase in food, and it has been marketed as an animal feed enzyme in US since 1996 [12]. All these factors have concurrently made it as the third largest feed enzyme [23].

There are various reports on phytase production by bacteria, yeast and fungi among which fungal phytases are widely employed in animal feed due to their acid tolerance and higher yield [29]. The existing commercial microbial phytases produced by submerged fermentation (Smf)

conditions are expensive because of diluted product, production using recombinant strains and high product recovery costs [11]. SSF provides a more economic alternative for enzyme production and application as compared to Smf.

There is no defined medium for optimum production of phytase from different microbial sources especially fungi because each fungus has its own special conditions and specific substrates for maximum enzyme production especially in SSF. Krishna and Nokes studied the effect of culture conditions, particularly inoculum age, media composition (wheat bran and full-fat soybean flour) and duration of SSF on the phytase production by *A. niger* [11]. Bogar et al reported phytase production by *A. ficuum* NRRL 3135, *M. racemosus* NRRL 1994 and *R. oligosporous* NRRL 5905 using various substrates such as canola meal, cracked corn, soybean meal, and wheat bran [2]. But the reports are few because of the low productivities and difficulties associated with operating and up scaling SSF conditions [3].

There is need for intensive research on SSF to develop a commercial process for phytase production with techno-economic feasibility. The intricacies in SSF technology can be understood through modeling, kinetics of growth of microbes, control of parameters, optimization, scale up and commercialization of the process for application. Before carrying out these studies it is however necessary to increase the phytase production and this can become significant by employing statistical optimization techniques rather than the conventional one variable at a time approach. This is because statistical optimization gives the optimum media formulation with minimum number of experiments in short time while also considering the interaction between selected components [25].

Earlier, we have reported phytase production by *A. niger* NCIM 563 under SSF using wheat bran with no additional nutrients [14]. The same fungus produces two dissimilar phytase under SmF [26]. In this work, our objective is to evaluate application of statistical methods to increase the phytase activity under SSF, partial characterization and up scaling to tray fermenter.

2. Materials and method

2.1 Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. All other chemicals used were of analytical grade. Various agriculture residues were purchased from a local market.

2.2 Identification of Fungal strain

For confirming the identification of the fungal strain, 5S ribotyping was carried out using universal fungal primers. The sequence analysis was done at Ribosomal Database Project (RDP) I & II and the Basic Local Alignment SearchTool (BLAST-n) analysis at National Centre for Biotechnology Information (NCBI) server. The alignment of the sequence was done using CLUSTALW program at European Bioinformatics site. The strain used throughout the present work was *Aspergillus niger* NCIM 563. It was maintained on Potato Dextrose Agar (PDA) slants. PDA contains (per litre distilled water: extract from 200 g potatoes; glucose, 20 g; Difco yeast extract, 1 g; and agar 20 g). At the same time slide culture was also done followed by microscopy.

2.3 Fungi and inoculum preparation

A. niger NCIM 563 was used in the present study from NCIM Resource Center, Pune, India. The stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C. Spores from 7 days old PDA slant were harvested using sterile distilled water containing 0.01% Tween 80 to obtain $5x10^7$spores/ml and used as inoculum for SSF.

2.4 SSF in Erlenmeyer flasks

The unoptimized medium contained 10g of agricultural residue moistened with 10ml distilled water in 250ml Erlenmeyer flask sterilized at 121^{o}C for 30min (Table 1). On cooling fermentation medium was inoculated with 1% spore suspension and incubated for 7days at 30°C. The optimized

fermentation medium for SSF contained 10g of wheat bran in 250ml Erlenmeyer flask plus glucose 3g, dextrin 1.25g, sodium nitrate 0.2g, magnesium sulphate 0.3g moistened with 20ml distilled water and sterilized by autoclaving at 121°C for 30min. On cooling fermentation medium was inoculated with 1% spore suspension and incubated for 4 days at 30°C. Enzyme production was expressed as enzyme activity IU/g DMB. All experiments were carried out in triplicates.

2.5 SSF in trays

50g, 100g, 200g and 1000g wheat bran was moistened with above mentioned optimized medium in Section 2.3 in enamel–coated metallic trays having dimensions 28x24x4cm, 45x30x4cm and 80x40x4cm. The trays were covered with aluminium foil and sterilized at 121°C for 20min. The cooled substrate was inoculated with 1% spore suspension of *A. niger* and incubated for 5days at 30°C. Samples were withdrawn aseptically each day from different parts of the tray to obtain representative composite samples.

2.6 Analytical methods

Phytase activity was measured at 50°C as described earlier [14]. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1μmol P per min under standard assay conditions.

Filter paper cellulase and xylanase activities were determined as reported earlier [10].α-amylase activity was determined by McCleary and Sheehan [16]. The reducing sugars were determined by using dinitrosalicylic acid (DNS) [15]. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of xylose, glucose or galacturonic acid/min under the assay conditions. Protein concentration in the culture filtrate was determined by the method of Lowry et al using bovine serum albumin as a standard [13]. Biomass determination was carried out by measuring the glucosamine content resulting from acid hydrolysis of the fermented substrate [27]. Glucosamine was determined by the method of Reissig and the dry mycelial weight was

calculated by assuming a mycelia glucosamine content of 139mg of glucosamine/g of dry mycelium [24]. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments.

2.7 Partial purification of phytase

The enzyme extraction from Koji was done as mentioned by Mandviwala [14].The filtrate obtained was subjected to ammonium sulphate precipitation (95% saturation) with constant stirring. The precipitate was collected by centrifugation (15000 xg, 20 min) and dissolved in minimum volume of above buffer. The enzyme was desalted by passing it through Sephadex G-25 column and fractions were estimated for phytase activity.

2.8 Electrophoresis

Native PAGE (8%) was performed at room temperature and 200 V for 2-3h [5]. Protein bands were visualized by silver staining [6].

2.9 Molecular weight determination

The molecular weight of native enzyme was estimated by gel filtration on Sephacryl S-200 (1x100cm) column equilibrated with 20mM acetate buffer, pH 5.5 using cytochrome C (12.4kDa), carbonic anhydrase (29kDa), bovine serum albumin (66KDa), alcohol dehydrogenase (150kDa) and β-amylase(200kDa) as standard proteins by the method of Andrews [1].

2.10 Characterization of phytase

The ammonium sulphate precipitated and desalted by Sephadex G-25 enzyme was used for characterization of partially purified phytase. The optimum pH was determined by measuring the activity between pH 2.0-10.0 using 200mM buffers; glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0–6.0), Tris-HCl (pH 7.0-8.0) and glycine-NaOH (pH 9.0-10.0) at 50°C. Stability assay was performed by incubating the partially purified enzyme at 30^{o}C for 24h in 50mM buffers of different

pH values glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0–6.0), Tris-HCl (pH 7.0-8.0) and glycine-NaOH (pH 9.0-10.0). The residual activity was then assayed using under standard assay conditions considering enzyme activity at zero time as 100%. The optimum temperature was determined over temperature range 45-60^{o}C. The thermal stability was studied up to 60^{o}C and the residual enzyme activity was determined using standard assay conditions and compared with the control without incubation.

2.11 Optimization using response surface methodology

The PBD was employed to identify the key ingredients and the conditions for the best yield of enzyme production [21]. Phytase production is influenced by media components, especially carbon and nitrogen sources, metal ions and physical variables such as pH, temperature, inoculum density, incubation time. The choice of variables was made based on studies employing Smf [26]. In the present study PBD was used to screen various variables, viz., glucose(A), dextrin(B), $NaNO_3$(C), distilled water(D), $MgSO_4.7H_2O$(E), $FeSO_4.7H_2O$(F), KCl(G), incubation period(H), inoculum level(J), Triton X(K) and $MnSO_4$(L). Total number of trials to be carried out were n+1 where n is the number of variables. Each independent variable was tested at two levels, a high (1) level and a low (2) level. Table 2 shows the variables and their levels used in the experimental design constructed using Design expert software (DES) Version 7.1.2, Stat-Ease, Minneapolis, MN, USA. The variables with more percent contribution were considered to influence phytase activity. It was calculated by adding the total sum of squares and then taking each term's sum of squares and dividing by the total to get a percentage.

Based on analysis of PBD results, five variables, viz., glucose(A), dextrin(B), $NaNO_3$(C), distilled water(D), $MgSO_4.7H_2O$(E) were chosen for further optimization by response surface methodology (RSM) using BBD. It is a good design for RSM studies because it permits estimation of the best fit parameters of the quadratic model, building of sequential designs and detection of lack of fit of the model [4]. The number of experiments (N) required for the development of BBD

is as $N=2k\ (k-1)+C_o$, (where k is number of variables and C_o is the number of central points). This was used to develop a mathematical correlation between five variables on production of phytase. Each variable was studied at three levels (1, 2, and 3). The values for each component at three levels studied in the BBD are shown in Table 3. The design matrix was constructed using DES to generate the response surface plot and optimum values for media formulation. The optimum values of the variables and the behavior of the system was studied using the quadratic equation model in DES. All experiments are carried out in triplicates and their mean values are presented.

3. Results and Discussion

3.1 Identification of the isolate

The 5S ribotyping and BLAST-n analysis of the 595 base pairs was done at the National Centre for Biotechnology Information (NCBI) server (Accession No. 68 DQ444287) which confirmed the identified organism as *Aspergillus niger*. Below is the sequence of the 5S r RNA gene fragment (595 base pair).

tccgtaggtgaacctccggaaggatcattaccgaatgtgggtctttgggcccaacctcccatccgtgtctattgtaccctgttg

cttcggcgggcccgccgcttgtcggccgccgggggggcgcctctgccccccgggcccgtgcccgccggagaccccaa

cacgaacactgtctgaaagcgtgcagtctgagttgattgaatgcaatcagttaaaactttcaacaatggatctcttggttccgg

catcgatgaagaacgcagcgagatgcgataactaatgtgaattgcagaattcagtgaatcatcgagtctttgaacgcacattg

cgccccctggtattccgggggggcatgcctgtccgagcgtcattgctgccctcaagcccggcttgtgtgttgggtcgccgtcc

ccctctccgggggggacgggcccgaaaggcagcggcggcaccgcgtccgatcctcgagcgtatggggctttgtcacatgc

tctgtaggattggccggcgcctgccgacgttttccaaccattctttaccaggttgacctcggatcaggtagggatacccgctg

aacttaagcatatcaataagcgg.

3.2 Phytase production using agricultural residues

Wheat bran gave highest phytase production of 50 IU/g DMB on 7thday of fermentation as compared to groundnut cake, coconut cake, cotton cake and rice bran (Table 1). As wheat bran is cheaper substrate, economical and supported maximum phytase production, it was selected for optimization experiments.

Table 1 Properties of crude and partially purified phytase from *A.niger* NCIM 563

Enzyme	Properties	Values
Crude	Production using agriculture residues	(IU/gm DMB)
	Ground nut cake	36.21
	Coconut cake	18.28
	Cotton cake	38.56
	Wheat bran	50.0
	Rice bran	6.8
	Production in statistically optimized media	
	Phytase	154.8
	Xylanase	133.2
	Cellulase	41.58
	Amylase	310.34
	Up scaling using optimized media	
	10g WB in 250 ml flask	154
	50g WB in 28x24x4cm tray	150
	100 g WB in 28x24x4cm tray	149
	200g WB in 45x30x4cm tray	151
	1000g WB in 80x40x4cm tray	148
Partially purified	pH	
	Optimum	6.0
	Stability	2.0-9.5
	Temperature	55^{o}C
	Optimum	20% residual activity
	Stability	at 60^{o}C after 1 hr
	Molecular mass	
	Gel filtration	87kDa

3.3 Optimization of phytase production by RSM

The eleven chosen variables (Table 2) likely to be influencing phytase production were optimized using PBD.

Table 2 PBD for selected factors and their assigned levels for phytase production with *A. niger* NCIM 563 in SSF

Code	Variable	Units	Level 1	Level 2
A	Glucose	w/w %	10	50
B	Dextrin	w/w %	5	20
C	$NaNO_3$	w/w %	2	8
D	DW	ml	100	200
E	$MgSO_4.7H_2O$	w/w %	1	5
F	$FeSO_4.H_2O$	w/w %	1	5
G	KCL	w/w %	1	5
H	Incubation period	days	4	8
J	Inocula level	ml	$1x\ 10^5$	$1x\ 10^9$
K	Triton X	v/v %	0.5	1
L	$MnSO_4$	w/w %	1	5

The PBD design matrix for experimental design of eleven selected variables is shown in Table 3 along with their responses on phytase production. Maximum phytase production of 110 IU/g DMB was observed in trial number 11. The variables showing high percent contribution were considered as significant variables influencing phytase production, by fitting to a linear model assuming the absence of interactions.

Table 3 Experimental designs used in PBD studies for eleven selected independent factors and also experimental values of phytase production

Trial number	Factor level											Phytase production (IU/gm DMB)
	A	B	C	D	E	F	G	H	J	K	L	
1	1	1	2	1	2	2	1	2	2	2	1	6.93
2	2	2	2	1	1	1	2	1	2	2	1	3.73
3	2	2	1	2	2	2	1	1	1	2	1	36.44
4	2	1	2	2	1	2	2	2	1	1	1	57.68
5	1	1	1	1	1	1	1	1	1	1	1	33.41
6	2	1	1	1	2	1	2	2	1	2	2	22.54
7	2	2	1	1	1	2	1	2	1	1	2	8.53
8	2	1	2	2	2	1	1	1	2	1	2	21.61
9	1	2	2	1	2	2	2	1	1	1	2	11.07
10	1	2	1	2	2	1	2	2	2	1	1	31.74
11	1	1	1	2	1	2	2	1	2	2	2	110.40
12	1	2	2	2	2	1	1	2	1	2	2	53.49

The culture was grown under solid state fermentation condition at 300C as described in Material and methods. The values given are the average of three independent experiments.

A-Glucose, B-Dextrin, C- $NaNO_3$, D-DW, E- $MgSO_4.7H_2O$, F- $FeSO_4.7H_2O$, G-KCL, H-Incubation period, J- Inoculum level, K-Triton X100, L- $MnSO_4$

Thus it was found that phytase production was influenced by glucose(A), dextrin(B), $NaNO_3$ (C), distilled water(D), $MgSO_4.7H_2O$(E) as seen from the analysis of half normal plot and the total sum of squares and percent contribution respectively (Table 4). The remaining variables were observed to have small percent contribution and they were therefore considered insignificant. Magnesium sulphate has also been identified as an important variable for phytase production by thermophilic mold *Sporotrichum thermophile* using PBD by Singh and Satyanarayana [29].

Moisture content of the medium in SSF is very important for the growth of microorganisms, production of enzyme and for enzyme activity [22]. It was found that distilled water contributed the most to phytase production as observed from the analysis of PBD results.

Table 4 Analysis of PBD for % contribution of each variable and its effect on phytase production by *A.niger* NCIM 563

Variable code	Variable	Sumof squares	% contribution
A	Glucose	776.23	7.85
B	Dextrin	964.15	9.75
C	$NaNO_3$	653.70	6.61
D	DW	4224.32	42.72
E	$MgSO_4.7H_2O$	1562.06	15.80
F	$FeSO_4.7H_2O$	347.06	3.51
G	KCL	490.85	4.96
H	Incubation period	106.46	1.08
J	Inoculum level	83.66	0.85
K	Triton X	402.44	4.07
L	$MnSO_4$	277.38	2.81

The best fit linear regression model used for modeling gave a model F-value of 5.75. This implies that there is only 0.01 % chance that this "Model F-Value" could occur due to noise. The coefficient of determination was obtained as R^2 = 0.873 and shows that the model used for analyzing the data is significant.

The five medium components (Table 5), identified above as significant variables for phytase production by PBD, were further optimized by RSM using BBD.

Table 5 Selected factors and their assigned levels by BBD for phytase production with *A.niger* NCIM 563 in SSF

Sr no	Variables with designate	Level 1	Level 2	Level 3
1	A Glucose (w/w %)	10	30	50
2	B Dextrin (w/w %)	5	12.5	20
3	C $NaNO_3$ (w/w %)	2	5	8
4	D Distilled water (ml)	100	150	200
5	E $MgSO_4.7H_2O$ (w/w %)	1	3	5

Table 6 represents the design matrix of the five significant variables in coded levels, and reports the experimentally obtained phytase activity. It may be seen that trial number 20 showed the highest phytase activity.

Table 6 BBD for optimization of selected variables and obtained experimental values of phytase production by *A.niger* NCIM 563 in SSF

Trial number	Factor level					Phytase production (IU/gm DMB)
	A Glucose	B Dextrin	C $NaNO_3$	D DW	E $MgSO_4$	
1	2	3	2	2	3	43.22
2	2	2	2	2	2	34.23
3	2	2	2	2	2	33.62
4	2	2	2	1	1	5.01
5	2	2	3	3	2	125.39
6	1	2	1	2	2	90.83
7	3	2	1	2	2	9.08
8	3	2	2	2	3	44.02
9	3	2	2	3	2	90.86
10	2	2	1	1	2	22.81
11	3	2	3	2	2	32.47
12	2	2	3	2	1	22.90
13	3	2	2	1	2	39.12
14	2	2	3	1	2	12.30
15	3	1	2	2	2	6.06
16	2	2	2	2	2	32.72
17	2	1	1	2	2	14.33
18	2	2	1	2	3	34.68
19	1	2	2	2	1	59.98
20	2	2	1	3	2	139.53
21	3	2	2	2	1	4.85
22	2	2	2	3	3	108.85
23	2	1	2	2	3	36.28
24	2	2	2	1	3	32.01
25	2	1	2	3	2	77.5
26	2	3	1	2	2	42.2
27	2	3	2	3	2	105.20
28	1	2	2	1	2	7.36
29	1	2	2	2	3	38.68
30	2	2	2	3	1	126.46
31	1	1	2	2	2	18.70
32	2	2	3	2	3	65.63
33	3	3	2	2	2	6.62
34	2	1	2	1	2	5.84
35	2	3	2	1	2	23.61
36	2	3	3	2	2	13.42

37	2	1	2	2	1	35.21
38	1	2	2	3	2	126.10
39	2	2	2	2	2	31.22
40	1	2	3	2	2	32.01
41	2	2	1	2	1	57.36
42	2	1	3	2	2	51.49
43	2	3	2	2	1	35.75
44	2	2	2	2	2	31.21
45	1	3	2	2	2	30.14
46	2	2	2	2	2	33.45

The culture was grown under SSF conditions at 300C as described in Material and Methods. The values given are the average of three independent experiments

The experimental results obtained for phytase production were fed into the DES and analysis was carried out using backward elimination regression. The calculated regression equation for the optimization of medium components showed the phytase activity (*Y)* as a function of these variables. By applying multiple regression analysis on the experimental data, the following quadratic model was found to explain phytase production:

$$Y=31.04-10.66A+3.42B-3.45C+46.99D+3.50E+20.54AC-6.75AD+15.10AE-16.48BC+16.35CE-11.15DE-9.30B^2+9.10\ C^2+32.56D^2+7.80E^2 \quad [a]$$

where, *Y* is the predicted response and *A, B, C, D* and *E* are variables.

The results were analyzed using ANOVA as appropriate to the experimental design used. The quality of the model was checked using various criteria. The coefficient of determination (R^2) is 0.9499 for phytase production, suggesting that 94.99% of the variability is explained in the model. The value of correlation coefficient (predicted R^2) for phytase production was 0·8685, which suggests a strong agreement between the experimental and predicted values of phytase production. The model *F* value of 37.94 and values of $p>F$ (<0.0001) indicated that the model terms are significant. For phytase production, *A, D, AC, AD, AE, BC, CE, DE,* B^2, C^2, D^2 and E^2 are significant model terms. The results of the ANOVA study are presented in the Table 7.

Table 7 ANOVA for response surface reduced quadratic model of BBD

Source	Sumof Squares	Mean Square	F-value	p-value Prob >F
Model	57056.16	3803.74	37.94	<0.0001
A	1819.43	1819.43	18.15	0.0002
B	187.26	187.26	1.87	0.1819
C	190.26	190.26	1.9	0.1785
D	35328.37	35328.37	352.39	<0.0001
E	195.54	195.54	1.95	0.1728
AC	1687.96	1687.96	16.84	0.0003
AD	1122.38	1122.38	11.2	0.0022
AE	911.44	911.44	9.09	0.0052
BC	1086.75	1086.75	10.84	0.0025
CE	1069.64	1069.64	10.67	0.0027
DE	497.44	497.44	4.96	0.0336
B^2	816.23	816.23	8.14	0.0078
C^2	780.18	780.18	7.78	0.0091
D^2	9995.29	9995.29	99.70	<0.0001
E^2	573.38	573.38	5.72	0.0233
Lack of fit	2999.40	119.98	73.45	<0.0001
Pure error	8.17	1.63		
Residual	3007.57	100.25		
Cor Total	60063.73			

The results show a strong agreement between the predicted and the experimental response. The optimum values of the tested variables are obtained as glucose 3g, dextrin 1.25g, $NaNO_3$ 0.2g, $MgSO_4.7H_2O$ 0.3g moistened with 20ml distilled water per 10g of wheat bran with the rest of the variables kept at mean value of the corresponding ranges in PBD. The enzyme production behavior was then studied under optimized fermentation conditions by monitoring in time for 6 days. An unoptimized media showed a phytase production of 50 IU/g DMB on 7^{th}day while optimization studies gave phytase production of 154 IU/g DMB on 4^{th}day itself. The rapid growth of fungus is corroborated by the corresponding increase in the mycelial weight (Fig 1).

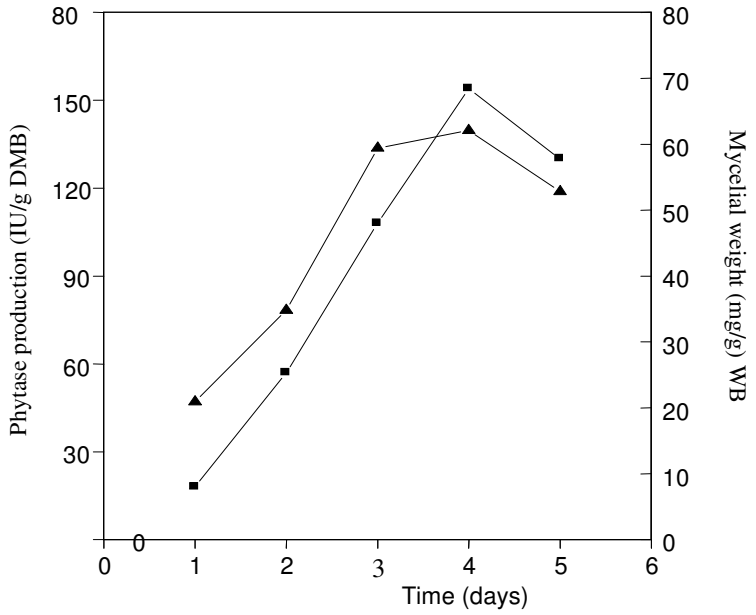

Fig1 Kinetics of phytase production in solid state fermentation by *A. niger* NCIM 563 under optimized conditions

Phytase production (IU/g DMB) (---■---); Mycelial wt (mg/g WB) (---▲---)

Thus the phytase activity is increased by 3.08 times with a simultaneous reduction of fermentation from 7 days to 4 days. The three-dimensional plots showing the optimal levels and nonlinear interactions among the variables for enzyme production is presented in Figs 2a and 2b. The maximum predicted value for phytase production was 123.14 IU/g DMB, while the experimental response was 139.53 IU/g DMB (Run number 20 in Table 6). It may also be noted that optimization using PBD and central composite design in case of *A. ficuum* gave phytase activity of only 15 IU/g using wheat bran (Table 8) [2].

Table 8 Comparison of phytase production by *A.niger* NCIM 563 with other fungal strains grown under SSF conditions

Phytase source	Phytase activity (IU/gm DMB)	Phytase productivity (IU/Kg/day)
Rhizopus pusilis	9.18	76
Rhizopus thailiandenism	3	38
Rhizopus oligosporous	5	75
Rhizopus microsporous	1	18
Mucor racemosus	26	361
Mucor hiemalis	12	160
Aspergillus niger	1008 [a]	4667 [a]
Aspergillus ficcium	15	159
A. niger CFR *335*	70	14000
*A. niger*NCIM *563*	**154**	**38500**

[a] Production using vegetative inoculum

The optimized results show a productivity of 38,500 IU/kg/day which is the highest as compared to other reported SSF using spore inoculum. Our experiments for phytase production using vegetative inocula did not give significant increase in production (data not included) but yet our productivity of 38,500 IU/kg/day is 8.3 fold more as compared to 4667 IU/kg/day reported for *A. niger* by Krishna and Nokes (Table 8). Moisture content in SSF plays a crucial role as can be seen from the above response surface analysis. But increasing the moisture content leading to semi-solid conditions did not enhance the activity any further. Thus the above optimized variables using response surface analysis gave the best conditions for maximum phytase production.

Fig 2a

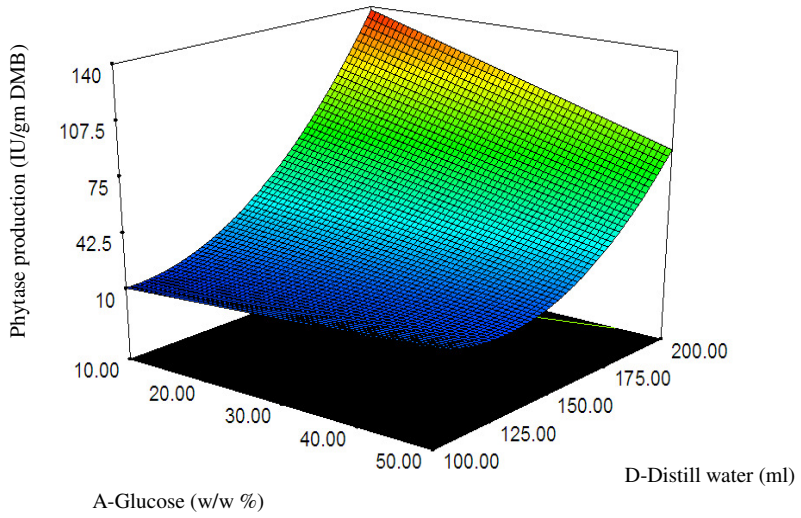

Fig 2b

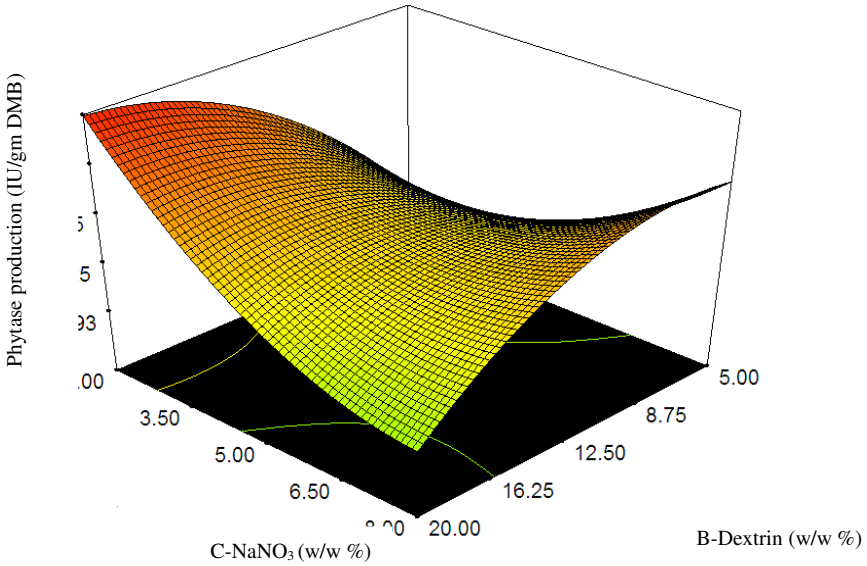

Fig 2 3D Surface plots reflecting the effect of glucose and distilled water (Fig 2a) and dextrin and sodium nitrate (Fig 2b) on phytase production of *A.niger* NCIM 563 in SSF.

A recommended 400 IU/kg feed supplementation would require only 2.59kg crude SSF enzyme of *A. niger* NCIM 563 to be added to each metric ton (MT) of feed which presently requires 16kg crude SSF enzyme of *A. ficuum* NRRL 3135. At present all available commercial phytase preparations are produced by recombinant strains using Smf. But they are costly as it requires concentration of diluted enzyme, extensive downstream procedures and treatment of generated effluents that do not make the process eco-friendly. All this restricts the use of Smf phytase in animal feed [20]. In contrast, as compared to Smf, the SSF enzyme as shown here is produced in large quantity along with hydrolytic enzymes and its application involves minimum downstream processing because the product can now be easily mixed with other ingredients in a feed ration [19]. So the process economy of the SSF enzyme appears to be favorable and eco-friendly. In this case a high productivity is achieved with a natural wild strain, and thus the possibility for genetic improvisation is also very good.

3.4 Up scaling and SSF in trays

SSF was performed in stationary trays analogous to conditions in traditional koji reactor as mentioned in Section 2.4. The procedure was up scaled from 10g wheat bran in 250ml Erlenmeyer flask to 1000g wheat bran in 80x40x4cm tray (Table 1). By scaling up from flasks to stationary trays, activities of 154 IU/g DMB were reproducibly obtained. These results are therefore encouraging for optimization under pilot scale conditions.

3.5 Production of accessory enzyme

The culture extract in addition to phytase consist of 133.2, 41.58 and 310.34 IU/g DMB of xylanase, cellulase and amylase respectively (Table 1). The supplementation of these enzymes along with phytase decrease viscosity, amount of loose droppings and increases mass gain, feed conversion and egg production [31]. The starch and nonstarch fractions of feed are efficiently hydrolyzed by hydrolytic enzymes improving prebiotic functional properties [18].

3.6 Partial purification and characterization of phytase

The ammonium sulphate precipitation and desalting by Sephadex G-25 procedure resulted in 69% enzyme recovery with purification of 2.5 folds and specific activity of 49.83 IU/mg of protein. The highest phytase activity was observed at pH 6.0. The enzyme retained ≈75 % activity over a wide pH range, 2.0-9.5 (Table 1). The pH optima and pH stability profile of phytase determines its ability to act efficiently in crop and stomach of digestive tract of poultry [8]. Fungal phytase acts efficiently in stomach (pH 2-5) and need reactivation to maintain activity in crop (pH 4-5) in digestive tract of poultry and bacterial phytases act vice versa [12]. The catalytic efficiency of *A. niger* NCIM 563 phytase will be more in both in crop and stomach of poultry because it retains activity over a wide pH range (2-9.5) and will not require reactivation.

The maximum of phytase activity was at 55^{o}C and declined thereafter (Table 1). Phytases from various *Aspergilli* show optimum temperature in the temperature range 40-65^{o}C [27]. Thermostability studies are performed to predict the stability of phytase during the dry pelleting process employed presently for commercial phytase produced under submerged condition. At 45^{o}C the phytase enzyme exhibited 90% of its original activity after 60 min. At 60^{o}C the enzyme exhibited 80% activity after 5 min and 20% activity after 60 min (Table 1). Phytase from *E. coli* (which is considered a candidate for commercial phytase) has also been reported to retain only 24 % activity at 60^{o}C for 1 hr [9]. This solid state product is therefore more efficient and cost effective than *E. coli* phytase. Because it does not require down streaming such as pelleting at high temperature and product formulation, the entire fermented product can be dried and ground and this also does not require high temperature. Therefore the step of pelleting at high temperature can be avoided and the dried product from food grade fungus can be sold as animal feed enzyme.

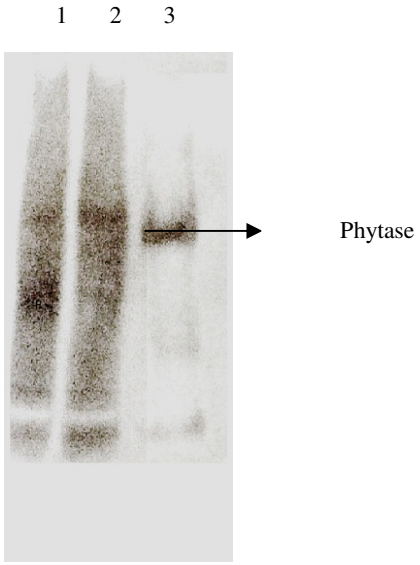

Fig 3 Native gel electrophoresis (8%) of partially purified phytase from *A. niger* NCIM 563

Lane 1- Crude; Lane 2- G25; Lane 3- partially purified

The partially purified phytase when subjected to gel filtration chromatography on Sephacryl S-200 along with standard markers showed its native molecular weight 87 kDa (Table 1). The partially purified phytase exhibited single band on non denaturing PAGE and is detected by activity staining (Fig 3).

4. **Conclusion**

The present work demonstrates that using response surface optimization employing PBD and BBD gave a high level of phytase production of 154 IU/g DMB along with accessory enzymes in SSF. Among the eleven chosen variables for optimization by PBD glucose, dextrin, $NaNO_3$, distilled water and $MgSO_4.7H_2O$ were found to influence phytase production more significantly. Some fungi are known to produce phytase and accessory enzymes by SSF but their low productivities are not comparable with the highest phytase productivity of 38500 IU/kg/day by *A. niger* NCIM 563 as shown by studies here. Tray fermentation studies for up scaling also gave promising results from an industrial point of view. The process can be improved using advanced bioreactors that provide accurate moisture and temperature control, as well as optimized O_2 mass transfer [7]. Instead of low yield, high cost Smf, SSF by *A. niger* NCIM 563 for phytase production emerges as a more efficient, less costly and a more directly applicable process. All these factors corroborate the commercial potential and industrial application of phytase produced by *A. niger* NCIM 563 as a solid state culture product.

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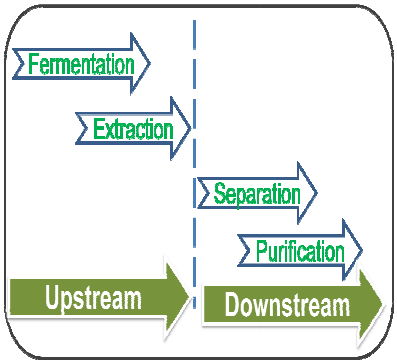

Chapter 3

Downstream processing of solid state phytase from *Aspergillus niger* NCIM 563

This chapter discusses the application of single step aqueous two-phase extraction (ATPE) for the downstream processing of phytase from *Aspergillus niger* NCIM 563, produced under solid state fermentation and compares it with the traditional multi-step procedure involving salt precipitation and column chromatography. High phytase recovery (98.5%) within a short time (3hrs) and improved thermostability was attained by ATPE in comparison to 20% recovery in 96hrs by chromatography process. The ATPE method, therefore, seems to be an interesting alternative for simultaneous partitioning and purification of phytase. The influence of system parameters; such as, phase forming salts, polymer molecular weight and system pH on the partitioning behavior of phytase was evaluated. The ATPE system consisting of combination of polyethylene glycol (PEG) 6000 and 8000 (10.5%) and sodium citrate (20.5%) resulted in one-sided partitioning of phytase in bottom phase with a purification factor of 2.5. This is the first report on phytase purification using liquid-liquid extraction and the results are likely to be beneficial in the poultry feed industry.

Part of the work presented in this chapter is published

1. Bhavsar KP, Ravi Kumar V, Khire JM (2012) Downstream processing of extracellular phytase from *Aspergillus niger*: Chromatography process vs. aqueous two phase extraction for its simultaneous partitioning and purification. Proc Biochem 47:1066-1072

1. Introduction

The cycling of P, a biocritical element in short supply, in nature is an important but slow biogeochemical process. P is a vital mineral important for bone and tissue growth in poultry. The massive growth of livestock production has made it the third most expensive nutrient in poultry production after energy and protein. Plants store P in the form of phytate (inositol 6-phosphate) carrying 6 phosphate groups. But this P present in seed grain as phytate is not readily available to mono-gastric animals, as they lack phytase activity. Phytate also acts as an antinutrient by chelating metal ions and reducing energy uptake [41]. To meet the P requirement, animal diets are generally supplemented with excess of commercial synthetic fertilizers. Human influences on the phosphate cycle come mainly from the introduction and use of these fertilizers. Strict norms for the excretion of large quantities of P effluents, human interference, decomposition of underutilized phytate cause phosphate pollution and price hike in synthetic fertilizers have currently led to the use of microbial phytase in animal feed [18].

Phytase (EC3.1.3.8) is an enzyme that acts by cleaving off 6 phosphate groups from inositol 6-phosphate and hence when added in feed has multiple benefits, mainly in increasing mineral, phosphorous and energy uptake. This decreases the necessity to fortify the fodder with above substances. It is estimated that 10kg dicalcium phosphate can be replaced by just 0.25kg of phytase and even then the worldwide demand for phytase in cattle feed is expected to be approximately 4000 tonnes/annum. In addition, they produce different myo-inositol phosphates that have novel metabolic effects, such as amelioration of heart disease by controlling hypercholesterolemia and atherosclerosis, prevention of renal stone formation, and reduced risk of colon cancer [15].

Hence efforts are needed to produce cost effective phytase with fast and economic downstream processing. The intricacies in phytase production technology can be understood through modeling, kinetics of growth of microbes, control of parameters, optimization, scale-up

and commercialization of the process. We have reported high level phytase production along with up-scaling studies under SSF by using response surface methodology [6]. Likewise production, downstream processing also is an integral part of any product development as the final cost of the product largely depends on the cost incurred in extraction and purification. Conventional procedures, including precipitation or chromatography, are currently employed for phytase purification. They have several limitations, such as, dilute concentration of enzyme, extensive downstream procedures and treatment of generated effluents. The process is also expensive, time consuming and difficult to scale-up. These traditional approaches are currently employed due to lack of alternative methods [27,32,38].

Thus there is a clear need for efficient, scalable and economical process for phytase bioseparation. One such purification method that meets all these criteria is liquid-liquid extraction [24, 37]. ATPE systems are an ideal technology where clarification, concentration, and partial purification can be integrated in just one-step. ATPE is composed of two different polymers or one polymer and one salt mixed at certain concentrations in an aqueous solution. The solution separates into two immiscible phases, with each dissolved component predominating in one or the other phase with water as a solvent in both phases. Due to higher water content, the ATPE systems have several advantages as compared to the commonly used separation and purification techniques, e.g., low interfacial tension, non-toxicity, non-flammability and biocompatibility [29, 5].

Polyethylene glycol (PEG)-salts systems and PEG-dextran systems have been introduced for large-scale protein separation. However, PEG-salt leads to faster separation than PEG-dextran because of the larger droplet size, greater difference in density between the phases, low viscosity and low cost. The industrial application of PEG-salt system is improved by the availability of commercial separators, which allows faster continuous protein separations [33]. PEG–potassium phosphate and PEG–magnesium sulfate are among the other frequently used polymer–salt systems.

These salts, however, lead to high phosphate or sulfate concentration in effluent streams, and are of environmental concern.

One way to reduce the amount of salt discharged into the wastewater is to substitute these inorganic salts by citrate, which is biodegradable and non-toxic and hence can be discharged directly into a wastewater treatment plant [39]. PEG-citrate systems have been applied to the recovery of few biomolecules [42], namely, for the recovery of α-amylase [43], penicillin acylase [1,22,23,], hexokinase [26], insulin [3], plasmid DNA [11,13,30] and monoclonal antibodies [4]. For the above reasons, we chose to study the suitability of ATPE system for phytase purification and to find the appropriate conditions concerning PEG molecular mass and citrate concentration.

The present work reports the use of ATPE for separation and purification of phytase and compares it with the conventional chromatography process. To our knowledge; this is the first attempt to evaluate purification methods for downstream processing of phytase from *A. niger* NCIM 563.

2. Materials and Methods

2.1. Materials

PEG of molecular weights 6000 and 8000 were procured from Himedia. All other chemicals were of analytical grade and Millipore water was used in all experiments. Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA.

2.2. Phytase production and extraction

The SSF medium contained 10g of wheat bran in 250ml Erlenmeyer flask plus glucose 3g, dextrin 1.25g, sodium nitrate 0.2g, magnesium sulphate 0.3g moistened with 20ml distilled water and sterilized by autoclaving at 121^{o}C for 30min. On cooling fermentation medium was inoculated

with 1% spore suspension of *A. niger* NCIM 563 and incubated for 4 days at 30°C. Enzyme production was expressed as enzyme activity IU/g DMB. Phytase extraction from koji was done as mentioned [21] and the specific activity of crude enzyme was approximately up to 5 U/mg of protein. The crude extract was stored at 4°C and used as and when required for the experiments.

2.3. Analytical methods

Phytase measurements were carried out at 50°C. The reaction mixture consisted of 3mM sodium phytate buffered with 100mM acetate buffer (pH 5.5). Enzymatic reactions were started by the addition of 50ml of enzyme solution. After 30min at 50°C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [14]. A freshly prepared solution of acetone: 5 N H_2SO_4: 10mM ammonium molybdate (2:1:1 v/v/v) and 400µl 1M citric acid was added to the assay mixture. Absorbance was measured at 370nm. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1µm P/min under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3–5% variability was observed. Concentration of protein was determined using Lowry method [19] using bovine serum albumin as standard.

2.4. Purification of phytase by ATPE

Predetermined and weighed quantities of polymer (PEG), salt (sodium citrate) and citric acid were used for obtaining the binodal curves estimated using cloud point method as described by Albertsson [2]. ATPE system is prepared in 15ml centrifuge tubes by adding the appropriate amount of PEG, salt and crude phytase enzyme. Distilled water was added to obtain 5g of the final weight. The contents were mixed thoroughly for 1h using magnetic stirrer for equilibration and was allowed to stand for phase separation. After clear phase separation, the top and bottom phases were collected and analyzed for protein concentration and enzyme activity according to the procedures

reported in the following sections. The experiments were conducted on micro-scale (5g in centrifuge tube) and macro-scale (100g in separatory funnel). All the experiments were repeated three times and average values are reported.

The partition coefficient, purification factor, activity recovery and phase volume ratios were calculated as follows:

Partition Coefficient

The partition coefficient in the aqueous two phase systems is defined as

$$Partition\ coefficient\ (K_e) = A_b/A_i \qquad (1)$$

where, A_b and A_i are the activities of phytase (IU/ml) in bottom phase and crude extract, respectively. Proteins were not partitioned in top phase but precipitated at the interface. The K_e is therefore calculated using the activities of protein and crude extract as employed in [28].

Purification Factor

The purification factor of phytase is defined as the ratio of specific activity of phytase in bottom phase to that of crude extract and calculated by the following equation:

$$Purification\ factor\ (PF) = \frac{A_b}{A_i} \times \frac{P_i}{P_b} \qquad (2)$$

where, P_i and P_b is the protein concentration in the crude extract and bottom phase, respectively and A_i and A_b are the activities of phytase (IU/ml) in crude extract and bottom phase, respectively [25].

Percentage Enzyme Activity Recovery

The enzyme activity recovery was calculated for the bottom phase because enzyme was preferentially partitioned to this phase.

$$Activity\ recovery\ (\%) = \frac{A_b V_b}{A_i V_i} \times 100 \qquad (3)$$

where, A_i and A_b are the phytase activities (U/ml) in crude extract and bottom phase, respectively. V_i and V_b are the corresponding volumes [25].

Phase volume ratio

The phase volume ratio is defined as the ratio of volume of the top and bottom phases

$$Phase\ volume\ ratio\ (V_r) = \frac{V_t}{V_b} \qquad (4)$$

where, V_t and V_b are the volumes of top and bottom phases, respectively [25].

2.5. Purification of phytase by chromatographic method

The purification of phytase by chromatography protocol was carried out in five steps:

Step 1- In the first step, 50ml of 2% aqueous solution of $CaCl_2 \cdot 2H_2O$ was added to fermented koji in flask and kept at 200 rpm for 2h at room temperature for extraction of enzyme. The suspension was squeezed through a double layer of muslin cloth and centrifuged at 5000xg for 20 min at 4^{o}C. The clear supernatant was designated as the crude enzyme preparation.

Step 2- Crude enzyme obtained was subjected to ammonium sulphate precipitation (95% saturation) with constant stirring. The precipitate was collected by centrifugation (5000xg, 20min) and dissolved in minimum volume of 50mM sodium acetate buffer, pH 5.5.

Step 3- The enzyme was desalted by passing it through Sephadex G-25 column and fractions were estimated for phytase activity and dialyzed overnight at 4^{o}C against the same buffer.

Step 4- The dialyzed solution was subjected to hydrophobic column chromatography using Phenyl-Sepharose CL-4B (30ml bed volume), previously equilibrated with 30% ammonium sulphate in 20mM acetate buffer, pH 5.5. The column was washed thoroughly with 20 bed volumes of the above buffer and eluted with a 120ml linear decreasing gradient of ammonium sulphate (30–0%) with a flow rate of 20ml/h and approximately 3ml fractions were collected. Fractions showing activity at pH 5.5 were pooled and estimated for phytase activity.

Step 5- The fractions were concentrated by rotavapor and loaded on a Sephacryl S-200 gel filtration column with a flow rate of 12 ml/h and 2ml fractions were collected. All the purification procedures were carried out at 4^{o}C.

2.6. SDS-PAGE

The purified samples obtained from ATPE and chromatography separation were analyzed by SDS-PAGE. The samples were boiled for 5min with the presence of 1% SDS, 80mM 2-mercaptoethanol, 100mM Tris–HCl buffer (pH 6.8) and 15% glycerol and loaded onto a 12% SDS polyacrylamide gel. After electrophoresis, the gels were stained with silver staining protocol [8].

2.7. Thermal stability of chromatography phytase versus ATPE phytase

The thermal stability was studied up to 60-80^{o}C and the residual enzyme activity was determined using standard assay conditions and compared with the control without incubation.

3. Results and Discussion

Phytase partitioning in a PEG-citrate ATPE system was studied systematically by varying one variable at a time (OVAT). Since solid state phytase has a pH optimum at around 5.6 and is stable

up to 40°C; all experiments were carried out at pH 5.6 and at room temperature (25°C). Using this approach, the effects of variables, viz., concentration of citrate, molecular weight and concentration of PEG, and the influence of process parameters such as pH, protein loading on phytase partitioning behavior was studied. Multistage extraction and polymer recycling experiments were also studied for the purification of phytase. The results obtained are discussed below.

3.1. Effect of sodium citrate concentration on phytase partitioning

In order to identify the citrate concentration suitable for purification of phytase, ATPE experiments were carried out by adding predetermined weighed quantity of PEG 6000 and different concentrations of citrate salt to crude phytase extract (1ml) making the total weight of the system 5g in 15ml centrifuge tube. The results are shown in Table 1. Phytase is selectively partitioned to bottom phase and the activity recovery was 86.8% with a *PF* and V_r value of 1.63 and 1.0, respectively. Due to the high molecular weight of PEG and molecular exclusion mechanism, phytase is preferentially driven to the salt phase while the other proteins precipitate at the interface because the solubility in PEG-rich phase is low.

Table 1 Effect of salt on phytase partitioning

Citrate concentration (%)	**Partition coefficient (K_e)**	**Specific activity (U/mg)**	**Activity recovery (%)**	**Purification factor (*PF*)**
25	0.76	16.0	76.0	1.52
20.5	0.87	16.3	86.8	1.63
18	0.71	14.3	71.0	1.35
15.2	0.62	13	61.9	1.30

Systems comprising high concentrations of polymer and salt have been reported to partition biomolecules at the interface due to the combined influence of both volume exclusion and salting out effects. Such effects have been observed and discussed for whey proteins [31] and alcohol dehydrogenase [20].

3.2. Effect of PEG molecular weight on phytase partitioning

It has been demonstrated that the molecular weight of PEG affects its distribution in the two phases and polymer–protein interactions [9]. In order to make selection of suitable molecular weight of PEG for purification of phytase, ATPE was performed with different molecular weights of PEG (MW 6000, 8000 and a combination of 6000 and 8000). Other parameters such as citrate, phase volume ratio, temperature and pH of system were kept constant as in Section 3.1. The partitioning coefficient, activity recovery, specific activity, and purification factor for phytase in bottom phase of ATPE with different PEG are presented in Table 2.

Combination of PEG 6000 and 8000 exhibits a better partition coefficient (K_e) of 0.96 with the highest activity recovery of 96% (Table 2). However PEG 6000 and 8000 at 10.5% exhibits lower activity recovery 90.5% and 86.8%, respectively.

Table 2 Effect of polymer molecular weight on phytase partitioning

Run number	PEG molecular mass	PEG Concentration (%)	Partition coefficient (K_e)	Activity Recovery (%)	Purification Factor (*PF*)
1	6000	9.5	0.42	41.8	0.68
2		10.5	0.90	90.5	2.05
3		11.5	0.39	39.9	0.66
4	8000	9.5	0.62	62.7	0.60
5		10.5	0.86	86.8	1.63
6		11.5	0.47	47.1	0.72
7	6000+ 8000	10.5	0.96	96.0	2.30

Phase system: PEG-citrate (20.5%), pH 5.6 at 25±2°C.

Lowering the average molecular weight of PEG is a strategy often employed to increase partition coefficient for the protein of interest and presumably occurs by lowering the hydrophobicity of PEG-rich phase. The molecular weight distribution of PEG can also be easily manipulated by mixing fractions of different average molecular weight PEG and was therefore studied. The mixture of PEG of different molecular weight and its distribution in ATPE influences the phase diagram [16] and results in reduction of free volume and low solubility for other proteins that now precipitate at the interface. Advantageously, phytase which has a high affinity for citrate is selectively partitioned to the bottom phase with high recovery of 96% and *PF* value of 2.3.

3.3. Effect of protein load on phytase partitioning

The protein content of crude extract loaded in ATPE can alter the partition behavior of target protein, i.e., phytase. Therefore, ATPE experiments were carried out by varying the protein loads up to 2.8mg protein/5gm ATPE (PEG 6000+8000) in centrifuge tube. Figure 1 illustrates the effect of protein load on phytase purification factor. Based on the results, crude protein load of 1.68mg gave the highest *PF* value of 2.4. Further, increase in protein content of crude extract results in a decrease of the ATPE performance as observed by the decrease in *PF* to a value of 2.2.

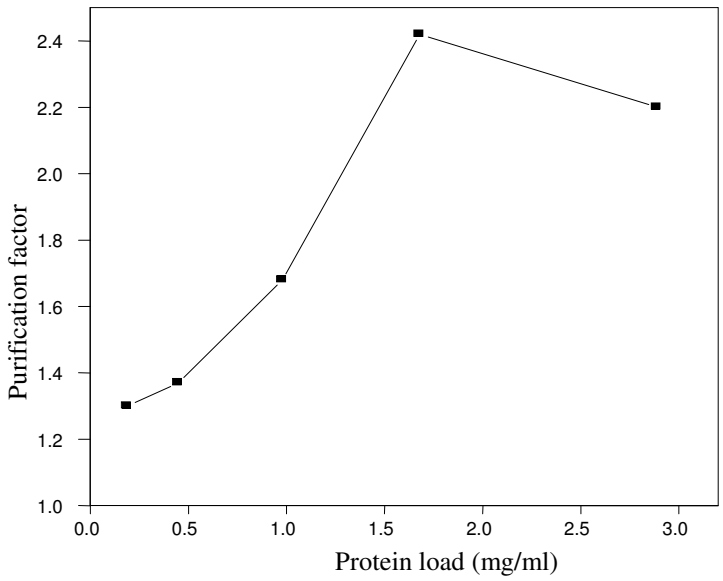

Fig 1 Effect of protein load on phytase partitioning

Purification factor (---■---)

Phase system: PEG (6000+8000)-citrate [10.5/20.5%] at 25±2°C. ATPE system was comprised of crude protein load ranging from 0.1 to 3.0 mg/ml.

This behavior can be explained by the increasing accumulation of precipitate at the interface that affect protein and phytase partitioning and gives low *PF* value for phytase. The process studied on micro-scale (5g) was successfully scaled-up to 100g on macro-scale in a separatory funnel. It is

clear that a maximum of 33mg crude enzyme in 100g ATPE gives the maximum recovery of phytase and this result is therefore encouraging from the industrial point of view.

3.4. Effect of pH on partitioning of phytase

The pH could affect the partition either by changing the charge of the solute or by altering the ratio of the charged species present. To study the influence of pH on phytase partitioning for the selected phase system (PEG 6000+8000-citrate), experiments were performed in the pH range 5.0-6.5 and the results are presented in Table 3. These pH values were chosen so that the aqueous phase is neither too acidic nor basic because the solutions cannot be discharged to the environment without further treatment. The pH of ATPE was controlled by adjusting the ratio of sodium citrate to citric acid and thus regulating the ratio of trivalent to divalent citrate ions.

Table 3 Effect of pH on phytase partitioning

pH	Specific activity (IU/mg)	Activity recovery (%)	Purification factor (*PF*)
5.0	9.2	50.0	1.75
5.6	14	98.5	2.50
6.0	6.12	92.6	1.20
6.5	6.0	90.6	0.99

Phase system: PEG 6000+8000-citrate, 10.5 / 20.5 (%) at 25±2°C

The iso-electric point (pI) of phytase obtained from solid state fermentation is unknown. But all the fungal, bacterial and plant phytase enzymes investigated so far have acidic pI values in the range of 3.65-5.2 for *Aspergillus* spp [40]. The PEG-citrate system are known to have almost no ability for resolving proteins on the basis of surface charge differences because no relation is found

between K_e and charge density. Instead the trivalent to divalent ratio of citrate ions in PEG-citrate system play a major role in separation [10]. Therefore it may be concluded that the enhanced affinity for the bottom phase is due to decrease of the salting-out effect in the lower phase and less hydrophobic interaction in upper PEG-rich phase rather than the effects of surface charge on the protein. At pH 6.5 and 5, the activity recovery is 90% and 50%; respectively, with an optimum pH 5.6 for the efficient separation and purification of phytase as seen in Table 3.

Thus, the optimal ATPE system for phytase purification consisted of 10.5% of PEG 6000+8000 and 20.5% of citrate at pH 5.6 as seen in Table 3. The same system also provided the best activity recovery (98.5%) in the bottom phase with a *PF* value of 2.5. The addition of different inorganic salts, such as NaCl and KCl was studied as these salts are often used to improve and direct selectivity partitioning between the phases. These salts help in increasing the hydrophobicity and promote the partitioning of hydrophobic proteins in polymer-rich phase [34, 36]. But no further improvement in phytase partitioning is observed upon addition of neutral salts in optimized ATPE indicating that the composition of the PEG-citrate is not influenced by them.

3.5 Multistage extraction and recycling of PEG for the purification of phytase

Multistage extractions were carried out to increase the purity of phytase where beginning from the optimized ATPE system (Table 3, pH 5.6) repeated extractions were performed. In the second extraction, 10.5% PEG (6000+8000) was added to the bottom phase of the first extraction. The concentration of PEG was chosen to obtain a V_r of 1.

Table 4 Multistage extractions for purification of phytase

Extraction	Partition coefficient (K_e)	Specific activity (IU/mg)	Activity recovery (%)	Purification factor (*PF*)
1	0.98	14	98.5	2.50
2	0.97	15.5	97.0	3.10
3	0.80	18	80.0	3.6
Crude	-	5	-	-

Phase system: PEG 6000+8000-citrate, 10.5 / 20.5 (%) at 25±2°C

The aliquots of the separated phases after the second extraction were analyzed for *PF* of phytase. In the third extraction, the same procedure was followed using the bottom phase of the second extraction. It was found that in the third extraction, the *PF* increased to 3.6 from 2.5 (i.e., by a factor of 1.4) with 80% enzyme activity recovery (Table 4). Further extraction attempts resulted in decreased activity recovery due to lower concentrations of citrate salt affecting the pH.

Recycling the components of both phases is important to assure the low-cost of this process and provides an environment-friendly way to avoid unnecessary disposal of chemicals. As phytase is preferentially extracted into the salt phase, the PEG-rich phase may be reused. To study the effect of polymer recycling, used polymer (PEG 6000+8000) was subjected to reuse for subsequent extractions, i.e., up to 8 cycles. It was observed that in the latter cycles. i.e., 5–8, partitioning of phytase to the salt-rich phase was affected with low activity recovery. However, limited recycling of the PEG-rich top phase, i.e., up to four cycles, has a minimum effect on the enzyme activity recovery (98.5%). The result shows that recycling of PEG is possible and this feature can help in process economics.

3.6. Chromatography protocol

The enzyme was extracted from the fermented koji and subjected to ammonium sulphate precipitation and desalting by Sephadex G-25 procedure which resulted in 69% enzyme recovery with purification factor of 2.5 and specific activity of 49.83 IU/mg of protein. The enzyme was further purified by adsorption and elution ion-exchange chromatography on Phenyl-Sepharose CL-4B, followed by gel filtration on Sephacryl S-200. A purification factor of 24.89 was possible although with a low phytase yield of 20%.

3.7. Comparison of ATPE and chromatographic phytase: Separation and purification

It was found that the obtained purification parameter for phytase is higher in ATPE protocol (Table 5). The chromatographic separation involves four major steps (ammonium sulphate precipitation, desalting, hydrophobic ion chromatography and gel filtration), two steps of dialysis and one step of conditioning for obtaining the required concentration.

Table 5 Comparison of down streaming process of phytase by chromatography and ATPE protocol

Property	Chromatography protocol	ATPE protocol
Purification process	Commonly employed	First report
Basis of enzyme separation	Ionic interactions and molecular weight	**Enzyme:** Size, shape, charge, pI, MW, hydrophobicity. **Phase system:** Concentration and MW of polymer/salt, pH, protein load, temperature, addition of salt/specific ligand
Pre-treatment of crude enzyme	Repeated centrifugation due to presence of solid particles	Not required
Capacity	Low	High
Operation Temp	Low	Room temperature
Operation Time (hrs)	96	3
Phytase recovery (%)	20	98.5
Maintenance	Cleaning in place (CIP), Storage and sanitization is mandatory	Less
Unit operations	7	1
Phytase enzyme	Thermo-labile	Thermo-stable

The multi-step chromatography process with long processing times (96hrs) however appears to less suitable for phytase purification. Besides it has several other shortcomings such as enzyme

pretreatment, low yield (20%), complex scale-up, high material cost, low capacity and high labor cost.

In contrast, the ATPE system involves a single step operation and has several advantages such as no pretreatment, high yield (98.5%), easy scale-up, low material cost, high capacity and low labor cost. Furthermore, volume reduction, good reproducibility, shorter process time (3hrs), polymer recycling and scope for continuous operation are additional features. Thus, ATPE has considerable potential for the separation and purification of phytase as compared to chromatography process.

The purity of phytase in ATPE process was also comparable to that obtained from the chromatographic separation. Figure 2 shows the SDS-PAGE analysis of phytase obtained by ATPE and column chromatography. The purified enzyme from ATPE in bottom phase and chromatography methods appears as a single band on silver stained SDS-PAGE, corresponding to a molecular mass of 85kDa. The reduction in number of bands compared to the crude extract indicates the purification of phytase. Also, the top phase of ATPE did not show any protein bands as discussed earlier.

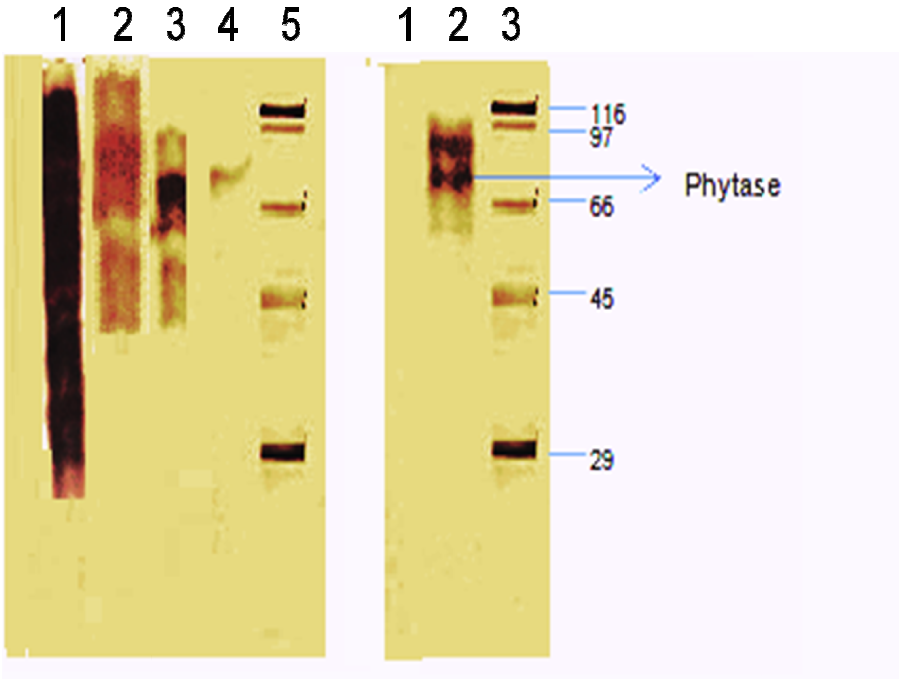

Fig 2 SDS PAGE

a) Purification steps of phytase using chromatography protocol.

Lane 1-Sample from crude extract; Lane 2-Sample from G25 column; Lane 3-Sample from Phenyl sepharose CL-4B column; Lane 4-Sample from Sephacryl column; Lane 5-Molecular standard markers

b) Purified enzyme by ATPE containing PEG 6000+8000- citrate (10.5 /20.5 %)

Lane 1-Top phase of ATPE system; Lane 2-Bottom phase of ATPE system; Lane 3-Molecular standard markers

The other known methods [12, 35] studied for the recovery and purification of phytase from different sources are compiled in Table 6. It clearly shows that the chromatography method seems to be less suitable for downstream processing of phytase and improvements are especially needed with respect to yield, purity, and energy consumption. In comparison, ATPE of phytase from *A. niger* NCIM 563 as shown here represents a simple and efficient process that has considerably improved upon all the above features.

Table 6 Recovery and purification of phytase using different separation techniques

Type	Source of phytase	Purification method	Number of steps	Phytase Recovery (%)
Bacteria	*Escherichia coli*	ASF, DS, IEC, HIC & GF	5	7
	Klebsiella terrigena	ASF, DS, IEC & GF	4	28
Yeast	*Schwanniomyces castellii*	Conc, Anion exchange & GF	3	-
	Candida krusei WZ-001	UF, IEC,HIC & GF	4	6.00
	Pichia. anomala	AP, IEC and DEAE-Sephadex	3	20.0
Plant	Oat	ASF, AP, IEC & GF	7	23.0
	Faba bean	ASF, AP, IEC and GF	7	6.0
Fungal	*A. ficuum*	UF, IEC &chromatofocusing.	3	58.0
	A. oryzae	UF, IEC 2 types and GF	4	17.7
	A. niger SK 57	UF, IEC 2 types, GF & CF	5	26.0
	A. niger ATCC 9142	UF, IEC, GF & CF	4	26.0
	Thermomyces lanuginosus	Lyophilization, DS, DEAE sepharose & GF	4	3.44
	A. ficuum NTG 23	Conc, IEC,DEAE cellulose, CM cellulose & GF	5	23.0
	***A. niger* NCIM 563**	**ASF, Desalting, HIC, and GF**	**4**	**20.0**
	(Present work)	**ATPE**	**1**	**98.5**

Acetone precipitation-AP, Ultra filtration-UF, Concentration-Conc, Desalting-DS, IEC-Ion exchange chromatography; HIC Hydrophobic ion chromatography; GF-Gel filtration; ASF-Ammonium sulphate fractionation; CF-Chromatography focusing

3.8. Thermostability studies

Thermostability is prerequisite for the successful application of phytase in animal feed because of exposure at 60–90°C during pelleting process for a few min. The poor thermostability of existing phytase enzymes is still a major concern for animal feed applications. Engineering of phytase and search for the determinants of its thermostability is of current research interest [7].

Phytase from *A. niger* is thermo-labile and it is assumed that the fermented koji be dried and then used in animal feed. In practice, the step of drying at high temperature and the presence of proteases lowers the phytase activity in the dried product. Our results show that the chromatography purified phytase is less thermo-stable at 60°C and 80°C as compared to ATPE purified phytase which exhibits maximum of 90% and 93% activities after 60 and 1min respectively (Fig 3).

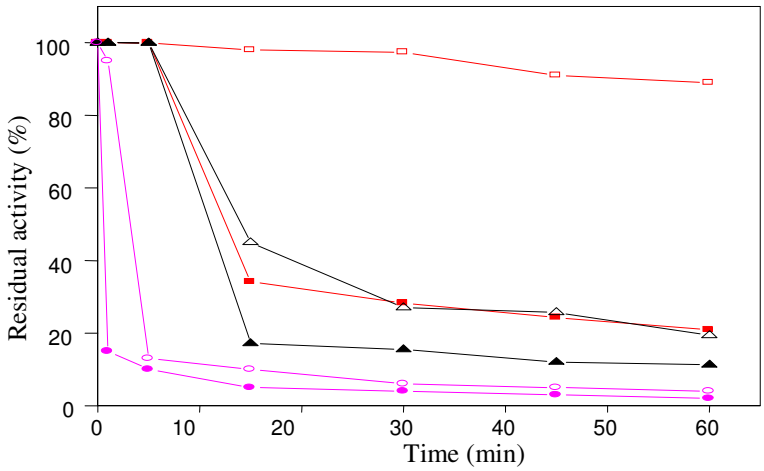

Fig 3 Thermostability studies

Residual activity of phytase purified by chromatography separation (---■---, ---▲--- and ---●---) and ATPE (---□---, ---Δ--- and ---○---) at 60°C, 70°C and 80°C, respectively.

In fact, ATPE purified phytase exhibits 4 times better thermostability profile at 60^{o}C as compared to chromatography purified process and is therefore likely to withstand the high temperature required for product formulation. The improvement in the thermostability of enzymes has earlier been reported [17] and ascribed to presence of salts and PEG which increases the heat transfer resistance and consequently protects the enzyme from heat.

4. Conclusion

Development of a viable process for phytase recovery and purification with techno-economic feasibility is necessary as the available methods have several limitations. This is the first report to show phytase extraction in a single step from fermentation broth by a liquid–liquid extraction process using ATPE. The speed and simplicity of this eco-friendly process with high throughput, improved thermostability and recycling of polymer are additional advantages. The results presented in this work show that the ATPE technique has considerable potential for the commercial development of an efficient process for separation and purification of phytase obtained from *A. niger*.

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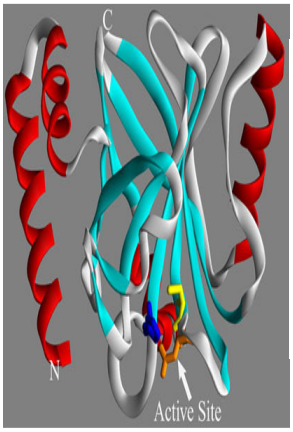

Chapter 4

Characterization and application of solid state Phy III from *Aspergillus niger* NCIM 563

This chapter discusses the biochemical and molecular characterization and application of phytase in synthesis of hollow silica nanocontainers in ionic liquids. A novel solid state phytase III was isolated from *Aspergillus niger* NCIM 563 with a procedure involving liquid liquid extraction and column chromatography. It possessed an optimal pH of 5.5 and an optimal temperature of 60^{o}C, and manifested a K_m and V_{max} of 0.156 mM and 220µm/min/mg. Phytase activity was moderately stimulated in presence of 5mM Ca^{2+}, Fe^{2+}, $Fe^{3+,}$ $Ba^{2+,}$ Pb^{2+} and inhibited in presence of 1mM Hg^{2}+, Ni^{2}+, Zn^{2}+, Cu^{2}+ and Ag^{2}+.The protein is a monomer and exhibited a molecular mass of 85kDa in gel filtration and SDS–PAGE. The aqueous two phase extracted phytase exhibited improved thermostability as compared to column chromatography. Phy III exhibited broad substrate specificity but had high affinity for sodium phytate. It was markedly inhibited by N-bromosuccinimide suggesting a possible role of tryptophan in its catalysis. Based on MalDI-LC-MS/MS identification amino acid sequences of the peptides, the enzyme did not show homology with any other known phytases from the literature suggesting its unique nature.

Part of the work presented in this chapter is communicated

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1. Introduction

P is an essential nutrient to biological systems. Its requirement is mainly seen in nucleic acids, cell membrane, bones and teeth. It is a main constituent of energy rich compounds such as ATP, ADP, GTP etc and serves as an energy conduit in various metabolic reactions. Despite its importance, P production, utilization and recycling is a slow process due to absence of gaseous phase and thus is therefore well-known as an imperfect cycle [20] .

Phytase are a special class of phosphatase that catalyzes the sequential hydrolysis of phytate to less phosphorylated *myo*-inositol derivatives and inorganic phosphate. Phytic acid (*myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) and mixed cation salts of phytic acid, designated as phytates, are a group of organic P (P) compounds found widely in nature [18]. Phytate phosphorous represents approximately 75-80% of total phosphorous in plant seeds and is largely unavailable to monogastric like poultry birds , pigs, fishes and humans due to lack of adequate levels of phytases. The phytic acid in plant derived food acts as an antinutritional factor since it causes mineral deficiency due to chelation of metal ions such as Ca^{2+}, Mg^{2+},Zn^{2+} and Fe^{2+} which form complexes with proteins and thus affect their digestion and also inhibit certain digestive enzymes like alpha amylase, trypsin, acid phosphatase and tyroninsae. Phytic acid excreted in faeces is degraded by soil microorganisms release P in soil which on reaching aquatic bodies that causes eutrophication [15]. Hence dephosphorylation of phytate by phytase is a critical process which overcomes the drawbacks of slow recycling and at the same time eliminating the anti nutritional effects of phytate. They can be produced from sources including plants, animals and microbes. Microbial sources however are promising for their commercial exploitation. Micro-organisms produce low levels of phytase and it would be beneficial if these production rates be improved with desirable process features by employing statistical techniques and strain improvement programs.

The available phytase preparations used as feed additives are of fungal origin, produced by recombinant strains under SmF and have shortcomings especially with regard to obtaining diluted product, sensitivity to heat, inactivation under low pH conditions present in the stomach of animals [2] and high product recovery costs. These disadvantages need to be alleviated while at the same time producing phytase with high yield and purity. Due to these limitations, the recovery, yield and purification of phytase represent a technological challenge that needs to be overcome to make the process economically viable for industrial application.

Based on the amino acid residue in the active site, phytate degrading enzymes can be referred to HAP, BPP, CP and PAP [19]. The phosphate residue of phytate is released by phytate degrading organisms at different rates and in different order. During the last few decades phytases have been studied extensively because of great interest in it application as feed additive and enviromental protection [25]. Current phytase research and its relationship to a contemporary environmental concern provide insights into budding applications that will promote additional research and development of this key biocatalyst. The potential of phytase in processing of human food and synthesis of lower inositol phosphate thereby improving human health have gained increasingly attention. Physicochemical properties of phytases, namely, broad pH range to survive under varied pH conditions in animal digestive tract, resistance to proteolytic degradation, thermal stability to resist higher temperatures during feed pelleting and substrate specificity, and so on need thorough evaluations to design versatile “second-generation” phytases with wider applicability. Further active research must, therefore, be directed to identify new native phytase proteins from diverse micro flora and plants [27] that would form the basis of creating consensus phytases using genetic and protein engineering approaches.

The immobilization of technologically important phytase onto solid supports is an area of extensive research because of their widespread applications in biomedicine as therapeutic peptide

loaded nanovehicles and in various industries as reusable biocatalysts [1, 10, 21]. However, one of the major challenges associated with enzyme immobilization is the retention of enzymatic activity after their immobilization onto a substrate. Previous studies have indicated that the enzyme activity can be preserved in ionic liquids (ILs) because of their unique solvent properties Bioscience is among the most interesting areas where ILs are just beginning to play an important role, with demonstrated applications in enzyme stabilization, protein crystallization, and biofuel cells. The unique solvent properties of ILs have led to the exploration of different biocatalytic reactions in Ils [28-30] as well as self-assembly of nonbiological amphiphiles (e.g., surfactants, lipids, block copolymers, etc.), However rhere are few reports exploring the potential of ILs as designer solvents for self-assembly of amphiphilic biomacromolecules [22].

We have earlier reported high levels of phytase by solid state fermentation using statistical techniques that involve a combination of PBD and BBD [5]**.** Te present investigation deals with characterization and immobilization of SSF phytase (Phy III) in silica hollow nanocontainers.

2. Materials and Methods

2.1. Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo. SDS–PAGE and gel filtration markers, Coomassie Brilliant Blue R-250 and Bromophenol Blue were purchased from Sigma Chemical Company, USA Sephacryl S-300, Phenyl-Sepharose CL-4B were obtained from Sigma.

2.2 Fungi and inoculum preparation

A. niger NCIM 563 was used in the present study from NCIM Resource Center, Pune, India. The stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4^{o}C.

Spores from 7 days old PDA slant were harvested using sterile distilled water containing 0.01% Tween 80 to obtain $5x10^7$ spores/ml and used as inoculum for solid state fermentation.

2.3. Phytase production and extraction

The SSF medium contained 10g of wheat bran in 250ml Erlenmeyer flask plus glucose 3g, dextrin 1.25g, sodium nitrate 0.2g, magnesium sulphate 0.3g moistened with 20ml distilled water and sterilized by autoclaving at 121°C for 30min. On cooling fermentation medium was inoculated with 1% spore suspension of *A. niger* NCIM 563 and incubated for 4 days at 30°C. Enzyme production was expressed as enzyme activity IU/g DMB. Phytase extraction from koji was done as mentioned and the specific activity of crude enzyme was approximately up to 5U/mg of protein. The crude extract was stored at 4°C and used as and when required for the experiments.

2.4. Analytical methods

Phytase measurements were carried out at 50°C. The reaction mixture consisted of 3mM sodium phytate buffered with 100 mM acetate buffer (pH 5.5). Enzymatic reactions were started by the addition of 50ml of enzyme solution. After 30min at 50°C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [9]. A freshly prepared solution of acetone: 5N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400µl 1M citric acid was added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1µm P/min under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3–5% variability was observed. Concentration of protein was determined using Lowry method using bovine serum albumin as standard [17].

2.5. Purification of Phy III

Purification of phytase was done by aqueous two phase extraction system as described earlier [6]. The system consisted of combination of polyethylene glycol (PEG) 6000 and 8000 (10.5%) and sodium citrate (20.5%) for partitioning of phytase.

2.6 Gel permeation chromatography

The molecular mass of purified enzyme was estimated by gel filtration. The gel filtration column of Sephacryl S-200 column equilibrated with 50mM sodium acetate buffer, pH 5.5 was calibrated using gel filtration standard molecular mass markers; cytochrome c (12.4kDa), bovine serum albumin (66kDa), alcohol dehydrogenase (150kDa), b-amylase (200kDa), apoferritin (443kDa) and thyroglobulin (669kDa).

2.7 Electrophoretic techniques, Zymogram analysis and mass spectrometry

Native PAGE (8%) was performed in a vertical gel apparatus at pH 7.5. Samples containing approximately 5-10µg of protein were applied to the gel and electrophoresis was carried out at 200V for 3–4h. Protein bands were visualized by silver staining (0.4% w/v) [7].

The subunit molecular mass was determined by SDS–PAGE in a vertical slab gel apparatus at pH 8.3 using Sigma high molecular mass markers [14]. The samples were boiled for 5 min with the presence of 1% SDS, 80mM 2-mercaptoethanol, 100mM Tris–HCl buffer (pH 6.8) and 15%glycerol and loaded onto a 12% SDS polyacrylamide gel. After electrophoresis, the gels were stained with silver staining protocol. Protein bands were detected either by Coomassie Brilliant Blue R-250 (0.2% w/v) or by silver staining (0.2% w/v).

Non-denaturing electrophoresis was carried out in the same manner, but with omission of SDS from the gel running and loading buffers, and the sample was not pre-treated under

denaturing conditions. Staining was carried out using Blue Coomassie R-250 or by zymogram analysis. The latter entailed overlay of the gel with molten agar (1.5% w/v) in 200mM sodium acetate buffer, pH 5.5, containing phytic acid (0.15% w/v). After overlay solidification, the gel was incubated at 55^{o}C for 12h, followed by flooding with calcium chloride (10% w/v). Bands of phytase activity were detected as translucent zones against an opaque background [3].

Mass spectral analysis was performed on a Voyager-De-STR (Applied Biosystems) MALDI-TOF. A nitrogen laser (337 nm) was used for desorption and ionization. Spectra were acquired in the range of 10–100 kDa, in linear mode with delayed ion extraction and with an accelerating voltage of 25kV. The low mass ion gate was set at 4,500Da. All the analyses were performed in four replications. The instrument was calibrated with myoglobulin and bovine serum albumin. In-gel tryptic digestion and analysis of peptides was done accordingly [4].

2.8 Effect of pH and temperature on phytase activity and stability

The optimum pH was determined by measuring the activity between pH 2.0-10.0 using 200mM buffers; glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0–6.0), Tris-HCl (pH 7.0-8.0) and glycine-NaOH (pH 9.0-10.0) at 50°C. Stability assay was performed by incubating the partially purified enzyme at 30^{o}C for 24h in 50mM buffers of different pH values glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0–6.0), Tris-HCl (pH 7.0-8.0) and glycine-NaOH (pH 9.0-10.0). The residual activity was then assayed using under standard assay conditions considering enzyme activity at zero time as 100%. The optimum temperature was determined over temperature range 45-60^{o}C. The thermal stability was studied up to 60^{o}C and the residual enzyme activity was determined using standard assay conditions and compared with the control without incubation.

2.9 Hydrolysis of soybean meal phytate in simulated gastric fluid

One gram soybean meal was dissolved in 9 ml simulated gastric fluid (SGF) [0.25 M glycine-HCl containing 2.0 mg/ml NaCl and 3.2 mg/ml pepsin] and pH was adjusted with HCl or NaOH to a final pH of 1.5, 2.0, 2.5, 3.5, 5.5 or 6.5. The solutions were incubated with agitation at 37°C for 30min, and pH was adjusted to the corresponding values again. Then 1ml partially purified enzyme was added to the solutions and incubated by agitation at 37°C for 60min. The amount of released P was determined by modified ammonium molybdate method as described in Section 2.5.

2.10 Effect of metal ions, inhibitors, detergents and organic solvents on phytase activity

The effect of various metal ions (1, 5 mM), sulfurhydryl compounds (1, 5 mM), chelating agents (1, 5 mM), detergents (0.1, 0.5%) and organic solvents (10%) on the enzyme activity by incorporating these into the reaction mixture.

2.11 Substrate specificity

Substrate specificity was examined by measuring the phytase activity with different phosphorylated substrates in place of sodium phytate. Enzyme activity was determined after incubating the enzyme with 3mM of each substrate under standard assay conditions.

2.12 Enzyme kinetics

The kinetic rate constants, V_{max} and K_m were determined with sodium phytate (0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mM) as substrate using Lineweaver–Burk plot. Data obtained were analyzed by creating: (i) a plot of initial velocities versus substrate concentrations (V_0 versus [S]); and (ii) a reciprocal plot of V_0 and [S] to make a Lineweaver-Burk plot for calculation of K_m and V_{max} [16].

2.13 Peptide sequencing

The enzyme was sent to Sutton Bonington Proteomics Facility, United Kingdom for MALDI-LC-MS/MS peptide sequence analysis. In-gel tryptic digestion was performed and the digested mixture was subjected to MALTI-TOF and LC-MS/MS for peptide mass fingerprinting and peptide sequencing. The data thus obtained was searched against the public database Swiss-Prot using the MS/MSIONS search tool on the MASCOT web site.

2.14 Synthesis of Hollow silica spheres using phytase enzyme as a template-

Ionic-Liquid-Mediated Synthesis of Phytase Capsules **-**0.5mL volume=490µL of the respective ionic liquid (IL) 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF4] +10µL purified phytase enzyme (1mg/ml dissolved in water), thus achieving a final enzyme concentration of 20µg/ml in the reaction. The IL-phytase mixture was incubated at 37^{o}C (24h with gentle reciprocal shaking, after which samples were centrifuged (14000rpm), followed by washing with deionized water and acetonitrile to remove the viscous IL. Phytase capsules thus obtained were further analyzed by TEM.

Ionic-Liquid-Mediated Synthesis of Silica Nanospheres-To obtain silica nanospheres, 20mM TEOS (silica precursor, tetraethyl orthosilicate) stock solution was prepared in the respective Ils. TEOS stock solution (500µL, 20mM) in ILs was added to 500µL of the reaction mixture containing IL and phytase capsules. The 1mL reaction contents were incubated at 37^{o}C (24h under stirring conditions, during which all reactions involving phytase became turbid, indicating TEOS hydrolysis. Samples were centrifuged at 14000rpm, followed by washing with deionized water and acetonitrile to remove the viscous IL. Silica nanostructures thus obtained were further analyzed by TEM.

3. Results and discussion

3.1 Purification of Phytase

The enzyme extracted from the SSF koji was subjected to aqueous two phase extraction resulting in one-sided partitioning of phytase in bottom phase with recovery of 98.5% and purification factor 2.5 within 3hrs.

3.2 Determination of molecular mass

Purification to homogeneity was confirmed by SDS-PAGE analysis and a single band was also observed on nondenaturing electrophoretic gels. The identity of this band on the non-denaturing gels as phytase was confirmed by zymogram analysis (Fig. 1). The subunit molecular mass of the purified phytase was estimated to be 85kDa by SDS-PAGE (Table 1).

Table 1 Biochemical properties of Phy III from *A. niger* NCIM 563

Property		Value
pH	Optimum	5.6
	Stability	2.0-9.5
Temperature	Optimum	60°C
	Stability	20% residual activity at 60°C after 1 hr
Molecular mass	Gel filtration	85kDa
	SDS-PAGE	85kDa
	MALDI-TOF	50.58kDa
Effect of metal ions	Stimulated (5mM)	Ca^{2+}, Fe^{2+}, Fe^{3+}, Ba^{2+}, Pb^{2}
	Inhibited (1mM)	Hg^{2+}, Ni^{2+}, Zn^{2+}, $Cu^{2+,}$ Ag^{2+}
Kinetic constants	K_m	0.156 mM
	V_{max}	220µm/min/mg

This molecular mass is similar to that of the purified phytase (phy A) from *A. ficuum* NRRL 3135, and falls well within the molecular mass range of previously purified phytases (38–200kDa) [8, 26]. The molecular masses of the native enzyme were determined to be 87 kDa on a calibrated 16/60 Sephacryl S-200 HR column with elution position being measured by determination of enzyme activity. These suggest that Phy III is a monomer of 85kDa, however, MALDI data revealed the molecular weight of Phy III to be 50.58 kDa (Table 1 and Fig. 1). This could be because of the doubly charged species of phytase during ionization process of MALDI-TOF spectrometry [13].

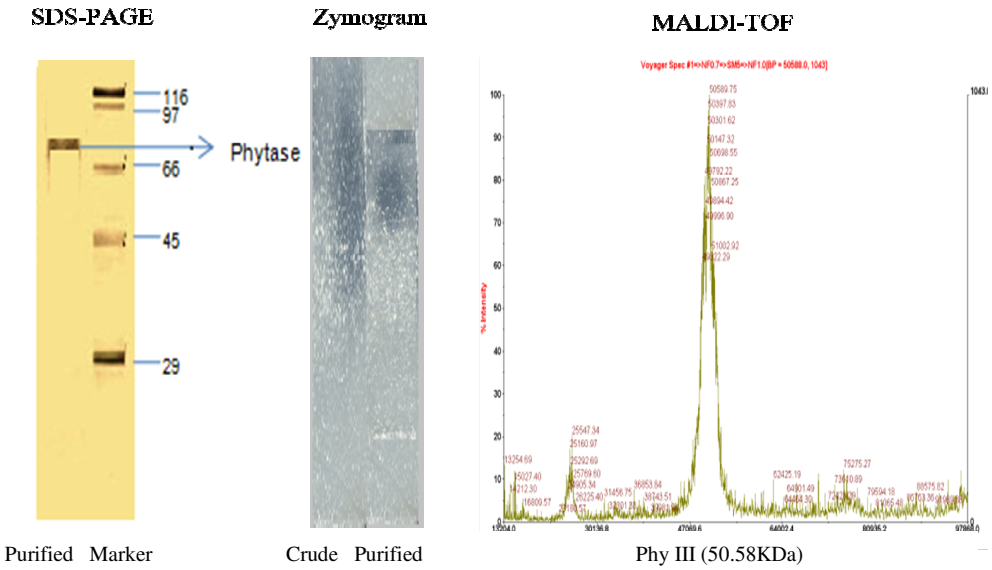

Fig. 1 SDS-PAGE, Zymogram and MALDI-TOF

3.3 Characterization of phytase from A. niger NCIM 563

The highest phytase activity was observed at pH 6.0. The enzyme retained ≈75% activity over a wide pH range, 2.0-9.5 (Table 1). The pH optima and pH stability profile of phytase determines its ability to act efficiently in crop and stomach of digestive tract of poultry. Fungal phytase acts efficiently in stomach (pH 2.0-5.0) and need reactivation to maintain activity in crop (pH 4.0-5.0) in digestive tract of poultry and bacterial phytases act vice versa. The catalytic efficiency of *A. niger* NCIM 563 phytase will be more in both in crop and stomach of poultry because it retains activity over a wide pH range (2.0-9.5) and will not require reactivation.

The maximum of phytase activity was at 55^{o}C and declined thereafter (Table 1). Phytases from various *Aspergilli* show optimum temperature in the temperature range 40-65^{o}C. Thermostability is prerequisite for the successful application of phytase in animal feed because of

exposure at 60–90^{o}C during pelleting process for a few minutes. The poor thermostability of existing phytase enzymes is still a major concern for animal feed applications. Phytase from *A. niger* is thermo-labile and exhibits 80% activity at 60^{o}C after 5min and 20% activity after 60min. It is assumed that the fermented koji be dried and then used in animal feed. In practice, the step of drying at high temperature and the presence of proteases lowers the phytase activity in the dried product. The improvement in thermostability using aqueous two phase extraction has been reported (Refer Section 2.5) and it exhibits 4 times better thermostability profile at 60^{o}C as compared to chromatography purified process and is therefore likely to withstand the high temperature required for product formulation.

Phytase activity was moderately stimulated in presence of 5mM Ca^{2+}, Fe^{2+}, $Fe^{3+,}$ $Ba^{2+,}$ Pb^{2+} and inhibited in presence of 1mM Hg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} and Ag^{2+} (Table 1). The enzyme retained 63% and 43% activity in presence of Zn^{2+} and Cu^{2+} in contrast to most phytate-degrading enzymes that are greatly inhibited by Cu^{2+} and Zn^{2+}. Actually the influence of zinc, iron, copper and calcium is potentially significant from an applied perspective with respect to phytase in animal feed. Retention of phytase activity in presence of Pb^{2+} (125% for 5mM) and Ag^{2+} (50% for 1mM) provides an opportunity for phytate hydrolysis in soils contaminated with heavy metals.

Table 2 Effect of various solvents, detergents and inhibitors on Phy III activity

Effect	Relative enzyme activity (%)	
Control	100	
Organic solvents	10% v/v	
Acetone	80.9	
Isoamyl alcohol	110.4	
Ethanol	94.5	
Hexane	105.6	
Acetic acid	98.7	
Glycerol	80	
Toluene	114.7	
Ethylene glycol	102.5	
Detergents	0.1%	0.5%
Tween 20	104.3	115
Tween 80	103.8	108.6
Triton X-100	100.5	102.5
SDS	18.9	31.7
Inhibitors	1Mm	5Mm
EDTA	102	100.9
Dithiothretiol	100.4	99.8
N-ethylmalemide	100	100
N-bromosuccinimide	0	0
PMSF	70.5	82.6
Iodoacetate	100	98.5

K_m and V_{max} was 0.156mM and 220µm/min/mg using Lineweaver Burk plot. These values fall well within the range previously reported for microbial phytases. The enzyme was insensitive to PMSF, ethylmaleimide and iodoacetate (Table 2). The reducing agents like DTT had no effect on enzyme activity suggesting that –SH groups are not involved in the catalytic activity or this enzyme does not have free and accessible –SH groups. Acetone, glycerol inhibited phytase

activity slightly while other solvents stabilized it. Among the non-ionic detergents, Tween 20, Tween 80, Triton-X 100 stabilized the enzyme activity, while the anionic detergent (SDS), even at low concentration severely inhibited the activity phytase. The SSF phytase of *A. niger* NCIM 563 was inhibited by NBS suggesting the possible role of tryptophan in catalysis. The amino acid in active domain of phytase from *A. ficuum* is arginine, histidine, and tryptophan [24]. In the phytase of *B. amyloliquefaciens,* lysine, histidine, and tryptophan is related to active domain [11].

The chelating agent EDTA did not show any observable effect on the catalytic properties of phytase. None of the characterized phytases are known to require metal ion for the activity except that of *Bacillus spp* which is calcium dependent [12].

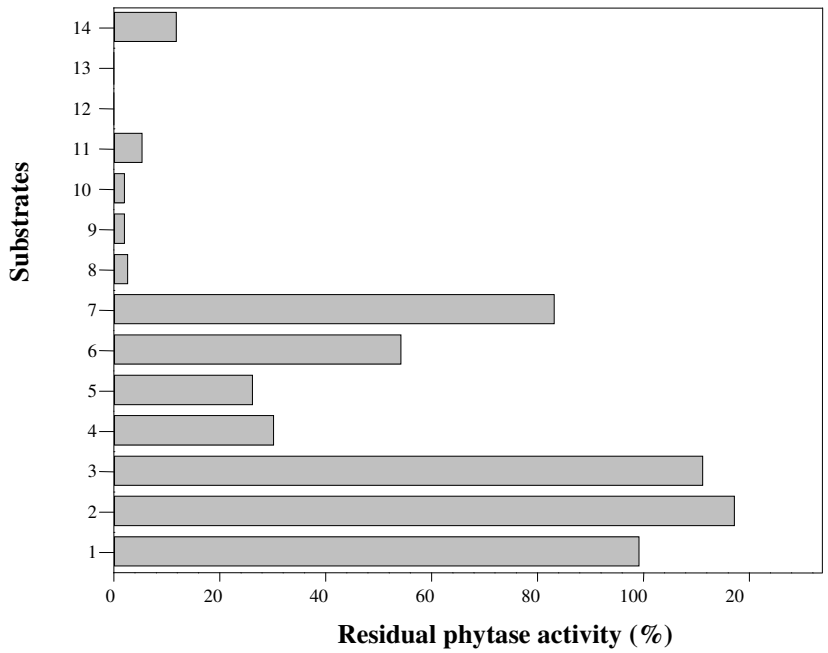

Fig. 2 Substrate specificity studies of SSF phytase from *A. niger* NCIM 563

---- ------ Phytase activity (%)

1- Sodium phytate, 2- *p*-nitro-phenyl phosphate, 3- Sodium phenyl phosphate, 4- α-D-Glucose-1-phosphate , 5- D-Glucose-6-phosphate, 6- 1-Napthyl phosphate,7- 2-Napthyl phosphate, 8- ATP, 9- ADP, 10- AMP, 11- β-NADP, 12- Sodium pyrophosphate, 13- Glycerol-1-phosphate, 14- Phenyl phosphate

Phytase has a broad substrate specificity and hydrolyzed most of the substrates used (Fig. 2). The MALDI-LC-MS/MS generated peptide sequences when compared and searched against the public database Swiss-Prot using the MS/MSIONS search tool on the MASCOT web site did not homology with the known microbial phytases.

3.4 Self-assembled phytase capsules in IL

Phytase enzyme was initially purified to homogeneity from the SSF broth of *A. niger*, as mentioned in section 3.1. In a typical reaction, phytase enzyme (20µg/mL) was added to IL [BMIM][BF4] and left at room temperature for 24h under shaking conditions. Fig. 3 shows the transmission electron microscopy (TEM) micrograph of phytase capsules obtained after self-assembly of phytase molecules in the IL [BMIM] [BF4]. The protein capsules are quasi-spherical in morphology with an apparently rough surface and an average diameter of 150-200nm. In a control experiment in water, no phytase self-assembly was observed. Controlled self assembly of Phy III enzyme in the IL ([BMIM] [BF4]), lead to the formation of phytase capsule.

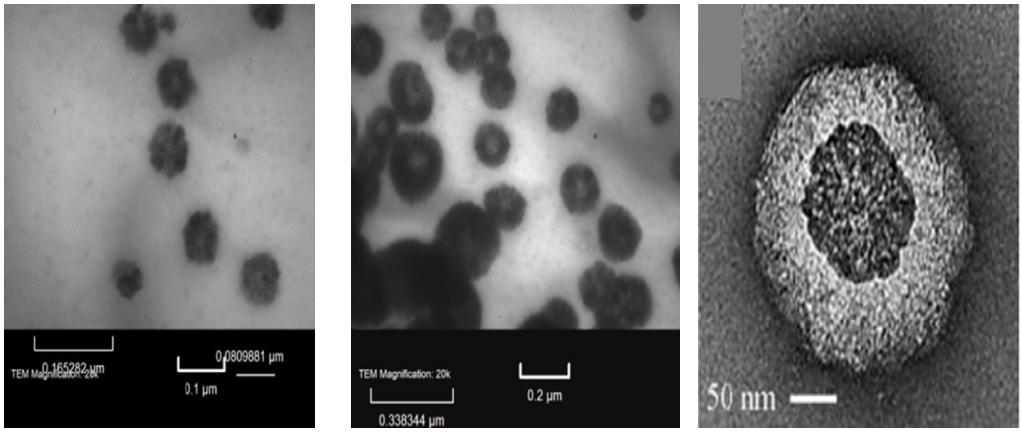

Fig 3 (A & B) Lower and (C) higher magnification TEM images of self-assembled phytase (20µg/mL) capsules synthesized in ionic liquid [BMIM] [BF4].

3.5 Silica nanoparticles

Phytase capsules obtained via self-assembly in [BMIM] [BF4] have been further utilized as functional templates for the spontaneous growth of hollow silica nanocontainers. To obtain hollow silica nanoparticles, TEOS was added to IL [BMIM] [BF4] containing phytase capsules.

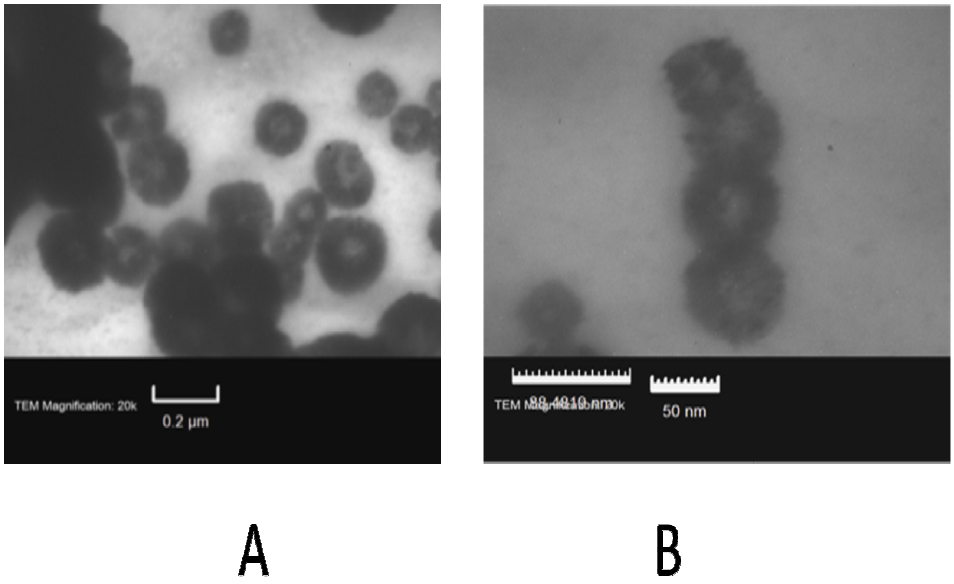

Fig. 4 (A) Lower and (B) higher magnification TEM images of solid silica nanoparticles synthesized in ionic liquid [BMIM] [PF6] using 20 µg/mL self-assembled phytase

Fig. 4A is the TEM image of hollow silica nanoparticles synthesized in [BMIM] [BF4]. The hollow silica nanoparticles are 120-150 nm in diameter with a rough surface and quasi-spherical morphology. The higher magnification Fig. 4B TEM micrograph of one of the hollow silica nanoparticles indicates that these hollow silica nanoparticles are ~50 nm thick. The hollow silica nanoparticle synthesized by in situ self-assembly and templating of phytase capsule in [BMIM] [BF4] thus acts as an enzyme nanocontainer, in which phytase molecules are encapsulated during its synthesis while retaining the native activity of enzyme molecules for at least up to four cycles.

The self-assembly of phytase molecules in IL [BMIM][BF4] leading to the formation of enzyme capsules and their utilization as self-templating nanoreactors for the synthesis of hollow silica nanoparticles with controllable wall thickness is quite interesting and has not been previously demonstrated for SSF phytase. Phytase enzyme used in this study has been isolated from the fungus *A. niger* and belongs to the histidine acid phosphatase (HAP) family of proteins. A common catalytically active site motif, RHGXRXP (Arg-His-Gly-X-Arg-X-Pro), is shared by all HAPs, wherein the catalytic histidine in this sequence is known to initiate a two-step reaction that results in the hydrolysis of substrate [23] Such catalytically active motifs that are present in phytase seem to be responsible for TEOS hydrolysis, leading to hollow silica nanoparticles. The hollow silica nanoparticle synthesized by in situ self-assembly and templating of phytase capsule in [BMIM] [BF4] thus acts as an enzyme nanocontainer, in which phytase molecules are encapsulated during its synthesis while retaining the native activity of enzyme molecules for at least up to four cycles.

Conclusions

The extracellular Phy III from *A. niger* NCIM 563 was purified to homogeneity. Characterization studies reveal some exclusive biochemical properties that bring out its potential for use as an animal feed additive. Among them is stability over broad pH range, high efficacy in hydrolyzing phytate P under emulated gastric conditions and phytate hydrolysis of heavy metal contaminated soils. We show that enzyme (phytase) molecules self-assembled in the presence of an ionic liquid, resulting in the formation of enzyme capsules. Phytase capsules synthesized using this approach was further used as templating nanoreactors for the synthesis of enzyme-containing hollow silica nanocontainers. In situ immobilized phytase enzyme in the silica nanocontainers, when subjected to enzyme-reusability application, establishes them as excellent reusable biocatalysts. This controlled templating of bionanomaterials in ILs of different properties will thus lead to applications in drug encapsulation and controlled release, biocatalysis, biosensing, and bioelectronics.

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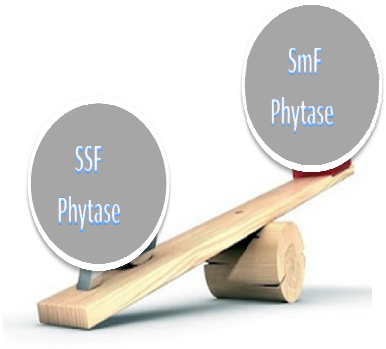


Chapter 5

Correlation studies of solid state Phy III with submerged (Phy I and II) produced by *Aspergillus niger* NCIM 563

A. niger NCIM 563 produces dissimilar phytase Phy I and Phy II (SmF) and Phy III (SSF) under different fermentation conditions. This chapter focuses on research work allowing comparison of the culture conditions, biochemical characters, purification techniques and molecular characteristics of these phytases.

A. niger in SSF has a higher metabolic potential as compared to SmF. Phy I and II were purified to homogeneity using column chromatography, while Phy III was purified using liquid liquid extraction. The molecular mass of Phy I, II and III as determined by SDS–PAGE were 66, 150, 87 and gel filtration were 264, 148, and 85 kDa respectively, indicating that Phy I consists of four identical subunits and Phy II and III is a monomer. Phy I was highly acidic with optimum pH of 2.5 and was stable over a broad pH range (1.5–9.0), Phy II showed a pH optimum of 5.0 with stability in the range of pH 3.5–9.0 and Phy III showed a pH optimum of 5.6 with stability in a broad pH range of 2.5-9.5 Phy I and III exhibited very broad substrate specificity while Phy II was more specific for sodium phytate. SSF phytase is less thermostable as compared to SmF phytase.

Peptide analysis by Mass Spectrometry (MS) MALDI-TOF also indicated that the three proteins were totally different. Based on the MALDI–LC–MS/MS identified amino acid sequences of the peptides, Phy I and II show homology with other known phytases while Phy III showed no homology with SmF phytases and any other known phytases from the literature which suggests its unique nature. This is the first report about structural differences among phytase produced under SSF and SmF by *A. niger* and this study provides basis for explanation of the stability and catalytic differences observed for these three phytase.

1. Introduction

Although phosphorous is a basic component and energy conduit of life, it does not have a cycle to constantly replenish its supply. So dephosphorylation of phytate, a phosphorus locking molecule, by phytase is a crucial process. Phytases have emerged as key enzymes in swiftly growing white biotechnology due to their multifarious application in animal nutrition, human health and environmental protection. It is most likely that its use will expand as the need to conserve the world phosphate reserves increases [11].

Phytases can be produced from a host of micro-organisms including bacteria, yeasts and fungi. Generally, phytase activity and bio-efficacy of plant and animal phytases is negligible when compared to their microbial counterparts. The use of filamentous fungi for the production of industrial enzymes has rapidly increased and phytases are no exception to this. Although phytases are widely distributed in nature, the production in wild-type organisms is far from an economically viable level [18]. The existing commercial microbial phytases produced by submerged fermentation (SmF) conditions are expensive because of diluted product, production using recombinant strains and high product recovery costs. However, industrial applications of phytase are still very limited due to its high production cost. Thus, there is a growing interest in the production, recovery, and purification of this enzyme [16]. Thus, to obtain better and alternative source of phytases, there is an ongoing interest in screening new organisms producing novel and efficient phytases with the ultimate aim to produce this enzyme to cost effective level and establish the suitability for its industrial application.

Phytase production has been studied under submerged and solid-state fermentation (SSF); literature reports that enzymatic production under SSF has many advantages in comparison to that of SmF. Among them are the higher titers of enzyme production, extracellular nature of enzyme, and the low protease production [17]. But the reports are few because of the low productivities and

difficulties associated with operating and up scaling SSF conditions. So at industrial level, the use of SmF is advantageous because of ease of sterilization, and process control is easier to engineer in this system [15].

Several authors have compared phytase productivity values in different fermentation systems trying to explain how the fermentation system affects fungi physiology [6, 10, 19, 21]. In such comparisons various aspects have been included such as medium composition, morphology, and diffusion of nutrients, growth patterns, titers of enzymatic productivity culture conditions, type of strain, and nature of substrate [23]. However, there is no information about structural differences among phytase produced under both systems.

The complexity of gene regulation in *Aspergillus* makes it highly adaptable, able to exploit a wide range of environmental conditions and nutrient sources; again this presents both problems and opportunities [13]. There is, however, a complex relationship between the morphology of these microorganisms, transport phenomena, the viscosity of the cultivation broth, and related productivity. The morphological characteristics vary between freely dispersed mycelia and distinct pellets of aggregated biomass, every growth form having a distinct influence on broth rheology. Hence, the advantages and disadvantages for mycelial or pellet cultivation have to be balanced out carefully. Because of the still inadequate understanding of the morphogenesis of filamentous microorganisms, fungal morphology is often a bottleneck of productivity in industrial production [24].

There is abundant proof in literature that the product spectrum from SSF is very different from that obtained in submerged fermentation (SmF). However, the mechanisms underlying these differences are not at all understood. Therefore rational new design of SSF processes to make new products and optimise the production of existing products is not possible [20]. Only recently, significant advances have been made in understanding the physical (process engineering) aspects

of SSF but the information on physiology and molecular genetics is limited. To obtain an optimized production process, it is of great importance to gain a better understanding of the molecular and cell biology of these microorganisms as well as the relevant approaches in biochemical engineering.

This study reports important differences in biochemical and molecular properties of phytase produced under SmF and SSF by a wild *Aspergillus niger* to evidence the effect of the fermentation system on enzyme functionality and specificity.

2. Materials and Methods

2.1. Materials and Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo. SDS–PAGE and gel filtration markers, Coomassie Brilliant Blue R-250 and Bromophenol Blue were purchased from Sigma Chemical Company, USA Sephacryl S-300, Phenyl-Sepharose CL-4B were obtained from Sigma.

2.2 Fungi and inoculum preparation

A. niger NCIM 563 was used in the present study from NCIM Resource Center, Pune, India. The stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C. Spores from 7 days old PDA slant were harvested using sterile distilled water containing 0.01% Tween 80 to obtain $5x10^7$spores/ml and used as inoculum for solid state fermentation.

2.3. Phytase production and extraction

The solid state fermentation medium contained 10g of wheat bran in 250ml Erlenmeyer flask plus glucose 3g, dextrin 1.25g, $NaNO_3$ 0.2g, magnesium sulphate 0.3g moistened with 20ml

distilled water and sterilized by autoclaving at 121°C for 30min. On cooling fermentation medium was inoculated with 1 % spore suspension of *A. niger* NCIM 563 and incubated for 4 days at 30°C. Enzyme production was expressed as enzyme activity IU/g DMB. Phytase extraction from koji was done as mentioned [14] and crude extract was stored at 4°C and used as and when required for the experiments.

The SmF medium for phytase production contained (g/100 ml): rice bran 1.15, glucose 8, $NaNO_3$ 0.86, KCl 0.1, $MgSO_4 \cdot 7H_2O$ 0.1, $MnSO_4$ 0.005, $FeSO_4 \cdot 7H_2O$ 0.01, pH 5.5 before sterilization. Fermentation medium (100ml in 250ml Erlenmeyer flask) was inoculated with 1% (v/v) of spore suspension and flasks were incubated at 30°C at 200rpm. Samples were removed after every 24h and enzyme production was expressed as enzyme activity IU/ml.

2.4. Analytical methods

Phytase measurements were carried out at 50°C. The reaction mixture consisted of 3mM sodium phytate buffered with 100mM acetate buffer (pH 5.5) and glycine-HCl buffer (pH2.5). Enzymatic reactions were started by the addition of 50µl of enzyme solution. After 30min at 50°C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [8]. A freshly prepared solution of acetone: 5 N H_2SO_4: 10mM ammonium molybdate (2:1:1 v/v/v) and 400µl 1M citric acid was added to the assay mixture. Absorbance was measured at 370nm. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1µm phosphorus/min under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3–5% variability was observed. Concentration of protein was determined using Lowry method [12] using bovine serum albumin as standard.

2.5. Purification of phytase

Purification of Phy I and Phy II

Phy I and Phy II were purified as described by Soni et al [22]. After fermentation, mycelium was separated by filtration, centrifugation (10,000xg for 30min) and the clear supernatant was further concentrated (50%) by Rotavapor rotary evaporation at 40^{o}C under vacuum. It was then subjected to hydrophobic column chromatography using Phenyl-Sepharose CL-4B (30ml bed volume), previously equilibrated with 30% ammonium sulphate in 20mM acetate buffer, pH 2.5. The column was washed thoroughly with 20 bed volumes of the above buffer and eluted with a 120ml linear decreasing gradient of ammonium sulphate (30–0%) with a flow rate of 20ml/hr and approximately 3.0ml fractions were collected. Fractions showing activity at pH 2.5 (Phy I) and pH 5.0 (Phy II) were pooled separately, concentrated by rota vapor and loaded on a Sephacryl S-300 gel filtration column with a flow rate of 12ml/h and 2ml fractions were collected. All the purification procedures were carried out at 4^{o}C.

Purification of Phy III

Enzyme was extracted from the fermented koji by addition of 50ml of 2% aqueous solution of $CaCl_2 \cdot 2H_2O$ at 200 rpm for 2h at room temperature. The suspension was squeezed through a double layer of muslin cloth, centrifuged (5000xg for 20min). The filtrate obtained was subjected to simultaneous partitioning and purification in a single step by ATPE consisting of PEG-citrate (10.5/20.5%) as described by Bhavsar et al [3]. The fractions were concentrated by rotavapor and loaded on a Sephacryl S-200 gel filtration column with a flow rate of 12ml/h and 2ml fractions were collected. All the purification procedures were carried out at 4^{o}C.

2.6 Peptide sequencing

The enzymes were sent to Sutton Bonington Proteomics Facility, United Kingdom for MALDI-LC-MS/MS peptide sequence analysis. In-gel tryptic digestion was performed and the digested mixture was subjected to MALTI-TOF and LC-MS/MS for peptide mass fingerprinting and peptide sequencing. The data thus obtained was searched against the public database Swiss-Prot using the MS/MSIONS search tool on the MASCOT web site.

2.7 Comparative studies of SSF vs SmF

This study involves a comparison of the culture conditions, biochemical characters, purification techniques and molecular characteristics of these phytases.

3. Results and Discussion

3.1 Production of Phy I, II (SmF) and Phy III (SSF)

Our preliminary study on the effect of different process parameters on phytase I and II production was carried using a classical OVAT (One variable at a time) approach. Among the various agriculture residues tested, rice bran showed maximal phytase activity. Optimization by OVAT showed that *A. niger* NCIM 563 produced best phytase activity Phy I (68,000 U/L) at low pH 2.5 and Phy II (17,000) on the 11^{th} day under SmF conditions in basal medium. The basal fermentation medium (100ml in 250ml Erlenmeyer flask) consisted of 1g rice bran, 5g glucose, 0.86g $NaNO_3$, 0.05g KCl, 0.05g $MgSO_4$, and 0.01g $FeSO_4$ with inoculum level 1xE+07 spores/ml. Other components, viz., dextrin, $MnSO_4$ and surfactant (Tween 80) also have a positive effect on phytase production [4] and were thus included in the further studies for optimization of media formulation.

Optimization of SmF culture conditions was studied using response surface methodology. First the Plackett-Burman (PBD) technique was used to evaluate the effects of 11 variables for phytase production. The results showed that glucose, $MgSO_4$, KCl, incubation period and $MnSO_4$ are the most significant variables affecting enzyme production. Further optimization in these variables, using a central composite design technique, resulted in 3.74 fold increase in the yield of phytase production to 254,500 U/L when compared with the activity observed with basal media (68,000 U/L) in shake flask [1].

Wheat bran gave highest phytase production (Phy III) in SSF 50 IU/g DMB on 7^{th}day of fermentation as compared to groundnut cake, coconut cake, cotton cake and rice bran. As wheat bran is cheaper substrate, economical and supported maximum phytase production, it was selected for optimization experiments.

Using response surface methodology involving a combination of PBD and a Box-Behnken design (BBD) high levels of phytase III of 154 IU/gm DMB was obtained along with accessory enzymes [2]. Phytase production improved from 50 IU/g dry moldy bran (DMB) to 154 IU/g DMB indicating 3.08 fold increase after optimization. A simultaneous reduction in fermentation time from 7days to 4days shows a high productivity of 38500 IU/kg/day.

The differential expression of proteins in SSF and SmF can be attributed to the effect of low aw and physical barriers to the hyphal extension on the enzyme expression (Table 1).

Table1 Differences between SSF and SmF fermentation techniques.

SSF	SmF
Medium is not free flowing	Medium free flowing
Shallow depth	Greater
Single solid substrate provides C, N, Minerals and energy	Not a single substrate
Medium absorbs water up-takes nutrients	Dissolved in water
Gradients of T, pH, concentration	Uniform
Minimum water, (less Volume).	More water ,more volume
3 phase system	2 phase system
Temp, O_2, H_20 control (critical)	Temp, O_2 control
Inoculum ratio large	Low
Intra particle resistances	No such resistance
Fungal cells adhere to solid and grow	Uniformly distributed
Highly concentrated product	Low concentration product

- One of the differences between SSF and SmF cultures is that in the former, the moisture content of the substrate is low, resulting in a limitation of growth and metabolism of the microorganism.
- The concept of water availability in a substrate, thus, becomes very important.
- This difference is one of the crucial factors that govern the processes that occur during fermentation.

- Moisture content being related to many factors can greatly influence the path of enrichment, leading to products that differ both **quantitatively and qualitatively** [5].

Table 2 Comparison of phytases from *A. niger* NCIM 563 under SSF and SmF

Properties	**Phy I**	**Phy II**	**Phy III**
Type of fermentation	Submerged		Solid state
Substrate	Rice bran		Wheat bran
Prodcution (OVAT)			
Phytase	68 (IU/ml)	17(IU/ml)	50(IU/g)
Xylanase	-		High
Cellulase	Low		High
Amylase	Low		High
Phosphatase	Low		High
Statistical optimization			
Techniques used	PB+CCD		PB+BBD
PB (Five variables)	Incubation period, glucose, $MnSO_4$ $MgSO_4$, and KCL.		glucose, dextrin, $NaNO_3$, DW and $MgSO_4$
Incubation period	Increased by 2 days		Decreased by 3 days
Phytase production	268(IU/ml)	68.21 (IU/ml)	154 (IU/g)
Phytase Productivity	20615.3 (IU/L/day)	5246.9 (IU/L/day)	38500 (IU/kg/day)

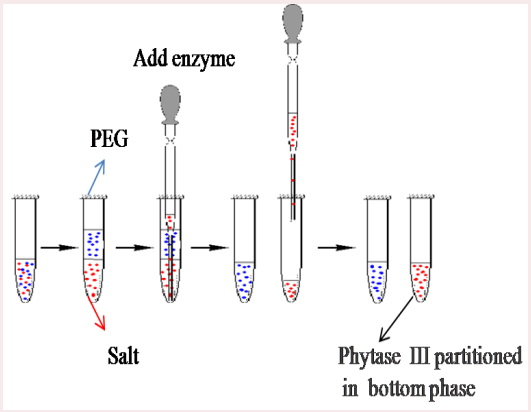
Differences in the osmotic pressure, water content and dissolved oxygen may contribute to the observed variations between the SmF and SSF type of cultures since the interactions between biomass and the culture media are different in each case. From our studies, it can be seen that wheat bran and rice bran supported maximum phytase production under SSF and SmF with least production with WB in SmF and RB in SSF (Table 2). This may due to the reason that the fungus is confronted with gradients in concentration of substrates and enzymes, the presence of a substrate-air interface, and gradients in water content and temperature. Production of accessory enzymes was higher in SSF when compared with SmF. This may be due to the conditions of SSF being more similar to fungal growth conditions in nature. Different mechanisms have been proposed for the observed difference in protein productivity during submerged growth and growth on a solid substrate. It is suggested that during filamentous fungal growth on a solid substrate, catabolite repression is less pronounced than during submerged fermentation The accessory enzymes under SSF may be improving the metabolic potential of *A. niger* in SSF as compared to SmF. The process of both SSF and SmF are up scaled to tray level and 14 L (New Brunswick, USA) with a working volume of 10L. Although a direct comparison between SSF and SmF is very difficult due to the different consistencies of the fungal cultures in the two technologies, *A. niger* in SSF has a higher metabolic potential since they proliferate in an almost natural environment, i.e. under conditions of limited free water and with a solid support for growth. The accessory enzymes under SSF may be improving the metabolic potential of *A. niger* in SSF as compared to SmF.

3.2 Purification of phytases from A. niger NCIM 563

A. niger NCIM 563 produced two different extracellular phytases (Phy I and Phy II) under SmF at 30^{o}C in medium containing rice bran-glucose-sodium nitrate-salts. Both the SmF enzymes were purified to homogeneity using Rotavapor concentration, Phenyl-Sepharose column chromatography and Sephacryl S-200 gel filtration. *A. niger* produced Phy III under SSF at 30^{o}C in

medium containing wheat bran-glucose-salts. SSF enzyme was purified to homogeneity using liquid liquid extraction. This is the first report to show phytase (Phy III) extraction in a single step from fermentation broth by a liquid–liquid extraction process using ATPE. The speed and simplicity of this eco-friendly process with high throughput, improved thermostability and recycling of polymer are additional advantages. The SmF phytases did not exhibit the partitioning in aqueous two phase systems. Phy I and II were purified to homogeneity using column chromatography, while Phy III was purified using liquid liquid extraction. The molecular mass of Phy I, II and III as determined by SDS–PAGE and gel filtration were 66, 264, 150, 148, and 87, 85 kDa respectively, indicating that Phy I consists of four identical subunits and Phy II and III is a monomer. A comparative account of purification of all three phytases is given in Table 3.

Table 3 Purification of SSF and SmF phytases

<table>
<tr><td colspan="2">SMF</td><td colspan="2">SSF</td></tr>
<tr><td>PHY I</td><td>PHY II</td><td colspan="2">PHY III</td></tr>
<tr><td colspan="2">Column Chromatography</td><td>Column Chromatography</td><td>Liquid Liquid Extraction</td></tr>
<tr><td colspan="2">Crude(Smf)</td><td>Crude (SSF)</td><td>Aqueous two phase extraction (PEG+Sodium citrate)</td></tr>
<tr><td colspan="2">Heating 90 min. at 50^0C</td><td>95%$(NH_4)_2SO_4$ Precipitation</td><td rowspan="4"> 
</td></tr>
<tr><td colspan="2">Phenyl sepharose Binding by 30% $(NH_4)_2SO_4$</td><td>Phenyl sepharose Binding by 35% $(NH_4)_2SO_4$</td></tr>
<tr><td>Eluted by 5% $(NH_4)_2SO_4$</td><td>Eluted by 15% $(NH_4)_2SO_4$</td><td>Eluted by 20% $(NH_4)_2SO_4$</td></tr>
<tr><td colspan="2">Gel Filtration Chromatography (S- 200)</td><td colspan="2">Gel Filtration Chromatography (S- 200)</td></tr>
<tr><td>PHY I Tetramer 264kDa Monomer 66kDa</td><td>PHY II Monomer 120 kDa</td><td colspan="2">PHY III Monomer 85 kDa</td></tr>
</table>

Phy I was highly acidic with optimum pH of 2.5 and was stable over a broad pH range (1.5–9.0), Phy II showed a pH optimum of 5.0 with stability in the range of pH 3.5–9.0 and Phy III showed a pH optimum of 5.6 with stability in a broad pH range of 2.5-9.5 Phy I and III exhibited very broad substrate specificity while Phy II was more specific for sodium phytate. SSF phytase is less thermostable as compared to SmF phytase. But the purification using liquid liquid extraction improved its thermostability 4 times at 60^{o}C as compared to chromatography purified process and is therefore likely to withstand the high temperature required for product formulation.

Phy II was strongly inhibited by Hg^{2+}, while Phy I and Phy III were not inhibited. Retention of Phy III activity in presence of Pb^{2+} (125% for 5mM) and Ag^{2+} (50% for 1mM) provides an opportunity for phytate hydrolysis in soils contaminated with heavy metals. Phy I hydrolyzes phytate phosphorus only up to pH 3.5, but Phy III was more resistant to pepsin and released more inorganic phosphorus from soybean meal under emulated gastric conditions over a much broader pH range upto 6.5. This determines the efficacy of *A. niger* NCIM 563 phytase and is exceptional as compared to SmF phytases and other reported phytases.

The 1D gel bands were diced into cubes (~1 mm^3), placed into individual wells of a micro titre plate, processed (destained, reduced, alkylated) and trypsin digested using standard procedures on the MassPREP station. Digestion buffer was 25 mM ammonium bicarbonate; trypsin gold was diluted in this buffer at 10 ng/µl and 25 µl of the enzyme/buffer mixture was added to each well. Trypsin was allowed to absorb into gel pieces at 6°C for 15 minutes, then digestion proceeded at 40°C for 5 hours.

Resulting peptides were delivered via nanoLC to the Q-ToF2 for tandem MS analysis.

An automated experiment (DDA = data dependent acquisition) was run where selected peptides automatically enter MSMS for fragmentation.

The data was searched against the public database Swiss-Prot using the MS/MSIONS search tool on the MASCOT web site (http://www.matrixscience.com/search_form_select.html) using the standard default settings (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS)

with standard variable modifications of carbamidomethylation of Cysteine and oxidation of Methionine. Peptide analysis by Mass Spectrometry (MS) MALDI-TOF also indicated that the three proteins were totally different. Phy I show 24% homology with Phy B from *A. niger* and Phy II show 20% homology with acid phosphatase from *A. ficuum.* Phy III shows no homology with other phytases but partial peptide sequence matches 1,4-beta-D-glucan cellobiohydrolase and is unique. Based on the MALDI–LC–MS/MS identified amino acid sequences of the peptides, Phy I and II show homology with other known phytases while Phy III is novel and showed no homology with SmF phytases and any other known phytases from the literature which suggests its unique nature (Annexure-I).

This is the first report about structural differences among phytase produced under SSF and SmF by *A. niger* and this study provides basis for explanation of the stability and catalytic differences observed for these three phytase (Table 4). In fact, only two reports on the comparative production of phytase by these two fermentation processes are available fungal [7] and bacterial [9]. But our studies with *A. niger* show that different proteins are produced and genes are differentially transcribed in SmF and SSF and is the first report that aims at elucidating the mechanisms behind the differences in SSF and SmF and aim to explore the biotechnological applications.

Table 4 Comparative account of biochemical characterization of phytases from *A. niger*

Properties	**SMF**		**SSF**
	Phy I	**Phy II**	**Phy III**
Optimum pH	2.5	5.0	5.6
Optimum temp	55 ^{o}C	55 ^{o}C	60 ^{o}C
Molecular weight (Gel filtration)	264	150	85-87
pH stability	2-9	4-8.5	2-9.5
Temp stability	40% residual activity at 65 ^{o}C (1.5 hr)	55% residual activity at 65 ^{o}C (1.5 hr)	20% residual activity at 55 ^{o}C (1 hr)
Improved thermostability (80^{o}C for 1 min)	Presence of PVP (100%)	Presence of PVP (100%)	Presence of PEG+ citrate salt (93%)
Purification technique	Column chromatography	Column chromatography	Column chromatography /LLE-ATPS
Effect of metal ions (1mM) Hg^{2+}	71	0	22
Cu^{2+}	95	11	43
Fe^{3}	15	65	70
Pb^{2+}	74	65	125(5 mM)
$Zn2^{+}$	85	52	63
Ca^{2+}	95	90	108(5 mM)
Fe^{2+}	92	73	91
Efficacy in SGF	2.0-3.5	3.5-5.5	2.5-6.5

4. Conclusions

Phytases have emerged as key enzymes in expanding white biotechnology yet there is a need of versatile "second-generation" phytases with wider applicability to meet the real-world demand. There is a need for thorough evaluations to design versatile "second-generation" phytases which is usually achieved by employing genetic and protein engineering. In this situation, exclusive characteristics and multilevel application of native phytase Phy III determines its efficacy and is exceptional. Our production, biochemical and molecular characterization studies with *A. niger* NCIM 563 show that different proteins are produced and genes are differentially transcribed in liquid fermentation vs. SSF. This is the first report exhibiting correlation for phytase production under SmF and SSF and can provide new insights to the existing "black box" of SSf/SmF biotechnology for phytase production. Significant development in SSF/SmF processing, regarding both biochemical engineering and reactor design with the goal of scaling up the process is done. On the basis of productivity, pH profiles, higher thermostability and hydrolysis of soyabean in simulated gastric fluid, these phytases could be ideal candidates for application in animal nutrition, enviromental protection and human health.

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Chapter 6

Conclusions

This chapter details the salient feature of the work presented in the thesis and emphasized on possible future potential developments in the area.

1 General Discussion

P is an essential nutrient to biological systems. Its requirement is mainly seen in nucleic acids, cell membrane, bones and teeth. It is a main constituent of energy rich compounds such as ATP, ADP, GTP etc and serves as an energy conduit in various metabolic reactions. Although P is Life's bottleneck for existence on earth, it is nonrenewable and current reserves will diminish by 2030. Despite its importance, P production, utilization and recycling is a slow process due to absence of gaseous phase and thus is therefore well-known as an imperfect cycle . So dephosphorylation of phytate, a P locking molecule, by phytase is a crucial process and its use will expand as the need to conserve the world phosphate reserves increases.

Bound P (18-88% of total P content) in fact exists as phytate which is already present in animal feed. But this phytate P is not utilized by food producing animals due to lack of intrinsic phytase in their gastrointestinal tracts. Therefore, inorganic phosphate is frequently added to their feed to facilitate optimal growth for these animals. Phytate in addition acts as an antinutrient by chelating various cations such as Ca^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+} and thereby reducing their bioavailability. This approach stimulates algal blooms and eutrophication of surface water, due to the high content of phytate and inorganic phosphate in the animal excreta.

Hence dephosphorylation of antinutrient phytate by phytase is a critical process which overcomes the drawbacks of slow recycling and at the same time eliminating the anti nutritional effects of phytate. They can be produced from sources including plants, animals and microbes. icrobial sources however are promising for their commercial exploitation. Few phytases have been reported and studied, and but existing knowledge of phytases has yet to yield a solution to meet the nutritional and environmental requirements that a real-world solution demands. The available phytase preparations used as feed additives are of fungal

origin, produced by recombinant strains under SmF have shortcomings especially with regard to obtaining diluted product, sensitivity to heat, inactivation under low pH conditions present in the stomach of animals and high product recovery costs. So further research into identifying new phytases, engineering better phytases and developing more cost-effective expression systems is needed and should be continued.

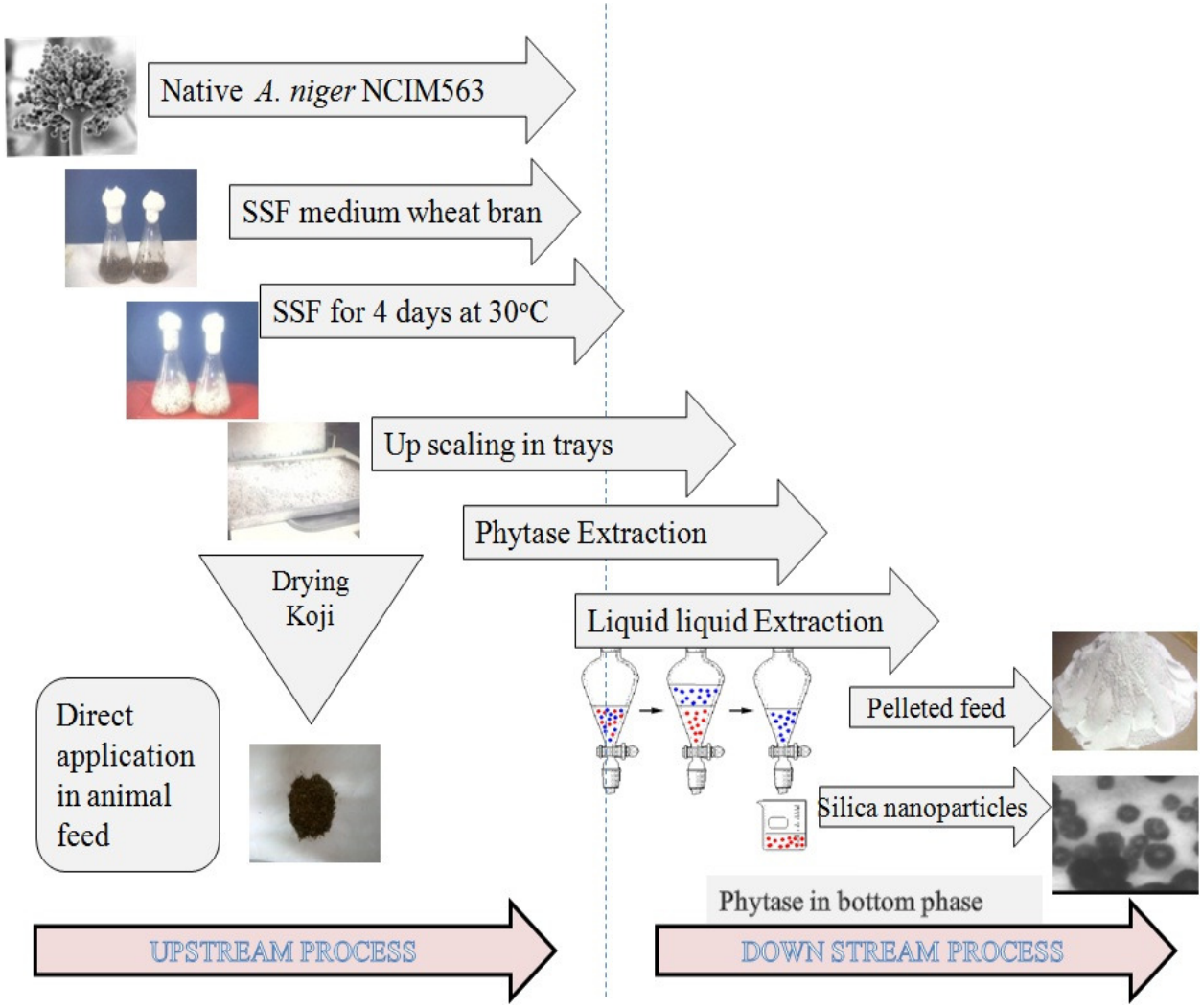
The disadvantages of existing phytases need to be alleviated while at the same time producing it with high yield and purity. Likewise production, downstream processing also is an integral part of any product development as the final cost of the product largely depends on the cost incurred in extraction and purification. Hence development of a viable process for phytase production, recovery and purification with techno-economic feasibility is necessary as the available SmF and chromatographic methods have several limitations.

Phytases have emerged as key enzymes in expanding white biotechnology but with varied properties. Theoretically, an 'ideal' phytase should be catalytically efficient, proteolysis-resistant, thermostable and cheap but phytases possessing all of these qualities is not found or generated. Therefore there is a need of versatile "second-generation" phytases with wider applicability in animal nutrition, human health and environmental protection.

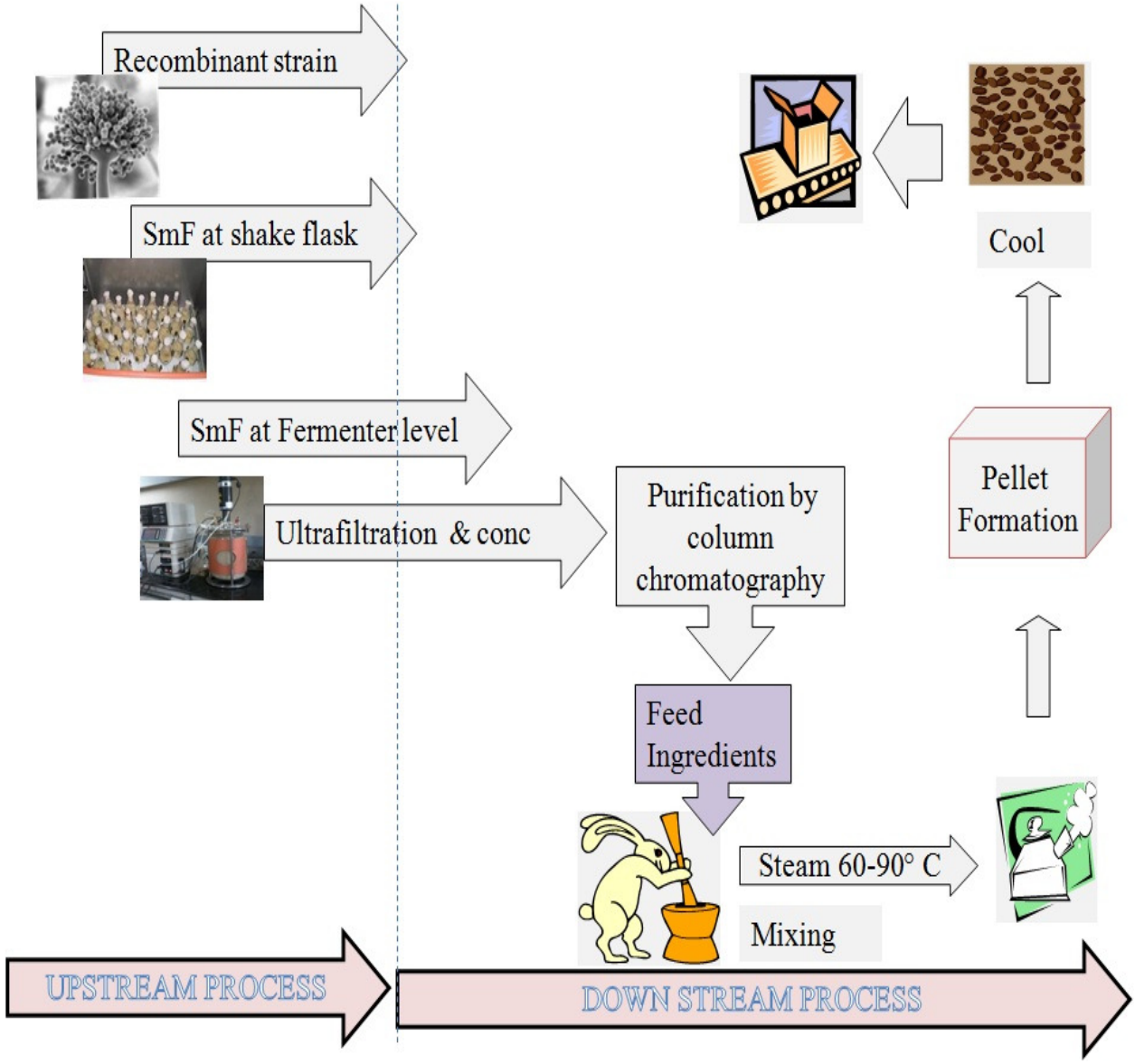
In this work, our objective is to evaluate application of statistical methods to increase the phytase activity under SSF and up- scaling to tray fermenter. The present work also reports the use of ATPE for separation and purification of phytase and compares it with the conventional chromatography process. The same fungus produces two dissimilar phytase Phy I and Phy II under SmF [129]**.** Many studies on SSF and SmF for phytase have focused on process and fermenter design while the organism has been considered as a black box. The role of the physiological and genetic properties of the microorganisms producing phytase used during growth on solid substrates compared with aqueous solutions has so far been all but neglected.

Hence we have tried to correlate different protein secretion in Smf and SSF and these studies can provide new insights to the existing "black box" of SSf/SmF biotechnology for phytase production.

Process flow chart for SSF phytase production, down streaming and application



Available commercial process for phytase up streaming and down streaming



2 Salient features of Phy III from A. niger NCIM 563

1. The Phy III enzyme is derived from a GRAS classified microorganism, allowing its use as a feed additive.

2. Stability over broad pH range and proteolysis resistance up to pH 6.5 in simulated gastric fluid for this fungal enzyme determines its efficacy and is exceptional.

3. Highest productivity is achieved with a natural wild strain, and thus the possibility for genetic improvisation is also very good.

4. Instead of low yield, high cost Smf, SSF by *A. niger* NCIM 563 for phytase III production emerges as a more efficient, less costly and a more directly applicable process.

5. Above features, along with up scaling in trays and hydrolytic enzymes corroborate the commercial potential of SSF phytase as a solid state culture product.

6. Retention of phytase activity in presence of heavy metals provides an opportunity for phytate hydrolysis in soils contaminated with heavy metals.

7. Development of a viable ATPE process for phytase III recovery and purification with techno-economic feasibility overcoming the limitations of existing processes.

8. This is the first report to show phytase extraction in a single step from fermentation broth by a liquid–liquid extraction process using ATPE.

9. The speed and simplicity of this eco-friendly process with high throughput, improved thermostability and recycling of polymer are additional advantages.

10. Broad pH range, resistant to proteolysis at high pH and multilevel application of native phytase Phy III determines its effectiveness and is incomparable.

11. Phy III showed no homology with SmF (Phy I and Phy II) phytases and any other known phytases from the literature which suggests its unique nature.

12. SSF phytase is less thermostable as compared to SmF phytase. This result of thermophily is in contrast to available reports for other enzymes under SSF/SmF.

13. This is the first report about structural differences among phytase produced under SSF (Phy III) and SmF (Phy I and Phy II) by *A. niger NCIM* 563.

14. Significant development in SSF/SmF processing, regarding both biochemical engineering and reactor design with the goal of scaling up the process is done.

15. These studies of fungal cultivation for phytase production can provide new insights to the existing “black box” of SSf/SmF biotechnology for phytase production.

16. This may, and will, be changed by more focused consideration of the biological parameters applicable to SSF and SmF.

3 Scope for future work

- Tray fermentation studies for up scaling also gave promising results from an industrial point of view. The process can be improved using advanced bioreactors that provide accurate moisture and temperature control, as well as optimized O_2 mass transfer.

- Plant growth promotion and formation of silica nanoparticles are the foreseeable applications of Phy III.

- Proteomic profiling to identify proteins produced under the specific conditions of SSF and SmF.

- Engineering of Phy III in order to optimise its catalytic features as a promising strategy to enhance of thermal tolerance and increase in specific activity

- Differences might be expected in the pattern of secreted proteins when *A. niger* NCIM 563 grown in SmF and SSF.

List of Publications

1. Bhavsar KP, Shah P, Khire JM (2008) Influence of pretreatment of agriculture residues on phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. African J Biotechnol 7:1101-1106.

2. Shah P, Bhavsar KP, Soni SK, Khire JM (2009) Strain improvement and up scaling of phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. J Ind Microbiol Biotechnol 36:373-380.

3. Bhavsar KP, Ravi Kumar V, Khire JM (2011) High level phytase production by *A. niger* NCIM 563 in solid state culture: response surface optimization, up scaling and its partial characterization. J Ind Microbiol Biotech 38:1407-1417.

4. Bhavsar et al (2012) Combinatorial approach of statistical optimization and mutagenesis for improved production of acidic phytase by *Aspergillus niger* NCIM 563 under submerged fermentation condition. Appl Microbiol Biotechnol DOI: 10.1007/s00253-012-3965-8

5. Bhavsar et al (2012) Downstream processing of extracellular phytase from *Aspergillus niger*: Chromatography process vs. aqueous two phase extraction for its simultaneous partitioning and purification. Process Biochem 47:1066-1072.

6. Bhavsar KP, Soni SK, Depan D, Sarkar S, Singh RP, Khire JM (2012) Phytase from *Aspergillus niger* NCIM 563: Biochemical Characterization and its role in biosynthesis of nanosized hydroxyapatite and its polymorphs. Biochim Biophy Acta (Communicated).

Review

7. Phytase from *Aspergillus niger* NCIM 563(Review under preparation)

Annexure-I

Peptide summary report

1E_MP1511 (PHY I)
phytase B

1F_MP1511 (PHY II)
acid phosphatase

1G_MP1511 (PHYTASE III)
1,4-beta-D-glucan cellobiohydrolase A
Glucoamylase

1E_MP1511 (PHY I) phytase B

Swiss-prot database search
http://www.matrixscience.com/cgi/master_results.pl?file=../data/20111104/FtomSfYEe.dat

this search gives the basic result of identification of the protein as **phytase B** (with the best match to a sequence from *Aspergillus awamori*). However, a more similar version of the protein sequence to your data is present in the NCBInr database from a different species *A. niger*). The scores, peptides and coverage levels are all higher for this version of the protein, therefore I report it rather than the swissprot entry match(es). However, the swissprot and the NCBInr search data outputs are all fully available from the links provided.

Ncbinr search
http://www.matrixscience.com/cgi/master_results.pl?file=..%2Fdata%2F20110728%2FFtotlaTOm.dat&REPTYPE=peptide&_sigthreshold=0.05&REPORT=AUTO&_server_mudpit_switch=99999999&_ignoreionsscorebelow=10&_showsubsets=1&_showpopups=TRUE&_sortunassigned=scoredown&_requireboldred=0

1. gi|145241119 **Mass:** 52453 **Score:** 666 **Matches:** 25(9) **Sequences:** 9(4)
3-phytase B [Aspergillus niger CBS 513.88]

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
10	403.7043	805.3940	805.3970	-0.0030	0	41	7.3	1		R.YPSPSAGK.S
11	403.7052	805.3958	805.3970	-0.0012	0	(41)	8.1	1		R.YPSPSAGK.S
23	471.2248	940.4350	940.4403	-0.0052	0	45	2.3	4		K.QFSQEFR.D
26	618.7672	1235.5198	1235.5319	-0.0121	0	36	20	1		K.HYGGNGPYSER.V
28	429.2082	1284.6028	1284.6211	-0.0183	1	54	0.32	1		R.HGERYPSPSAGK.S
29	676.7743	1351.5340	1351.5384	-0.0044	0	33	31	1	U	R.SSPIACQEGAAMD.- + Carbamidomethyl (C); Oxidation (M)
40	851.4106	1700.8066	1700.8114	-0.0047	0	(103)	3.1e-06	1	U	R.DPPTGCEVDQVIMIK.R + Carbamidomethyl (C)
43	859.4066	1716.7986	1716.8063	-0.0076	0	128	1.1e-08	1	U	R.DPPTGCEVDQVIMIK.R + Carbamidomethyl (C); Oxidation (M)
50	885.9133	1769.8120	1769.8254	-0.0134	0	(24)	2.2e+02	1		R.LSCQATALSDEGTYVR.L + Carbamidomethyl (C)
51	885.9187	1769.8228	1769.8254	-0.0026	0	117	1.3e-07	1		R.LSCQATALSDEGTYVR.L + Carbamidomethyl (C)

☑	52	885.9203	1769.8260	1769.8254	0.0006	0	(50)	0.66	1	R.LSCQATALSDEGTYVR.L + Carbamidomethyl (C)
☑	53	885.9206	1769.8266	1769.8254	0.0012	0	(44)	2.4	1	R.LSCQATALSDEGTYVR.L + Carbamidomethyl (C)
☑	57	758.6859	2273.0359	2273.0542	-0.0183	0	(12)	3.1e+03	1 U	R.YGHLWDGETVVPFFSSGYGR.V
☑	59	758.6878	2273.0416	2273.0542	-0.0126	0	(83)	0.00026	1 U	R.YGHLWDGETVVPFFSSGYGR.V
☑	60	758.6881	2273.0425	2273.0542	-0.0117	0	(53)	0.23	1 U	R.YGHLWDGETVVPFFSSGYGR.V
☑	61	758.6893	2273.0461	2273.0542	-0.0081	0	(10)	4.7e+03	1 U	R.YGHLWDGETVVPFFSSGYGR.V
☑	62	1137.5370	2273.0594	2273.0542	0.0052	0	(19)	6.5e+02	1 U	R.YGHLWDGETVVPFFSSGYGR.V
☑	63	1137.5381	2273.0616	2273.0542	0.0074	0	96	1.2e-05	1 U	R.YGHLWDGETVVPFFSSGYGR.V
☑	77	809.7274	2426.1604	2426.2343	-0.0739	0	119	6.2e-08	1 U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	79	809.7424	2426.2054	2426.2343	-0.0289	0	(23)	2.4e+02	1 U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	80	1214.1191	2426.2236	2426.2343	-0.0106	0	(117)	9e-08	1 U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	81	809.7496	2426.2270	2426.2343	-0.0073	0	(51)	0.35	1 U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	82	809.7505	2426.2297	2426.2343	-0.0046	0	(72)	0.0027	1 U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	83	809.7507	2426.2303	2426.2343	-0.0040	0	(11)	3.8e+03	1 U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	85	809.7511	2426.2315	2426.2343	-0.0028	0	(92)	2.8e-05	1 U	R.VAFGNPYSTGNIVPQGGHLTIER.L

Proteins matching a subset of these peptides:

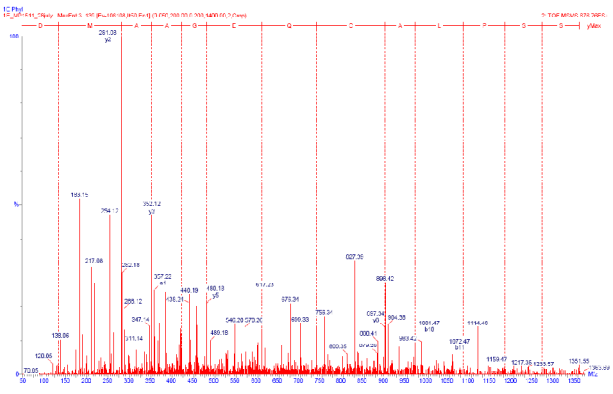
gi|148530276 **Mass:** 52725 **Score:** 388 **Matches:** 15(3) **Sequences:** 6(2)
phytase B precursor [synthetic construct]
gi|154550830 **Mass:** 52765 **Score:** 388 **Matches:** 15(3) **Sequences:** 6(2)
pH 2.5 acid phosphatase [synthetic construct]
gi|154550832 **Mass:** 52721 **Score:** 388 **Matches:** 15(3) **Sequences:** 6(2)
pH 2.5 acid phosphatase [synthetic construct]
gi|294960286 **Mass:** 50734 **Score:** 388 **Matches:** 15(3) **Sequences:** 6(2)
phytase B [Aspergillus ficuum]
gi|83655609 **Mass:** 52725 **Score:** 353 **Matches:** 14(3) **Sequences:** 5(2)
acid phosphatase [Aspergillus niger]
gi|152143417 **Mass:** 50649 **Score:** 343 **Matches:** 14(3) **Sequences:** 5(2)
acid phosphatase [Aspergillus niger]

Match to: **gi|145241119** Score: **666**
3-phytase B [Aspergillus niger CBS 513.88]
Found in search of 1E_MP1511_28july.pkl
Nominal mass (M_r): **52453**; Calculated pI value: **4.63**
NCBI BLAST search of gi|145241119 against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus niger CBS 513.88
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|134077735 from Aspergillus niger
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **24%**
Matched peptides shown in **Bold Red**

```
  1 MPRTSLLTLA CALATGASAF SYGAAIPQST QEKQFSQEFR DGYSILKHYG
 51 GNGPYSERVS YGIARDPPTG CEVDQVIMIK RHGERYPSPS AGKSIEEALA
101 KVYSINTTEY KGDLAFLNDW TYYVPNECYY NAETTSGPYA GLLDAYNHGN
151 EYKARYGHLW DGETVVPFFS SGYGRVINTA RKFGEGFFGY NYSTNAALNI
201 ISESEVMGAD SLTPTCDTDN DQTTCDNLTN QLPQFKIAAA RLNSQNPGMN
251 LTASDVYNLM VMASFELNAR PFSNWINAFT QDEWVSFGYV EDLNYYYCAG
301 PGDKNMAAVG AVYANASLTL LNQGPKEAGS LFFNFAHDTN ITPILAALGV
351 LIPTEDLPLD RVAFGNPYST GNIVPQGGHL TIERLSCQAT ALSDEGTYVR
401 LVLNEAVLPF NDCTSGPGYS CPLANYTAIL NKDLPDYTTT CNVSASYPQH
451 LSFWWNYNTT TELNYRSSPI ACQEGAAMD
```

Note that you are fortunate enough to have the C-terminal peptide in this case (676). This is relatively infrequently seen, unless a specific type of experiment has been undertaken, it is more or less chance if the N or C termini are detected. In this case I thought the mascot data was a reasonable match but decided to go ahead and perform de novo sequencing on it manually to verify the match.

In this the sequence I obtained was SSPIACQEGAAMD.



I am content that this sequence is correct. Please note that at one position, this is a relatively uncommon version of this peptide for the proteins that have been reported in the databases to date. I made a multiple sequence alignment shown below, which reveals that the 4th final residue is more commonly found to be **D**, in your sequence it is an **A**.

```
P34754          SSPIACQEGDAMD  479
P34755          SSPIACQEGDAMD  479
ABG88857        SSPIACQEGDAMD  463
1QFX_A          SSPIACQEGDAMD  460
EHA18670        SSPIACQEGAAMD  479
ABS83554        SSPIACQEGDAMD  479
ABS83553        SSPIACQEGDAMD  479
ABQ82276        SSPIACQEGDAMD  479
XP_001393206    SSPIACQEGAAMD  479
ABC39588        SSPIACQEGDAMD  479
ADF49635        SSPIACQEGDAMD  460
ABS29445        SSPIACQEGDAMD  459
```

1F_MP1511 (PHY II) acid phosphatase

Swiss-prot database search standard (trypsin)
http://www.matrixscience.com/cgi/master_results.pl?file=..%2Fdata%2F20110728%2FFtotlisnR.dat&REPTYPE=peptide&_sigthreshold=0.05&REPORT=AUTO&_server_mudpit_switch=99999999&_ignoreionsscorebelow=0&_showsubsets=1&_showpopups=TRUE&_sortunassigned=scoredown&_requireboldred=0

Swiss-prot database search semiTrypsin
http://www.matrixscience.com/cgi/master_results.pl?file=../data/20110728/FtotlieOS.dat

swissprot standard search results

1. PPA_ASPFI **Mass:** 67169 **Score:** 293 **Matches:** 14(4) **Sequences:** 7(3)

Acid phosphatase OS=Aspergillus ficuum GN=aphA PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
6	419.2119	836.4092	836.4181	-0.0088	0	17	60	1	U	K.VHYGFSK.L
8	453.7188	905.4230	905.4277	-0.0046	0	(16)	1e+02	1	U	R.TPSCSQVK.A + Carbamidomethyl (C)
9	453.7220	905.4294	905.4277	0.0018	0	32	2.2	1	U	R.TPSCSQVK.A + Carbamidomethyl (C)
12	510.2882	1018.5618	1018.5699	-0.0080	0	30	4	1	U	R.EAFEGLLLK.Y
19	730.9037	1459.7928	1459.8035	-0.0106	0	(47)	0.062	1	U	R.NNVNVISLSYIPK.G
20	730.9056	1459.7966	1459.8035	-0.0068	0	64	0.001	1	U	R.NNVNVISLSYIPK.G
21	730.9072	1459.7998	1459.8035	-0.0036	0	(14)	1.2e+02	1	U	R.NNVNVISLSYIPK.G
22	730.9084	1459.8022	1459.8035	-0.0012	0	(36)	0.68	1	U	R.NNVNVISLSYIPK.G
23	731.4087	1460.8028	1459.8035	0.9994	0	(38)	0.48	1	U	R.NNVNVISLSYIPK.G
24	520.6296	1558.8670	1558.8831	-0.0161	0	71	0.00019	1	U	R.LVEPPAVKPATANPR.N
25	520.6307	1558.8703	1558.8831	-0.0128	0	(59)	0.0037	1	U	R.LVEPPAVKPATANPR.N
31	535.2729	2137.0625	2137.0891	-0.0266	0	(39)	0.24	1	U	K.GMHIHYQTPFGLGQLPAVR.W + Oxidation (M)
32	713.3653	2137.0741	2137.0891	-0.0151	0	67	0.00036	1	U	K.GMHIHYQTPFGLGQLPAVR.W + Oxidation (M)
35	1208.1052	2414.1958	2414.1907	0.0052	0	11	1.5e+02	1	U	R.FPYTGPAVPIGDWVDPTINGNGK.G

Proteins matching a subset of these peptides:
PPA1_ASPNG **Mass:** 47965 **Score:** 214 **Matches:** 10(4) **Sequences:** 4(3)
Phosphate-repressible acid phosphatase OS=Aspergillus niger GN=pacA PE=4 SV=1

Match to: **PPA_ASPFI** Score: **293**
Acid phosphatase OS=Aspergillus ficuum GN=aphA PE=1 SV=1
Found in search of 1F_MP1511_28july.pkl
Nominal mass (M_r): **67169**; Calculated pI value: **5.37**
NCBI BLAST search of PPA_ASPFI against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus ficuum
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **15%**
Matched peptides shown in **Bold Red**

```
  1 MKGTAASALL VALSATAAQA RPVVDERFPY TGPAVPIGDW VDPTINGNGK
 51 GFPRLVEPPA VKPATANPRN NVNVISLSYI PKGMHIHYQT PFGLGQLPAV
101 RWGKDPRNLN STAQGYSHTY DRTPSCSQVK AVTQCSQFFH EVSIDGLEPD
151 TTYYYQIPAA NGTTQSEVLS FKTSRPAGHP GSFSVAVLND MGYTNAHGTH
```

```
201 KQLVKAATEG TAFAWHGGDL SYADDWYSGI LACADDWPVC YNGTSSTLPG
251 GGPLPEEYKK PLPAGEIPDQ GGPQGGDMSV LYESNWDLWQ QWLNNVTLKI
301 PYMVLPGNHE ASCAEFDGPH NILTAYLNDD IANGTAPTDN LTYYSCPPSQ
351 RNFTAYQHRF RMPGPETGGV GNFWYSFDYG LAHFVSIDGE TDFANSPEWN
401 FAEDVTGNET LPSESETFIT DSGPFGNVNG SVHETKSYEQ WHWLQQDLAK
451 VDRSKTPWVI VMSHRPMYSS AYSSYQLHVR EAFEGLLLKY GVDAYLSGHI
501 HWYERLYPLG ANGTIDTAAI VNNNTYYAHN GKSITHIING MAGNIESHSE
551 FSDGEGLTNI TALLDKVHYG FSKLTIFNET ALKWELIRGD DGTVGDSLTL
601 LKPSHVAGGK KLHS
```

A few more peptides can be mapped onto the sequence if the search parameters are extended to include peptides that have been incompletely cleaved by trypsin – as shown below. However, note that some of these peptides are of "rank" 2nd and 3rd and therefore these matches are of lower statistical significance. For reporting purposes (in publications), the standard search is better to use since the data is of higher statistical merit – however, for some studies the extra information in the extra peptides can be useful to know about.

1. PPA_ASPFI **Mass:** 67169 **Score:** 412 **Matches:** 20(4) **Sequences:** 11(4)

Acid phosphatase OS=Aspergillus ficuum GN=aphA PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
6	419.2119	836.4092	836.4181	-0.0088	0	17	9.7e+02	1	U	K.VHYGFSK.L
8	453.7188	905.4230	905.4277	-0.0046	0	(16)	1.6e+03	3	U	R.TPSCSQVK.A + Carbamidomethyl (C)
9	453.7220	905.4294	905.4277	0.0018	0	32	34	2	U	R.TPSCSQVK.A + Carbamidomethyl (C)
12	510.2882	1018.5618	1018.5699	-0.0080	0	30	63	1	U	R.EAFEGLLLK.Y
15	426.2029	1275.5869	1275.5673	0.0196	0	23	2.8e+02	1	U	K.SYEQWHWLQ.Q
17	674.3637	1346.7128	1346.7306	-0.0178	0	45	1.7	1	U	V.EPPAVKPATANPR.N
19	730.9037	1459.7928	1459.8035	-0.0106	0	(47)	1.1	1	U	R.NNVNVISLSYIPK.G
20	730.9056	1459.7966	1459.8035	-0.0068	0	64	0.018	1	U	R.NNVNVISLSYIPK.G
21	730.9072	1459.7998	1459.8035	-0.0036	0	(14)	2e+03	1	U	R.NNVNVISLSYIPK.G
22	730.9084	1459.8022	1459.8035	-0.0012	0	(36)	12	1	U	R.NNVNVISLSYIPK.G
23	731.4087	1460.8028	1459.8035	0.9994	0	(38)	8.3	1	U	R.NNVNVISLSYIPK.G
24	520.6296	1558.8670	1558.8831	-0.0161	0	71	0.0033	1	U	R.LVEPPAVKPATANPR.N
25	520.6307	1558.8703	1558.8831	-0.0128	0	(59)	0.064	1	U	R.LVEPPAVKPATANPR.N
27	627.9809	1880.9209	1880.9480	-0.0271	0	(35)	14	1	U	R.GDDGTVGDSLTLLKPSHVA.G
28	627.9830	1880.9272	1880.9480	-0.0208	0	(11)	2.9e+03	1	U	R.GDDGTVGDSLTLLKPSHVA.G
29	941.4803	1880.9460	1880.9480	-0.0019	0	62	0.027	1	U	R.GDDGTVGDSLTLLKPSHVA.G
31	535.2729	2137.0625	2137.0891	-0.0266	0	(39)	4.6	1	U	K.GMHIHYQTPFGLGQLPAVR.W + Oxidation (M)
32	713.3653	2137.0741	2137.0891	-0.0151	0	67	0.0067	1	U	K.GMHIHYQTPFGLGQLPAVR.W + Oxidation (M)
35	1208.1052	2414.1958	2414.1907	0.0052	0	11	2.8e+03	3	U	R.FPYTGPAVPIGDWVDPTINGNGK.G
48	1005.1747	3012.5023	3012.4982	0.0041	1	32	15	1	U	P.VVDERFPYTGPAVPIGDWVDPTINGNGK.G

Match to: **PPA_ASPFI** Score: **412**
Acid phosphatase OS=Aspergillus ficuum GN=aphA PE=1 SV=1
Found in search of 1F_MP1511_28july.pkl
Nominal mass (M_r): **67169**; Calculated pI value: **5.37**

NCBI BLAST search of PPA_ASPFI against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus ficuum
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **20%**

Matched peptides shown in **Bold Red**

```
  1 MKGTAASALL VALSATAAQA RPVVDERFPY TGPAVPIGDW VDPTINGNGK
 51 GFPRLVEPPA VKPATANPRN NVNVISLSYI PKGMHIHYQT PFGLGQLPAV
101 RWGKDPRNLN STAQGYSHTY DRTPSCSQVK AVTQCSQFFH EVSIDGLEPD
151 TTYYYQIPAA NGTTQSEVLS FKTSRPAGHP GSFSVAVLND MGYTNAHGTH
201 KQLVKAATEG TAFAWHGGDL SYADDWYSGI LACADDWPVC YNGTSSTLPG
251 GGPLPEEYKK PLPAGEIPDQ GGPQGGDMSV LYESNWDLWQ QWLNNVTLKI
301 PYMVLPGNHE ASCAEFDGPH NILTAYLNDD IANGTAPTDN LTYYSCPPSQ
351 RNFTAYQHRF RMPGPETGGV GNFWYSFDYG LAHFVSIDGE TDFANSPEWN
401 FAEDVTGNET LPSESETFIT DSGPFGNVNG SVHETKSYEQ WHWLQQDLAK
451 VDRSKTPWVI VMSHRPMYSS AYSSYQLHVR EAFEGLLLKY GVDAYLSGHI
501 HWYERLYPLG ANGTIDTAAI VNNNTYYAHN GKSITHIING MAGNIESHSE
551 FSDGEGLTNI TALLDKVHYG FSKLTIFNET ALKWELIRGD DGTVGDSLTL
601 LKPSHVAGGK KLHS
```

1G_MP1511 (PHY III) 1,4-beta-D-glucan cellobiohydrolase A Glucoamylase

Swiss-prot database search

http://www.matrixscience.com/cgi/master_results.pl?file=..%2Fdata%2F20110729%2FFtotleEaR.dat&REPTYPE=peptide&_sigthreshold=0.05&REPORT=AUTO&percolate=0&_server_mudpit_switch=99999999&_ignoreionsscorebelow=0&_showsubsets=1&_showpopups=TRUE&_sortunassigned=scoredown&_requireboldred=0

1. CBHA_ASPNC **Mass:** 48216 **Score:** 268 **Matches:** 6(4) **Sequences:** 3(2) **emPAI:** 0.25

Probable 1,4-beta-D-glucan cellobiohydrolase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=cbhA PE=3 SV=1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
52	512.2601	1022.5056	1022.5145	-0.0089	0	61	0.0023	1	U	K.FVTGSNVGSR.L
53	512.2618	1022.5090	1022.5145	-0.0055	0	(49)	0.034	1	U	K.FVTGSNVGSR.L
88	1015.0117	2028.0088	2028.0085	0.0003	0	44	0.078	1	U	K.MTVVTQFITDGSGSLSEIK.R + Oxidation (M)
95	729.0407	2184.1003	2184.1096	-0.0094	1	163	1e-13	1	U	K.MTVVTQFITDGSGSLSEIKR.Y + Oxidation (M)
96	729.0408	2184.1006	2184.1096	-0.0091	1	(97)	4.4e-07	1	U	K.MTVVTQFITDGSGSLSEIKR.Y + Oxidation (M)
97	729.0424	2184.1054	2184.1096	-0.0043	1	(15)	67	1	U	K.MTVVTQFITDGSGSLSEIKR.Y + Oxidation (M)

Proteins matching the same set of peptides:
CBHA_ASPNG **Mass:** 48228 **Score:** 268 **Matches:** 6(4) **Sequences:** 3(2)
1,4-beta-D-glucan cellobiohydrolase A OS=Aspergillus niger GN=cbhA PE=2 SV=1

Proteins matching a subset of these peptides:
CBHA_EMENI **Mass:** 47607 **Score:** 61 **Matches:** 2(2) **Sequences:** 1(1)
Probable 1,4-beta-D-glucan cellobiohydrolase A OS=Emericella nidulans

Match to: **CBHA_ASPNC** Score: **268**
Probable 1,4-beta-D-glucan cellobiohydrolase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=cbhA PE=3 SV=1
Found in search of 1G_MP1511_29july.pkl
Nominal mass (M_r): **48216**; Calculated pI value: **4.09**
NCBI BLAST search of CBHA_ASPNC against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus niger CBS 513.88
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **6%**
Matched peptides shown in **Bold Red**

```
  1 MHQRALLFSA LLTAVRAQQA GTLTEEVHPS LTWQKCTSEG SCTEQSGSVV
 51 IDSNWRWTHS VNDSTNCYTG NTWDATLCPD DETCATNCAL DGADYESTYG
101 VTTDGDSLTL KFVTGSNVGS RLYLMDTSDE GYQTFNLLDA EFTFDVDVSN
151 LPCGLNGALY FTAMDADGGA SKYPANKAGA KYGTGYCDSQ CPRDLKFIDG
201 QANVDGWEPS SNNDNTGIGN HGSCCPEMDI WEANKISTAL TPHPCDSSEQ
251 TMCEGNDCGG TYSDDRYGGT CDPDGCDFNP YRMGNDSFYG PGKTIDTGSK
301 MTVVTQFITD GSGSLSEIKR YYVQNGNVIA NADSNISGVT GNSITTDFCT
351 AQKKAFGDDD IFAEHNGLAG ISDAMSSMVL ILSLWDDYYA SMEWLDSDYP
401 ENATATDPGV ARGTCDSESG VPATVEGAHP DSSVTFSNIK FGPINSTFSA
451 SA
```

2. AMYG_ASPAW **Mass:** 68267 **Score:** 197 **Matches:** 5(2) **Sequences:** 4(2) **emPAI:** 0.17

Glucoamylase OS=Aspergillus awamori GN=GLAA PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
39	426.2148	850.4150	850.4185	-0.0034	0	9	3.9e+02	3	U	K.EVVDSFR.S
45	481.7271	961.4396	961.4465	-0.0068	0	78	6e-05	1	U	K.SDGEQLSAR.D
72	495.9279	1484.7619	1484.7736	-0.0117	1	31	2.1	1	U	R.ALANHKEVVDSFR.S
80	842.9265	1683.8384	1683.8356	0.0029	0	(3)	1.2e+03	1	U	K.QGSLEVTDVSLDFFK.A
81	842.9292	1683.8438	1683.8356	0.0083	0	79	3.4e-05	1	U	K.QGSLEVTDVSLDFFK.A

Proteins matching the same set of peptides:
AMYG_ASPNG **Mass:** 68267 **Score:** 197 **Matches:** 5(2) **Sequences:** 4(2)
Glucoamylase OS=Aspergillus niger GN=GLAA PE=1 SV=1

Proteins matching a subset of these peptides:
AMYG_ASPKA **Mass:** 68230 **Score:** 41 **Matches:** 2(0) **Sequences:** 2(0)
Glucoamylase I OS=Aspergillus kawachi GN=gaI PE=1 SV=1
AMYG_ASPSH **Mass:** 68089 **Score:** 41 **Matches:** 2(0) **Sequences:** 2(0)
Glucoamylase OS=Aspergillus shirousami GN=glaA PE=3 SV=1

Match to: **AMYG_ASPAW** Score: **197**
Glucoamylase OS=Aspergillus awamori GN=GLAA PE=1 SV=1
Found in search of 1G_MP1511_29july.pkl
Nominal mass (M_r): **68267**; Calculated pI value: **4.25**
NCBI BLAST search of AMYG_ASPAW against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus awamori
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **5%**
Matched peptides shown in **Bold Red**

```
   1 MSFRSLLALS GLVCTGLANV ISKRATLDSW LSNEATVART AILNNIGADG
  51 AWVSGADSGI VVASPSTDNP DYFYTWTRDS GLVLKTLVDL FRNGDTSLLS
 101 TIENYISAQA IVQGISNPSG DLSSGAGLGE PKFNVDETAY TGSWGRPQRD
 151 GPALRATAMI GFGQWLLDNG YTSTATDIVW PLVRNDLSYV AQYWNQTGYD
 201 LWEEVNGSSF FTIAVQHRAL VEGSAFATAV GSSCSWCDSQ APEILCYLQS
 251 FWTGSFILAN FDSSRSGKDA NTLLGSIHTF DPEAACDDST FQPCSPRALA
 301 NHKEVVDSFR SIYTLNDGLS DSEAVAVGRY PEDTYYNGNP WFLCTLAAAE
 351 QLYDALYQWD KQGSLEVTDV SLDFFKALYS DAATGTYSSS SSTYSSIVDA
 401 VKTFADGFVS IVETHAASNG SMSEQYDKSD GEQLSARDLT WSYAALLTAN
 451 NRRNSVVPAS WGETSASSVP GTCAATSAIG TYSSVTVTSW PSIVATGGTT
 501 TTATPTGSGS VTSTSKTTAT ASKTSTSTSS TSCTTPTAVA VTFDLTATTT
 551 YGENIYLVGS ISQLGDWETS DGIALSADKY TSSDPLWYVT VTLPAGESFE
 601 YKFIRIESDD SVEWESDPNR EYTVPQACGT STATVTDTWR
```

de novo sequenced peptide

1109.9 +2 ------TLNDGLSDSEAVAVGR partial peptide sequence.

Matches 1,4-beta-D-glucan cellobiohydrolase A