Phytase from *Aspergillus niger* NCIM 563: Isolation, Purification, Characterization and its Applications.

A THESIS
SUBMITTED TO THE UNIVERSITY
OF PUNE FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
THE DEPARTMENT OF MICROBIOLOGY

By

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Dedications

This thesis is dedicated to my family who I couldn't have survived through my stay in Pune without. First, to my father Jai prakash soni and mother Geeta rani for never doubting in my ability to achieve a goal and being very supportive of every endeavor I have ventured to take. Second, to my brothers Brijesh and Bhartesh soni their wives Vinita, Parul and both of my niece Vanshika and Rhidhima., who instilled in me the desire to learn and confidence to achieve.

"My mind is still tender, my thoughts are still young
My dreams are still soar, there are battles to be won"

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DECLARATION

This is to certify that the work incorporated in the thesis entitled **"Phytase from *Aspergillus niger* NCIM 563 : Isolation, purification, characterization and its applications"** submitted by **Sarvesh Kumar Soni** was carried out under my supervision at NCIM Resource centre, National Chemical Laboratory, Pune 411008, Maharashtra, India. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. J. M. Khire
(Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled **"Phytase from *Aspergillus niger* NCIM 563: Isolation, purification, characterization and its applications",** submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at NCIM Resource centre, National Chemical Laboratory, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. J.M. Khire (Research supervisor). The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Sarvesh Kumar Soni
(Research Scholar)

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ABSTRACT

Phytic acid (myo-inositol 1,2,3,4,5,6 hexa kis di hydrogen phosphate) is the principle storage form of phosphorus comprising 1-5% by weight in cereals, legumes, oil seeds and nuts. It is the mixture of calcium –magnesium salts of inositol hexa phosphophoric acid considered to be an anti nutritional part of human and animal diet because:

- Negatively charged phytic acid chelates with positively charged divalent cations as Fe^{+2}, Ca^{+2}, Mg^{+2}, and Zn^{+2} and thus interferes with the assimilation of important trace metals. This is partially attributed to the wide spreading human nutritional deficiencies of calcium, iron and zinc in developing countries, where the staple foods are of plant origin.
- It binds to proteins and make them more resistant to proteolytic digestion.
- Phytate phosphorus is poorly available to monogastrics (because of phytase absence)

Thus inorganic phosphorus is supplemented in diets for poultry, fish and swine to meet their nutritional requirement for phosphorus, after digestion the unutilized phytate phosphorus from plant feeds is excreted, becoming an environmental pollutants in areas of intensive animal agriculture; excessive phosphorus in soil runs off in lakes and the seas causing eutrofication and stimulating growth of aquatic organisms that may produce neurotoxins, injurious to humans.

Phytase (EC 3.1.3.8) (myo-inositol hexa kis phosphate phosphohydrolase) catalyzes the hydrolysis of myo- inositol hexakis phosphate (phytic acid, myo – inositol –p6) to inorganic monophosphate and lower phosphoric esters of myo-inositol, in some cases to free myo-inositols.

Due to its selectivity for phytic acid, phytase can be used not only as feed additive but also for selective phytate hydrolysis leading to lower myo-inositol phosphates and myo-inositol, those are being used extensively in cell signaling pathways, blood glucose response, lowering of cholesterol and triglycerides, in treatment of Parkinson's disease, Alzheimer's disease and Multiple sclerosis.

Thermo tolerant fungus, *Aspergillus niger* NCIM 563, was found to produce novel, highly acidic extracellular phytase at 30°C under submerged fermentation conditions. Phytase plays an important role in biochemistry of inositol phosphates, so our interest

has concentrated to large-scale production of this commercially interesting enzyme, especially microbial phytases that are secreted extracellularly.

As the report of Abelson, P.H.,(Science. 283(1999), pp.2015) annual sale of phytase as an animal food additive were estimated in 1999 to be 500 $ million and rising.

The thermo stability of Phytase enzyme suggests potential biotechnological applications in pulp and paper industry as a novel biological agent to remove plant phytic acids. Enzyme may also be used via coupling for diagnostic purposes in clinical medicine such as diagnosis of Hyperinositolemia.

Hydroxyapatite is an element contained in bones and is compatible with the human body, an artificial bone material and which in nano form can be used as good tool for localized drug (antibiotics, anti cancer drugs) delivery. So far there is no report for biological synthesis of hydroxyapatite. In the present study biomimetic synthesis of Hydroxyapatite and its polymorphs (β Tri calcium phosphate(β TCP) and Di calcium phosphate(DCP)) using cheap agro-based waste materials i.e. wheat bran and extra cellular enzyme phytase, produced by fungus *Aspergillus niger* NCIM 563 is demonstrated.

So the major potential uses of Phytase in these three areas: -

1. Animal nutrition
2. Environmental protection
3. Human health

has induced the fast emerging of phytase science and biotechnology.

This thesis entitled "**Phytase from *Aspergillus niger* NCIM: 563 Isolation, purification, characterization and its applications** " was designed to study the Phytases production from fungi(*Aspergillus*), purification and characterization of two types of phytases under submerged fermentation condition and their applications in synthesis of artificial bone material (calcium phosphate), production of myo-inositol and in different cell culture studies.

Chapter 1: General Introduction

This chapter covers the literature on phytase enzyme production, different sources, applications and recent advancements in phytase research. It also covers biomimetic synthesis of bone materials and different chemical methods of synthesis of

Hydroxyapatite and its biocompatibility studies on cell lines. The significance and objectives of the study is included in this chapter.

Chapter 2: Production of Phytase I (Highly Acidic), II by *Aspergillus niger* (NCIM 563) under submerged fermentation condition.

This chapter describes Novel extracellular phytase (I) was produced by *Aspergillus niger* NCIM 563 under submerged fermentation conditions at 30^0C in medium containing dextrin and glucose as carbon sources along with sodium nitrate as nitrogen source. Maximum phytase activity (41.47 IU/ml at pH 2.5 and 10.71 IU/ml at pH 4.0) was obtained when dextrin was used as carbon source along with glucose and sodium nitrate as nitrogen source. Nearly 13 times increase in phytase activity was observed when phosphate in the form of KH_2PO_4 (0.004 g/100 ml) was added in the fermentation medium. Physic-chemical properties of partially purified enzyme indicate the possibility of two distinct forms of phytases, Phy I and Phy II. Optimum pH and temperature for Phy I was 2.5 and 60^0C while Phy II was 4.0 and 60^0C, respectively. Phy I was stable in the pH range 1.5–3.5 while Phy II was stable in the wider pH range, 2.0–7.0. Molecular weight of Phy I and Phy II on Sephacryl S-200 was approximately 304 kDa and 183 kDa, respectively. Phy I activity was moderately stimulated in the presence of 1 mM Mg^{2+}, Mn^{2+}, Ca^{2+} and Fe^{3+} ions and inhibited by Zn^{2+} and Cd^{2+} ions while Phy II activity was moderately stimulated by Fe^{3+} ions and was inhibited by Hg^{2+}, Mn^{2+} and Zn^{2+} ions at 1 mM concentration in reaction mixture. The Km for Phy I and II was 3.18 and 0.514 mM while Vmax was 331.16 and 59.47 µmols/min/ mg protein, respectively.

Chapter 3: Purification and characterization of phytase I (Highly Acidic) and II from *Aspergillus niger* (NCIM 563).

Biochemical characteristics suggest the enzyme isolated (Acidic) to be a novel phytase (I). Optimum pH, pH stability studies and purified protein bands on SDS – PAGE and Native page confirms two different phytases under submerged fermentation conditions viz. active at pH 2.5 and 5.0. So we purified these 2 phytases Phy I and Phy II by using hydrophobic chromatography (phenyl sepharose) and gel filtration chromatography (S-200). Both the enzymes were characterized for optimum pH and stability, optimum temp.& stability, molecular weight (determined by SDS-PAGE,

MALDI- TOF, Gel filtration chromatography), isoelectric point, peptide mass finger printing, N-terminal sequencing, substrate specificity and kinetics, total amino acid analysis, effect of different metal ions & solvents, effect of gastric enzymes (protease, pepsin, trypsin) and glycosylation.

Chapter 4: Application of phytase of *Aspergillus niger* (NCIM 563) in biomimetic synthesis of Hydroxyapatite and its polymorphs

Wheat bran is a cheap agro-based waste material, which have a substantial amount of bound phosphorous in the form of phytic acid. However, there have been no attempts at harnessing the enormous amount of phosphorous present in most of the agro wastes included wheat bran in to nanosized hydroxyapatite particle and its polymorphs. In this chapter, the exciting possibility of a novel bio-inspired enzymatic synthesis of nanosized Hydroxy apatite (HAP) $Ca_{10}(PO_4)_6(OH)_2$ and its polymorphs (β Tri calcium phosphate(β TCP) and Di calcium phosphate(DCP)) using cheap agro-based waste materials i.e. wheat bran and a novel extra cellular enzyme phytase, produced by fungus *Aspergillus niger* NCIM 563 is demonstrated. The as prepared apatite powder with ~100 nm interconnected hollow spheres capped by stabilizing proteins devoid of any contamination of carbonate was synthesized by the hydro catalytic action of phytase on phytic acid i.e. extracted from wheat bran and calcium ions at 50ºC and pH of 5.5, under inert environment of nitrogen. Calcination of white solid precipitate leads to loss of occluded protein and converts it to highly crystalline material comprising HAP, β TCP and DCP.

Biomimic synthesis of nanosized biocomposites using novel enzymes from micro organisms starting from potential cheap agro-industrial waste materials is an exciting possibility and could lead to an energy-conserving and economically viable green approach towards the large-scale synthesis of crystalline artificial bone nanomaterials.

Chapter 5: Biocompatibility studies of enzymatically synthesized Hydroxyapatite and its polymorphs on Human Osteoblast like MG-63 cell line

This chapter describes the studies of artificial bone material Hydroxyapatite (HAP) synthesized by phytase enzyme and commercially available HAP (Sigma) on Osteosarcoma MG-63 cell line for its biocompatibility. Human Osteosarcoma cells (MG-63 cells) readily seed over 90% of the available biomaterials under static culture conditions, and the attached cells proliferate to extensively cover the biomaterials as

seen by DAPI staining and scanning electron microscopy. Cell growth on biomaterials was also monitored by total protein assay. The attached cells are over 90% viable after 7 days in culture as assessed by MTT staining. MG-63 cells also show significant increase in alkaline phosphatase activity 14 days post-addition of oestrogenic supplement. This biomaterial (enzymatically synthesized HAP) show promise for use as vehicles for cell delivery to place large numbers of cells directly into a wound site or onto a tissue engineering scaffold and can potentially be used as artificial bone material.

Chapter 6: Application of phytase in cell culture studies and myo-inositol production

This chapter describes the effect of phytase on cell line and its stability in different cell culture media. Partially purified phytase was stable up to 89 to 92 % till 72 hour in different cancer cell line culture media i.e. DMEM, MEM and RPMI 1640, showed 19.23 % proliferation of cancerous HL-60 cell line with 0.8 IU/3ml of phytase in MEM media. Inorganic phosphorus (98.45μg/ml) also released in culture media from cells after 24 hour of incubation at 37^0C.

Myo-inositol is well-known to play a major role in many cell signaling pathways of Ca^{+2} uptake. HPLC analysis of complete phytate degradation by phytase produced by *A. niger* NCIM: 563 showed myo-inositol as the main product. Produced myo-inositol and intermediates are very cheap in comparison to those synthesized by chemical methods since we are getting them from very cheap raw agricultural products like wheat bran.

ABBREVIATIONS

EDTA	Ethylene diamine tetraacetic acid
NCIM	National Collection of Industrial Microorganisms
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulphate
WRK	Woodward's Reagent K
ADP	Adenosine diphosphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
E. coli	*Escherichia coli*
MW	Molecular Weight
NADP	Nicotinamide adenine dinucleotide phosphate
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
MG-63	Human osteosarcoma Fibroblast cell line
HAP	Hydroxyapatite
DAPI	4', 6-diamidino-2-phenylindole, dilactate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ALP	Alkaline phosphatase
DMEM	Dulbecco's modified Eagle's medium
PBS	phosphate buffered saline
RT	Room temperature
BCA	Bicinchoninic acid
PNPP	p-nitrophenyl phosphate, disodium salt
CM	Conditioned media
PLAGA	Poly lactideco- glycolide
DMEM	Dulbecco's modified Eagle's medium
MEM	Minimum Essential Medium
RPMI 1640	Roswell Park Memorial Institute 1640
HL-60	Human promyelocytic leukemia cells
HPLC	High-performance liquid chromatography
GRAS	Generally Recognised as Safe
PDA	Potato Dextrose Agar
A 431	Human epithelial carcinoma cell line
MCF-7	Human breast adenocarcinoma cell line
THP1	Human acute monocytic leukemia cell line
IU	International Unit

CHAPTER 1

Introduction

In biological systems, phosphomonoester hydrolysis is an important reaction for energy metabolism, metabolic regulation, and signal transduction pathways. Phosphorus is an essential element for the growth of all organisms and in livestock production; feed must be supplemented with inorganic phosphorus (1). Cereals, legumes and oilseed crops are grown over 90% of the world's harvested area. Together they serve as a major source of nutrients for the animal kingdom. An important constituent of these crops is phytic acid (*myo*-inositol hexakisphosphate). In forage, one-third of phosphorus is present as digestible inorganics and two-thirds as organic phosphorus in the form of Phytin, which is a mixture of calcium–magnesium salts of inositol hexaphosphoric acid, known as phytic acid. The salt form, phytate, is an anhydrous storage form of phosphate accounting for more than 80% of the total phosphorus in cereals and legumes. Phytic acid is also a storage form of *myo*-inositol – an important growth factor. In addition phytic acid, and *myo*-inositol derivatives derived from it, serves several other important physiological functions in plants (1).

Due to its chemical structure phytic acid is a very stable molecule. It differs from other organo-phosphate molecules in having a high phosphate content, which results in a high negative charge over a wide pH range. Under normal physiological conditions phytic acid chelates essential minerals such as calcium, magnesium, iron and zinc. Phytic acid also binds to amino acids and proteins and inhibits digestive enzymes (3). Thus, phytic acid is an antinutritive component in plant-derived food and feed, and therefore enzymatic hydrolysis of phytic acid is desirable.

Phosphatases are a diverse class of enzymes catalyzing the cleavage of monophosphoester bonds in various organo-phosphate compounds. However, these enzymes are virtually unable to hydrolyze the monophosphoester bonds in phytic acid. Since the hydrolysis of phytic acid is of great importance a special class of enzymes hydrolyzing phytic acid has evolved – the phytases. These enzymes (*myo*-inositol hexakisphosphate phosphohydrolases) hydrolyze phytic

acid to less phosphorylated *myo*-inositol derivatives (in some cases to free *myo*-inositol), releasing inorganic phosphate. Phytase is widespread in nature, occurring in microorganisms, plants, as well as in some animal tissues. Several phytases have been cloned and characterized, such as fungal phytase from *Aspergillus ficuum* (4), bacterial phytase from *Escherichia coli* (5) and a mammalian phytase (6). These enzymes share a highly conserved sequence motif that is found at the active sites of acid phosphatases (7, 8). The reaction mechanism of *E. coli* phytase has been revealed (9, 10) and is likely to be common for most phytases. Therefore, these enzymes are said to form the phytase subfamily of histidine acid phosphatases (11).

The ruminants digest phytic acid through the action of phytases produced by the anaerobic gut fungi and bacteria present in their rumenal microflora. However, monogastric animals such as pig, poultry and fish utilize phytate phosphorus poorly because they are deficient in gastrointestinal tract phytases. Therefore, supplemental inorganic phosphate is added to their feed to meet the phosphate requirement and to ensure good growth. However, supplemental inorganic phosphate does not diminish the antinutritive effect of phytic acid. The antinutritive effect of phytic acid is especially problematic in the feeding of fish (12), due to their short gastrointestinal tract. This hinders the use of plant-derived protein in fish feed.

The problems mentioned above could be solved by hydrolysis of phytate using supplemental phytase (13). Therefore, phytase has become an important industrial enzyme and is the object of extensive research. By working efficiently on the substrate in the prevailing conditions, supplemental phytase could diminish the antinutritive effects of phytic acid and reduce the cost of diets by removing or reducing the need for supplemental inorganic phosphate.

In addition, phytase would be an environmentally friendly product, reducing the amount of phosphorus entering the environment. The Netherlands, Germany, Korea and Taiwan have enacted or are enacting legislation to reduce the phosphorus pollution created by monogastric livestock production (14).

Myo-inositol phosphates are also found in animal cells. However, the primary function of these compounds in animal cells is not to serve as a storage form of

phosphorus or *myo*inositol. Instead, their major role is in transmembrane signalling and mobilization of calcium from intracellular reserves. Therefore, these *myo*-inositol phosphates can be used as enzyme substrates for metabolic investigation, as enzyme inhibitors and therefore potentially as drugs (15). Chemical synthesis of these compounds is difficult, requiring protection and deprotection steps (16). Thus phytase, which converts phytic acid to lower *myo*-inositol phosphates, could be used for industrial production of these special *myo*-inositol phosphate derivatives. The enzyme was first discovered by Suzuki (17) during the course of rice bran hydrolysis studies, which found that the phosphatidylinositols exhibiting varying degree of phosphorylation were generated as intermediates or in some cases as end products.

The first commercial preparation of phytase came to the market in Europe in 1994 via Gist-Brocades. This required not only a practical use and delivery of enzyme but also the ability to produce the enzyme economically. Society's awareness and increasingly demanding recent regulations worldwide on controlling the agricultural pollution, particularly phosphorus pollution with limits on the phosphorus content in manure, have intensified the phytase research. The focus has mainly been on its production and use as a means of reducing inorganic phosphorus supplementation. At the close of 20th century, annual sales of phytase as an animal feed additive were estimated to be $500 million, and these are rising further.3 The growth of the market for phosphate to supplement animal feed fostered a critical step in the commercial development of phytase. The data based on the most recent livestock production showed that if phytase were used as a feed ingredient in the diets of all of the monogastric animals reared in the United States, it would release phosphorus with a value $\$1.68 \times 10^8$.

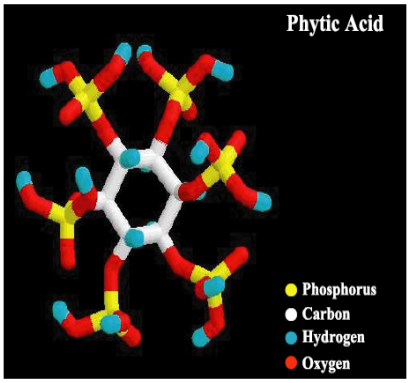
1.1 Phytic acid

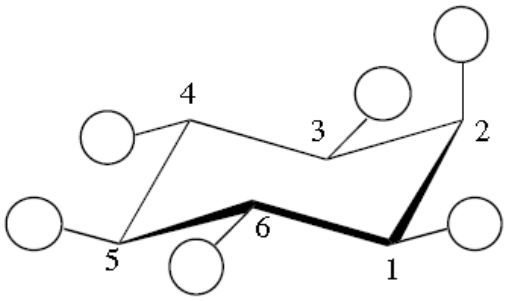
Phytic acid, which was discovered in 1903, has been found to be a nearly ubiquitous component in cereals and grains (18). Phytic acid is the major storage form of phosphorus in cereals, legumes and oilseeds. It serves several physiological functions and also significantly influences the functional and nutritional properties of cereals, legumes and oilseed (and food and feed derived thereof) by forming complexes with proteins and minerals. The correct chemical

description of phytic acid is *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (IUPAC-IUB, 1977). The salts of phytic acid are described as phytates. More accurately, phytate is a mixed potassium-, magnesium- and calcium salt of phytic acid that is present as a chelate in cereals, legumes and oilseed.

1.1.1 Chemical Structure of Phytic acid

The conformational structures for phytic acid have been derived from 31P-NMR (19) X-ray analysis (20). Johnson and Tate (19) suggested that the phosphate at 2-position is in axial position, the other phosphates being in an equatorial position. In contrast, Blank et al (20) concluded that the phosphate groups at the 1-, 3-, 4-, 5-, and 6- positions are axial, that at the 2-position being equatorial. Data of Costello et al (21) supports the conformation suggested by Johnson and Tate (19). This energetically most favourable conformation of phytic acid is shown in Figure 1. Costello and co-workers (21) also determined pKa values for dissociating groups of phytic acid using 31P-NMR and pH titration methods. They concluded that six groups were in the strong acid range (pKa 1.1 to 2.1), one in the weak acid range (pKa 5.70), two with pKa 6.80 to 7.60, and three in the very weak acid range (pKa 10.0 to 12.0). This suggests that phytic acid has a strong potential for complexing multivalent cations and positively charged proteins, since it exists as a strongly negatively charged molecule over a wide pH range.





(a) **(b)**

Figure 1(a). Phytic acid, the predominate storage form of phosphorus in mature seeds (figure courtesy of W. Schmidt – USDA/ARS)

(b) Energetically the most favourable conformation of phytic acid (*myo*-inositol hexakisphosphate). Circles represents the phosphate groups. The carbon atoms are numbered for D-configuration.

1.1.2 Physiological Functions of Phytic acid

Several physiological roles have been suggested for phytic acid in seeds and grains. These include functioning (i) as a phosphorus store, (ii) as an energy store, (iii) as a source of cations, (iv) as a source of *myo*-inositol (a cell wall precursor), and (v) initiation of dormancy. In addition phytic acid probably serves several other unknown functions in seeds (2). The role of phytic acid as a natural antioxidant in seeds during dormancy was suggested by Graf et al (22). The antioxidant property of phytic acid is based on the assumption that phytic acid effectively blocks iron-driven hydroxyl radical formation. Phytic acid has been shown to exert an antineoplastic effect in animal models of both colon and breast carcinomas.

The presence of undigested phytic acid in the colon may protect against the development of colonic carcinoma (23). Studies in the late 1980s and early 1990s have established the role of inositol phosphate intermediates in the transport of materials into the cell. Their role, especially that of inositol triphosphates, in signal transduction and regulation of cell functions in plant and animal cells is a very active area of research (14). An antagonist-stimulated increase in inositol (1,4,5)-triphosphate (and inositol (1,3,4,5)-tetraphosphate) is often associated with an increase in cytosolic free Ca^{2+}, which subsequently triggers a variety of physiological events. Many reviews on inositol phosphates are available in the literature (16, 24).

1.1.3 Occurrence, Distribution and Content of Phytic acid

Phytic acid occurs primarily as salts of mono- and divalent cations (e.g. potassium magnesium salt in rice and calcium-magnesium-potassium salt in soybeans) in discrete regions of cereal grains and legumes. It accumulates in seeds and grains during ripening, accompanied by other storage substances such as starch and lipids. In cereals and legumes phytic acid accumulates in the aleurone particles and globoid crystals, respectively (2). The endosperm of wheat and rice kernels is almost devoid of phytate, as it concentrates in the germ and aleurone layers of the cells of the kernel. Ferguson and Bollard (25) found that 99% of the phytate in dry peas was in the cotyledons and 1% in the embryo taxis. The highest amount of

phytate among cereals is found in maize (0.83 - 2.22%) and among legumes in dolique beans (5.92 - 9.15%) (2).

Table 1. Phytin-phosphorus (PP) content of feed ingredients as a percent of total phosphorus (TP)

Ingredient	Total P	PP, % (SD)	PP, % of TP
Soy beans *G max*	0.59	0.41 (0.22)	69.5
G soja	0.77	0.56 (0.18)	72.7
SBM (50% protein)	0.52	0.37 (0.03)	71
Corn	0.25	0.17 (0.02)	66
Corn Gluten Meal	0.58	0.36	62
Wheat Middlings	0.47	0.35	74
Cereals/Millets			
Maize	0.39	0.25	64
Rice	0.15	0.09	60
Wheat	0.44	0.27	61
Sorghum	0.30	0.22	73
Barley	0.33	0.20	61
Bajara	0.31	0.23	74
Oilseed meals			
Groundnut meals	0.60	0.46	77
Soybean meal	0.88	0.56	64
Cotton seed meal	0.93	0.78	82
Sunflower meal	0.90	0.45	51

1.1.4 Antinutritive Effects of Phytic acid

Phytic acid has been shown to have a strong antinutritive effect (3). This effect is based on the unusual molecular structure of phytic acid. At complete

dissociation, the six phoshate groups of phytic acid carry a total of twelve negative charges. Therefore, phytic acid effectively binds different mono-, di-, and trivalent cations and their mixtures, forming insoluble complexes (2). The formation of insoluble phytate mineral complexes in the intestinal tract prevents mineral absorption. This reduces the bioavailability of essential minerals (26). Zinc appears to be the trace element of which the bioavailability is most influenced by phytic acid. Rimbach and Pallauf (27) showed that graduated phytic acid supplementations had a negative influence on apparent Zn^{2+} absorption and lifeweight gain of growing rats.

Phytic acid interacts with proteins over a wide pH range, forming phytate-protein complexes. At a low acidic pH, phytic acid has a strong negative charge due to total dissociation of phosphate groups. Under these conditions a negative influence of phytic acid on the solubility of proteins can be expected because of the ionic binding between the basic phosphate groups of phytic acid and protonized amino acid (lysyl, histidyl and arginyl) residues (28, 29). Under acidic conditions phytic acid is likely to bind tightly to plant proteins, since the isoelectric point of plant proteins is generally around pH 4.0 - 5.0. In the intermediate pH range (6.0 to 8.0) both phytic acid and plant proteins have a net negative charge. However, under these conditions complex formation occurs between phytic acid and proteins. Possible mechanisms include direct binding of phytic acid to protonated α-NH_2 terminal groups and ε-NH_2 groups of lysine residues, and a multivalent cation-mediated interaction (30). By binding to plant proteins, phytic acid decreases their solubility and digestability, therefore also reducing their nutritive value.

In addition to complexing with minerals and proteins, phytic acid interacts with enzymes such as trypsin, pepsin, α-amylase and β-galactosidase, resulting in a decrease in the activity of these important digestive enzymes (31, 34, 35).

1.2 Phytase

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) catalyzes the hydrolysis of *myo*-inositol hexakisphosphate (phytic acid) to inorganic monophosphate and lower *myo-inositol* phosphates, and in some cases to free *myo*-inositol. The Enzyme NomenclatureCommittee of the International Union of Biochemistry distinguishes two types of phytase: 3- phytase (EC 3.1.3.8) and 6-

phytase (EC 3.1.3.26). This classification is based on the first phosphate group attacked by the enzyme (see numbering in Fig. 1). 3-Phytase is typical for microorganisms and 6-phytase for plants. Phytase is widespread in nature. Phytase activity has been reported in plant and animal tissues and in a variety of microorganisms. Some of the reported phytases from various sources are summarized in Table 2 and 3**.**

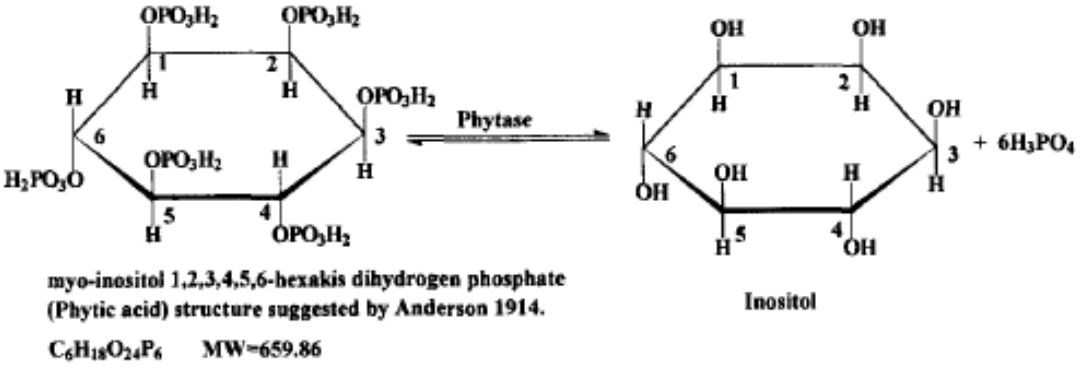
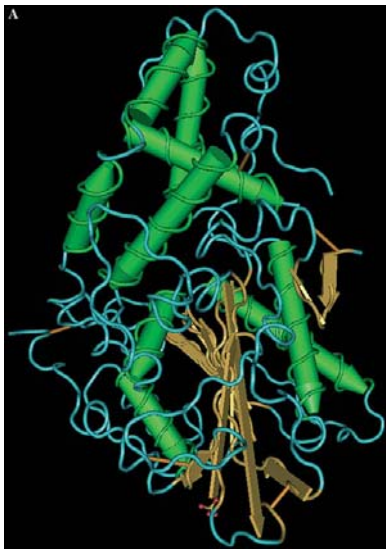


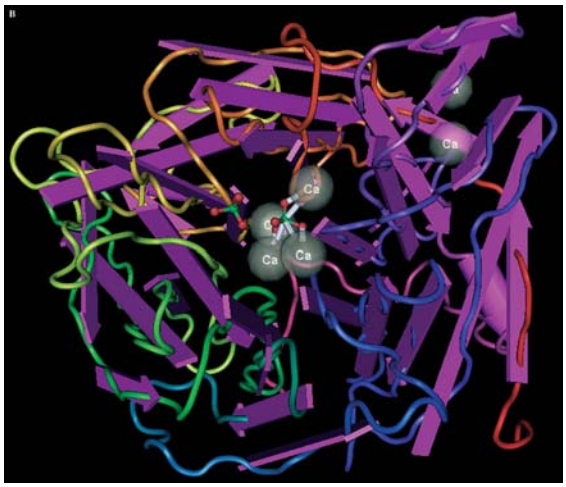
Figure 2. Hydrolysis of phytic acid to inositol and phosphoric acid by phytase

In order for an enzyme to be a phytase it must display phosphatase activity. Depending on the pH versus activity profile and the optimum pH for catalysis, these enzymes are further broadly classified as acid, neutral, or alkaline phosphatases. Since most of the recent interest generated in phytase research had centered on identifying an enzyme that would function effectively in the digestive tract of monogastric animals, most of these studies have focused on acid phosphatases with high specific activity for the preferred substrate, phytic acid. Within this subdivision, three structurally distinct classes of enzymes have been described to date as phytases. These three classes include representatives of histidine acid phosphatases (HAP), β propeller phytase (BPP), and purple acid phosphatases (PAP) (36).

Table 2. Survey of Microbial acid optimum histidine phosphatases

Enzyme	Source	EC No	NCBI Seq. ID	Gene . loci	AA	RHG motif	HD motif	Reference
Periplasmic phosphoan, hydride phosphohydrolase, 6-phytase, pH 2.5 optimum APase	*E. coli*	3.1.3.2 3.1.3.26	130735		432	Present	Present	(37)
3-Phytase	*A. niger* NRRL 3135	3.1.3.8	464382	phyA	467	Present	Present	(28)
3-Phytase	*A. niger*	3.1.3.8	484414	phyA	467	Present	Present	(38)
3-Phytase	*A. niger (awamori)*	3.1.3.8	166518	phyA	467	Present	Present	(39)
acid phosphatase (pH 2.5 optimum)	*A. niger* NRRL 3135	3.1.3.2, 3.1.3.8	464385	phyB	479	Present	Present	(40)
3-Phytase	*A. niger (awamori)*	3.1.3.8	464384	phyB	479	Present	Present	(39)
Acid phosphatase	*Schizosac. pombe*	3.1.3.2	130719	phol	453	Present	Present	(41)
Acid phosphatase	*Sacch. cerevisiae*	3.1.3.2	130721	pho3	467	Present	Present	(42)
Acid phosphatase, thiamine repressible	*Schizosac. pombe*	3.1.3.2	400839	pho4	463	Present	Present	(43)





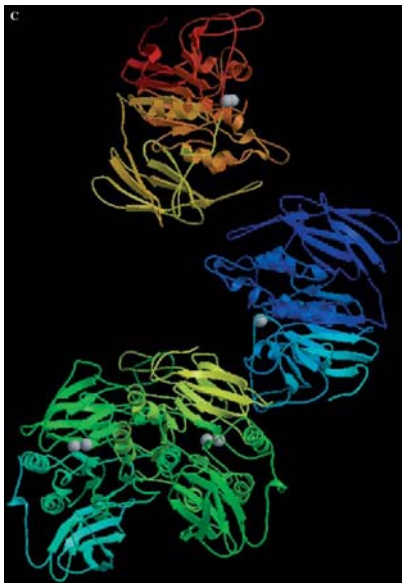


Figure 3. Computer-generated molecular models from National Center for Biotechnology Information (NCBI)'s Web site (www.ncbi.nlm.-nih.gov) of representatives from three classes of phosphatases: (A)1IHP, PhyA, a histidine acid phosphatase; (B) 1H6L, Ts-Phy, a β propeller phytase; (C) 1KBP, KSPAP, a purple acid phosphatase.

Histidine acid phosphatase (HAP): Phytases belonging to this class are the most widely studied and utilized today. Representatives of this large class of enzymes are known to occur in animals, plants and microorganisms (14). A common active site motif, RHGXRXP, is shared by all HAPs (7). The catalytic histidine in this sequence initiates a two-step reaction that results in the hydrolysis of phosphate monoesters. The term Histidine Acid Phytase (HAPhy) has been advanced to designate the HAPs that can accommodate phytate as a substrate (44). Both prokaryotic and eukaryotic HAPhys are known and they share little sequence homology other than the conserved active site motif. Among prokaryotic phytases, the one produced by *Escherichia coli* is the best-characterized HAPhy (5). A 3-D molecular model of its structure is available(Figure 3 a), and a eukaryotic version of the enzyme from *Aspergillus niger* has been advanced for use as an animal feed additive. In eukaryotes, HAPhys have been cloned in maize and in a number of fungal isolates. The most widely studied fungal phytases are from *A. niger* and *A. fumigatus*. Theses studies revealed that there are two classes of HAPhys. The first class has broad substrate specificity but a lower specific activity for phytic acid;

the second class has narrow substrate specificity but a high specific activity for phytase (45). Evidence from site-directed mutagenesis studies established the importance of certain amino acid residues that make up the substrate specificity site in fungal HAPhys. Mutating these key amino acids leads to changes in substrate affinity and the pH profile (46). While not directly involved in the catalytic mechanism of HAPhys, the conservation of an eight-cysteine motif appears to be essential to maintain the proper molecular structure necessary for the enzyme activity in fungal phytases (47). Today, the major application for HAPhys is in the hydrolysis of phytate in cereal and grains in animal feed. Future applications extend from the development of plant cultivars that require less P fertilizer to modification for use as a peroxidase.

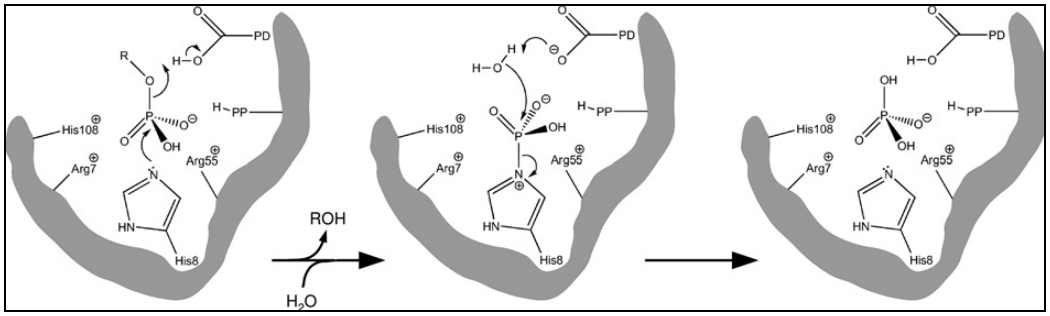


Figure 4. Catalytic mechanism of the histidine phosphatase superfamily

The essentially invariant four residues of the catalytic core are shown (Figure 4) numbered as in *E. coli* SixA. His8 is phosphorylated during the course of the reaction. The other three residues interact electrostatically with the phospho group before, during and after its transfer and form most or all of the 'phosphate pocket'. Additional neutral or positive residues, represented as PP in the diagram, may also contribute to the 'phosphate pocket' by hydrogen-bonding to the phospho group. The proton donor, an aspartate or glutamate residue whose position varies in different families, is shown as PD (Figure 4).

β−Propeller Phytase (BPP) : A wide range of catalytic functions has been dascribed to proteins possessing the β-propeller molecular architecture (48). A 3-D drawing of BPP molecule (Figure 3 b) shows a shape that resembles a propeller

with six blades (49). A novel calcium dependent *Bacillus* phytase that has this configuration has been cloned and characterized. It lacks the RHGXRXP sequence motif and therefore, it is not a member of HAP. It requires Ca^{2+} for both activity and thermostability (50). This phytase employs two phosphate-binding sites, a cleavage site for substrate hydrolysis and an affinity site to bind the substrate (51). β−propeller phytases share an optimum pH range with some alkaline plant phytases. The molecular structure of these plant phytases has yet to be determined, but they display some common traits with β-propeller phytases. They both have a narrow substrate range while requiring calcium for activity and only remove three phosphates from phytic acid to yield inositol trisphospahte as a final product. No commercial applications are available thus far for BPP, but it has been advanced as an animal feed additive and as a means to promote plant growth under phosphate limiting conditions.

Cysteine Phosphatase (CP): Recently, another class of phytase has been reported from an anaerobic ruminal bacterium, *Selenomonas ruminantium* (52). Its optimum temperature ranged between 50–55 ^{o}C with optimal activity in the pH range of 4.0–5.0 depending on the buffer used. Lead cations enhance activity, while Fe^{2+}, Fe^{3+}, Hg^{2+}, and Zn^{2+} ions strongly inhibited the enzyme. Sequence homology studies support similarities between this phytase and the catalytic domain found in the cysteine phosphatase superfamily. Its 3-D structure's accession number is 1U24. The structure of this phytase consists of one large and one small domain. Towards the C-terminal, near the edge of the large domain is a shallow pocket containing a two loop structure similar to the active site found in protein tyrosine phosphatase with the catalytically important HCXXGXXR(T/S). This enzyme catalyzes dephosphorylation of phytic acid to *myo*-inositol monophosphate.

Purple acid phosphatase (PAP): Characterization of a soybean (*Glycine max* L. Merr.) phytase has revealed the purple acid phosphatases sequence motif, DXG..GDXXY. .GNH(E,D)..VXXH..GHXH. The *GmPhy* phytase, found in germinating soybean seedlings, apparently contains the catalytic mechanism associated with this large class of metalloenzymes (53). This and a putative rice

(*Oryza sativa*) phytase are the only PAP phytases currently deposited in GenBank. As compared to fungal phytase, this soybean seed phytase has a relatively low specific activity for phytic acid. It has been proposed that the low catalytic activity of *GmPhy* may be advantageous in plant seed where a slow and balanced breakdown of phytate during germination could be efficacious. No 3-D model of soybean phytase is available, and no commercial applicant is envisioned.

1.2.1 Microbial Sources

Microbial phytase activity is most frequently detected in fungi, particularly in *Aspergillus* species. Shieh and Ware (54) screened over 2000 microorganisms isolated from soil for phytase production. Most of the positive isolates produced only intracellular phytase. Extracellular phytase activity was observed only in 30 isolates. All extracellular phytase producers were filamentous fungi. Twenty-eight belonged to the genus *Aspergillus*, one to *Penicillum* and one to *Mucor*. Of the 28 phytase-producing *Aspergillus* isolates 21 belonged to the *A. niger* group. Other studies (55-59) confirmed *A. niger* strains to be the best producers of extracellular phytase. Phytase has also been detected in various bacteria, e.g. *Aerobacter aerogenes* (60), *Pseudomonas* sp. (61), *Bacillus subtilis* (62), *Klebsiella* sp. (63), *B. subtilis* (natto) (64), *Escherichia coli* (5), *Enterobacter* sp. 4 (65) and *Bacillus* sp. DS 11 (later designated as *B. amyloliquefaciens*) (50). The only bacteria producing extracellular phytase are those of the genera *Bacillus* and *Enterobacter*. *E. coli* phytase is a periplasmic enzyme.

Some yeasts, such as *Saccharomyces cerevisiae*, *Candida tropicalis*, *Torulopsis candida*, *Debaryomyces castelii*, *Debaryomyces occidentalis, Kluyveromyces fragilis* and *Schwanniomyces castelii*, have also been shown to produce phytase (66-70).

Recently Phytase production has been reported from *Sachharomyces cerevisiae* CY (70), *Pedobacter nyackensis* MJ11 CGMCC 2503 (71), *Yersinia rohdei* (2008)), *Penicillium expansum* (72), rabbit cecal bacteria (73), lactic acid bacteria (74), *Sporotrichum thermophile* (75), *Megasphaera elsdenii* (76), *Bifidobacterium animalis* (77), *Aspergillus niger van Teighem* (78), *Selenomonas*

lacticifex (79), Marine Yeast *Kodamea ohmeri* BG3 (80), *Mucor indicus* MTCC 6333 (81), *Debaryomyces castellii* CBS 2923 (82), marine yeast *Kodamaea ohmeri* BG3 (83), antarctic yeast strain *Cryptococcus laurentii* AL27 (84), *Mucor hiemalis* (85), *Streptomyces hygroscopicus* NRRL B-1476 (86).

1.2.2 Plant Sources

Phytase occurs widely in the plant kingdom. Phytase has been isolated and characterized from cereals such as triticale, wheat, maize, barley and rice and from beans such as navy beans, mung beans, dwarf beans and California small white beans. Phytase activity has also been detected in white mustard, potato, radish, lettuce, spinach, grass and lily pollen (23). Laboure et al (87) purified and characterized phytase from germinating maize seedlings (Zea mays), and the cDNA coding for this phytase was cloned (88). This cDNA was used to screen a maize genomic library and two distinct genes were isolated and sequenced.

1.2.3 Animal Sources

Phytase has been found to exist in monogastric animals (89-92).Generally, however, intestinal phytase does not play a significant role in food-derived phytate digestion in monogastrics (93). Craxton et al (6) cloned and expressed a rat hepatic multiple inositol polyphosphate phosphatase (MIPP) having phytase activity. The MIPP mRNA was present in all rat tissues examined, but was most highly expressed in kidney and liver. A phytase-like enzyme was also decribed in the protozoan *Paramecium* (94).

1.2.4 Sequence Homology of phytases

The primary sequences of several fungal phytases have been determined in recent years. A phytase cloned from *A. niger* var. *awamori* had over 97% identity to *A. niger* NRRL 3135 phytase (*phyA*). Less homologous to the *A. niger* NRRL

3135 phytase are the phytases from *A. fumigatus* (65%), *A. terrus* (62%), *E. nidulans* (62%), *T. thermophilus* (61%) and *M. thermophila* (46%). The PhyB from *A. niger* NRRL 3135 shows 99% identity to the corresponding protein from *A. niger* var. awamori. Surprisingly, two phytases (PhyA and PhyB) from *A. niger* NRRL 3135 are only 25% homologous. Bacterial phytase from *Escherichia coli* and a mammalian phytase (rat hepatic MIPP) do not exhibit apparent sequence similarity to *A. niger* NRRL 3135 phytase. However, they share a highly conserved sequence motif - RHG - that is found at the active sites of acid phosphatases (7, 95). Furthermore, they contain a remote C-terminal motif with histidine and aspartic acid residues that probably take part in the catalysis. Therefore, these phytases are said to form the phytase subfamily of histidine acid phosphatases (11). The two plant phytases from Zea mays (PHYT I and PHYT II) are practically identical, but do not show any homology to other phytases or to any phosphatases. However, a region of 33 amino acids was detected that showed similarity to *A. niger* NRRL 3135 phytase. This region is probably the acceptor site for phosphate (88). The phytase from *B. amyloliquefaciens* (88, 96) shows 72% identity to an open reading frame revealed in the genomic sequencing of the *Bacillus subtilis* (97), but is not homologous to any phytases or to any phosphatases. Similarly, the phytase from *Enterobacter* sp. is not homologous to any phytases or histidine acid phosphatases. However, it is 30-38% homologous to low molecular weight acid phosphatases from *Chryseobacterium meningosepticum* and *Streptococcus equisimilis*. Especially certain lysine and tryptophan residues appears to be conserved.

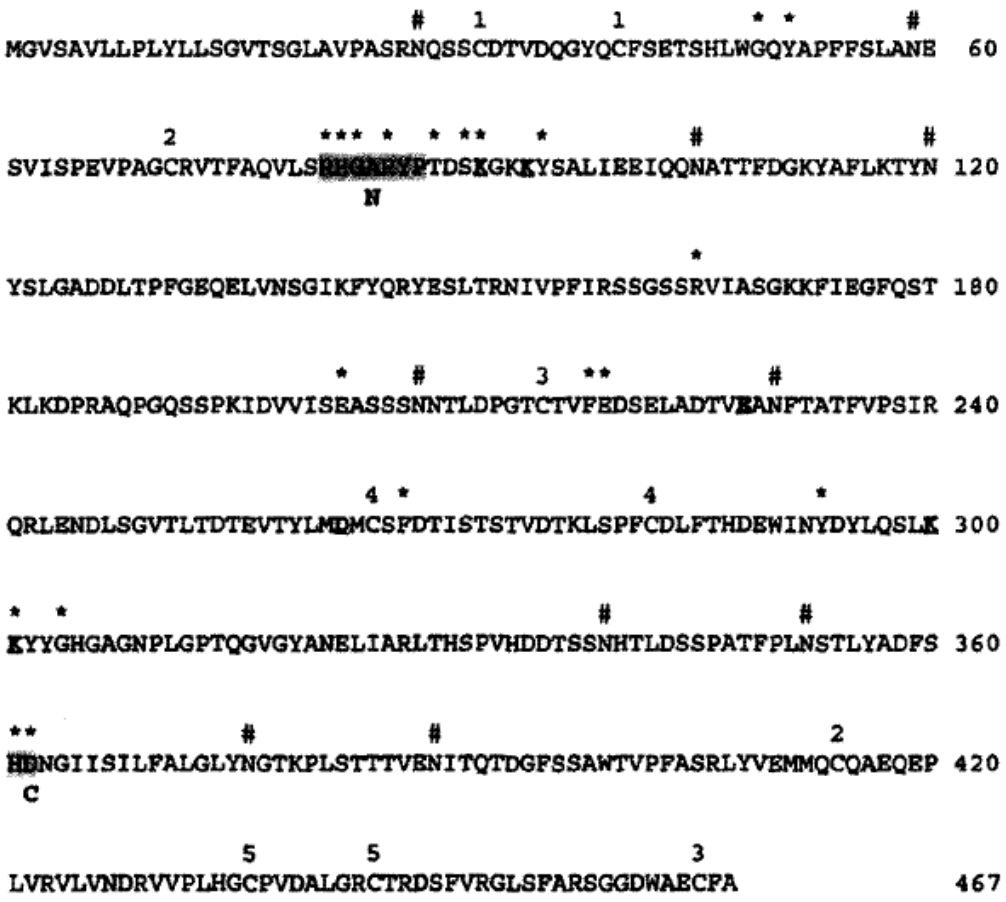


Figure 5. The *A. niger* NRRL 3135 phyA amino-acid sequence (NCBI Accession No. JN0656). The conserved residues having sidechains protruding into the reaction cavity have an asterisk (*) above them (98) and the # above the 10 Asn residues denotes glycosylation. The N-terminal (N) (RHGXRXP) and C-terminal (C) (HD) motifs found in histidine acid phosphates are highlighted. The number above each of the 10 cysteine residues refers to the individual disulfide bridge to which it belongs (99). The two acidic and four basic amino acids, respectively, that compose the substrate specificity site (100)--Glu228, Asp262, Lys91, Lys94, Lys300, and Lys301--are bold underlined.

Figure. 6 The amino-acid sequence from the crystal structure study of the *A. niger* T213 phyB phytase gene (100). The N-terminal (N) (RHGXRXP1 and C-terminal (C) (HD) motifs found in histidine acid phosphatase are underlined. The acidic amino acids of its substrate specificity site are highlighted. The number above each of the 10 cysteine residues refers to the individual disulfide bridge to which it belongs.

1.2.5 Induction of phytases

Shieh et al (101) observed that the production of extracellular fungal phytase was induced by a limiting concentration of inorganic phosphate in the growth medium. In contrast to fungal phytases, *B. subtilis* phytase is induced by phytate in the cultivation medium (62). The enzyme is also induced by wheat bran extract, which is known to contain phytate. Yoon et al (65) isolated and identified a phytase-producing bacterium using a synthetic medium containing phytate as the sole source of phosphate. Kim et al (50) also used phytate as the sole source of phosphate to isolate a phytase-producing *Bacillus* sp. strain DS 11. They produced phytase in a medium containing wheat bran, casein hydrolysate and mineral salts, and reached the maximum phytase activity after 24 hours of cultivation. On the

basis of these results it is difficult to say whether the production of these two enzymes is induced by phytate itself or by phosphate starvation. *Klebsiella* phytase production is induced by phytate (63, 102, 103). This situation is different from the production of phytate-degrading enzymes in *E. coli*, the synthesis of which has been shown to be stimulated by phosphate starvation or anaerobiosis (5, 103).

Various investigators have reported that in plants, during seed germination, phytate is rapidly degraded and that the levels of phytase increase by several orders of magnitude. It is not clear whether the increase in phytase activity is a result of expression of phytase genes or simple activation of existing enzyme. Nayini and Markakis (66) concluded that seeds contain both constitutive and germination-inducible phytases. Northern blot analysis and *in situ* hybridization showed a high accumulation of phytase mRNA during the early steps of germination in coleorhiza, radical cortex and coleoptile parenchyma (104).This indicates germination-induced synthesis of maize phytase.

1.3 Enzymatic properties of phytases

Table 3: Physicochemical and kinetic properties of phytases from different sources

Sources	Mole. Wt. (kDa)	(p*I*)	Optimum pH	Temp (^{0}C)	Subst. specificity	*K*cat (s−1)	*K*m (mM)	Ref.
Bacteria								
Bacillus sp. DS11	44	5.3	7.0	70	Specific	–	0.55	(50, 96)
Bacillus subtilis	36–38	6.25	6.0– 6.5	60	Specific	5.5	0.50	(105, 106)
Bacillus licheniformis (168 phyA, *phyL*)	44, 47	5.0, 5.1	4.5–6.0	55, 65	Specific	--	--	(107)
Escherichia coli	42	6.3–6.5	4.5	60	Specific	6209	0.13	(5)
Klebsiella terrigena	40	–	5.0	58	–	–	–	(103)
Pseudomonas syringae	45	–	5.5	40	Specific	–	0.38	(108)
Lactobacillus sanfrancesis	50	–	4.0	45	Broad	–	–	(109)
Citrobacter braakii	47	–	4.0	50	–	–	0.46	(110)

Yeasts								
Arxula *adeninivorans adeninivorans*	–	–	4.5	75	Specific	–	0.23	(111)
Schwanniomyces castellii	490	--		77				(112)
Sacch. cerevisiae	120	–	2.0–2.5, 5.0–5.5	55–60	–	–	–	(113)
Pichia pastoris	95	–	2.5, 5.5	60	--	--	--	(114)
Fungi								
Aspergillus ficuum (phyA)	85	4.5	2.5, 5.0	58	Specific	348	0.027	(115)
A. ficuum (phyB)	68	4.0	2.5	63	Broad	628	0.103	(116, 117)
A. oryzae	120	4.15	5.5	50	Broad	–	0.33	(118)
Aspergillus niger SK-57	60	–	2.5,5.5	50	Specific	--	0.019	(119)
A. niger ATCC 9142	84	–	5.0	65	Broad	–	0.10	(120)
Peniophora lycii (phyA)	72	3.61	4–4.5	50–55	Specific	--	--	(121)

1.3.1 Biophysical characteristics

Published molecular size and the calculated theoretical molecular size of the mature protein, and the isoelectric point (p*I*) of phytases from various sources are shown in Table 3. Most phytases hitherto characterized are monomeric enzymes. This is the case with fungal phytases (115, 122, 123) with *E. coli* and *K. terrigena* phytases (5, 103) and with *B. subtilis* (natto) Phytase (64). However, some plant and animal phytases appear to be built up of, multiple subunits. A phytase accumulating in maize seedlings during germination is a dimeric enzyme built up from two 38 kDa subunits (87). Purified rat intestinal phytase exhibited two protein bands in SDS-PAGE with estimated molecular masses of 70 and 90 kDa (124). However, since only the 90 kDa subunit is induced by phytic acid, it is likely that these protein bands represent two different enzymes (alkaline phosphatase and phytase, respectively). An inositol hexakisphosphate dephosphorylating enzyme from the protozoan *Paramecium* has been proposed to have a hexameric structure (94).

Two different forms of *Klebsiella aerogenes* phytase have been reported. One, possibly the native enzyme, has an exceptionally large size (700 kDa). The other is probably a fraction of the native enzyme, with a full complement of activity and an exceedingly low molecular weight between 10 and 13 kDa (102). Bacterial phytases are generally smaller than their fungal counterparts. The predicted size of fungal phytases is around 50 kDa and the experimental size is between 65 and 70 kDa, indicating heavy glycosylation. *A. niger* NRRL 3135 native phytase is 27% glycosylated. It contains a substantial proportion of N-linked mannose chains and galactose (4, 45) reported that glycosylation of recombinant phytases was highly variable. Whereas glycosylation was moderate in *A. niger*, it was excessive and highly variable in *Hansenula polymorpha* and *Saccharomyces cerevisiae*. Surprisingly, glycosylation differed not only between the different expression systems used but also between different batches of a phytase produced in the same expression system. Analysis of the glycosylation pattern of *A. niger* phytase showed that the heterogeneity was due to incomplete glycosylation of two out of ten potential N-glycosylation sites.

In general, glycosylation may have several effects on the properties of an enzyme. Firstly, it may influence the catalytic properties or have an impact on the stability of the enzyme. Secondly, it may influence the pI of the protein. Thirdly, by consuming metabolic energy it may lower the level of expression of the protein. Surprisingly, different extents of glycosylation had no effect on the catalytic properties, thermostability or refolding properties of *A. niger*phytase (122). The importance of glycosylation for the structure and function of phytase is further brought into question by the fact that only two potential N-glycolysation sites are conserved in fungal phytases (125). Han and Lei (126) studied the role of glycosylation in the functional expression of *A. niger* phytase (*phyA*) in *Pichia pastoris*. Their results indicated an identical capacity of phytic acid hydrolysis and slightly improved thermostability in glycosylated enzyme produced in *P. pastoris* compared to the same enzyme overexpressed in *A. niger*. Deglycosylation of the phytase resulted in 34% reduction in thermostability. Suppression of glycosylation by tunicamycin during expression resulted in significant reduction of phytase production, indicating that glycosylation is vital for the biosynthesis of

recombinant PhyA in *P. pastoris*. However, tunicamycin might also impair the production by other means. Because there was no accumulation of intracellular phytase protein, the impairment did not appear to occur at the level of translocation of phytase. On the other hand, studies by Wyss et al (122) suggest that glycosylation has no or only a minor effect on the pI of the fungal phytases tested. The only exceptions were the phytases expressed in *H. polymorpha*, in which a pronounced shift to acidic pI values was observed. All the fungal, bacterial, and plant phytases hitherto investigated have acidic pI values, with the exception of *A. fumigatus* phytase, which has a basic pI. 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. *A. fumigatus*, *Emericella nidulans*, *A. terrus*, and *Myceliophthora thermophila* phytases have a tendency to undergo proteolytic degradation when expressed in *A. niger* and stored as concentrated culture supernatants at 4^{o}C (122). The activity of phytase from *B. subtilis* is unaffected by proteases such as trypsin, papain and elastase (62), indicating a stronger protease resistance than that of fungal phytases.

1.3.2 Temperature and pH stabilities and optima

The pH and temperature optima of phytases from various sources are presented in Table 3. The pH optimums of phytases vary from 2.2 to 8. Most microbial phytases, especially those of fungal orgin, have a pH optimum between 4.5 and 5.6. In contrast to most fungal phytases, *A. fumigatus* phytase has a broad pH optimum; at least 80% of the maximal activity is observed at pH values between 4.0 and 7.3. Some bacterial phytases, especially those from *Bacillus*, have a pH optimum at 6.5 - 7.5. The pH optima of plant seed phytases range from 4.0 to 7.5, most having an optimum between 4.0 and 5.6. Two alkaline plant phytases having a pH optimum at about 8.0 have been described in legume seed (127) and lily pollen (128). *A. niger* NRRL 3135 and *Citrobacter freundii* phytases differ from other phytases in having two pH optima. The temperature optima of phytases vary from 45 to 77oC. Wyss and co-workers (129) studied the thermostability of three acid phosphatases of fungal orgin (*A. fumigatus* and *A.niger* phytase, and *A. niger* pH 2.5 optimum acid phophatase) by circular dichroism (CD) spectroscopy and fluorescence, and by measuring the enzymatic activity. They concluded that *A.*

niger phytase was not thermostable, neither did it have the capacity to refold after heat denaturation. At temperatures between 50 and 55^{o}C it underwent an irreversible conformational change that resulted in 70-80%loss of enzyme activity. The *A. fumigatus* phytase was not thermostable, but had the remarkable property of being able to refold completely into native like, fully active conformation after 20 min heat denaturation at 90^{o}C. Compared to two phytases, *A. niger* pH 2.5 acid phosphatase had higher intrinsic thermostability. At temperatures up to 80^{o}C, only minor changes in CD spectral characteristics and only slight, but irreversible enzyme inactivation were observed. However, exposure to 90^{o}C resulted in an irreversible conformational change and complete loss of activity. *Bacillus* sp. strain DS11 phytase (50) had a temperature optimum at 70^{o}C, which is higher than the temperature optimum of phytases in general. It was also very thermostable: 100% residual activity after 10 min incubation at 70^{o}C (in the presence of CaCl2). The enzyme stability of *Bacillus* sp. strain DS11 phytase was drastically reduced above 50^{o}C in the absence of CaCl2, whereas it was rather stable up to 90^{o}C in the presence of $CaCl_2$. After incubation at 90^{o}C for 10 min, the residual enzyme activity was approximately 50% of the initial activity. This indicates that the Ca^{2+} ion has a strong protecting effect on the enzyme against thermal denaturation.

1.3.3 Modulators of Enzyme activity

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 phytic acid and therefore decrease the active substrate concentration. Phytase from *Enterobacter* sp. 4 was greatly inhibited by Zn2+, Ba^{2+}, Cu^{2+} and Al^{3+} (65).Similarly, the phytase from *B. subtilis* (natto) N- 77 was greatly inhibited by metal ions such as Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+}, and Al^{3+} (64). Both of these enzymes, as well as two other *Bacillus* phytases (50, 62), were greatly inhibited by EDTA, indicating that a metal ion (calcium) is needed for the activity. Wyss et al (45) reported that Cu^{2+} considerably depressed the enzyme activities of *E. nidulans* and *A. terrus* phytases, and that several metal ions inhibited *A. fumigatus* phytase. The activity of *A. fumigatus* phytase was stimulated up to 50% by EDTA, whereas EDTA had no major effects on the enzymatic

activities of other fungal phytases tested (*E. nidulans*, *A. niger* and *A. terrus*). The effects of metal ions and the fact that EDTA either has no effect or even stimulates phytase activity indicates that fungal phytases clearly differ from Ca^{2+}dependent *Bacillus* phytases that are readily inhibited by EDTA. This conclusion is supported by the lack of metal ions in the crystal structure of *A. niger* Phytase (99). In addition to calcium-dependent *Bacillus* phytases, a phytase from pollen of *Typha latifolia* and phytases from some other plants require Ca^{2+} for optimal activity (87, 128, 130, 131).

Reducing reagents, such as 2-mercaptoethanol, dithiotreitol and reduced glutathione have no major effects on microbial phytases. This suggests that these enzymes either have no free and accessible sulfhydryl groups or that the free sulfhydryl groups play a negligible role in the enzyme activity and structure. This interpretation is supported by the fact that most mature phytases have an even number of cysteine residues that might be implicated in disulfide bridges, as is the case with *A. niger* phytase (99). The function of disulfide bonds in *A. ficuum* phytase was elucidated by unfolding studies performed by Ullah and Mullaney (132). These authors concluded that disulfide bonds are necessary for the structure and activity of the enzyme and play an important role in the folding of the protein. Mature *Bacillus* phytases appear to have no cysteine residues.

A structural analog of the substrate, *myo*-inositol hexasulfate, has been shown to be a potent competitive inhibitor of both PhyA and PhyB enzymes from *A. ficuum* (133). The Ki of inhibiton for the PhyA and PhyB enzymes were estimated to be 4.6 and 0.2 μM, respectively. Fluoride is a known inhibitor of different phytases and phosphatases (56, 134). The phytase from cotyledons of germinating soybean seeds was strongly inhibited by fluoride, vanadate and inorganic phosphate (130). Inorganic phosphate was a competitive inhibitor of soybean seed phytase. The fact that soybean seed phytase is competitively inhibited by orthophosphate with a Ki value of 28 μM implies that the activity of the enzyme is tightly regulated. Competitive product -inhibiton of phytate hydrolysis caused by inorganic phosphate is recognized for bacterial, fungal and oat spelt phytases (5, 55, 135). Fluoride also inhibited the alkaline phytase from lily pollen (131, 136) and competitively inhibited the phytase from *K. terrigena* (103) and phosphate,

molybdate and vanadate. Molybdate and vanadate are known to inhibit phosphatase enzymes. It has been suggested that these transition metal oxoanions inhibit phosphomonoesterases by forming complexes that resemble the trigonal bipyramidal geometry of the transition state (137).

Substrate concentrations above 300 μM have been reported to be inhibitory for the phytase like enzyme from *Paramecium* (94). The *Klebsiella* sp. and *Rhizopus oligosporus* phytases were also inhibited by the substrate (63, 138), but only in higher substrate concentrations. Fungal phytase activity has been shown to be inhibited by substrate concentrations exceeding 1 mM (139). Maize root and soybean phytases were found to be inhibited at 300 μM and 20 mM substrate concentration, respectively (140, 141). In high substrate concentrations, the charge due to the phosphate groups may affect the local environment of the catalytic domain of the enzyme. This might inhibit conversion of the enzyme-substrate complex to enzyme and product (142), although inhibition due to the formation of poorly soluble protein-phytate complex cannot be ruled out.

1.3.4 Substrate Specificity and Kinetic parameters

Phytases show relatively broad substrate specificity. ADP, ATP, p-nitrophenyl phosphate, phenyl phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate, α-, and β-glycerophosphate and 3-phosphoglycerate, that are not structurally similar to phytic acid, are frequently hydrolyzed by phytases. Only a few phytases have been described as highly specific for phytic acid: the *Bacillus* phytases (62, 64) and alkaline phytase isolated from lily pollen (136). Most phytases hitherto studied follow Michaelis- Menten kinetics, with the exceptions of *M. thermophila* and *E. nidulans* phytases which display non-Michaelis-Menten behavior. It should be noted that under the standard assay conditions (i.e. 2 mM phytic acid), only the rate of the reaction from *myo*-inositol hexakisphosphate to pentakisphosphate is measured. Ullah and Phillippy (142) determined the kinetic parameters of *A. ficuum* phytase and two acid phosphatases for *myo*-inositol hexa-, penta-, tetra, and triphosphates. Phytate had the lowest Km value for all three enzymes. They concluded that both phytase and pH 2.5 optimum acid phosphatase effectively hydrolyzed the tested *myo-inositol* phosphates. Poor hydrolysis of tested forms of *myo*-inositol phosphates by pH 6.5 optimum acid phosphatase was

demostrated by low Vmax and Kcat values. The kinetic efficiency of an enzyme is validated by means of the Kcat/Km values for a substrate. The highest Kcat/Km values for phytase and pH 2.5 optimum acid phosphatase were those for phytic acid (1.29×10^7 and 6.10×10^6 M^{-1} s-1, respectively). *E. coli* phytase has a Kcat/Km value of 4.78×10^7 M^{-1} S^{-1} (5), which is the highest value reported for a phytase. The specific activities for fungal phytases with phytic acid as substrate vary almost 10- fold, from 23 to 198 U mg^{-1} (*A. fumigatus* and *A. terrus*, respectively). The different extent and patterns of glycosylation have no significant effect on the specific activities of fungal phytases (45). Specific activities reported for bacterial phytases vary almost 100-fold, from 8.7 to 811 U mg-1, (*B. subtilis* and *E. coli*, respectively). On the basis of substrate specificity, phytases can be divided into two classes - phytases with broad substrate specificity (e.g. *A. fumigatus*, *E. nidulans* and *M. thermophila*) and phytases with rather high specificity for phytic acid (e.g. *A. niger*, *A. terrus* and *E. coli*). Phytases with broad substrate specificity inherently have rather low specific activity for phytic acid (23 to 43 U mg^{-1}), whereas phytases with narrow substrate specificity have specific activities of 103 - 811 U mg^{-1}. *Bacillus* phytases do not fit into this classification. They appear to be very specific for phytic acid, but have apparently low specific activity. The low specific activity is likely to hinder their use in industrial applications. The Km values and specific activities of some published phytases for phytic acid are presented in Table 4.

1.3.5 Kinetics and End Products of Phytic acid degradation

Phytic acid has six phosphate groups that may be released by phytases at different rates and in different order. Wyss et al (45) investigated the kinetics of phosphate release and the kinetics of accumulation of reaction intermediates, as well as the end products of phytic acid 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 stereospecificity 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 phytic acid 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 pronouced accumulation of *myo*-inositol tetrakisphosphate during phytic acid 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 was detected. Therefore, lower *myo*-inositol phosphates appears to be less suitable substrates for *A. niger*, *A. terrus* and especially *E. coli* phytases than phytic acid. The stereoisomer assignment of the reaction intermediates and degradation pathway was not determined for these enzymes. The fact that the end products of phytic acid hydrolysis for most phytases is identical does not necessarely mean that the degradation pathways for phytic acid 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 (103, 143). 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 (131) was shown to start the hydrolysis of phytic acid at position D-5, with two subsequent dephosphorylation steps to yield Ins(1,2,3)P3 as the final product (136). Inositol triphosphate is also the end product of phytic acid hydrolysis for the phytase from *Typha latifolia* pollen (128). 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 (6). A detailed characterization of the phytase from the protozoan *Paramecium* by Freund et al (94) revealed that this enzyme degrades phytic acid 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 preceeds 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 (62) showed that *myo*-inositol monophosphate (phosphate position not determined) is the end product for *B. subtilis* phytase. Kinetics, reaction intermediates and degradation pathways of phytic acid degradation have not been reported for *Bacillus* phytases, neither is it known whether these enzymes are 3- or 6-phytases. The strong stereospecificity 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 equatorically from the inositol ring can access the catalytic sites of these enzymes.

1.3.6 Active Site and Reaction mechanism

Acid phosphatases are a heterologous group of enzymes that hydrolyze phosphate esters, optimally at low pH. A number of acid phosphatases, from both prokaryotes and eukaryotes, share two regions of sequence similarity, each centered around a conserved histidine residue (in bold) (95). The consensus pattern for these two regions reported in the SWISS-PROT protein domain data base are [LIVM]-X(2)-[LIVMA]-X(2)-[LIVM]-X-R-**H**- [GN]-X-R-X-[PAS] and [LIVMF]-X-[LIVMFFAG]-X(2)-[STAGI]-**H**-D-[STANQ]-X- [LIVM]-X(2)-[LIVMFY]-X(2)-[STA]. Sequence alignment of *pho3* and *pho5* gene products in yeast, human prostatic and lysosomal acid phosphatase, and PhyA and PhyB from *A. niger* NRRL 3153 reveals a conserved heptapeptide of R**H**GXRXP near the N-terminus (compare to the former consensus pattern). The acid phosphatases containing this active site motif are grouped as histidine acid phosphatases. This active site motif is totally conserved in all fungal phytases and is also present in the *E. coli* phytase. Sequence alignment of fungal and *E. coli* phytases reveals a conserved **H**D-motif near the C-terminus (compare to the latter consensus pattern). Protein data base searches for the sequence motifs RHG and HD reveal that they are present in a number of acid phosphatases. In general, two classes of acid phosphatases can be identified in terms of molecular mass. A low molecular weight form lacks both motifs. A high molecular form is divided into two subclasses. One exhibits either the RHG or the HD motif, the other both (144). Therefore, phytases are said to form the phytase sub-family of high molecular weight histidine acid phosphatases (11). Ullah and co-workers have used amino acid residue specific modifying

reagents to probe the active sites of fungal phytases (7, 8). Their results clearly establish the crucial role of histidine and arginine residues for the activity of phytase. Ullah and Dischinger (144) showed that some tryptophan residues might also be involved in the phosphohydrolytic cleavage of phytic acid.

The results of Ullah and co-workers correlate with the site-directed mutagenesis studies on *E. coli* Phytase (9, 10). On the basis of these results the following two-step reaction mechanism for the high molecular weight histidine acid phosphatases has been suggested: the positive charge of the guanido group of the arginine residue in tripeptide RHG interacts directly with the phosphate group in the substrate, making it more susceptible to nucleophilic attack, while the histidine residue serves as a nucleophile in the formation of covalent phosphohistidine intermediate; the aspartic acid residue (from the C-terminal HD-motif) protonates the group leaving the substrate. The histidine residue in the HD-motif also has a critical role in the enzyme activity. Due to the existence of these motifs in fungal phytases and in the *E. coli* phytase, the proposed reaction mechanism is likely to be characteristic to the members of the phytase sub-family of histidine acid phosphatases.

The active site of phytases shows remarkable homology to the active site residues of the members of a particular class of acid phosphatase termed "histidine phosphatase" (7, 95). Chemical probing at the active site of human prostatic acid phosphatase suggested that an arginine residue is involved in catalysis (145). A similar observation was also made in A. *niger* NRRL 3135 (7). Sequence similarity search among diverse phosphate metabolizing enzymes, i.e., fructose 2,6-biphosphatase, phosphate glycerate mutase, and acid phosphatase, led to the identification of a tripeptidic region with the sequence RHG (146). When A. *niger* NRRL 3135 N-terminal amino acid sequence was compared with these enzymes, it was noted that the conserved tripeptidic region was also present not only in fungal phyA but also in the N-terminal region of phyB (147). Further chemical probing of the fungal phytase also suggested a sensitive histidine at the active site (8). On close examination of the active site residues of phyA and phyB in A. *niger* NRRL 3135, pH 2.5 optimum acid phosphatase in *E. coli,* pho3 and pho5 gene products in yeast, human prostatic, and lysosomal acid phosphatase, it was observed that the

most conserved sequence is RHGXRXP (Table). The acid phosphatases and phytases containing this active site motif in the N-terminal segment of the protein are grouped under "histidine phosphatase"; a survey of the protein and DNA databases revealed 14 members belonging to this group of acid phosphatases (Table VII). The positive charge of the guanido group of arginine is probably responsible for the recognition and anchoring of the negatively charged phosphate group to the proximity of a histidyl residue in the active site. The phosphate group is transiently

Table 4. Alignment of fungal Phy A and Phy B Active–site Sequence with published acid phosphatases, Phosphoglycerate Mutase And Fructose-2, 6- Biphosphatase

Enzyme	Active- site sequence
AfphyA	C R V T F A Q V L S R H G A R Y P T D S K G K
AfphyB	C E V D T V I M V K R H G E R Y P S P S A G K
YscACP3	C E M K Q L Q M L A R **H** G E R Y P T Y S K G A
YscACP5	C E M K Q L Q M L A R H G E R Y P T V S L A K
YscpACPl	C K I K Q V H T L Q R H G S R N P T G G N A A
ECACP	L K L E S V V I V S R H G V R A P T K A T Q L
YePGM	* * * * P K L V L V R H G Q S E W N E K N L F

transferred to the histidine group to form an unstable phosphoenzyme complex before hydrolytic cleavage to form orthophosphate (145). This mechanism is reasonable because it is known that a phosphate group attaches to the solitary histidine residue in phosphocarrier protein, HPr of gram negative bacteria (148). In *A*. *niger* NRRL **3135** phyA inactivation of tryptophan led to catalytic demise (8). Of the four tryptophans, only Trp25 and Trp267 are in the hydrophilic region; the other two residues are in the hydrophobic region and may not play a role in active site formation.

1.3.7 Crystal structure of phytase

In 1996, a process for the deglycosylation of proteins for crystallization using a recombinant glycosidase fusion protein was developed (149) that enabled Kostrewa et al (99) to first crystallize *A. niger* NRRL 3135 phyA and then determine its structure by X-ray crystallography. The X-ray crystal structure of phyA established how the 5 disulfide bridges are formed from the 10 Cys residues: Cys31-Cys40, Cys71-Cys414, Cys215-Cys465, Cys264- Cys282, and Cys436-Cys444 (Fig 5). The structure can be subdivided into a large α/β and a smaller α-domain. A deep indentation that contains the catalytically essential amino acids Arg81 and His82 is formed at the interface of these two domains.

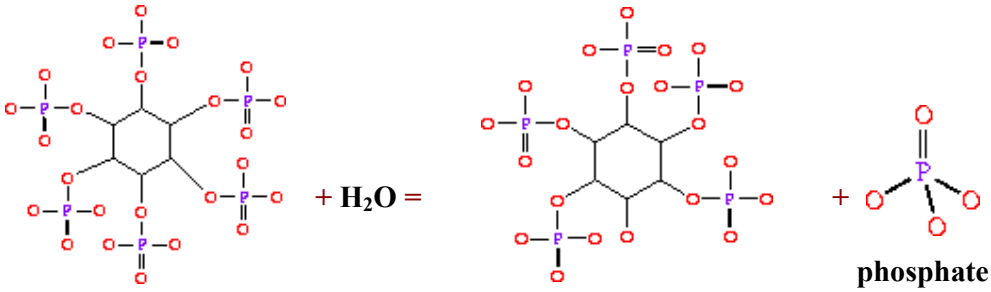
The X-ray crystal structure of A. niger T213 phyB is now known (100). Unlike *A. niger* phyA, it is a tetramer formed by two dimmers (Figure 7 &8). As in phyA, phyB also shows five disulfide bridges in its X-ray crystal structure. The three bridges analogous to Cys71-Cys414, Cys215- Cys465, and Cys436-Cys444 (Fig.5) in phyA are conserved in phyB. The N terminus of phyB stretches out to allow for interfacing with its neighbor, while in phyA the N terminus is a disulfide bridge formed by Cys31-Cys40, which results in a compact loop configuration in this region.

Despite having almost identical catalytic centers, phyA and phyB have different pH profiles for hydrolysis of phytate. PhyA hydrolyzes phytate at both pH 2.5 and 5.0, but phyB displays optimum phytase activity at pH 2.5 and lacks activity at pH 5.0. Kostrewa et al (100) attribute this variation to differences in the charge distribution at the substrate specificity sites. In the *A. niger* T213 phyB site, there are only two acidic amino acids, Asp75 and Glu272 (Fig. 6). At the *A. niger* NRRL 3135 phyA substrate specificity site, there are two acidic and four basic amino acids: Glu228, Asp262, Lys91, Lys94, Lys300, and Lys301 (Fig. 5) [the last two Lys residues were erroneously given as 250 (227) [1] and 251 (228) [1] in the report of Kostrewa et al (100). The active site of phyB is thus more acidic than the active site of phyA.

Recently, a recombinant form of *E. coli* phytase was purified and crystallized to provide a three-dimensional structure of a 6-phytase (150). This X-ray-deduced model can be used with the crystal structure of A. niger phyA, a 3-phytase (99) to define the structural basis for their different catalytic pathways.

[1]The amino-acid residue location in the abbreviated sequence in Fig. 6 (100)

Enzyme reaction for phytase E.C.3.1.3.8



Myo-inositol hexakisphosphate

1D-myo-inositol 1,2,4,5,6-pentakisphosphate

phosphate

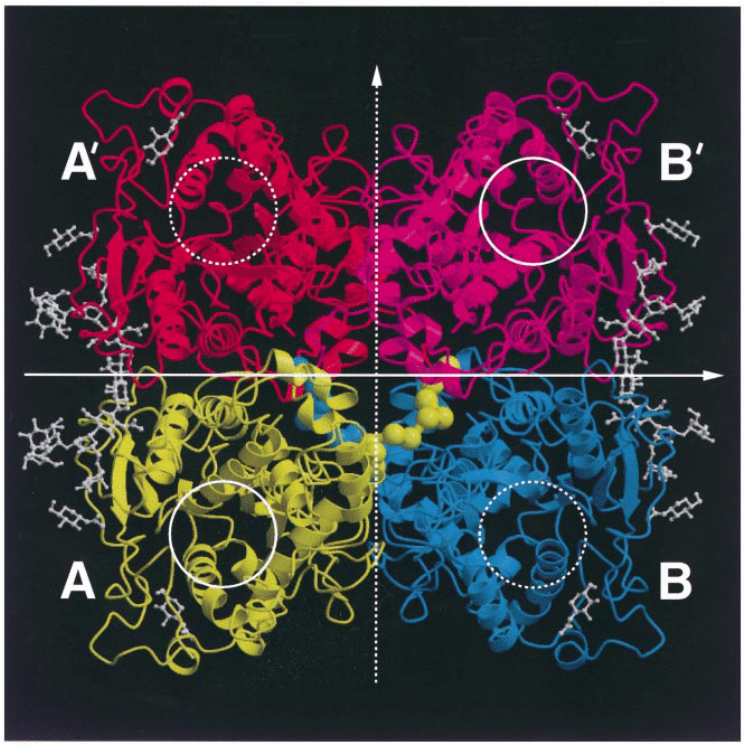


Figure 7. Schematic diagram of the tetramer of *A. niger* pH 2.5 acid phosphatase with almost exact 222 (D2) symmetry. The tetramer is built from two dimers with a crys- tallographic dyad (indicated by a horizontal white filled arrow) lying perpendicular to the non-crystallo- graphic dyad (indicated by a verti- cal white broken arrow). One dimer is shown in yellow (A) and blue (B), and its crystallographi- cally equivalent dimer is shown in red (A0) and purple (B0).

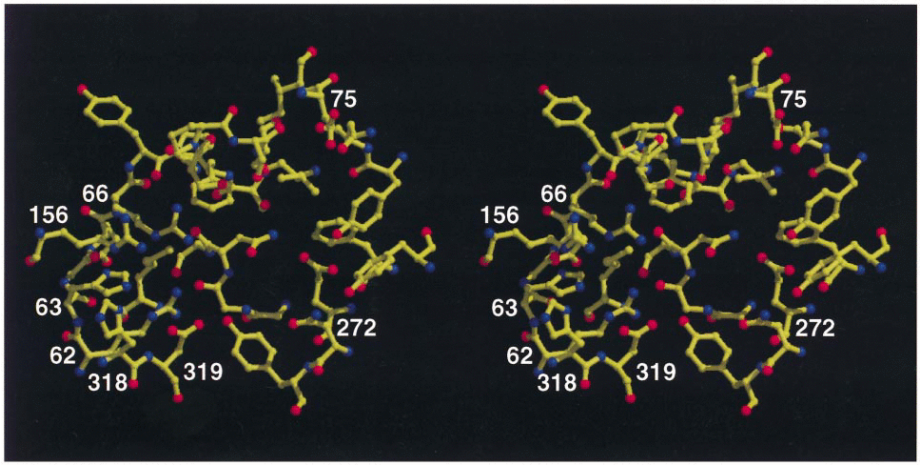


Figure 8. Stereo diagram of the active site of *A. niger* pH 2.5 acid phosphatase. The active site can be subdivided into a catalytic center (left-hand side) and a substrate specificity site (right-hand side).

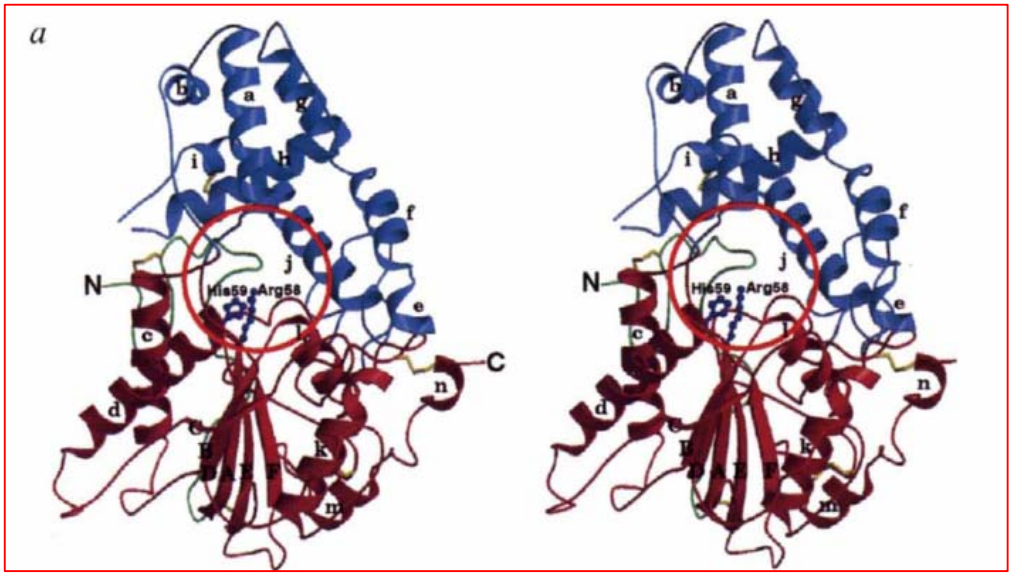


Figure 9. Stereo picture of the three- dimensional fold of *A. ficcum* phytase. The α/β- domain is coloured red, the α-domain blue, the N- terminal lid green, and the disulphide bridges yellow. There is a gap on the left hand side of the α-domain for residues 249-252, which were not visible in the electron density. The active site is indicated with a red circle and the catalytically essential amino acid residues Arg 58 and His 59 are shown in blue ball-and-stick representation. This figure was prepared with the programs MOLSCRIPT (151) and RASTER3D (152).

1.4. Application of Phytases

1.4.1 Phytase as Feed Additive

Since phytic acid can not be metabolized by monogastric animals due to lack of phytate-degrading enzyme in their gastrointestinal tract, feeds for pigs and poultry are commonly supplemented with inorganic phosphate in order to meet the phosphorus requirements. It is seen that the use of phytase in feed fodder improves the phosphorus bioavailability and reduces the chelating ability of phytic acid, thus resulting in reduction of phosphorus excretion in the areas of intensive livestock. The superior activity of *Aspergillus niger* NRRL 3135 phytase and its practical application to animal feed for the removal of phytic acid were demonstrated in several studies (153, 154) collaborated to confirm the earlier feed studies by

feeding solvent precipitated phytase to chick diets, besides demonstrating the efficacy of feeding phytase to swine. They concluded that the addition of enzyme (1000 μmol P/h · ml phytase/kg diet) was sufficient to improve performance further than that obtained by adding supplemental inorganic phosphate, thus establishing the role of phytase as feed-additive in combating phosphorus pollution in soil and water. The Natuphos produced by Gist-Brocades, when supplemented in feed, resulted in enhanced utilization of phytin phosphorus by monogastric animals.8 Alko Biotech tested Finase F for its use in improving the phosphorus availability in swine and found results parallel to those obtained by phytases from other sources (155-157). It was observed that the units of enzyme required for the hydrolysis of set amount of phytin phosphorus were fairly consistent and one can estimate, within a narrow range, the amount of phytase required to hydrolyze phytin-P present in any commercial diet used in livestock units. Depending on the specific diet, 380–1000 μmol P/h (6408– 16,600 nkat/ml) phytase is required to replace 1 g phosphorus supplied by inorganic source. It was seen from the livestock production statistics of the USDA that if phytase were used as a feed ingredient in the diets of all the monogastric animals in United States, it would release phosphorus with a value of $\$1.68 \times 10^8$ per year, besides diminishing the amount of phosphate in the manure and subsequently entering the environment. The quantitative determination of released phosphate upon phytase supplementation indicated that if the phytase were used in the diets of all monogastric animals in the United States, it would preclude 8.23×10^7 kg phosphorus from entering the environment (14).

1.4.2 Food Application

A diet rich in cereal fibers, legumes and soy protein results in an increased intake of phytate. Vegetarians, eldery people consuming unbalanced food with high amounts of cereals, people in undeveloped countries who eat unleavened bread, and babies eating soy-based infant formulas take in large amounts of phytate (13). Undigested phytate in the small intestine negatively affects the absorption of zinc, calcium, magnesium and iron. It also reduces the digestability of dietary protein and inhibits digestive enzymes. Using phytase, Simell and co-workers (13) reported the preparation of a phytate-free soy protein isolate with increased

solubility at low pH (pH 3) compared to the control soy protein isolate (158) phytate from soybean milk using wheat phytase. Additions of *A. niger* phytase to flour containing wheat bran increased iron absorption in humans (159). However, more studies should be performed before accepting phytase as a food additive.

1.4.3 Pulp and Paper industry

Aging of papers is one of the main problems faced by the pulp and paper industries. Lignin-free paper degraded faster than the lignin-containing paper, even when it was sized with acid alum. Lignin acts as an antioxidant because it is oxidized faster than cellulose and forms stable radicals; this shows that the process aging is oxidative. Attempts to prevent oxidation by use of ethylenediamine tetraacetic acid (EDTA) only accelerated the degradation process. Only a few complexing agents block the Fenton reaction (formation of hydroxyl radicals); one of them is phytic acid, found in seeds. A thermostable phytase can have potential as a novel biological agent to degrade phytic acid during pulp and paper processing. The enzymatic degradation of phytic acid would not result in the production of toxic and mutagenic by-products, and at the same time aging of paper can be prevented. Therefore, the exploitation of phytases in the pulp and paper process could be environmentally friendly and would assist in the development of cleaner technologies (160).

1.4.4 Phytase as Soil amendment

Phytic acid and its derivatives represent up to 50% of the total organic phosphorus in the soil at certain locations, suggesting the possibility of phytase addition that might stimulate plant growth in these soils. Findenegg and Nelemans (161) studied the effect of phytase (phyA) on the availability of phosphorus from phytic acid in the soil for maize plants. It was seen that phytin hydrolysis increased when phytase was added to the soil, which further resulted in stimulation of plant growth. However, the amount of phytase necessary for a significant effect indicated that it is not a practical technique. This further indicated that the expression of phytase in the roots of transgenic plants might increase the

availability of phosphorus to plants. Thus, expression of phytase in the roots of plants might increase the phosphorus bioavailability (162).

1.4.5 Semisynthesis of Peroxidase

Based on structural similarity between the active sites of vanadium dependent haloperoxidase, fungal phytase, and acid-phosphatase, a semisynthetic peroxidase was designed.91 The Delft group incorporated vanadate ion into the active site of *A. niger* NRRL 3135 phytase, thus transforming native phosphohydrolase into semisynthetic peroxidase. The "new" enzyme was able to catalyse enantioselective oxidation of prochiral sulphides and was found to be stable for 3 days with only slight loss in turnover number. Being water miscible, the enzyme could be used in the presence of organic solvents up to 30% concentration (v/v) with only a slight loss in enzyme activity. It was seen that of all the phytases and acid phosphatases tested, only histidine acid phosphatase (HAPs) with "RHGXRXP" active site sequence could function as a peroxidase when vanadate ion was incorporated into the active site.

1.4.6 Preparation of myo-Inositol phosphates

Besides the adverse effects of phytate and other highly phosphorylated inositolphosphates on mineral bioavailability, some novel metabolic effects of some of its degradation products have been recognized. The most extensively studied positive aspect of *myo*-inositol phosphate (InsP1,4,5 and InsP1,3,4,5) is the potential for reducing the risk of colon cancer. InsP5 plays an important role in modulation of the oxygen-binding affinity of hemoglobin in the erythrocytes of avian, amphibian, and reptilian species,5 while InsP4 and InsP3 was found to act as secondary messenger in synergistically controlling intracellular calcium level.6 . Surprisingly, the esters of inositol triphosphate have been shown to exert significant inhibitory effects against retroviral infections including HIV (163)**.** The position of phosphate group on inositol ring is thereby of great significance for their physiological function. To investigate the physiological effects of defined *myo*-inositol phosphate isomers, these compounds have to be available in pure form and sufficient quantity. . The chemical syntheses of *myo*-inositol phosphates

include difficult protection and deprotection steps, and are performed at extreme temperatures and pressures (16).

Attempts to produce them nonenzymatically results in a mixture of isomers, making the purification very uneconomical and arduous. Since phytases hydrolyze *myo*-inositol hexaphosphate sequentially, the production of *myo*-inositol phosphate derivates and free *myo-inositol* using phytase is a potential alternative to chemical synthesis. Phytases are distributed widely in nature and different types of phytases are known, 3-phytases, 4-phytases, and 6- phytases, indicating the predominant attack at the susceptible phosphoester bond. Thus, using phytases of different origin may lead to the production of different isomers. Siren (164) prepared *myo*-inositol-1,2,6-triphosphate and *myo*inositol- 1,2,5-triphosphate by enzymatic hydrolysis of phytic acid using *S.cerevisiae* phytase. The esters of inositol triphosphate were found to alleviate conditions associated with abnormal levels of neuropeptideY (NPY), such as arthritis and asthma, besides acting as a pain killer (163). Greiner and Konietzny reported the synthesis of Ins-1,2,3,4,5-P, Ins-2,3,4,5-P, Ins-2,4,5-P, and Ins-2,5- P using *E. coli* phytase covalently immobilized on NHS-activated Sepharose. As only one major isomer of each InsP was formed, further purification could be easily achieved by ion-exchange chromatography. Using a combination of high-performance liquid chromatography (HPLC) and kinetic studies, the stereospecificity of *E. coli* phytase P2 was established, indicating sequential removal of phosphate groups via the 6/1/3/4/5 route (165).

Naturally, the advantages of enzymatic hydrolysis are stereospecificity and mild reaction conditions. In addition to usage as drugs, *myo*-inositol phosphate derivatives can be used as enzyme substrates for biochemical and metabolic investigations and as chiral building blocks (15).

1.4.7 Environmental Benefits of Enzymes as Additives to Animal feed

The major enzyme application in the animal feed industry is the use of phytase, which releases the phosphate bound in the grain and thus diminishes the need for addition of inorganic phosphate to the feed. Phytase was developed for use in animal feed based on the assumption that the substitution of inorganic

phosphate with phytase would offer a significant environmental advantage. To check this assumption, an LCA study has been carried out on the use of phytase for intensive pig farming. The study builds on Danish conditions, which means that the average phosphate binding capacity for agricultural soils has been assumed to be 95%. The results are shown in the Table 15. Most significant is the reduction of the pollution of the aquatic environment with phosphate, which in Denmark due to the intensive pig farming is a major problem. It can be calculated that the effect of using phytase for all the pigs in Denmark (23 million) reduces the emission of P to the aquatic environment with 260 t P. This corresponds to approximately 25% of the diffuse emission of P from Danish agriculture. Another significant benefit of phytase is the saving of the consumption of phosphate, which is an essential, but limited resource. The phosphate saving from feeding all Danish pigs phytase corresponds to the annual consumption of phosphate from 1 million people. Most other enzyme applications within animal feed address the efficiency of digestion of the grain nutrients. It is expected that such efficiency gains will drive significant environmental benefits – just as it has been demonstrated for the food industry. (**C**)

1.5 Market trends and future prospects

The growth of the market for phosphate to supplement animal feed fostered a critical step in the commercial development of phytase. Recent trends in market have clearly shown the phytases to be an important enzyme as feed supplement. Feed enzymes (protease, xylanase, phytase, amylase, cellulase, lipase) form an important segment of animal nutrition market. Presently, only about 6% of manufactured animal feeds contain enzymes. Due to concerns about environmental pollution, 22 countries have adopted the use of phyA, produced from *Aspergillus niger* NRRL 3135, as a feed additive. Gist-Brocades cloned multiple copies of *A. niger* NRRL 3135 *phyA* gene into their PluGBug system resulting in the high yields of phytase and marketing it under the trade name Natuphos. The largest market share (~ 40%) in feed enzymes was of Finnfeeds International followed by BASF. Finnfeeds recently developed phytase for commercial applications. Novo-Nordisk too is marketing phytase in Europe under the trade name Phytase Novo and is replacing it with another phytase cloned from *Peniophora lycii,* a

basidomycete. This phytase will be overexpressed in the *A. niger* expression system and sold under the trade name Bio-Feed Phytase. Cenzone is producing a concentrate of digestive enzymes, Cenzyme, which is a unique blend of various digestive enzymes besides phytase from a fungal source. Alltech, Inc., produces another phytase,Allzyme Phytase (166), from a wild-type isolate. Researchers at the University of Wisconsin cloned and expressed fungal phytase gene in alfalfa plants to commercially produce phytase.

1.6 Synthesis of Calcium phosphate

The development of new biomaterials for medical applications is one of the challenging tasks for materials science today. In particular, there is an obvious need for better implants as well as the manufacturing of artificial tissues. A characteristic feature of this research area is the importance of biologically inspired approaches. Bio-inspired materials open new models for manufacturing implants for bone replacement. Bone is the major calcification present in a human body (167a), which serves as structural (mechanical) support for the body. Bone is an inorganic–bioorganic composite material consisting mainly of collagen proteins and hydroxyapatite, and its properties depend intimately on its nano-scale structures (168a). From the chemical point of view, bone is a composite material of calcium phosphate and collagen (168a, 169a, 170). Different routes for new bone implant materials are presented using the principle of functional gradation. The scaffold has to be functionally graded in order to create an optimized mechanical behavior as well as the intended improvement of the bone in growth (171). Consider the example of orthopedic implants for shoulder and knee joint replacements (171). In order to improve the acceptance of artificial implants by living tissues, a functionally graded interlayer of biopolymers is a favorable approach (171). The development and optimization of bone regeneration techniques represent a major clinical need (172). A large number of bone fractures have been treated by bone grafting. High amounts of autogenous bone grafts are needed in revision surgery with severe loss of bone (173). Bone fractures are usually treated with metallic wires, nails, screws and plates; joints are replaced by

artificial endoprostheses (hip or knee), and lost teeth are replaced by metallic implants in the jaw, to name a few examples (167a). As soon as foreign materials come into internal contact with the body, the question of biocompatibility becomes paramount, as any adverse effect (namely toxicity, allergy, inflammation, corrosion, and mechanical failure) must be strictly avoided. The search for optimally designed biomaterials is still ongoing as a joint effort of physicians, engineers, chemists, and physicists (174). Calcium phosphate ceramics have received much attention as potential bone graft substitutes during the past decades primarily because of their biocompatibility, bioactivity, and osteoconduction characteristics (175a).

Biomedical applications of polymeric materials have faced many critical obstacles such as undesirable protein adsorption and cell adhesion, due to the poor biocompatibility of conventional polymer surfaces. To solve these problems, an enormous number of basic and applied researches have been devoted to the surface modifications of polymeric materials (176). Synthetic polymers have the advantage of sustaining the release of the encapsulated therapeutic agent over a period of days to several weeks compared to natural polymers, which have a relatively short duration of drug release; they are in general limited by the use of relatively harsher formulation conditions (177). The synthetic polymeric scaffold for medical applications like implants and tissue engineering matrices must have sufficient mechanical stability and elasticity as well as desired stability towards degradation, and should be non-toxic (178). It has been shown that methods of implant surface preparation can significantly affect the resultant properties of the surface and subsequently the biologic responses (179).

The mineral-protein biocomposites (Hydroxyapatite – enzymes) produced by enzymatic (chapter 4) protocols has been characterized by a host of techniques such as Fourier Transform Infrared Spectroscopy (FTIR), UV-visible Spectroscopy (UV-vis), Thermal Gravimetric Analysis (TGA), Scanning Electron Microscopy (SEM), Energy Dispersive Analysis of X-Rays (EDAX), Transmission Electron Microscopy (TEM), X-ray diffraction (XRD), and Gel Electrophoresis. This section is devoted in explaining the basic principles used for characterization.

1.6.1 Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a characterization method by which infrared radiation is passed through a sample. The main importance of IR spectroscopic analysis is to determine the chemical functional groups and molecular orientations in the sample. Different functional groups absorb characteristic frequencies of IR radiation. IR spectroscopy is also an important and popular tool for structural elucidation and compound identification. It is bound by the red end of the visible region at high frequencies and the microwave region at low frequencies. IR absorption positions are generally presented as wavenumbers. Samples may be prepared in a solid, liquid, or gas form. The nature of the sample determines which technique should be used. Common sample preparation methods include: the salt pellet technique (sample powder is diluted in an IR-transparent salt like KBr); Nujol Mull method (sample powder is diluted in an IR-transparent oil); thin sample technique; attenuated total reflectance (the sample is sandwiched between two IR-transparent crystals); and diffuse reflectance infrared FT (optics focus beam on the top surface of the sample) (180). .

1.6.2 Scanning electron microscopy

Scanning electron microscope (SEM) is designed for direct studying the surfaces of solid objects. By scanning with an electron beam that has been generated and focused by the operation of the microscope, an image is formed in much the same way as a television. SEM gives better resolution and depth of field than optical microscope. For this reason the SEM can produce an image that is a good representation of the three dimensional sample. In this thesis we have extensively used SEM for observing the morphology of minerals synthesized via various methods described in the following chapters. SEM measurements were performed on a Leica Stereoscan-440 scanning electron microscope instrument equipped with a Phoenix EDAX attachment.

1.6.3 Energy dispersive analysis of X-rays

Energy Dispersive analysis of X-rays (EDAX) is a chemical microanalysis technique. The technique utilizes X-rays that are emitted from the sample during bombardment by the electron beam to characterize the elemental composition of the analyzed volume. Features or phases as small as about 1 μm can be analyzed.

When the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms comprising the sample's surface. A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted to balance the energy difference between the two electrons (Fig.2.6). The EDAX X-ray detector measures the number of emitted X-rays versus their energy. The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum of the energy versus relative counts of the detected X-rays is obtained and evaluated for qualitative and quantitative determinations of the elements present in the sampled volume. In this thesis we have extensively used EDAX measurements in ordered to determine the chemical composition (both qualitative and quantitative) of mineral-surfactant and mineral-protein composites.

1.6.4 Transmission electron microscopy

This technique requires that the thickness of the films under study will be less than 300 Å; however, direct observation of one monolayer is not possible because of lack of contrast. The electron energy in TEM is very high (100 KeV) and the resolution ranges from 1000 Å in to a few tens of Angstroms. We have used TEM for observing the crystal morphologies with finer details. Samples for TEM analysis were prepared by dropcoating films of the re dispersed powder in deionized water on carbon-coated copper TEM grids, allowing the grid to stand for 2 minutes following which the extra solution was removed using a blotting paper. TEM analysis was performed on a JEOL model 1200EX instrument operated at an accelerating voltage at 120 kV.

1.6.5 X-ray diffraction

X-ray Diffraction (XRD) is one of the primary techniques used by mineralogists and solid state chemists to examine the physico-chemical make-up of unknown solids. It is the most effective way of observing the atomic structure of crystals. X-ray diffraction is scattering of x-rays by periodic arrangement of atoms forming lattice or crystals. X rays are only a small part of the electromagnetic spectrum with wavelengths (λ) ranging from 0.02 Å to 100 Å. X-rays used to study crystals have λ on the order of 1 to 2 Å (i.e. copper Kα = 1.5418 Å). Visible light has much larger λ's (4000-7200 Å) and thus, x-rays are much more energetic (i.e. can penetrate deeper into a material). This can easily be seen by inspection of the

Einstein equation ($E = h\nu = hc/\lambda$; E is Energy, ν frequency, c speed of light which is constant for electromagnetic radiation, λ wavelength, h Plank's constant).

1.6.6 Thermal gravimetric analysis

The determination of changes in chemical or physical properties of material as a function of temperature in a controlled atmosphere can be obtained by a thermal analysis. Thermal analysis is a good analytical tool to measure thermal decomposition of solids and liquids, solid-solid and solid-gas chemical reactions, material specification, purity and identification, inorganic solid material adsorption and phase transitions. Thermo gravimetric analysis (TGA) is based on the measurement of the weight loss of the material as a function of temperature. TGA operates on a null-balance principle, using an electromechanical transducer coupled to a taut-band suspension system. The sensitivity of the balance is 0.1 mg. TGA curve provides information concerning the thermal stability of the initial sample, intermediate compounds that may be formed and of the residue if any. In addition to thermal stability, the weight losses observed in TGA can be quantified to predict the pathway of degradation or to obtain compositional information. The ability to vary atmosphere during the TGA evaluation, particularly from an inert to a reactive gas, provides additional information about a material composition and its stability. The experimental data offer more sophisticated understanding of reactions occurring at materials heating. This ability to obtain measurements at higher temperatures is most useful for inorganic materials such as minerals used in this thesis.

1.7 Future Studies

Future studies will probably be concentrated on (i) enzyme engineering to improve the heat stability of the enzyme, reduce the molecular mass, and construct a chimeric enzyme with the acid phosphatase; (ii) elucidation of the 3-dimensional structure of the enzyme and precise glycosylation of the enzyme especially in different plant and microbial systems; (iii) increasing yields in microbial and plant

systems by use of various promoters and leader sequences: (iv) application research to find additional uses of the enzyme; (v) basic research on inositol intermediates in plant and animal systems that may create demand for an immobilized enzyme to produce those intermediates; (vi) additional titration of the enzyme in animals for use of least-cost formulations; and (vii) research on delivery systems for the enzyme for use in animal feeds (cloning of the enzyme into various plants high in phytin-P that are used in commercial diets).

Despite the considerable economic interest, low yield and high cost of enzyme production are the limiting factors in using this enzyme in animal diet. However, with the development of heterologous microbial expression systems, large-scale phytase production for animal feed is now possible at relatively low cost. India is presently using dicalcium phosphate (DCP) in animal feeds and it was seen that the phytase supplementation could replace 50–60% dicalcium phosphate. It is estimated that 10 kg DCP can be replaced by 250 g of phytase enzyme, thus, the potential demand for phytase in cattle and poultry feed will be around 4000 tonnes per annum . In India all the livestock units do not depend upon commercial feed, therefore, the actual demand level is approximately 200 tonnes per annum. Thus, to obtain better and alternative source of phytases, there is an on going 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.

1.8 Aims and objectives*:*

Due to our interest in thermostable, acid tolerant phytase with reference to the required properties for animal feed applications, we decided to study further the enzyme accounting for Phytic acid hydrolyzing activity. Although number of phytases has been studied from plants, animals and microorganisms, only few are available commercially. A commercial enzyme should be thermo stable and fast reacting during reaction, should have a high acid tolerant and should be produced by organisms free of toxicity. *Aspergillus niger* have been widely used in food fermentation in the orient for hundreds of years. They are generally recognized as safe (GRAS) by many investigators. Therefore we decided to isolate and study the phytase from *Aspergillus niger* NCIM 563.

Objectives of the study: "Phytase from *Aspergillus niger* NCIM: 563 Isolation, purification, characterization and its applications" was taken up with the following objectives.

1. Screening of microorganism producing Phytase.
2. Media optimization and effect of culture conditions on production of phytase I (highly acidic) and II by *Aspergillus niger* NCIM 563 strain in submerged fermentation.
3. Purification and characterization of both the enzymes i.e. Phytase I and II.
4. Application of phytase in biomimetic synthesis of Hydroxyapatite and polymorphs.
5. Biocompatibility studies of enzymatically synthesized Hydroxyapatite & Polymorphs and commercially available Hydroxyapatite on human Osteosarcoma MG-63 cell line.
6. Study of phytase on different cancer cell lines and myo-inositol production

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CHAPTER 2

Production of phytase (Phy I and Phy II) by *Aspergillus niger* NCIM 563 under submerged fermentation conditions

2.1 Summary

Aspergillus niger NCIM 563 produced two types of phytase under submerged fermentation condition at 30°C in medium containing dextrin and glucose as carbon sources along with sodium nitrate as nitrogen source. These two enzymes were purified to near homogeneity using heat treatment, ion-exchange and gel-filtration chromatography. Physico-chemical properties of purified enzymes indicate the presence of two distinct forms of phytases, Phy I and Phy II. Optimum pH and temperature for Phy I was 2.5 and 60°C while Phy II was 4.0 and 60°C, respectively. Phy I was stable in the pH range 1.5 to 3.5 while Phy II was stable in the wider pH range, 2.0 to 7.0. Molecular weight of Phy I and Phy II on Sephacryl S-200 was approximately 304 kDa and 183 kDa, respectively. Phy I activity was moderately stimulated in the presence of 1 mM Mg^{2+}, Mn^{2+}, Ca^{2+} and Fe^{3+} ions and inhibited by Zn^{2+} and Cd^{2+} ions while Phy II activity was moderately stimulated by Fe^{3+} ions and was inhibited by Hg^{2+}, Mn^{2+} and Zn^{2+} ions at 1 mM concentration in reaction mixture. The *Km* for Phy I and II was 3.18 and 0.514 mM while *V*max was 331.16 and 59.47 μmols/min/mg protein, respectively.

2.2 Introduction

Phytic acid, myo-inositolhexakisphosphate, is a major storage form of phosphorus in cereals and legumes, representing 18-88 % of total phosphorus content (1). Phytases (EC 3.1.3.8 and EC 3.1.3.26) belong to the family of histidine acid phosphatases which catalyses the hydrolytic degradation of phytic acid and its salts (phytates), generally yielding inositol, inositol monophosphate and inorganic phosphate (2). Monogastric animals are incapable of digesting phytate phosphorus due to lack or low level of the phytase activity in intestine (3, 4). Consequently, the phytate in animal feeds is discharged in the feces of these animals into rivers and seas,

resulting in severe pollution of water resources (2, 5). Similarly, phytic acid is an anti-nutrient, which complexes with protein and nutritionally important metals such as calcium, zinc, magnesium and iron, decreasing their bioavailability (6, 7). To overcome this difficulty the feed has to be supplemented with inorganic phosphate to meet the nutritional requirements of the animals (8). Source of phosphates are non-renewable so addition or treatment of feed with phytase is a biotechnological challenge. Thus supplementation of feed with phytase will increase the bioavailability of phosphate, decreasing phosphorus pollution in areas of intensive animal agriculture (9, 10).

Although large number of micro-organisms viz. bacteria, yeast and fungi are reported to produce phytase (11, 12) fungal phytases are widely used in animal feed due to their acid tolerance and higher yield (13) in comparison to the bacterial phytase. Moreover in poultry the gut pH varies from 2.5 to 6.0, thus phytase active and stable in acidic environment is highly preferred (14). Among fungi many *Aspergilli* (11, 12, 15-18) are known to be active phytase producers. As *Aspergillus niger* is Generally Recognized as Safe (GRAS) it is frequently used in food and feed applications. Earlier we have reported phytase production by solid state fermentation (SSF) of agriculture residue using *Aspergillus niger* NCIM 563 which was highly active at pH 5.0 (19). The present communication reports culture conditions for production of two novel phytases (Phy I and Phy II) under submerged fermentation condition and their partial characterization, which according to us, is the first report of two distinct forms of phytase produced under submerged fermentation.

2.3 Materials and Methods

Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, U.S.A. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo.

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).

Scanning Electron Microscopy study

Vegetative mycelium of the organism was collected, washed thrice with physiological saline and spread on an aluminium foil and air dried. The samples were then fixed with gluteraldehyde (2.0 % v/v), for 4 hours at 25ºC. The fixed samples were then washed serially with 10-90 % of acetone; each fraction of acetone was kept for the period of 10 minutes. The film was air dried and coated with thin layer of gold in a gold coating unit (model E-5000, Polaron equipment Ltd.) and viewed with SEM Leica Stereoscan 440 model (Leica Cambridge Ltd. UK) at an accelerating voltage of 10 kV and beam current of 25 Pa. The photographs were recorded by a 35 mm camera attached to the resolution recording unit.

Medium and Culture conditions

Fermentation medium for phytase production was according to Shieh and Ware (20) with few modifications. Soluble starch was replaced with dextrin and potassium dihydrogen phosphate was used as phosphorus source. Thus modified fermentation medium contained (per 100 ml): Dextrin 5 g; Glucose 2.5 g; $NaNO_3$ 0.86 g; KH_2PO_4

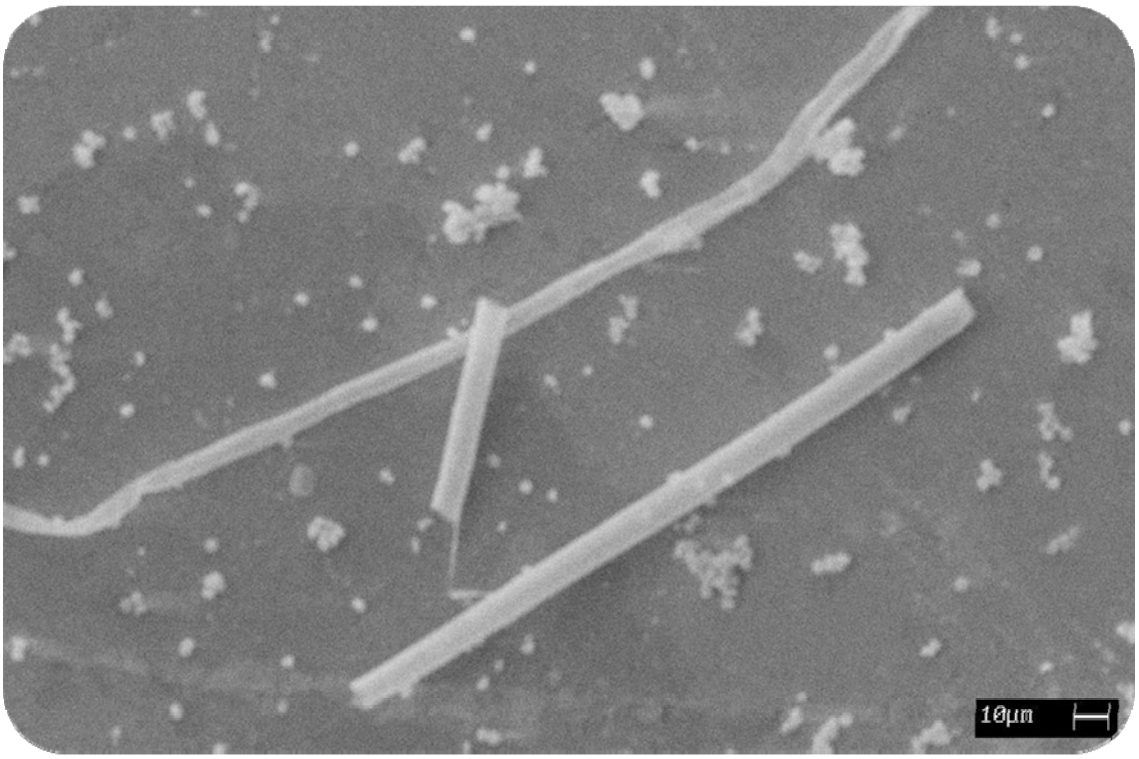

Figure a. Scanning electron Micrograph of Vegetative mycelium of *Aspergillus niger* NCIM 563.

0.004 g; KCl 0.05g; $MgSO_4.7H_2O$ 0.05g; $FeSO_4.7H_2O$ 0.01g. pH 5.5 before sterilization.

Fermentation medium (100 ml in 250 ml Erlenmeyer flask) was inoculated with 1 % (v/v) of spore suspension (5 x 10^7 spores per ml) prepared by suspending the spores from 7 day old sporulated slant of *Aspergillus niger* NCIM 563 grown on PDA in 10 ml of sterile distilled water containing 0.01 % (v/v) Tween 80 and incubated at 30°C at 200 rpm. Samples were removed after every 24 h and checked for pH, growth, total residual reducing sugar, extra cellular protein and phytase activity. Various carbon (different starches) and nitrogen (organic and inorganic) sources and metabolizable sugars were supplemented in the fermentation medium to study their effect on production of phytase. The effect of inorganic phosphorus (end product of phytic acid degradation) on the phytase was checked at conc. range between 0.001 to 0.006 g/ 100 ml medium. The effect of various surfactants on phytase production was checked at 0.5 ml/100 ml medium. Effect of various volumes of fermentation medium in either 250 or 500 ml Erlenmeyer flasks was also studied. Effect of water or buffer for preparation of fermentation medium was studied.

Phytase assay

Phytase activity was measured at 50°C as described earlier (19). The reaction for Phy I and Phy II was carried out at pH 2.5 (100 mM Glycine-HCl buffer) and pH 4.0 (100 mM acetate buffer) at 50°C for 30 min, respectively. The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (21). A freshly prepared four ml solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1µmol phosphorus per minute 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.

Protein estimation

Protein concentration in the culture filtrate was determined by the method of Lowry et al (22), using Bovine serum albumin as a standard. The biomass was measured after drying at 105°C for 24 hour.

Partial purification of Phy I and Phy II

After fermentation mycelium was separated by filtration followed by centrifugation at 10,000 x g for 30 min and the clear supernatant was collected. Solid ammonium sulphate was added to the supernatant to 95 % saturation with constant stirring. The precipitate was collected by centrifugation at 15, 000 x g for 20 min and dissolved in smallest possible volume of Glycine HCl buffer (100 mM, pH 2.5) and salt was removed by passing through Sephadex G-25 column and active fractions were concentrated through YM-30 membrane (Millipore) and used for phytase activity measurement.

Electrophoresis

Native PAGE (8 %) was performed in a vertical gel apparatus at pH 7.5, according to the method described by Davis (23). Samples containing approximately 5-10 μg of protein were applied to the gel and electrophoresis was carried out at 200 V for 3-4 hours. Protein bands were visualized by Silver staining (0.4 % w/v) (24).

Molecular weight determination by Gel filtration

The molecular weight of native enzyme was estimated by gel filtration on Sephacryl S-200 (1 x 150 cm) column equilibrated with 50 mM Glycine-HCl buffer, pH 2.5 using cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa) as standard proteins by the method of Andrews (25).

Sugar content

Total residual reducing sugar concentration was estimated by DNSA method (26) and HPLC system (Dionex India Limited, Mumbai, India) equipped with UV- or RI-detectors. An ion exclusion column (Aminex HPX-87H; Bio-Rad, Hercules, CA, USA) was used at a temperature of 38^0C with 8 mmol H_2SO_4 as a mobile phase at a flow rate of 6 ml per min.

Characterization of phytase

Ammonium sulphate precipitated and desalted by Sephadex G-25 enzyme was used for characterization of phytase. The effect of pH on phytase activity was determined at different pH values, by using 100 mM glycine-HCl buffer (pH 1.5-3.5), acetate buffer (pH 4.0-6.0) and tris-HCl buffer (6.5-7.0). The pH stability of phytase was determined by incubating suitable concentration of enzyme in above buffers, at 4°C for 18 h. After incubation, small aliquots were removed and checked for residual activity. The optimum temperature was determined over the temperature range 40-65°C and thermal stability was investigated up to 70°C for 60 min.

The effect of various metal ions on phytase activity was carried out at 50°C for 30 min as described earlier by adding metal ion (1 mM final concentration) to the phytase reaction mixture viz. 100 mM Glycine-HCl buffer, pH 2.5 and acetate buffer, pH 4.0 containing 3 mM sodium phytate along with control, i.e. phytase reaction without metal ion at 50°C.

The rate of hydrolysis of substrate (sodium phytate) was determined by varying the concentrations in the range of 0.5-10 mM. Assays were carried out at 50 °C under standard assay conditions. The kinetic rate constants, *Km* and *V*max were determined graphically from Lineweaver-Burk plotting (27).

2.4 Results and Discussions

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.

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
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cgccccctggtattccgggggggcatgcctgtccgagcgtcattgctgccctcaagcccggcttgtgtgttgggtcgccgtcc
ccctctccgggggggacgggcccgaaaggcagcggcggcaccgcgtccgatcctcgagcgtatggggctttgtcacatgc
tctgtaggattggccggcgcctgccgacgttttccaaccattctttaccaggttgacctcggatcaggtagggatacccgctg
aacttaagcatatcaataagcgg.

Optimization of phytase production under shake flask conditions

Preliminary results indicate that *Aspergillus niger* NCIM 563 grew well and produced phytase in modified basal medium (100 ml in 250 ml flask) containing 5 g dextrin and 2.5 g glucose as carbon source and 0.86 g $NaNO_3$, 0.004 g KH_2PO_4 with mineral salts. There was no increase in phytase activity with use of soluble starch, corn starch or potato starch when used as starch source (less significant data is not shown). Reports of phytase production from various *Aspergillus* sp. indicate use of soluble starch or corn starch as a source of carbon for fermentation (17, 28). Dextrin (partially hydrolyzed starch) was utilized by present strain of *Aspergillus niger* NCIM 563 for phytase production with specific activity of 224 IU/mg.

Time course of phytase production

The time course of phytase production under submerged fermentation conditions in medium (100 ml in 250 ml flask) containing 5 g dextrin and 2.5 g glucose and inorganic salts are shown in Fig. 1. The fungus grew rapidly as indicated by an increase in biomass (2.24 g/100 ml) and extracellular protein (199.5 μg/ml) with corresponding increase in phytase activity up to fifteen day of submerged fermentation. Phytase showed high activity at pH 2.5 (41.47 IU/ml) than at pH 4.0 (10.71 IU/ml). About 80 % of glucose was utilized rapidly with decrease in pH of the fermentation medium to 2.80. After fifteen day, fermentation medium started to turn black due to formation of spores by the fungus and resulted in decline in phytase

activity. Phytase activity reported during present work on fifteen day of submerged fermentation is very high i.e. 41.47 IU/ml i.e. 688 nkat/ml at pH 2.5 as compared to 184 nkat/ml by *Aspergillus niger* van Teigham after seventeen days of submerged fermentation (18) and 110 nkat/ml by *Aspergillus niger syn. Aspergillus ficuum* NRRL 3135 (29).

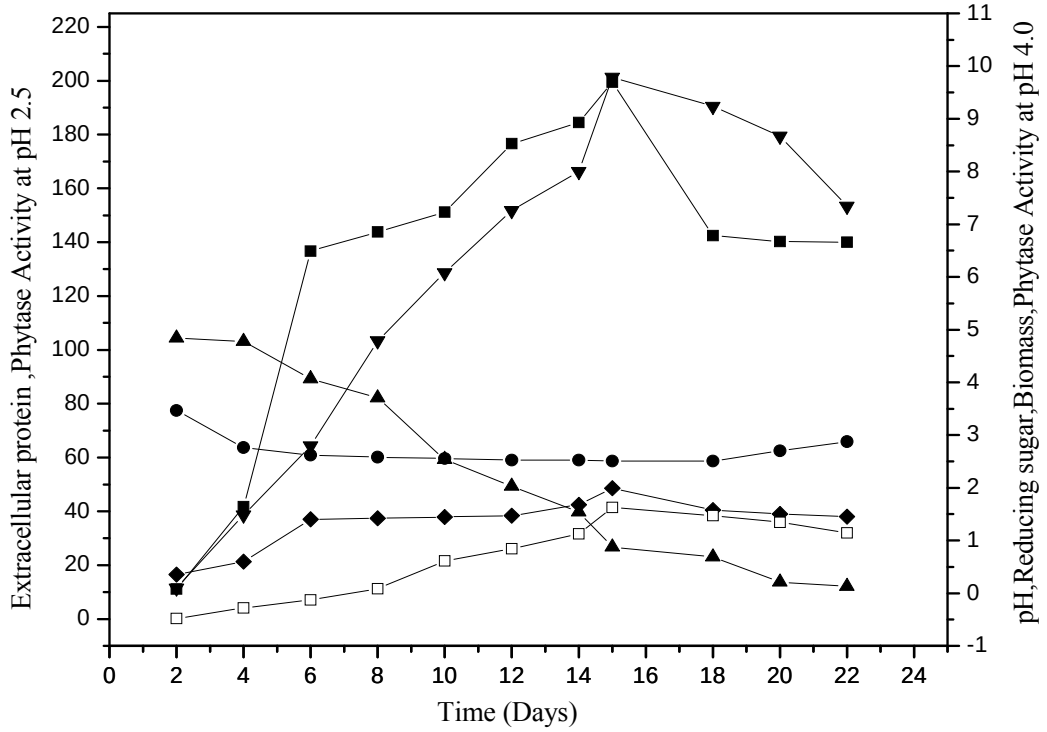

Figure 1. Time Course of phytase production during shake flask : —♦— Phytase activity (IU/ml) at pH 2.5, —▼— Phytase activity (IU/ml) at pH 4.0, —□— Biomass (g/100ml), —▲— Reducing sugar (%), — ● — pH, — ■—Extra cellular protein (µg/ml)

Effect of easily metabolizable sugars on phytase production

To study the effect of various easily metabolizable sugars on phytase production, fermentation medium was supplied either with glucose, sucrose, lactose, maltose and fructose at 2.5 g/100 ml medium. All these sugars had positive effect on phytase activity with more pronounced effect on activity was observed with glucose or fructose in fermentation medium. With 2.5 g glucose in 100 ml fermentation medium phytase activity at pH 2.5 was 2.8 times higher (41.47 IU/ml) while phytase activity at

pH 4.0 was 3.5 times higher (10.71 IU/ml) than fermentation medium without sugar. Varying the concentration of either glucose or fructose from 0.5 to 5 g/100 ml medium did not show increase in phytase activity (Table 1). As most of the extracellular metabolites secreted during fermentation are directly related to biomass, addition of easily metabolizable sugars may help the organism to generate rapid biomass. Easily metabolizable sugar e.g. glucose has been reported to increase phytase production by *Aspergillus niger* during in submerged and/or solid state fermentation (12,17,18).

Table 1: Effect of easily metabolizable sugars on phytase production

Carbon Source (2.5 g/100 ml)	Phytase activity (IU/ml) Phy I	Phy II	Biomass (gm/100ml)	Protein (μg/ml)
Without sugar	14.37	3.06	1.62	149.3
Glucose (0.5 %)	20.40	5.07	1.68	154.8
Glucose (1.5 %)	27.95	7.11	1.70	178.3
Glucose (2.5 %)	41.47	10.71	2.24	199.5
Glucose (3.5 %)	31.68	8.00	1.91	182.9
Glucose (4.5 %)	26.87	6.74	1.69	176.5
Fructose	40.27	9.97	2.14	197.4
Sucrose	32.32	8.20	1.88	187.1
Maltose	37.84	9.74	2.05	191.4
Lactose	29.76	8.61	1.78	182.0
Xylose	30.92	8.69	1.75	183.5
Galactose	33.18	9.24	1.87	188.3

The culture was grown under submerged fermentation condition at 30^0C with shaking (200 rpm) as described in Material and Methods .The values given in the table are the average of three independent experiments with 3-5% variation.

Effect of organic and inorganic nitrogen sources on phytase production

Among the various nitrogen sources (0.86 g/100 ml) added to fermentation medium containing dextrin and glucose as carbon sources, sodium nitrate and peptone favoured maximum phytase production, followed by potassium nitrate while ammonium sulphate, ammonium hydrogen phosphate, yeast extract, beef extract, liver extract and tryptone did not support high phytase production by *Aspergillus niger*

NCIM 563 (Table 2). Yeast extract, peptone and tryptone have been reported to stimulate maximum phytase production by *Aspergillus niger* van Teigham (18).

Table 2: Effect of various organic and inorganic nitrogen sources on phytase production

Nitrogen source (0.86 g/100 ml)	Phytase activity (IU/ml) pH 2.5	pH 4.0
$NaNO_3$	41.47	10.71
KNO_3	34.25	9.11
$(NH_4)_2SO_4$	1.32	0.46
$(NH_4)_2HPO_4$	1.58	0.57
Urea	1.02	0.23
$NaNO_2$	0.26	0.06
Yeast extract	1.25	0.94
Beef extract	1.37	0.75
Liver extract	1.18	0.44
Peptone	39.43	10.37
Tryptone	1.77	0.46
Corn Steep Liquor	19.14	4.17

The culture was grown under submerged fermentation condition at 30^0C with shaking (200 rpm) as described in Material and Methods .The values given in the table are the average of three independent experiments with 3-5% variation.

Effect of phosphorus on phytase production

Addition of various inorganic phosphorus sources in fermentation medium at 0.004 g/100 ml concentrations increases the phytase activity. Nearly 13 times increase in phytase activity at pH 2.5 (41.47 IU/ml) and pH 4.0 (10.71 IU/ml) was found by addition of potassium dihydrogen phosphate as compared to fermentation medium without inorganic phosphate. However increasing the concentration of potassium dihydrogen phosphate to 0.006 g/100 ml medium had adverse effect on phytase activity (Table 3). Various reports established that the phytase production by submerged or solid state fermentation is limited by the amount of inorganic

phosphorus in fermentation medium (1, 2, 10, 11, 20). High levels of inorganic phosphorus repress the biosynthesis of phytase. However, according to Vats et al (18) phytase production by *Aspergillus niger* van Teighem in seven litre laboratory scale fermenter was influenced by acidic pH (1.5-1.8) of the medium than phosphorus concentration.

Table 3: Effect of phosphates on phytase production

Phosphate source (0.004 g/100 ml medium)	Phytase activity (IU/ml) pH 2.5	pH 4.0
No phosphate	3.03	0.78
KH_2PO_4 (1 mg%)	8.19	2.06
KH_2PO_4 (2 mg%)	18.89	4.56
KH_2PO_4 (4 mg%)	41.47	10.71
KH_2PO_4 (6 mg%)	26.07	6.48
K_2HPO_4	39.73	10.64
Na_2HPO_4	26.14	6.93
NaH_2PO_4	22.71	5.91
NH_4HPO_4	23.95	6.23
$CaHPO_4$	24.78	6.45
$Ca_3(PO_4)_2$	26.17	7.11

The culture was grown submerged fermentation condition at 30^0C with shaking (200 rpm) as described in Material and Methods .The values given in the table are the average of three independent experiments with 3-5% variation.

Effect of surfactant on phytase production

To check the effect of surfactants on phytase production the fermentation medium was supplied with either Tween 80 or Triton X-100 (0.5 ml/100 ml) on either third or fifth day of fermentation.

Addition of surfactants increased the release of phytase activity. Tween 80 was found much superior to Triton X100. Addition of Tween-80 on the third day of fermentation resulted in 35 % increase in phytase activity at pH 2.5 (56.24 IU/ml) and 43 % increase (15.31 IU/ml) in phytase activity at pH 4.0 as compared to fermentation medium without surfactant (Fig 2). Similar effect was observed during lipase production by *Aspergillus niger* NCIM 1207 when Triton X-100 (0.5 ml/100 ml

medium) was added to fermentation medium. (30). Surfactants increased the cell wall/cell membrane permeability, which may be the reason for increase rate of metabolite secretion by the cells in fermentation medium.

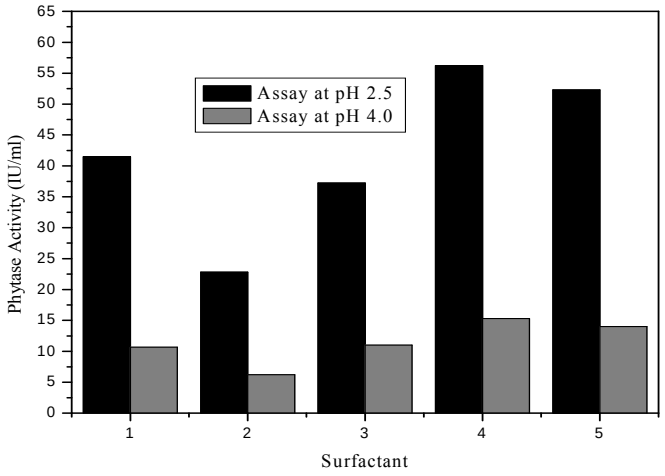

Figure 2. Effect of surfactants on phytase production
1: No Surfactant , 2: Triton X 100 Added on 3rd day,
3: Triton x 100 Added on 5th day, 4: Tween 80 Added on 3rd day,
5: Tween 80 Added on 5th day

Effect of volume of fermentation medium during shake flask studies

The effect of volume of fermentation medium in either 250 or 500 ml Erlenmeyer flasks indicate that maximum phytase activity at pH 2.5 (41.47 IU/ml) and pH 4.0 (10.71 IU/ml) produced when 100 ml fermentation medium was taken in 250 ml flask as compared to 50 ml medium in the same flask. Similar trend was found when fermentation was carried out in 500 ml Erlenmeyer flasks with 100 and 200 ml fermentation medium (Fig 3). This may be due to perhaps requirement of microaerophilic atmosphere for fungus, *Aspergillus niger* NCIM 563 to secrete higher amount of phytase.

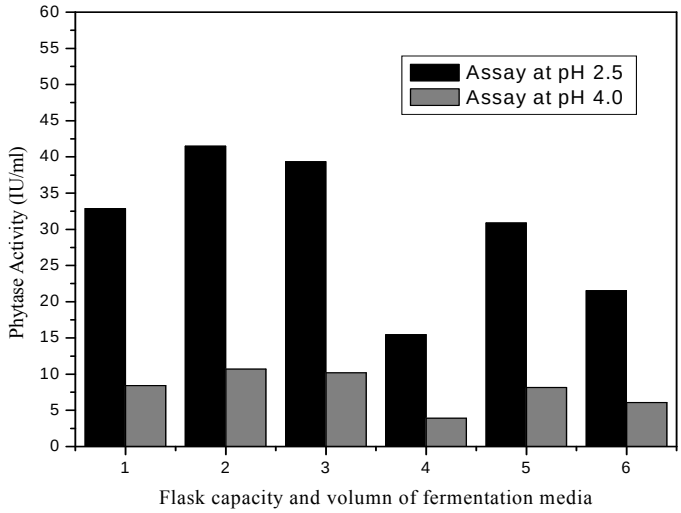

Figure 3. Effect of volume of fermentation media, to flask capacity and type
1: 50 ml medium in 250 ml flask, 2: 100 ml medium in 250 ml flask
3:150 ml medium in 250 ml flask, 4: 50 ml medium in 500 ml flask
5: 100 ml medium in 500 ml flask, 6: 200 ml medium in 500 ml flask

Effect of buffer on phytase production

Preparation of fermentation medium in buffer (100 mM glycine-HCl pH 2.5 or acetate buffer pH 5.0) resulted in 35-37 % increase in phytase activity at pH 2.5 (57.06 IU/ml) and pH 4.0 (14.90 IU/ml) as compared to fermentation medium prepared in distilled water. (Fig 4). This may be due to acidic pH of the buffer may support for fungal growth more than distilled water.

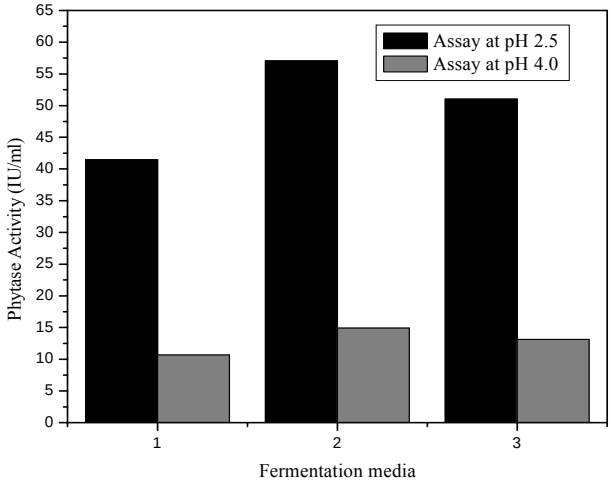

Figure. 4. Effect of fermentation Media prepared in **1.** Distilled Water, **2**: 100 mM Gly – HCl buffer, pH 2.5, **3**: 100 mM Acetate buffer, pH 5.0 on Phytase production

Characterization of partially purified phytase

The ammonium sulphate precipitation procedure resulted in 95 % enzyme recovery with purification of 3.0-fold. The enzyme showed two pH optima i.e. 2.5 and 4.0 (Fig 5). According to Oh et al (31) phytases can be categorized into two major classes, the histidine acid phytases (HAP) and alkaline phytases and based on their pH optima HAP is further divided into two groups, PhyA showing two pH optima viz. 2.5 and 5.5 with higher activity at pH 5.5 while PhyB show only single pH optima of 2.5. The phytase from *Aspergillus niger* NCIM 563 in the present communication belongs to PhyA with two pH optima with unusual high activity at pH 2.5 than at pH 4.0. The maximum enzyme activity at pH 2.5 was (41.47 IU/ml) and 10.71 IU/ml at pH 4.0. Thus activity at pH 4.0 was 25 % as compared to at pH 2.5. On the other hand extracellular phytase from *Aspergillus ficuum* NRRL 3135 even though exhibit two pH optima of 2.5 and 5.0, shows 48 % less activity at pH 2.5 than at pH 5.0 (15).

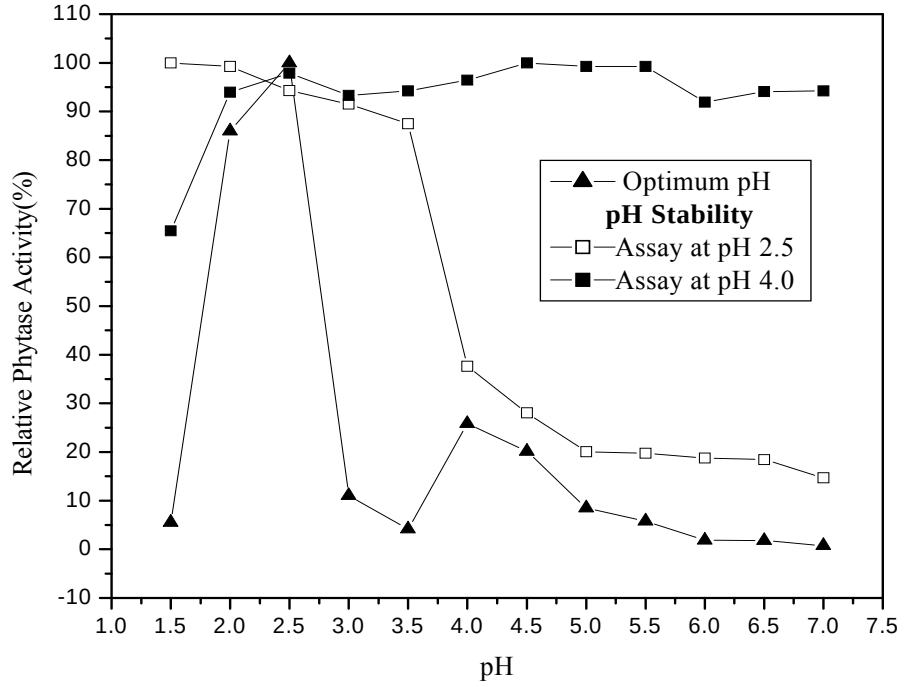

Figure 5 Effect of pH on phytase activity and stability: —▲— Optimum pH —□— Assay at pH 2.5, — ■—Assay at pH 4.0

Storage of phytase at various pHs for 18 h at 4°C reveled that phytase activity at pH 2.5 was stable at pH range 1.5-3.5 whereas phytase activity at pH 4.0 was more stable in the pH range 2.0-7.0 (Fig. 5). The optimum temperature for phytase activity was 60°C for both the activities at pH 2.5 and 4.0 (Fig. 6). Phytase from various *Aspergilli* show optimum temperature in the temperature range 40-65°C (11, 12). Thermostability of phytase active at pH 2.5 and 4.0 was different (Fig 4a and 4b). At 65°C phytase active at pH 2.5 (Fig. 7a) retained 80 % of its original activity after 15 min but at 70°C activity decreased sharply with only 40 % of its original activity remained after 15 min while phytase active at pH 4.0 retained only 40 % of its original activity after 15 min exposure at 65°C and 36 % of its original activity could be detected after 5 min exposure at 70°C (Fig.7b).

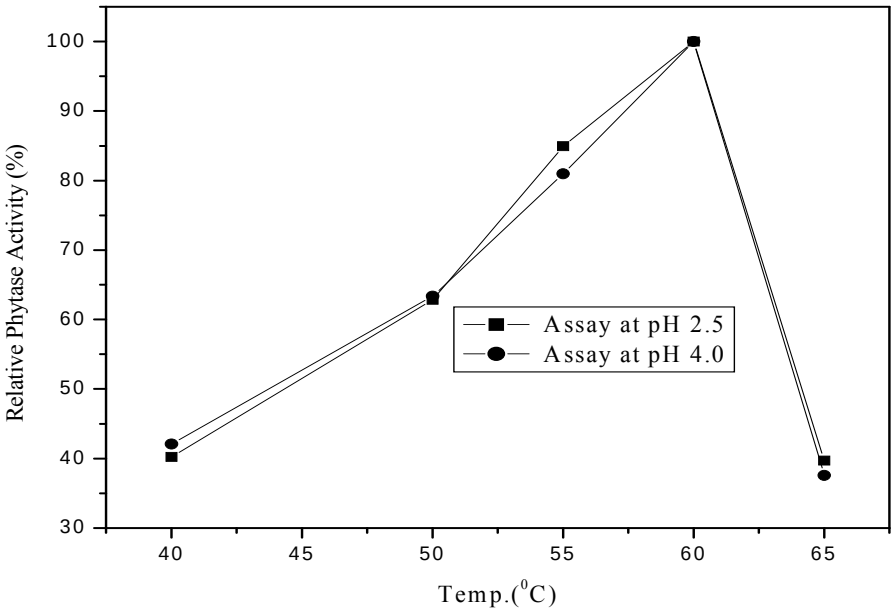

Figure 6. Effect of temperature on phytase activity: —■— Assay at pH 2.5, —♦— Assay at pH 4.0

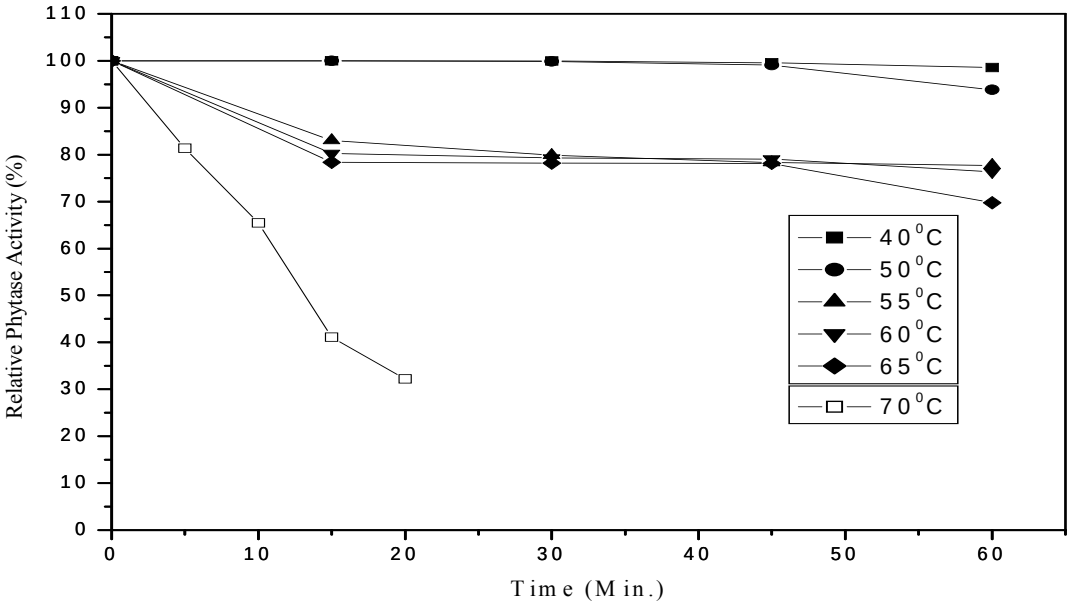

Figure 7a Effect of temperature on stability of phytase activity (pH 2.5)

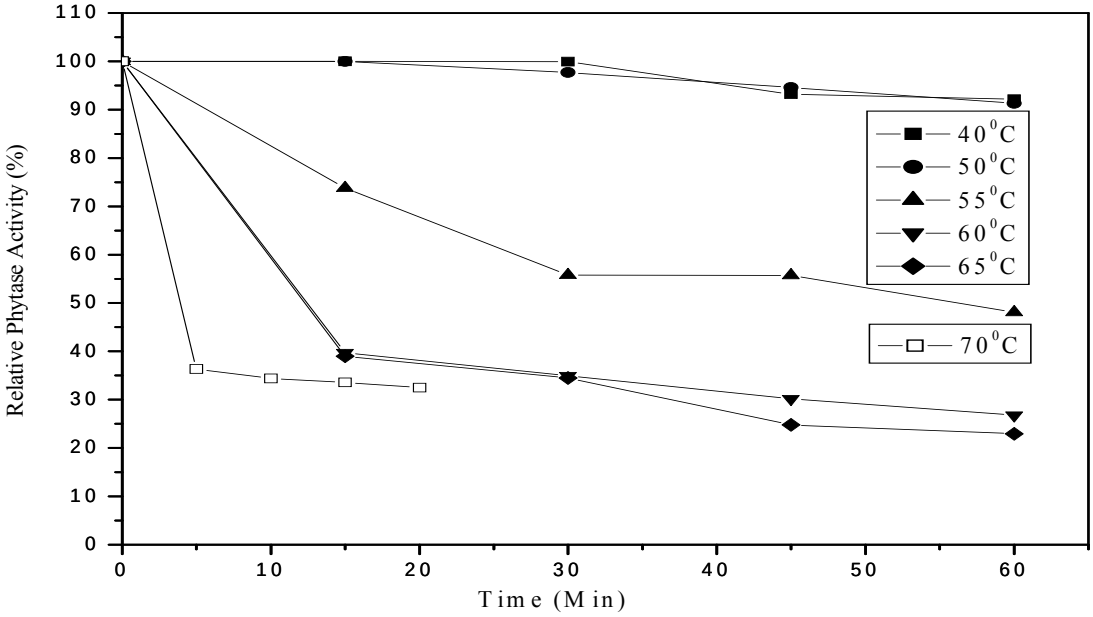

Figure 7b Effect of temperature on stability of phytase activity (pH 4.0)

—■— 40^{0}C, —●— 50^{0}C, —▲— 55^{0}C, —▼— 60^{0}C, —♦— 65^{0}C, —□— 70^{0}C

The effect of metal ions was studied by adding metal ion at 1 mM final concentration in a reaction mixture using 100 mM Glycine-HCl buffer, pH 2.5 and acetate buffer, pH 4.0 at 50°C (Table 4). The enzyme retained 79 % of its activity at pH 4.0 in presence of 1 mM Zn^{2+} while at pH 2.5 it retained 61 %. Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{3+} had stimulatory effect at pH 4.0 while Fe^{2+} and Cu^{2+} had stimulatory effect at pH 2.5. Zn^{2+} and Cd^{2+} had more inhibitory effect at pH 2.5 than at pH 4.0.

Table 4: Effect of metal ions on phytase activity

Metal ions (1 mM in reaction mixture)	Residual Relative activity (%) pH 2.5	pH 4.0
None	100	100
Hg^{2+}	82.69	74.38
Ni^{2+}	101	91.66
Mg^{2+}	103.48	91.56
Mn^{2+}	111.60	76.62
Ca^{2+}	104.23	90.47
Zn^{2+}	61.06	79.54
Fe^{2+}	98.66	109.0
Fe^{3+}	106.66	100.0
Cu^{2+}	94.08	105.5
Cd^{2+}	66.35	95.11

100 µl of enzyme was incubated with different metal ions (1mM final concentration) as the method described in Material and Methods, and the residual activity was calculated.

The partially purified enzyme was subjected to gel filtration chromatography on Sephacryl S-200 along with standard markers resulted in separation of two phytase activities viz Phy I active at pH 2.5 and Phy II active at 4.0 with molecular weight 304 and 183 kDa, respectively (Fig. 8). Moreover electrophoresis of partially purified enzyme on non-denaturing PAGE revealed two separate bands of phytase activity, which were active at pH 2.5 and 4.0 (Fig. 9).

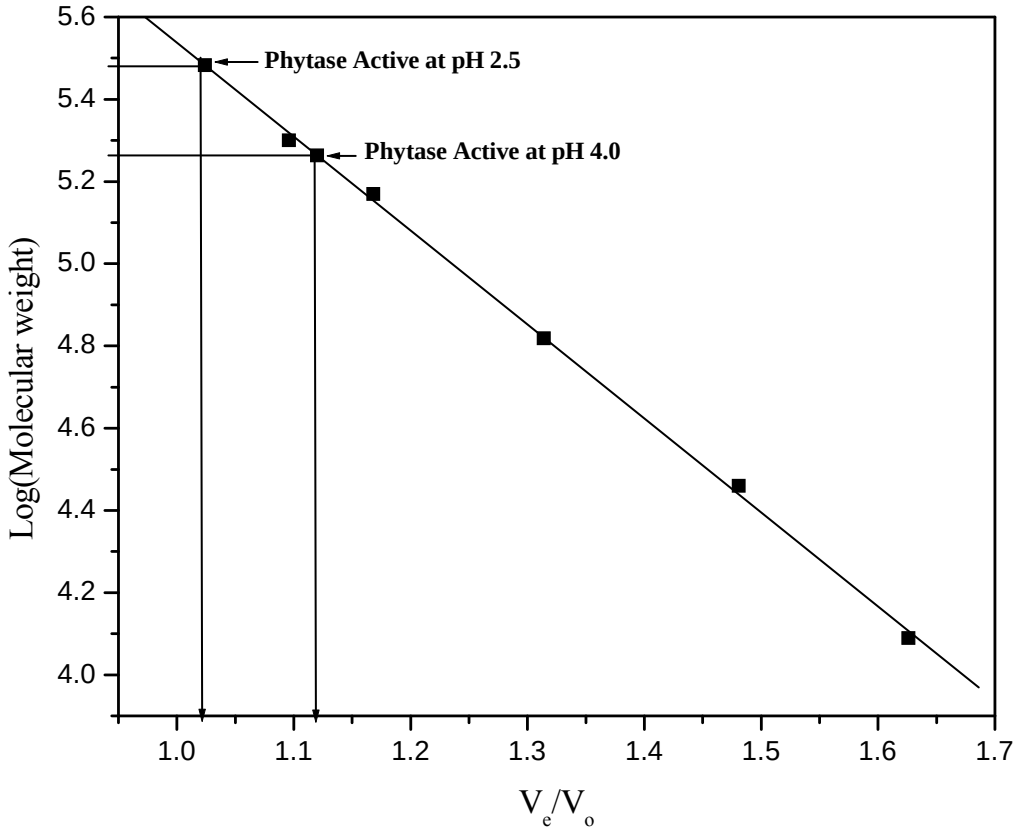

Figure 8. Molecular weight determination by gel filtration on Sephacryl S-200 column.
Markers used are**:** Cytochrome C (12.4 kDa), Carbonic anhydrase (29 kDa), Bovine serum albumin (66 kDa), Alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa).

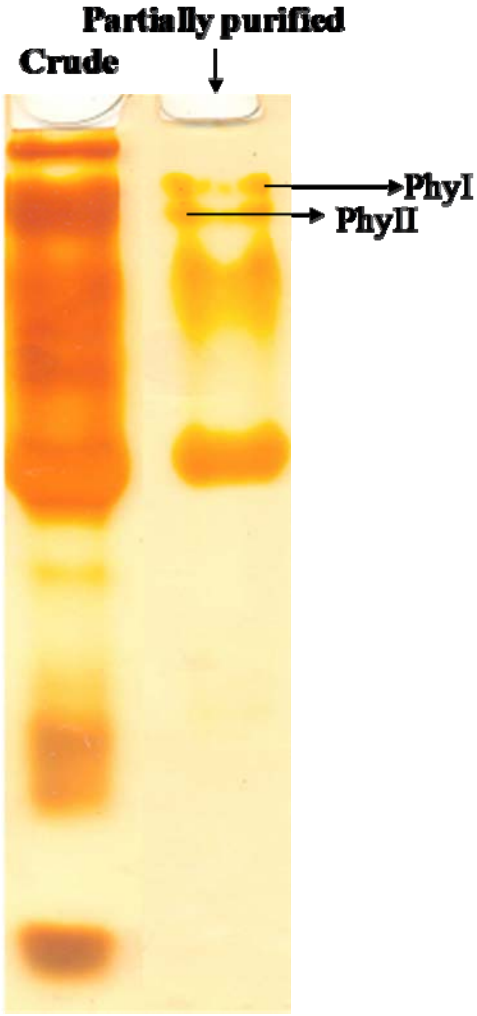

Figure 9. Native gel electrophoresis (8 %) of partially purified enzyme showing multiple forms of phytase

The *Km* for Phy I and II was 3.18 and 0.514 mM, respectively while *V*max was 331.16 and 59.47 μmols/min/mg when sodium phytate was used as substrate which is slightly higher than the *Km* (0.04-0.44 mM) reported from purified phytase of *Aspergillus niger* 92 (10) and *Aspergillus niger* NRRL 3135 (32). Fujita et al (33) also reported two types of phytase from *Aspergillus oryzae* under solidstate fermentation, which show same pH optima (5.0), thermal stability (100 % stable up to 50^0C) but differ in their temperature optima (65 ^{0}C and 55 ^{0}C, respectively) and molecular weight (120 and 116 kDa, respectively).

2.5 Conclusions

Earlier we have reported production of phytase by solid state fermentation using *Aspergillus niger* NCIM 563 with pH optimum 5.0 (19). Present studies on phytase production under submerged fermentation conditions by *Aspergillus niger* NCIM 563 indicate the possibility of two different forms of phytases, Phy I and Phy II which differ in their molecular weight, pH optima, pH stability and their inhibition by metal ions. High activity at pH 2.5 than at pH 4.0 indicates its application in poultry feed supplement.

2.6 References

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CHAPTER 3

Purification and characterization of phytase I (Highly Acidic) and II from *Aspergillus niger* (NCIM 563)

3.1 Summary

An extracellular acid tolerant phytase (Phy I) from *Aspergillus niger* (NCIM-563) has been purified along with totally different phytase (Phy II) to homogeneity by two chromatographic steps, using Phenyl Sepharose CL4B and Sephacryl 200, with total yield of 30% and 26% respectively. The molecular mass of the enzymes as determined by SDS-PAGE and gel filtration column were 66, 264 kDa and 150, 148 respectively, suggested that Phy I consists of four identical subunits and Phy II is a monomer. The purified phytase I is a glycosylated (2.01%) protein with a p*I* value of 3.55, Phy II is a nonglycosylated protein with a pI value of 3.91. The pH and temperature optima for the purified enzymes are 2.5, 5.0 and 55 ºC, respectively. The Phy I was stable over a broad pH range (1.5-9) and its half-life of inactivation ($t_{1/2}$) at 70ºC was 2-3 minutes. Phy II was stable (3.5-9.0) and $t_{1/2}$ at 70 ºC was 2-2.5 hour. The Phy I exhibited very broad substrate specificity and Phy II was more specific for sodium phytate. The relative substrate specificity of Phytase I towards the various synthetic substrates is in the order of Na-pyro-phosphate> ATP> p-Nitrophenyl phosphate> Sodium phenyl phosphate > β-NADP > 1-Naphthyl phosphate > 2-Naphthyl phosphate > ADP> D-Glucose 6-phosphate > α-D-Glucose 1-phosphate > Phenyl phosphate > Sodium phytate > Calcium phytate > Glycerol-1-phosphate > AMP.

The kinetics of hydrolysis of number of substrates by Phy I showed that Na-pyro-phosphate and Sodium phytate has lower K_m (higher affinity) and Calcium phytate and ADP has higher K_{cat} (higher catalytic efficiency). For Phy II Sodium phytate has lowest K_m and ADP and *p*-Nitrophenyl phosphate has highest K_{cat} among all the substrates. For sodium phytate K_m is 2.01, .145 mM and V_{max} is 5018, 1671 μmoles min^{-1} mg^{-1} for Phy I and II respectively. Phy II was completely inhibited by Ag^{+}, Hg^{2+} metal ions, and Phy I was partially inhibited. Peptide analysis of both the purified proteins by Mass Spectrometry (MS)

(MALDI-TOF) after in gel trypsin digestion showed totally different mass (m/z) pattern, proves that both the proteins are totally different. The N-terminal amino acid sequences of Phy I and Phy II were **FSYGAAIPQQ** and **GVDERFPYTG**, respectively. Activity and stability of both phytases in acidic range suggests their potential application in poultry feed.

3.2 Introduction

Phytase- a phosphomonoesterase- is an enzyme capable of hydrolyzing phytate [*myo*-inositol [1,2,3,4,5,6] hexakisphosphate], the major storage form of phosphorus in cereals and legumes, representing 18-88 % of total phosphorus content (1). Phytases (EC 3.1.3.8 and EC 3.1.3.26) belong to the family of histidine acid phosphatases which hydrolyses phytate to liberate inositol and inorganic phosphorus (2). Phytate phosphorus is biologically unavailable to nonruminants as they lack or secrete very low level of phytase activity in intestine. Consequently, the phytate in animal feeds is discharged in the faeces of these animals into rivers and seas, resulting in severe pollution of water resources (2). Similarly, phytic acid is an anti-nutrient; due to its strong chelating potential can bind essential minerals such as calcium, zinc and copper, rendering them unavailable or poorly available for absorption (3). To overcome this difficulty the feed has to be supplemented with inorganic phosphate to meet the nutritional requirements of the animals (4). Supplementation of feed with phytases increases the bioavailability of phytic acid bound phosphate, facilitating a reduction in the addition of inorganic phosphate to the feed and decrease phosphorus excretion in areas of intensive animal agriculture. Although large number of bacteria, yeast and fungi are reported to produce phytase (5-7) fungal phytases are preferred in animal feed due to their high yield and more acid tolerance as compared to phytases from bacteria (8). Similarly pH in poultry gut varies from 2.5 to 6.0, thus phytase active and stable in acidic environment is highly preferred (9). Several phytases have been cloned and characterized, such as fungal phytase from *Aspergillus ficuum* (10), bacterial phytase from *Escherichia coli* (11) and a mammalian phytase (12). These enzymes share a highly conserved sequence motif that is found at the active sites of acid phosphatases (13, 14). The reaction mechanism of *E. coli* phytase has been

revealed (15, 16) and is likely to be common for most phytases. Therefore, these enzymes are said to form the phytase subfamily of histidine acid phosphatases (17).

Among fungi many *Aspergilli* (5, 6,18, 19) are known to be active phytase producers. As *Aspergillus niger* is Generally Recognised as Safe (GRAS) it is frequently used in food and feed applications. Earlier we have reported phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation (20-22) which includes production and partial characterization of two types of phytase from *Aspergillus niger* NCIM 563.

In order to study structure function relationship of enzyme, it is necessary to obtain it in a pure form and in large quantity through simple and efficient method of purification. In the present study we have described simple and single method of chromatographic step purification of phytases (I and II) from *Aspergillus niger* (NCIM 563). The purified enzyme has been extensively characterized for its biochemical and molecular properties and also for its substrate specificity towards the different substrates comprising phosphorus.

3.3 Material & Methods

Chemicals

Sodium phytate, Calcium phytate, *p*-Nitrophenyl phosphate, Sodium phenyl phosphate, α-D-Glucose 1-phosphate , D-Glucose 6-phosphate, 1-Naphthyl phosphate, 2-Naphthyl phosphate, ATP, ADP, AMP, β-NADP, Na-pyro-phosphate, Glycerol-1-phosphate, Phenyl phosphate, SDS and gel filtration markers, Coomassie Brilliant Blue R 250, Bromophenol Blue, Phenyl Sepharose CL-4B, Sephacryl-300, Sephacryl-200 were purchased from Sigma Chemical company , U.S.A.. External mass standards Calmix 1 and 2 obtained from Applied Biosystems. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Hi media and Glaxo.

Organism and culture condition

The strain used throughout the present work was *Aspergillus niger* NCIM 563. It was maintained as described earlier (20). For purification of Phytases (I and II), isolate was grown in a medium containing (per100 mL): Dextrin 5 g; Glucose 2.5 g; $NaNO_3$ 0.86 g; KH_2PO_4 0.004 g; KCl 0.05 g; $MgSO_4.7H_2O$ 0.05 g; $FeSO_4.7H_2O$ 0.01 g. pH 5.5 before sterilization. Fermentation medium (100 mL in 250 mL Erlenmeyer flask) was inoculated with 1% (v/v) of spore suspension ($5X10^7$ spores per mL) prepared by suspending the spores from 7 day old sporulated slant of *Aspergillus niger* NCIM 563 grown on PDA in 10 mL of sterile distilled water containing 0.01% (v/v) Tween 80 and incubated at 30^0C at 200 rpm for 10-12 days. After fermentation, the fungal biomass was separated by filtration and then centrifugation (10,000 x g, 10 minutes) and clear supernatant was used as source of extracellular enzyme.

Phytase assay and protein, glycosylation determination

Phytase activity was measured at 55^0C as described earlier (20). The reaction for Phy I and Phy II was carried out at pH 2.5 (100 mM Glycine-HCl buffer) and pH 5.0 (100 mM acetate buffer) at 55^0 C for 30 min, respectively. The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (23). A freshly prepared 4 mL solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1µmol phosphorus per minute 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. Protein concentration in the culture filtrate was determined by the method of Lowry et al. (24) as well as measurement of absorbance at 280 nm using Bovine serum albumin as a standard. The neutral sugar content (glycosylation) of the purified enzyme preparation was determined by modified phenol sulphuric acid method (25).

Purification of Phytase I and II

Mycelia free culture broth was concentrated 10 times by rota vapour at 40^0C (both the enzymes are 100% stable at this temperature) under vacuum. The same enzyme sample was then applied to hydrophobic column chromatography using Phenyl Sepharose CL-4B (30 ml bed volume), previously equilibrated with 30% Ammonium sulphate in 20 mM Acetate buffer (pH 2.5). The column was washed thoroughly with 20 bed volumes of above buffer. Elution was done with 120 ml linear decreasing gradient of ammonium sulphate (30-0 %) with a flow rate of 20 ml per hour and approximately 3.0 ml fractions were collected. Fractions showing activity at pH 2.5 (Phy I) and pH 5.0 (Phy II) were pooled separately, concentrated by rota vapour and loaded on gel filtration Sephacryl (S-200) column with a flow rate of 12 ml per hour and 2ml fractions were collected. Unless otherwise mentioned all the purification procedures were carried out at 4 °C.

Electrophoretic techniques and N-terminal sequence

SDS-PAGE (10%) was performed in a vertical slab gel apparatus at pH 8.3, according to the method described by Laemmli (26). Native PAGE (8%) was done at pH 7.0 under non-denaturing conditions Davis (27). Protein bands were detected either by coomassie Brilliant Blue R-250 (0.2%, w/v) or by Silver staining (0.2% w/v), The Isoelectric Focusing Polyacrylamide Gel Electrophoresis (IEF-PAGE) was performed in a precasted acrylamide gel 7.5% (Biorad) using wide range Ampholytes (pH, 3-10) by the method of Chinnathambi et al (28) using a mini scale density gradient isoelectric focusing unit. Protein bands were detected either by Coomassie Brilliant Blue R-250 (0.2 % w/v) or by Silver staining (0.2 % w/v) and phytase band was revealed by sodium phytate as a substrate.

For N-terminal sequence analysis, the purified enzyme was electrophoresed on 10% SDS-PAGE at pH 8.8. After electrophoresis, the gel and polyvinylidenedifluoride (PVDF) membrane were sandwiched between Whatman filter paper strips. The strips and membrane were previously equilibrated with CAPS buffer (10 mM, pH 11.0) containing 10 % methanol. The protein was electrotransfered using a semi dry blotting system (Pharmacia Biotech, Nova Blot)

under a constant current of 52 mA for 30 minutes. After transfer, the membrane was removed and washed thoroughly with milli-Q water and protein band was visualized by Coomassie Blue R-250 stain. The N-terminal amino acid sequence of the first 10 residues of the both enzymes was determined by Edman degradation on an Applied Bio systems Procise sequencer at commercial services (Commonwealth Biotechnologies, Inc., Richmond, Virginia, USA). Reported sequences of Phytases from various sources were searched from GenBank and SwissProt Databases and a sequence alignment were performed using a Clustal-W sequence alignment program (29).

Determination of molecular mass (Mr)

The native molecular mass of α-galactosidase was estimated by gel filtration, according to the method described by Andrews (30). The gel filtration column (Sephacryl, S-300, 1x150 cm), was equilibrated in phosphate buffer (50 mM, pH 7.0) and calibrated using gel filtration standard molecular weight markers**:** Bovine serum albumin (*M*r 66 kDa); Alcohol dehydrogenase (*M*r 150 kDa); β-amylase (*M*r 200 kDa), Apoferetin (*M*r 443 kDa) and Thyroglobulin (*M*r 669 kDa). The column void volume was determined with Blue dextran (*M*r 2000 kDa). The subunit molecular mass of the purified Phytases was determined by SDS-PAGE according to Weber and Osborn (31) using sigma high molecular mass markers.

MALDI-TOF analysis

Mass spectral analysis was performed on a Voyager-De-STR (Applied Bio systems) MALDI-TOF. A nitrogen laser (337 nm) was used for desorption and ionization. Spectra were acquired in the range of 10 to 100 kDa, in linear mode with delayed ion extraction and with an accelerating voltage of 25 kV. The low mass ion gate was set at 4500 Da. 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 according to Bar et al (32).

pH and temperature effect

pH optimum of phytase was determined by assaying phytase activity over a pH range 1.5-9.0, using 50 mM Gly-HCL buffer (pH, 1.5-4.5),Acetate buffer (pH, 4.0-6.5) and Tris- HCL buffer(pH, 6.5-9.0) by standard assay method. The pH stability was also determined in a same pH range by pre-incubating enzyme samples in above buffers at room temperature for the period of 12 hours. Small aliquots (10 µl) were withdrawn from all the samples and residual enzyme activity was determined by standard assay method.

The optimum temperature of phytase was determined by performing assays at temperatures, 30 to 70°C. The Temperature stability was also determined by incubating enzyme samples over a same temperature range for the period of one hour. Aliquots (100 µl) were withdrawn after suitable time interval and residual enzyme activity was determined by standard assay method.

Effect of various metal ions and inhibitor reagents

A suitably diluted enzyme (100 µl) in 50 mM phosphate buffer, pH 7.0, was pre incubated with various metal salts, and inhibitor reagents at room temperature for 10 minutes; subsequently residual activity of the samples was determined by standard assay method.

A suitably diluted enzyme was pre-incubated with various Metal ion solutions at 4^0C for 24 hour and subsequently residual activity of phytase was determined to check the stability of enzymes under different metal ion solutions.

Effect of different additives on thermal stability of phytases

Purified enzyme samples (5.0 U/ml) were incubated at 70ºC with different additives (10 % W/V). Aliquots (~10 µl) were removed after 0, 10 and 30 min and assayed for residual enzyme activity. Enzyme activity of 0 min sample was taken as 100 %.

Substrate specificity and kinetic studies

The relative substrate specificity of both the phytases towards various substrates was determined at final concentration of 3.0 mM. The initial rate of hydrolysis of various substrates was determined by varying the substrate concentrations in different ranges (.1mM to 10mM). Assays were carried out at 55°C under standard assay conditions. The kinetic rate constants, K_m, V_{max}, K_{cat} and K_{cat} / K_m were determined graphically from Lineweaver-Burk plotting (33).

3.4 Result and discussion

Purification of Phytase I and II

The purification of Phytase I and Phytase II was carried out as summarized in Table 1a and 1b. The crude culture supernatant was first concentrated (50%) on rota vapour followed by adsorption and elution ion exchange chromatography on Phenyl Sepharose CL-4B followed by molecular sieving on Sephacryl S-300. Major activities were eluted in two peaks with a reverse gradient of ammonium sulphate (30-0 %) in 20 mM acetate buffer, pH 2.5. First peak was obtained at 15 % ammonium sulphate with higher enzyme activity at pH 5.0 (Phy II) while second peak was obtained at 5-0 % ammonium sulphate concentration in 20 mM acetate buffer with higher enzyme activity at pH 2.5 (Phy I). These fractions were dialyzed, concentrated and applied on gel filtration column. The purified Phy I and II had a specific activity of 3436.37 and 1215.33 Umg-1 with 16.67 and 14.29 fold purification, respectively. Nagashima et al (34) purified phytase from *A. niger* SK-57 to homogeneity in four steps by using ion-exchange chromatography, gel filtration chromatography and chromatofocussing. Recently Xiaoyu Li et al (35) reported purification of extracellular phytase from marine yeast, *Kodamaea ohmeri* BG3 by ammonium sulphate fractionation, gel filtration on Sephadex G-75 followed by fast flow anion exchange chromatography on DEAE Sepharose.

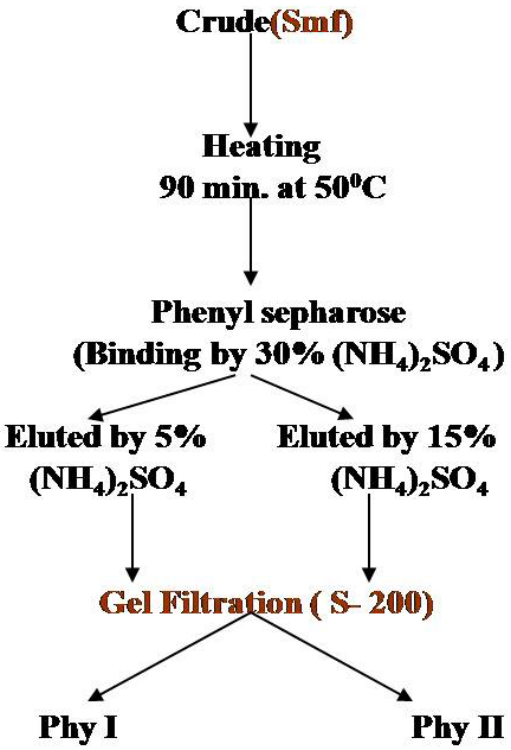


Figure 1. Schematic representation of the comparative analysis of the purification steps used for the purification of Phytase I and II.

Table 1A. Summary of purification of Phy I from *Aspergillus niger* NCIM 563

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification (Fold)	Recovery (%)
Culture filtrate	242	50,000	206	1	100
Rota vapour Conc.	169	41,130	243.3	1.18	82.20
Phenyl Sepharose CL-4B (5 %)	17.6	31,240	1775	8.61	62.48
Sephacryl S200	4.4	15,120	3436.36	16.67	30.24

Table 1B. Summary of purifications for Phy II from *Aspergillus niger* NCIM 563

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification (Fold)	Recovery (%)
Culture filtrate	242	20,600	85.12	1	100
Rota vapour Conc.	169	16,312	96.5	1.13	79.10
Phenyl Sepharose CL-4B (5 %)	14.0	10,520	751	8.83	51.06
Sephacryl S200	4.5	5,469	1215.33	14.29	26.55

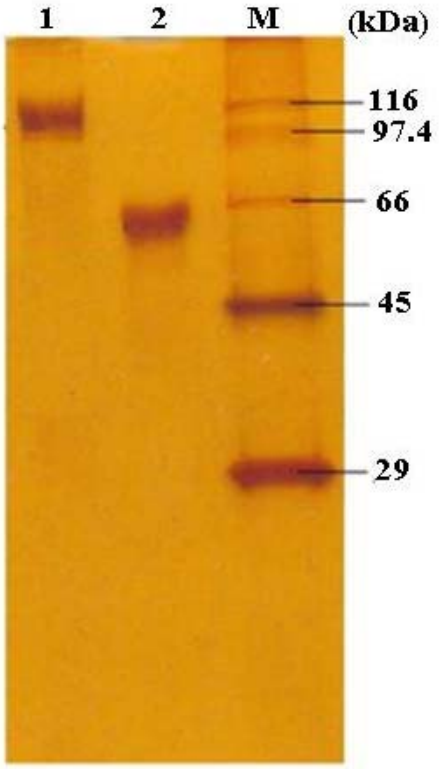


Figure 2. **(A)** SDS-PAGE of purified Phy I and Phy II from *Aspergillus niger* NCIM 563
Lane M:Molecular weight markers, Lane 1: Purified Phy II, Lane 2: Purified Phy I.

Molecular properties of phytase

The subunit molecular mass of purified Phy I and II was determined to be 66 kDa and 150-160 kDa, respectively, by SDS-PAGE analysis (Fig 2 A), while that of native enzyme was found to be 264 kDa and 150-160 kDa, respectively, by gel filtration on Sephacryl S300 (Figure 3). This suggests that Phy I is tetramer and Phy II is monomer. Dimeric Phy B has been reported from *Aspergillus niger* NRRL 3135 which has molecular mass of 68 kDa (36). However MALDI data revealed the molecular weight of Phy I and II to be 66 kDa and 74 kDa [Fig 4 A & B (inset)]. Thus molecular weight of Phy II by MALDI-TOF was half the molecular weight shown by SDS gel electrophoresis. This could be because of the

doubly charge species of Phy II during ionization process of MALDI-TOF spectrometry (37).

Isoelectric point (pI)

Single protein bands were observed on IEF-PAGE (Figure 2B) indicated that the both the enzymes are homogenous and contain single molecular form of Phytases. The p*I* of the Phytase I and II estimated by Isoelectric focusing was ~ 3.65 and 3.95 respectively (Fig 2b) revealing that the both the enzymes are acidic in nature. Golovan et al.(38) purified phytase (45 kDa) from *E. coli* of 45 kDa molecular weight and further separated it into two isoforms of identical size with pI of 6.5 and 6.3 by chromate focussing. The isoforms showed similar optimum temperature and pH 4.5 of 60 ◦C, respectively.

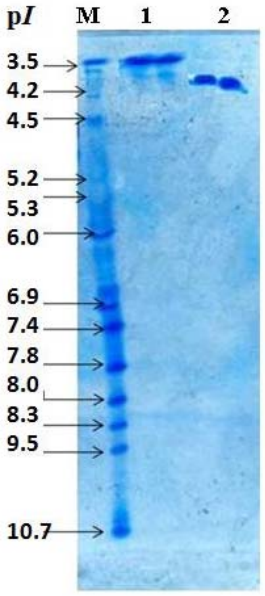


Figure 2 (B) Iso-electric focusing of purified Phy I and Phy II.Lane M: Standard p*I* markers (Pharmacia), Lane 1: Purified Phy I Lane 2: Purified Phy IIIsoelectric point of purified enzymes M- Marker, I- Phytase I, II- Phytase II

Protein (source)	**p*I***	**Protein (source)**	**p*I***
Cytochrome C (horse, heart)	10 .7	Ribonuclease A (bovine, pancreas)	9.5
Lectin (Lens culinaris)	8.3, 8.0, 7.8	Myoglobin (horse, muscle)	7.4, 6.9
Carbonic anhydrase (bovine, erythrocytes)	6.0	ß-Lactoglobulin (bovine, milk)	5.3, 5.2
		Trypsin inhibitor (soybean)	4.5
Glucose oxidase (*A. niger*)	4.2	Amyloglucosidase (*A. niger*)	3.5

Molecular mass (M_r)

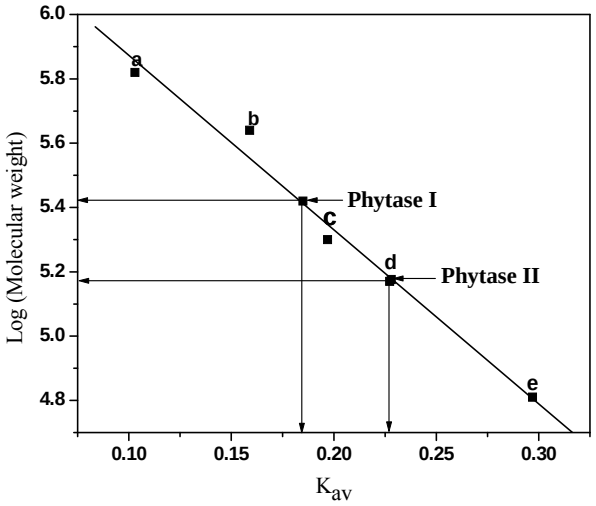


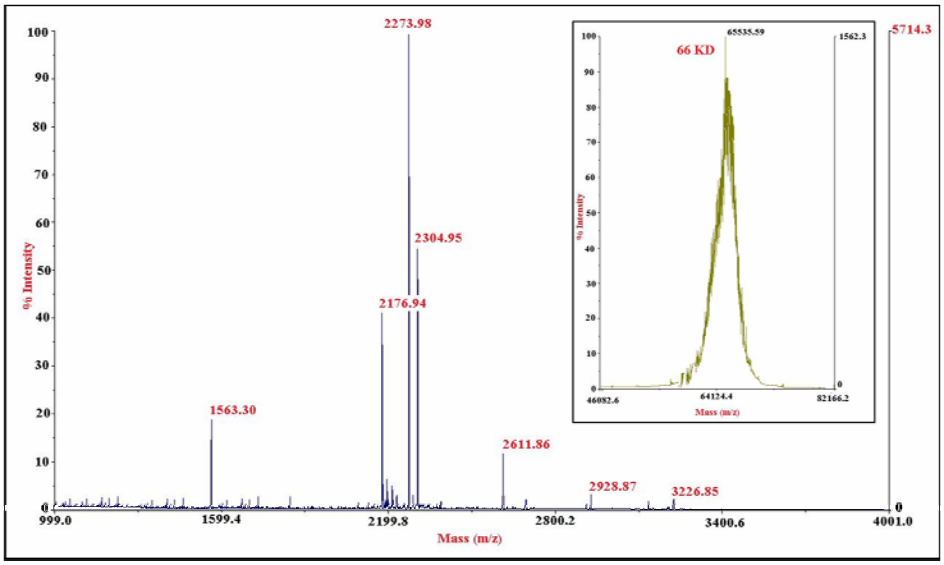
Figure: 3 Native molecular mass of Phytase I and II estimated by gel filtration column : Sephacryl, S-300 column (1x150 cm) calibrated using standard gel filtration molecular weight markers as described in Methods. Partition coefficient (Kav) of the proteins was determined by the formula as given in the text and values are plotted against the log molecular mass (Mr). The best-fit line was drawn using Origin 7.5 (MicroCal).
Markers are, a: Thyroglobulin (669.0 KDa), b: Apoferritin (443.0 KDa), c: β amylase (200.0 KDa), d: Alcohol dehydrogenase (150 KDa), e: Albumin bovine serum (66.0 KDa)

Matrix Assisted Laser Desorption /Ionization- Time Of Flight (MALDI-TOF) mass spectrometry analysis

MALDI-TOF analysis of peptides after tryptic digestion for phytase I (1563.3011, 2273.9826, 2304.9520, 2611.8672, 2928.8716, 3226.8563) (Fig 4A) and Phytase II (2176.9194, 2214.9406, 2464.9820, 2693.8450, 3494.7825) (Fig 4B) showed different molecular weights (m/z). Peptides were analyzed by different proteomics softwares and databases. Molecular weights of purified phytases i.e. I-66 KDa (Inset Fig 4A), II-74 KDa (Inset Fig 4B) determined by MALDI- TOF were also totally different, that proved both the enzymes are different. Phytase I showed the same molecular weight (66 KD) of single subunit showed by SDS-PAGE (66 KD) (Fig 2A) but Phytase II showed half (74 KD) of it molecular weight (m/z) showed by SDS PAGE (150KD) (Fig 2B), this could be because of

the doubly charge species of Phytase II during ionisation process of MALDI-TOF spectrometry (37).

A



B

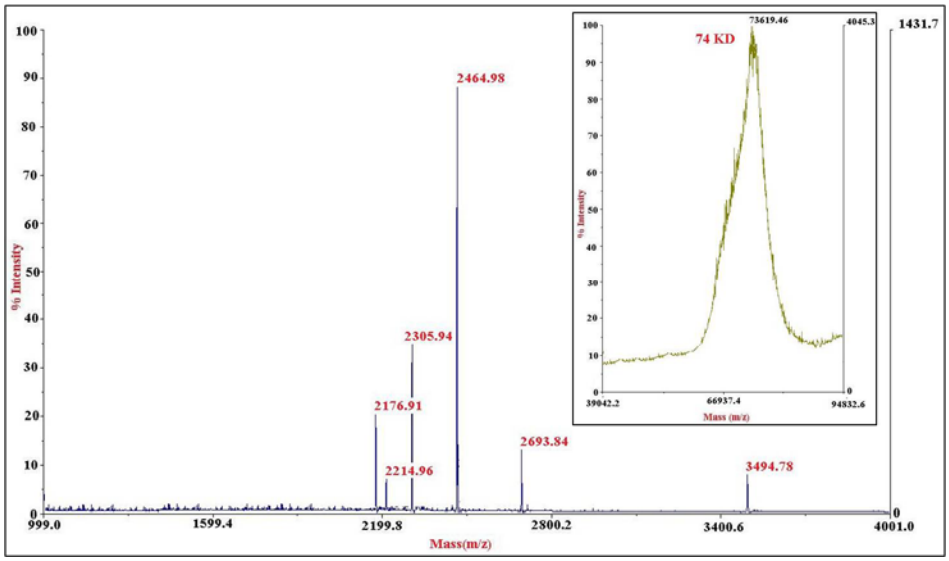


Figure 4. MALDI-TOF analysis of peptides after in gel tryptic digestion of Phy I and Phy II **A**. Peptides of Phy I [Molecular weight of peptides (Da) 1563.3011, 2273.9826, 2304.9520, 2611.8672, 2928.8716, 3226.8563] Inset: - Molecular weight of Phy I by MALDI-TOF.
B. Peptides of Phy II [Molecular weight of peptides (Da) 2176.9194, 2214.9406, 2464.9820, 2693.8450, 3494.7825] Inset: - Molecular weight of Phy II by MALDI-TOF.

Amino acid analysis

Table 2. Amino Acid composition of Purified Phytases (I & II) from *Aspergillus niger* NCIM 563.

Amino acids [a]	**Phytase I**	**Phytase II**
As(X)	324	174
Gl(X)	232	112
Ser	184	120
His	36	40
Gly	236	188
Thr	176	126
Ala	236	116
Arg	76	32
Tyr	148	52
Cys[b]	0*	0*
Val	120	90
met	32	18
Trp[c]	55*	32*
Phe	108	58
Ile	100	60
Leu	196	92
Lys	64	48
Pro	148	90
Total	2471	1448
Mol. wt(kDa)[d]	**271.8**	**159.3**

[a]Values are given in residues/mole, [b] Habeeb A. F. S. A., [c] Spande T. F and Witkop B [387), [d] Calculated molecular weight, *Determined by spectrophotometrically.

The amino acid composition of the purified phyatses I and II revealed the enzyme of 2471, 1448 amino acid residues and a calculated molecular weight of about 271.81, 159.29 kDa respectively. The content of hydrophobic or apolar amino acids (Leucine, Isoleucine, Valine, Methionine, Proline, Alanine , Phenylalanine, Tryptophane) of Phy I and II is about 40.26% & 38.39% (residues/mole). Polar amino acids (Gly, Ser, Thr, Cys, Asx, Glx, Tyr) content is 52.61 & 53.31%. The Asx & Glx content is about 22.5 % & 19.75% and outnumbered the basic amino acids (Lys, Arg, His) about 7.1% & 8.28%. The enzymes had higher Proline content (5.98 & 6.21 %).

N-terminal sequence

The N-terminal amino acid sequence of first 10 amino acid residues of purified Phy I and II was found to be **FSYGAAIPQQ** and **GVDERFPYTG,** respectively. The comparison of N-terminal amino acid sequence of Phy I and PhyII from other *Aspergill* with other selected examples available in a literature is given in Table 3. The sequence of Phy II was different from known phytases. However, Phy I showed strong homology with N-terminal sequence of *Aspergillus ficuum* pH 2.5 optimum acid phosphatase which later referred to be as *phyB* phytase (38).

Carbohydrate content

Phytase I is glycosylated and Phytase II is not glycosylated. *A. niger* phytase is extensively glycosylated, as pointed out earlier by the banding pattern in SDS-PAGE (Ullah and Gibson (39).

Table 3: N-terminal sequence alignment of Phytases from *Aspergillus niger* (NCIM 563) with some of the known sequences of Phosphohydrolase (Phytases and acid phosphatase)

Source and gene name	N-terminal Sequence										% Similarity		(Ref)
	1	2	3	4	5	6	7	8	9	10	Phy I	Phy II	
***Aspergillus niger* NCIM 563 Phy I**	F	S	Y	G	A	A	I	P	Q	Q	100	0	P I*
***Aspergillus niger* NCIM 563 Phy II**	G	V	D	E	R	F	P	Y	T	G	0	100	P II*
***Aspergillus niger* Acid phosphatase aph, 3-phytase phyB-**	F	S	Y	G	A	A	I	P	Q	Q	100	0	1
Aspergillus niger CBS 513.88 Phosphate-repressible acid phosphatase precursor (Acid phosphatase PII)	-	V	D	E	R	F	P	Y	T	G	0	90	2
Aspergillus fumigatus Af293 Acid phosphatase	M	K	K	L	Y	N	G	R	R	V	0	0	3
Aspergillus clavatus NRRL 1 Acid phosphatase	M	G	T	F	A	F	L	L	S	V	10	10	4
Aspergillus clavatus NRRL 1 histidine acid phosphatase, putative	M	E	S	N	D	S	L	Q	S	S	0	0	5
Aspergillus flavus NRRL3357 acid phosphatase, putative	M	Q	D	M	V	F	A	L	P	L	0	0	6
Aspergillus flavus NRRL 3357 histidine acid phosphatase	M	T	S	L	N	P	R	D	P	Y	0	0	7
Aspergillus oryzae 3-phytase	M	A	V	L	S	V	L	L	P	I	0	0	8
Aspergillus fumigatus Af293 acid phosphatase	M	V	R	N	T	P	V	I	M	A	0	0	9
Escherichia coli C str. ATCC 8739 4-phytase	M	S	D	M	K	S	G	N	I	S	10	10	10

*** P I** Present work Phytase I , **P II** Present work Phytase II
Similarity with Phytase I is in Red colour and with II is in Brown colour.

Ref.: **1**. NCBI Reference Sequence: XP_001393206.1, **2**. Swiss-Prot: P20584.1, **3**. NCBI Reference Sequence: XP_751964.2, **4**. NCBI Reference Sequence: XP_001271757.1, **5**. NCBI Reference Sequence: XP_001273523.1, **6**. GenBank: EQ963472.1, EED57470.1, **7**. GenBank: EED57611.1, EQ963472.1, **8**. NCBI Reference Sequence: XP_001821210.1, **9**. NCBI Reference Sequence: XP_001481417.1, **10**. NCBI Reference Sequence: YP_001725574.1

Biochemical properties of phytases

Figure 7 and 9 indicates that optimum temperature of Phy I and II was 55ºC but optimum pH was 2.5 and 5.0, respectively (Fig 5 & 6). Purified phytase from *Aspergillus niger* van Teighem also exhibited maximum activity at pH 2.5 and 52-55ºC (6). However phytase produced from *Aspergillus niger* CFR335 either under solid state or submerged fermentation showed phytase activity at pH 4.5 only (40). Similarly Phy I had half life of 6-8 h at 55ºC while Phy II had 10-12 h. Several other microbial phytases (1) and various commercial phytases display optimum temperature range of 45-55ºC (41). Phy I showed broad pH stability (pH 2-9) while Phy II was stable between pH 4.0 to 8.5 (Fig. 5&6). At 65º C for 1.5 h Phy I retained 40 % activity while Phy II retained 55 % original activity (Fig.8 &10). However crude phytase was found to retain 100 % original enzyme activity in presence of 12 % skim milk after treatment at 70ºC for 3 min (22).

pH optimum and stability

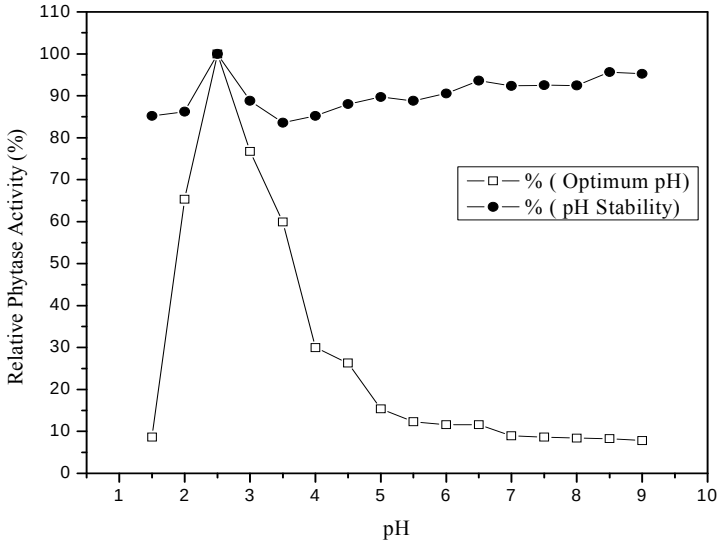


Figure: 5 Effect of pH on activity and stability of purified Acidic Phytase(Phy I) from *Aspergillus niger* (NCIM 563)

pH Optimum (□) **:** A Suitably diluted enzyme (~ 0.05 U) was assayed in a series of pH (1.5-9) at 55 °C as described in Methods and the relative enzyme activity was determined. Maximum enzyme activity obtained was taken as 100 %.
pH Stability (●) : A suitable concentration of purified enzyme (~ 5.0 U/ml) was incubated in a series of pH (1.5-9) at R.T. for the period of 12 hours. Aliquots (10 µl) were removed and residual enzyme activity was determined under standard assay conditions. Maximum activity obtained was taken as 100 %.

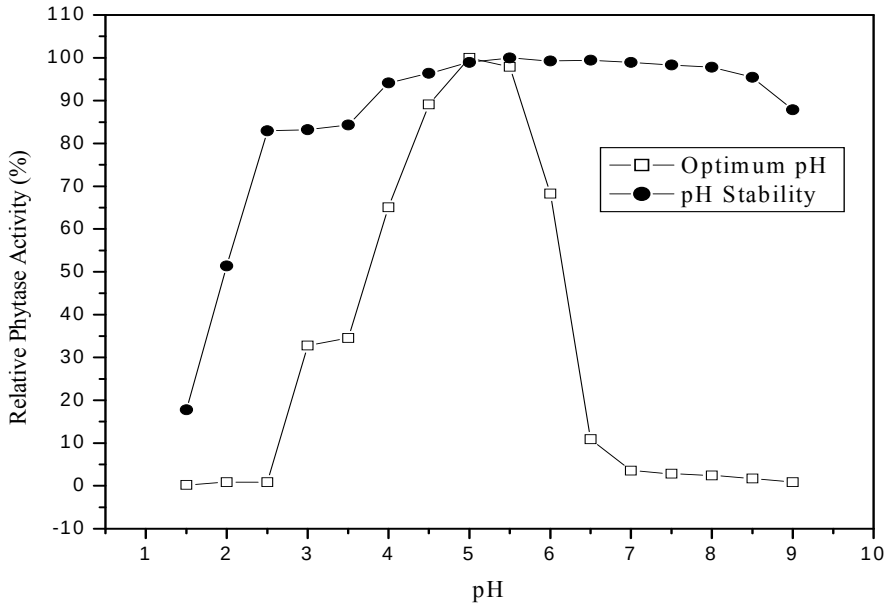


Figure 6. Effect of pH on activity and stability of purified Phytase(Phy II) from A*spergillus niger* (NCIM 563)

pH Optimum (□) **:** A Suitably diluted enzyme (~ 0.1 U) was assayed in a series of pH (1.5-9) at 55 °C as described in Methods and the relative enzyme activity was determined. Maximum enzyme activity obtained was taken as 100 %.

pH Stability (•) **:** A suitable concentration of purified enzyme (~10.0 U /ml)was incubated in a series of pH (1.5-9) at R.T for the period of 12 hours. Aliquots (10 µl) were removed and residual enzyme activity was determined under standard assay conditions. Maximum activity obtained was taken as 100 %.

Temperature optimum and stability

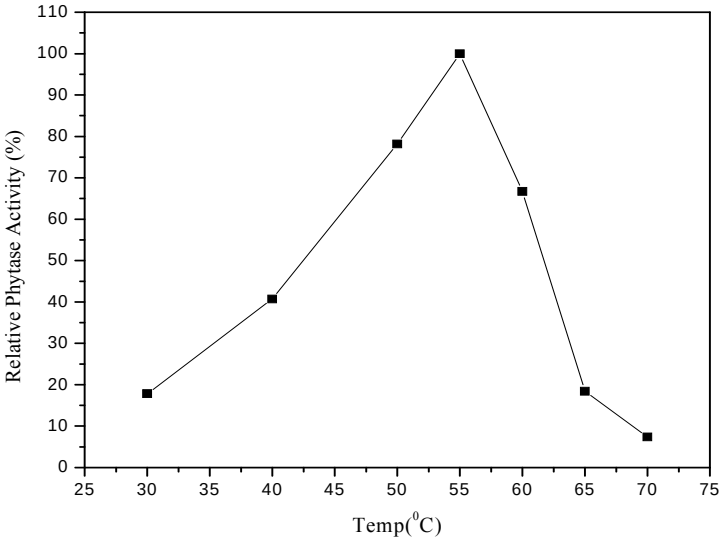


Figure 7. Temperature optimum of purified acidic phytase (Phy I) Purified phytase (~ 0.05U) was assayed at different temperatures ranging from 30-70 °C at optimum pH as described in Methods and relative enzyme activity was determined. Maximum enzyme activity obtained was considered as 100 %.

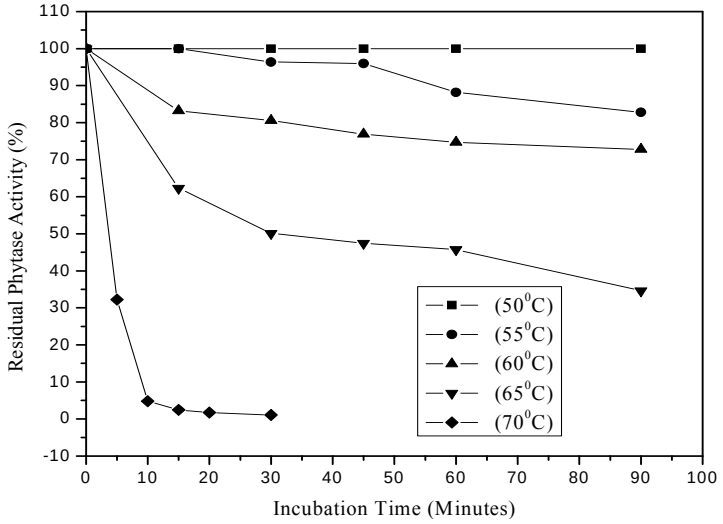


Figure 8. Temperature stability of purified Acidic Phytase (Phy I): Purified enzyme samples (5.0 U/ml) were incubated at various temperatures ranging from 30-70 °C. Aliquots (~10 µl) were removed at suitable time interval and assayed for residual enzyme activity. Maximum enzyme activity obtained was taken as 100 %.

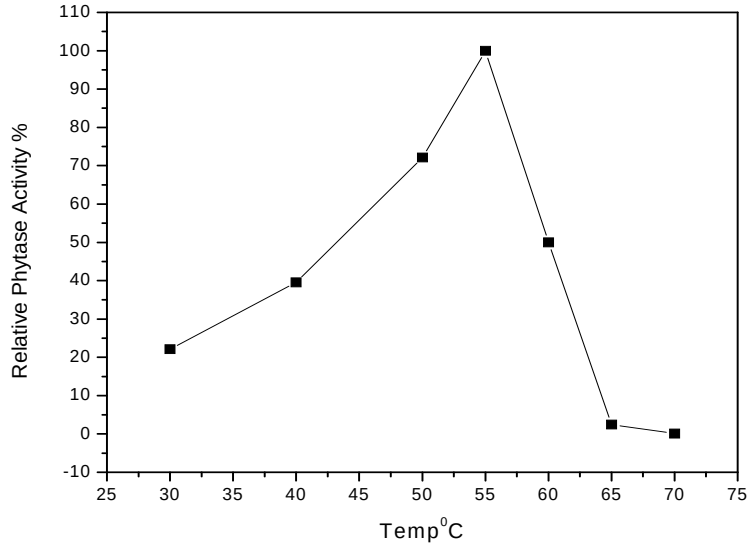


Figure 9. Temperature optimum of purified Phytase (Phy II) Purified phytase (~ 0.1U) was assayed at different temperatures ranging from 30-70 °C at optimum pH as described in Methods and relative enzyme activity was determined. Maximum enzyme activity obtained was considered as 100 %.

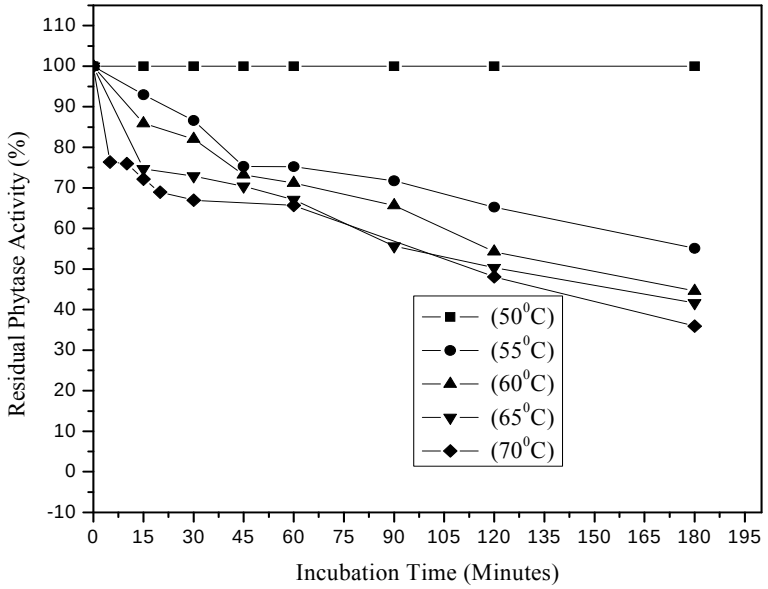


Figure 10. Temperature stability of purified phytase (Phy II) Purified enzyme samples (10.0 U/ml) were incubated at various temperatures ranging from 30-70 °C. Aliquots (~10 µl) were removed at suitable time interval and assayed for residual enzyme activity. Maximum enzyme activity obtained was taken as 100 %.

Table 4. $t_{1/2}$ of Phytases at various temperatures.

Temp. ^{0}C	Phytase I	Phytase II
50	24-30 hour	48-56 hour
55	6-8 hour	10-12 hour
60	3.5 hour	4 hour
65	30 min	3 hour
70	2-3 min	2-2.5 hour

Substrate specificity

The hydrolysis of several phosphate esters (3 mM) by purified Phy I and II was examined by measuring the release of free phosphate. The relative rate of hydrolysis of various substrates is summarized in Table 4. Phy I showed considerably broad substrate specificity and was noticeably specific for other phosphate esters including *p*-nitrophenyl phosphate, a general substrate for acidic phosphatase. Similar results were also recorded by phytase from *Saccharomyces cerevisiae* CY strain (42) and *Mucor hiemalis* (43). Phytases derived from *Aspergillus niger*, namely AllzymeTM and Natuphos® also displayed broad substrate specificity (44). Many other fungal phytases also exhibit broad substrate specificity which is desirable in terms of animal feed application (45).

Kinetics

Results of the kinetic studies (Table 5) indicated that Km values for sodium phytate were lower than calcium phytate indicating that Phy I and II showed more affinity towards sodium phytate. However Phy II showed higher Vmax (26674 μmoles min-1 mg-1) than Phy I (5018 μmoles min-1 mg-1). Lower Km (0.04 –

0.44 mM) for purified phytases from *Aspergillus niger* 92 and *Aspergillus niger* NRRL 3135 (39) has been reported. Similarly among all substrates for phytase activity the turn over number (Kcat) values of both phytases (Phy I and II) was higher with calcium phytate as substrate. Furthermore the ratio of Kcat/Km i.e. catalytic efficiency showed that Phy II degraded sodium phytate and calcium phytate, 2.62 and 1.53 folds more efficiently than Phy I.

Table: 5 Substrate specificity and Kinetic parameters of the hydrolysis of different substrates by Phy I and Phy II

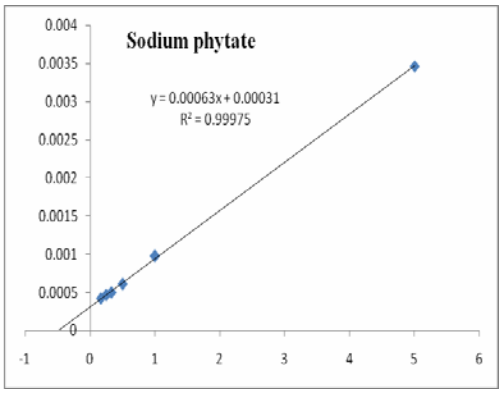
Sr. No	Substrates (3mM)	Relative Phytase Activity (%)		K_m		V_{max}		K_{cat}		K_{cat}/K_m	
		I	II	I	II	I	II	I	II	I	II
1	Sodium phytate	100	100	2.01	0.145	5018	1675	1324.7	251.25	659.08	1732.75
2	Calcium phytate	72.98	92.49	37.27	1.14	26674	2205	7041.9	330.75	188.94	290.13
3	*p*-Nitrophenyl phosphate	229.00	126.99	4.07	1.18	15029	2169	3967.6	325.35	974.85	275.72
4	Sodium phenyl phosphate	224.41	62.99	5.34	0.69	18463	1056	4874.2	158.4	912.77	229.56
5	α-D-Glucose 1-phosphate	137.77	--	3.65	NH	8704	NH	2297.8	NH	629.54	NH
6	D-Glucose 6-phosphate	166.36	--	5.5	NH	14338	NH	3785.2	NH	688.22	NH

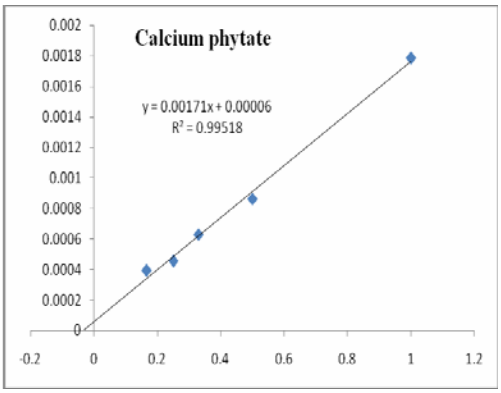
7	1-Naphthyl phosphate	196.39	24.49	6.86	NH	19153	NH	5056.3	NH	737.08	NH
8	2-Naphthyl phosphate	195.39	64.66	3.98	1.28	12376	1673	3267.2	250.95	820.92	196.05
9	ATP	262.91	46.16	2.45	1.12	13476	1022	3557.6	153.3	1452.10	136.87
10	ADP	168.95	51.66	11.65	5.71	24203	2316	6389.5	347.4	548.46	60.84
11	AMP	20.97	48.33	5.79	NH	1970	NH	520.0	NH	89.82	NH
12	β-NADP	215.50	--	2.79	NH	8980	NH	2370.7	NH	849.72	NH
13	Na-pyro-phosphate	293.37	31.83	1.72	2.31	12792	962	3377.0	144.3	1963.42	62.46
14	Glycerol-1-phosphate	28.44	--	5.71	NH	2890	NH	762.9	NH	133.61	NH
15	Phenyl phosphate	132.32	31.83	8.32	1.51	15470	1436	4084.0	215.4	490.87	142.64

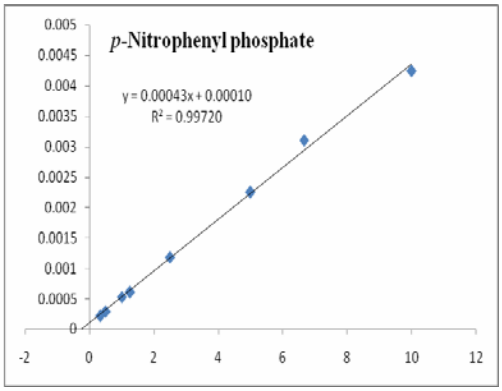
* The hydrolysis rate of Sodium phytate (3mM) was taken as 100% for comparison.

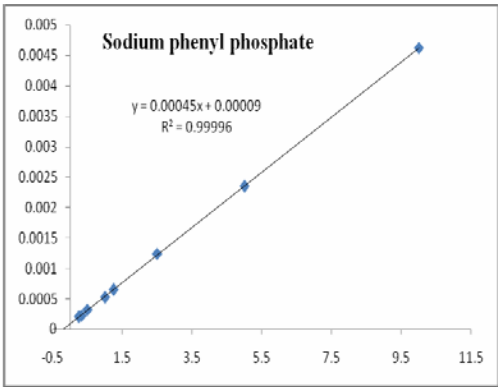
*1-Naphthyl Phosphate, D-Glucose 1-Phosphate and D-Glucose 6-Phosphate, AMP, β-NADP, Glycerol-1-phosphate were not hydrolyzed by Phy II (pH 5.0 Active).

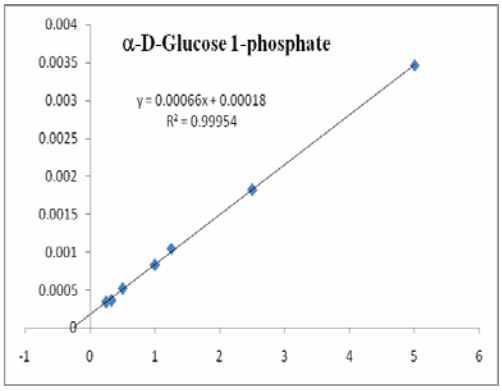
*Determined at 55 ºC under standard assay conditions, incubation time was 30 minutes. Units of the kinetic parameters are K_m = mM, V_{max} = μmoles min^{-1} mg^{-1}, K_{cat} = min^{-1}, K_{cat}/K_m= mM^{-1} min^{-1}.

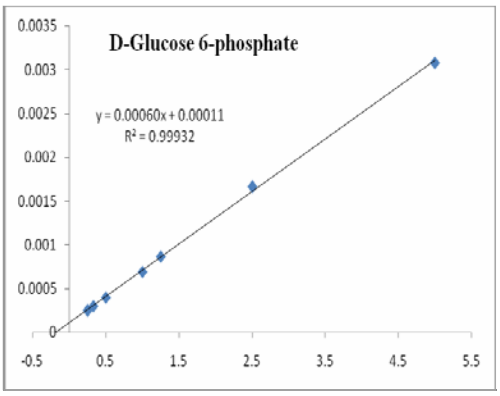

Sodium phytate
y = 0.00063x + 0.00031
R² = 0.99975

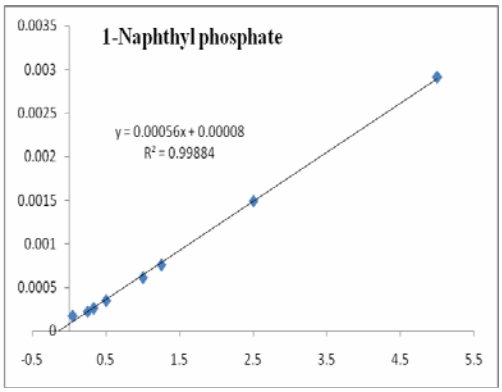

Calcium phytate
y = 0.00171x + 0.00006
R² = 0.99518

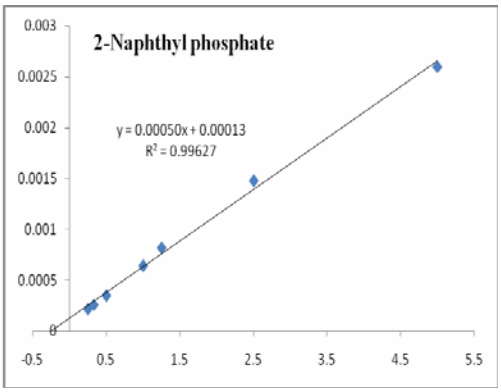

p-Nitrophenyl phosphate
y = 0.00043x + 0.00010
R² = 0.99720

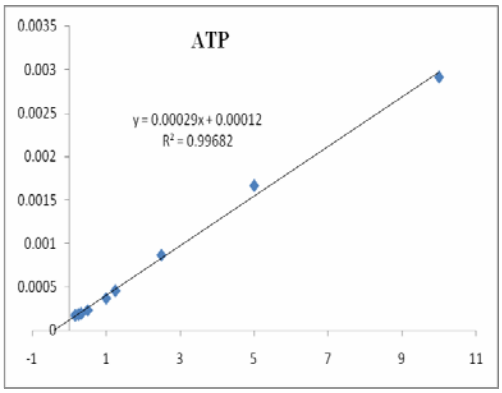

Sodium phenyl phosphate
y = 0.00045x + 0.00009
R² = 0.99996

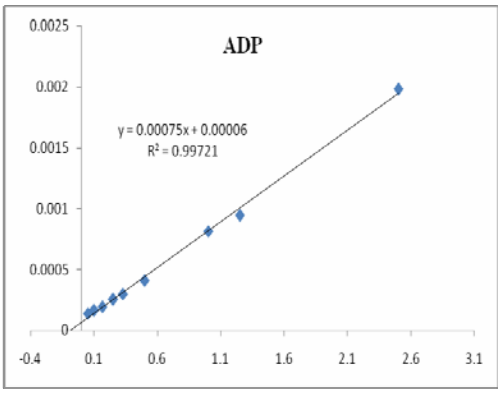

α-D-Glucose 1-phosphate
y = 0.00066x + 0.00018
R² = 0.99954

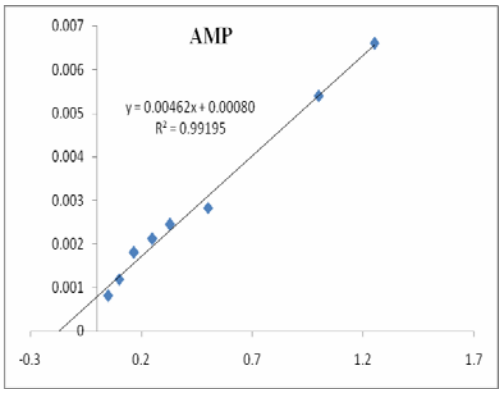

D-Glucose 6-phosphate
y = 0.00060x + 0.00011
R² = 0.99932

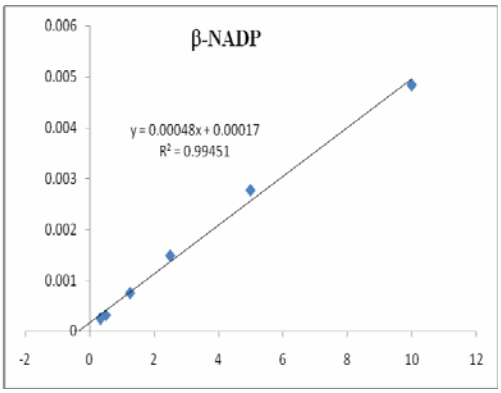

1-Naphthyl phosphate
y = 0.00056x + 0.00008
R² = 0.99884


2-Naphthyl phosphate
y = 0.00050x + 0.00013
R² = 0.99627


ATP
y = 0.00029x + 0.00012
R² = 0.99682


ADP
y = 0.00075x + 0.00006
R² = 0.99721


AMP
y = 0.00462x + 0.00080
R² = 0.99195


β-NADP
y = 0.00048x + 0.00017
R² = 0.99451

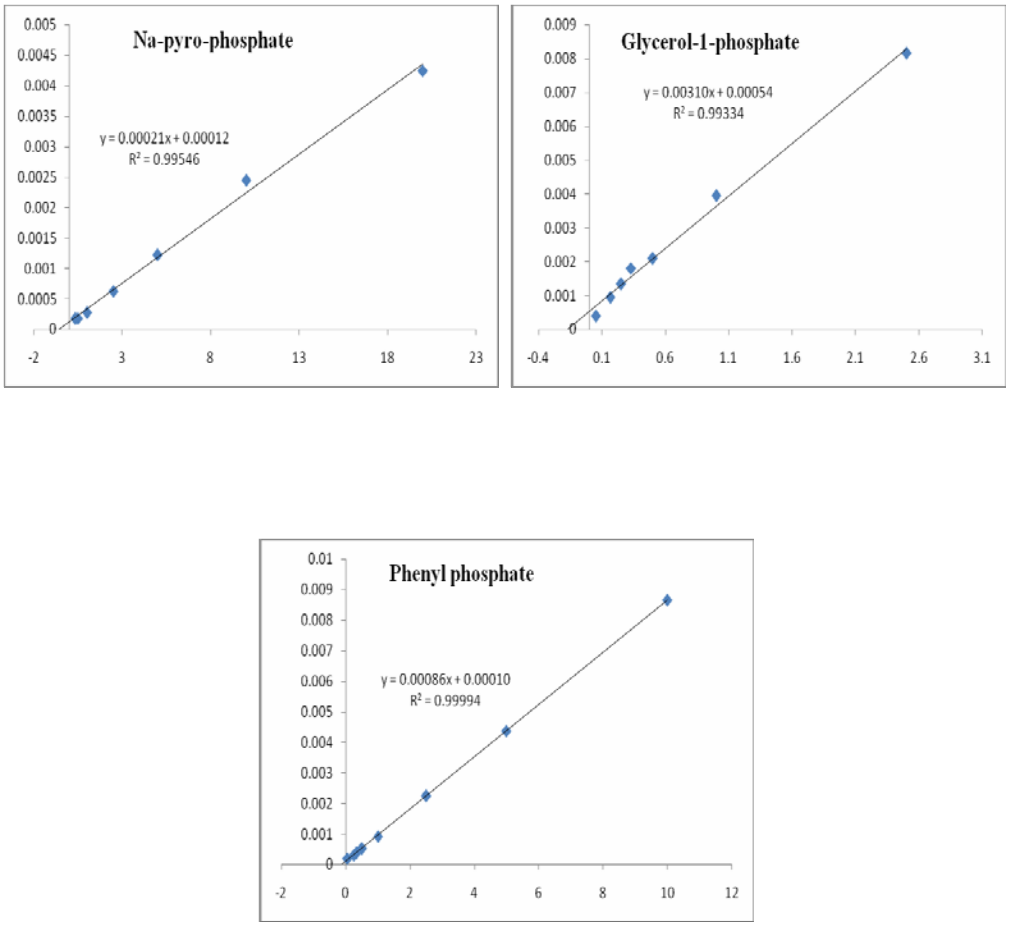
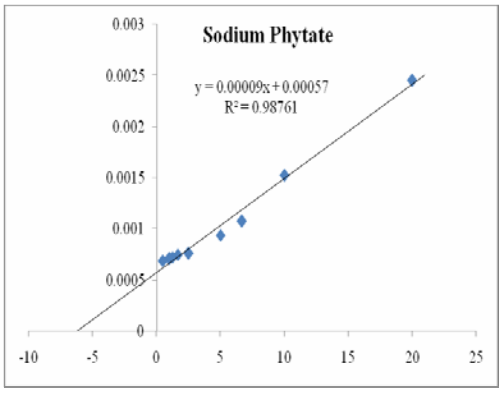
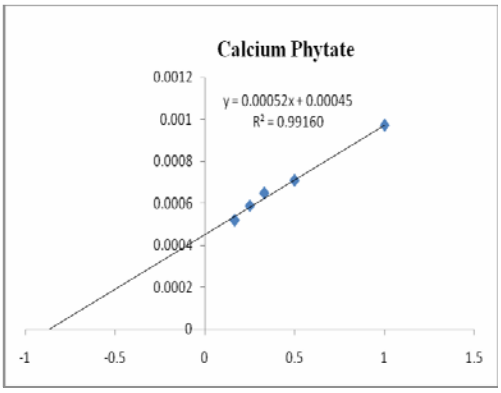
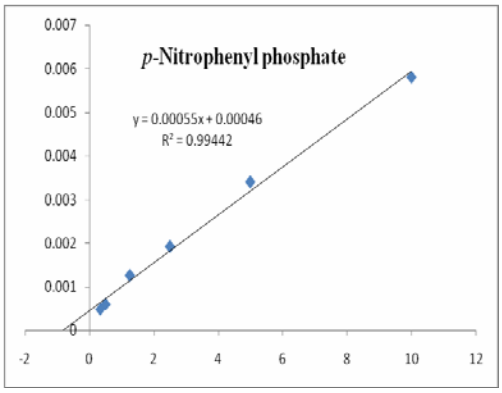
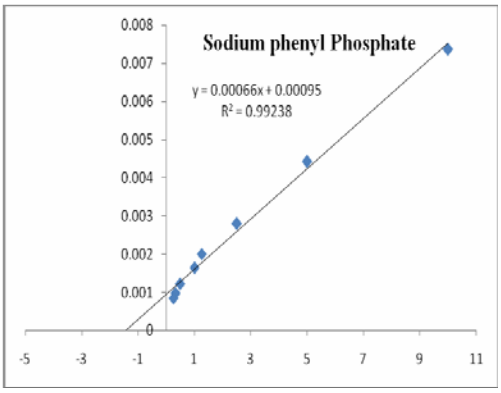


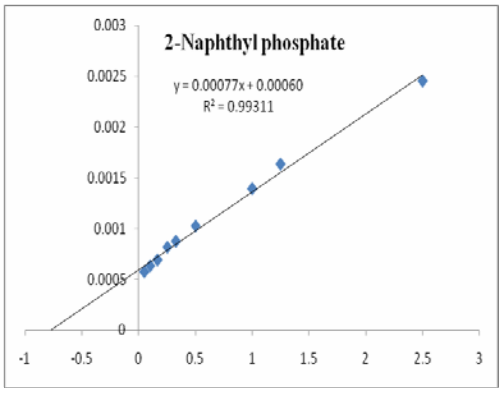
Figure 11a. Lineweaver-Burk plots for the hydrolysis of several substrates by Phytase I of NCIM-563. The assays were carried out at different substrate concentrations as described in methods under the standard assay conditions. Vmax is expressed as μmoles min-1mg-1. Data was fitted to linear regression using Lineweaver-Burk plot in Microsoft Excel.

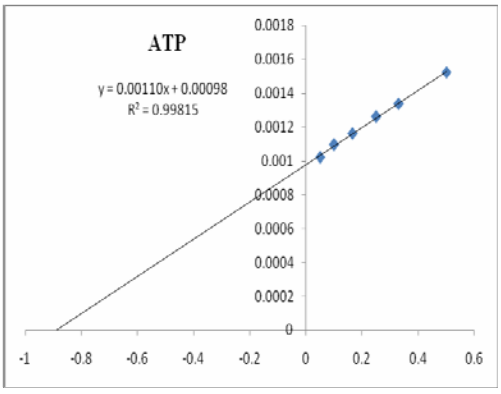

Sodium Phytate
y = 0.00009x + 0.00057
R² = 0.98761


Calcium Phytate
y = 0.00052x + 0.00045
R² = 0.99160


p-Nitrophenyl phosphate
y = 0.00055x + 0.00046
R² = 0.99442


Sodium phenyl Phosphate
y = 0.00066x + 0.00095
R² = 0.99238


2-Naphthyl phosphate
y = 0.00077x + 0.00060
R² = 0.99311


ATP
y = 0.00110x + 0.00098
R² = 0.99815

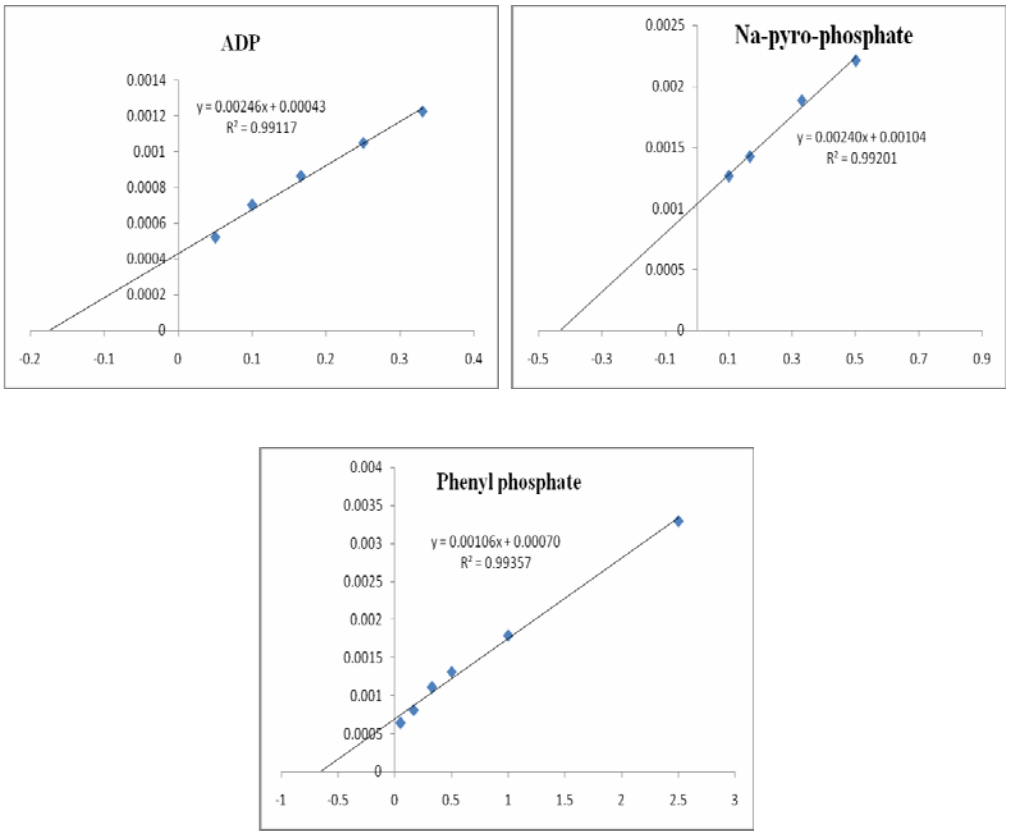


Figure 11b. Lineweaver-Burk plots for the hydrolysis of several substrates by Phytase (Phy II) of NCIM-563. The assays were carried out at different substrate concentrations as described in methods under the standard assay conditions. Vmax is expressed as μmoles min-1mg-1. Data was fitted to linear regression using Lineweaver-Burk plot in Microsoft Excel.

Effect of various metal ions, sugars and reagents

The effect of various metal ions (Figure 3) and other reagents (Table 3) on Phy I and II were investigated. It was observed that Phy II activity was completely inhibited Hg^{2+} at 1mM concentrations while Phy I retained 71 % of its original activity. Fe^{2+} showed negative effect (85 %inhibition) on Phy I activity at 1 mM concentrations. Phy II was strongly inhibited by Cu^{2+} (retained 11 %). The phytase from *Saccharomyces cerevisiae* was totally inhibited by Hg^{2+} and strongly inhibited by Fe^{2+} at 5 mM concentrations (42). The presence of EDTA stimulated Phy I activity while showed slight inhibition of Phy II activity. Phytase from marine yeast *Kodamaea ohmeri* BG3 also showed slight activation in phytase activity at conc. upto 5 mM (35). However SDS showed negative effect on both

Phy I and II. Oxalic acid and EDTA also showed stimulating effect on Phy I activity. Phytase from *Aspergillus niger* van Teighem, however, was not affected by most metal ions, inhibitors and organic solvents (6).

Table 6. Effect of different metal ion solutions on Phytase activity: -

		Phy I			**PhyII**		
	Effective Conc. in Enzyme Assay	1mM	5mM	10mM	1mM	5mM	10mM
Sr.No.	Metal ions	Residual Phytase Activity (%)					
1	None	100	100	100	100	100	100
2	Co^{2+}	103	72	35	100	101	106
3	Cu^{2+}	94	94	94	65	45	32
4	Ba^{2+}	98	ppt	ppt	93	83	73
5	Fe^{3+}	108	100	97	60	70	80
6	Fe^{2+}	98	57	ppt	58	73	94
7	Ca^{2+}	100	94	93	73	87	104
8	Mg^{2+}	101	89	75	82	93	116
9	Ni^{2+}	100	98	93	77	82	94
10	K^{+}	100	100	101	73	80	91
11	Na^{+}	100	100	102	70	82	98
12	Mn^{2+}	100	83	72	80	85	91
13	Pb^{+}	ppt	ppt	ppt	65	46	36
14	Hg^{2+}	100	100	87	00	00	00
15	Ag^{+}	92	51	20	00	00	00
16	Cd^{2+}	89	85	80	81	80	80
17	Zn^{2+}	98	91	85	43	39	32
18	NH_4VO_3	ppt	ppt	ppt	11	8	4
19	$K_2Cr_2O_7$	86	56	10	93	51	25

A suitably diluted enzyme is assayed with varying final concentrations of Metal ion solutions i.e. 1mM, 5mM and 10mM in reaction mixture. Activities are expressed as percentage of activity compare to the control sample.

Table 7. Stability of purified enzymes under different metal ion solutions.

Sr.No.	Metal ions (1mM)	**PhyI**	**PhyII**
1	None	100	100
2	Co^{2+}	100	94
3	Cu^{2+}	95	11
4	Ba^{2+}	100	91
5	Fe^{3+}	94	74
6	Fe^{2+}	15	65
7	Ca^{2+}	94	89
8	Mg^{2+}	90	104
9	Ni^{2+}	100	93
10	K^{+}	97	98
11	Na^{+}	98	84
12	Mn^{2+}	80	89
13	Pb^{+}	73	64
14	Hg^{2+}	71	00
15	Ag^{+}	80	53
16	Cd^{2+}	89	84
17	Zn^{2+}	86	54
18	NH_4VO_3	15	46
19	$K_2Cr_2O_7$	90	104

A suitably diluted enzyme was pre-incubated with various Metal ion solutions at 4^0C for 24 hour and subsequently residual activity of Phytase was determined as described in methods. Activities are expressed as percentage of activity compare to the control sample.

Table 8. Effect of different solvents and reagents on phytase activity.

Sr.No.	Solvents and reagents		**PhyI**	**PhyII**
1	None (Control)		100	100
2	Acetic acid	(0.1%)	95	92
3	Oxalic acid	(1mM)	194	91
		(10mM)	208	82
4	DTT	(1mM)	101	97
5	EDTA	(1mM)	181	88
		(10mM)	187	93
6	Triton-X-100	(0.1%)	101	93
7	Tween 80	(0.1%)	113	89
8	Tween 20	(0.1%)	114	94
9	SDS	(1mM)	4	4
10	Glycerol	(0.1%)	97	95
11	Toluene	(0.1%)	105	91
12	Sodium azide	(1mM)	101	97
13	β-mercepto ethanol	(0.1%)	105	93
14	Ethylene Glycol	(0.1%)	104	92

A suitably diluted enzyme is assayed with different solvents (0.1%) and reagents (1mM) in reaction mixture. Activities are expressed as percentage of activity compare to the control sample.

Table 9. Effect of different additives on thermal stability of Acidic Phytase (PhyI):

Sr.No.	Additive (10%W/V)	Remaining Phytase Activity (%)	
		70^0C 10 min	70^0C 30 Min
1	70^0C 0 min.	100	100
2	Control (with out additive)	5.08	2.92
3	Maltose	6.28	4.48
4	Xylose	2.67	2.11
5	Glucose	9.94	4.50
6	2-Deoxy Glucose	3.64	2.50
7	Lactose	10.98	7.39
8	Galactose	3.11	1.14
9	Sucrose	6.23	3.14
10	Cellobiose	5.62	2.55
11	Stachyose	2.98	1.14
12	$CaCl_2.2H_2O$	2.23	1.04
13	PEG	3.56	2.11
14	Glycerol	2.56	1.45
15	Mannose	2.34	1.23

Purified enzyme samples (5.0 U/ml) were incubated at 70^0C with different additives (10 % W/V). Aliquots (~10 µl) were removed after 0, 10 and 30 min and assayed for residual enzyme activity. Enzyme activity of 0 min sample was taken as 100 %.

Table 10. Effect of different additives on thermal stability of Phytase II

Sr.No.	Additive (10%W/V)	Remaining Phytase Activity (%)	
		70^0C 2 hour	70^0C 4 hour
1	70^0C 0 min.	100	100
2	Control (without additive)	63.68	35.15
3	Maltose	49.77	15.79
4	Xylose	42.27	7.89
5	Glucose	52.70	22.56
6	2-Deoxy Glucose	64.41	33.08
7	Lactose	62.03	35.15
8	Galactose	5.49	2.45
9	Sucrose	57.09	28.38
10	Cellobiose	48.67	26.50
11	Stachyose	23.86	12.56
12	$CaCl_2.2H_2O$	12.07	4.67
13	PEG	28.18	12.88
14	Glycerol	56.91	29.51
15	Mannose	38.24	.376

Purified enzyme samples (10.0 U/ml) were incubated at 70^0C with different additives (10 % W/V). Aliquots (~10 µl) were removed after 0, 2 hour and 4 hour and assayed for residual enzyme activity. Enzyme activity of 0 min sample was taken as 100 %.

3.5. Conclusion

Fujita et al (46) reported three types of acid phosphatases in which one was phytase from *Aspergillus oryzae* under submerged fermentation conditions. Extracellular phytase with two pH optima (2.5 and 5.0) has been reported from *Aspergillus niger* (18) and commercial *Aspergillus niger phytase* (Natuphos) (45). Earlier we have reported production and partial characterization of two types of phytase from *Aspergillus niger* NCIM 563 under submerged fermentation (20). In the present studies we were able to purify both phytases (Phy I active at pH 2.5 and Phy II active at pH 5.0) from *Aspergillus niger* NCIM 563 produced under submerged fermentation to homogeneity and found that they differ with each other in molecular and biochemical properties including pH optima, temperature and pH stability, effect of various metal ions and other reagents, MALDI-TOF analysis, affinity towards various substrates and N-terminal amino acid analysis. This, according to our knowledge, is the first report of two distinct phytases from *Aspergillus niger* produced under submerged fermentation conditions. High phytase activity at pH 2.5 and stability under acidic conditions (pH 1.5 to 9.0) suggests its application in poultry feed. However, further work is needed to improve its thermostability by immobilization and/or micro encapsulation along with site directed mutagenesis for its application in pelleted poultry feed which is in progress.

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CHAPTER 4

Application of phytase of *Aspergillus niger* (NCIM 563) in biomimetic synthesis of Hydroxyapatite and its polymorphs

4.1 Summary

Wheat bran is a cheap agro-based waste material, which has a substantial amount of bound phosphorous in the form of phytic acid. However, surprisingly there have been no attempts at harnessing such phosphorous in wheat bran or other agro wastes into nanosized hydroxyapatite particles and its polymorphs. In the present study, we demonstrate novel bio-inspired enzymatic synthesis of nanosized Hydroxy apatite (HAP) $Ca_{10}(PO_4)_6(OH)_2$ and its polymorphs (β Tri calcium phosphate(β TCP) and Di calcium phosphate(DCP)) using wheat bran and a novel extra cellular enzyme phytase, produced by solid state fermentation of fungus *Aspergillus niger* NCIM 563. The as prepared apatite powder with ~100 nm interconnected hollow spheres capped by stabilising proteins and devoid of any carbonate contamination was synthesized by the hydro catalytic action of phytase on phytic acid i.e. extracted from wheat bran and calcium ions at 50ºC and pH of 5.5 under inert environment of nitrogen. Calcination of white solid precipitate leads to loss of occluded protein and converts it to highly crystalline material comprising HAP, β TCP and DCP.

Biomimic synthesis of nanosized biocomposites using novel enzymes from micro organisms starting from cheap agro-industrial waste materials is an exciting possibility that could lead to an energy-conserving and economically viable green approach towards the large-scale synthesis of crystalline artificial bone nanomaterials.

4.2 Introduction

There is a high demand of fabrication of bone like biomaterials for tissue revitalization for orthopaedic and dental applications. Biomimetic mineralization of advanced man-made materials with properties similar to bone is a challenge

because of their complex nature. Synthetic calcium hydroxyapatite [HAP, $Ca_{10}(PO_4)_6(OH)_2$], is a bioactive material that is chemically similar to biological apatite, the mineral component of bone (1).

HAP has immense applications as artificial bone material, supportive media for drug delivery, purification of protein (2), nucleic acid, isolation of virus etc. The calcium phosphate matrix is capable of various chemical interactions with DNA and proteins, including charge repulsion by crystal phosphates and metal affinity interactions with crystalline calcium (3-7). Indeed, human bone is a natural composite comprising of nano-apatite rods (which are ~100 nm) arranged in lamellae and bound to collagen (8). Thus, synthetic HAP is of interest as biocompatible phase reinforcement in biomedical composites, for bulk filling, bone defects and for coatings on metal implants (9). HA and other calcium phosphates (calcium deficient hydroxyapatite, CDHA) are also of interest as components in injectable bone cements; controlling particle properties (*e.g.* size and shape) and are often used to modulate cement setting behaviour (10).

HAP powders and coatings can be synthesized using a number of methods including sol-gel processing (11), co-precipitation (12, 13), emulsion techniques (14-17), mechano-chemical methods (18) and chemical vapour deposition (19). The disadvantages of these methods include requirement of very precise control over reaction conditions, expensive starting materials, large amounts of toxic organic solvents and time consuming processes. For example, in wet chemical synthesis of HAP, a maturation step (>18 h), followed by a heat treatment of 650°C, is required (20). Failure to allow sufficient maturation, gives a phase-separated product upon heat treatment, which can adversely affect biological properties *in vivo*. Furthermore, the aforementioned synthesis approaches give little or no control over HAP particle characteristics i.e. particle size, agglomeration surface area, etc (21, 22).

To gain a better understanding of the factors that produce particles with the desired properties, current synthesis methods are essentially too slow and unpredictable. Thus, there is interest in developing faster synthesis techniques for synthetic apatite that allow a greater degree of control over particle properties.

Wheat (*Triticum sativum*) is a member of family Gramineace, typically constitutes 20-25% of its total product in the form of wheat bran and a great deal of wheat bran is either used as fodder or disposed off by farmers as waste. Wheat bran has a substantial amount of bound phosphorous in the form of phytic acid; a very well known fact. However, to our knowledge there have been no attempts at harnessing this enormous amount of phosphorous present in most of the agro wastes including wheat bran in-to nanosized hydroxyapatite particles.

Phytases (EC 3.1.3.8 and EC 3.1.3.26) are a class of phosphohydrolases that catalyzes the hydrolysis of phytic acid (myo- inositol hexakis phosphate) to inorganic monophosphate and lower phosphoric esters of myo-inositol, in some cases to free myo-inositols (23, 24, 25). Our approach involves the use of GRAS (Generally recognised as safe) fungus *Aspergillus niger* for the extracellular production of phytase enzyme by solid state fermentation of wheat bran.

The present Chapter describes the rapid, single step synthesis of crystalline nano-particle of HAP, β TCP and DCP by a wet biomimetic method. Briefly, aqueous 50mM solution of calcium chloride was mixed with microbial phytase and phytic acid, under continuous stirring at 50°C in acetate buffer of 50mM at pH 5.5. The precipitation of calcium phosphate particles started in the biocatalysed reaction, which was aged for 24 h, filtered, washed with deionized water, and dried at 40°C. This is the first report, to our knowledge, of biomimetic synthesis of nanosized hydroxyapatite and its precursors starting from wheat bran.

4.3 Materials and Methods

Chemicals

The wheat bran used in this study was obtained from an animal feed stuff outlet at Pune, Maharashtra, India. Phytic acid sodium salt from corn was purchased from Sigma Chemical Company, St Louis, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma, and Glaxo.

Fungal strain and maintenance

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).

Production of phytase, culture conditions and Extraction of phytate from agriculture residue

Submerged fermentation for production of phytase was carried out accordingly to a reported work (25). Fermentation medium was inoculated with 1 % (v/v) of spore suspension (5 x 10^7 spores per ml) prepared by suspending the spores from 7 day old sporulated slant of *Aspergillus niger* NCIM 563 grown on PDA in 10 ml of sterile distilled water containing 0.01 % Tween 80 and incubated at 30°C at stationary condition. Phytase produced by the same strain under submerged fermentation condition (25) was having optimum pH 2.5 so it wasn't used in the present study. Extraction of phytate from wheat bran was carried out according to reported by Harland (27) and Latta (28).

Partial purification of phytase II produced by submerged fermentation

After fermentation mycelium was separated by filtration followed by centrifugation at 10,000g for 30 min and the clear supernatant was collected. Solid ammonium sulphate was added to the supernatant to 95% saturation with constant stirring. The precipitate was collected by centrifugation at 15,000g for 20 min and dissolved in smallest possible volume of Glycine HCl buffer (100 mM, pH 2.5) and salt was removed by passing through Sephadex G-25column and active fractions were concentrated through YM-30 membrane (Millipore) and used for phytase activity measurement.

Enzyme assay

Phytase activity was measured at 50°C and using acetate buffer of pH 5.5 100mM as described earlier (26). The liberated inorganic phosphate was measured

by a modification of the ammonium molybdate method (29). A freshly prepared solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1µmol phosphorus per minute under standard assay conditions.

Synthesis of HAP and reaction conditions

Reaction was carried out strictly under inert environment of nitrogen and solutions were prepared in distilled deionized water (resistivity 18.2 MΩ. cm). Deionised water was obtained with a Millipore ultra pure water system, which was previously distilled and decarbonised by boiling and bubbling N_2. Reaction was carried out in acetate buffer (pH 5.5, 50 mM) while keeping the constant temperature of 50°C (optimum temperature for enzyme activity) (26) with continuous stirring for 4 hrs. Extracted phytate from wheat bran (3mM final concentration) and partially purified phytase (17.19 IU/ml) was added in a three neck round bottom (RB) flask. Calcium chloride (50mM) was dissolved in distilled water, under inert atmosphere and was added into this round bottom flask with an addition funnel. The whole assembly (Fig.1) was kept under N_2 atmosphere, to avoid the carbonate contamination. A white precipitate was obtained after reaction and aged in the mother liquid for 24h. The white solid products were isolated by repeated centrifuging and washing with decarbonated water and finally dried at 40°C. During the centrifuging, sealed containers were used to avoid contact with air. The deionised-distilled water used for synthesis and washing, was decarbonated prior to use by boiling and subsequent cooling in the absence of CO_2 by means of a gas-washing bottle filled with KOH solution. During the washing process, some of the HAP particles that remained suspended in the supernatant after centrifuging were discarded. Dried samples were calcined at 1200^0C for 2 hour. Reaction was also carried out with out enzyme as control experiment keeping all the conditions same.

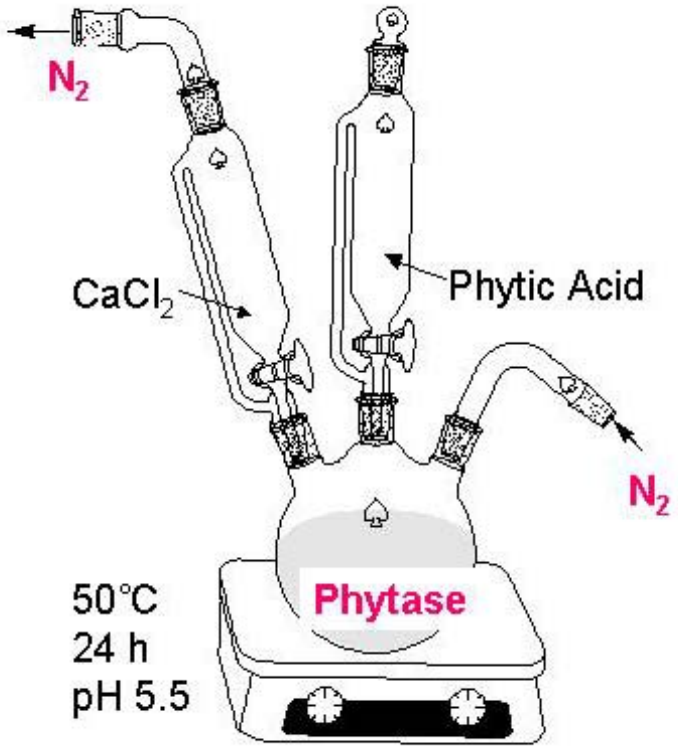

Figure1. Line diagram of reaction set up for biomimetic synthesis of Hydroxyapatite and precursors.

Characterisation of material

The Wide Angle X-ray Diffractometer (WXRD) patterns of the samples were obtained by Rigaku (Japan) X-Ray diffractometer with Cu-Kα radiation at 50kV between the scan ranges of 2θ from 2-60 ° at the scan rate of 5°/min. The d-spacing was calculated by Bragg's formula where the λ was 0.154nm.Samples for Fourier transform infrared (FTIR) spectroscopy analysis were taken in KBr pellets and analyzed on a Perkin-Elmer Spectrum One instrument at a resolution of 2 cm^{-1}. The mass of materials used in the FTIR and XRD analysis were 2 mg and 10 mg respectively to obtain acceptable signal to noise ratios. Samples for transmission electron microscopy (TEM) were prepared by drop coating films of nanosized

HAP powder dispersed in water onto carbon-coated copper grids. TEM and SAED patterns of synthesized products were obtained on a JEOL 1200 EX instrument operated at an accelerating voltage of 120 kV. For scanning electron microscopy (SEM) analysis, solution cast film of biogenic calcium phosphate was made on Si (111) substrates. SEM measurements were performed on a Leica Stereoscan-440 instrument equipped with a Phoenix energy dispersive analysis of X-rays (EDAX) attachment. Thermo gravimetric analysis (TGA) profile of carefully weighed quantity of the as prepared powder of calcium phosphate was recorded on a Seiko Instruments model TG/DTA 32 instrument from 50 °C to 900°C with a heating rate of 10°C min^{-1} under nitrogen with flow rate 20ml min^{-1}. Solid state ^{31}P nuclear magnetic resonance (NMR) spectra were obtained at 162 MHz on a Bruker spectrometer Avance 400 (rotor 4mm, spinning rate 2-12 kHz) using Magic Angle Spinning (MAS). Phosphoric acid (H_3PO_4) was used a reference sample.

Gel Electrophoresis

The biomolecules occluded in the nanostructures were analyzed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure published (34). For protein analysis, the as synthesized particles of HAP, along with occluded proteins were treated with 14.4mM β-mercapto ethanol (2-mercapto ethanol) dissolved in double distilled water; for 5-10 min in boiling water bath. Free proteins were removed by repeated washing and centrifugation at 10000 rpm for 30 min in supernatant. Following dissolution, the protein sample was dialyzed against deionized water for 48 h, lyophilized and analyzed using SDS-PAGE along with protein molecular weight markers.

4.4 Results and Discussions

Production and partial purification of Phyatse II

The partial purified phytase enzyme preparation from the submerged fermentation by fungus *Aspergillus niger* NCIM 563 used for the reaction was having activity 20.6 IU/ml and specific activity 150.04 IU/mg. The purification

process showed 70.11 % recovery with 1.76 fold purification of crude enzyme preparation.

Table 1. Purification table for phytase II produced by *Aspergillus niger* NCIM 563

Purification steps Phytase II	Total protein (mg)	Total activity (IU)/L	Specific activity U/mg	Fold purification	Yield %
Culture filtrate	242	20,600	85.12	1	100
Rota vapour conc.	169	16,312	96.5	1.13	79.10
95% Ammonium sulphate ppt. and desalting	96.26	14,443	150.04	1.76	70.11

Possible mechanism of calcium phosphate production

On mixing of partial purified enzyme preparation to phytic acid (Extracted from wheat bran), inorganic monophosphate is released due to the hydrocatalysis by phytase on the ester bond present between phosphorus and carbon of myo-inositol ring. The released phosphate bind with the calcium ion and the white precipitate of calcium phosphate was formed at 50°C and pH 5.5. Remaining proteins along with phytase and reaction end products like myo-inositol, lower phosphoric esters of myo-inositol were separated from calcium phosphate precipitate by subsequent washing with deionised double distilled water. (Fig.2)

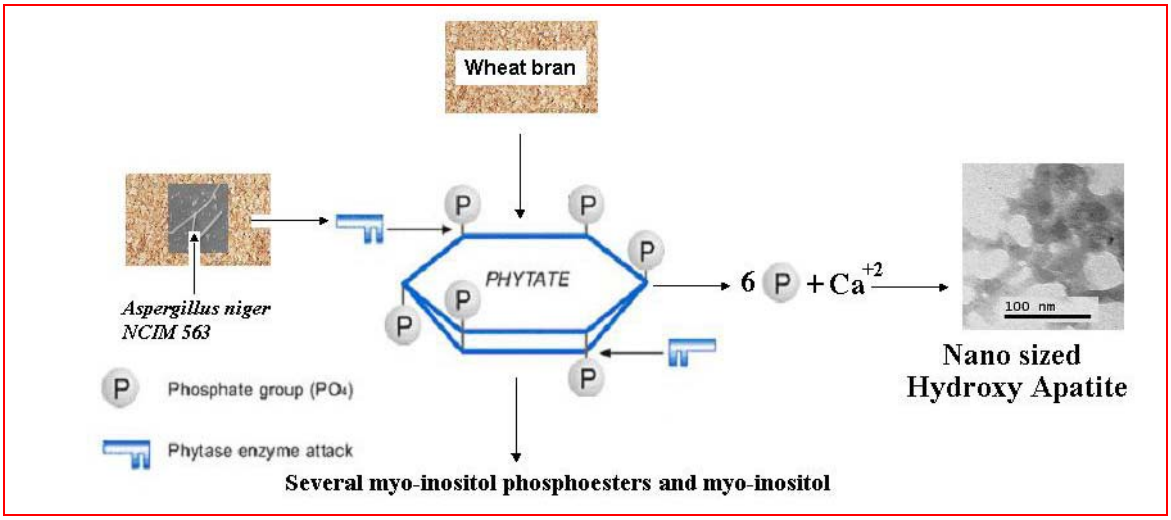

Figure.2. Possible mechanism of production of calcium phosphate (Hydroxy apatite, β TCP, DCP) by phytase from *Aspergillus niger* NCIM 563 produced under submerged fermentation

X- ray diffraction studies

The crystallization of biomimetic product formed in the enzyme-phytic acid reaction was studied by X-ray diffraction (XRD) analysis at 2 h of reaction (Figure 1A). XRD traces showed amorphous powders of low crystallinity, represented by broad diffraction peak at around 28.16 and at 5.32, and free of secondary phases owing to its low temperature processing. However, the obtained results are comparable to the diffraction pattern of the biological apatite and are in good agreement with the previous results (35).

To further verify the crystallinity of particles, XRD analysis of calcined particles was performed. The results are shown in Fig 1B. The room temperature XRD profile (2θ value) of the calcined material matches very well with HAP, β TCP (Tri calcium phosphate), DCP (Di calcium phosphate) that reported in the literature (36). The peaks of the resulting diffractogram were observed near 29.60 and 31.10 and are diagnostic for the presence of calcium hydroxyapatite (JCPDS No. 09-0 432) (37). In addition, the presence of organic molecules greatly influences the crystalline behaviour of the HAP.

We revealed the PC-PDF no., Space Group, unit cell parameters and crystal structure for HAP, β-TCP and DCP, respectively: (34-0010, $P6_3/m$, unit cell parameters a=b=9.414 A°, c=6.879 A°, Tetragonal, α=β= $\gamma=90^0$ [38]), (40.0008, $P2_1/n$, unit cell parameters a=9.332 A°, b=18.13 A°, c=7.841 A°, Monoclinic, α= γ≠ β $=106.69^0$. ≠γ), (41.0483, $P2_1/n$, a=7.667A°, b=12.88 A°, c=7.144 A°, Monoclinic, α= γ≠ β $=107.0^0$. ≠γ). The material is polyphasic in nature .. The XRD pattern of the HAP powder resembles closely to the diffractogram of bone material (39).

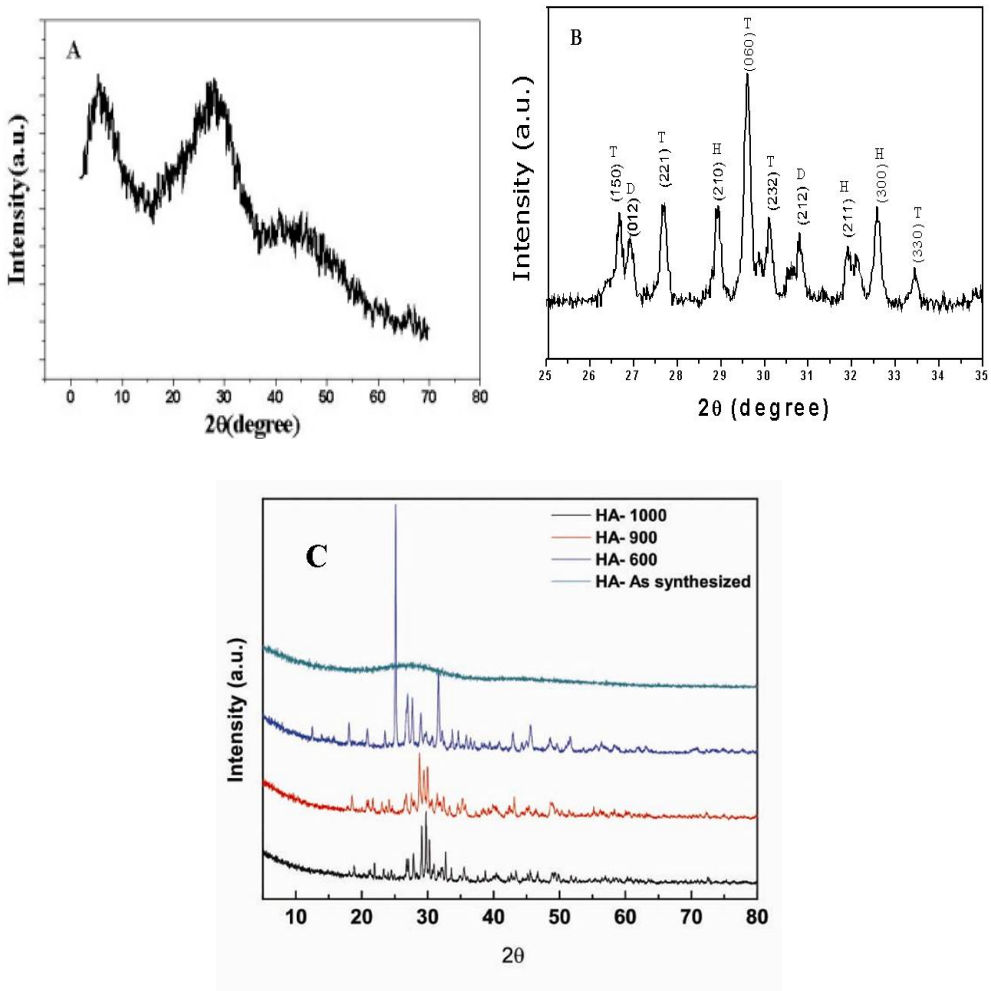

Figure 3. Powder X-ray diffraction pattern of (A) as prepared and (B) calcined material. T- β-Tri calcium phosphate, H - Hydroxy apatite, D - Di calcium phosphate.(C) Calcination on different Temp.

Transmission electron microscopy and selected area electron diffraction studies

The Figure 4 shows the transmission electron micrograph (TEM) of HAP and its polymorphs, demonstrating its nanosized regular structure. The as prepared HAP was ~100 nm in size and formed a dense network. We observed interconnected hollow spheres like structures; those were having 3D, polyhedral and ring-like shape. The TEM of calcined HAP (Fig. 5) showed that after heat treatment (at 600 ^{0}C for 2 hours), due to loss of occluded proteins the morphology changed to an irregular interconnected lattice type structure. The smallest particles were 100 nm in size (Fig 5D), but in general, they were larger, up to tens of microns (Fig 5A-C). Transmission Electron Micrograph of the enzymatically as prepared hydroxyapatite and its precursors A, B, C, D (Inset A, C, D Higher magnification images).

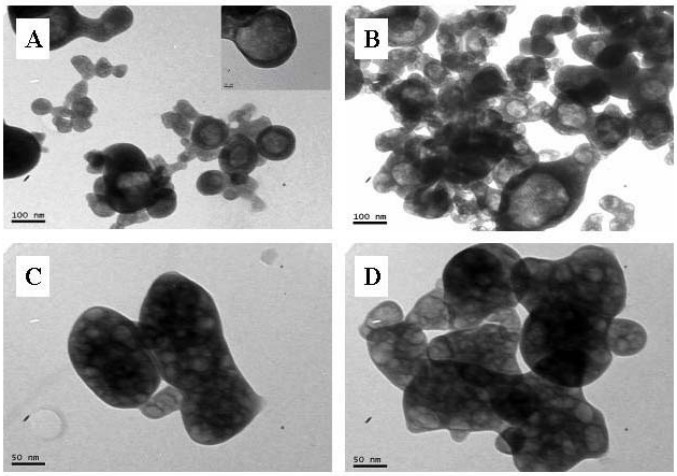

Figure 4. Transmission Electron Micrograph of the enzymatically as prepared hydroxyapatite and its precursors A, B, C, D (Inset A, C, D Higher magnification images).

SAED analysis (Figure 5A inset) of calcined HAP particles shows sharp diffused ring patterns confirming the crystallinity of the calcined calcium phosphate, where the lattice planes exhibit spacing of ~ 2.077 A° and ~ 1.347 A° corresponding to the lattice planes (113) and (314) respectively. The as synthesized protein coated uncalcined HAP sample did not show any discernable diffraction. SAED pattern for calcined HAP suggested that material was polycrystalline in nature. (INSET Figure 5).

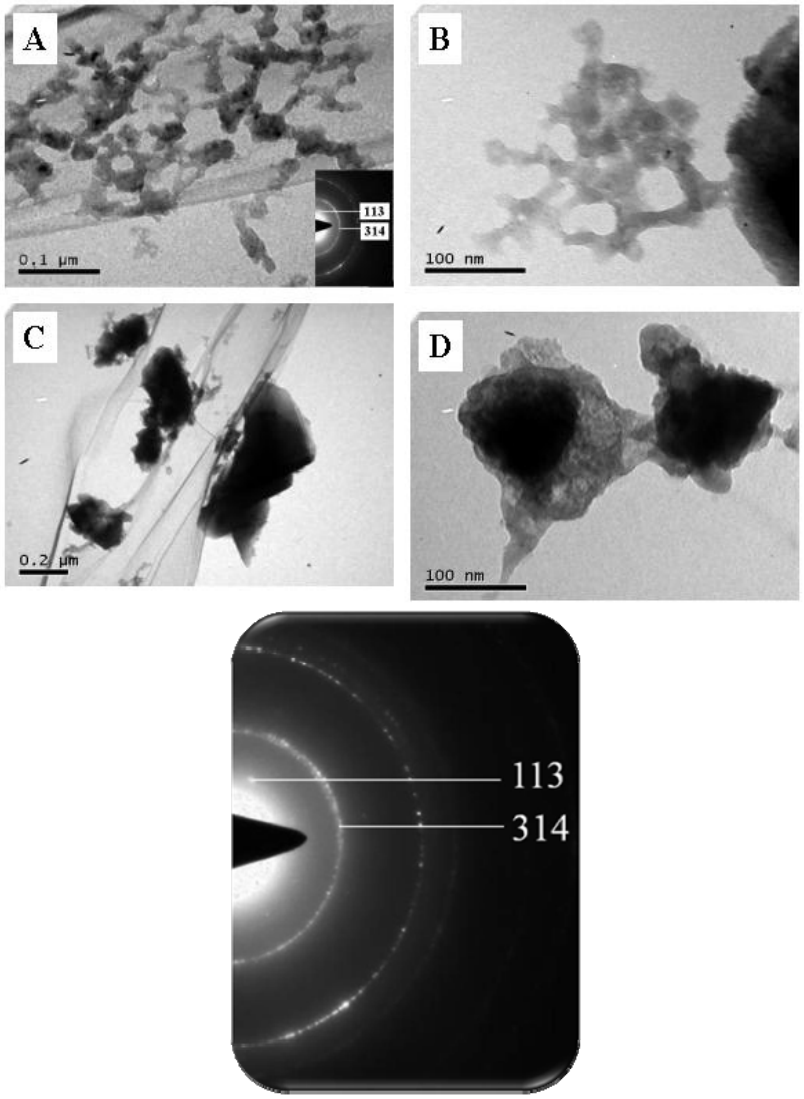

Figure 5. Transmission Electron Micrograph of calcined material (Hydroxyapatite and its precursors). B,D Higher magnification images. Inset(A) shows selected area electron diffraction curve.

FTIR Analysis

The FT-IR spectrums of as prepared particles in Fig. 6(1), calcined in Fig. 6(2) and with commercial available HAP in Fig. 6(3) are given in Figure 6. An intense band observed at 3451 cm^{-1} corresponds to the combination of OH group (HAP phase). The apatitic $PO4^{3-}$ ν_4 modes, in the region of 600- 500 cm^{-1} and ν_1 and ν_3 modes at 1150-1000cm^{-1}, which corresponds to the factor group splitting of

$PO4^{3-}$ tetrahedral, were also detected [40]. The bands at ~953-961 cm^{-1} corresponds to ν_1 symmetric and ν_4 P-O stretching vibrations of the $PO4^{3-}$ ion, respectively (41). Samples have not been contaminated by atmospheric carbon dioxide, as no peak corresponding to a secondary carbonate is recorded. The presence of intense amide I and II bands at 1644 and 1547 cm-1 respectively are also observed in the FTIR spectrum (1). Observation in Fig. 6(1) indicates that the particles in the hollow spherical/circular morphology are present with proteins that are possibly occluded into the particles or are bound to the surface of the particles (42). When the as prepared HAP particles were calcined at 600^0C for 2 h, the amide signatures as well as the signatures from organic molecules around ca. 1400 cm^{-1} in FTIR spectra fade away, indicating the removal of most of the biomolecules during calcinations (43) as shown in Figure 6(2).

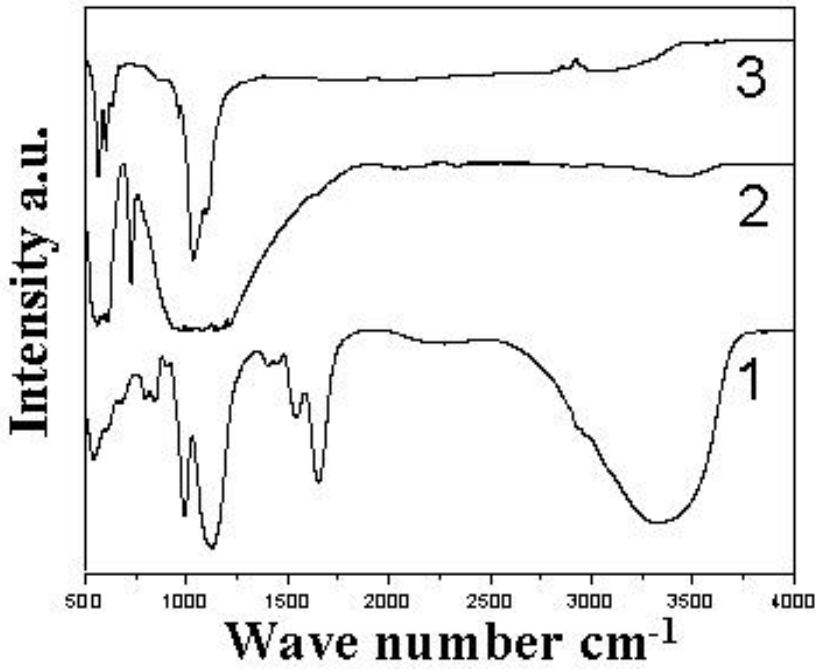

Figure 6. FTIR spectra of (1) as prepared material, (2) Calcined and (3) commercially available hydroxyapatite.

Nuclear Magnetic Resonance Spectra

To further confirm the synthesis of HAP and its polymorphs NMR analysis was done. For HAP a sharp signal was observed at +2.65 ppm (Fig. 7) corresponding to the phosphate (PO_4^{3-}) anion of nanocrystalline HAP (44, 45).

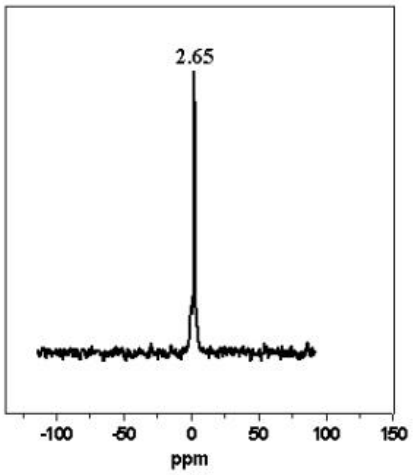

Figure 7. Solid state ^{31}P nuclear magnetic resonance spectra of calcined material

Scanning electron Microscopy and EDAX studies

In order to study the surface morphology of HAP, crystal orientation, and growth of apatite, a scanning electron microscopy was done. A representative micrograph of crystalline calcined biogenic powder is shown in Figure 8A, which provides a good evidence for the nucleation and bionic growth of HAP synthesized by a novel phytase of *Aspergillus niger* NCIM: 563. The calcined HAP possesses large amount of ultrafine crystals with irregular shape and surface and irregular surface texture. Majority of the crystals are spread out on the substrate and few of them agglomerated with a broad size distribution, owing to their high surface area and thus occasionally appear to be in cluster like deposits. The mean aggregating size of the calcined grains was approximately 500 nm to 5 μm. These results suggest the feasibility of *in situ* nucleation of HAP crystals.

The presence of Ca and P, with the Ca/P ratio of 1.55 is revealed by EDAX analysis, which is shown in Figure 8B. EDAX analysis showed strong peaks of calcium (Ca) and Phosphorous (P) and oxygen (O), but Ca and P are primary elements in HAP, β TCP and DCP, confirming the presence of hydroxyapatite.

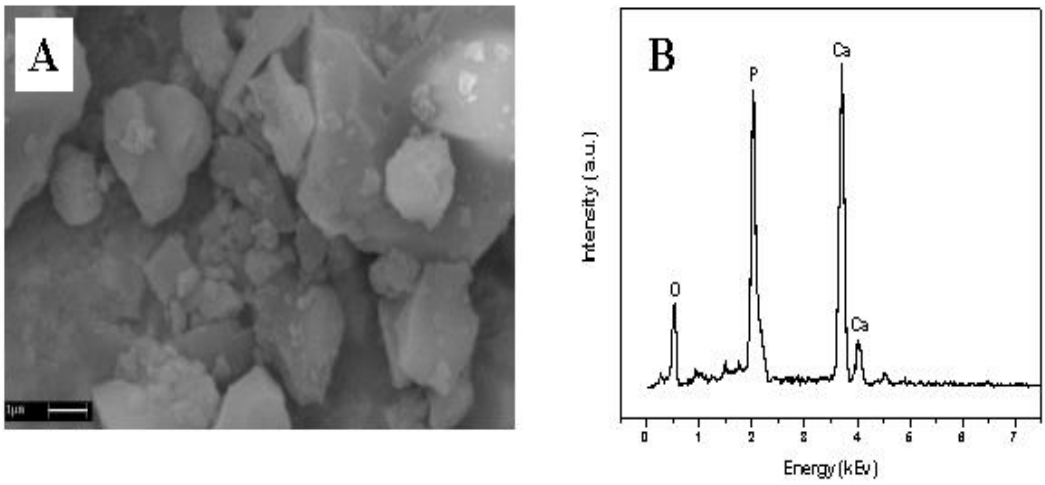

Figure 8. (A) Scanning electron micrograph of material (B) EDX spectra of calcined material.

Thermo gravimetric analysis (TGA)

To understand better the nature of protein occlusion into the particles, TGA measurements of the as prepared powder were done. Fig 9 shows the TGA curve obtained from power of biogenic calcium phosphate. It is observed that there are three prominent weight losses of material 15, 7.39 and 8.57 % at 100-220, 330-410 and 620-830°C, respectively. The first weight loss is clearly due to release of water entrapped in protein-calcium phosphate biocomposite, whereas the higher temperature weight loss is attributed to decomposition/desorption of proteins bound to the material. A third additional weight loss is due to the further decomposition (in to what??) of HAP at higher temperature. The TGA curve shows an over all loss in weight of 31%. Considering the above results, the thermal stability of the biogenic as prepared product seems to be not high. The calcined

material (HAP, β TCP, DCP) was prepared at 1200°C, so it did not show any weight loss in TGA measurements.

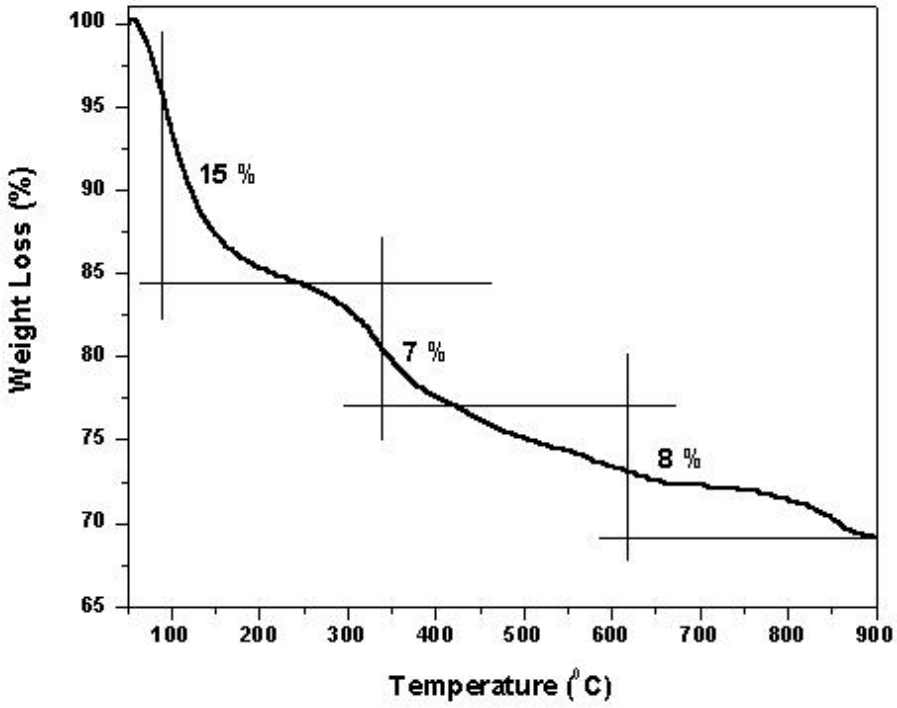

Figure 9. Thermo gravimetric analysis graph of as prepared material.

Study of attached proteins by SDS Gel Electrophoresis

To identify the biomolecules bound to As prepared nanoparticles, the purified calcium phosphate particles were treated with β-mercapto ethanol (2-mercapto ethanol) for 5-10 min in boiling water bath which breaks the disulfide bond present in the quaternary structure of proteins, that leads to conformational changes and finally detachment of occluded proteins from particles. Proteins when analyzed using 10% SDS-PAGE (Figure 7), four major proteins were identified (Line P). Molecular weights of band 1-3 varied between 116 to 29 KDa (line M) and these proteins also present in the partial purified enzyme preparation (line A). The proposed role of these proteins may be to stabilize the size and shape of nanoparticles during synthesis (43). Fourth broad band likely to be the mixture of low molecular weight peptide traces (3-5 KDa) of protein band 1-3, those may be

produced during the detachment process. These results suggest the possible application of synthesized bionic particles for purification of certain proteins.

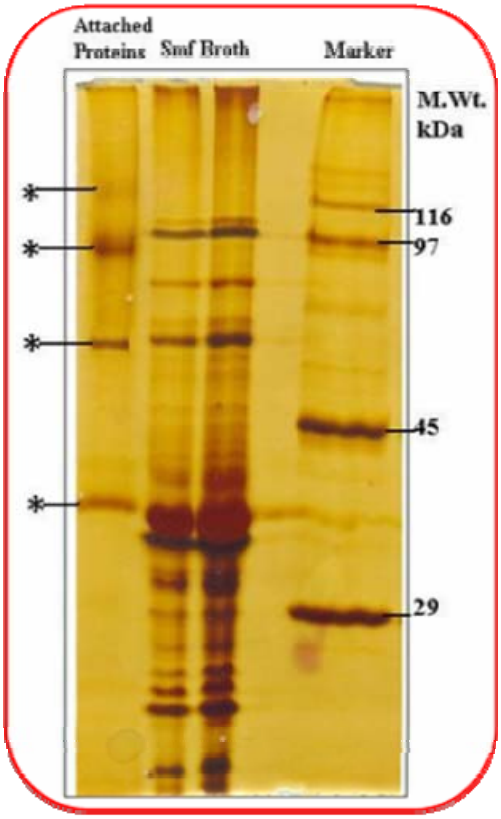

Figure 10. SDS-PAGE (10 %) data showing the silver stained fungal extracellular proteins bound onto the surface of synthesized uncalcined particles.

Lane A: Partially purified phytase along with other extracellular proteins after sub merged fermentation of fungus *Aspergillus niger* NCIM 563.

Lane M: Standard protein molecular weight markers indicated in kDa.

Lane P: Corresponds to the proteins bound to synthesized particles.

4.5 Conclusions

Hydroxyapatite and polymorphs have been successfully synthesized by various chemical methods. According to current study, it is possible that nanoscale and microscale mineral Hydroxyapatite can be synthesizes by biomimetic enzymatic process. Pure synthesis of HAP nano particles by the direct use of fungus biomass and understanding of the role of fungal mechanism would definitely be an issue of great interest in future investigations. Strictly anaerobic archaeobacterial microorganism (56) methanogans i.e. *Methanobacterium, Methanoccus* etc. and sulfur metabolizers i.e. *Desulfurococcus, Methanopyrus, Sulfolobus* etc. may have great potential for biosynthesis of pure hydroxyapatite nanoparticles using their phosphohydrolase enzymes. The overall results therefore suggest that the biomimetic synthesized nanospheres can be a good choice for bone grafting and protein purification applications. This bionic approach could be envisioned as an energy-conserving and economically viable green approach towards the large-scale synthesis of crystalline artificial bone, using potential cheap agro-industrial waste materials like wheat bran.

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CHAPTER 5

Biocompatibility studies of enzymatically synthesized Hydroxyapatite and its polymorphs on Human Osteoblast like MG-63 cell line

5.1 Summary

This chapter describes the studies of artificial bone material Hydroxyapatite (HAP) synthesized by phytase enzyme and commercially available HAP (Sigma) on osteosarcoma MG-63 cell line for its biocompatibility. Human osteosarcoma cells (MG-63 cells) readily seed over 90% of the available biomaterials under static culture conditions, and the attached cells proliferate to extensively cover the biomaterials as seen by DAPI staining and scanning electron microscopy. Cell growth on biomaterials was also monitored by total protein assay. The attached cells are over 90% viable after 7 days in culture as assessed by MTT staining. MG-63 cells also show significant increase in alkaline phosphatase activity 7 days post-addition of oestrogenic supplement. This biomaterial (enzymatically synthesized HAP) show promise for use as vehicles for cell delivery to place large numbers of cells directly into a wound site or onto a tissue engineering scaffold and can potentially be used as artificial bone material.

5.2 Introduction

New materials have been developed for orthopaedic implants. Among these materials, HAP which is known for its high biological activity and its unique property to integrates with, has attracted attention .The most common tissue for transplant procedures is bone tissue. Out of the 500,000 bone graft procedures per year, half relate to spinal surgeries (1). Synthetic materials are man-made materials such as metals, plastic or ceramics that mimic natural bone in its strength, flexibility and/or structure (2). Most are calcium composites designed to be resorbed within the body over time. Autografts, allografts and ceramics and the rationale, benefits and drawbacks of choosing them are discussed next.Bone tissue in itself is composed of four different cell types: osteoblasts, osteoclasts, bone lining cells on the surfaces of

bone, and osteocytes within the mineralized component (3). Osteoblasts lay down new bone while osteoclasts digest away old bone. Osteocytes are mature osteoblasts contained in a calcified matrix are responsible for intercellular communication. The bone lining cells are considered to be fairly inactive carrying out neither absorption nor formation. Little is known about their function but it is speculated that they could also be precursors for osteoblasts (4). Osteoblasts are cells that lie at the surface of the bone matrix that is already formed and they deposit new matrix or osteoid. They are fully differentiated cells. They lay down bone matrix, by secreting collagen Type I and other non-collagenous proteins (4, 5). The osteoblasts themselves often embed themselves in the new matrix that they form and are then termed osteocytes. The matrix hardens as calcium phosphate (hydroxyapatite) crystals are deposited in it. The osteocyte is considered to be the most terminally differentiated cell of the osteoblast lineage. Osteocytes are embedded in the bone matrix and communicate with other cells through gap junctions present in the canaliculi (6). Before becoming trapped in the matrix, the osteocytes test positive for the presence of alkaline phosphatase activity, as functional mature osteoblasts (6). These osteoblasts can also be identified by the other products they produce such as Type I collagen, osteopontin, osteonectin, osteocalcin and bone sialoprotein (6). Osteoblasts that are active show alkaline phosphatase (ALP) activity that can be detected with a histochemical stain (7- 9) and by a biochemical assay that can quantify enzyme activity (10, 11).

This chapter describes the high bioactivity and biocompatibility of artificial bone material Hydroxyapatite (HAP) synthesized by phytase enzyme and commercially available HAP (Sigma) on MG-63 cells, an osteosarcoma cell line that is commonly used in osteoblast and osteoblast/biomaterial research (12-17). A number of osteoblast cell lines and osteoblast precursor cell lines are used in the study of bone development, physio logy and bone-tissue engineering. The MG-63 cell line is derived from a human osteosarcoma (isolated from a 14 year old boy) (18). When induced to differentiate with 1, 25-dihydroxyvitamin D3 (1,25D3), they express increased alkaline phosphatase activity and secrete osteocalcin (19). Another point to note is that the MG-63 cells differ from osteoblasts in that they do not mineralize their

extracellular matrix upon addition of osteogenicinducing media but express all other markers of differentiation. Also, in a study that evaluated these cells and another osteosarcoma cell line, both cell lines showed adhesion or differentiation characteristics similar to those of human osteoblast- like cells (20). The MG-63 cells exhibit features of an undifferentiated Osteoblast and express increased alkaline phosphatase activity when they are induced to differentiate (21). The MG-63 cell line cannot be used for *in vivo* applications in humans, but are a useful *in vitro* model to assess attachment, proliferation and differentiation similar to that of osteoblasts.

HAP which is known for its high biological activity and its unique property to integrate with bones has attracted attention. Human Osteosarcoma cells (MG-63 cells) readily seed over 90% of the available biomaterials under static culture conditions, and the attached cells proliferate to extensively cover the biomaterials as seen by DAPI staining and scanning electron microscopy. Cell growth on biomaterials was also monitored by total protein assay. The attached cells are over 90% viable after 7 days in culture as assessed by MTT staining. MG-63 cells also show significant increase in alkaline phosphatase activity 7 days post-addition of oestrogenic supplement. This biomaterial (enzymatically synthesized HAP) show promise for use as vehicles for cell delivery to place large numbers of cells directly into a wound site or onto a tissue engineering scaffold and can potentially be used as artificial bone material.

5.3 Materials and Methods

MG-63 osteosarcoma cell line

The osteosarcoma MG-63 cell line was obtained from National Centre for Cell Science **(**NCCS**),** Pune, India. Cells obtained were at passage numbers 90-93. Cells were cultured at 37°C with 5% CO2 and 90% humidity in T-75 tissue culture flasks (Corning 430641). Cells were maintained in the culture medium used include 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM) with out phenol red with 2.5 mM L-glutamine (Invitrogen, US), 0.3 mg/ml G418

(Sigma, US) and supplemented with 10% fetal bovine serum (FBS; Gibco 10082-139)

In vitro culture of osteoblasts

As synthesized material was converted in pallets by pressure and pallets were autoclaved and imbedded in 70 % ethanol twice for a period of 20 minutes at each time for sterilization purposes. The pallets were then washed with phosphate buffered saline (PBS) thrice to remove any residual ethanol. Human osteosarcoma cells (MG-63) cultured until passage 5, were seeded on the HA surface at a cell concentration of 10^4 cells/sample. The constituents of the culture medium used include 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM) with out phenol red with 2.5 mM L-glutamine (Invitrogen, US), 0.3 mg/ml G418 (Sigma, US) and 10% fetal bovine serum (Invirogen, US). The medium was changed every two days.

Cell attachment study

One mL of the solution containing 10,000 cells osteosarcoma was added to each of the wells containing the samples. The cells were incubated with the test sample in co2 incubator supplied with 5% co2, 95% humidity at 37ºC for 7days. At the end of the incubation period, the solution containing the unattached cells was discarded and each well containing the test samples was washed thrice with 1 ml of PBS. For the purpose of cell counting, the nuclei of the attached cells were stained with DAPI (4', 6-diamidino-2-phenylindole, dilactate) (Invitrogen^{TM}) using the following protocol. Pre-chilled methanol was added to the wells containing the attached cells and allowed for fixation at room temperature for 10 minutes. Next, the nuclei of the attached cells were stained with 0.25 mL/well of 1µg/mL DAPI for 30 minutes at room temperature. Each test sample was photographed using a fluorescence microscope Leica DM IRB) at 100 times magnification. For each test sample, the number of cells in 5 predetermined rectangular areas (top, centre, bottom, left and right portion of the culture well) was counted based on the nuclei count. This cell

count method was also used to calculate the cell number at the day 0, day 3, day 5 and day 7 for cell proliferation quantification.

Cell proliferation quantification

Cell count method was calculate the cell number at the day 0, day 3, day 5 and day 7 for cell proliferation quantification by the MTT cytotoxicity assay. One mL of the solution containing 10,000 cells osteosarcoma was added to each of the wells containing the samples. The cells were incubated with the test sample in CO_2 incubator supplied with 5% CO_2, 95% humidity at 37ºC for 7days. At the end of the incubation period, the solution containing the unattached cells was discarded and each well containing the test samples was washed thrice with 1 ml of PBS. For the purpose of cell counting, via the MTT cytotoxicity assay is the conversion of the tetrazolium salt (MTT) to the colored product formazan. The concentration can be measured photo metrically at 490 nm. The formation of formazan takes place via intact mitochondria. MTT assay was performed by using the following protocol. Pre-chilled methanol was added to the wells containing the attached cells and allowed for fixation at room temperature for 10 minutes. Next, add MTT reagent (5mg/ml), incubate at 37ºC for 1hr.add 200µl of 100% isopropyl alcohol and keep it at RT for minimum 4hrs.After incubation ensure that formazan precipitate is dissolved by pipetting up and down until any precipitate is visible. Take the observance at 490nm.

Total protein assay

Osteoblasts (10,000 cells/sample) were seeded on blank controls, commercial HA and as prepared HA samples, respectively, in three 24 well plates and cultured in osteosarcoma cell culture medium under standard cell culture conditions for 1, and 7 days respectively. At the end of the prescribed times, the culture medium was removed from culture well, and washed by PBS for 3 times, Samples were stained by bicinchoninic acid (BCA) protein assay working reagent (BCA Kit No. 23225, Pierce) at 37ºC for 30 minutes. Once room tempetature was reached, the protein content was

determined at 590 nm using a FLUO star optima microplate reader (BMG Labtech Gmbh). The total intracellular protein (expressed in mg) synthesized by the osteoblast cells cultured in the medium was determined from standard curve of absorbance versus known concentrations of albumin by the BCA kit.

Alkaline phosphatase activity assay

The prescribed cells were cultured on the materials, and alkaline phosphatase (ALP) activity was measured at days 0, 3, 5 and 7. Four hundred mL of p-nitrophenyl phosphate, disodium salt (PNPP) solution (Phosphatase Substrate Kit, No. 37620, Pierce Biotechnology) was added to the 24-well culture plate and incubated at room temperature for 1 h. The reaction of p-nitrophenol conversion to p-nitrophenylate was stopped by the addition of 200 mL of 2M of sodium hydroxide. Light absorbance of these samples was measured at 405nm on a FLUO Star Optima microplate reader.

SEM of cell morphology

Scaffolds with cells that were cultured for 0, 3, 5 and 7 days were fixed in 4% paraformaldehyde solution for 30 min at room temperature. Scaffolds were dehydrated in 50%, 70%, 95%, and 100% (twice) ethanol (5 cycles with increasing concentrations of ethanol) for 10 min. Samples were subsequently air-dried overnight and observed using SEM.

5.4 Results and discussion

Approach in evaluating the attachment, proliferation, and differentiation of MG-63 cells on HAP in this work is outlined by the following diagram (Fig1).

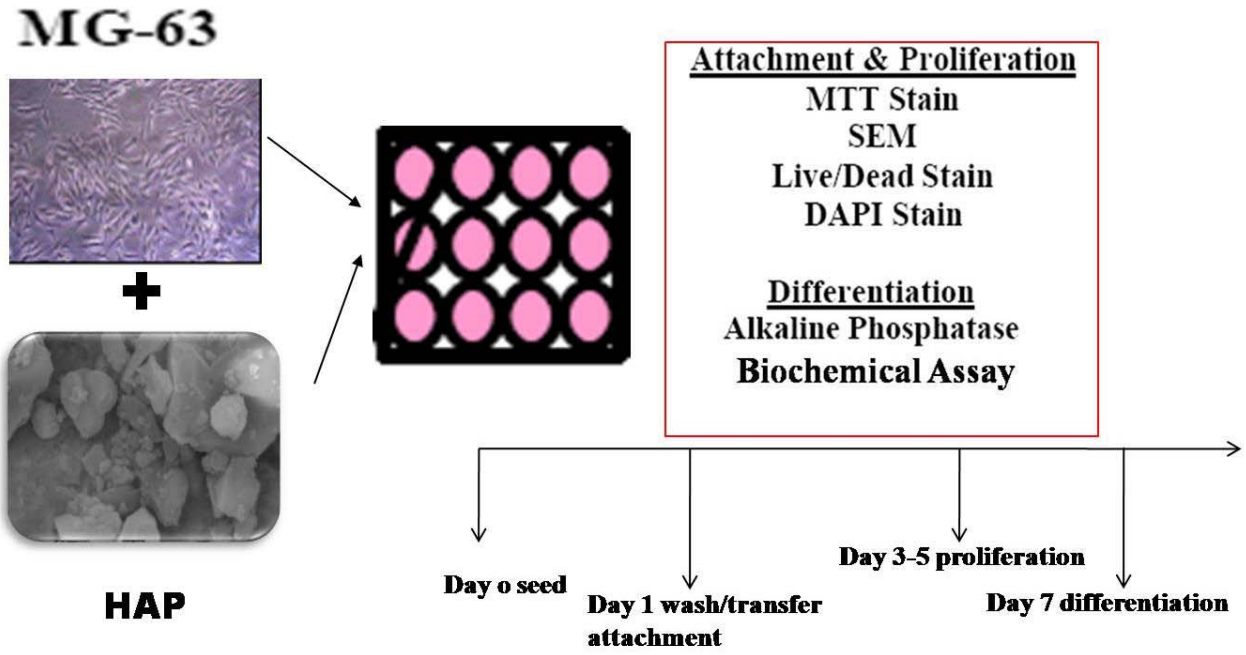

Figure 1. Flow Chart of Experimental Design

The attachment, viability, proliferation and the ability to differentiate into osteoblasts were evaluated.

HAP is not cytotoxic MG-63 cells

MG-63 cells, showed high viabilities when cultured in un-conditioned media, conditioned media (CM) and subsequent dilutions of CM (Table1). Five mL media was conditioned by soaking in one gram quantities of artificially synthesized HAP and commercially available HAP (Sigma) for five days. Cells were plated on glass cover slips and cultured overnight in the appropriate media and LIVE/DEAD staining (data not shown) was carried out to assess viability. Ten fields were measured on each cover slip and live and dead cells quantified. Approximate number of morsels in each field was 5. Percentage viabilities for MG-63 cells were greater than 90% in

all cases. These results indicate that the artificially synthesized HAP and commercially available HAP (Sigma) were not leaching cytotoxic components into the media.

Table 1: Percent viabilities of MG-63 cells on artificially synthesized HAP and commercially available HAP (Sigma) cytotoxicity testing**.**

	Unconditioned Media	Conditioned Media (CM)	1:5 CM	1:10 CM	1:15 CM
HAP	98%	97%	98%	98%	96%
HAP(from sigma)	99%	99%	95%	97%	97%

Percentage viabilities (Total live cells/Total number of cells) of MG-63 cells when cultured in un-conditioned media, conditioned media (CM) and subsequent dilutions of the CM. Viabilities represent the mean of ten fields. Cells were cultured overnight in the corresponding media types and LIVE/DEAD staining was carried out to assess viability. Ten fields were examined for each cover slip and live and dead cells quantified by LIVE/DEAD staining. Experiments were carried out more than three times

The MG-63 cells showed high percentage viabilities in the presence of conditioned media (CM) and subsequent dilutions of CM. The MG-63 cells showed greater than 90% viabilities .The attachment and proliferation of both kinds of cells was evaluated by MTT stain, scanning electron micrographs, DAPI and LIVE/DEAD stain to qualitatively assess their attachment and proliferation on days 1, 3, 5 and 7(Table1).

MG-63 cells attach and remain viable on HAP

Attachment of MG-63 was assessed one day after seeding by washing away unattached cells, staining viable cells with MTT, and imaging stained cells under the microscope. 25 mg/well of HAP fillers were seeded with 10,000 cells/sample and samples were assessed 24 hours post-seeding (Fig 2).

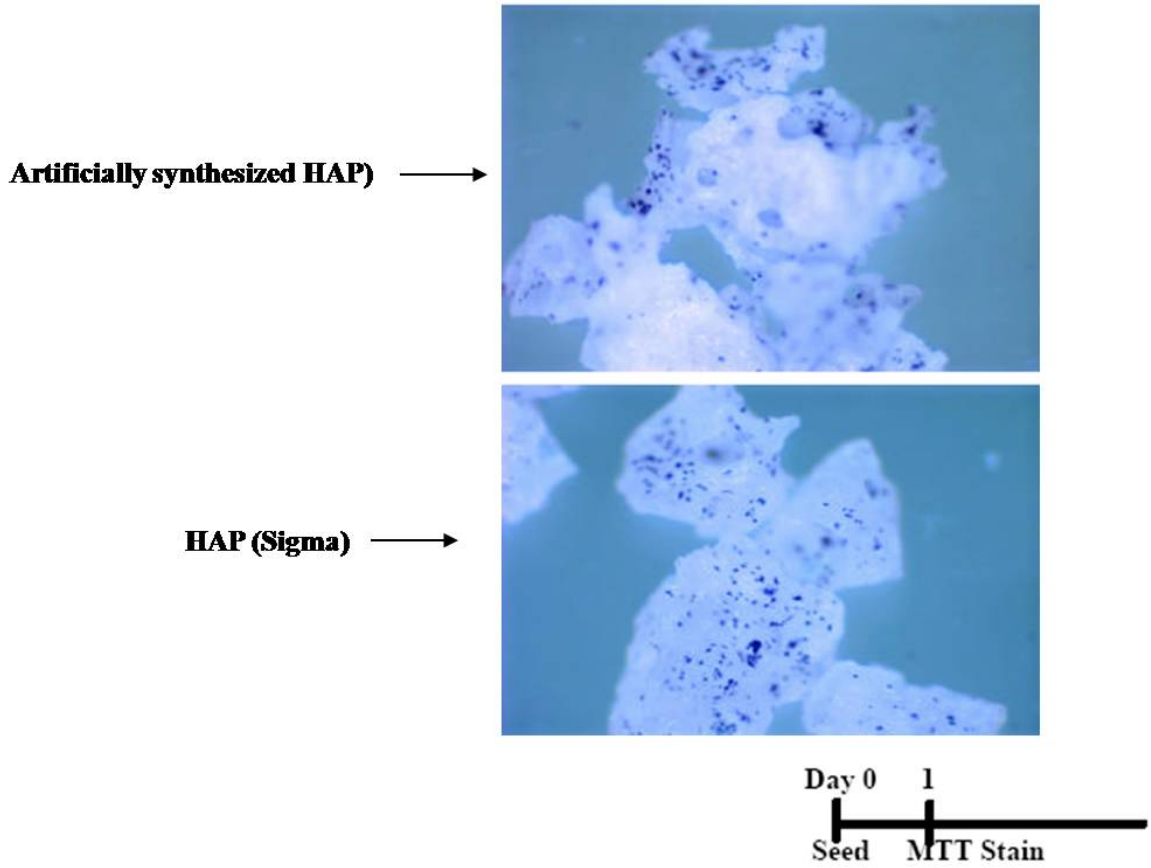

Figure 2. MG-63 cells attach to HAP as assessed by MTT staining (light scatter 10 x magnifications effect).

HAP were seeded with 10,000 cells/sample mg artificially synthesized HAP and commercially available HAP (Sigma) were taken on day 1 for MTT staining. Attachment of MG-63 cells on artificially synthesized HAP and commercially available HAP (Sigma) was visualized using MTT stain stained dark blue. Seeding efficiency (%artificially synthesized HAP and commercially available HAP (Sigma) with cells/total bone HAP morsels) was 100%.

Initially, the MTT stain was used to assess the number of cells attaching as well as proliferating on the ceramics. While it did stain cells that had attached to the HAP the assay was not sensitive enough to measure the proliferation quantitatively. The results varied highly possibly due to a light scatter (10 x magnifications) effect from the biomaterial and all efforts were abandoned (Fig 2).

Cell attachment over 0, 3, 5 and 7 days after seeding was examined via scanning electron microscopy for MG-63 on artificially synthesized HAP material.

Scanning electron microscopy (Fig3) revealed that MG-63 cells attach to the material surfaces of artificially synthesized HAP In all protocols unattached cells were washed away after the second day. The MG-63 cells populated all topographies on the surfaces including concavities and pores (Fig 3).

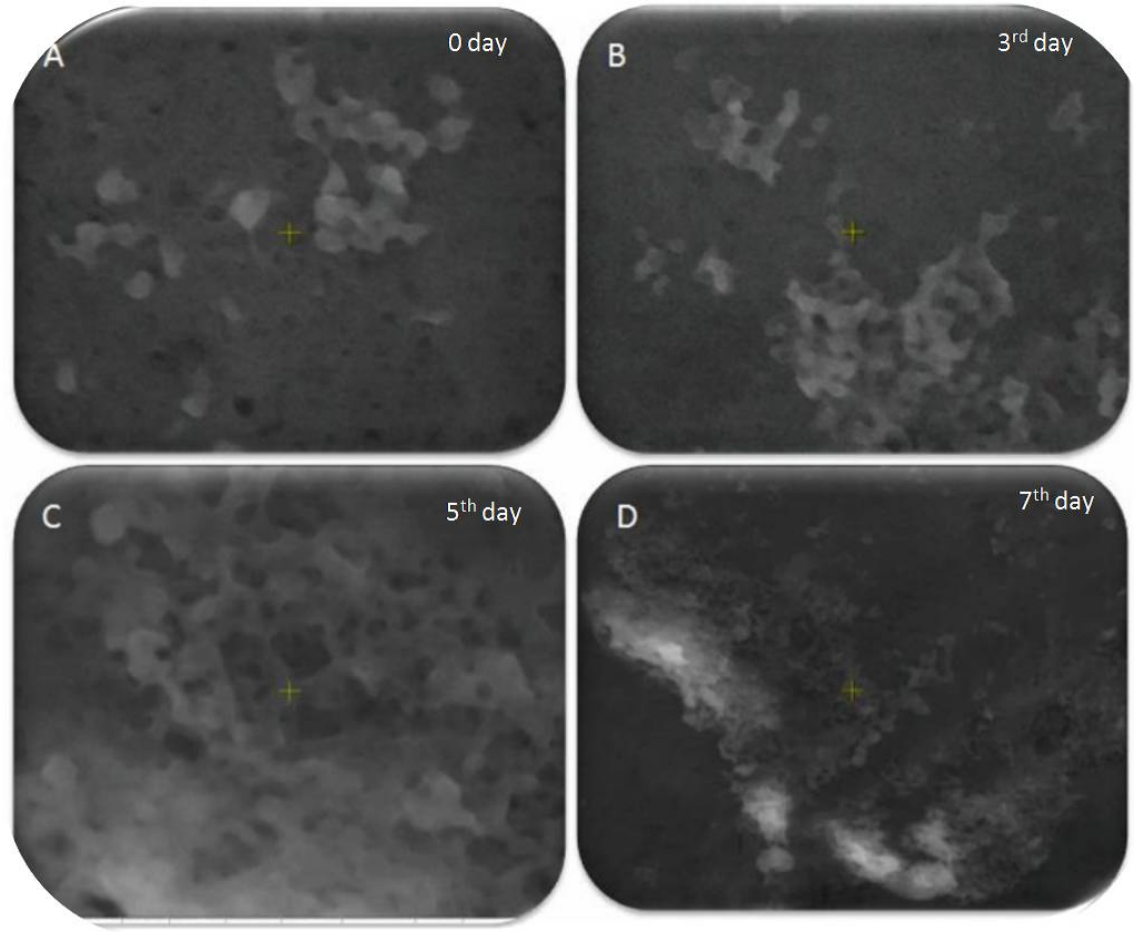

Figure 3. MG-63 cells attach to HAP**.**
Scanning electron microscopy shows MG-63 cells on the surfaces of HAP on day 0, 3, 5 and 7. 10,000 cells/sample HAP was seeded.

Scanning electron micrographs (SEM) showed that MG-63 cells can attach onto the surfaces as well as pores of HAP. SEM studies examining of attachment and proliferation of osteoblasts and fibroblasts on a copolymer poly (lactideco- glycolide (PLAGA) scaffold showed that cells grew in concentric patterns and had an ability to fill (100-250 μm) pores (22) (Fig 3).

Attachment and viability were also assessed by DAPI (Fig 4) and LIVE/DEAD (Fig 5) staining showing that MG-63 cells attach and maintain high viabilities up to seven days after seeding. The cells densely populated the surfaces as early as Day 1 making individual cell counts impossible. High viability (few dead cells) indicated high survival rates of the cells on HAP. The seeding efficiency of HAP populated with MG-63 cells was always 100%.

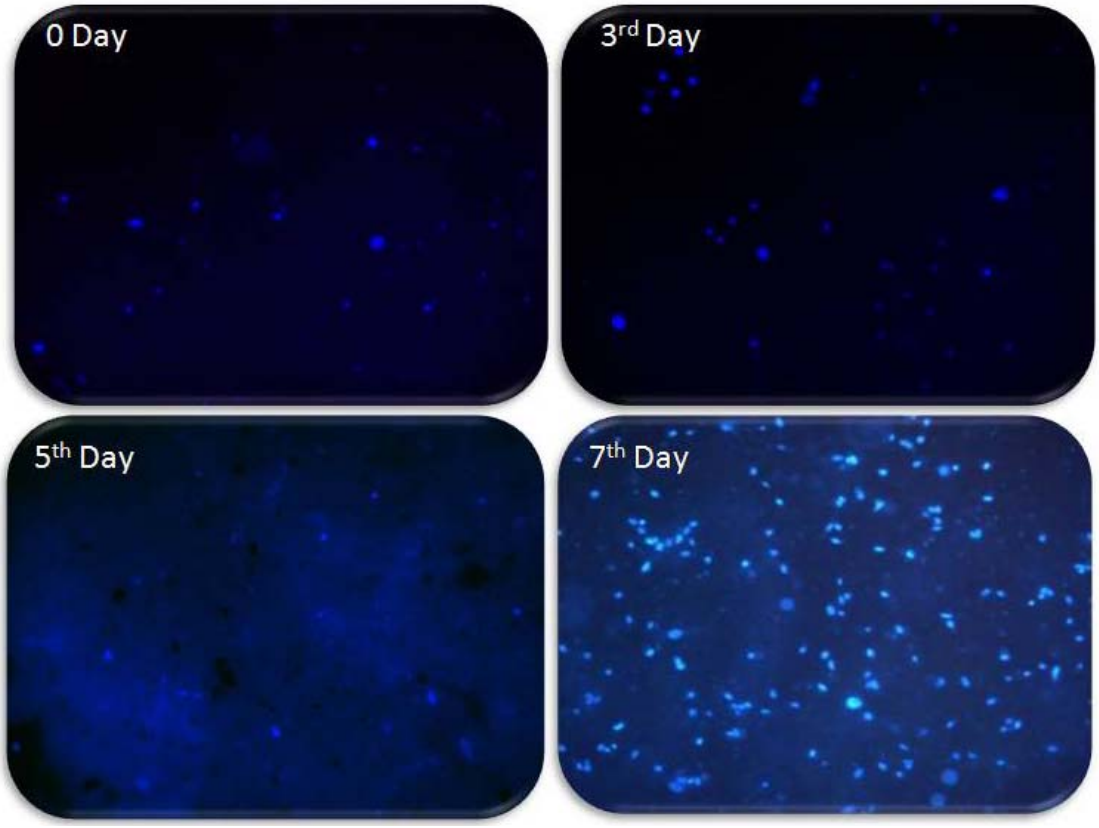

Figure 4. MG-63 cells attach and remain viable on HAP seen by DAPI stain**.**

HAP was seeded in each well and showed attachment and high viabilities through 7 days post-seeding. Unattached cells were washed away on day 1 after seeding .DAPI stained cellular nuclei blue, excitation358 nm and emission 461 nm.

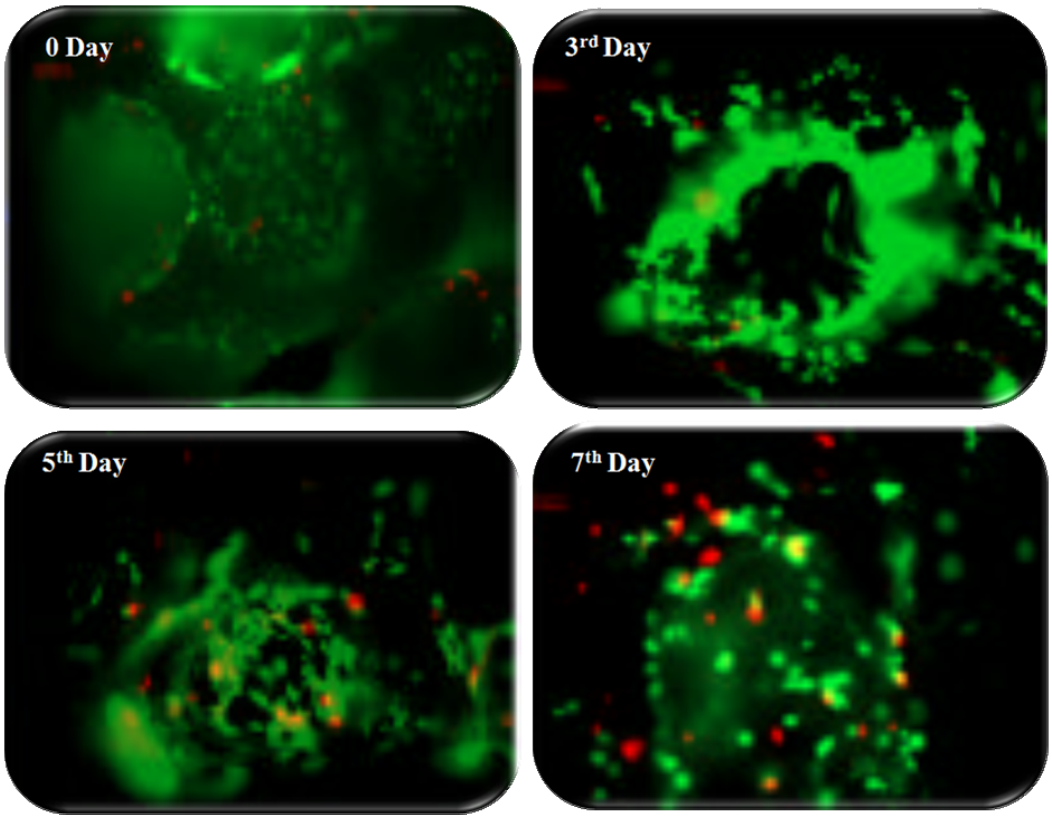

Figure 5. MG-63 cells attach and remain viable on HAP seen by stain LIVE/DEAD.

HAP was seeded in each well and showed attachment and high viabilities through 7 days post-seeding. Unattached cells were washed away on day 1 after seeding. LIVE/DEAD stained live cells green and dead cells red, thereby allowing for a semi-quantitative assessment of live/dead ratios under UV 350–360 nm excitation and emission read at 450 nm.

MG-63 cells also populated with high viabilities all topographies on the material surfaces including concavities and pores. Large numbers of live cells that fluoresced green showed up as intense green as cell boundaries blended and prevented individual cell recognition (Fig4).

The MG-63 cells were seeded with a density of 10,000 cells/ well onto HAP and stained with LIVE/DEAD stain and seemed to densely populate the surfaces making individual cell counts impossible. This gave a visual representation of high percentage viabilities (few dead cells) and thereby high survival rates of the cells on HAP (Fig5).

MG-63 cells attached to HAP express alkaline phosphatase activity (ALP)

Cell proliferation, total protein assay, and Alkaline phosphatase activity (ALP) is a phenotypic marker for differentiating osteoblasts. Expression was assessed on days 0, 3, 5 and 7 of culture. The cell proliferation results were agreed with the results of the cell capture study, as previously mentioned. The final protein concentrations (Fig 6) synthesized by the cells were obtained by dividing the initial results by the cell numbers (Fig. 7), ALP activity (Fig. 8) was comparable for all of the material groups.

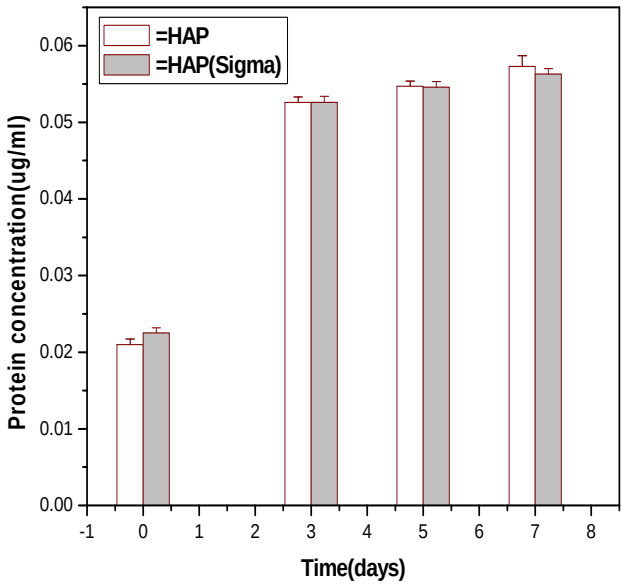

Figure 6. Total protein assay. MG-63 cells on HAP, HAP from sigma (control) from day 0, 3, 5 and 7. Experiments were carried out more than three times and results are mean ± SD

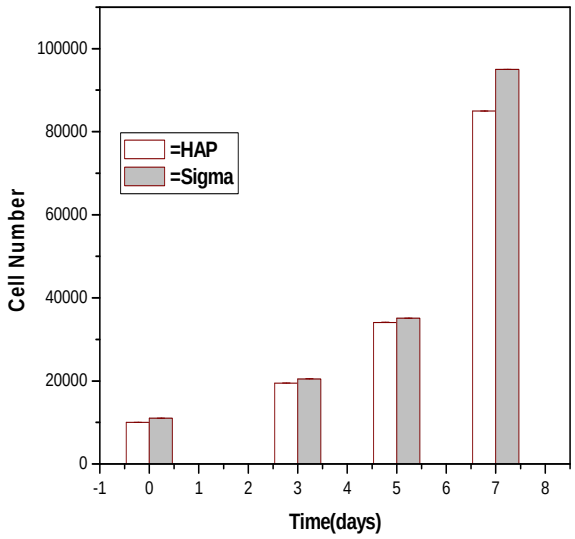

Figure 7. Cell proliferation .Number of MG-63 cells on HAP, HAP from sigma (control) from day 0, 3, 5 and 7 Experiments were carried out more than three times and results are mean ± SD.

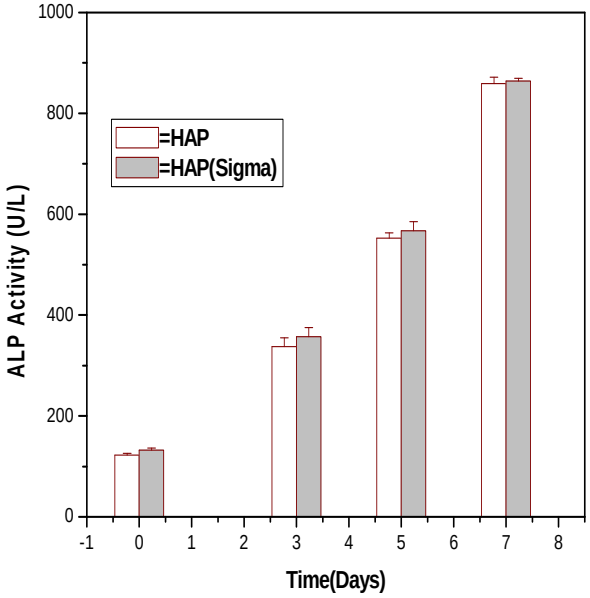

Figure 8. Alkaline phosphatase (ALP) expression. MG-63 cells express ALP on HAP, HAP from sigma (control) from day 0, 3, 5 and7. Experiments were carried out more than three times and results are mean ± SD.

ALP as a biochemical marker for osteogenesis was measured for MG- 63 cells. In my research, I evaluated the differentiation of mg-63 seeded on HAP; a significant increase in alkaline phosphatase activity (ALP) was seen (Fig 8).

5.5 Conclusions

The MG-63 cell line was used as an initial assessment to evaluate whether cells would survive, attach and proliferate on the HAP. Owing to the fact that the cell line is an osteosarcoma cell line, it cannot be implanted *in vivo* and was thus evaluated as a preliminary, qualitative assessment of cells cultured in the HAP *in vitro* environment. Most of the applications for using MG-63 cells in studies assess the cells as a preliminary investigation to see whether they attach to certain biomaterials, remain viable on the biomaterials or express morphological or biochemical characteristics similar to those of osteoblasts (21, 24, 17, and 23). We observed that

1) Artificially synthesized HAP does not exert a cytotoxic effect on MG-63 cells.

2) MG-63 cells attach and proliferate on artificially synthesized HAP.

3) MG-63 cells express alkaline phosphatase activity on artificially synthesized HAP.

More studies are necessary in order to assess HAP as a vehicle for delivery into bone defects. Our results evaluating the attachment, proliferation and differentiation of MG-63 on HAP. Granulat show great promise for their use. The analysis of further osteoblastic phenotypical markers such as calcium deposition is impossible due to the beta tri-calcium phosphate nature of the HAP, but measurement of osteocalcin, osteonectin or osteopontin mRNA would be useful.

Another interesting study would be to conduct studies of convex surfaces of pores when compared to concave surfaces and to investigate which surfaces are preferred by the MG-63. Observations made during LIVE/DEAD staining showed more cells colonizing the insides of concave pores. More studies need to be done in order to show whether the cells have a preference and such characteristics can be used to design better biomaterials and better hybrid therapies for bone healing. Thus, in-depth studies of surface undulations and its effect on cell attachment, proliferation and differentiation are needed. Further SEM studies could be carried out to evaluate how far into the centre of the bone void filler, the cells attach.

5.6 References

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CHAPTER 6

Application of phytase in cell culture studies and myo-inositol production.

6.1 Summary

This chapter describes the application of phytase in cell culture studies and myo-inositol production. Effect of phytase on cell line and its stability in different cell culture media was checked. Partially purified phytase was stable up to 89 to 92 % till 72 hour in different cancer cell line culture media i.e. DMEM, MEM and RPMI 1640, showed 19.23 % proliferation of cancerous HL-60 cell line with 0.8 IU/3ml of phytase in MEM media. Inorganic phosphorus (98.45µg/ml) also released in culture media from cells after 24 hour of incubation at 37^0C.

Myo-inositol is well-known to play a major role in many cell signalling pathways of Ca^{+2} uptake. HPLC analysis of complete phytate degradation by phytase produced by *A. niger* NCIM 563 showed myo-inositol as the main end product. Produced myo-inositol and intermediates are very cheap in comparison to those synthesized by chemical methods since we are getting them from very cheap raw agricultural products like wheat bran.

6.2 Introduction

Phytic acid, myo-inositol hexa kis phosphate, is a major storage form of phosphorus in cereals and legumes, representing 18-88 % of total phosphorus content (1). Phytases (EC 3.1.3.8 and EC 3.1.3.26) catalyses the hydrolytic degradation of phytic acid and its salts (phytates), yielding inositol, inositol monophosphate and inorganic phosphate (2). Phytic acid is also an anti-nutrient, which complexes with protein and nutritionally important metals such as calcium, zinc, magnesium and iron, decreasing their bioavailability (3, 4). To overcome this problem the feed has to be supplemented with inorganic phosphate to fulfill the nutritional requirements (5).

Many bacteria, yeast and fungi are reported to produce phytase (6, 7). Among them fungal phytases are widely used in animal feed due to their acid tolerance and higher yield (8). In poultry the gut pH is acidic (pH2.5 to 6.0) so phytase active and stable in acidic environment is highly preferred (9). Among fungi many *Aspergilli* (6, 7, 10-12) are known to be active phytase producers. As *Aspergillus niger* is Generally Recognised as Safe (GRAS) it is frequently used in food and feed applications.

The properties of *myo*-inositol phosphates strongly depend on the number and distribution of the phosphate residues on the *myo*-inositol ring. *myo*-Inositol phosphates containing the 1,2,3-trisphosphate cluster, for example, are iron binding anti-oxidants (13), and D-*myo*-inositol (1,2,3,6) tetra*kis*phosphate [D-Ins(1,2,3,6)P_4] is moderately effective in opening Ca channels (14). In addition, D-Ins(1,2,6)P_3 has anti-inflammatory and anti-secretory properties (15). Until now, the diversity and unavailability of individual *myo*-inositol phosphate intermediates precluded investigation of their bioactivity. Phytate-degrading enzymes, especially the histidine acid phytate-degrading enzymes (16), are good candidates for the production of lower *myo*-inositol phosphates from phytate, because they release only one major *myo*-inositol penta*kis*-, -tetra*kis*-, tris-, -bis-, and -monophosphate (17). Only the alkaline phytate-degrading enzymes from *Bacillus* species have been demonstrated to degrade phytate using a dual pathway (18). To exploit the full potential of naturally occurring phytate-degrading enzymes, identification of the *myo*-inositol phosphates generated during enzymatic phytate degradation is of great importance.

Myo-inositol is also known as inositol, hexahydroxycyclohexane, cyclohexanehexol, mouse antialopecia factor and, chemically, as cis-1,2,3,5-trans-4,6-cyclohexanehexol. *Myo*-inositol is abbreviated as Ins and sometimes as just I. It is represented by the following chemical structure (Fig. 1).

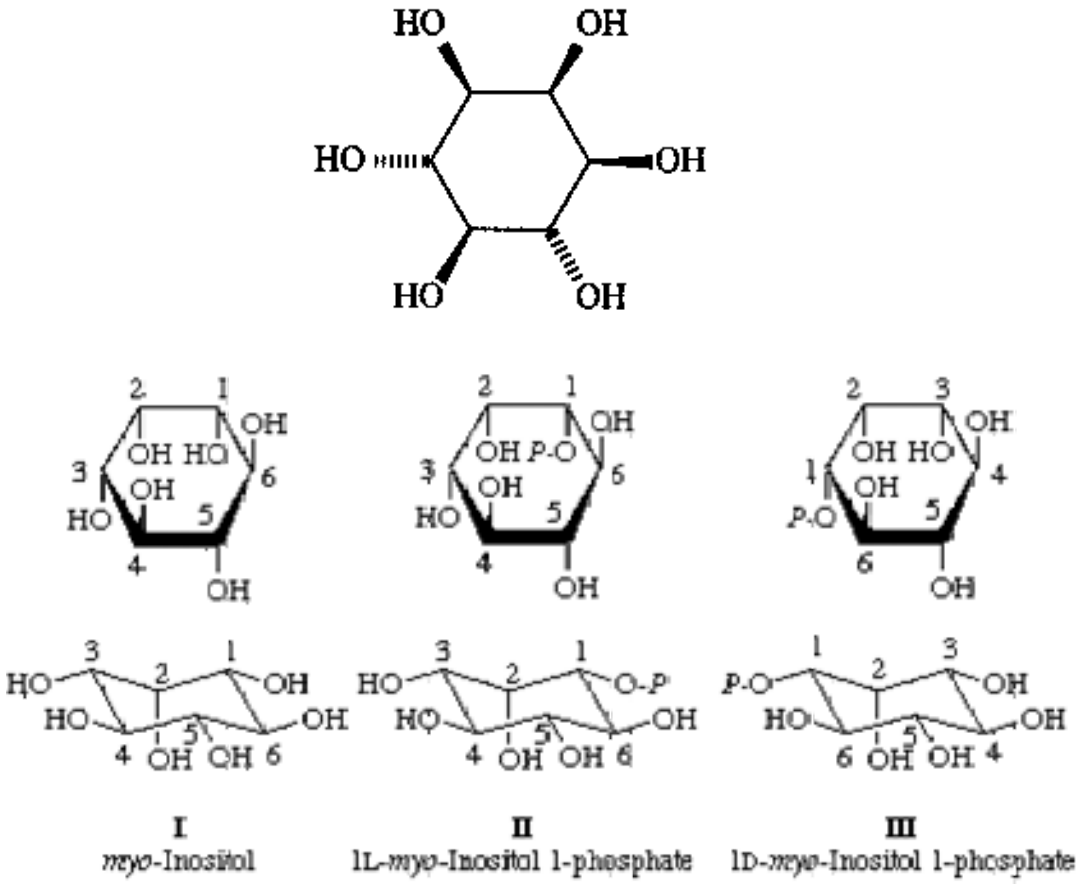

Figure 1. *myo*-Inositol

Myo-inositol phosphates are being extensively studied for their pharmacological and nutraceutical properties including their ability to reduce risk of colon cancer, affecting intracellular release of calcium in neuro degenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and dementia and also for their positive effects on the heart.

Synthesis of myo-inositol phosphates by chemical method is time consuming and expensive, requiring vigorous reaction conditions and leading to

optically impure product. Attempts to produce defined isomers of the different lower myo-inositol phosphates nonenzymatically have resulted in mixtures of myo-inositol pentakis-, tetrakis-, tris-, and bisphosphate isomers. Purification of these isomers from the mixtures is arduous and uneconomical.

Different metal ion chelators have major application in prevention of neurodegenerative diseases. Phytate consists of a myo-inositol ring and six symmetrically distributed phosphate moieties. It is remarkably stable and forms tight complexes with a variety of polyvalent metals such as iron, zinc, calcium and magnesium. This pronounced ability of phytate to form metal chelates lowers the nutritional bioavailability of minerals (19). However, in the last few years epidemiological evidences have strongly supported the hypothesis that phytate and degradation products of phytate, found in bioprocessed foods of plant origin, also have various physiological effects of importance for health (20). Certain myo-inositol phosphates have been proposed to have positive effects on heart disease by controlling hypercholesterolemia and atherosclerosis and also to prevent renal stone formation, diabetes complications and inflammation. The most extensively studied positive aspect of myo-inositol phosphates is their potential for reducing the risk of colon cancer. Much attention has been focused on lower myo-inositol phosphates, in particular the intracellular second messengers D-myo-inositol(l,4,5)trisphosphate and D-myo-inositol (1,3,4,5) tetrakisphosphate, stimulating intracellular release of calcium affecting cellular metabolism and secretion. The physiological role of different myo-inositol phosphates is presently undergoing extensive research.

Nicoletti (21) have demonstrated an increase of calcium ion influx in cerebellar neurons. They provide evidence for a direct activating effect of phytic acid and its salts on the functional activity of the central nervous system. There are not so many reports of effect of phytase enzyme on different cell lines. Different kind of phospholipids which are present in cell membrane (23), may affect phytase (phosphohydrolase) enzyme of these membrane phospholipids. The present chapter describes the application of purified phytase II in different cell line culture medium and its effect on growth of cells, which according to us, is the first of this kind of work.

6.3 Materials and Methods

Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. Different cell culture media e.g. Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), Roswell Park Memorial Institute (RPMI) 1640 were purchased from Bioresource (GIBCO). All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo.

Fungal strain

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).

Cell line and maintenance

HL60 human leukemia cell line procured from NCCS, Pune, India. The cells were cultured in MEM media supplemented with L-glutamine, sodium bicarbonate, glucose, sodium pyruvate and 10% fetal bovine serum. The cells were grown at 37^0C under 5%CO_2 and 95 % humidity. $1X10^6$cells/ml maintained in 25 cm^2 tissue culture flask and the media was replaced three times a week.

Table1. Commonly used cell lines and time for their maximum growth

Cell Lines	Specificity	Time for maximum growth
A 431	Skin Cancer	5 days
MCF-7	Breast cancer	8 days
HL-60	**Blood cancer**	**24 hours**
THP-1	Monocyte	72 hours

As HL-60 grows in 24 hours so this cell line is used in MEM media for further experiments.

Medium and Culture conditions

Production of enzyme was done by submerged fermentation. Medium and culture conditions were used according to the methods described in Chapter 2.

Enzyme assay

Phytase activity was measured at 50°C and using acetate buffer (100 mM, pH 5.5) as described earlier (24). The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (25). A freshly prepared solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1µmol phosphorus per minute 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.

Purification of Phytase II produced by submerged fermentation

Phytase II was purified according the process described in Chapter 3.

Stability of phytase in different cell culture medium

Suitably diluted purified phytase II obtained after different chromatography steps of concentrated broth was incubated in different animal cell culture medium (DMEM, MEM, RPMI 1640) for its stability at 37^0C for 72 hour. After every 24 hour, small aliquots (100µl) were removed, diluted accordingly and checked for residual phytase activity.

Effect of phytase on HL-60 cell line

Different concentrations of suitably diluted and filter sterilized partially purified phytase incubated with HL60 cell line in MEM media. After 24 hour the released free inorganic phosphorus was measured by Heinohen and Lathi (25) and number of cells were counted by haemocytometer Fisher scientific No 026715 (26).

Production of myo-inositol by enzymatic digestion of phytase II

Production of myo-inositol and different myo-inositol phospho esters can be understood by the Figure 2.

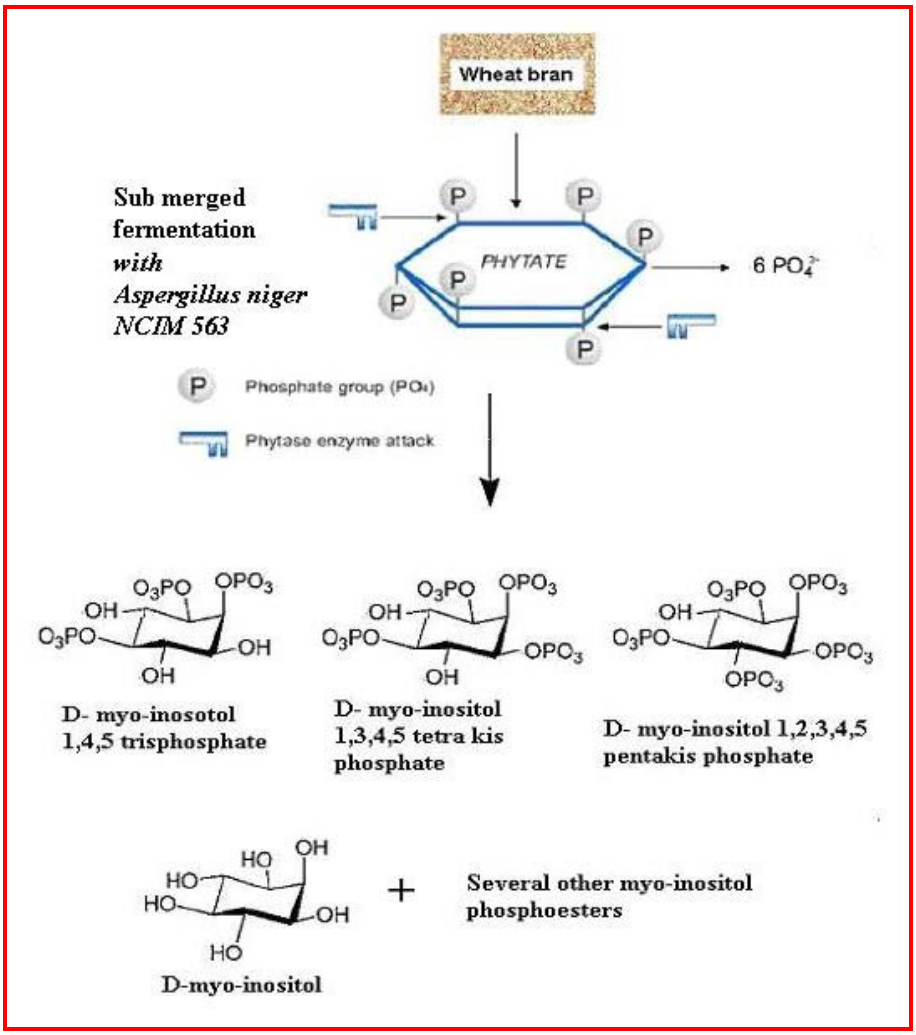

Figure 2. Schematic diagram for production of myo-inositol and myo-inositol phosphoesters

Purification of myo-inositol and other reaction products

Reactions of phytases from *Aspergillus niger* NCIM 563 and phytic acid (commercial and extracted from wheat bran) were performed in different buffer solutions and reaction product was then analysed and purified by HPLC system

(Dionex India Limited, Mumbai, India) equipped with UV- or RI detectors. An ion exclusion column (Aminex HPX-87H; Bio-Rad, Hercules, CA, USA) used at a temperature of 38 ^{0}C with 8 mmol H_2SO_4 as a mobile phase at a flow rate of 6 mL per min. The production of myo-inositol is confirmed by chromatogram of reaction end product devoid of proteins (Figure 3).

6.4 Results and discussions

Characterization of phytase II

Purification and characterization of phytase II has been described in detail in Chapter 3. Storage of suitably diluted phytase II in different cell culture medium revealed that it retained 97.55% and 92.45% of initial activity after 24 and 72 hours of incubation in MEM media (Table 2). As phytase II showed maximum stability in MEM media, which is having least free PO_4^{-3} among all other medium used (100 µg/ml) so MEM can be used efficiently in further cell line experiment (Table 3).

Table 2. Stability of Phytase I and Phytase II with different cell culture media.

Cell culture medium	% Stability of Enzyme					
	Phytase I Assay at (pH 2.5)			Phytase II Assay at (PH 5.0)		
	24 hr	48hr	72hr	24hr	48hr	72hr
DMEM	93.45%	87.77%	88.34%	94.56%	91.44%	89.78%
MEM	**96.09%**	**92.98%**	**90.56%**	**97.55%**	**94.67%**	**92.45%**
RPMI	94.67%	91.04%	87.66%	95.77%	92.88%	89.09%

The suitably diluted purified enzyme was incubated with different media and residual Phytase activity was determined as described in Materials and Methods. MEM Media is having the least free PO_4^{-3} (100µg/ml) and both the enzymes showed the highest stability in MEM media after the incubation of 72 hour (90-92%). So MEM media is used for further cell line experiments.

Effect of phytase on HL-60 cell line

After incubating filter sterilized purified phytase II (0.8 IU) with HL60 cell line for 24 hour in MEM media (3 ml), revealed 19.23% proliferation in HL-60 cell line with release of inorganic phosphorus (98.45 μg/ml) which indicates as a marker of phytase activity on cell surface in media. Mg^{2+} -independent phosphatidate phosphohydrolase dephosphorylated different phospholipids phosphatidate, ceramide 1-phosphate, lysophosphatidate and sphingosine 1-phosphate. The enzyme could thus play an important role in regulating cell activation and signal transduction (27). We used cancerous cell line instead of normal cell line due to its easier maintenance in laboratory and more regeneration frequency. Cell membrane permeability (28) increased due to action of phytase, which enhanced the nutrient uptake by the cell and resulted in cell proliferation. These results suggest the controlled use of phytase enzyme to enhance the cell membrane permeability that can be used in cell transformation (29, 30) and controlled drug release (31, 32). Increasing the phytase conc. from 0.8 IU to 6.0 IU/3ml of medium resulted in 73.07% decline in cell growth and released inorganic phosphate-increased to 142.25 μg/ml (Table 3), which strongly suggests that phytase reacted on the surface phosphate chains associated with membrane lipid or proteins that leads to disintegration of plasma membrane and resulted in declined cell growth. Curtis and Hill (33) used trypsin treatment of intact cells or isolated plasmalemmae from embryonic chick neural retinae leads to an accumulation of lysophospholipids in the plasmalemmae. Juckett and Rosenberg (34) incubated nucleases on mouse tumor cells to probe for the existence of surface nucleic acids. Nanjundan M. and Possmayer F. (35) examined the localization of lipid phosphate phosphohydrolase in lipid-rich signalling platforms from rat lung, isolated rat type II cells and mouse lung epithelial cell lines (MLE12 and MLE15cells). Sphingolipids are major components of eukaryotic plasma membrane which play major role in regulating signal transduction pathways, directing protein sorting and mediating cell-to-cell interactions. Recent studies have shown that sphingolipid-metabolizing enzymes function not only in

intracellular organelles but also in the extracellular spaces including the outer leaflets of the plasma membrane (36)

There may be some role of enzyme in cell growth with the relation of released phosphorus in culture media. When no. of cells per ml are maximum than released phosphorus is also maximum when 0.8 IU/ml of purified Phytase II filter sterilized enzyme was used.

Table 3. Effect of Phytase II on HL-60 (Human leukemia) Cell line after 24 hrs of incubation.

Enzyme concentration in media (IU/3ml)	Cell growth (Cells/ml)	Released PO_4^{--3} in media (μg/ml)
0.0	$5.2X10^4$	12.34
0.2	$5.2X10^4$	54.25
0.4	$5.8X10^4$	86.9
0.8	$6.2X10^4$	98.45
1.60	$4.84\ X10^4$	112.55
2.0	$2.2\ X10^4$	128.15
4.0	$1.8\ X10^4$	139.85
6.0	$1.4\ X10^4$	142.25

The suitably diluted and filter sterilized partially purified enzyme was incubated with HL 60 cell line. The released inorganic phosphorus and cell number was determined as described in Materials and Methods

Production of myo-inositol by enzymatic digestion of phytic acid

The phytate-degrading enzyme of *Aspergillus niger* NCIM 563 is a extracellular enzyme belonging to the group of histidine acid phosphatases (37). The final product of enzymatic phytate degradation was identified by High Performance Liquid Chromatography (HPLC) coupled with UV/IR detector as Inositol. The full sequence of phytate hydrolysis by the phytate-degrading enzyme from *A.niger* NCIM 563 was not elucidated but only the final concentration of myo-inositol was calculated. From 100 mg of Phytic acid after the reaction of phytase 8.6 mg of myo-inositol was recovered.

Phytic acid has six phosphate groups that may be released by phytases at different rates and in different order. Wyss et al. (38) investigated the kinetics of phosphate release and the kinetics of accumulation of reaction intermediates, as well as the end products of phytic acid 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 stereospecificity 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 phytic acid 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 pronouced accumulation of *myo*-inositol tetrakisphosphate during phytic acid 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 was detected. Therefore, lower *myo*-inositol phosphates appears to be less suitable substrates for *A. niger*, *A. terrus* and

especially *E. coli* phytases than phytic acid. The stereoisomer assignment of the reaction intermediates and degradation pathway was not determined for these enzymes. The fact that the end products of phytic acid hydrolysis for most phytases is identical does not necessarely mean that the degradation pathways for phytic acid are identical. The strong stereospecificity 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 equatorically from the inositol ring can access the catalytic sites of these enzymes.

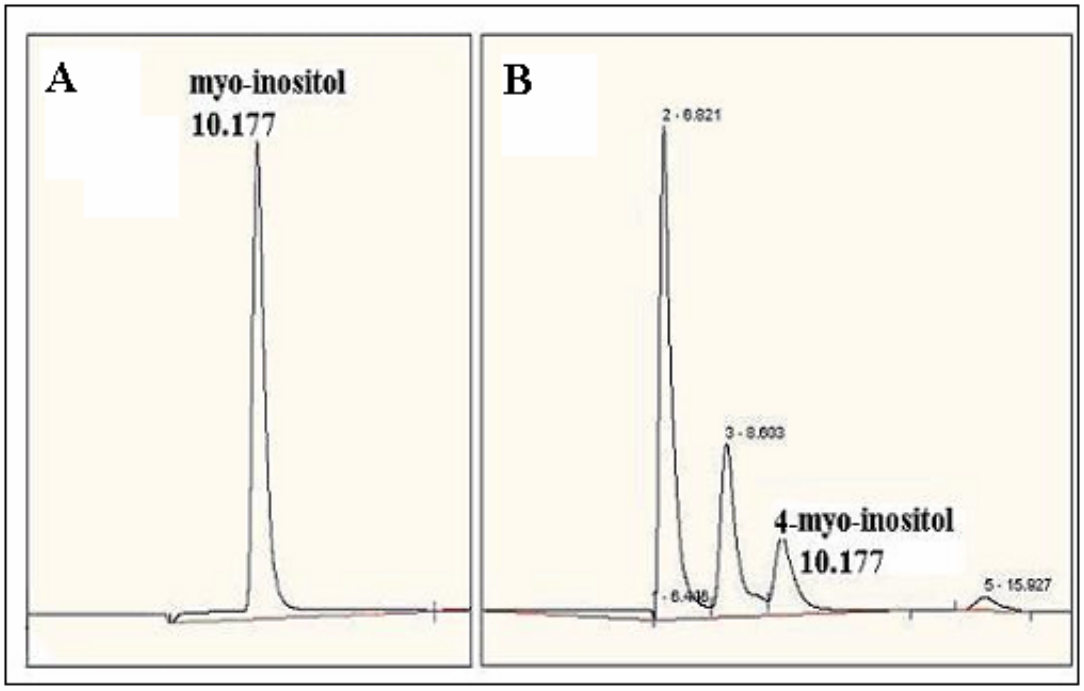

Figure 3. HPLC profiles of (A) myo-inositol standard, (B) Reaction mixture

The chemical syntheses of *myo*-inositol phosphates include difficult protection and deprotection steps, and are performed at extreme temperatures and pressures (39). Since phytases hydrolyze *myo*-inositol hexaphosphate sequentially, the production of *myo*-inositol phosphate derivates and free *myo-inositol* using phytase is a potential alternative to chemical synthesis. The preparation of D*myo*- inositol 1,2,6-trisphosphate, D-*myo*-inositol 1,2,5-trisphosphate, L-*myo*-inositol 1,3,4-

trisphosphate and *myo*-inositol 1,2,3-trisphosphate by enzymatic hydrolysis of phytic acid by *S. cerevisiae* phytase has been described (40). Immobilized phytases have been used to produce various *myo*-inositol phosphates (17, 41). Naturally, the advantages of enzymatic hydrolysis are stereospecificity and mild reaction conditions. In addition to usage as drugs, *myo*-inositol phosphate derivatives can be used as enzyme substrates for biochemical and metabolic investigations and as chiral building blocks (42).

6.5 Conclusions

Phytase from *Aspergillus niger* NCIM 563 indicates its application in different cell culture experiments to focus some light on phospho inositol lipids and role of phytase in cell membrane permeability.

The role of phytase enzyme in different cell culture experiments and controlled use of phytase enzyme to enhance the cell membrane permeability that can be used in cell transformation and controlled drug release. In future it can throw some light on phospholipid research associated with animal cell membrane.

Enzymatic production of important myo-inositol phosphate intermediates molecules from cheap agricultural residues, those are having several important roles in cell signaling pathways.

- Offers stereo specificity and cost-effective method of synthesis in mild reaction conditions.
- This bionic approach could be envisioned as an energy-conserving and economically viable green approach towards the large-scale synthesis of myo-inositol and myo-inositol phosphates; using potential cheap agro-industrial waste materials.

6.6 References

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Conclusions

1. *Aspergillus niger* (NCIM 563), produces high amount of two types of extracellular thermostable phytases i.e. Phytase I and Phytase II, under submerged fermentation conditions. Phytase I is highly acidic and show very high activity at pH 2.5. Phytase II exhibited broad pH stability and optimum activity at pH 5.0.

2. The crude phytase preparation has been purified to homogeneity by a simple chromatographic step; using Phenyl Sepharose CL-4B and sephacryl S-200, with final yield of 30.24 % and 26.55 % for Phytase I and Phytase II respectively. Both the enzymes differ with each other in molecular and biochemical properties including pH optima, temperature and pH stability, effect of various metal ions and other reagents, MALDI-TOF analysis, affinity towards various substrates and N-terminal amino acid analysis.

3. The purified Phytase I is a Glycosylated and II is a non-glycosylated protein. The native molecular mass of Phytase I is four times higher than its mass under denaturing conditions, confirms tetrameric nature of native protein. Phytase II is a monomer.

4. The partial N-terminal sequence of Phytase I from NCIM -563 exhibited homology with the earlier reported Phytase B (3-Phytase) from *Aspergillus ficuum* NRRL 3135. Phytase II shows similarity with the earlier reported Phosphate-repressible acid phosphatase precursor (Acid phosphatase PII) of *Aspergillus niger CBS 513.88*. Because of the homology with reported enzymes, we can say that both the enzymes are Histidine acid phosphatase.

5. Phytase I is having broad substrate specificity and it releases phosphorus from diverse group of substrate, in comparison to it, Phytase II exhibits

specific hydrolysis of substrates and shows maximum specificity toward the phytate.

6. Apart from its conventional use in animal feed, phytase preparation from *Aspergillus niger* NCIM 563 can be used in synthesis of Hydroxyapatite (an artificial bone material). As phytase II has its optimum pH 5.0 and synthesis of Hydroxyapatite is favourable under less acidic or basic environment, so Phytase II is a good candidate for synthesis of HAP, in comparison to Phytase I that is having optimum pH 2.5.

7. Enzymatically synthesized HAP does not exert a cytotoxic effect on Osteosarcoma MG-63 cells. MG-63 cells attach and proliferate on artificially synthesized HAP and express alkaline phosphatase activity on artificially synthesized HAP. Commercially available HAP and HAP synthesized in present study shows almost same biocompatibility.

8. The overall result of enzymatic synthesis therefore suggests that the biomimetic synthesized HAP nanospheres can be a good choice for bone grafting and protein purification applications. This bionic approach could be envisioned as an energy-conserving and economically viable green approach towards the large-scale synthesis of crystalline artificial bone, using potential cheap agro-industrial waste materials like wheat bran.

9. Phytase from *Aspergillus niger* NCIM 563 indicates its application in different cell culture experiments to focus some light on phospho inositol lipids and role of phytase in cell membrane permeability.

10. The role of phytase enzyme in different cell culture experiments and controlled use of phytase enzyme to enhance the cell membrane permeability that can be used in cell transformation and controlled drug release. In future it can throw some light on phospholipid research associated with animal cell membrane.

11. Enzymatic production of important myo-inositol phosphate intermediates molecules and finally myo-inositol from cheap agricultural residues, those

are having several important roles in cell signaling pathways, offers stereo specificity and cost-effective method of synthesis in mild reaction conditions.

12. Finally, since *Aspergillus niger* is generally recognized as a safe (GRAS) for food applications. Moreover the present enzyme preparation is thermostable and highly active at acidic pH, as per the requirement for poultry feed. It has potential applications as additive for animal feed, phytic acid hydrolysis, in other related industries and production of myo-inositol phospho intermediates of therapeutic importance.

Publications

Publications

1. **Sarvesh Kumar Soni**, J. M. Khire* Production and partial characterization of two types of phytase from *Aspergillus niger* NCIM 563 under submerged fermentation conditions. World J Microbiol Biotechnol (2007) 23:1585–1593.

2. Kavita Bhavasar , Parin Shah **, Sarvesh Kumar Soni**, J. M. Khire.*Influence of pretreatment of agricultural residues on phytase production by *Aspergillu niger* NCIM 563. African Journal of Biotechnology. (2008) 7: 1101-1108.

3. Strain improvement and upscaling of phytase production by Aspergillus nigerNCIM 563 under submerged fermentation conditions. Shah P., Bhavsar K., **Soni S.K**. & Khire J.M*. Journal of Industrial Microbiology and Biotechnology 2009 36: 373-380

4. **S. K. Soni**, A. Magdum and J. M. Khire* Purification and characterization of two distinct phytases from *Aspergillus niger* NCIM 563. Communicated

Patents

1. **Sarvesh kumar soni,** J.M. Khire. A process for preparation of novel acidic phytase (pH 2.5) by *Aspergillus niger* NCIM 563 under submerged fermentation condition. (Applied for US patent) Indian patent CSIR no. NF-180/2005.

2. **Sarvesh kumar Soni**, Dilip Depen, J.M.Khire, R.P.Singh. Novel biocatalytic synthesis of nanosized hydroxyapatite, myo-inositol and several myo-inositol phosphates simultaneously from agricultural residues. CSIR no. NF-0049/2008. Applied for international patent.

Abstract

1. 31st FEBS congress Abstract Title 'Highly acidic phytase from *Aspergillus Niger* NCIM563 under submerged fermentation.'
   http://www.blackwell-synergy.com/toc/ejb/273/s1, Poster presentations (pdf), PP832 (Page no. 225 of 291).