"Studies on Acidic Phytase from *Aspergillus niger* mutants"

A thesis submitted to The University of Pune for the Degree of

DOCTOR OF PHILOSOPHY

(In Biotechnology)

By

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Research Guide

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In loving memory of Late Mrs. Kusum Jagtap (Aai)

With all your love in my heart

I had promised to never part

Blinded by the mystery storm

All I know, I was wrong

For it was the one that took you along

Leaving me thrashed to pile upon

The blessed hands that cuddled me

Were cursed by the darkened 'three'

Far apart will they now be

Never to be back for me

-ρπαδηϒα

TABLE OF CONTENTS

CERTIFICATE

This is to certify that the work entitled **"Studies on acidic phytase from *Aspergillus niger* mutants"** is the result of investigations carried out by **Ms. Pradnya D. Gujar** at the NCIM Resource Centre, National Chemical Laboratory, Pune, India under my supervision and the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

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DECLARATION

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

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

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"We can only be said to be alive in those moments when our hearts are conscious of our treasures."

-Thornton Wilder

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ABBREVIATIONS

°C	Degree Centigrade
Å	Angstrom
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ANOVA	Analysis Of Variance
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
ATPE	Aqueous Twp Phase Extraction
BBD	Box Behnken Design
BPP	β-Propeller Phytase
CCD	Central Composite Design
CP	Cysteine Phosphatase
DCP	Dicalcium Phosphate
DDA	Data Dependent Acquisition
DMB	Dry Mouldy Bran
DTT	Dithiothretiol
EDTA	Ethylene Diamine Tetra acetic Acid
g, mg, µg, ng	Gram, Milligram, Microgram, Nanogram
GRAS	Generally Regarded As Safe
HAP	Histidine Acid Phosphatase
HCl	Hydrochloric Acid
IP1, IP2, IP3,IP4,IP5	Inositol Mono-, Bis-, Tris-, Tetrakis-, Pentakis-Phosphate
IP6	Phytic Acid
IU	International Unit
kDa	Kilodalton

L, mL, µL	Liter, Milliliter, Microliter
M, mM, µM	Molar, Millimolar, Micromolar
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time Of Flight
MCP	Mono Calcium Phosphate
MW	Molecular Weight
NCIM	National Collection Of Industrial Microorganisms
OVAT	One Variable Approach
P	Phosphorus
PAGE	Polyacrylamide Gel Electrophoresis
PAP	Purple Acid Phosphatases
PBD	Placket Burman Design
PDA	Potato Dextrose Agar
pI	Isoelectric Point
PNPP	p-nitrophenyl phosphate
RB	Rice Bran
RSM	Response Surface Methodology
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SmF	Submerged Fermentation
SSF	Solid State Fermentation
SEM	Scanning Electron Microscopy
WB	Wheat Bran

ABSTRACT

Phosphorus is an essential component of life. It forms the major backbone of nucleic acids (DNA and RNA), it also has significant role in signaling and energy metabolism (IP_2, IP_3, ATP, ADP etc.). The major source of phosphorus is rocks and volcanic sediments moreover its other sources are limited. Based on the current usage of phosphate supplements, it is estimated that the world reserves of phosphate will be depleted by the year 2050; also the excessive use of phosphate supplements has lead to the emergence of serious concern for phosphorus pollution in soil and water. Phytic acid is the principle storage form of phosphorus in cereals, legumes, oil seeds, and nuts, accounting for 60-90% of total phosphorus content in plants. In forage, one-third of phosphorus is present as digestible in-organic form 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. Chemically, phytic acid is a very stable molecule. It exists as a highly negatively charged molecule over a wide pH range due to its high phosphate content. In physiological conditions, essential minerals such as calcium, magnesium, iron and zinc etc. are chelated by phytic acid. It binds to different proteins and amino acids and also inhibits digestive enzymes. Due to its antioxidant nature it inhibits iron-driven hydroxyl radical formation. Many plants and monogastric animals cannot utilize phytate-phosphorus thus the need for supplementation arises. However, surplus supplementation and phytate phosphate excretion lead to pollution and possess a global environmental threat. Thus due to this anti-nutritive property of phytate enzymatic hydrolysis of phytic acid is a more eco-friendly approach.

Phytases (EC 3.1.3.8) belong to the family of histidine acid phosphatases and catalyze the hydrolysis of phytic acid to free inorganic phosphate (iP) and lower myo-inositol phosphate esters (Ins P5 to P1) or myo-inositol in some cases. Phytases are widespread in nature and are found in microbes, plants and some animal tissues. Several phytases have been cloned and characterized from various sources. Phytase can be used to hydrolyze phytate and thus proves to be an important industrial enzyme and an object of extensive research. In addition, phytase would be an environmentally friendly product, reducing the amount of phosphorus entering the environment. Though several phytases have been isolated, characterized and cloned the "phytase fairy-tale" is yet to be accomplished. Further research into discovering new approaches for commercialization of phytase and engineering better phytases based on various properties of the enzyme is desirable. This thesis mainly focuses on isolation of phytase hypersecretory mutants of *Aspergillus niger*, with respect to production under submerged fermentation conditions subsequently dealing with genetic, morphological and physiological profiling of the mutants followed by studies on application of phytase in plant growth promotion, soil amendment and enhancing mineral availability.

Chapter 1: Introduction

This chapter includes a brief overview of literature covering basic definitions of phytate and phytase enzyme, classification and sources and diversity of phytases, production and purification of phytase under submerged fermentation conditions, applications and recent advances in

phytase research. We conclude the first chapter with the objectives and scope of the thesis.

Chapter 2: Strain Improvement

Strain improvement by mutagenesis and selection is a highly developed technique and it plays a central role in the commercial development of microbial fermentation processes. This chapter includes studies on strain improvement of *Aspergillus niger* NCIM 563 for enhanced phytase production under submerged fermentation conditions. The use of classical approach for strain improvement of *A. niger* NCIM 563 included a combination of physical (UV) and chemical mutagenesis (EtBr and Hydroxyl amine) resulted in obtaining two mutants P16 (*A. niger* NCIM 1359) and P33 (*A. niger* NCIM 1360) which were found to be superior to parent strain as they produce 156 and 95 U/ml phytase activities on 10.5^{th} day and 7^{th} day, respectively as compared to 68 U/ml of phytase activity by parent on 11th day. Mutants *A. niger* NCIM 1359 and *A. niger* NCIM 1360 showed increase in activity up to 159.5 % and 312.78 %, with increase in productivity up to 2.4 and 2.2 times that of parent respectively.

Chapter 3: Production Of Phytase Under Submerged Fermentation Using Plant Based Media: A Comparative Account

Production of phytase under submerged fermentation conditions was studied using different media. Studies pertaining to production of phytase in synthetic medium containing dextrin and glucose as carbon sources along with sodium nitrate as nitrogen sources at 30 °C demonstrated

activities on 13th day to be 41.47, 51.92 and 50.57 IU/mL for *A. niger* NCIM 563, *A. niger* NCIM 1359 and *A. niger* NCIM 1360 respectively. RSM media optimized for *Aspergillus niger* NCIM 563 was used to study phytase production in mutants with the assumption that optimized media might benefit in enhancing phytase activity of strains thus maximize phytase production from under submerged fermentation (SmF). Optimization of culture conditions for parent strain using Plackett Burman and Central composite design technique had resulted in 3.74 fold increase in the yield of phytase production to 254.5 IU/mL when compared with the activity observed with basal media (68 IU/mL) in shake flask. The mutants *A. niger* NCIM 1359 and 1360 exhibited 407.2 IU/mL and 389.7 IU/mL phytase activity on 13th and 10th day, respectively using RSM optimized media suggest its potential for industrial application. Production of phytase was also studied on soybean flour medium wherein *A. niger* NCIM 563, *A. niger* NCIM 1359 and *A. niger* NCIM 1360 showed activities of 122.55 IU/mL, 294.12 IU/mL and 201.23 IU/mL on 13th and 10th day respectively. Exploitation of this observation could have tremendous value addition from the phytate feed-conversion and environmental point-of-view. Furthermore, the results of the present study provide an excellent basal medium formulation for studying phytase production with the mutant strain and assessing its properties.

Chapter 4: Morphological and Physiological Characterization Of Mutants: A Comparative Account

Characterization of mutants in comparison with the wild-type strain by different methods is vital for the assessment of morphological and

physiological changes with respect to applicability at industrial level. Variability studies showed remarkable difference between sporangium and mycelial morphology, sporulation time and extracellular enzyme profile amongst parent and mutant strains. The scanning electron micrographs showed variation in the morphology of the mutant strains compared to that of the parental strain. NCIM 1359 displayed high conidiospore density in the fruiting bodies compared to the other two strains. NCIM 1360 showed marked difference in the conidiophore morphology and decreased spore density when compared to the parental strain. The dimensions of the conidiospores and conidiophores displayed significant differences among the three strains. NCIM 1360 had the smallest conidiospore diameter (28.71 µm) and conidiophore width (4.94 µm) when compared to the other strains. NCIM 563 showed largest conidiospore diameter (118.52 µm), whereas the mutant NCIM 1359 displayed the widest conidiophore (14.78 µm) among the three strains. In addition, the parent and mutant strains showed significant differences in the activities of five secreted hydrolase enzymes. Xylanase activity was not detected in NCIM 563, whereas NCIM 1359 and NCIM 1360 showed 0.19 IU/ml and 0.34 IU/ml of xylanase activity correspondingly. The mutant strains NCIM 1359 and NCIM 1360 displayed 1.8- and 1.1-fold lower CMCase activity when weighed against NCIM 563. The activity of β-glucosidase was lowered by 1.3- and 37.8 folds in NCIM 1359 and NCIM 1360, respectively compared to that of the parental strain. Relative to NCIM 563, both amylase and acid phosphatase activities decreased by nearly 3-fold in NCIM 1359 and by 3.5-and 2.4-fold in case of NCIM 1360. Another remarkable difference among these strains was delay in the sporulation time of the mutant strains as compared to parent. Parent strain showed sporulation on 4^{th} day whereas that of mutant strains was

obtained on 7th day. MALDI-TOF/MS-MS analysis was performed as a part of partial characterization of phytases from parent and mutant strains.

Chapter 5: Genetic Profiling Of Mutant Strains: A Comparative Account

Genetic variation among *Aspergillus niger* wild type strain NCIM 563 and two phytase hyper secreting mutant strains were assessed using inter simple sequence repeats (ISSR) and sequence-related amplified polymorphism (SRAP) markers. ISSR primers amplified 116 loci out of which 113 were polymorphic while SRAP primers generated 135 amplification products of which 125 were polymorphic. To investigate the genetic basis of differences in phytase activity in the parental and mutant strains, the DNA sequence of the genes encoding phytase from the three strains was characterized. The 3-Phytase B gene sequences from NCIM 563, NCIM 1359 and NCIM 1360 showed 100% identity. However, comparison of these sequences with the *A. niger* CBS 513.88 3-Phytase B gene sequence (gi|145241119) indicated five changes. The changes in the 3-Phytase B genes from NCIM 563, NCIM 1359 and NCIM 1360 in comparison with 3-Phytase B gene from *A. niger* CBS 513.88 (gi|145241119) were in Exon 1 (C to T: position 863), Exon 3 (T to C: position 1208) and in the 3' UTR (ATA insertion: 1886-1888).The transversion in Exon 1 (position 863) resulted in an amino acid change from Alanine to Valine at the position 240 of the 479 amino acid phytase B protein. The other transversion in Exon 3 (position 1208) resulted in a silent mutation. The 3-Phytase B gene sequences from the *A. niger* strains NCIM 563, NCIM 1359 and NCIM 1360 could be used for cloning and over-expression to produce phytase enzyme that can be used as an

additive to upgrade the nutritional quality of phytate-rich seed-based animal feed.

Chapter 6: Application of Phytase In Plant Growth Promotion, Soil Amendment and Functional Foods

The application of phytase from *Aspergillus niger* NCIM 563 in degradation of phytate-phosphorus to benefit mineral release, plant growth and soil amendment is described in this chapter. Additionally, a study on phytase applicability in dephytinization and mineral release from soy based infant foods has been included in this chapter. An enzyme dose of 12 IU was sufficient to hydrolyze phytate and increase assimilation of phosphorus by about 74 %. Phytase supplementation leads to increase in shoot to total length ratio by about 200 %, indicating its growth promoting effect. Consistency in phytase induced growth was reflected at pot and tray levels, wherein shoot to total length ratio was observed to be 2.01 and 2.12 respectively. Mineral assimilation due to phytase was more efficient as compared to chemical fertilizers thus overcoming the constraints of practicability and economics of agriculture industry. Phytase was efficient in reducing the phytic acid content of soil by about 30 % while simultaneously increasing the phytate phosphate availability by 1.18 fold. The role of *A. niger* phytase in improving phytate-phosphorus uptake and its utilization will have significant impact on both agriculture and environment. Besides plant growth promontory effect, reduction in use of chemical fertilizers and soil improvement could be achieved simultaneously for maintaining the sustainability of agriculture. Similarly, degradation of phytic acid and release of mineral ions was studied in soy based infant food and soybean flour. Phytase was

capable of reducing phytic acid content of soybean flour and soy based infant food by about 82.8 % and 91.9 % respectively while enriching bound phosphorus by about 18.7 % and 28 % respectively at the same time enhancing the availability of metal ions such as Zn, Fe, Cu, Mn, Mg, and Ca.

Chapter 7: General Discussion and Conclusions

This chapter contains the summary of the work presented in the thesis. It also discusses the scope and future prospects of the work done in the thesis.

CHAPTER 1 INTRODUCTION

1 General introduction

Phosphorus is an essential component of life. It forms the major backbone of nucleic acids (DNA and RNA) and cell membrane phospholipids, it also has significant role in signaling and energy metabolism (IP_2, IP_3, ATP, ADP etc.) (Vats et al, 2005). Elemental phosphorus was historically first isolated from human urine, and bone ash was an important early phosphate. The major source of phosphorus is rocks and volcanic sediments. But the availability of inorganic phosphorus is limited since it mostly occurs in nature as phytate (major storage form of phosphorus in plants). Interestingly, about 60-90% of phosphorus in nature exists as phytate (Vats *et al.*, 2005). Plants and monogastric animals have limited ability to utilize phosphorus from phytate; hence the need for supplementation arises (Richardson et al, 2000). Based on the current usage of phosphate supplements, it is estimated that the world reserves of phosphate will be depleted by the year 2050 (Runge-Metzger, 1995; Vance *et al.*, 2003); also the excessive use of phosphate supplements has lead to the emergence of serious concern for phosphorus pollution in soil and water (Vats *et al.*, 2005). However, the increasing phosphorus deficiency is a major pin down for agriculture industry worldwide. Moreover, phytic acid being a chelating agent filches the metal ions essential to soil fertility and plant growth (Wodzinski et al, 1996). Phytases (EC 3.1.3.8) belong to the family of histidine acid phosphatases and are found primarily in microorganisms and plants (Piddington et al, 1993), they catalyze the hydrolysis of phytic acid to free inorganic phosphate (iP) and lower myo-inositol phosphate esters (Ins P5 to P1) or

myo-inositol in some cases. This makes phytase a noteworthy candidate for substitution of phosphate supplements. Phytase can also be exploited for its ability to solublize essential minerals in soil. These properties direct the use of phytase in plant growth promotion and soil amendment.

Phytic acid is the principle storage form of phosphorus in cereals, legumes, oil seeds, and nuts, accounting for 60–90% of total phosphorus content in plants (Tang *et al.*, 2006). In forage, one-third of phosphorus is present as digestible in-organic form 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. Being a natural antioxidant, it acts as a potent inhibitor of iron-driven hydroxyl radical formation (Harland and Oberleas, 1999). Since plants and monogastric animals lack the ability to utilize phytate-phosphorus, traditionally supplementation with inorganic phosphorus was considered. But excess phosphate supplementation along with the phytate phosphorus excretion, imposes a global ecological problem contributing to pollution. Phytases (myo-inositol hexakisphosphate 3-phosphohydrolase) are acid phosphatases, which efficiently cleave phosphate moieties from phytic acid (myo-inositol hexakisphosphate) and inorganic phosphate (Mitchell *et al.*, 1997). Thus, phytase seems to be a multi-utility solution for many problems. Phytic acid being an anti-nutrition factor it limits the absorption of essential minerals (Fe, Zn, Mg, Ca etc.) by plants from soil (Raboy, 2001) it is also a major source of phosphate pollution in animal manure (Wodzinski & Ullah, 1996; Vohra & Satyanarayana, 2003). The deficiencies of growth essential minerals in soil in turn specify the need for dephosphorylation of phytate using phytase as soil amendment consequently assisting in plant growth (Singh and Satyanarayana, 2010).

Excess addition of phosphorus from agriculture (Sharpley, 2000; Sims et al, 2000) and industrial sources contributes in pollution of fresh water bodies via erosion and surface runoff causing eutrophication (Boesch et al, 2001). Worst case scenario may lead to algal blooms (may be harmful), hypoxia, occasional catastrophic fish kills due to excess nutrition addition (Burkholder and Glasgow, 1997). One of the futuristic approaches includes replacement of phosphate supplements with phytase, thus minimizing surplus addition and facilitating reuse of phosphate in the environment. Many experiments and field trials have shown that 500 to 1000 units of phytase can replace approx. 1 g inorganic phosphorus supplementation and reduce total phosphorus excretion by 30–50% (Kemme et al, 1997; Liu et al, 1997; Yi et al, 1996). Phytic acid is used in pulp industry as an anti-oxidant to prevent aging of paper. Use of phytase in phytic acid degradation during pulp and paper processing, seems to be an eco-friendly way of waste management (Liu *et al.*, 1998). Various reports on phytase production by bacteria, yeast and fungi are available. (Vohra & Satyanarayana, 2003). Studies on developing new effective phytases with improved properties have also been considered. Importance of soil microbes for increasing availability of phytate-phosphorus to plant roots has been suggested by Tarafdar and Marschner (Richardson et al, 2001; Tarafdar & Marscner, 1995; Singh and Satyanarayana, 2010). Some studies on exploitation of phytase in sustainable agriculture are evident. Solublization of phytate-phosphorus and its effect on plant growth promotion has been studied (Singh and Satyanarayana, 2010). Recombinant expression of phytases in plants (*Tobacco* and *Arabidopsis*) has lead to increased phosphate uptake and enhanced plant growth (Lung et al, 2005; Lung et al, 2008; Yip et al, 2003; Yadav et al, 2003). Idris et al, 2002 has studied the role of culture

filtrates of phytase producing Bacillus on plant growth promotion in maize.

The sharp environmental awareness of phosphorus pollution has created the need for phytase at industrial scale. The annual sale of commercial supplemental phytase is estimated at US$ 50 million, which is one-third of the entire feed enzyme market (Sheppy, 2001) and recently increased to 150 million euro (Greiner and Konietzny, 2006). The commercial feasibility can be attributed to several strain improvement and recombinant cloning experiments. Stain improvement and up scaling of phytase production has been reported for *A. niger* under submerged and solid state fermentation conditions (Shah et al, 2009 and Bhavsar et al, 2010). A high level of functional expression of *A. fumigatus* phytase gene was achieved in *Pichia pastoris* (Rodriguez et al, 2000). With the aim of improving cost-effective phytase production, the gene phy A from *A. niger* var. *awamorii* ALKO243 cloned which resulted in several fold overproduction of phytase (Piddington et al, 1993). The phyA gene encoding heat-stable phytase was cloned from *A. fumigatus* and over expressed in *A. niger* (Pasamontes et al, 1997). Natuphos, Phytase Novo™ (Cao et al, 2007), Finase (Meittinen-Oinonen et al, 1997) etc. are some commercial phytases from different organisms. Further research into discovering new phytases, engineering better phytases based on three dimensional structures, and developing more cost-effective expression systems should be continued. As no single phytase or expression system is likely to be able to meet the diverse needs for this enzyme, a series of phytases specifically designed for different species at different physiological stages and feeding conditions should be developed.

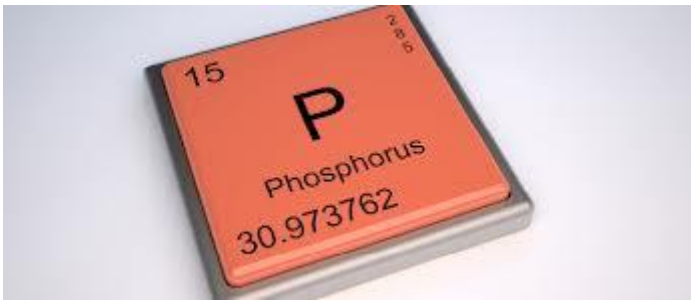
This thesis mainly focuses on isolation of hypersecretory mutants of phytase from *Aspergillus niger*, with respect to production under

submerged fermentation conditions and subsequently dealing with genetic, morphological and physiological profiling of the mutants followed by studies on application of phytase in plant growth promotion, soil amendment and enhancing mineral availability.

1.1 Phosphorus

- Introduction

  Phosphorus (also known as Devil's element) was the 13th element to be discovered. It is a non metallic element which exists in two major forms viz. red phosphorus and white phosphorus. Being highly reactive, it never exists as a free element in nature. Phosphorus is an essential bio-nutrient for all forms of life. It serves as a vital component of the structural scaffold of DNA and RNA. Phosphorus is also a critical component of ATP, ADP, NADP etc (the cellular energy currencies) as it serve as an energy release for organisms to use in building proteins or contacting muscles (Holleman and Wilberg, 1985).



- Cycling

  Phosphorus cycle, unlike other biogeochemical (water, carbon, nitrogen and sulfur) cycles lacks a gaseous phase (Fig 1). Diminutive quantities of phosphorus circulate in the atmosphere because at Earth's natural conditions of temperature and pressures, phosphorus and its other compounds are not in gaseous state, thus

contributing to infrequent acid rains. The principal reservoir of phosphorus is sedimentary rocks, which is also the means for it to enter the environment. Commercially available form of phosphorus is apatite. Guano formed from fossilized bone or bird dropping may also serve as a phosphorus deposit. Phosphorus is released as phosphate ions from rocks due to weathering and erosion, thus distributing it in soil and water. Phosphate is an important constituent of various molecules indispensable for life such as ATP, NADP, DNA and RNA. Plants assimilate the phosphate ions from soil. Herbivores and carnivores then acquire these phosphates through the food chain. In due course, phosphates from animal tissues absorbed by animal and plant tissues returns to soil by means of animal excreta and terminal decomposition; through bacterial action; after death. Similar process takes place within the aquatic ecosystem. Thus making the phosphorus released in the environment available for reprocess.

Phosphorus is sparingly soluble in water. It binds tightly to soil molecules and reaches water as soil run-off. Other means through which phosphate enters water bodies is through runoff from fertilizers, sewage, mineral deposits and industrial wastes. Phosphates tend to sediment at the bottom of lakes and oceans which may reenter the phosphorus cycle, upon stirring. Aquatic organisms get access to these nutrients through erosion. Aquatic plants assimilate phosphate from water, hence serving as a doorway to the aquatic food chain. Though phosphorus is considered as an essential bio-element, its extreme concentrations in water bodies are considered as pollution. Phosphate incites over-growth of plants and planktons.

Phosphate stimulates the growth of plankton and plants, favoring weedy species over others. Their surplus growth leads to blocking of sunlight to organisms residing at the bottom of water body, consumption of huge amounts of dissolved oxygen which leads to suffocation and death of marine organisms. All of these events are collectively referred to as eutrophication.

In contrast to N and C cycles, microorganisms and plants do not dominate the P cycle also it does not possess a prolonged gaseous phase; hence the physical transfer is significantly abridged thus blocking the flow of P from ocean to land subsequently constricting the atmospheric phosphorus reservoirs.

At a much expanded time frame, the phosphorus cycle appears as a one way flow disrupted by momentary absorption of minute quantities of phosphorus by organisms.

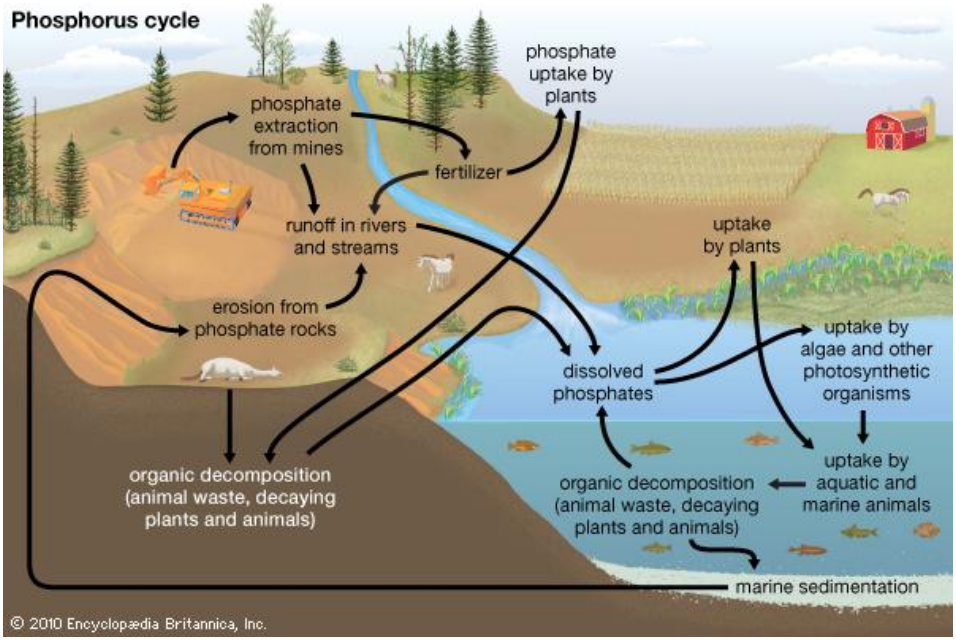


Figure 1: Phosphorus cycle.

Human Inputs to the Phosphorus Cycle:

There are several ways through which humans alter the phosphorus cycle viz. deforestation and use of fertilizers in agriculture (Fig 2). Apatite (deposit of calcium phosphate) is the source of mined phosphate. Hefty amounts of sulphuric acid are used for extraction of rock phosphate churn out 'super phosphate' which is a fertilizer. In efficient utilization of the fertilizers by plants causes surplus amount of fertilizer to runoff contributing to ever-increasing phosphate levels in water bodies. Till the recent past, laundry detergents were the most prominent source of phosphate pollution in water bodies, thus initiating use of non-phosphorus detergents. Application of animal waste or manure as fertilizer may also contribute to phosphate pool in soil. Misapplication of fertilizers on frozen land may lead to loss by spring thaw as runoff; contributing to overload of phosphates in water bodies. Similarly, ecosystems in the rain forests are balanced through recycling of nutrients since they lack. After deforestation, heavy rains sweep away nutrients making the land infertile. Other human alterations to phosphorus cycle include waste from municipal sewage treatment plants, which contains higher concentrations of phosphate following lack of costly tertiary sewage treatment options, thus adding to large amounts of phosphate entering the water bodies. In a nut shell, some of the troublesome environmental consequences arising from human interferences include speedy global warming, extensive acidification of soils and waters and increasing nitrogen fortification of ecosystems.

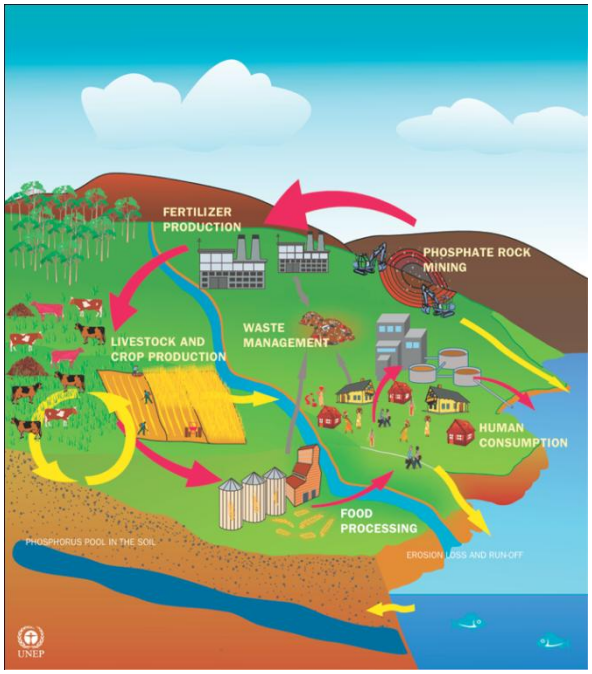


Figure 2: Human interferences in phosphorous cycle.

- Sources

  Phosphorus does not exist in a free form in nature; rather it mainly exists as phosphate minerals. Traditionally principal commercial organic sources of phosphorus included guano and bone ash (Fig 3). Today, the dominant source of phosphorus is inorganic rock phosphate which constitutes apatite (a mineral of impure tri calcium phosphate). Moreover, 50 percent of the world's phosphorus reserves are located in Arab countries, whereas bulky deposits of apatite are found in China, Russia, Morocco, Florida, Idaho, Tennessee, Utha etc (http://minerals.usgs.gov/minerals/pubs/commodity/phosphate_rock/). Phytic acid is the principal storage form of phosphorus in plants. The total phosphorus content of cereals, legumes and oil seeds ranges from about 60 to 90 % while that of nuts is 50 % and 24 % in cocoa and chocolate (Rao et al, 2009).

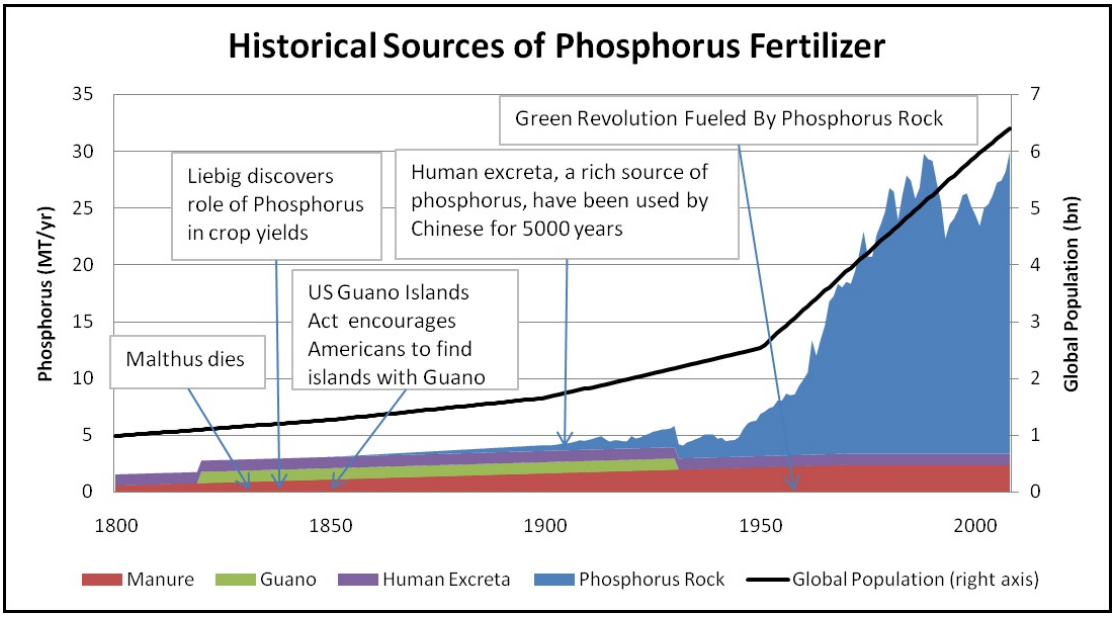
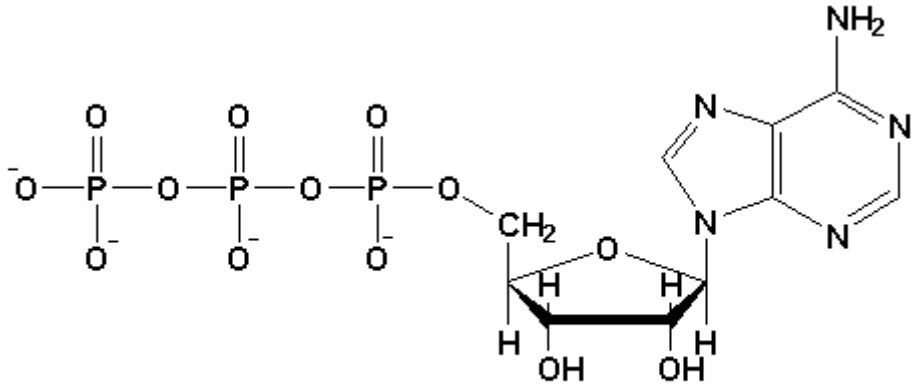


Figure 3: Traditional sources of phosphorus fertilizer (http://seekingalpha.com/article/182522-taking-stock-of-phosphorus-and-biofuels).

- Significance and biological importance

  Phosphorus is a bio-essential element for life whose supply is very limited due to sluggish biogeochemical cycling. It is an important constituent of several structural and catalytic compounds involved in various biochemical reactions. Its prominent role in photosynthesis is well documented. It is a key component of the genetic material or DNA, RNA, several proteins and lipids (Fig 4). It is also an imperative part of the "energy currency of the cell" or ATP. Noticeably hydrolysis of phosphomonoester linkages is a critical reaction for execution of energy, metabolic and signaling pathways. Phosphorus ranks only 2nd to Calcium for its abundance in human body. It accounts to 1% of total body weight mainly attributed to its presence in the skeleton, energy reactions and neural networks. Inadequate availability of phosphorus to organisms can lead to several health disorders such as hypophosphatemia, diarrhea, anorexia, osteomalacia, calcification,

lethargy, seizures, frail muscles, diminished performance and growth abnormalities. Phosphorus is an important additive in animal feed industry, making it the 3rd most expensive nutrient succeeding energy and protein. Compounds of phosphorus are chief constituents of various detergents, water softeners, chemical fertilizers, smoke screening agents; teeth care products, flame retardants, plastic stabilizers, corrosion protectors, paint accessories and metal coating agents etc.



Adenosine triphosphate (ATP)

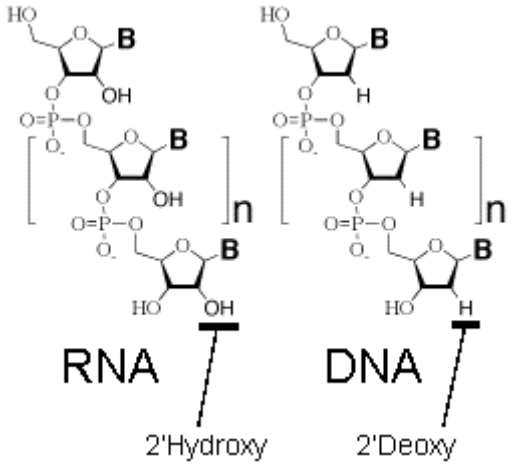


Figure 4: Biologically significant phosphorus compounds.

- Phosphorus reserves and its depletion

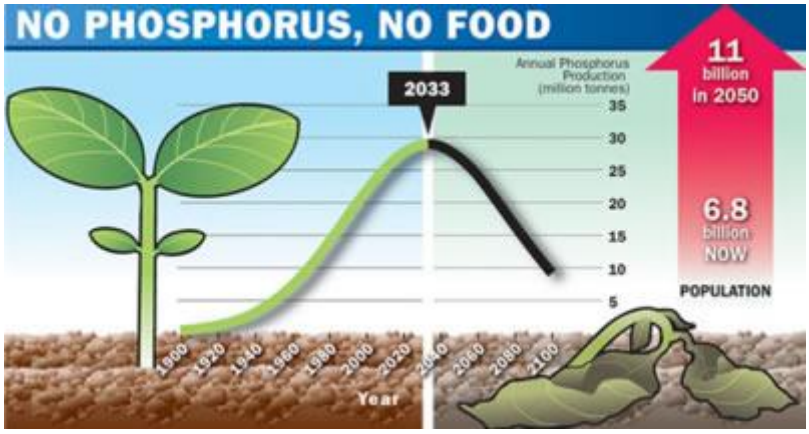


Figure 5: Trends in phosphorus production and pricing (http://tpuc.org/forum/viewtopic.php?f=4&t=57008&start=20).

Phosphorus is the chief growth essential element. It can neither be produced nor substituted and is crucial for food security in the near future. Traditionally, phosphorus was applied as manure in human and animal excreta, bone meal and guano which was later replaced by rock phosphorus following its discovery in 19th century; ever since the rising necessity for rock phosphorus has subjugated the fertilizer industry worldwide (Fig 5). At present, about 90% of the mined phosphorus is exploited for agro and food industry. Rock phosphate production is expected to reach its peak in the next few decades. Even though phosphorus ranks 11th for its abundance in earths crust its availability is not the same. To attain phosphorus safekeeping using sustainable approaches it is essential that optional phosphorus sources be made available with simultaneous reduction in phosphorus requirement (Stewart et al, 2005). There is a growing concern for availability of utilizable phosphorus since the mineable deposits of rock phosphates are limited. According to the US Geological Survey (Fig 6), the current total phosphorus content of rock phosphate reserves is projected to be 2358 million tonnes P and it is estimated that peak for worldwide phosphorus

production may occur by the year 2035 (Buckingham and Jasinski, 2006; Jasinski, 2007; Jasinski, 2008). The geographical allocation of phosphate reserves proposes a major challenge for its access to underprovided countries. According to the IDFC, five countries (China, Morocco, United States, Jordon and Russia) control about 90% of the world's phosphate reserves (Fig 7).

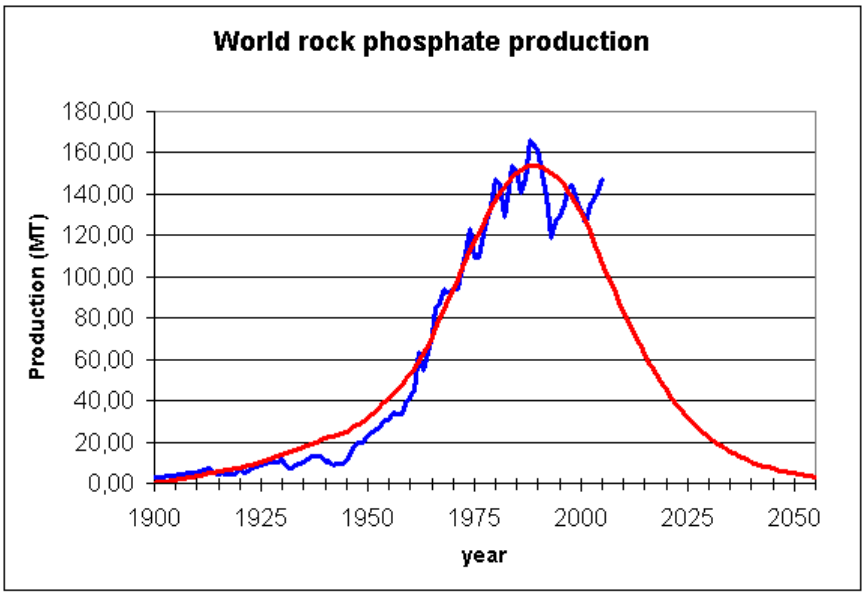


Figure 6: Trend of rock phosphate production across the world (Dery and Anderson, 2007).

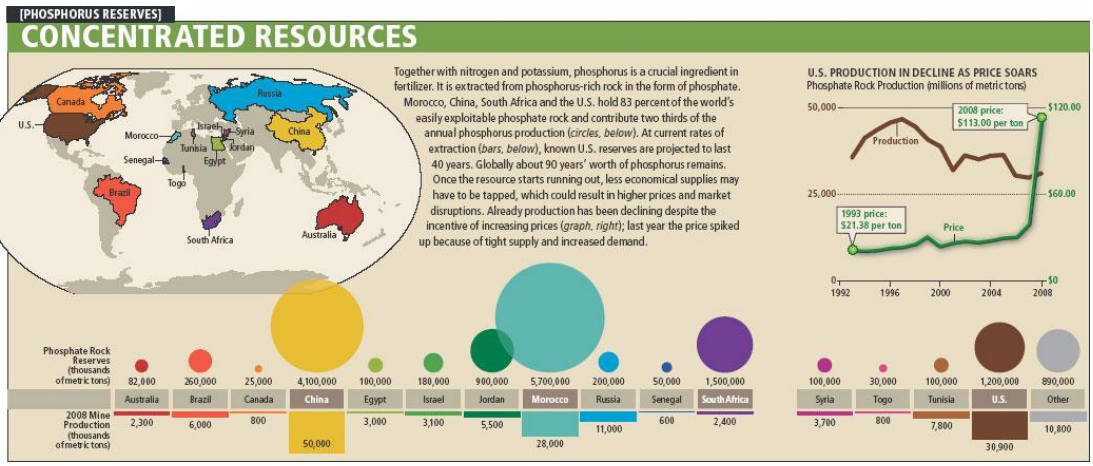


Figure 7: Overview of phosphorus reserves throughout the world (Vaccari, 2009).

Annual consumption of fertilizers in India was observed to rise from 0.7 million tons in 1951–52 to 264.86 million metric tons in 2009–10, and the per hectare consumption escalated to 135.27 kg in 2009–10 compared to 1 kg in 1951–52 (*Annual Report 2010–2011*). India's phosphate rock imports hit close to 3.8 million tonnes in 2011, up from 2.8 million tonnes in 2010. A bulk share of it is utilized in producing phosphate fertilizers (DAP, NPK and SSP) and, to a lesser extent, DCP and P4-derivatives (*Annual Report 2012–2013*). Due to stringent availability of phosphorus, requirement for fertilizers cannot be met thus affecting food accessibility of about two third of the world's population. 50 % of the world's population residing in most densely inhabited low-income countries having enormous requirement of phosphorus fertilizers have measly 5% of the reserves. This noticeably brings to light the dependence on Morocco which supplies only 15 % of the global demand stirring up the fear for racketeer monopoly.

- Market prices

In olden times, phosphate rock was a relatively low-value bulk commodity, but proliferation of agricultural industry led to extensive use of these mineral reserves as phosphate supplements in turn altering the economics of mineral costing (Elser and Bennett E, 2011). The price trend maintained a low profile until the sudden surge in the year 2007-08 (Fig 8). The abrupt soar of phosphorus price in 2008-09 from about $45/t in Feb 2008 to $430/t by Aug 2008. But today the phosphorus prices are maintaining a steady rate of $80-110/t (Evans, 2012).

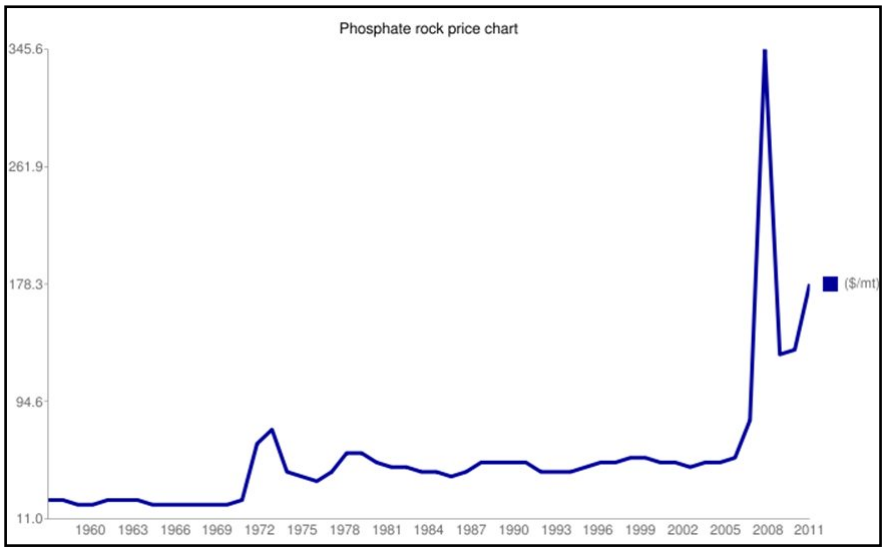


Figure 8: Trends in rock phosphate prices (http://www.mongabay.com).

This liability of global market for rock phosphate marks the need for implementation of sustainable alternatives to ensure food security. The demand for phosphorus is predicted to increase as a result of the growing fertilizer need. A foretaste of this was witnessed in year 2008 when the rock phosphate prices temporarily rose to about US$ 500 per ton which was about 800% increase from the price in the previous 18 months.

- Regulation amongst different countries

As a measure to curtail the approaching phosphorus calamity, several countries have implemented various approaches for reducing and recycling of phosphorus (Fig 9). Recycling of phosphorus has been encouraged by The Soil Association, the UK organic agriculture certification and pressure group, through a report published in year 2010 (Soil association, 2010). In Ireland an efforts are underway to minimize P surpluses on land by modifying maximum fertilizer application rates according to soil P

content and crop requirement. In an attempt to curb excess utilization of phosphatic fertilizers, Sweden, Belgium and Netherlands have made laws regulating the use of fertilizers. Germany has a regulation which controls the fertilization practice. To restrict the phosphorus pollution through animal husbandry, Korea and Taiwan have legalized enactments (Wodzinski and Ullah, 1996). Some European countries provide financial incentives for reducing the load of phosphorus manure (Leeson and Summers, 1997). Furthermore, stringent laws have been passed to limit the content of phosphorus in animal waste in European Union (Bogar et al, 2003).

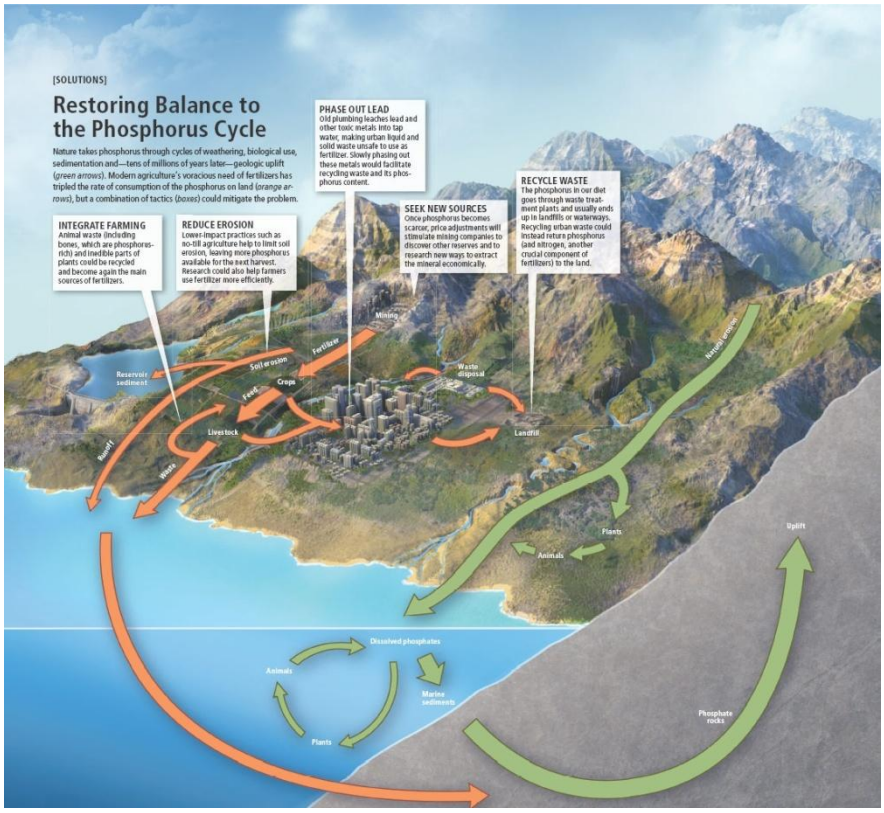


Figure 9: Solutions towards restoring phosphorus balance (Vaccari, 2009).

- Alternative sources

  Most of the researchers opine that alternative sources of phosphorus are a key to sustainable development. One of the approaches includes recycling and recovery of phosphates from water treatment plants. In Canada, it was estimated that, through the use of biological treatments, it can meet the fertilizer need of the state by up to 30%. Another mode includes recycling of phosphorus from the manure generated by poultry, cattle, dairy and pig farming since it is reported to contain about five times more phosphate than human excreta (Gilbert 2009). Phytic acid is one of the chief alternatives sources of phosphorus. It is the principle storage form of phosphorus and inositol in plants (cereals, legumes, oil seeds, and nuts etc.) and accounts for about 60–90% of total phosphorus content in plants (Tang et al, 2006). The liberation of phosphorus from phytic acid can be carried out by degradation or hydrolysis of ester bond using chemical processes or phytases (Singh, 2008).

1.2 Phytate

- Introduction

Myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (IP6) is an organic phosphate compound which serves as a primary store house for phosphorus and inositol in plant seeds (Fig 10). Phytic acid constitutes about 80% of the total seed phosphorus thus accounts to as much as 1.5% of the dry seed weight. At the time of germination, the phytate undergoes hydrolysis with the help of phytases and some phosphatases in turn liberating phosphates, inositol derivatives and

micronutrients assisting the growth of the seedling. Phytic acid also plays significant role in RNA trafficking, DNA repair, cell signaling, vesicular transport etc (Cosgrove, 1980).

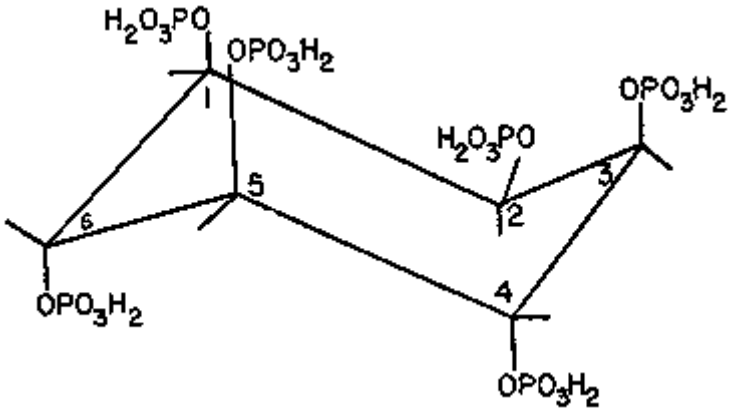


Figure 10: Chemical structure of phytic acid.

- Discovery

Phytate was first reported to be discovered in the year 1855 to 1856 by Hartig, who described circular particles, similar in size to that of potato grains, in several plant seeds (Hartig and Klebermehl, 1855; Hartig et al, 1856). Phytic acid was first described by Posternak in 1903 (Posternak 1903). Winterstein (Winterstein et al, 1897), Schulze and Winterstein (Schulze and Winterstein, 1896) and Posternak (Posternak 1903) demonstrated that hydrochloric acid hydrolysis of phytate yielded phosphoric acid and inositol. Molecular structure of phytic acid was put forward by Anderson in 1914 (Anderson 1914). Trivial names for D-*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate are IP6, InsP6 or phytic acid (PA).

- Structure and chemistry

Myo-inositol (1,2,3,4,5,6) hexa*kis*phosphate bears 6 phosphate groups attached to the inositol ring. The prefix "hexakis" signifies the

phosphates not being internally connected (Johnson and Tate, 1969) while the compound being a polydentate ligand, acts as a chelator binding to more than one metal ions. The phosphate groups are attached to the inositol ring via an ester linkage and can bind to about 12 protons. Six protons are strongly acidic, with pKa ranging from 1.5 to 2.0 while two being weakly acidic depicting a pKa of approximately 6.0. Four protons are found to be very weakly acidic with pKa of 9.0 to 11.0 (Costello et al, 1976; Erdman 1979). Phytic acid has a molecular formula of $C_6H_{18}O_{24}P_6$ while its molecular weight is 659.86 g/mol. The phosphate groups in phytic acid have about two negatively charged oxygen atoms at neutral pH, rendering the cations the ability to chelate strongly amongst two phosphate groups or weakly for a single phosphate group. The term ‘phytate’ describes salts of phytic acid mostly with magnesium, calcium, sodium and potassium whereas the term ‘phytin’ is generally used for calcium/ magnesium salts of phytic acid. The conformational structures of phytic acid have been obtained using ^{31}P NMR (Johnson and Tate, 1969) X-ray analysis (Blank et al, 1971).

Figure 11: Different plant based sources of phytate.

- Occurrences

Phytic acid or Phytate is well known to be present in plants (Fig 11) as a prime source of inositol and phosphorus (Selle et al, 2000). Phosphorus in the form of phytic acid amounts to a considerable fraction (about 60 to 80%) of total phosphorus content in plants (Table 1). The phytic acid content of plants is subject to variation with respect to level of maturity, extent of processing, plant variety, climate changes, location and growth period (Reddy et al, 1982; Manangi and Coon, 2006).

Table 1: Phytic acid content of some plants (Nach: Erdman JW, 1979; Harland B, Obeleas D, 1987; de Boland AR et al, 1975; Schlemmer U, 1993; Fretzdorff and Weipert,1986).

Name	Phytic acid content (g/100g of dry weight)
Beans (*Phaseolus*)	0.1 – 1.7
Green	0.1
Brown	1.0
Red kidney beans	1.2
Soya beans (*Glycine soja*)	1.2 – 1.7
Peas (*Pisum sativum*)	0.7 – 0.9
Green peas	0.7
Split peas	0.9
Lentils (*Lens culinaris*)	0.3 – 0.7
Red	0.9 – 2.1
Green	0.7
Oilseeds	
Sunflower seeds (*Heliantus an.)*	1.1 – 2.1
Rapeseed (*Brassica napus L.*)	0.9 – 2.1
Rapeseed – protein isolate	(5 – 7)
Sesame (*Sesamum indicum*) flour – after oil	1.7 (5.2)

extraction

Wheat (*Triticum aestivum*)	0.9 – 1.2
Wheat bran. spec. fraction	4.1 (8.4)
Whole meal wheat bread	1.0
Maize (*Zea mays*). seeds	0.9 (6.4)
Rye (*Secale cereale)*	0.8 – 1.2
Whole meal rye bread	0.0 – 0.4
Crisp bread - rich in dietary fibre	1.4
Oats *(Avena sativa*)	1.1
Rice (*Oryza sativa*)	0.3
Spelt (*Triticum spelta*)	1.4
Spelt grain (*Triticum spelta. unripe*)	1.0
Buckwheat (*Fagopyrum esc*.)	1.2
Linseed (*Linum usitatissimum L*.)	2.5
Peanuts (*Arachis hypogaea*)	1.3 – 1.8
Almonds (*Prunus dulcis*)	1.4
Hazelnuts (*Corylus avellana L.)*	1.0
Tomatoes (*Solanum lycopersicum)*	0.01
Asparagus (*Asparagus officinalis*)	0.4
Aubergines (*Solanum melongena*)	0.2
Potatos (*Solanum tuberosum*)	0.5

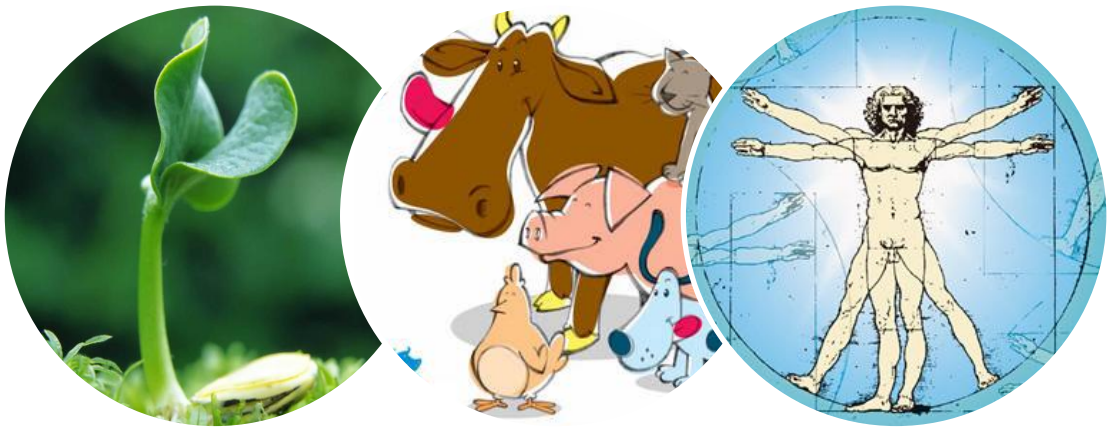
- Physiological significance

Phytic acid is assigned numerous functions (Fig 12) in plants and other organisms as mentioned in Table 2 (Reddy et al, 1989; Graf et al, 1987).

Table 2: Various biochemical functions of phytic acid.

Roles of Phytic acid
• Storage of phosphorus (Reddy et al, 1982). • Energy store (high energy phosphate bonding). • Source of cations (being a chelator of metal ions) (Torres et al, 2005; Veiga et al, 2006). • Source of *myo-inositol* (a cell wall precursor). • Initiation of dormancy in plant seeds. • Antioxidant (Iqbal et al, 1994). • Stress responses, membrane biogenesis and intracellular signaling (Storcksdieck et al, 2007; Loewus and Murthy, 2000). • Antineoplastic (Vucenik and Shamsuddin, 2006). • Cell signalling, trafficking and regulation (Wodzinski and Ullah, 1996). • DNA repair, RNA editing and mRNA transport (Hanakahi et al, 2000; York et al, 1999). • Inhibition of kidney stone calcification *(*Grases 1998) • Reduction of serum cholesterol and triglycerides (Jariwalla 1999). • Lowering effects on digestion and absorption of starch and reduction of the glycemic index (Yoon 1983, Thompson 1993).

Figure 12: Physiological role of phytic acid in various organisms.



- Applications of phytic acid

i. Food and beverage industry

Phytic acid is used as a food additive and preservative owing to its antioxidant or chelating property. For liquids such as wines, molasses and other beverages, phytic acid is used as a metal chelating agent. It is also employed as an anti-scum and antifoam supplement in instant coffee. It is used to prepare an edible collagen sausage skin and also used for preparing dairy products with high protein fibre. Phytic acid is used as a food chemical addition for reducing the time required to reconstitute dried vegetable products (viz. pastes, corn products, fruit and vegetables etc.) (Neth. Patent 7103153, 1972).

ii. Medicine and healthcare industry

Oral solutions of sodium, sulphur and bismuth phytates are reported to be efficient for treating gastric problems, gastroduodenitis, ulcers and diarrhea (Ger. Patent 1807733, 1969; Ft. Patent 7794, 1972; ltal. Patent 65968, 1982). It has a significant application in dental care. It is effectively used to improve X-ray imaging property of the contrasting agent barium sulphate. Phytic acid is also applied as an imaging agent during organ scintigraphy. Phytic acid can be a potential candidate for treating radical related diseases such as cirrhosis, Alzheimer's and Parkinson's diseases etc. (Benzie, 2003).

iii. Metallurgical applications

The anticorrosive property of phytic acid conferred by the chelating ability makes it a useful and inexpensive candidate for precipitation during extraction and separation of rare earth metals from ores (Jpn. Patent 7002832, 1970).

iv. Chemical industry

The tremendous affinity for cations leads to indispensable use of phytic acid for preparation of different cleaning formulations. It is also used as an antioxidant, chelating agent, catalyst and anti-static component during polymerization reactions. Phytic acid is also employed as a coacervate-forming agent during preparation of heat-sensitive image recording materials constituting gelatin microcapsules (Jpn. Patent 77145046, 1978).

v. Biotech/Pharma industry

Phytic acid is also exploited as an inhibitor of aflatoxin synthesis in *Aspergillus parasiticus*, attributed to its zinc chelating ability (Gupta and Venkitasubramanian, 1975; Demyers, 1979).

vi. Miscellaneous

Phytic acid is an ingredient of several cosmetics. It is also as instant hardener for anaerobic resins. It is aalso applied in historical method of document and paper preservation (Sala et al, 2006).

- Unfavourable/ antinutrient properties.

Phytic acid possesses a strong anti-nutritive property which can be attributed to its curious molecular structure (Fig 13). It contains six phosphates amounting to twelve negative charges which carryout effective binding to various mono-, di-, and trivalent cations resulting in formation of insoluble complexes (Reddy et al, 1989). Phytic acid hampers mineral assimilation due to formation of mineral phytate complexes (Davies 1982). Phytic acid is also reported to form phytate-protein complexes with proteins over a wide range of pH (De Rham and Jost, 1979; Fretzdorff et al, 1995) thus decreasing their solubility and digestibility in turn reducing the nutritive value of food. Mineral-phytate complexes in foods

remain unabsorbed in the gastro-intestinal tract thus reducing the bioavailability of these minerals. Furthermore, the absence of phytate degrading enzymes or microbes in the gut adds to the problem (Iqbal et al, 1994). Phytate also interacts with different enzymes (viz. trypsin, pepsin, α-amylase and β-galactosidase etc.) and reduce their activity (Deshpande and Cheryan, 1984; Singh and Krikorian, 1982; Inagawa et al, 1987). Phytate also binds to starch through hydrogen bonding or via interactions with starch associated proteins (Richard and Thompson, 1997). Phytate also interacts with lipids and its derivatives resulting in the formation of lipo-phytin complexes (Leeson 1993).

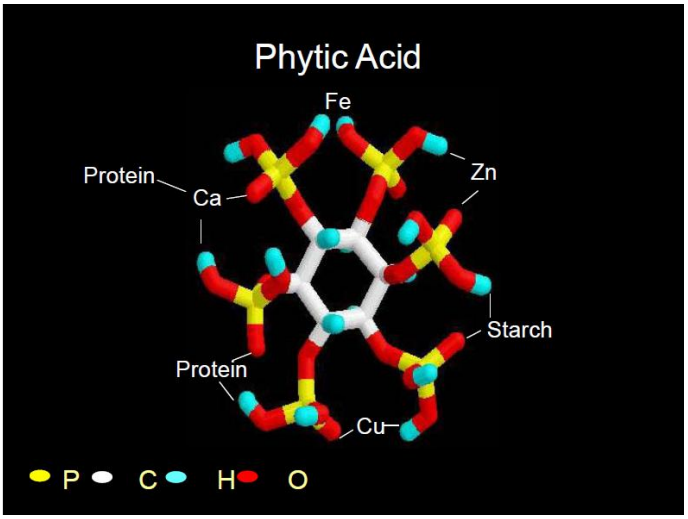


Figure 13: Interactions of phytic acid with different compounds.

- Environmental damages/impact

  Phytate bound phosphorus is already present in animal feed but remains unutilized due to unavailability of proper means of phytate hydrolysis in the gastro-intestinal tract. Moreover, being a chelating agent it inhibits the bioavailability of several cations (Ca^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+}) thus reinstating its anti-nutritive ability. Thus, the undigested phytate consequently becomes the source of

phosphorus pollution in areas subjected to intense livestock rearing and agriculture subsequently causing eutrophication and algal blooms (Ole et al, 2002).

- Degradation of phytate

  Phosphate dephosphorylation is an essential requirement to achieve better nutritive quality owing to decreased mineral binding capacity, in turn improving the bioavailability of minerals (Sandberg et al, 1999). Non-enzymatic method of phytate degradation includes harsh conditions of temperature (121°C for 1h) (Phillippy et al, 1987). Other treatment options such as milling, soaking or extracting are non-specific and alter the nutritive content of the food hence cannot be used as an effective way out for this dilemma (Hurrell, 2004). Therefore, enzyme mediated hydrolysis of phytic acid may prove to be a better option than other conventional methods.

- Enzymatic hydrolysis of phytate

  Enzymes mediating phytic acid hydrolysis are termed as 'phytases' or myo-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolases. They catalyze consecutive liberation of phosphate from phytate with myo-inositol phosphates intermediates as by-products (Debhnath et al, 2005). Phytase reverses the chelating and anti-nutritive properties of phytic acid (Fig 14) thus instigating the release of minerals, proteins, lipids and carbohydrates bound to phytic acid in food and feed stuff, indirectly avoiding phosphorus pollution in the environment (Baruah et al, 2007).

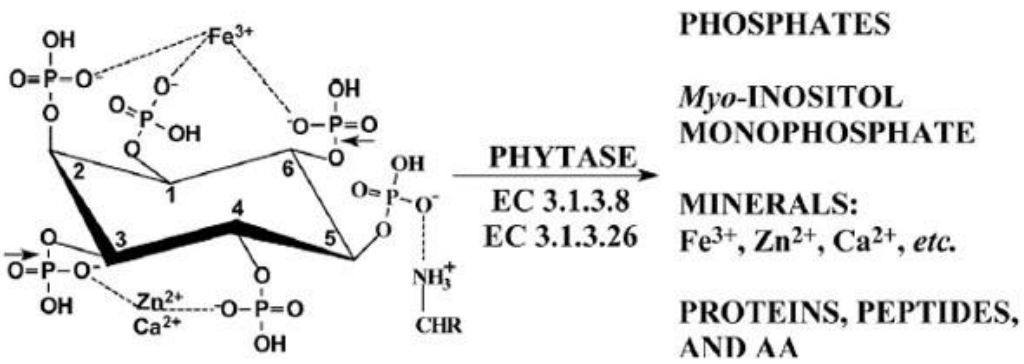


Figure 14: Phytate subjected to phytase hydrolysis and possible outcomes.

1.3 Phytase

- General information

  Phytase (myo-inositol hexakisphosphate phosphohydrolase) is categorized as a phosphoric monoester hydrolase. It mediated the hydro-catalysis of phytic acid or myo-inositol hexakisphosphate releasing inorganic phosphate and lower phosphate derivatives of myo-inositol, or unbound myo-inositol in some instances. Phytases also possesses ability to hydrolyze additional organic phosphate compounds such as lower inositol phosphates, nucleotides, etc. albeit varied efficiency and affinity (Konietzny and Greiner, 2002; Gibson and Ullah, 1990). Phytase was discovered in 1907 during some experiments on hydrolysis of rice bran (Suzuki et al, 1980).

- Classification

  Phytase catalyzes the stepwise breakdown of phytic acid to yield lower myo-inositol derivatives, inorganic phosphate and myo-inositol in some cases; belongs to a subclass of phosphatases and are categorized as members of histidine acid phosphatase family (Mitchell et al, 1997). According to recent classifications by the

International Union of Biochemists (IUB, 1979) phytases are grouped into three classes based on the inositol ring position (3, 4/6 or 5) at which the dephosphorylation begins (Fig 15).

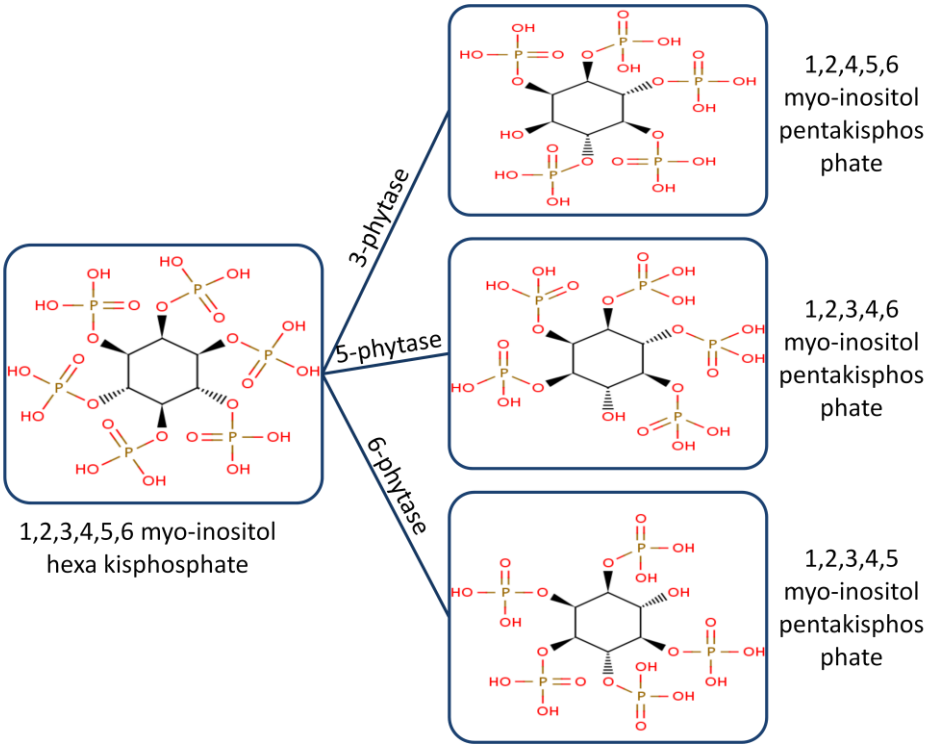


Figure 15: Schematic diagram demonstrating phytic acid hydrolysis with respect to different phytases.

i. 3-phytases (EC 3.1.3.8) catalyze hydrolysis of phytic acid to produce myo-inositol 1,2,4,5,6-pentakisphosphate. This phytase seldom performs absolute hydrolysis and is generally found in microorganism with *E. coli* being an exception as it produces a 6-phytase (Greiner et al, 1993).

ii. 4- or 6-phytases (EC 3.1.3.26) produce myo-inositol 1,2,3,4,5-pentakisphosphate as a first product. It performs complete hydrolysis every time and is generally found in plants. Some exceptions include phytase from soybean which is a 3-phytase (Nayani and Markakis, 1986; Persson et al, 1998; Reddy et al, 1982).

iii. 5-phytases (EC 3.1.3.72) commence the hydrolysis at 5^{th} position and is generally found in *Medicago sativa, Phaseolus vulgaris,* and *Pisum sativum* etc (Barrientos *et al.*, 1994).

Phytases are generally grouped as Histidine acid phosphatases and Alkaline phytases based upon the pH at which the enzyme is optimally functional. Other classification which considers differences such as structure and catalytic properties etc. further classifies phytases as Cysteine phytases, Histidine acid phosphatases, β-propeller phytases and Purple acid phosphatases (Mullaney and Ullah, 2003).

Histidine Acid Phosphatase (HAP)

The members of this family are characterized by a distinctive catalytic mechanism and an exclusive active site motif. The active site motif at N-terminal contains the sequence RHGXRXP while C-terminal motif possesses the sequence HD (Wodzinski and Ullah, 1996). Subsequent to appropriate folding of the enzyme polypeptide, the sequences collectively form the catalytic site of the enzyme, which instigate the hydrolysis of phosphoric monoesters (Van Etten et al, 1991). HAPs constitute a huge cluster of acid phosphatases and are capable of hydrolyzing a variety of substrates, hence it must be noted that all the members of HAPs cannot degrade phytate efficiently. The presence of an effective catalytic ability is a pre-requisite for efficient hydrolysis of phytate (strongly negatively charged substrate); which can be achieved by proper positioning of the active site and the substrate. In HAPs form prokaryotes and eukaryotes, the active site are predominantly

positively charged at acidic conditions thus enables competent substrate binding ensuring appropriate phytate hydrolysis. The term Histidine Acid Phytase (HAPhy) was recommended by Oh and co-workers in 2004 (Oh et al, 2004) referring to Histidine acid phosphatases capable of efficient phytate hydrolysis. Histidine acid phytases are reported to be present in prokaryotic as well as eukaryotic organisms. The *E. coli* phytase is the most studied HAPhy from prokaryotes with its structure being available as a 3-D molecular model (Greiner et al, 1993; Lim et al, 2000). *A. niger* and *A. fumigates* phytases are amongst extensively studied fungal phytases.

Beta-Propeller Phytase (BPPhy)

This class of phytases includes novel enzymes which lack presence of homology to any known phosphatases and were first identified in *Bacillus amyloliquefaciens* as thermostable phytase (Ha *et al*., 2000). Their nomenclature is based on structure of the enzyme; as it principally contains beta sheets and looks like a propeller with six blades (Shin et al, 2001). The enzymes in this class are characterized by their need for Ca^{2+} (since they create encouraging electrostatic conditions) for effective catalysis and thermostability. In BPPhys, the affinity site (substrate attraction) and cleavage site (phosphate hydrolysis) are the two most important modules critical in catalysis reactions (Shin et al, 2001). BPPhy requires two adjacent phosphate groups; each occupying the above mentioned sites to perform hydrolysis thus hydrolyzing every other phosphate group in phytate producing *myo*-inositol triphosphate as end product.

Cysteine Phosphatase (CP)

During some studies on anaerobic ruminal bacteria, a novel phytase was discovered from *Selenomonas ruminantium* which possessed properties such as monomeric existence, molecular weight of 46 kDa, pH optima of 4.0–5.5, temperature optima of 50–55 °C and cation inhibition (Yanke et al, 1999). Studies on this enzyme characterized it neither as HAPhy nor a BPPhy (Chu et al, 2004), but it had structural and catalytic properties similar to cysteine phosphatase (CP) superfamily. The deduced amino acid sequence shows presence of active site motif HCXXGXXR(T/S) along with other similarities to protein tyrosine phosphatase (PTP) a member of the CP family. Loop formation at active site mediating substrate binding which is exclusive to PTPs is also seen in BPPhys (Denu and Dixon, 1998). The favorable electrostatic environment present in the BPPhys confers the ability to bind to phytic acid while other members lack the same. Negative charge of the substrate is critical for mediating initial binding of phytate at the active site (Chu et al., 2004), hence the inhibition caused by cations can be attributed to formation of substrate-cation complexes (Yanke et al, 1999).

Purple Acid Phosphatase (PAP)

Purple acid phosphatases (PAP) belong to a class of metallophosphoesterases and are characterized by presence of distinctive set of seven amino acid residues capable of liganding metals. These residues (D, D, Y, N, H, H, H) together constitute a pattern of five general consensus motifs viz. .DxG/GDx2Y/GNH.E.D./Vx2H/GHxH. (Schenk et al, 2000). This group contains phosphatases from plants, mammals, fungi and

bacteria (Schenk et al, 2000; Olczak et al, 2003). The PAP lack the ability to efficiently utilize phytate in contrast to HAPs and CPs. In animal PAPs, the metallic center contains two iron ions whereas in plants one of them is replaced by zinc or manganese ion (Olczak et al, 2003).

- Sources

  Phytases are generally found in plants, animals, bacteria and fungi. Phytase is also secreted by mucosa of the small intestine and some ruminant microorganisms though the activity is significantly low (Cooper and Gowing, 1983). Animal phytases show lower activity as compared to plant and microbial sources (Weremko et al, 1997). Microbial phytases, fungi in particular are amongst the widely studied phytases (Stefan et al, 2005). Plant phytases were found to be present in extensive amounts but are less efficient due to lack of thermostability and weak pH profile. Microbial phytases clearly have an edge over plant phytases with respect to catalytic efficiency (Zimmermann et al, 2002).

- Physiological roles of phytases

  The evident presence of phytase in microbes, plants and animals, points towards the multiple functions it plays with respect to organism physiology in which they exist.

  Microorganisms

  Phytases from microbes are synthesized as a critical reaction induced upon starvation of phosphorus. Secretion of phytase leads to enhance availability of inorganic phosphorus in the surrounding or from phosphorus derivatives of inositol. Phytase degrading

enzymes were found to be excessively produced during stationary phase in *E. coli* when grown under anaerobic fermentation conditions (Greiner et al, 1993) while in case of *Klebsiella terrigena* phytase activity was reported to be enhanced in presence of phytate in growth medium (Greiner et al, 1997). In fungi (*A. niger* and *A. ficuum*) however, phytase production is maximum at optimally restricted concentration of phosphorus in growth medium (Shieh and Ware, 1968). Prokaryotes (such as *Xanthomonas oryzae, X. compestri* and *Salmonella dublin*) produce phytase to tackle various physiological roles such as pathogenicity and scavenging of phosphorus (Irving and Cosgrove, 1971; Powar and Jagannathan, 1982).

Plants

Plants produce phytase during seed germination to help degrade stored phytate for increasing availability of phosphates, lower inositol derivatives, myo-inositol and chelated cations (Shah and Parekh, 1990). Alkaline phytase produced constitutively in lily pollen and seeds degrade phytate to yield tri-phosphate derivatives of inositol which act as antioxidants (Hawkins et al, 1993; Brearley et al, 1996). Several intermediary compounds and terminal reaction products of phytic acid degradation act as signalling molecules in transduction as well as are critical in secondary messaging (Shears, 1998).

Animals

Animal phytases generally work to maintain the supply of inositol hexa phosphate and its other lower phosphate derivatives since

they are critical to cell signalling pathways such as Ca^{+2} mobilization (Yu et al, 2003; Chi et al, 1999).

- Commercial phytases

  Before commercial phytase was made available, it was known that the cost of supplementing phytase was tremendous (Nelson et al, 1971) also the environmental constraint was lacking which lead to delay in active research for commercializing phytase till late 1980s. Attempts to commercialize phytase as an alternative to utilize phytate phosphorus instead of rock phosphorus started as early as 1962, but due to successive futile attempts the mission was aborted in 1968 (Wodzinski and Ullah, 1996). Irving & Cosgrove (1972) successfully commercialized phytase from *Aspergillus (ficuum) niger* NRRL 3135 (ATCC 66876) in 1990s. In 1991, Natuphos® was launched as the first globally commercialized phytase (Rodriguez et al, 1999). Today, there are several commercial phytases available viz. Phyzyme, Ronozyme, Finase etc (Table 3).

Table 3: Commercial Phytases (Cao et al, 2007).

Commercial Name	Company	Phytase source	Producing strain
Finase	AB Enzymes	*A. awamori*	*Trichoderma reesei*
Sp, TP, SF	Alko Biotechnology	*A. oryzae*	*A. oryzae*
Allzyme Phytase	Alltech	*A. niger*	*A. niger*
Natuphos	BASF	*A. niger*	*A. niger*
AMAFERM	BioZyme	*A. oryzae*	*A. oryzae*
Bio Feed Phytase	DSM	*P. lycii*	*A. oryzae*

Phyzyme	Fermic	*A. oryzae*	*A. oryzae*
Avizyme	Finnfeeds International	*A. awamori*	*T. reesei*
ROVABIO	Genencor International	*P. simplicissimum*	*Penicillium funiculosum*
Finase	Roal	*A. awamori*	*T. reesei*
Ronozyme®	Novozymes	*A. oryzae*	*A. oryzae*
Roxazyme®	Novozymes	*A. oryzae*	*A. oryzae*

- Market trend

  Feed enzymes such as protease, xylanase, phytase, amylase, cellulase, lipase, alpha/beta-glucanase etc. have proved to be a recent boom for the animal feed industry amounting to about $5 billion of the market value. In 1991, first phytase to enter the feed market was Natuphos, which was produced by Gist Brocades (now DSM) and marketed by BASF. It is now available in variety of preparations such as powder, granules, or liquid. The first International phytase summit in 2010 held at Washington predicted that the present worldwide market for phytase accounts to about 60% of the total feed enzyme revenue and is expected to be $350 million of worth annually. Currently, about 70% of the swine and poultry feed is estimated to contain phytase supplementation (Lei et al, 2013). The FDA has approved "generally recognized as safe (GRAS)" status for phytase to be used in food and feed preparations since 1996.

  Economics of phytase supplementation

  Phosphorus being an expensive nutrient, efforts for its substitution in animal feed so as to make it economical was at its peak during late 1990s. Replacing dicalcium phosphate in plant based feed with

phytase as 25 g/100 kg made the broiler meat by cheaper by about INR 1/kg (Vinil et al, 2000). Several reports suggested economical advantages of phytase supplementation in broilers Kundu et al., 2000; Singh and Khatta, 2004). Before 2007, substituting inorganic phosphorus by phytase was not much economical as the cost of dicalcium phosphate was about $200-250/ton, but the prices soared to $1200/ton which made alternative use of phytase much more reasonable (Lei et al, 2013).

Phosphorus Equivalence

About 500–1000 units/kg of phytase can replace 1% of DCP or 0.18% of phosphorus in feed. Supplementing phytase (1000 units/kg) costs about $0.5–$2/ton (Radcliffe and Kornegay, 1998; Esteve-Garcia et al, 2005), but may vary depending on the unit differences in phytase activity among various products (Kerr et al, 2010). Supplementation of phytase can lead to decrease in fecal phosphorus excretion by about 50% which can be improved to about 80% depending on the enzyme dosage (Lei et al, 2013). The overall worldwide yield of phytate from plants is more than 51 million metric tons per year which accounts for about 65% of inorganic phosphorus vended as fertilizers. Exploitation of phytase as a supplement may lead to a better social and economical influence.

1.4 Microbial production of phytase

- Isolation and screening

  Screening is an important step in isolating phytase producing organisms. A method for screening phytase secreting microbes,

which exploited *Corynebacterium glutamicum* as an indicator was made available (Chen, 1998). Phytase isolated from museum and soil sources (about 71% soil isolates exhibited more than 0.01 U/ml activity) concluded superiority of mould phytases to that of bacterial ones (Lissitskaya et al, 1999). A two step method to screen phytase producing fungi was employed by Gargova et al (1997). Another easy and speedy method based on quantification of phosphorus released upon degradation of sodium phytate at pH 5.5 was put forth by Engelen et al (1994). Plate based assay system was devised by Bae et al (1999) wherein the phytase activity was measured based upon the disappearance of sodium/calcium phytate precipitates. The major drawback of this technique was its inability to differentiate between zones of hydrolysis caused by acid to those of phytases as is the case of ruminant microbes.

- Production technique

  Phytases have been produced by different microbes such as bacteria, fungi, yeast etc. under submerged (SmF) (Irving and Cosgrove, 1971), semisolid fermentation (Han et al, 1987) as well as solid state (SSF) fermentation (Howson and Davis, 1983). There is no optimal method or medium composition to ensure maximum production of phytase by the microbe. The choice of method of production depends upon characteristics such as culture strain, growth conditions, type of substrate and nutrient assimilation, yield, production time, downstream processing, economical aspects etc. extracellular phytases are more significant when production at industrial scale is considered. A large share of extracellular microbial phytases is owned by filamentous fungi. Most of the phytases currently in use as feed additives are fungal derived,

either produced by wild type or recombinant strains. *Aspergillus niger* is the most extensively studied and commercialized phytase producing fungus (Howson and Davis, 1983; Volfova et al, 1994; Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004; Vats et al, 2004). Several bacterial species have also been reported to produce phytase viz. *A. aerogenes* (Greaves et al, 1967), *Pseudomonas* sp. (Irving and Cosgrove, 1971), *Klebsiella* sp. (Shah and Parekh, 1990), *B. subtilis* (natto) (Shimizu, 1992), *E. coli* (Greiner et al, 1993), *Enterobacter* sp. 4 (Yoon et al, 1996) and *B. amyloliquefaciens* sp. DS 11 (later designated as *B. amyloliquefaciens*) (Kim et al, 1998a). Most bacteria produce intracellular or cell bound phytases with the exception of *Bacillus* and *Enterobacter* genera who produce extracellular phytases and *E. coli* who produce periplasmic phytase. Various yeasts (*S. cerevisiae*, *C. tropicalis*, *T. candida*, *D. castelii*, *D. occidentalis*, *K. fragilis* and *S. castelii*) have also been shown to produce phytase (Nayini and Markakis, 1986; Lambrechts et al, 1992; Mochizuki et al, 1999; Kaur et al, 2007; In et al, 2009). Phytase production was studied on different agricultural residues and wastes such as canola meal (Ebune et al, 1995), wheat bran and full-fat soybean flour (Krishna and Nokes, 2001), cracked corn, soybean meal (Bogar et al, 2003) under submerged and solid state fermentations. Phytase production in E. coli was studied using fed batch method wherein low concentration of oxygen and constant concentration of glucose supported maximum phytase production (120 U/ml) with reduced fermentation time (14 h) (Kleist et al, 2003). Bacillus sp. DS11 grown on wheat bran and casein hydrolysate medium secreted thermostable phytase at 37 °C (Kim et al, 1998a; Kim et al, 1998b), the gene for which was later cloned into *B. subtilis* resulting in

increased phytase production (Kim et al, 1999a). Recombinant *H. polymorpha* was exploited for economical phytase production at pilot level (2000 l) (Mayer et al, 1999) using wild type genes from *A. fumigates* (Ehrlich et al, 1993) and *A. terreus* (Mitchell et al, 1997). Phosphate concentration affects phytase production, thus optimal concentration is quintessential for high phytase production. *A. ficuum* phytase production increased to 113 nkat/ml whilst the phosphorus concentration of 0.4 mg/100 ml with 8% cornstarch was maintained (Wodzinski and Ullah, 1996). Several reports confirmed the observation of high phosphorus inhibiting phytase production (Han and Gallagher, 1987; Gibson, 1987; Vats and Banerjee, 2002). Tween-80, Triton-X-100, Na-oleate and other surfactants were studied for their positive influence on phytase production (Al-Asheh and Duvnjak, 1994) based on their ability to alter cell permeability. Inoculum properties also govern phytase production (Wodzinski and Ullah, 1996); inoculum age, size, growth phase and quality affect the fermentation behavior of the organism thus affecting enzyme synthesis (Krishna and Nokes, 2001). Glucose, maltose and yeast extract act as carbon and nitrogen sources in commonly used submerged fermentation media for phytase production by various bacteria (wild and modified strains) viz. *B. subtilis*, *E. coli*, *Lactobacillus amylovorus*, *B. amyloliquefaciens*, *Klebsiella sp*. A recombinant strain of *E. coli* (BL21) was subjected to statistical optimization to produce phytase activity of 2250 U/l, the media contained glucose, tryptone, NaCl and yeast extract (Sunitha et al, 1999). Submerged fermentation is also the preferred choice for production of phytase in yeasts. Yeasts such as *Pichia*, *S. castellii*, *A. adeninivorans* and *C. kruzei* etc. produce phytase with preference for glucose and galactose as

carbon sources (Angelis et al, 2003). Phytase production is also extensively studied in *P. anomala* using statistical method. Maltodextrin and soya flour were found to be better sources of carbon with respect to phytase production in *Peniophora lycii* (Lassen et al, 2001). Several carbon sources (e.g. starch, maltodextrin, glucose, rice bran, coconut oil cake, wheat bran, etc) have been exploited for phytase production in fungi (Singh et al, 2011). Though numerous yeasts and bacteria fungi are currently reported to produce phytase under various conditions, filamentous fungus *Aspergillus niger* is the indubitable favorite for phytase production at commercial level (Haefner et al, 2005).

- Strain improvement

  Since phytase is an industrial enzyme, it is desirable that the production strain must possess some properties such as hyper secretion of desired product with reduced fermentation time, ability to assimilate low cost complex substrates, lesser byproduct interference, insensitive to end product inhibition etc (Parekh, 2000). A very small number of publications discussing strain improvement for phytase production are available. *A. niger* NRRL 3135 was subjected to mutagenesis using UV radiation to isolate a catalytic mutant producing phytase (phy A) by about 3.3 fold times of that produced in wild type strain (Chelius and Wodzinski, 1994). Though hypersecretion was achieved, specificity and sensitivity were hampered. A novel method was applied for enhancing phytase production in *Rhizopus oryzae* (Rani and Ghosh, 2011).

- Transgenic studies

Even though phytases are produced by various organisms, the use of wild type strain at industrial level is remotely possible due to commercial implications. Thus, phytase gene from different organisms is cloned and expressed in appropriate microbe to achieve desired yield (Table 4). According to the economical restrictions at industrial level, the choice of host organism and recombination machinery is made. Plants, fungi, yeast and bacteria can be used as hosts for phytase gene expression. Some examples of heterologus phytase expression with corresponding pros and cons are mentioned below:

1. Plants and Animals: Phytase from *A. niger* has been expressed in several plants viz. soybean (Li et al, 1997), tobacco (Pen et al, 1993; Verwoerd et al, 1995; Ullah et al, 1999), alfalfa (Gutknecht 1997), wheat (Brinch-Pedersen et al, 2000), and canola (Ponstein et al, 2002) etc. Several properties of the thus produced phytases including shifts in pH optima, glycosylation, thermotolerance, extracellular secretion etc. were found to be altered, hence compelling researchers to look for suitable alternatives. Genetically modified pigs, called as EnviroPig, have been developed by expressing phytase gene (AppA) from *E. coli* in salivary glands (Golovan et al, 2001).

2. Yeast: Among the several reports on yeast phytases, very few are available mentioning yeast phytase expression in heterologous host system. But numerous publications are available for use of yeast as an efficient host organism for heterologous phytase gene expression (Rodriguez et al, 2000).

3. Bacteria: Bacterial systems have been classically used for simple and efficient expression of heterologous genes, however due to limitations such as lack of secretion ability and post translational modification their applicability in phytase transgenics has been limited. Several studies on expression of fungal phytases in bacteria have lead to loss of activity, intracellular accumulation or under glycosylation of the said protein (e.g. phyA expression in *E.coli*) (Phillipy and Mullaney, 1997).

4. Fungi: Numerous reports for heterologous expression of phytase genes in fungi are available. *A. niger* has been the most exploited host organism for recombinant expression of phytase genes (*A. niger*, *A. terreus*, *A. fumigatus*, *E. nidulans*, and *M. thermophila*). *A. niger* can secrete functional protein without much alterations in enzyme properties. A major disadvantage for use of fungi as heterologous host is the higher concentrations of proteases produced along with the desired product. Hence, studies to minimize the loss due to proteolysis are necessary.

Table 4: Heterologous expression of phytases (Haefner et al, 2005).

Phytase source	Production strain[a]	Phytase activity (U ml−1)[b]	Phytase productivity (U l−1 h−1)[b]	References
B. amyloliquefaciens	*B. subtilis*	2	167	Kim et al, 1999a,b
B. licheniformis	*B. subtilis*	28		Tye et al, 2002
E. coli	*Streptomyces lividans*	950	19792	Stahl et al, 2003

E. coli	*P. pastoris*	114		Rodriguez et al, 1999
E. coli	*P. pastoris*	117	2438	Stahl et al, 2003
E. coli	*P. pastoris*	4946	25760	Chen et al, 2004
A. fumigatus	*P. pastoris*	55		Rodriguez et al, 2000a,b
A. fumigatus	*A. awamori*	62	369	Martin et al, 2003
A. fumigatus	*H. polymorpha*	7.6	30	Mayer et al, 1999
A. niger	*E. coli*	0.2		Phillipy and Mullaney, 1997
A. niger	*S. cerevisiae*	3186		Han et al, 1999
A. niger	*P. pastoris*	39	279	Xiong et al, 2004
A. niger	*P. pastoris*	64	593	Han and Lei, 1999
Aspergillus terreus	*H. polymorpha*	4.5	15	Mayer et al, 1999
Consensus[d]	*H. polymorpha*	13.5	46	Mayer et al, 1999

1.5 Molecular and biochemical characteristics of phytase

Phytases, based upon their source or origin, are high molecular weight proteins with molecular weight ranging from about 35–700 kDa. Based upon their classification, phytases possess different pH optima but most phytases are active over a pH range of 4.5-6.0.

Phytases are generally monomeric in nature with the exception of phytase B (produced by *A. niger*) which is a tetramer. Most phytases are found to be glycosylated in eukaryotes such as fungi, yeasts, animals and plants resulting in them being high molecular weight enzymes viz. plant and animal phytases (50-150 kDa), yeast (500 kDa), fungal phytases (85-150 kDa); while those of bacterial origin depict 40-55 kDa of molecular mass due to absence of glycosylation (Table 5). A majority of phytases are optimally active over a temperature range of 44–60 °C with the exception of phytases from *A. fumigatus* and *B. amyloliquefaciens* who demonstrate the temperature optima of about 70 °C. *A. fumigatus* and *Schwanniomyces castellii* phytases have been reported to be the best thermotolerant phytases isolated so far (Segueilha et al, 1992). Microbial phytases have been found to be superior to plant phytases in terms of thermotolerance and susceptibility to extreme pH. Bacterial phytases have alkaline to neutral pH optima while that of fungi range from pH 2.5-6.0. The isoelectric pH of all phytases (bacterial, plant and fungal) fall in the acidic range with the exception of *A. fumigates* phytase (pI 8.6). All phytases have broad substrate specificity along with high affinity for phytate however few phytases have been reported to be highly specific for phytate viz. *A. niger* phytase, alkaline phytases from *B. subtilis*, *E. coli* phytase and *A. terreus* CBS. Phytases generally show *Km* values ranging between 10 and 650 μM. Several reports exists describing alterations in phytase activity due to presence of metal ions, which can be attributed to either direct metal binding to enzyme or due to formation of ion-substrate complexes. Bacillus phytases have been found to be dependent on Ca^{+2} for its optimal activity (Kerovuo et al, 1998). Fungal phytases are more

susceptible to proteolytic digestion as compared to bacterial phytases (Rodriguez et al, 1999; Igbasab et al, 2000).

Fungal and yeast phytases (mostly grouped as 3-phytases) belong to histidine acid phosphatase category are generally glycosylated and have a broad substrate specificity (Wyss et al, 1999a). Phytase from *Aspergillus niger* (PhyA) is a most studied phytase which is also exploited at commercial scale. The monomeric enzyme has a molecular weight of 80 kDa and displays two pH optima at 2.5 and 5 respectively. Its temperature optima being 55-60 °C and has a high affinity for phytic acid (Han et al, 1999). Another phytase from *Aspergillus fumigatus* exhibits 66% sequence similarity to *A. niger* PhyA phytase, has a broad pH range and low specific activity against phytate (Wyss et al, 1999a; Rodriguez et al, 2000a) nevertheless displays much superior thermo-tolerance (Pasamontes et al, 1997a; Wyss et al, 1998). Yet another commercialized phytase includes PhyA from *Peniophora lycii*. It is classified as a 6- phytase demonstrating pH optima of 4.0–4.5 and temperature optima of 50–55 °C and is prone to pH, thermal and enzymatic denaturation (Lassen et al, 2001; Simon and Igbasan, 2002). Many phytases with superior thermal tolerance and catalytic ability have been isolated from T. *lanuginosus, Mucor pusillus* (Chadha et al, 2004). *S. cerevisiae* etc also produce phytases which are glycosylated and function at pH and temperature conditions of acidic and 40 °C respectively (Quan et al, 2002; Türk et al, 2000).

Bacterial phytases belong to histidine acid phosphatases or alkaline phytases and possess a ß-propeller structure. They are non-glycosylated monomeric enzymes with molecular mass of about 38–47 kDa, pH optima about 7 and temperature optima of about

55–70 °C (Kerovuo et al, 1998). *E. coli* phytase (AppA) has been found to be superior to *A. niger* phytase for use in swine and poultry feed due to its acidic pH optima, pepsin resistance, high specific activity for phytate (Wyss et al, 1999a; Augspurger et al, 2003a). Quite a lot of phytases with novel properties have been isolated viz. *Pseudomonas syringae* phytase (45 kDa, 649 U/mg specific activity, pH optima of 5.5 and temperature optima of 40 °C) and *Citrobacter braaki* (highly specific for phytic acid, pH optima 4 and temperature optima 50 °C) (Cho et al, 2003; Kim et al, 2003).

Plant phytases are generally grouped as histidine acid phosphatases, however some are found to belong to alkaline or purple acid phosphatases category (Jog et al, 2005). They can be grouped as either 3 or 6-phytases, their pH optima ranges from 4.5 to 6.0, the specific activity also varies from 43 to 636 U/mg protein while the temperature optima is about 38–55 °C. The Km and Kcat fluctuate from 30 to 300 mM and 43 to 704 s−1 respectively. Phytases have been isolated from several legumes, oilseeds, cereals, fruits and vegetables (Viveros et al, 2000; Phillippy and Wyatt, 2001). Intrinsic phytases from cereals and grains have been exploited for use in animal feed (Han et al, 1997; Fredlund et al, 2003; Porres et al, 1999). Though several reports on animal phytases exist, very few have been characterized (Bitar and Reinhold, 1972). Animal phytases are active at physiological temperatures and neutral to alkaline pH. They show Km of about 0.03 to 2.6 mM. Intestinal phytase from poultry demonstrated pH optima of 5.5-6.0 (Maenz and Classen, 1998) and 3.5–4.5 in hybrid striped bass (Ellestad et al, 2002a; Maenz and Classen, 1998);

however its in situ implication has been neglected due to availability of advanced supplemental phytases (Maenz and Classen, 1998; Ellestad et al, 2003). Some of the ruminal microbial phytases have been misinterpreted as that of animals (Wise and Gilburt, 1982; Yanke et al, 1998).

Table 5: Overview of biochemical properties of different phytases.

Phytase source	**Mol wt (kDa)**	**Temp opt**	**pH opt**	**Km (mM)**	**pI**	**Specificity**
Fungi						
A. fumigatus	75	58	5.0	-	8.6	-
A. niger	85	58	2.5, 5.0	0.040	4.5	P
A. niger SK-57	60	50	5.5, 2.5	0.0187	-	P
A. niger	-	55	5.5	0.33	4.2	-
A. niger	353	55	2.5	0.606	-	P
A. oryzae	120-140	50	5.5	-	-	B
A. nidulans	77.8	55	5.5	-	-	-
R. oligosporous	-	55	4.5	0.150	-	-
A. niger ATCC 9142	84	65	5.0	0.10	-	B
R. oligosporous	124	65	5.0	0.01	-	B
P. lycii	72	50-55	4.0-4.5	-	3.6	-
Ceriporia sp	59	55-60	5.5-6.0	-	7.36-8.01	-
Agrobyce pediades	59	50	5.0-6.0	-	4.15-5.2	B
Trametes pubescens	62	50	5.0-5.5	-	3.6	-

Thermomyces lanuginosus	60	65	7.0	0.11	4.7-5.2	B
Theroascus aurantiacus	-	55	-	-	-	-
Rhizomucor pusilis	-	70	5.4	-	-	B
Myceliopthora thermophile	-	37	6.0	-	-	B
Sporotrichum thermophile	456	60	5.5	0.15	4.9	B
Yeast						
Saccharomyces cerevisiae	-	45	4.6	-	-	-
Schwanomyces castelli	490	77	4.4	0.038	-	B
Arxula adeninivorans	-	75	4.5	0.25	-	P
Candida krusei WZ OO1	330	40	4.6	-	-	-
Pichia anomala	64	60	4.0	0.20	-	B
P. rhodanesis	-	70-75	4.0-4.5	0.25	-	-
P. spartinae	-	75-80	4.5-5.0	0.135	-	-
Bacteria						
Aerobacter aeerogenes	-	25	4.0-5.0	0.135	-	-
Bacillus sp DS 11	-	70	7.0	0.55	5.3	P
Bacillus subtilis	37	60	7.5	-	-	-
B. subtilis	38	60	6.0-6.5	-	-	-
B. subtilis	43	55	7.0-7.5	-	6.5	P
B. subtilis	44	55	6.0-	-	5.0	P

			7.0			
B. licheniformis	47	65	6.0-7.0	-	5.1	-
B. amyloliquefaciens	44	70	7.0-7.5	-	-	-
E. coli	42	55	4.5	0.13	6.3-6.5	P
Klebsiella oxytoca	40	55	5.0-6.0	-	-	-
K. aerogenes	700	65	4.5	-	3.7	P
Pseuedomonas syringe	47	40	5.5	0.38	-	P
L. sanfranciscensis	50	45	4.0	-	5.0	B

B-broad spectrum, P-phytate specific

- Purification

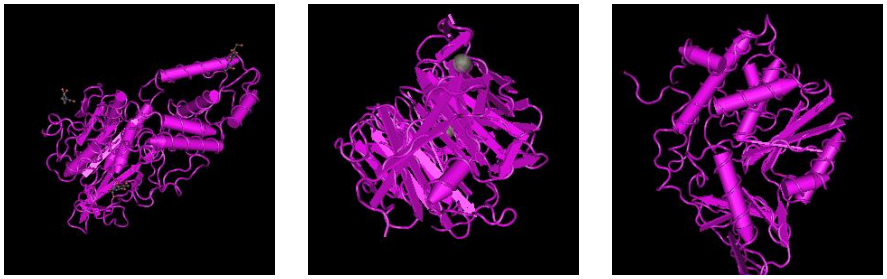
In order to study properties and applicability of phytases, it was essential to purify phytase from various sources, since the properties tend to change with respect to the source of phytase. Not many phytases have been purifies to homogeneity. Generally, purification procedures for phytases involves common protocols such as ammonium salt or acetone precipitation, gel filtration, chromatography (affinity, hydrophobic or ion-exchange) etc. among many difficulties in phytase purification, the most important one is removal of non-specific acidic phosphatase enzymes acting as contaminants (Konietzny et al, 1995). Several different methods have been used for purifying and recovering phytases from various sources. Phytase from *Mucor hiemalis* was purified in five steps to achieve a purification factor of 14.1 and recovery of 51% (Boyce and Walsh, 2007). Purification of phytase was achieved in three steps viz. SP sepharose, Mono Q and single pass in *Aspergillus*

niger (Spier et al, 2011), the recovery was found to be 6.35 % and purification factor was calculated as 10.1. Similarly, two phytases (phytase 1 and 2) from *Rhizopus oligosporus* were purified to yield recovery of 1.3 % and 1.6 % respectively whereas the purification factor was obtained to be 75 and 46 respectively (Azeke et al, 2011). 98.5 % phytase recovery was obtained by Bhavsar et al (2012) by using aqueous two phase system. The purification system consisted of a combination of polyethylene glycol (PEG) 6000 and 8000 (10.5%) and sodium citrate (20.5%) which was successful in partitioning of phytase with a purification factor of 2.5.

- Crystal structure

  Crystallographic studies of phytases have revealed significant information with respect to structure and its biophysical characteristics. Studies on crystal structure of phytase from *A. ficuum* indicated presence of three different domains, viz. a large α-helical domain (consisting of 5 α-helices), β-sheet domain (containing eight β-sheets), and a small α-helical domain (containing four α-helices) visualized at a resolution 2.5 A° (Kostrewa et al, 1997). Crystallographic studies on *Escherichia coli* phytase under a resolution of 2.5 A° revealed presence of two domains with one domain consisting of five α-helices and two β-sheets while the other possesses six α-helices and nine β-sheets (Lim et al, 2000). *B. amyloliquefaciens* phytase (TsPhy) crystal structure possesses a six-bladed β-propeller where each blade contains four or five-stranded antiparallel β-sheets. This enzyme has seven Ca^{+2} binding sites (two near periphery, one at channel in center and four near top of molecule). It lacks presence of

conserved sequence repeat regions like several β-propeller structures. Crystallographic studies have been proved vital in studying mechanism of Ca^{+2} binding to the TsPhy and its effect on thermostability of the enzyme. Crystal structure also revealed another dual specificity phosphatase from *Selenomonas ruminantium* phosphatase (SrPhy). It contains a conserved cysteine (C241) in its P loop, with presence of two diverse crystal packing arrangements of the SrPhy-inhibitor (myo-inositol hexasulfate) complex. The inhibitor can bind to two conformations viz. inhibited and standby conformations. Several studies indicated that the phytase in the P loop pulls the substrate towards itself and subsequently hydrolyses it via a yet unknown mechanism (Chu et al, 2004). The aforementioned classes of enzymes are depicted in figure 16.



Phy A Propeller phytase SrPhy

Figure 16: 3D structures of different phytases.

With the invention of deglycosylation methodology in 1996 during studies on recombinant glycosidase the new windows for detailed studies on enzyme crystals were made open (Wyss et al, 1999). Kostrewa et al (1999) was the first to exploit this method and obtain crystals of *A. niger* NRRL 3135 phyA and successfully determine its crystal structure. The formation of disulfide bonds (among 10 Cys residues) was elucidated following the availability

of phy A crystal structure. A large α/β and a smaller α- domain are characteristics of the structure. Amino acids crucial for catalysis are contained in a cavernous serration which is located at the interface of two domains. Phytase (phy B) from *A. niger* T213 has also been crystallographically studied (Kostrewa et al, 1999). It is a tetramer (consisting of two dimmers) unlike other phytases. It also contains five conserved disulfide bridges amongst which three resemble those found in phytase A. Regardless of the evident similarity among phytases A and B, their pH profiles show variability. PhyA displays the pH optima of 2.5 and 5.0, while phyB shows optima at pH 2.5 with absence of activity at pH 5.0, this can be attributed to the charge differences at substrate specificity sites of the enzyme (Kostrewa et al, 1999). Studies in recent times regarding crystal structures of 6-phytase from *E. coli* and 3-phytase from *A. niger* phy A could prove to be useful in elucidating the mechanism of catalytic variation amongst these enzymes (Jia et al, 1998).

1.6 The Quest for invincible phytases

Due to public understanding of environmental damage caused by animal husbandry many countries have started regulating the excretory phosphorus concentrations. The intensification of this movement will lead to increased exploitation of phytases in animal diets to improve mineral phosphate bioavailability thus reducing pollution via excretion. Even though phytase research has grown and flourished among last 15 years, very few phytases with desired properties have been discovered. To meet the current requirements of environment and nutrition a large amount of significant research

in phytases is needed. Advanced studies to identify new, efficient and cost friendly phytases must be taken up or else approaches such as identification of new native phytases from microorganisms or plants can be exploited or alternatively genetic modification of available phytases can be tried out.

- Exploiting new native phytases

  Though phytases are known to be produced by several plants, bacteria, fungi and animal tissues; microbial phytases (*A. ficuum* (Gibson, 1987), *a fumigatus* (Pasamontes et al, 1997) or *Mucor piriformis* (Howson and Davis, 1983), *Rhizopus oligosporous* (Casey and Walsh, 2004) and *Cladosporium* species (Promdonkoy et al, 2009)) are the ones widely studied and applied at a large scale. The quest for superior enzyme characteristics such as thermostability, catalytic efficiency, wide pH profile etc acted as a driving force for several research activities such as cloning, production and purification of *A. fumigatus* phytase gene to obtain enzyme capable of retaining 90 % activity after treating at 100 °C for 20 min; another example is that of cloning and expression of a novel phytase from *A. niger* N-3 in *P. pastoris* wherein the enzyme retained about 45% activity after treating at 90 °C for 5 min furthermore, the enzyme exhibited more affinity for sodium phytate than for *p*-nitrophenyl phosphate. It also showed two pH optima (2 and 5.5); the activity at pH 2 being more than that of at pH 5.5 by 30 %. A set of novel genes indicative of superior thermostability and tremendous commercial potential was detected in *A. japonicas* viz. BCC18313 (TR86) and BCC18081 (TR170), respectively (Phillippy and Mullaney, 1997). Due to enhanced applicability at commercial scale other microbial phytases have

also been cloned and identified. The search for phytase highly active at low temperature and neutral pH lead to isolation of two phytases from *Pedobacter nyackensis* MJ11 CGMCC 2503 and *Erwinia carotovora var. carotovota* ACCC 10276. The phytase from *Pedobacter* shares 28.5 % similarity with phytase from *B. subtilis* (BPP family). A recombinant *E. coli* phytase showed higher activity and greater efficiency of soybean meal digestion at neutral pH (temperature of reaction being 25 °C) than *B. subtilis* and various commercial phytases; suggestive of its tremendous potential in aquatic feed supplements. Other phytases with prospective application in aquaculture (Greiner and Konietzny, 2006) belong to *Erwinia* and *Klebsiella pneumoniae* who share about 50 % amino acid identity and contain a conserved active site hepta-peptide motif (RHGXRXP) and the catalytically active dipeptide (HD) characteristic of HAPs (Huang et al, 2009a,b). Furthermore, due to its low temperature activity, it can be used as a model to study the structure-function relationship of the enzyme. The phytase showing wide pH stability (pH 1.5-11) from *Yersinia kristeensenii* was cloned and expressed in *P. pastoris*. This recombinant phytase possesses remarkable potential for applicability at commercial scale (Fu et al, 2008). Owing to growing popularity of phytases in animal feed industry (enhance phytate phosphorus availability in monogastrics), several yeast phytases have been isolated and identified to (*Kodamaea ohmeri* BG3 (Li et al, 2009), *Pichia anomala* (Kaur et al, 2010) and wastewater treatment yeast *Hansenula fabianii* J640 (Watanabe et al, 2009)).

- In pursuit of ideal phytase

With the increasing availability of diverse phytases the quest is on for a single or ideal phytase possessing abilities such as thermostability, resistance to proteolysis, enhanced catalytic efficacy and most importantly commercial viability (Lei and Stahl, 2001), though realization of this dream is yet to be achieved. It can be accomplished by following two methodologies viz. coherent protein design and planned molecular evolution to yield enzymes with desired characteristics (Bottcher and Bornscheuer, 2010; Chica et al, 2005).

1. Thermostability: due to high pelleting temperature (60-80 °C) requirement of animal feeds it is essential that all feed enzymes retain significant activity after the process. An interesting approach to enhance the thermostability of enzyme is by exchanging multiple amino acids thus increasing the unfolding temperature of protein. Traditional approach to improve thermostability is based on sequence analysis with other thermostable enzymes and subsequently replacing the chosen amino acids. To confer thermostability to an enzyme various approaches have been tried including phasing in extra disulfide bonds, stabilizing the bonding of the hydrophobic core thus strengthening it, modifying surface salt bridges or helical characters such as dipole interactions, propensity and entropy (Haney et al, 1999; Tomschy et al, 2000a,b).

2. Catalytic activity: In an attempt to improve the catalytic activity, 300^{th} amino acid residue of phytase from *A. niger* NRRL 3135 was subjected to site directed mutagenesis. The single mutagenesis resulted in enhancing the phytase activity at pH 3.0 and 5.0 whereas another mutation (K300E) in which amino acid lysine was

replaced by an acidic amino acid lead to increase in hydrolysis of phytate at pH 4.0 and 5.0 but interestingly, no change in activity (at pH 4.0) was visible when lysine was replaced by a basic amino acid though decrease in activity was evident when lysine was replaced by arginine (over a pH range of 2.0 to 6.0) (Mullaney et al, 2002).

3. Resistance to proteolysis: An important pre-requisite for a feed enzyme is stability in digestive tract of the animal, which can only be achieved through possession of powerful proteolytic resistance for enzymes present in the gastro-intestinal tract of the animals. Phytases from various origins exhibit distinctive sensitivity to digestive enzymes such as pepsin and trypsin. While bacterial phytases possess superior proteolytic resistance, fungal phytases aren't much privileged. The enzyme sites responsible for conferring proteolytic resistance are situated in exposed loops at the enzyme surface and can be targeted for improving resistance to proteolysis in fungi (Wyss et al, 1999). Structure directed mutagenesis approach was used for improving the pH profile in *A. niger* phytase (phyA) (Lim et al, 2000). The recombinant *A. niger* phytases expressed in different hosts reported increased sensitivity to protease digestion due to exposure of loop structures on the molecular surface. Thus site directed mutagenesis was used to modify susceptibility to protease digestion in several phytases from *Aspergillus fumigates*, *Emericella nidulans* etc. with no changes in the enzyme specific activity. The recent developments in molecular design and engineering have lead to achieving desired enzyme properties to be brought together into a single enzyme molecule. Some efforts on improving the activity of phytase by studying the

role of amino acids near catalytic or substrate binding site have yielded success with the use of site directed mutagenesis (Tian et al, 2010). Molecular approaches such as error-prone PCR have also been used in E. coli to improve the thermostability, thus enhancing its commercial importance (Zhu et al, 2010). Mutagenesis approach was also applied for *Penicillium sp*. Phytases to increase thermostability, pH and temperature optima (Zhao et al, 2010). Structure based approach was also applied for increasing optimal pH range in β-propeller phytases (Viader-Salvado et al, 2010).

- Immobilization

  Phytases catalyze sequential hydrolysis of *myo*-inositol hexakisphosphate to produce lower phosphate derivatives. Hence, the idea of continuous utilization of this ability of phytase was thought of, moreover fungal phytases have tremendous catalytic turnover number (220-1000 per second) to liberate various lower isomers, consequently exploiting this ability at industrial scale could be achieved through immobilization of the enzyme. A very small number of reports of phytase immobilization are available (46, 139). Catalytic characteristic such as pH dependence is not affected after immobilization however; thermal stability was found to be enhanced in several cases. Furthermore it can also be regarded as an alternative to improve storage stability for a period of several months. Immobilization of phytase from *A. ficuum* in gelatin gels hardened by formaldehyde lead to increase in pH optima from 58 °C to 80 °C, increase in *Km* while the residual activity was found to be 34.6 % (Liu et al, 1999).

1.7 Applications of phytases

Phytases have multiple applications ranging from human to plants and other environmental advantages.

- Probiotics

Live microbes thought to be helpful for host organisms are termed as probiotics. They are generally ingested as a component or supplement to fermented foods specially designed to contain live active cultures of microbes. The microbes generally used for human and animal consumption have a perquisite to be regarded as safe, as are several phytase producing yeasts and fungi. *S. cerevisiae* is the most commonly used probiotic ingredient and can be also used for enhancing phosphorus assimilation (Nayani and Markakis, 1984). *Candida tropicalis* a common pathogen in humans is not regarded as safe for probiotic use however; it is found to produce phytase which can either be cloned or expressed in a "safe" organism (eg. *Lactobacillus*, *Brevibacterium casei* and *Brevibacterium epidermidis* etc.). To be applied as probiotic or else efforts to lessen their pathogenicity must be undertaken (Hirimuthugoda et al, 2007).

- Human nutrition

Several developing countries have been battling mineral paucity in diets which are caused due to changing food lifestyle. Manufacturing and processing of foods is also an area of phytase application. The market is yet to receive a significant phytase food product. Several studies improving mineral content and its assimilation are in progress. Due to its characteristics, presence of phytate in foods is found to prevent mineral uptake (Hurrell et al,

2003). Due to limited phytate digestion potential of the small intestine, humans suffer several nutritional adversities. Phytate chelates various mineral ions at acidic pH of the stomach making them insoluble thus altering nutrient uptake in the digestive tract (Costello et al, 1976). The phytases present in human gut viz. alkaline phosphatase and mucosal phytases do not contribute much towards phytate breakdown, hence the need for dietary phytase arises (Sandberg and Anderson, 1988).

- Bakery

  Phytase have been applied in bread-making processes since a long time. Several studies on application of phytase in bread making have yielded significant role of fungal phytase in improving bread volume, quality and texture as well as reducing bread fermentation time without pH alterations (Haros et al, 2001). Due to emerging trends for use of fibre rich foods, whole grain derived breads are in demand. These are derived from bran and retain high phytic acid content (Brune et al, 1992). Phytase supplementation reduces phytic acid content of dough as well as improves bread shape and crumb softness, with significant increase in nutrient bioavailability.

- Iron absorption

  Iron deficiency is among the major causes of malnutrition all over the world. Cereal and legume based diets are significantly loaded with phytic acid, which inhibits iron absorption thus evoking the need for a more sustainable approach. Using phytase as food supplements or alternatively cloning phytase gene in plants used as staple foods is suggested. One such example includes that of

transgenic rice endosperm containing phytase gene (phy) from *A. fumigatus* (Lucca et al, 2001).

- Animal nutrition

  Monogastrics lack phytases in their gut consequently making them inadequate assimilators of phosphorus and other minerals, hence calling for phytase supplementation in animal feeds as a better alternative. Phytases are given the impression of being eco-friendly alternatives for reducing phosphorus pollution from undigested phytate in manure from animal husbandry industry (Vats et al, 2005). Several countries have approved the use of phytase as a feed supplement while the FDA has granted it a GRAS status (Wodzinski and Ullah, 1996). Phytase has been widely used as a feed supplement in various animals such as pig, poultry and fish diets. Innumerable reports are available demonstrating the effect of phytase supplementation in animal diets (Zyla et al, 2004). Enhancing nutrient assimilation and elimination of its deficiency could be more promising areas for combined application studies of bacterial and fungal phytases. However, a pre-requisite for use of phytase in animal feed include resistance to high temperature, since during pelleting of feed the temperature reaches about 90 °C. Phytase supplementation can reduce phosphorus excretion by about 25 to 50 % when factors such as diet, species and supplemental phytase concentration are considered (Kornegay, 1999). To circumvent the effect of dietary phytate, supplementation of animal feed with phytase is suggested to improve phosphate assimilation by animals. Many commercial phytase preparations are available for use in animal feed from *A. ficuum* (Natuphos), *A. niger* (Allyzyme), *A. awamori* (Finase and Avizyme), *A. oryzae* (SP, TP,

SF, AMAFERM and Phyzyme) and *P. lycii* (Ronozyme, Roxazyme and BioFeed phytase).

- Fish feed

  Phytases in aquaculture

  As in the case of monogastric animals, dietary phosphorus also plays a significant role in aquaculture. Optimal fish growth owing to effective utilization of aqua feed is the indicator of flourishing global aquaculture. Several reports on studies involving supplementation of phytase in aqua feed indicated positive interference leading to enhanced assimilation of biominerals and in turn decreasing the phosphorus load in the excreta thus proving to be an eco friendly alternative. (Nwanna and Schwarz, 2007; Vielma et al, 2004).

  Aqua culture industry is one of the most promptly growing industries. Traditionally used feed such as fishmeal is very costly and thus an alternative is essential. Replacing fishmeal with plant derived foods or by-products are a viable option. But the inevitable presence of phytic acid is bound to cause some anti nutritional problems. Alternatively, supplementing the plant based diet with phytase can help alleviate the problem of mineral bioavailability and also reduce environmental phosphorus pollution. One such example is that of rainbow trout wherein phytase supplementation improved nutrient content of canola protein concentrate in addition to decreasing phosphorus excretion (Forster et al, 1999). Some other reports are also available from channel catfish (Li and Robinson, 1997), African catfish (van Weerd et al, 1999) and *Pangasius pangasius* (Debnath et al, 2005).

Poultry and pig feed

Phytase supplementation enhances mineral bioavailability and phosphorus assimilation (Selle et al, 2007; Ahmadi et al, 2008). It also improves feed digestibility and gross energy (Cheng and Hardy, 2002). Studies on broiler diets supplemented with phytase increased phosphate retention and decreased phosphorus excretion in environment (Pillai et al, 2009). Similar observations were recorded for studies in pig (Hill et al, 2009).

- Plant growth promotion

  Many plants have adapted several means to enhance the assimilation of phosphorus and other minerals in the soil. Since rhizospheric phytase activity is very low, plants cannot efficiently digest phytate, thus limit phosphorus uptake (Richardson et al, 2000; Hayes et al, 2000). Phytase producing organisms can be used as biofertilizers to assist phytate degradation thus increasing phosphorus assimilation in plants. Some fungi producing phytases and phosphatases were exploited as bioinoculants to enhance phosphorus nutrition in plants grown in high phytate soils (Yadav and Tarafdar, 2003). *S.* thermophile producing phytase has been used for promoting plant growth in wheat (Singh and Satyanarayana, 2010). *B. amyloliquefaciens* and *B. mucilaginosus* have been reported to promote plant growth in maize and tobacco respectively (Li et al, 2007; Idris et al, 2002). Another interesting approach is to use phytase producing microbes to degrade phytate in poultry and fish waste, which may lead to improving the phosphorus mineral availability in manures. Yet another idea could be cloning of phytase genes in conventionally used biofertilizers

such as *Pseudomonas*, *Rhizobium* etc, thus equipping them with absolute fertilizer ability. Alternatively, plants can be directly subjected to phytase gene cloning, thus making them equipped to digest phytate on their own. More detailed description of this approach is discussed under transgenic plants.

- Transgenic plants

  Transgenic plants expressing higher amounts of phytases are turning out to be a precondition for improving phosphorus availability in feeds or in supplementation to microbial phytases. Several studies have shown comparable results for use of transgenic plants expressing phytase to be similar if not better when compared with direct feed supplementation of microbial phytase with respect to phosphorus assimilation. Phytase gene from *E. coli* was over-expressed in micro alga *C. Reinhardtii* and used as a food additive (Yoon et al, 2011). With commercial viability considerations, transgenic plants have been thought to be potential bioreactors for recombinant phytase production at industrial level. The presence of post translational machinery in plants proves to be a boon for producing functional proteins as compared to prokaryotic counterparts (Gontia et al, 2012). Besides, tissue specific secretion of phytases in plants can enable targeted approach to overcome phosphorus limiting conditions. Expression of phytases from *A. niger* in roots tissues of *Arabidopsis* (Mudge et al, 2003) and in soybean plants from *A. ficuum* (Li et al, 2009b) helped increase phosphorus assimilation in these plants, thus provided a potential alternative for use as phosphorus fertilizer in agriculture.

- Transgenic animals

  Environmental phosphorus pollution caused due to manure generating from animal husbandry is a major cause of concern throughout the world. One of the approaches to overcome this problem was developing transgenic animals such as mice and pig expressing phytase gene from *E. coli* (appA) in salivary glands (Golovan et al, 2001a; Golovan et al, 2001b). This method was capable of reducing phosphorus load in manure by about 11 % and 75 % in transgenic mice and pigs respectively. Hence, this approach was found to be a promising alternative for dietary supplementation of phosphorus.

- Industrial applications

  Paper and pulp industry

  Phytase has been used to remove phytate from plant raw materials used in paper and pulp industry (Liu et al, 1998). However, phytases active at higher temperatures could be of much more significance for the process of phytate hydrolysis in this industry. Phytase can also be used in multienzyme blends used for treating pulp (Maheshwari and Chandra, 2000). Moreover, phytase based enzymatic degradation of phytate would not produce carcinogenic or toxic by-products thus proving to be an eco-friendly approach.

  Fractionation of cereal bran

  Phytase has also been applied bran milling which is a by-product of flour making from cereal grains. Being very nutritious, a huge amount of research focuses on producing distinct fractions consisting of high protein content, soluble carbohydrates, oil and

insoluble fibre etc. Several enzymatic processes using phytases followed by wet milling and extraction are done on the bran to obtain the desired industrially and commercially viable product (Greiner and Konietzny, 2006). Phytate is an unwanted ingredient in corn steep liquor interfering in concentration process and other processes such as production of enzymes, amino acids, alcohol, polysaccharides, antibiotics as well as energy based animal liquid foods (Greiner and Konietzny, 2006). Phytase together with other hydrolytic enzymes can be successfully used to phytate free liquor along with value added by-products such as animal feed (Caransa et al, 1988).

- Myo-inositol production and its pharmaceutical applications

  Phytases for the production of lower myo-inositol phosphates

  Lower myo-inositol derivatives have tremendous significance in plant and animal cells with respect to membrane transport and signaling. (Berridge and Irvine, 1984; Michell, 1975; Dasgupta et al, 1996; Krystofova et al, 1994; Samanta et al, 1993). Extensive research and growing need for inositol derivatives generated the need for their large scale production. But enzymatic synthesis of derivatives was preferred due to several factors such as high cost, harsh reaction conditions, stereospecific purification and yield of the desired product (Billington 1993). Various applications of myo-inositol derivatives include prevention of diabetic complications (Carrington et al, 1993), anti-inflamatory properties (Claxon et al, 1990), anti-cancerous (Maffucci et al, 2005), cholesterol regulation, atherosclerosis (Jariwalla et al, 1990) and kidney stone formation inhibition (Grases et al, 2000).

Phytate being the store house of myo-inositol can be exploited as a value added product. Myo-inositol is used as an emulsifying agent and is an important secondary messenger (Thorsell et al, 2008). Deficiency of myo-inositol may lead to buildup of triglycerides and abnormal fatty acids (Holub 1992). Myo-inositol is also used in infant foods due to its nutritive value (Pereira, 1990). Myo-inositol is very costly nutrient supplement with price for 750 mg quoted to be as $16.95 (http://www.vitasprings.com/inositol-myoinositol-100-caps-jarrow.html.). Several attempts to chemically synthesize phosphate isomers using chemical means have resulted in production of a racemic mixture; further isolation and purification of desired derivatives being tedious, costly and thermo-sensitive. In contrast, phytases from various microbial sources can be effectively exploited to synthesize the desired product. Different phytases synthesize various position specific isomers of phosphate derivatives of myo-inositol (Chen and Betty, 2003). Use of immobilized phytases followed by anion exchanger can also be a different but promising approach for isolation of pure desired products.

- Semi synthesis of peroxidase

  Peroxidases catalyze several oxidation reactions using primary oxidant hydrogen peroxide. The structural similarity of haloperoxidases with acid phosphatases lead to development of semi synthetic peroxidises. The active site of phytases was modified by insertion of vandate ion enabling it to selectively oxidise prochiral sulphides (van de Velde et al, 2000). Several phytases from *A. niger, A. fumigates, A. nidulans* have been incorporated with vanadium to produce semi synthetic peroxidises.

- Phytase as a soil amendment

  Phosphorus is an essential limiting nutrient which governs the agricultural productivity worldwide. Organic phosphorus accounts to about 30–80% of the total phosphorus in soils (Harrison, 1987). 50 % of the total organic phosphorus in soil is in the form of phytate which is not properly utilized by plants (Anderson, 1980). Several plants viz. tobacco (Lung and Lim, 2006), tomato, barley (Asmar, 1997), alfalfa etc (Li et al, 1997) have been reported to secrete phytase in response to stress conditions. Some reports have confirmed the ability of plants to utilize phytate phosphorus upon supplementation of phytase or phytase producing microbes to plants grown under limiting phosphorus or phytate containing media.

- Other applications

  Ever since its discovery and commercialization, the primary application of phytase has been as an animal feed additive to enhance phosphorus nutrition of plant based diets in animals by enzyme hydrolysis of phytate. It also reduces the phosphorus load in animal excreta thus proving to be an effective environmental friendly approach. Use of phytase based approach leads to decreasing the need for inorganic phosphorus supplementation in the feed thus reducing the overall costing of feed.

  Food regulations in European countries such as restrictions on dietary supplementation of meat and bone meal have embarked the economic as well as social significance of phytase as a feed additive. Plant phytate yield accounted to about 51 million metric

tons in year 2000 which amounts to 65 % of the sale of inorganic phosphorus fertilizers globally (Lott et al, 2000). Interestingly, phytase can convert phytate from plant origin to value added phosphorus moieties with enhanced bioavailability. Many countries including Netherlands and Denmark have introduced policies for encouraging phytase use. The potential of phytase in improving phosphorus availability has lead to emergence of various biotechnological innovations. Even though the role of phytases in biotechnological industry has been highlighted, very few phytases have been studied and commercialized; hence the need for more research on phytases is emphasized.

1.8 Future prospects

- P: Boon and Bane

  Phosphorus is a natural nutrient whose supply is very limited attributing to its biogeochemical cycle which lacks a gaseous phase. To add to this problem, some scientists have predicted the exhaustion of natural phosphate reserves in the coming 50 years. Being an essential element for life, phosphorus resources have forever been badly managed keeping food and water safety on the line. Phosphorus resource depletion has been put at par to oil depletion which was termed as a sensational catastrophe some years ago. Being an important ingredient of foods and fertilizers phosphorus starvation is estimated to affect about two third of global population. Phosphorus cannot be substituted for nor can it be synthesized. Even though phosphorus is an essential nutrient for life to exist, its scarcity or surpluses have deleterious effects on

living organisms and environment. Thus the importance of phosphorus recycling to uphold the survival is envisaged. Use of existing but unexploited alternative sources of phosphorus such as phytate etc might prove to be a significant approach. In the view of that, phytase may substantiate into a larger share.

- Phytase on its part

  Phytases have gained importance as ecofriendly alternatives to reduce phosphorus load in the environment along with decreasing artificial phosphorus nutrient supplementation in animal diets. Moreover since phytase is capable of hydrolyzing inositol hexa kis phosphate and its derivatives, it has been highly acknowledged for reversing the antinutrient properties of phytate. Phytases also have tremendous potential for applicability in cell signaling pathways and its regulation because several reactions such as transmembrane signaling and calcium mobilization are driven by inositol phosphate derivatives. Use of phytase in aquaculture industry is looked towards as the next boom in the coming years to facilitate use of economical plant based diets. Some other areas of phytase applicability include that of plant growth promoters, soil amendment and use as peroxidase. The increasing popularity of phytases has lead to the quest for new and novel phytases from various sources. In order to be exploited at an economical scale, phytases need to be improved with respect to biochemical properties (pH and temperature optima, substrate specificity etc.), product yield (production cost) and physiological characters (pH range, thermostability, proteolytic resistance etc.); which can be effected through use of protein and genetic engineering techniques.

- Need for more research

  Even though several bacteria and yeast are reported to produce phytase, fungal phytases are the ones mostly used in animal diets due to its properties such as high yield and physiological suitability (acidic tolerance). Most of the commercial preparations available are fungal phytases produced recombinantly under submerged fermentation conditions and are active at pH 5 but those active at pH 2.5 are scarce. However, several shortcomings encircle them including cost of product recovery, low yields, thermosensitivity and low pH susceptibility. Thus these phytases have a limited applicability in animal feed due to inadequate performance in the physiological environment of the animal body.

  Though fungal phytases are the ones extensively applied at industrial scale, the problem of production cost with respect to product yield persists. Hence there is a need for hypersecretory strains capable of producing phytase in a low cost high phytate media such as plant based raw materials.

  Submerged fermentation provides higher productivities, easy operation and scale-up. It can also be combined with simple downstream processing such as filtration wherein the filtrate can be used for further applications such as mixing with feed or encapsulation in matrix or as a biofertilizer.

  Phytase production from *Aspergillus niger* NCIM 563 was reported by my colleagues under both SmF and SSF using rice bran and wheat bran respectively, however their productivities were very low and thus the need for strain improvement arose (Mandviwala and Khire, 2000; Soni and Khire, 2007). Furthermore,

improvement of production of phytase would be highly desirable considering its economical significance.

In this work, our objective is to appraise the relevance of combinatorial mutagenesis approach for strain improvement to enhance phytase activity under submerged fermentation conditions. The present work also reports the production of phytase by the mutant strains on various plant based media and its potential for use at industrial scale. Since application at industrial scale require complete profiling of the production strain studies on genetic, morphological and physiological properties of mutants are also included in this thesis as a comparative account. Keeping in mind the emerging trend of genetic engineering and recombinant technology, the gene for phytase synthesis was isolated and studied from both parental and mutant strains and is included in the thesis as a comparative account. Purification and characterization of phytase along with studies of its application are also included in the thesis.

1.9 Aims and Objectives

For an organism to be industrially applicable, it should possess characters such as high productivity, amenable to scale up, non-toxic or eco-safe. Since *A. niger* has been bestowed by GRAS (generally recognized as safe) status, it has been exploited at industrial scale by many scientists ever since. The immense curiosity revolving around acidic phytase from *A. niger* NCIM 563 and its prospective commercialization drove us to rummage around for exploiting an alternative approach of combinatorial mutagenesis to isolate hypersecretory mutants with tremendous industrial potential. With several phytases being reported and studied from various organisms, their commercial availability has been scarce; moreover acidic phytases are yet to be industrialized.

Objectives of the study: "Studies on acidic phytase from *Aspergillus niger* mutants" was taken up with the following objectives.

1. Strain improvement, screening and isolation of mutant capable of enhanced phytase production.
2. Production of phytase under submerged fermentation conditions using different plant based media: A comparative account.
3. Genetic, morphological and physiological characterization of mutants: A comparative account.
4. Purification and characterization of phytase from mutants.
5. Gene profiling, isolation and partial characterization.
6. Application of phytase in plant growth promotion, soil amendment and functional foods.

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CHAPTER 2 STRAIN IMPROVEMENT

Abstract

Strain improvement by mutagenesis and selection is a highly developed technique and it plays a central role in the commercial development of microbial fermentation processes. This chapter includes studies on strain improvement of *Aspergillus niger* NCIM 563 for enhanced phytase production under submerged fermentation conditions. The use of classical approach for strain improvement of *A. niger* NCIM 563 included a combination of physical (UV) and chemical mutagenesis (EtBr and Hydroxyl amine) which resulted in obtaining two mutants P16 (*A. niger* NCIM 1359) and P33 (*A. niger* NCIM 1360) which were found to be superior to parent strain as they produce 156 and 95 U/ml phytase activities on 10.5^{th} day and 7^{th} day, respectively as compared to 68 U/ml of phytase activity by parent on 11th day. Mutants *A. niger* NCIM 1359 and *A. niger* NCIM 1360 showed increase in activity up to 230 % and 145 %, with increase in productivity up to 2.4 and 2.2 times that of parent respectively.

1. Introduction

Importance of microbial genetics was realized in 1940s during the global bloom of penicillin production. At the beginning the studies pertained to creation and assessment of mutants, however, effortlessness of the technique and its ability to change phenotypic expression of characters lead to unhindered exploitation of microbial genetics. Increase in productivity at industrial scale by thousand folds has been an outcome of

mutagenesis moreover it is also responsible for flourishing of the fermentation industry. Strain improvement plays a central role in the commercial development of microbial fermentation processes. The current practice of strain improvement by mutagenesis and selection is a highly developed technique (Parekh et al, 2000). Mutagenic procedures can be carried out in terms of type of mutagen and its dosage or concentration so as to obtain mutant types that may be screened for improved activity. Microorganisms do not overproduce any metabolite naturally, unless made to do so. For the strain to be used at industrial scale, it must be hypersecreting; so as to make the process economical. Strain improvement carried out through mutagenesis followed by screening or using recombinant DNA technology has lead to higher industrial productivities and simultaneous reduction in production costs in the last few years (Vinci and Byng, 1999). Strain improvement can be achieved by creating strains (i) with ability to utilize inexpensive complex raw materials; (ii) increasing product concentrations or reducing by-product interferences; (iii) extracellular secretion of product; (iv) insensitivity to product inhibition; (v) reduction in fermentation time etc (Parekh et al, 2000).

Two ways by which microbes can create new characters are mutation and genetic recombination. Mutations can either be spontaneous or induced. Mutations can be harmful or beneficial for the organism the former being eliminated by selection. Mutagenesis can be carried out by either physical or chemical methods, while protoplast fusion, conjugation, transformation and recombinant DNA technology are other means of genetic recombination. Mutagenesis approach is widely applied at industrial level to alter concentrations of the metabolite of interest (Parekh et al, 2000), along with other applications such as studies on metabolite pathways,

search for new/novel compounds etc. some of the most potent mutagens include hydroxyl amine, ethyl methyl sulphonate, methyl methane sulphonate (MMS), nitrosoguanidine (NTG), 4-nitroquinolone- 1-oxide and ultraviolet light (UV) (Table 1).

Table 1: Mutagens and their mode of action (Parekh et al 2000).

Mutagen	Characteristic of mutation induced
X rays, Gamma rays	These ionizing radiations causes strand breakage in DNA leading to deletions and structural changes in DNA.
Ultraviolet rays	These short wavelength radiations cause pyrimidine dimerization and crosslinking leading to transversions, deletions and frame shift mutations in DNA.
5-chloro/uracil, 5-bromouracil	Results in faulty pairing leading to AT to GC transitions.
2-aminopurine	Causes errors in DNA replication.
deaminating agents	
Hydroxyl amine	Causes deamination of cytosine leading to GC to AT transition.
Nitrous acid	Causes deamination of A, G and C leading to bidirectional translation and deletion.
N-methy, N'-nitro	Causes methylation leading to GC to AT transition.
N-nitroso guanidine	Causes alkylation of C and A leading to GC to AT transition.
Methyl methyl sulphonate, Ethyl methyl sulphonate	Causes alkylation of C and A leading to GC to AT transition.
Ethidium bromide, acrydine dyes	Causes intercalation between two base pairs leading to frameshifts and microdeletions.

Generally, mutants are obtained by subjecting the organisms to the mutagenic agent for a defined period of time known as kill time (95-99.9 % kill) followed by culture in media, screening and selection for the metabolite of interest (Simpson and Caten, 1979). This mutant screening process ensures isolation of best quality of mutants sans its low success rate (Baltz, 1999).

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is a major storage form of phosphorus in many cereals and legumes, representing 18-88 % of total phosphorus content (Dvorakova et al, 1998; Reddy et al, 1982). Phytic acid acts as an antinutrient as it binds to essential dietary minerals such as calcium, zinc, magnesium and iron decreasing their bioavailability (Ford et al, 1978). Phytases (EC 3.1.3.8 for 3-phytase and EC 3.1.3.26 for 6-phytase) hydrolyse the phytic acid in a stepwise manner to get inositol phosphates, myo-inositol and inorganic phosphate. A large number of micro-organisms produce phytases (Selle & Ravindran, 2007). Since phytase is absent or not present in sufficient amounts in the gut of monogastric animals (Bitar & Reinhold, 1972; Common et al, 1989) the supplementation of phytase in poultry feed is essential to improve phosphorus bioavailability. It also reduces the phosphorus pollution in the areas of livestock production due to decomposition of underutilized phytate (Ravindran et al, 2001). Thus because of its industrial importance the ultimate objective here has been to produce this enzyme at a cost effective level.

Many experiments and field trials have shown that 500 to 1000 units of phytase can replace approx. 1 g inorganic phosphorus supplementation and reduce total phosphorus excretion by 30–50 % (Kemme et al, 1997, Lei et al, 1993, Liu et al, 1997, Yi et al, 1996). Phytic acid is used in pulp industry as an anti-oxidant to prevent aging of paper. Use of phytase in

phytic acid degradation during pulp and paper processing, seems to be an eco-friendly way of waste management (Liu et al, 1998). Importance of soil microbes for increasing availability of phytate-phosphorus to plant roots has been suggested by several researchers (Richardson et al, 2001; Tarafdar & Marscner, 1995; Singh and Satyanarayana, 2010).

Several efforts were made to obtain phytase overproducing strains of industrial value, however, success for acidic phytase (phyB) from *A. niger* are yet to be achieved. A high level of functional expression of *A. fumigatus* phytase gene was achieved in *Pichia pastoris* (Rodriguez et al, 2000). With the aim of improving cost-effective phytase production, the gene phy A from *A. niger* var. *awamorii* ALKO243 was cloned which resulted in several fold overproduction of phytase (Piddington et al, 1993). The phyA gene encoding heat-stable phytase was cloned from *A. fumigatus* and overexpressed in *A. niger* (Pasamontes et al, 1997). Chelius and Wodzinski (1994) carried out strain improvement of *A. niger* for phytase production using UV as a mutagen and resistance to hygromycin B as the selection criteria. However, they could not obtain significant changes in acidic phytase (phyB) production levels as compared to 3.3 fold increase obtained for phytase I (phyA).

Aspergillus niger phytase (PhyA) has been used as a feed supplement to improve the bioavailability of phytate phosphorus in monogastric animals. Due to inability of phytase enzyme to survive at pelleting temperatures, several workers across the globe have been working on improving the thermal stability and other biochemical properties of phytase. Yan Liao and coworkers (2013) used site directed mutagenesis approach to improve catalytic efficiency and thermostability of phytase of *A. niger* N25 wherein they achieved a 20 % improvement in

thermostability in mutants when compared to the wild type strain. They were also able to improve the catalytic efficiency of phytase significantly.

Natuphos, Phytase Novo™ (Cao et al, 2007), Finase (Meittinen-Oinonen et al, 1997) etc. are some commercial phytases obtained from different organisms. All available phytase preparations used as feed additives today are of fungal origin and produced by submerged fermentation (SmF) and are active at pH 5.0. Fungal phytases have serious shortcomings, especially with regard to their sensitivity to heat and inactivation under low pH conditions generally present in the stomach (Afinah et al, 2010). These disadvantages need to be alleviated in the use of fungal phytase as feed enzyme in animal diet. This work is directed towards realizing these aims while simultaneously increasing phytase production rates.

2. Materials and Methods

2.1 Organism

The organism used *Aspergillus niger* NCIM 563 was used throughout the experiments. It was obtained from NCIM Resource Center, India and was maintained on Potato Dextrose Agar (PDA) slants at 4 °C.

2.2 Medium and culture conditions

Fermentation medium for phytase production contained (per 100 ml): rice bran 1 g; glucose 5 g; $NaNO_3$ 0.86 g; KCl 0.05 g; $MgSO_4 \cdot 7H_2O$ 0.05 g; $FeSO_4 \cdot 7H_2O$ 0.01 g, pH 5.5 before sterilization. Fermentation medium (100 in 250 ml Erlenmeyer Flask) was inoculated with 1 % (v/v) of spore suspension (1×10^7 spores/ml) prepared by suspending the spores from 7 day old sporulated slant of *A. niger* NCIM 563 grown on PDA in 10 ml of

sterile saline 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.

2.3 Selection of 3 log kill mutant dose by kill curve

The spore suspension (10^7/ml) was obtained from 7 days old slant of *A. niger* NCIM 563 grown on PDA, by scraping in 10 ml sterile saline containing 0.01 % Tween 80. The spore suspension was treated with mutagen for different time intervals. The appropriately diluted samples were then plated on PDA plates. The Colony Forming Unit per ml (CFU/ml) was used to calculate the 99% kill time.

2.4 Analytical methods

2.4.1 Phytase activity

Phytase activity was measured using 100 mM Glycine-HCl buffer, pH 2.5 at 50 °C for 30 min as described earlier (Bhavsar et al, 2008). One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 μmol phosphorus/min under standard assay conditions.

2.4.2 Protein estimation

Protein concentration in the culture filtrate was determined using the method of Lowry et al (1951) using Bovine serum albumin as standard.

2.4.3 Sugar content

Total residual reducing sugar concentration was estimated by DNSA method (Miller 1959).

2.5 Isolation of A. niger mutant

The spore suspension (10^7 spores ml) was treated with mutagens, both physical (UV) and chemical viz. Ethidium bromide (0.1 mg/ml) and Hydroxyl amine (0.1 %) for different time intervals. The appropriately diluted samples were then plated on PDA plates. The Colony Forming Unit per ml (CFU/ml) was used to calculate the 99 % kill time. Mutants obtained were selected by spreading treated spore suspension on slightly modified phytase screening medium (PSM) agar plates containing 0.5 % calcium phytate and 0.05 % $NaNO_3$. The selection of mutants was based on the enhanced zone of calcium phytate hydrolysis.

3. Results

3.1 Kill Curve

Kill curve calculation is the basic step of mutagenesis experiments. It helps researchers' detemine the concentration or time period of exposure of the mutagenic agent in order for the mutagenesis to succeed. Time interval for 99 % kill was obtained to be 45 min (Fig 1).

3.2 Isolation of phytase producing A. niger mutant

Mutants were selected on the basis of small compact colony with larger zone of hydrolysis on calcium phytate plate as compared to parent strain (Fig 2). All the positive mutants were eveluated for phytase production using rice bran–glucose–salt medium in shake flask condition.

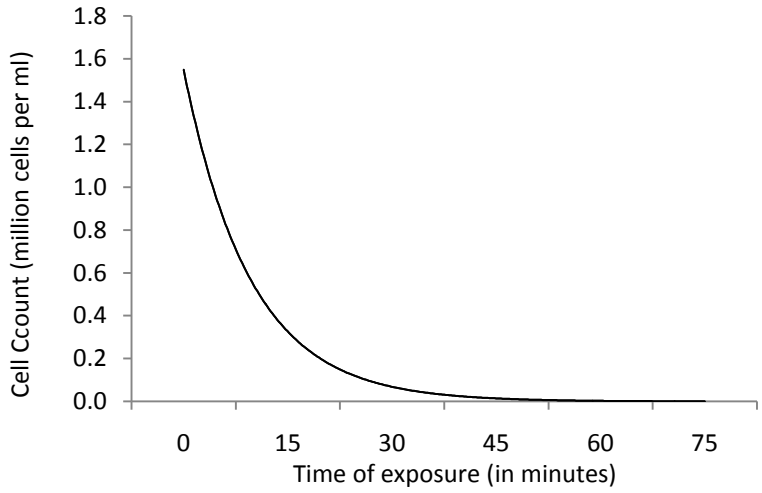

Figure 1: Kill curve of Fungal spores

Mutants P-16 and P-33 were found to be superior to parent strain as they produce 156 and 95 U/ml phytase activities on 10.5th day and 7th day, respectively as compared to 68 U/ml of phytase activity by parent on 11th day (Fig 3). P16 and P33 mutants isolated showed increase in activity up to 230 % and 145 %, with increase in productivity up to 2.4 and 2.2 times that of parent respectively (Table 2).

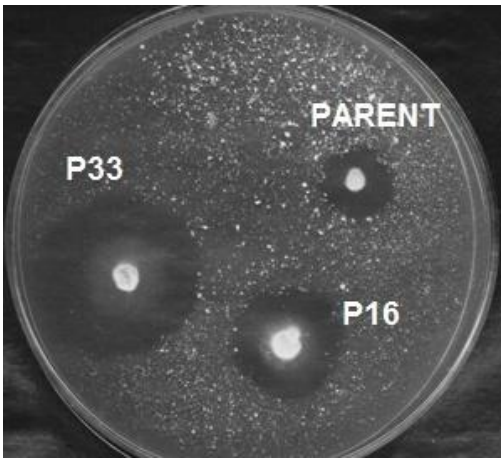

Figure 2: Plate based screening

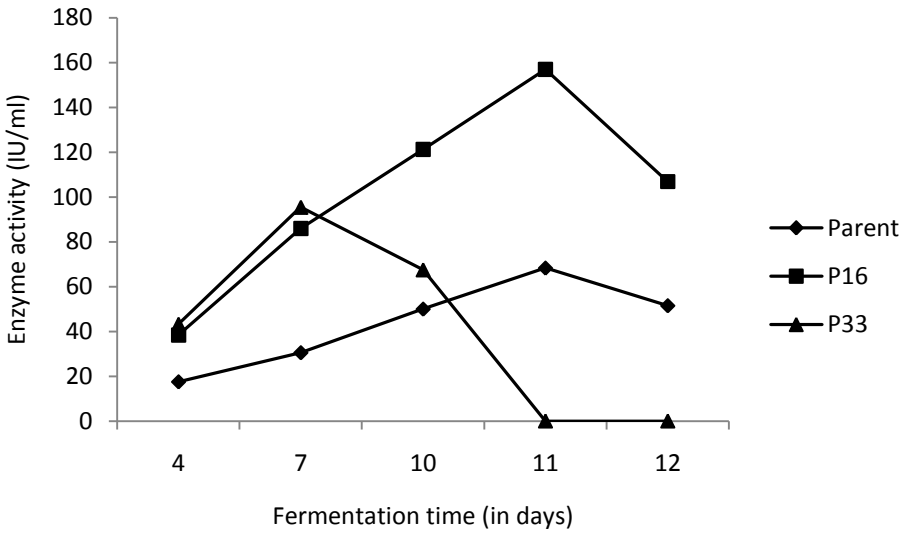

Figure 3: Activity profile under submerged fermentation conditions

Table 2: Productivity of Phytase

Strain	Productivity (U/l/d)	Fold increase
Parent	6,181	1
P-16	14,857	2.4
P-33	13,571	2.2

The morphological patterns of the mutant strains were evaluated by using microscopy. Mutant and parent strains showed striking differences in mycelial morphology, sporangium shape and sporangium size (Fig 4). Another remarkable difference among these strains was delay in the sporulation time of the mutant strains as compared to parent. Parent strain

showed sporulation on 4th day whereas that of mutant strains was obtained on 7th day.

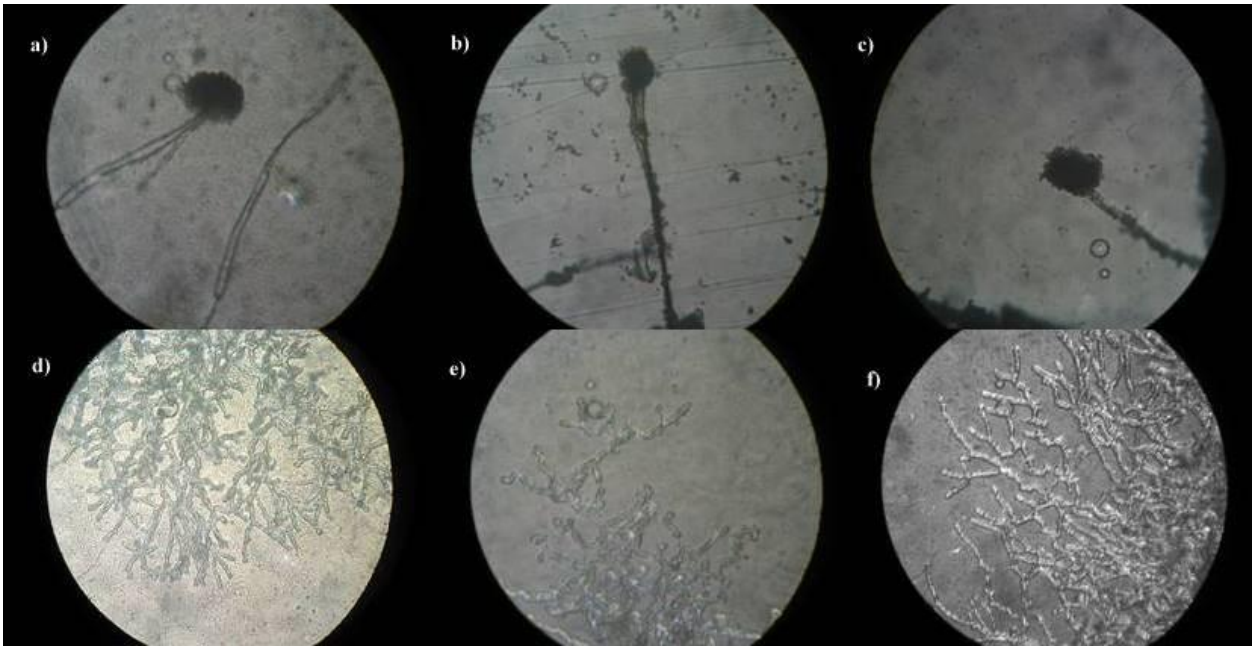

Figure 4: Morphological variations (Upper row: Sporangium morphology a) Parent; b) P-16; c) P-33 Lower row: Mycelial morphology d) Parent; e) P-16; f) P-33)

4. Discussion

Though several organisms have been reported to produce phytase, the expression levels are far from the idea of commercialization with an economical point of view. Combinatorial mutagenesis studies were conducted, using different combinations of ethidium bromide or hydroxyl amine with that of ultraviolet light exposure, for obtaining hypersecretory mutant strains. The mutants were selected based on the enhanced zone of hydrolysis of phytate on phytase screening medium. However, the method is unable to differentiate between phytase activity and acid production. Hence, all the positive mutants were quantified and confirmed for phytase production using the statistically optimized media for the parent strain in shake flask condition. Among the hypersecretory

mutants, the mutant *A. niger* NCIM 1359 exhibited the highest phytase productivity (14857 U/l/d) and improved the yield 2.4 fold as compared to the productivity obtained from parent strain. Interestingly, another mutant *A. niger* NCIM 1360 exhibited equally improved productivity (13571 U/l/d) and yield (2.2 fold) with reduced fermentation time (7 days). These observations could have tremendous value addition to phytate feed-conversion and environmental aspects of phytate biology. Strain improvement has its own pros and cons. Even if mutants with the desired phenotype are isolated, there is no guarantee that the mutation has occurred in the gene of interest also low mutation frequency in the desired gene is another difficulty associated with strain improvement. Overcoming these shortcomings and exploitation of recent developments in gene cloning and recombination can be applied to study the site and type of mutagenesis. From a simpler viewpoint, however, we can reasonably assume that genetic variability amongst parent and mutant strains exist if morphological differences are seen through mutagenesis studies. In the present study, we exploit this observation. Low yield, high production costs, and lack of desirable characteristics in the currently available commercial phytases have limited its widespread use (Krishna and Nokes 2001). Thus there is a need for identifying novel phytases with high yield and improved desired enzymatic characteristics. The results of mutagenesis show a remarkable improvement in phytase production rates which is suggestive of its potential for industrial application. *A. niger* mutants outperform the phytase production rates in comparison to other organisms, viz., *Sporotrichum thermophile* with 10,100 U/l (Singh and Satyanarayana 2006) and 12,500 U/l (Singh and Satyanarayana 2008) for two different media formulations and recombinant *Escherichia coli* with 2,250 U/l (Sunitha et al, 1999). Currently *A. niger* is "Generally Recognized as Safe (GRAS)" in food and feed applications (Bhavsar et

al, 2011). Along with the high yield, pH tolerance, and temperature stability characteristics, it appears to be a viable option when compared to other available commercial phytase supplements.

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CHAPTER 3 PRODUCTION OF PHYTASE UNDER SUBMERGED FERMENTATION USING PLANT BASED MEDIA: A COMPARATIVE ACCOUNT

Abstract

Production of phytase under submerged fermentation conditions was studied using different media. Studies pertaining to production of phytase in by fungal strains synthetic medium containing dextrin and glucose as carbon sources along with sodium nitrate as nitrogen sources at 30 °C were performed. *A. niger* NCIM 563, *A. niger* NCIM 1359 and *A. niger* NCIM 1360 produced 41.47, 51.92 and 50.57 IU/mL respectively on 13th day. *Aspergillus niger* NCIM 563 and its mutants were evaluated for phytase production on RSM optimized media under submerged fermentation (SmF). Optimization of culture conditions for parent strain using Plackett Burman and Central composite design technique had resulted in 3.74 fold increase in the yield of phytase production to 254.5 IU/mL when compared with the activity observed with basal media (68 IU/mL) in shake flask. The mutants *A. niger* NCIM 1359 and 1360 exhibited 407.2 IU/mL and 389.7 IU/mL phytase activity on 13th and 10th day, respectively using RSM optimized media suggesting its potential use in industrial application. Production of phytase was also studied on soybean flour medium wherein *A. niger* NCIM 563, *A. niger* NCIM 1359 and *A. niger* NCIM 1360 showed activities of 122.55 IU/mL, 294.12 IU/mL and 201.23 IU/mL on 13th and 10th day respectively. Exploitation of this observation could have tremendous value addition from the phytate feed-conversion and environmental point-of-view. Furthermore, the results of the present study provide an excellent basal medium

formulation for studying phytase production with the mutant strain and assessing its properties.

1. Introduction

Phytic acid occurs in nature frequently, it constitutes the primary storage form of phosphorus (60–90 %) in plants, legumes and oil seeds (Reddy et al, 1982). Phytic acid is thus a common constituent of plant based foods. It exists as a highly negatively charged ion over a broad pH range and therefore has a tremendous affinity for food components with positive charge(s), such as minerals, trace elements and proteins (Greiner and Konietzny 2006). It is also considered an anti-nutrient because it acts as a strong chelator of divalent and trivalent minerals such as Mn^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}/Fe^{3+} (Harland and Oberleas 1999). The formation of insoluble mineral-phytate complexes at physiological pH is the main reason for the poor mineral bioavailability, because these complexes are difficult to digest in the animal and human gastrointestinal tract (Greiner and Konietzny 2006). Minerals are essential for several intracellular and extracellular reactions of the cell. Deficiency of mineral may lead to severe metabolic disorders and might compromise the health of the organism (Lopez et al, 2002). Hence, enzymatic degradation of phytate is desirable to increase the bioavailability of minerals (Sandberg et al, 1999).

Though phosphorous is an essential bionutrient and energy moiety of life, it lacks a complete biogeochemical cycle. Dephosphorylation of phytate mediated by phytase can prove to be a crucial phenomenon in phosphorus cycling. Phytases have gained popularity as emerging enzymes with miscellaneous applications in white biotechnology. Extensive use of

phytase has been predicted with a vision to conserve world's phosphate reserves (Lei and Stahl, 2001). Phytases are known to be obtained from different micro-organisms including bacteria, yeasts and fungi as well as some plants and animals; however the efficacy and activity of animal and plant phytases is insignificant in comparison to microbial strains. Filamentous fungi have been widely used for production of enzymes at commercial scale, phytase not being an exception. Conversely the industrial exploitation of phytase is limited due to its high cost of production. Hence, tremendous research is underway for encouraging economical implications with respect to industrial production, recovery, and purification of phytase (Oh et al, 2004). Phytase production has been studied under submerged (SmF) and solid-state fermentation (SSF). At industrial level, the use of SmF is advantageous because of ease of sterilization and process control (Mitchell et al, 2000).

There are various reports on phytase production by bacteria, yeast and fungi (Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004). Among them, strains of *Aspergillus niger* (syn. *A. ficuum*) produce large amounts of extracellular phytase (Chelius and Wodzinski, 1994) and show more acid tolerance than bacteria and yeasts (Kim et al, 1998). In view of its industrial importance the ultimate objective is to produce this enzyme at cost effective level and establish conditions for its industrial application.

In our preliminary studies, for development of production medium for phytase production at low pH, a large number of variables including rice bran, glucose, $NaNO_3$, KCl, $MgSO_4$ and $FeSO_4$ were found to be important factors (Bhavsar et al, 2008). Application of a conventional one-variable-at-a-time (OVAT) method for optimization is tedious, time consuming and will not take into consideration the interactions that exist between the key factors that can affect the activity and production

drastically (Castro et al, 1992). To overcome the difficulties of OVAT experimentation, the use of response surface methods (RSM) was suggested for process optimization by statistical methods (Abdel-Fattah et al, 2005). Using RSM, the optimal operating conditions obtained gave a phytase yield of 254.5 IU/mL from *A. niger* NCIM 563 (Bhavsar et al, 2012). Soy bean flour has not much been exploited for phytase production in fungi. Soy bean has been reported to have a very high content of phytic acid; hence it was assumed to prove as a better substrate for phytase production and is thus used for further studies (Lönnerdal et al, 1999).

2. Materials and methodology

2.1 Chemicals

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

2.2 Fungal strain

The strains used throughout the present work were *Aspergillus niger* NCIM 563 and its mutants *Aspergillus niger* NCIM 1359 and 1360 (earlier referred to as Mutant P16 and P33 respectively). The strains were maintained on Potato Dextrose Agar (PDA) slants. PDA contains (per litre distilled water): extract from 200 g potatoes; glucose, 20 g; yeast extract, 1 g; and agar 20 g.

2.3 Culture conditions

Fermentation medium for phytase production was according to Bhavsar et al, 2008. The medium contained (per 100 mL): soy bean flour 0.25, 0.5 or 1 g (as per the experimental design); glucose 5 g; $NaNO_3$ 0.86 g; KCl 0.05 g; $MgSO_4 \cdot 7H_2O$ 0.05 g; $FeSO_4 \cdot 7H_2O$ 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 (1 x 10^7 spores per mL) obtained by suspending the spores from 7 day old sporulated slants of *Aspergillus niger* NCIM 563, 1359 and 1360 grown on PDA in 10 mL of sterile distilled water containing 0.01% (v/v) Tween 80 and incubated at 30 ºC at 200 rpm. Samples were removed after every 24 h and checked for pH, growth, total residual sugar, extracellular protein and phytase activity.

2.4 Analytical methods

2.4.1 Phytase assay

Phytase activity was measured at 50 ºC as described earlier (Bhavsar et al, 2012). The reaction was carried out at pH 2.5 (100 mM Glycine-HCl buffer) at 50 ºC for 30 min. The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (Heinohen and Lathi, 1981). 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 l µmol 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.

2.4.2 Protein estimation

Protein concentration in the culture filtrate was determined by the method of Lowry et al (1951), using Bovine serum albumin as a standard.

2.4.3 Electrophoresis

Native PAGE (8%) was performed in a vertical gel apparatus at pH 8.0, according to the method described by Davis (1964). Samples containing approximately 5–10 µg of protein were applied to the gel and electrophoresis was carried out at 200 V for 3–4 h. Protein bands were visualized by Silver staining (0.4% w/v) (Deutscher 1990).

2.4.4 Sugar content

Total residual reducing sugar concentration was estimated by DNSA method (Miller 1959).

2.5 RSM medium and culture conditions

Fermentation medium for phytase production contained 8% glucose, 0.1% $MgSO_4$, 0.1% KCl, 0.005% $MnSO_4$. Fermentation medium (100 mL in 250 mL Erlenmeyer Flask) was inoculated with 1% (v/v) of spore suspension (1 x 10^7 spores/mL) prepared by suspending the spores from 7 day old sporulated slant of *A. niger* NCIM 563, 1359 and 1360 grown on PDA in 10 mL of sterile saline 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.

2.6 Extracellular protein profiling

The extracellular culture filtrates from parent and mutant strains were used to study the protein expression profile of the strain on the highest activity day. About 20 µg of protein was loaded on the gel for each strain.

3. Results

3.1 Production on soy bean flour

Out of three concentrations of soy bean flour tested viz. 0.25 %, 0.5 % and 1 %; use of 0.5 % was found to be appropriate for yielding maximum phytase activities from parent and mutant strains. Production studies on 0.5 % soy bean flour medium yielded activities of 122.55 IU/mL, 294.12 IU/mL and 201.23 IU/mL for *A. niger* NCIM 563, *A. niger* NCIM 1359 and *A. niger* NCIM 1360 on 13th and 10th day respectively (Fig 1).

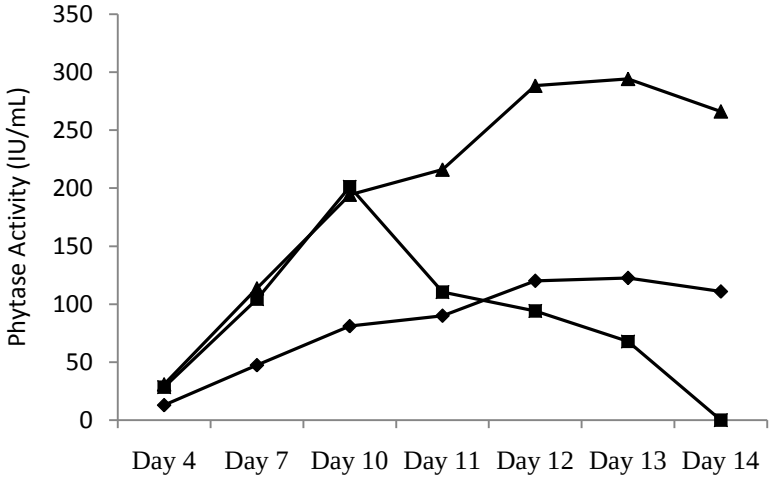

Figure 1: Production on 0.5 % soy flour medium

3.2 Production on RSM media

The mutants *A. niger* NCIM 1359 and 1360 exhibited 407.2 and 389.7 IU/mL phytase activity on 13^{th} and 10^{th} day, respectively using RSM optimized media (Fig 2) suggesting the potentiality of the strains for industrial application. Exploitation of this observation could have tremendous value addition from the phytate feed-conversion and environmental point-of-view. Furthermore, the results of the present study provide an excellent basal medium formulation for studying phytase production with the mutant strain. The efficiency of combinatorial approach followed is summarized in Table 1.

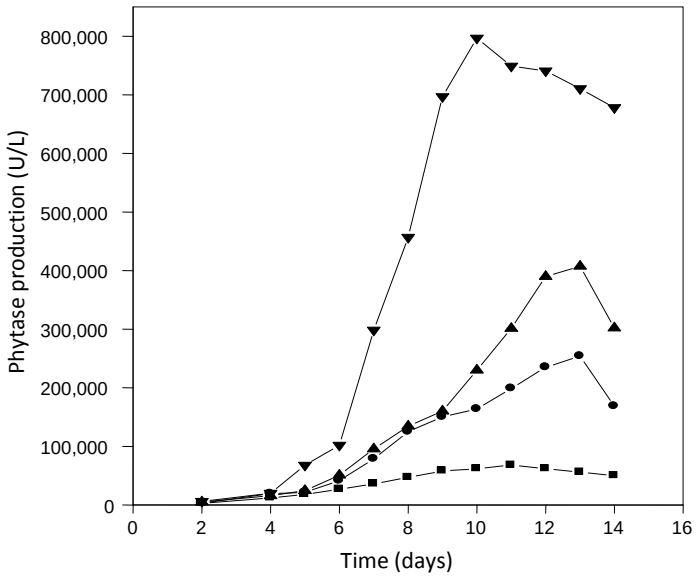

Figure 2: Time course of phytase production under SmF by *A. niger* NCIM 563 and mutants using OVAT and RSM optimized media. ─▼— Production in RSM media by Mutant P-33 —▲─ Production in RSM media by Mutant P-16 —●— Production in RSM media by parent strain —■— Production in OVAT media by parent strain

Table 1: Comparison of phytase production by different strains using statistical technique under submerged fermentation condition

Strain	Phytase Activity (IU/mL)	Fold Increase
Aspergillus niger NCIM 563 (Parent)	68[a]	1
	259.8[b]	3.8
Aspergillus niger NCIM 1359 Mutant P16	407.2[b]	5.9
Aspergillus niger NCIM 1360 Mutant P33	796.9[b]	11.71

Phytase production using OVAT[a] media and RSM[b] media

3.3 Extracellular protein profiling

The protein bands obtained showed striking variation in pattern of extracellular expression which is indicative of differential protein expression in parent and mutant strains. It could be further analyzed using 2D gel electrophoresis followed by MALDI analysis to exploit the changes in the proteome of the mutant cultures. The presence and absence of protein bands amongst the three strains studied is summarized in Figure 3.

4. Discussion

Soy based products are emerging as a preferable choice in the food and feed industry, thus exploitation of soy based media for phytase production also proved to be a promising area. A major percentage of the world's populations who use soy based foods suffer from several mineral

deficiencies. (Bentley et al, 1997; Ohri-Vachaspati and Swindale, 1999; Tatala et al, 1998). Similarly infant soy formula and other soy protein products also contain high levels of phytate (Lönnerdal et al, 1988). The potential of supplemental phytases in enhancing mineral assimilation and reducing phytate content of plant derived foods has been well documented (Fredrikson et al, 2001; Greiner and Konietzny 1999; Lei et al, 1993; Stahl et al, 1999; Porres et al, 2001; Sandberg et al, 1996).

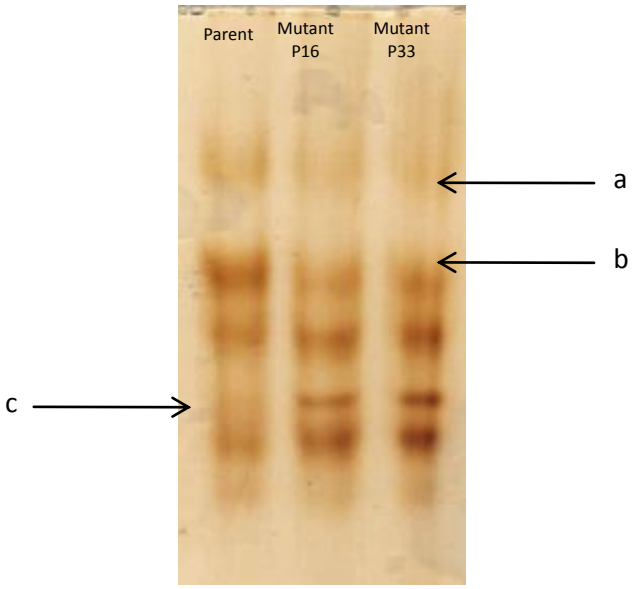

Figure 3: Comparison of protein profile of extracellular culture filtrate of parent and mutants on native gel electrophoresis (8%) Lane 1: Parent, lane 2: Mutant P16, lane 3: Mutant P33. Expression of proteins a and b is enhanced in parent while protein c is more expressed in mutants.

Most of the studies involving use of soy based media have been carried out under solid or semi solid fermentation conditions where as only few reports are available for production of phytase under submerged fermentation using soy based media (Krishna and Nokes, 2001; Bogar et

al, 2003; Han et al, 1987). Similarly, recombinant studies to enhance phosphorus assimilation from soy based foods in animals involving cloning of phytase gene to reduce manure phosphate excretion in animals have also been reported (Cromwell 2001). However, lack of appropriate research pertaining to exploitation of soy based media for production of phytase has proved to be a stumbling block in phytase commercialization. Use of soy based media for production as per our work might prove to be an innovative and economical approach for phytase production at industrial level.

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CHAPTER 4 MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERIZATION OF MUTANTS: A COMPARATIVE ACCOUNT

Abstract

Characterization of mutants in comparison with the wild-type strain by different methods is vital for the assessment of morphological and physiological changes with respect to applicability at industrial level. Variability studies showed remarkable difference between sporangium and mycelial morphology, sporulation time and extracellular enzyme profile amongst parent and mutant strains. The scanning electron micrographs showed variation in the morphology of the mutant strains compared to that of the parental strain. NCIM 1359 displayed high conidiospore density in the fruiting bodies compared to the other two strains. NCIM 1360 showed marked difference in the conidiophore morphology and decreased spore density when compared to the parental strain. The dimensions of the conidiospores and conidiophores displayed significant differences among the three strains. NCIM 1360 had the smallest conidiospore diameter (28.71 µm) and conidiophore width (4.94 µm) when compared to the other strains. NCIM 563 showed largest conidiospore diameter (118.52 µm), whereas the mutant NCIM 1359 displayed the widest conidiophore (14.78 µm) among the three strains. In addition, the parent and mutant strains showed significant differences in the activities of five secreted hydrolase enzymes. Xylanase activity was not detected in NCIM 563, whereas NCIM 1359 and NCIM 1360 showed 0.19 IU/ml and 0.34 IU/ml of xylanase activity correspondingly. The mutant strains NCIM 1359 and NCIM 1360 displayed 1.8- and 1.1-fold

lower CMCase activity when weighed against NCIM 563. The activity of β-glucosidase was lowered by 1.3- and 37.8 folds in NCIM 1359 and NCIM 1360, respectively compared to that of the parental strain. Relative to NCIM 563, both amylase and acid phosphatase activities decreased by nearly 3-fold in NCIM 1359 and by 3.5-and 2.4-fold in case of NCIM 1360. Another remarkable difference among these strains was delay in the sporulation time of the mutant strains as compared to parent. Parent strain showed sporulation on 4^{th} day whereas that of mutant strains was obtained on 7^{th} day. MALDI-TOF/MS-MS analysis was performed as a part of partial characterization of phytases from parent and mutant strains.

1. Introduction

Phytic acid (myo-inositol hexakisphosphate) is the principal storage form of phosphate in cereals, legumes and oilseeds. Phytic acid causes inefficient utilization of nutrients since it is feebly digested by monogastric animals and has the capacity to chelate metal ions (Reddy et al, 1982). Phytase (myo-inositol hexaphosphate phosphohydrolase, E.C. 3.1.3.8) hydrolyzes phytic acid to inorganic phosphate and other myoinositol derivatives (mono- to penta-phosphates). Hence, phytases are used as animal feed supplements. The filamentous Ascomycete fungus *Aspergillus niger* produces the most active extracellular phytase (Gunashree and Venkateswaran, 2008) and due to its GRAS status and efficient secretory potential, is a preferred candidate for use at industrial level. Mutagenesis can help improve phytase production and activity and help make its industrial production cost-effective (Chelius and Wodzinski, 1994; Shivanna and Govindarajulu, 2009). Previously, mutagenesis of the *A. niger* strain NCIM 563 using a combination of

physical and chemical mutagens was carried out by us to obtain two strains namely, NCIM 1359 and NCIM 1360 that exhibited 1.6 and 3.13 fold increase in phytase activity, respectively compared to that of the parental strain (Bhavsar et al, 2012).

Characterization of mutants in comparison with the wild-type strain by different methods is vital for the assessment of genetic and physiological changes. Various methods for characterization are in practice such as morphological, biochemical, immunological and molecular marker profiling (Zubke et al, 1977). Fungal morphological features are critical in determining the rheology of the fermentation medium, which in turn influences parameters such as mass transfer, aeration, and agitation in a bioreactor (Papagianni, 2004). Scanning electron microscopy (SEM) allows visualization of cellular structures at high resolution and can help in assessing the effects of mutagenesis. Hence three-dimensional visualization of mycelia and fruiting body morphology by scanning electron microscopy (SEM) was carried out for NCIM 563, NCIM 1359 and NCIM 1360.

In animal feed materials of plant origin like wheat bran, corn meal, soybean meal etc. apart from phytic acid, non-starch polysaccharides are usually present in significant amounts. These complex polysaccharides are hard to digest and can reduce nutrient utilization by encapsulating nutrients and/or depressing overall nutrient digestibility through gastro-intestinal modifications (Bedford et al, 1992). A mixture of phytase and polysaccharide hydrolases such as amylase, glucosidase, xylanase etc. in animal feed can improve its quality (Lei and Porres, 2003; Gulati et al, 2007). It has previously been suggested that polysaccharide hydrolases can hydrolyze non-starch polysaccharides in cell walls to increase the accessibility of phytase to phytic acid present in the cell. Hence, phytase

and polysaccharide hydrolases can act synergistically in improving nutrient utilization (Woyengo and Nyachoti, 2011).When random mutagenesis is performed with the aim of improving phytase production, its fortuitous effect on feed enzymes other than phytase cannot be denied (Prabhakaran et al, 2009). Therefore, in addition to phytase, the activities of five secreted enzymes with potential biotechnological importance were also compared for the three *A. niger* strains in the preset study.

In plant-based foods, inorganic phosphate is present in large amounts (up to 80%) in the form of phytate (myo-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate). The bioavailability of phosphorus from phytate is inefficient. In addition, it can also chelate calcium, zinc and copper ions and inhibit their absorption. The enzyme phytase can release inorganic phosphorus from phytic acid by hydrolysis. Seed-based feeds for swine and poultry can be supplemented with phytase enzyme to increase the bioavailability of inorganic phosphate. Extracellular phytases from fungi possess immense biotechnological potential because of their high yield and optimum activity in acidic pH similar to that found in the guts of swine and poultry. *Aspergillus niger* can secrete phytase in acidic environments and is commercially important due to its Generally Recognized as Safe (GRAS) status. A phytase secreting *A. niger* strain NCIM 563 was mutagenized to isolate two strains namely, NCIM 1359 and NCIM 1360 that exhibited 1.6 and 3.13 fold increase in phytase activity, compared to that of the parental strain. To investigate the genetic basis of differences in phytase activity in the parental and mutant strains, the DNA sequence of the genes encoding phytase from the three strains was characterized.

2. Materials and Methods

2.1 Microorganism and culture conditions

The *A. niger* strains NCIM 563, 1359 and 1360 deposited at the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India (Bhavsar et al, 2012) were subjected to detailed characterization. For enzyme assays, Erlenmeyer flasks (250 ml) containing 100 ml production medium (1 % rice bran, 5 % glucose, 0.86 % $NaNO_3$, 0.05 % KCl, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.01 % $FeSO_4 \cdot 7H_2O$; pH5.5) were inoculated with 1% spore suspension (1 x 10^7 spores per ml) and incubated at 28 $^{\circ}$C at 200 rev min^{-1} for 11 days. The fermented broth was harvested by centrifugation at 8500 x *g* for 10 min at 4 $^{\circ}$C.

2.2 Purification of phytase

After fermentation, mycelium was separated by filtration followed by centrifugation at 10,000 x *g* for 30 min and the clear supernatant was collected. It was further concentrated (50 %) by rotary evaporation using a rota vapour at 40 °C under vacuum and subjected to hydrophobic column chromatography using Phenyl-Sepharose CL-4B (30 ml bed volume), previously equilibrated with 30% ammonium sulphate in 20 mM glycine-HCl buffer, pH 2.5. The column was washed thoroughly with 20 bed volumes of the above buffer and eluted with a 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) were pooled followed by concentration by rota vapor and loaded on a Sephacryl S-200 gel filtration column with a flow rate of 12 ml per hour and 2 ml fractions were collected. Unless otherwise mentioned, all the purification procedures were carried out at 4°C.

2.3 Enzyme assays

Phytase activity in the culture supernatant was measured as described previously (Bhavsar et al, 2008). One unit of phytase activity (IU) indicated the amount of enzyme that liberated 1 µmol phosphorus/min under standard assay conditions. For Acid phosphatase assay, 100 µL of suitably diluted culture supernatant was added to 900 µL of 2.69 mM *p*-nitro phenyl phosphate prepared in 0.2 M Glycine-HCl buffer (pH 2.5). After incubation at 50 $^{\circ}$C for 30 min, the reaction was terminated by addition of 2 ml 1N NaOH and the released *p*-nitro phenol was estimated at 405 nm. Xylanase, β-glucosidase and carboxy methyl cellulase (CMCase) activities were determined as reported earlier (Krishna and Nokes, 2001). The activity of *α*-amylase was determined as described by Sheehan and McCleary (1988). The reducing sugars were determined using dinitrosalicylic acid (DNS) method (Miller 1959). One unit of xylanase, *α*-amylase, CMCase and β-glucosidase enzyme activity was defined as the amount of enzyme that produces 1 µmol of the reducing sugars xylose or glucose respectively per minute under the standard assay conditions. Student's *t*-Tests were performed using MS Excel 2007 to evaluate if the differences in the enzyme activities among the strains were significant.

2.4 Electrophoresis

SDS-PAGE (10%) was performed in a vertical slab gel apparatus at pH 8.3, according to the method described by Laemmli (1970). Samples containing approximately 5–10 µg of protein were applied to the gel and electrophoresis was carried out at 200 V for 3–4 h. Protein bands were visualized by Silver staining (0.4% w/v) (Deutscher 1990).

2.5 Scanning electron microscopy (SEM)

The parental and the mutant strains were inoculated in Petri dishes containing PDA and incubated at 30 ^{o}C for 7 days. The fungal biomass was mounted on microscopic stubs containing carbon tape and visualized under environmental mode using SEM (200 3D-Dual beam ESEM with EDAX, FEI Quanta, USA). Conidiospore and conidiophore dimensions were measured as per the micrograph scale and mean values of triplicate measurements were recorded.

2.6 MALDI-TOF analysis

Mass spectral analysis was performed on a Voyager-De-STR (Applied Biosystems) MALDI-TOF. A nitrogen laser (337 nm) was used for desorption and ionization. Spectra were acquired in the range of 10 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 myo-globulin and bovine serum albumin. In-gel tryptic digestion and analysis of peptides was done according to Bar et al (2007).

3. Results

3.1 Purification of phytase

The purification of phytase was carried out as summarized in Table 1. 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-200. Major activities were eluted in two peaks with a reverse gradient of ammonium sulphate (30-0 %) in 20 mM glycine HCl 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 glycine HCl buffer with higher enzyme activity at pH 2.5 (Phy I). The fractions containing phytase I were dialyzed, concentrated and applied on gel filtration column. The purified Phy I had a specific activity of 813.69, 815 and 905 U/mg with 26.21, 33.86 and 34.01 fold purification, for NCIM 563, 1359 and 1360 respectively. The SDS PAGE analysis of purified proteins from parent and mutant strains are depicted in Figure 1. The molecular mass of the phytase subunit was found to be 66 kDa.

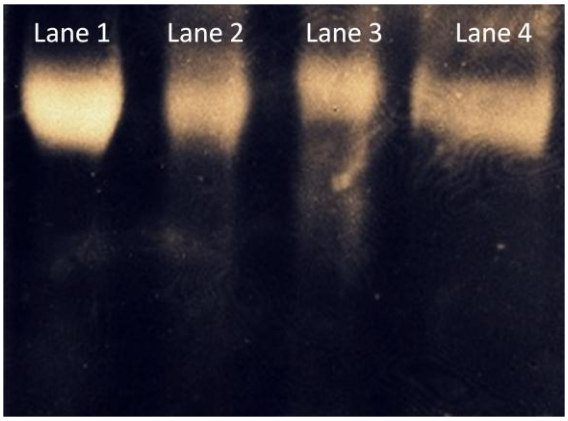


Figure 1: SDS-PAGE of purified Phy I from parent and mutant strains. Lane 1: Marker (66 kDa) Lane 2, 3 and 4: Purified phytases from *Aspergillus niger* NCIM 563, NCIM 1359 and NCIM 1360 respectively.

Table 1: Summary of purification of Phy I from *A. niger* NCIM 563, NCIM 1359 and NCIM 1360

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
A. niger NCIM 563					
Culture filtrate	1033.05	32073.05	31.05	1	100
Rotavapour concentration	525	26334	50.16	1.62	82.11
Phenyl sepharose CL-4B (5 %)	51	19750	387.25	12.47	61.58
Sephacryl S-200	13	10578	813.69	26.21	32.98
A. niger NCIM 1359					
Culture filtrate	2580	62092.5	24.07	1	100
Rotavapour concentration	1020	53946.5	52.89	2.20	86.88
Phenyl sepharose CL-4B (5 %)	103.7	40459.88	390.16	16.21	65.16
Sephacryl S-200	26.60	21769	815	33.86	34.91
A. niger NCIM 1360					
Culture filtrate	2121	56448	26.61	1	100
Rotavapour concentration	727.7	44054.4	60.54	2.28	78.04
Phenyl sepharose CL-4B (5 %)	74	33540	453.25	17.03	59.42
Sephacryl S-200	19.3	17465.7	904.96	34.01	30.94

3.2 Comparison of activities of extracellular enzymes

The mutant strains indicated reduced activities for four of the six extracellular enzymes assayed, while that of xylanase and phytase indicated enhanced activities compared to the parental strain (Table 2). Phytase activity showed 2.3- and 1.4 fold enhancement in NCIM 1359 and NCIM 1360 respectively in comparison with NCIM 563. Xylanase activity was not detected in NCIM 563, whereas NCIM 1359 and NCIM 1360 showed 0.19 IU/ml and 0.34 IU/ml of xylanase activity correspondingly. The mutant strains NCIM 1359 and NCIM 1360 displayed 1.8- and 1.1-fold lower CMCase activity when weighed against NCIM 563. The activity of β-glucosidase was lowered by 1.3- and 37.8 folds in NCIM 1359 and NCIM 1360, respectively compared to that of the parental strain. Relative to NCIM 563, both amylase and acid phosphatase activities decreased by nearly 3-fold in NCIM 1359 and by 3.5-and 2.4-fold in case of NCIM 1360 (Refer Annexure for statistical data).

3.3 SEM studies

The scanning electron micrographs showed variation in the morphology of the mutant strains compared to that of the parental strain (Fig 2). NCIM 1359 displayed high conidiospore density in the fruiting bodies compared to the other two strains. NCIM 1360 showed marked difference in the conidiophore morphology and decreased spore density when compared to the parental strain. The dimensions of the conidiospores and conidiophores displayed significant differences among the three strains (Table 3). NCIM 1360 had the smallest conodiospore diameter (28.71 µm) and conidiophore width (4.94 µm) when compared to the other strains. NCIM 563 showed largest conidiospore diameter (118.52 µm),

whereas the mutant NCIM 1359 displayed the widest conidiophore (14.78 µm) among the three strains (Refer Annexure for Statistical data).

Table 2: Activities of various enzymes in the culture filtrate of *A. niger* NCIM 563, NCIM 1359 and NCIM 1360.

Enzyme	Enzyme activity (IU/ml)		
	A. niger NCIM 563	*A. niger* NCIM 1359	*A. niger* NCIM 1360
Carboxymethyl cellulase (CMCase)	0.17	0.10	0.16
Xylanase	0	0.19	0.34
β-glucosidase	2.04	1.60	0.05
Acid Phosphatase	197.66	53.36	57.19
α-amylase	7.41	2.41	3.14
Phytase	68	156	95

Table 3: Conidiospore and conidiophore dimensions of *A. niger* NCIM 563, NCIM 1359 and NCIM 1360 derived by scanning electron microscopy

Culture	Average conidiospore diameter (µm)	Standard deviation	Average conidiophore width (µm)	Standard deviation
NCIM 563	118.52	1.96	9.73	0.66
NCIM 1359	68.34	0.73	14.78	0.44
NCIM 1360	28.71	1.88	4.94	0.19

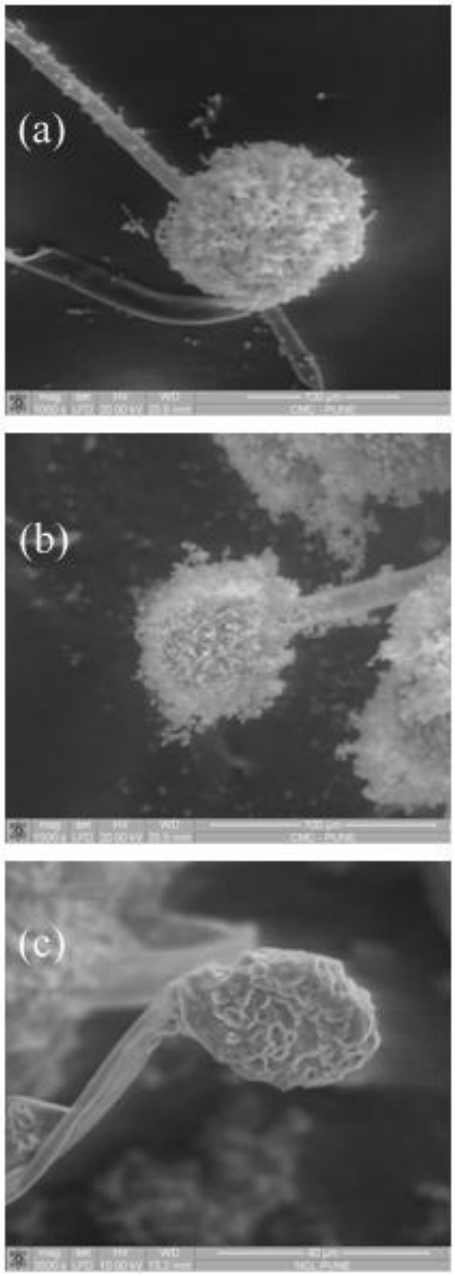
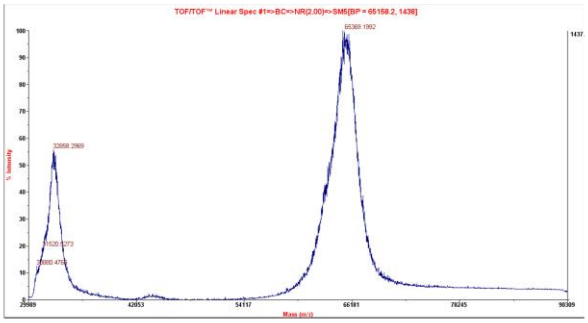


Figure 2: *Aspergillus niger*. Scanning electron micrographs (SEM) of a fruiting body of the fungus *A. niger*, on the tip of a conidiophore. The small, spiky spheres are spores (reproductive cells), about to be released into the air. (a) Parent *A. niger* NCIM 563 (b) Mutant *A. niger* NCIM 1359 (c) Mutant *A. niger* NCIM 1360

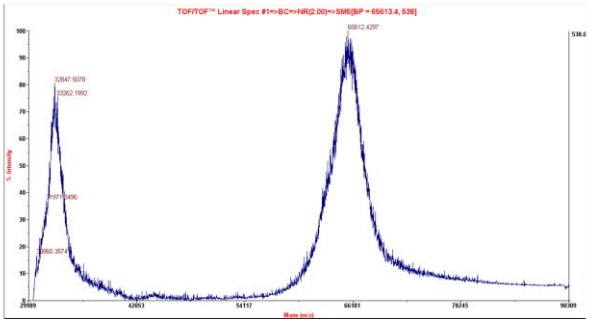
3.4 MALDI TOF analysis

MALDI-TOF analysis of phytase showed similar molecular weights (m/z) for both parental and mutant derived phytases. Proteins were analyzed by different proteomics software and databases. Molecular weight of purified phytases was found to be approximately to be 66 KDa (Fig 3) as determined by MALDI- TOF which was consistent with the molecular weight (66 KD) of single subunit showed by SDS PAGE (Fig 1).

Aspergillus niger NCIM 563



Aspergillus niger NCIM 1359



Aspergillus niger NCIM 1360

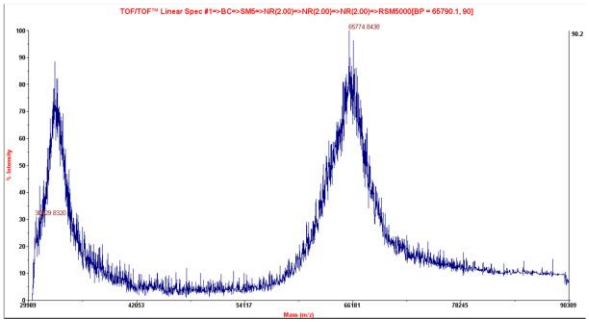


Figure 3: MALDI-TOF analysis of phytase from parent and mutant strains

4. Discussion

Multienzyme blends produced by fungal strains have been demonstrated earlier to increase the digestibility of fodder and poultry feed (Bedford, 1995; Saki et al, 2005). Significant differences were observed in activities of CMCase, xylanase, β-glucosidase, acid phosphatase and amylase among the parent and the mutant *A. niger* strains in the present study. In addition to high-phytase activity, the mutant strains showed increased xylanase and acid phosphatase activities compared to the parental strain. With the secretion of enzymes with cellulolytic, phytase and xylanase activities, these mutants exhibited a potential for being industrial sources of multi-enzyme blends to hydrolyze both phytic acid and non-starch carbohydrates in animal feed.

Scanning electron microscopy revealed noteworthy alterations in morphology of mutant strains compared to NCIM 563. Previous studies have suggested the role of morphological parameters in influencing enzyme production. Papagianni et al (1999) reported that smaller pellicle size of the culture resulted in increased phytase activity in the fermentation broth. Smaller conidiospores might form smaller pellets as compared to larger conidiospores and alter pellicle formation. Hence the increased phytase activity in the mutants might possibly be to some extent due to reduced conidiospore size when compared to the parent. Furthermore, the mutant strain NCIM 1360 had the smallest conidiospore diameter while simultaneously depicting highest phytase productivity followed by the mutant NCIM 1359 and parent *A. niger* NCIM 563 which showed higher in conidiospore size and lower phytase activities. Furthermore, it was observed that sporulation in the mutant strains was delayed as compared to parental strain which might be one of the factors affecting phytase production in these strains. Previously, Swe et al

(2009) also demonstrated the influence of sporulation time on phytase activity.

Phytase was purified from both mutant strains with a Nagashima et al (1999) purified phytase from *A. niger* SK- 57 to homogeneity in four steps by using ion-exchange chromatography, gel filtration chromatography and chromatofocussing. Recently Xiaoyu et al (2008) 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. Some reports suggest that only 5- to 25- fold purification was required to achieve near homogeneity using ion-exchange chromatography and chromatofocussing (Ullah and Cummins 1987, 1988; Ullah and Gibson 1987). MALDI TOF was able to successfully predict the molecular mass of phytase subunit to be 66 kDa. However, there were no differences observed among the phytase enzymes from parent and mutant strains in MALDI TOF experiment; which indicates that no mutations exist in sequence of phytase polypeptide among mutant strains. However, occurrence of mutations in regulatory regions of the phytase gene cannot be neglected.

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extracellular phytase from a marine yeast *Kodamaea ohmeri* BG3. Marine Biotechnol 10: 190-197.

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CHAPTER 5 GENETIC PROFILING OF MUTANT STRAINS: A COMPARATIVE ACCOUNT

Abstract

Genetic variation among *Aspergillus niger* wild type strain NCIM 563 and two phytase hyper secreting mutant strains (NCIM 1359 and NCIM 1360) was assessed using inter simple sequence repeats (ISSR) and sequence-related amplified polymorphism (SRAP) markers. ISSR primers amplified 116 loci of which 113 were polymorphic; while SRAP primers generated 135 amplification products of which 125 were polymorphic. To investigate the genetic basis of differences in phytase activity in the parental and mutant strains, the DNA sequence of the genes encoding phytase from the three strains was characterized. The 3-Phytase B gene sequences from NCIM 563, NCIM 1359 and NCIM 1360 showed 100% identity. However, comparison of these sequences with the *A. niger* CBS 513.88 3-Phytase B gene sequence (gi|145241119) indicated five changes. The changes in the 3-Phytase B genes from NCIM 563, NCIM 1359 and NCIM 1360 in comparison with 3-Phytase B gene from *A. niger* CBS 513.88 (gi|145241119) were in Exon 1 (C863T), Exon 3 (T1208C) and in the 3' UTR (ATA insertion at: 1887).The transversion in Exon 1 (863) resulted in amino acid change from (A240V) in the 479 amino acid phytase B protein. The other transversion in Exon 3 (1208) resulted in a silent mutation. The 3-Phytase B gene sequences from the *A. niger* strains NCIM 563, NCIM 1359 and NCIM 1360 could be used for cloning and over-expression to produce phytase enzyme that can be used as an additive to upgrade the nutritional quality of phytate-rich seed-based animal feed.

1. Introduction

Characterization of mutants in comparison with the wild-type strain by different methods is vital for the assessment of genetic and physiological changes due to the mutation treatment. Various methods for characterization are in practice such as morphological, biochemical, immunological and molecular marker profiling (Zubke et al, 1977).

Molecular markers are distinguishing DNA sequences, found at defined locations of the genome, which abide by the standard laws of inheritance by passing from one generation to the next. These can be considered as permanent landmarks in the genome that usually do not have any biological effect. Molecular markers have a number of advantages over the traditional phenotypic markers such as they are not environmentally influenced and are, therefore, unaffected by the conditions of growth. Molecular markers are detectable at all stages of growth and are useful in complementing morphological and phenological characters because of their abundance (Aneja et al, 2012). Various molecular marker types have been used earlier such as RAPD, ISSR, SSR, and AFLP etc. in earlier studies for characterization of fungi (Siddique et al, 2011).

In Inter-simple sequence repeat (ISSR) markers microsatellites are used as primers (usually 16-25 bp long) in a single primer PCR, which target multiple genomic loci and essentially amplify the inter-SSR sequences of various sizes (Meyer et al, 1993; Gupta et al, 1994; Wu et al, 1994). The primers are usually anchored at 3’ or 5’ end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz et al, 1994). ISSR markers are technically simple to use. They are inherited as dominant loci

and offer a reliable marker system for genotyping many organisms. These markers are extremely polymorphic and are therefore utilized in studies associated with genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology.

Sequence-related amplified polymorphism (SRAP) is a more recently established molecular marker system which potentially targets the coding regions. SRAP is an ORF-based marker system which specifically targets random coding regions. The SRAP technique preferably amplifies the ORFs using two primers a forward primer (consisting of 17 nucleotides) and a reverse primer (consisting of 18 nucleotides). The forward primers contain a GC rich region of 14 bases at the 5' end (mostly fixed sequence also known as the "core" sequence) and three selective bases in the 3' end. The first ten bases at the start of 5' end are "filler" sequences (with no specific constitution) followed by the sequence CCGG and then by three selective nucleotides at the 3' end. A set of primers sharing the same core sequence is obtained by varying these three selective nucleotides. The reverse primers have similar components as the forward primers but with slight variations; AATT sequence follows the filler sequence instead of the CCGG. Three selective bases follow the 3′ end of the primer after the AATT sequence (Li and Quiros, 2001, Aneja et al, 2012).

The SRAP marker system has been used earlier for construction of linkage map (Yeboah et al 2007), genomic and cDNA fingerprinting, gene tagging (Li and Quiros 2001), genetic diversity analysis (Li et al 2009) and map-based cloning (Zhang et al 2010). Some other advantages of SRAP include utilizing relatively small amount of template genomic DNA, and good levels of polymorphism in numerous species. Above all, these types of DNA markers can be detected without any prior knowledge

of the genome sequence, and hence pointing towards the uniqueness in the sense that it can permit mapping of gene expression markers or gene expression quantitative loci (i.e. QTL). SRAP has also been used to study genetic variations in fungi (Sun et al, 2006, Yu et al, 2008). Acknowledging its effectiveness and reliability in identifying genetic variation, this technique can provide a practical option for studying genetic variability amongst parent and mutant strains.

Both the ISSR and SRAP marker systems are technically simple and generate large number of amplicons. They are highly polymorphic and are inherited as dominant markers. Therefore, we performed ISSR and SRAP marker analyses to assess the variability among the parent *A. niger* strain NCIM 563 and the mutant strains NCIM 1359 and NCIM 1360.

2. Materials and Methods

2.1 Genomic DNA isolation

For each *A. niger* strain, 100 mg mycelia were mixed with 0.5 ml of CTAB extraction buffer Saghai-Maroof et al (1984) and 4-6 zirconium beads and homogenized using a bead beater (Biospec Products, USA) for 5 min. Further processing was as described by Saghai-Maroof et al (1984) with minor modifications. Genomic DNA was dissolved in sterile deionized water and checked spectrophotometrically for quality and quantity.

2.2 Inter simple sequence repeat polymorphism

ISSR amplification as described by Ammiraju et al (2001) was followed with slight modifications. Twenty randomly selected ISSR primers [University of British Columbia (UBC), Canada] were used for

polymorphism survey among the three *A. niger* strains. All PCR reactions were 25 µL in volume containing 10 mM Tris-HCl buffer (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of dATP, dGTP, dCTP, and dTTP (Amersham Biosciences, USA), 0.2 µM primer, 0.4 mM Spermidine, 0.5 units of Taq DNA Polymerase (Bangalore Genei, India) and 10 ng genomic DNA. PCR conditions were as follows: initial denaturation of 94 °C for 5 min, followed by 45 cycles of 30 s at 94 °C, 45 sec at 50 or 60 °C (depending on the primer) and 2 min at 72 °C, followed by a final extension for 5 min at 72 °C.

2.3 Sequence Related Amplified polymorphism

The forward SRAP primers contain ten "filler" nucleotides followed by the sequence CCGG constituting the core and then by three selective nucleotides at the 3' end. A set of primers sharing the same core sequence is obtained by varying these three selective nucleotides. The reverse primers have similar components as the forward primers but AATT sequence follows the filler sequence instead of the CCGG (Li and Quiros, 2001). A total of twenty different combinations of SRAP primers using ten forward primers (me) and ten reverse primers (em) were employed for polymorphism survey among the three *A. niger* strains. The sequences of the SRAP primers used are listed in the Annexure. PCRs (25 µL) were performed using 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 100 mM of dNTPs, 0.5 mM of each primer, 0.36 unit of Taq polymerase, and 10 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, five cycles of three steps: 94 °C for 1 min, 36 °C for 1 min, 72 °C for 20 s, further 38 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 20 s and a final extension at 72 °C for 5 min.

Both ISSR and SRAP PCR were performed in a MJ 9700 thermal cycler (MJ Research, USA) and carried out twice to check for reproducibility. Further, PCR products were resolved using 2% agarose gels, visualized using Gel Red (Biotium, USA) and documented on ImageMaster VDS gel documentation system (Amersham Biosciences, USA).

2.4 Data analysis

For both ISSR and SRAP analyses, the presence or absence of each amplicon among the parent and mutant genotypes was assessed and scored as 1 or 0, respectively to generate a binary matrix. The Multi Variate Statistical Package (MVSP) v 3.13 was used to perform cluster analysis (Kovach, 1998). A dendrogram was derived on the basis of similarity matrix using Unweighted Pair Group Method with Arithematic mean (UPGMA) wherein Jaccard's coefficient was used as the measure of similarity or distance. Polymorphism information content (PIC) was calculated using the formula PIC= $1-p^2-q^2$, where p is the frequency of positive allele and q is the frequency of null allele (Rajwade et al, 2010).

2.5 Gene isolation and Characterization

The parental strain NCIM 563 was grown in submerged fermentation medium and the culture filtrate was subjected to hydrophobic column chromatography followed by gel filtration column chromatography (as mentioned in Chapter 4). Fractions showing phytase activity at pH 2.5 were pooled, concentrated and SDS-PAGE was used to resolve the 66 kDa band. This protein was subjected to trypsin proteolysis and nano-liquid chromatography-high definition mass spectrometry, which indicated its similarity to 3-phytase B protein from *A. niger* CBS 513.88(gi|145241119) with 24 % sequence coverage (Refer Annexure for details). The gene sequence of 3-phytase B from CBS 513.88

(gi|145241358, supercontig An08) was used to design four sets of primers to generate overlapping polymerase chain reaction (PCR) amplicons (Refer Annexure for primer details). Genomic DNA from the parental *A. niger* strain NCIM 563 and the mutants NCIM 1359 and NCIM 1360 was used for PCR using a high-fidelity DNA polymerase.

All PCRs were 50 µL in volume containing 1X Accuprime reaction mix, 1 µM primer (forward and reverse each), 0.4 units of Accuprime Pfx Polymerase (Invitrogen-Life Technologies, India) and 10 ng genomic DNA. PCR conditions were as follows: initial denaturation of 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 or 60 °C (depending on the primer) and 2 min at 72 °C, followed by a final extension for 5 min at 72 °C.

The resulting amplicons were sequenced, assessed for quality and the full-length gene sequences were assembled for each strain. BLAST searches were performed using the full-length gene sequences and the specificity of the primer targets was confirmed. Sequence alignment was performed to compare the gene sequences from CBS 513.88, NCIM 563, NCIM 1359 and NCIM 1360. The protein sequences for the genes from the four strains were derived *in-silico* and compared.

3. Results

3.1 ISSR and SRAP analysis

Among the 20 ISSR primers used for amplification, 12 yielded clear and reproducible amplifications (Refer Annexure for gel images). These 12 primers generated a total of 116 loci of which 113 were polymorphic (94.9%) with an average of 9.42 polymorphic bands per primer. The size

of amplified products ranged from 0.1 to 2.0 kb. The mean PIC for polymorphic ISSR primers was 0.47, while the highest was 0.50 (UBC 822 and 841) (Table 1).

All the 20 SRAP primer combinations yielded reproducible polymorphic amplification (Refer Annexure for gel images). In total, 135 fragments were generated of which 125 were polymorphic (92.6%) with an average of 6.25 polymorphic bands per primer combination. Similar to ISSR, the size of amplified products ranged from 0.1 to 2.0 kb. The average PIC value of the SRAP primer combination was 0.47, while the highest and lowest PIC values were 0.50 (Primer combinations em6 - me22, em5 - me12, em4 - me21, em11 - me19, em2 - me13, em3 - me16 and em9 - me24) and 0.39 (Primer combination em5 - me19), respectively (Table 2).

The UPGMA cluster analysis of ISSR (Fig 1a) and SRAP (Fig 1b) markers divided the *A. niger* strains into two distinct clusters. The first cluster solely included NCIM 1359 whereas the second cluster contained NCIM 563 and NCIM 1360.

The similarity coefficients for the strains with the 12 polymorphic ISSR primers, ranged from 0.115 (between NCIM 1359 and NCIM 1360) to 0.207 (between NCIM 563 and NCIM 1360) (Table 3). For SRAP analysis, the similarity coefficients for NCIM 1359 and NCIM 1360 was 0.118 and 0.307 for NCIM 563 and NCIM 1360(Table 3). The similarity coefficients between the two clusters were 0.106 and 0.161 by ISSR and SRAP markers respectively. Cluster analysis performed from combining data from both markers generated a dendrogram similar to that of individual marker analyses (Fig 1c). The Cophenetic correlation coefficient among the two marker systems was significant ($r = 0.954$).

Table 1: Summary of polymorphism obtained by inter simple sequence repeat marker analysis of *A. niger* NCIM 563, NCIM 1359 and NCIM 1360

Primer	Primer sequence 5'-3'*	Number of amplicons	Number of polymorphic amplicons	Percent polymorphism	Polymorphism information content
813	$(CT)_8T$	15	15	100.00	0.49
814	$(CT)_8A$	11	11	100.00	0.46
815	$(CT)_8G$	15	15	100.00	0.49
818	$(CA)_8G$	12	12	100.00	0.44
822	$(TC)_8A$	11	10	90.91	0.50
823	$(TC)_8C$	3	3	100.00	0.44
841	$(GA)_8YC$	9	9	100.00	0.50
842	$(GA)_8YG$	9	7	77.78	0.48
843	$(CT)_8RA$	4	4	100.00	0.44
845	$(CT)_8RG$	13	13	100.00	0.49
846	$(CA)_8RT$	12	12	100.00	0.48
850	$(GT)_8YC$	2	2	100.00	0.44
Total		116	113		
Average per primer		9.67	9.42	97.39	0.47

*Y can be T or C and R can be A or G

Table 2: Summary of polymorphism obtained by sequence related amplification polymorphism marker analysis of *A. niger* NCIM 563, NCIM 1359 and NCIM 1360

Primers	Number of amplicons	Number of polymorphic amplicons	Percent polymorphism	Polymorphism information content
em 9 + me 25	3	2	66.67	0.49
em 8 + me 24	3	2	66.67	0.49
em 17 + me 23	8	6	75.00	0.49
em 6 + me 22	9	9	100.00	0.50
em 5 + me 12	5	4	80.00	0.50
em 4 + me 21	2	2	100.00	0.50
em 11 + me 19	4	4	100.00	0.50
em 3 + me 18	4	4	100.00	0.44
em 2 + me 16	7	7	100.00	0.44
em 1 + me 13	3	3	100.00	0.44
em 1 + me 12	5	5	100.00	0.48
em 2 + me 13	7	7	100.00	0.50
em 3 + me 16	6	6	100.00	0.50
em 4 + me 18	13	11	84.62	0.49
em 5 + me 19	10	7	70.00	0.39
em 6 + me 21	10	10	100.00	0.44
em 17 + me 22	1	1	100.00	0.44
em 8 + me 23	7	7	100.00	0.44
em 9 + me 24	22	22	100.00	0.50
em 11 + me 25	6	6	100.00	0.48
Total	135	125		
Average	6.75	6.25	92.15	0.47

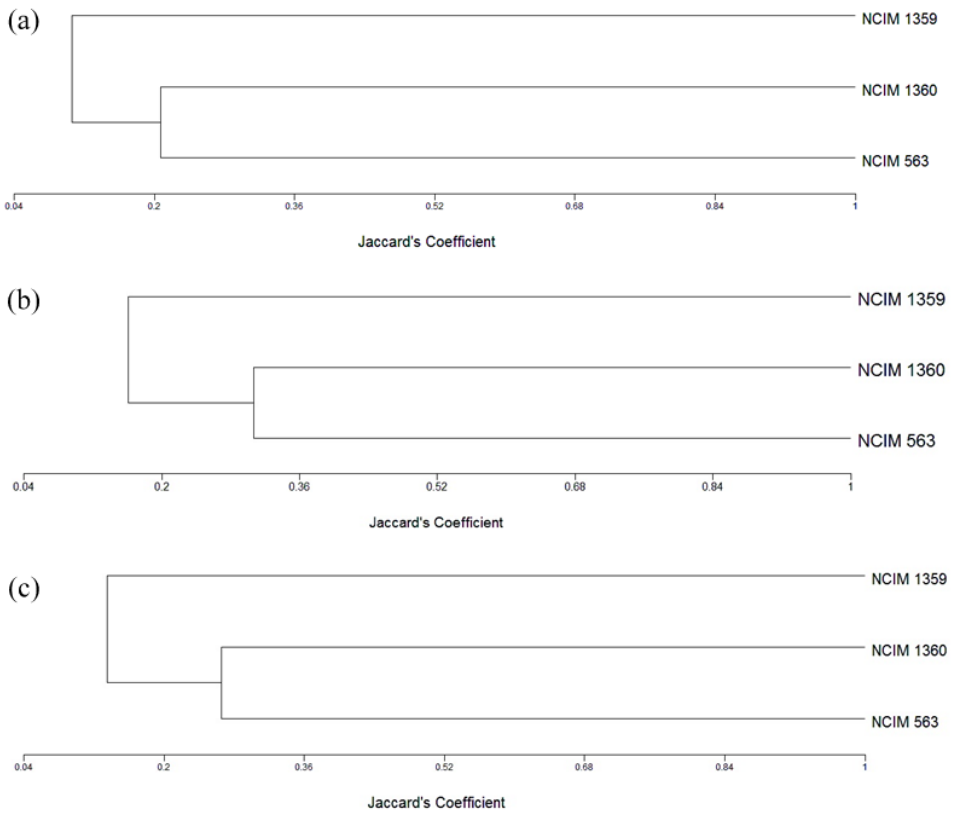

Figure 1: Dendrograms generated by UPGMA cluster analysis for NCIM 563, NCIM 1359 and NCIM 1360 using (a) ISSR and (b) SRAP markers. (c) Combined data for ISSR and SRAP markers

Table 3: Similarity matrix for UPGMA cluster analysis for *A. niger* NCIM 563, NCIM 1359 and NCIM 1360 using ISSR and SRAP markers

Strains	NCIM 563	NCIM 1359	NCIM 1360
ISSR similarity matrix			
NCIM 563	1.000		
NCIM 1359	0.097	1.000	
NCIM 1360	0.207	0.115	1.000
SRAP similarity matrix			
NCIM 563	1.000		
NCIM 1359	0.118	1.000	
NCIM 1360	0.307	0.204	1.000
ISSR-SRAP combined similarity matrix			
NCIM 563	1.000		
NCIM 1359	0.109	1.000	
NCIM 1360	0.265	0.162	1.000

3.2 Gene isolation and characterization

Figure 2 shows the PCR amplification of the genes from the parental and mutant strains. The 3-Phytase B gene sequences from NCIM 563, NCIM 1359 and NCIM 1360 showed 100% identity. However, comparison of these sequences with the *A. niger* CBS 513.88 3-Phytase B gene sequence (gi|145241119) indicated five changes. The 2004 bp sequences of NCIM 563, NCIM 1359 and NCIM 1360 strains comprised 1839 bp (92…1930) full-length sequence of 3-Phytase B gene containing four exons and three small introns. The lengths of the four exons were 834 bp (92…925), 118 bp (990…1107), 105 bp (1169…1273) and 598 bp (1333…1930), respectively; while the lengths of the introns were 64 bp (926…989), 61 bp (1108…1168) and 59 bp (1274…1332). The CDS was 1440 bp with the start and stop codon positions at 145 and 1766, respectively. The

changes in the 3-Phytase B genes from NCIM 563, NCIM 1359 and NCIM 1360 in comparison with 3-Phytase B gene from *A.niger* CBS 513.88 (gi|145241119) were in Exon 1 (C863T), Exon 3 (T1208C) and in the 3' UTR (ATA insertion at: 1887). The transversion in Exon 1 (863) resulted in an amino acid change (A240V) of the 479 amino acid phytase B protein. The other transversion in Exon 3 (1208) resulted in a silent mutation.

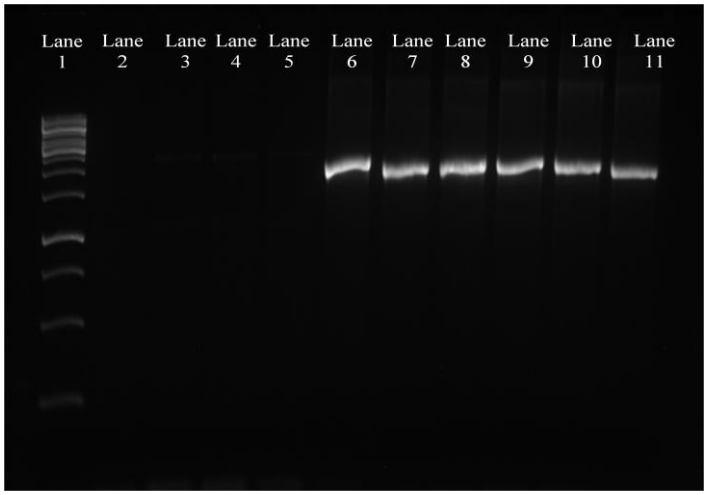

Figure 2: 2% Agarose gel image showing phytase gene amplification (1.7 kb) from parent and mutant strains. Lane 1: 200 bp Marker ladder; Lane 2, 3, 4, 5: Negative controls; Lane 6, 7: PCR product obtained using Primer PFL 563; Lane 8, 9: PCR product obtained using Primer PFL 1359; Lane 10, 11: PCR product obtained using Primer PFL 1360.

4. Discussion

In the present study, the ISSR and SRAP markers were used to evaluate polymorphism among the parental and two high-phytase activity mutants of *A. niger*. Both ISSR and SRAP marker systems revealed high levels of polymorphism among the parent and mutant strains indicating successful

mutagenesis. Even though the two mutants were obtained from a single *A. niger* parental strain NCIM 563, high levels of polymorphism was observed among the three strains. For mutagenesis, the parental strain was exposed to Ethidium bromide (0.1%) and hydroxyl amine (0.1%) overnight followed by exposure to UV for 30 min to isolate mutants NCIM 1359 and NCIM 1360 respectively. The severe mutagenesis conditions using both chemical and physical mutagens possibly resulted in higher levels of polymorphism. Both the marker systems indicated higher similarity of NCIM 1360 to the parental strain compared to NCIM 1359. However, there was slight difference in the similarity coefficients between the two clusters in the dendrogram generated using ISSR and SRAP marker data. The possible reason could be the difference in the target genomic regions for the two marker techniques. The target regions for ISSR marker amplification are located in the nucleotide repeat containing regions, while SRAP aims the coding regions.

In the view of the fact that, the studies involve mutants obtained from a single strain, the magnitude of the variability amongst them could be low. Alternatively, the identification of mutations altering the physiological, morphological or genetic characteristics in these strains would be challenging. In this study, ISSR and SRAP markers were exploited to evaluate the level and pattern of genetic relationship among the three strains of *Aspergillus niger*. The results indicated that ISSR and SRAP systems were highly efficient in demonstrating the genetic diversity among the parent and mutant strains. The percentage of polymorphic bands generated by ISSR primers (58.43%) was lower than that of SRAP primers (92.15%). Moreover, the average PIC (polymorphism information content) of SRAP makers (0.4735) was higher than that of ISSR analysis (0.283). The dendrograms were used to predict the

similarity among the parent and mutant strains. The ISSR marker system was able to predict 80% similarity whereas that obtained from SRAP marker system was about 75%. The possible reason for this observation may be the difference in target regions of the genome for both ISSR and SRAP marker systems. The target regions for ISSR marker amplification are located between simple sequence repeats (SSRs), which is not a general transcription region whereas for SRAP markers, the target region for amplification is the ORF which includes intron(s) and promoter regions. Therefore, combining ISSR and SRAP analysis were effective and more reliable for accurately assessing the genetic variability among the parent and mutant strains of *Aspergillus niger*.

The cluster results based on ISSR, SRAP and combined ISSR and SRAP analyses showed that the strains could confidently be clustered into two major groups. Ferriol et al, (2003) reported that the information obtained from SRAP markers was more concordant with the morphological variations and the evolutionary history of the morphotypes than that found with AFLP markers. The results of the present study show that the SRAP and ISSR marker systems are effective and reliable methods for analyzing the genetic relationships and similarities among *Aspergillus niger* strains.

Nevertheless, the mutants are phytase hypersecreting, indicating functional genetic changes due to the mutation treatment. Considering this, the 3-Phytase B gene from these three strains was sequenced. However, it was surprising to find identical sequence of the gene from all the three strains. Hence, it is most likely that the promoter region of the 3-Phytase B gene might harbor mutations leading to higher expression of the gene. Alternatively, the 3-Phytase B protein from the mutant strains might have higher phytase activity. Both these hypothesis need to be

evaluated to know the genetic basis of phytase hyper secretion by both the mutant strains.

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CHAPTER 6 APPLICATION OF PHYTASE IN PLANT GROWTH PROMOTION, SOIL AMENDMENT AND FUNCTIONAL FOODS

Abstract

The application of phytase from *Aspergillus niger* NCIM 563 in degradation of phytate-phosphorus to benefit mineral release, plant growth and soil amendment is described in this chapter. Additionally, a study on phytase applicability in dephytinization and mineral release from soy based infant foods has been included in this chapter. An enzyme dose of 12 IU was sufficient to hydrolyze phytate and increase assimilation of phosphorus by about 74 %. Phytase supplementation leads to increase in shoot to total length ratio by about 200 %, indicating its growth promoting effect. Consistency in phytase induced growth was reflected at pot and tray levels, wherein shoot to total length ratio was observed to be 2.01 and 2.12 respectively. Mineral assimilation due to phytase was more efficient as compared to chemical fertilizers thus overcoming the constraints of practicability and economics of agriculture industry. Phytase was efficient in reducing the phytic acid content of soil by about 30 % while simultaneously increasing the phytate phosphate availability by 1.18 fold. The role of *A. niger* phytase in improving phytate-phosphorus uptake and its utilization will have significant impact on both agriculture and environment. Besides plant growth promontory effect, reduction in use of chemical fertilizers and soil improvement could be achieved simultaneously for maintaining the sustainability of agriculture. Similarly, degradation of phytic acid and release of mineral ions was studied in soy based infant food and soybean flour. Phytase was capable of reducing phytic acid content of soybean flour and soy based

infant food by about 82.8 % and 91.9 % respectively while enriching bound phosphorus by about 18.7 % and 28 % respectively at the same time enhancing the availability of metal ions such as Zn, Fe, Cu, Mn, Mg, and Ca.

1. Introduction

Phosphorus is an essential component of life. It is a major component for synthesis of nucleic acids (DNA and RNA) moreover it plays important role in signaling and energy metabolisms viz IP_2, IP_3, ATP, ADP etc (Vats and Banerjee 2006). Reycling of phosphorous is a slow biogeochemical process in nature and this limits its availability from natural sources such as rocks and volcanic sediments. The intensification in agriculture industry has led to extensive use of these non-replenishable mineral reserves as phosphate supplements (Elser and Bennett 2011). Based on the current usage, it is estimated that these phosphate reserves will be depleted in the next 50 years (Vance et al, 2003); furthermore their excessive use has led to the emergence of serious concern for phosphorus pollution in soil and water (Vats and Banerjee 2006).

In India, the annual consumption of fertilizers has increased from 0.7 million metric tons in 1951-52 to 264.86 million metric tons in 2009-10, while per hectare consumption, has increased to about 135.27 kg in 2009-10 compared to that of 1 kg in 1951-52 (Annual Report 2010-11 Department of Fertilizers, Ministry of Chemicals & Fertilizers Government of India). Phosphorus deficiency in plants leads to several morphological and physiological changes which in turn affect plant growth, productivity and survival (Tran et al, 2010) and hence is a major pin down for agriculture industry worldwide. All of the abovementioned

facts raise concerns for the need of sustainable agriculture and alternative phosphorus source.

Interestingly, phytate is a primary storage form of phosphorus (60-90 %) in plants (Vats and Banerjee 2006). Phytate is an anti-nutrient and a major source of phosphorus pollution in animal manure (Sajjadi and Carter 2004; Vohra and Satyanarayana 2003). Different forms of phytate exist as adsorbed to clays or precipitated as insoluble salts of iron and aluminum in acidic soils or as insoluble calcium salts in alkaline soils, consequently denoting a need for soil amendment (Tang et al, 2006). Moreover, phytate being a chelating agent filches the metal ions essential to soil fertility and plant growth (Wodzinski and Ullah 1996). Plants and monogastric animals have limited ability to utilize phytate-phosphorus; hence the need for phytase supplementation arises (Richardson et al, 2001).

Phytases catalyze the hydrolysis of phytate to free inorganic phosphate (iP) and different myo-inositol phosphate esters (Ins P5 to P1) or myo-inositol in some cases (Piddington et al, 1993). This makes phytase a noteworthy candidate for substitution of existing phosphate supplements. Phytase can also be exploited for its ability to solubilize essential minerals in soil as it eliminates the anti-nutritional effect of phytate.

Justus von Liebig and others pioneered the theory that minerals such as nitrogen, phosphorous and potassium were required for plant growth (McNeill and Winiwarter 2004). Furthermore, he also proposed a law of the minimum which stated that the availability of the scarcest nutrient limits plant growth (Janick et al, 1976). Importance of soil microbes for increasing availability of phytate-phosphorus to plant roots has also been suggested (Richardson et al, 2001; Singh and Satyanarayana 2010;

Tarafdar and Marschner 1995). Solubilization of phytate-phosphorus and its effect on plant growth promotion *in vitro* has been studied by Singh and Satyanarayana (2010). Recombinant expression of phytases in plants (*Tobacco* and *Arabidopsis*) has led to increased phosphate uptake and enhanced plant growth (Lung et al, 2008; Yadav and Tarafdar 2003; Yip et al, 2003). Idris et al, (2002) studied the role of culture filtrates of phytase producing *Bacillus* on plant growth promotion in maize.

Many experiments and field trials have shown that 500 to 1000 units of phytase can replace approx. 1 g inorganic phosphorus supplementation and reduce total phosphorus excretion by 30-50 % (Kemme et al, 1997; Liu et al, 1997). Even though large amount of research is focused on improving phosphorus availability from soil its primary applicability at a broader scale is misled. Richardson and Simpson (2011) have documented the need for projection of small scale promises to substantial farm levels.

Phytate frequently occurs in nature and constitutes the principal storage form of phosphorus (60–90 %) in plants, legumes and oil seeds (Reddy et al, 1982). It usually exists as a salt of monovalent and divalent cations (Fe^{2+}, Mn^{2+}, K^{+}, Mg^{2+} and Ca^{2+}) and builds up in seeds during the ripening. Phytic acid is thus a common constituent of plant based foods. It exists as a highly negatively charged ion over a broad pH range and therefore has a tremendous affinity for food components with positive charge(s), such as minerals, trace elements and proteins (Greiner and Konietzny 2006). It is also considered as an anti-nutrient because it acts as a strong chelator of divalent and trivalent minerals such as Mn^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}/Fe^{3+} (Harland and Oberleas 1999). The formation of insoluble mineral–phytate complexes at physiological pH is the main reason for the poor mineral bioavailability, because these complexes are

difficult to digest in the animal and human gastrointestinal tract (Greiner and Konietzny 2006). Minerals are essential for several intracellular and extracellular reactions of the cell. Minerals deficiency may lead to severe metabolic disorders and compromise the health of the organism (Lopez et al, 2002).

Legumes and cereals are rich sources of proteins and dietary minerals. The content of Fe^{2+}, Zn^{2+} and other minerals is generally high in legumes (Jambunathan and Singh 1981). However, their applications as food are limited due to phytate leading to reduction in nutritive value of these legumes. Individual supplementation of minerals in human foods and feeds such as iron etc may lead to undesirable organoleptic changes in the product (Davidson et al, 2000). Supplementation of ascorbic acid is also another alternative to enhance mineral absorption, but may lead to pH or taste changes in food, moreover ascorbic acid addition requires food to be superiorly packed and stored, thus adding to the cost of production which is not bearable in developing countries (Hurrel et al, 2004). Hence, enzymatic degradation of phytate is desirable to increase the bioavailability of minerals (Sandberg et al, 1999).

Phytases (myoinositol hexaphosphate phosphohydrolase EC 3.1.3.8 and 3.1.3.26) are phytic acid degrading enzymes present in plants, animal tissues and also produced by a large number of bacteria and fungi (Vohra and Satyanarayana 2003). However, monogastric animals lack of adequate levels of phytases (Rao et al, 2009) thus phytase is used to enhance the nutritive value of foods. The great potential for use of phytase in processing and manufacturing of food for human consumption has been proposed (Haros et al, 2001; Hurrell et al, 2003), but it has not been applied till date.

Here we have studied the effect of phytase on plant growth at pot and tray level and compared it with commercial fertilizers pertaining to chemical and physiological parameters. We have also studied the use of phytase as a soil amendment relating to the augmentation of fertility and combating pollution.

2. Materials and Methods

2.1 Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company (St Louis, MO, USA). All the other chemicals used were of analytical grade. Fertilizers (NPK 19:19:19, NPK 13:40:13 and Diammonium phosphate) were purchased from the local market.

2.2 Microorganism, culture media and enzyme production

A. niger NCIM 563, used in the present study, was obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune India. The stock culture was maintained on Potato Dextrose Agar (PDA) slant and stored at 4 $^{\circ}$C. Spores for inoculation were obtained by culturing the strain at 30 $^{\circ}$C on a PDA slant for 7 days, followed by washing with 10 ml sterile saline containing 0.01 % Tween 80. *A. niger* was cultivated in production medium (Soni and Khire 2007) at room temperature for 15 days. After fermentation mycelium was separated by filtration followed by centrifugation at 10,000 × *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 × *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). The enzyme (specific activity approximately 625 IU/mg) thus obtained was used for further studies (Soni and Khire 2007).

2.3 Phytase assay

Phytase activity was measured at 50 °C; the reaction was carried out at pH 2.5 using 100 mM Glycine-HCl buffer at 50 °C for 30 min. The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (Heinonen and Lahti 1981), wherein 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 1 M citric acid were added to the assay mixture and absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 µmol of phosphorus per min under standard assay conditions.

2.4 Plant material

Wheat (*Triticum aestivum* L.) grains used for the experiments were obtained from local market.

2.5 Soil

Soil was collected from local farm (within an area of 1 m^2) ploughed to a depth of 20 cm. It was air-dried, sorted from coarse organic residues and ground to pass through a 2 mm sieve, sterilized and used for further experimentation.

2.6 Effect of A. niger spores and phytase on plant growth

Sterilized soil (100 g) was distributed in pots equally and seed of wheat were sown at a depth of 2 cm each. The pots were supplemented with spore suspension (10^7 spores per ml), phytase enzyme (6 and 12 IU) along with sodium phytate (1.32 and 3.30 mg), watered periodically and monitored for height and growth of plant compared to that of control. The experimental pots were placed in the green house to ensure stable conditions for the plants. The 15 day old seedlings were uprooted and measured for plant height, root and shoot length, subsequently these were subjected to drying in a hot oven at 60 °C, the dried parts were ashed in an incinerator at 400 °C for 4 h and dissolved in 0.9 M H_2SO_4 and used for further chemical analysis (Ryan et al, 2001).

2.7 Up scaling to tray level

The pot level combination exhibiting best results was chosen for further experimentation. A scale up experiment was designed to check the potential of phytase as a plant growth promoter on a larger scale. Two levels of scale up for combination B included initial studies at 0.5 kg tray level followed by 2 kg tray level. For 0.5 kg level, sterilized soil (500 g) was laid in trays and wheat seeds (50 g) were sown at a depth of 2 cm each. Similarly, for 2 kg tray level, sterilized soil (2 kg) was laid in trays and wheat seeds (200 g) were sown at a depth of 2 cm each. The trays were supplemented with phytase enzyme and sodium phytate as mentioned earlier and placed in the greenhouse to ensure stable conditions for the plants where they were watered periodically and monitored for height and growth of plant compared to that of control. After 15 days the seedlings were uprooted and measured for weight and height, subsequently subjected to drying in a hot oven at 60 °C, the dried

parts were ashed in an incinerator at 400 °C for 4 h and dissolved in 0.9 M H_2SO_4 and used for further analysis (Ryan et al, 2001).

2.8 Comparative effect of phytase and other fertilizers on plant growth

The potential of phytase as a plant growth promoter was analyzed in contrast with chemical fertilizers at 2 kg tray level. Trays with control and enzyme combination were prepared as mentioned earlier. Fertilizer combination included addition of 2 g fertilizers (19:19:19, 13:40:13 and DAP) each to the trays. The trays were incubated in greenhouse, watered periodically and monitored for growth of plants. 15 day seedlings were uprooted and prepared for analysis as mentioned above (Ryan et al, 2001).

2.9 Role of phytase as a soil amendment

The efficacy of phytase as a soil amendment was studied where in, 100 g sterile soil was mixed thoroughly with phytase enzyme (12 IU). The pots were incubated in green house for 15 days after which it was collected, dried and used for further physical and chemical analysis (Ryan et al, 2001).

2.10 Estimation of phytic acid

The estimation of phytate content was done as proposed by Latta and Eskin (1980). Phytic acid was extracted from 5 g of soil sample with 100 ml HCl (2.4 %) for 1 hour at room temperature, followed by centrifugation at 3000 × *g* for 10 minutes. 1 ml of the supernatant was diluted to 25 ml in a volumetric flask with distilled water. 10 ml of diluted sample was subjected to column with anion exchange resin AG1-X8, and eluted initially with 15 ml of 0.1 M NaCl solution and finally with 15 ml of 0.7 M NaCl solution. To 3 ml of the extracted sample, 1 ml

of Wade reagent (0.03 % $FeCl_3.6H_2O$ and 0.3 % Sulphosalicylic acid in distilled water) was added. The samples were incubated at room temperature for 15 minutes and then read at 500 nm. The standard curve was prepared with sodium phytate. Each experiment was carried out in triplicate and the mean values are reported with a maximum of 3-5 % variability.

2.11 Soy bean experiment

2.11.1 Phosphate Liberation from SBF

To estimate the amount of phosphate liberated from SBF after phytase treatment, 1 g of SBF was suspended in 10 mL Glycine HCL buffer (0.2 M, pH 2.5). The suspensions were incubated with and without the supplementation of 10 U of phytase at 50 °C on a rotary shaker (100 rpm). Aliquots were obtained at fixed intervals. The liberated inorganic phosphate was quantified by standard procedure as mentioned earlier.

2.11.2 Mineral Analysis

The ability of phytase to increase bioavailability of PA bound minerals (Ca^{2+}, Fe^{2+}, and Zn^{2+}) from SBF was quantified using an inductively coupled plasma atomic emission spectroscopy (ICP-AES). Digestion of SBF for total mineral analysis was carried out according to Friel and Ngyuen (1986), with slight modification. SBF was dried in oven at 80 ºC until constant weight was obtained. One gram of dried SBF was weighed in crucible and heated at 500 ºC for 4.30 h. After cooling at room temperature, samples in crucibles were mixed with 5 mL of HNO_3 : $HClO_4$ (4:1) and heated at 120–140 ºC until a clear solution was obtained, which was filtered through Whatman filter paper (0.2 mm, Pall Corporation,

Port Washington, NY). This filtrate was used for total mineral analysis in untreated SBF. For determination of mineral mobilization after phytase treatment, 1 g of dried SBF was suspended in 10 mL of 0.2 M Glycine HCl buffer pH 2.5, containing 10 U of crude phytase and incubated at 50 ºC on a rotary shaker at 100 rpm for 2 h. Samples were centrifuged at 10,000 x g for 10 min at 4 ºC, supernatant was filtered (0.2 mm, Pall Corporation) and filtrate was collected. Filtrate was diluted with distilled water for adjusting the signals in the calibration ranges of each element. Zn^{2+}, Fe^{2+} and Ca^{2+} contents in the samples were measured by ICPAES. The experiment was conducted in duplicate, and the mean values and standard errors reported. Mobilization (%) was calculated as follows: Mobilization (%) = 100 ¥ *Y*/*Z*, where *Y* is the element content of the bioavailable fraction (mg mineral element / 100 g SBF), and *Z* is the total Zn^{+2}, Fe^{+2} and Ca^{+2} content (mg mineral element/ 100 g SBF).

2.11.3 Determination of phytic acid

The leftover pellet of SBF after phytase treatment was used to estimate the residual PA content. PA extraction and cleanup were carried out on the basis of the modified procedure of Latta and Eskin (1980). Briefly, 1 g of phytase treated and untreated SBF were extracted with 2.4 % HCl (20 mL) by incubating on rotary shaker at 150 rpm for 1 h at room temperature. After centrifugation, the supernatant was diluted with double volume of distilled water, and 10 mL of the diluted supernatant was loaded on to a column containing an AG-1-X8 anion exchange resin (200–400 mesh chloride form). Interfering compounds and inorganic phosphorus were removed by washing with distilled water (10 mL)

followed by 0.1 M NaCl (10 mL). Bound PA was eluted with 0.7 M NaCl (10 mL). The elutant was evaporated to dryness with a Speedvac concentrator at 40 ºC, and the residue was dissolved in mobile phase (0.008 N, H_2SO_4).The PA content in SBF before and after phytase treatments were determined using a high-performance liquid chromatography(HPLC) system (Dionex India Ltd, Mumbai, Maharashtra, India) equipped with UV/RI-detectors. Anion exclusion column (Aminex, HPX-87H, Biorad, Hercules, CA) was used at a temperature of 30 ºC with 0.008 NH_2SO_4 as a mobile phase at flow rate of 0.6 mL/min. The solution was centrifuged at 10,000 x g for 5 min to remove any suspended material prior to injection of 50 mL into HPLC. The concentration of PA was quantified based on standard curve created with Na–phytate (100–1,000 mg).The experiment was conducted in duplicate; the mean values and standard deviation are presented.

3. Results

3.1 Plant growth promotion

Enzyme for the experiments was obtained as per mentioned above. Enhanced growth was observed in seedlings grown under phytase supplementation as compared to control. The root and shoot length of the wheat plants was higher in pots supplemented with phytase and sodium phytate when compared to that of control. Combination B was found to be the best in all parameters measured to correlate plant growth amongst all other combinations studied (Fig 1). Figure 2 depicts the shoot to total length ratio and it can be seen that phytase promoted plant growth up to 200 % (combination B). An enzyme dose of 12 IU per plant was found

adequate to liberate enough amount of inorganic phosphate required for supporting plant growth. However, the results for the spore based combinations were not comparable with that of enzyme extracts (Data not shown). Further analysis of plant samples from combination B depicted an improved mineral assimilation as compared to that of control. The phosphate content of plantlets was found to be 0.12 % and 0.86 % for control and supplemented plantlets respectively, depicting comprehensible distinction in assimilation of phosphorus.

Figure 1: Image showing difference in growth of wheat plants obtained after cultivation under different combinations. (a)Control- Without sodium phytate and phytase (b)Combination B-1.32 mg sodium phytate + 12 IU phytase (c) Combination A -1.32 mg sodium phytate + 6 IU phytase (d) Combination C-3.3 mg sodium phytate + 6 IU phytase (e) Combination D-3.3 mg sodium phytate + 12 IU phytase

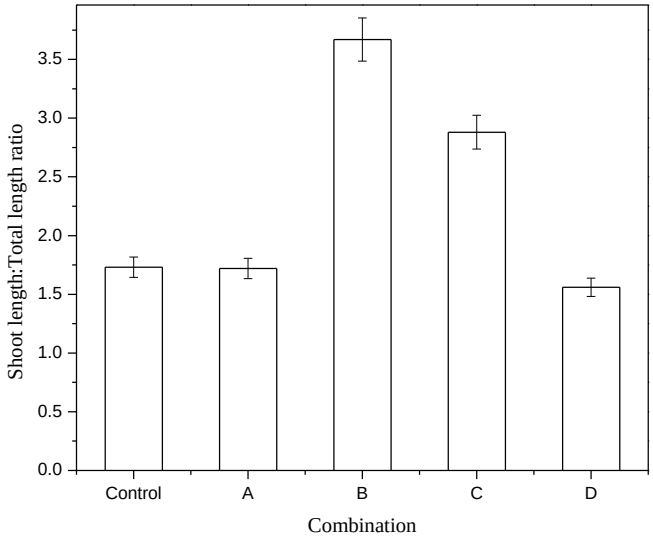


Figure 2: Effect of phytase and sodium phytate on growth of wheat seedlings. Control- Without sodium phytate and phytase A -1.32 mg sodium phytate + 6 IU phytase B-1.32 mg sodium phytate + 12 IU phytase C-3.3 mg sodium phytate + 6 IU phytase D-3.3 mg sodium phytate + 12 IU phytase

3.2 Up scaling to tray level

Supplementation of phytase at a larger scale also contributed to increase in plant growth irrespective of the experiment scale (0.5 kg and 2 kg). Figure 3 shows the difference in plant growth obtained after supplementation at various scale levels. The shoot to total length ratio for the test samples was found to be about 2.01 and 2.12 times that of the control in case of 0.5 kg and 2 kg trays respectively.

Figure 3: Tray level scale-up for comparative analysis of phytase in promoting plant growth (a) 0.5 kg level (b) 2 kg level Control (Without sodium phytate and phytase) v/s Combination B (1.32 mg sodium phytate+12 IU phytase)

3.3 Effect of phytase and fertilizers on plant growth

The growth of plants obtained using phytase supplementation was comparable to that of the fertilizers 19:19:19 and 13:40:13, except for DAP which exhibited moderate growth (Fig 4). Further analysis of plantlets revealed the potential of phytase in assisting the absorption of mineral nutrients in plants a cut above than fertilizers. Nitrogen assimilation by plants was higher in DAP than others, whereas phosphorus content of plants was higher in 13:40:13 which can be attributed to its high phosphate content. Potassium content of plants was found to be highest in 19:19:19 fertilizer combination (Fig 5a). Similarly, enhanced micronutrient assimilation in plants augmented with phytase was observed in contrast with other fertilizer combinations (Fig 5b).

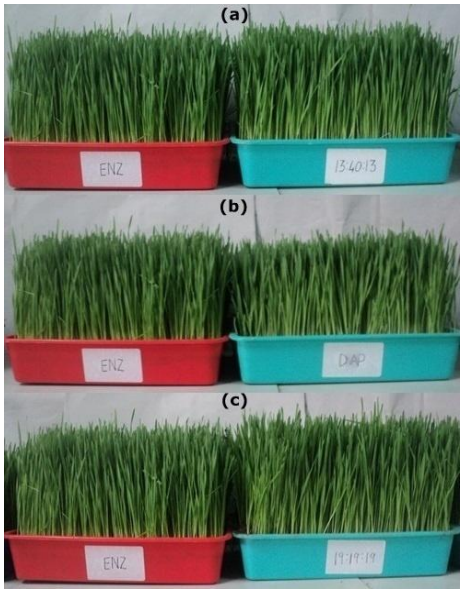
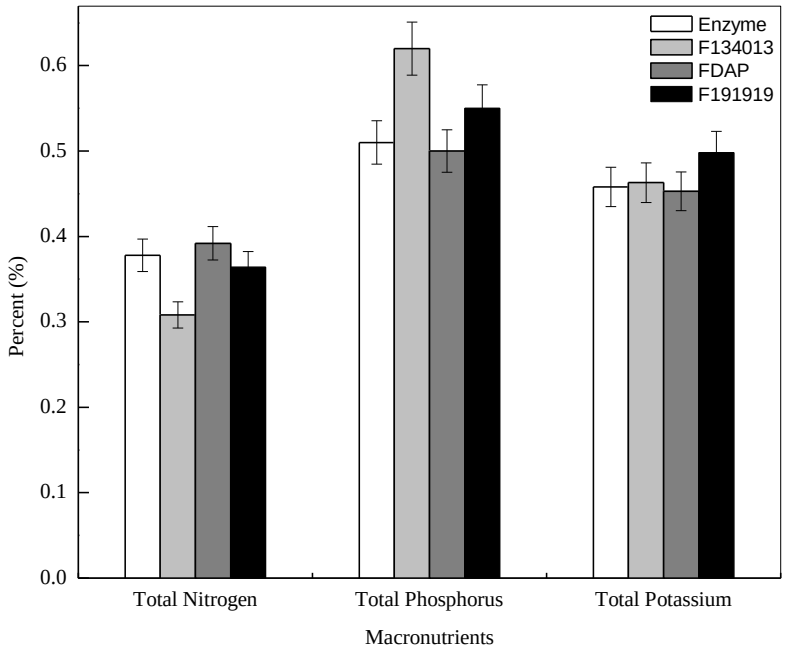
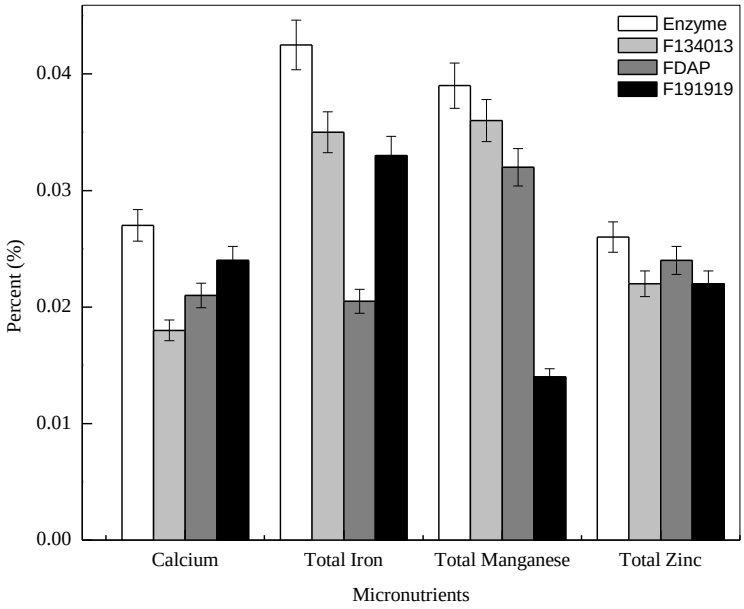


Figure 4: Comparative effect of phytase and fertilizers on plant growth (a)Enzyme v/s F 13:40:13 (b) Enzyme v/s F DAP (c) Enzyme v/s F 19:19:19



(a) Macronutrients



(b) Micronutrients

Figure 5: Comparison of nutrient assimilation by plants

3.4 Phytase as a soil amendment

Addition of phytase in soil has led to increase in its fertility blatant with decrease in pH, $CaCO_3$ and increase in electrical conductivity (Table 1). Comparison of macronutrient and micronutrient availability suggests that phytase has ascendancy in soil improvement. Phytic acid content of soil was reduced by about 4.45 mg/g of soil under phytase supplementation coherently evident with the increase in phosphorus content of the soil by about 1.18 fold (Table 1), suggesting the positive intervention of phytase in soil productivity.

Table 1: Effect of phytase on soil nutrient content

Parameter	Control	Test
pH (1:2.5)	8.4	8.06
Electrical Conductivity (dSm^{-1})	0.32	0.47
Organic Carbon (%)	1.53	1.79
$CaCO_3$ (%)	10.38	6.5
Nitrogen (kg/ha)	276	301
Phosphorus (kg/ha)	105	124
Potassium (kg/ha)	1030	997
Iron (kg/ha)	0.47	0.43
Manganese (kg/ha)	8.63	3.63
Zinc (kg/ha)	2.45	2.31
Copper (kg/ha)	2.72	2.98
Phytic acid (mg/g)	18.51	13.06

3.5 Dephytinization and mineral release

Phytase was capable of reducing phytic acid content of soybean flour and soy based infant food by about 82.8 % and 91.9 % respectively (Table 2) while enriching bound phosphorus by about 18.7 % and 28 % respectively (Table 3a, b) which highlights its potential for use as infant supplements.

Table 2: Estimation of Phytic acid content before and after phytase treatment

Sr. No.	Substrate	Initial Phytic acid content (mg/g)	Final Phytic acid content (mg/g)
1.	Soy bean flour	25.6	4.43
2.	Soy based infant food	39.07	3.14

Phytase was also found to enhance the bioavailability of metal ions such as Zn, Fe, Cu, Mn, Mg, and Ca (Table 3a, b) which can be exploited for use as supplements to increase mineral assimilation in humans.

Table 3: (a) Quantitative estimation of minerals released after phytase treatment from Soy bean flour

Sr. No.	Metal Ion	Soy bean flour	
		Initial concentration (µg/g of substrate)	Final concentration (µg/g of substrate)
1.	P	506.63	601.47
2.	Zn	19.33	21.59
3.	Fe	2.59	3.04
4.	Cu	4.15	4.76
5.	Mg	65.67	76.06
6.	Ca	482.9	542.24
7.	Mn	13.83	15.36

Table 3: (b) Quantitative estimation of minerals released after phytase treatment from Soy based infant food

Sr. No.	Metal ion	Soy based infant food	
		Initial concentration (µg/g of substrate)	Final concentration (µg/g of substrate)
1.	P	773.2	989.1
2.	Zn	24.8	26.8
3.	Fe	2.9	3.2
4.	Cu	0.4	0.5
5.	Mg	48.6	51.1
6.	Ca	89.84	89.85
7.	Mn	24.0	26.2

4. Discussion

In our experiments, phytase was capable of enhancing uptake of phosphorus (by approximately 74 %) and promoting plant growth (up to 200 %). The replication of these observations at higher levels (0.5 kg and 2 kg) highlights the potential of phytase as soil amendments. Furthermore, 12 IU (*in situ*) enzyme was sufficient to enable release of sufficient phosphorus to promote plant growth. These observations substantiate the findings of Shen et al (2011) that phosphate uptake and its utilization in plants determine the final crop yield. The *Fertilizer Consumption Report* by the International Fertilizer Industry Association (Soh and Prudhomme, 2000) revealed that farmers across the world were using about 10 times more fertilizer in 2001 as compared to that in 1950, but it has barely contributed to about a threefold increase in food production. It is estimated that the worldwide consumption of fertilizers will increase by about 1.0 % per year, rising from 136 million metric tons

per year in 2008/2009 (Lal 2004) to 188 million metric tons per year in 2030 (Bruinsma 2003). Considering that interruption in fertilizer supply across the world would lead to a drop in food production by about 40 % (Brown 1989) and the decline in accessibility of fertilizers by about 22 % since 1991 (Sundquist 2012) it can be stated that the concern to find alternatives has increased. Phytase promoted mineral assimilation much more efficiently when compared to the chemical fertilizers studied. The exhibition of moderate growth by plants supplemented with DAP (mainly a phosphate and nitrogen fertilizer) when compared to other combinations emphasizes the significance of potassium in plant growth. Higher nitrogen assimilation by plants supplemented with DAP could be attributed to the significant quantity of ammonium in it, which makes DAP a valuable nitrogen fertilizer. Furthermore, the highest assimilation of phosphorus and potassium was found in combinations 13:40:13 and 19:19:19 respectively, which can be attributed to their high concentration in the fertilizers. Improved micronutrient assimilation in plants could be attributed to reversal of phytate chelation upon supplementation with phytase (Wodzinski and Ullah, 1996). According to our investigation, it is evident that the ability of phytase as a plant growth promoter is comparable to that of chemical fertilizers; therefore it can be shown that 12 IU phytase can be used to replace 2 g chemical fertilizer, suggesting the potential of phytase in replacing chemical fertilizers with eco-friendly alternatives. Stutter et al (2012) have reported the necessity for use of newer approaches in exploiting soil phosphorus resources as an ecofriendly perspective towards sustainable agronomy. Phytase can be used as a potential tool to harvest phosphorus from organic esters, which contribute to a large fraction of the phosphorus reserves in soil. Alkaline pH of the soil limits assimilation of phosphorus, in accordance with fixation and precipitation being highly dependent on soil pH and ionic

strength (Rodriguez and Fragga, 1999) which makes plant growth reliant on soil character. In this paper, contrasting observations suggest that intervention of phytase is significant in fixation of phosphorus by plants even at high soil pH. Since phytase from *A. niger* is active over a broad pH range (Soni et al, 2010) its suitability as a plant growth promoter in alkaline soils is significant. The efficiency of phytase in increasing availability of soil phosphorus is evident through changes in soil conductivity and phosphate availability from these experiments. Phytase was able to degrade about 30 % of the phytic acid in the soil. The increase in nitrogen availability in soil can be attributed to release of ammonium ions bound to phytate following degradation by phytase. Moreover, phytase releases phosphorus from phytate, leading to the loss of ability of phytate to bind or chelate minerals, starch or proteins either directly or via ionic bridges, and consequently amend the availability of nutrients (nitrogen, calcium, magnesium, zinc and iron) in soil (Pallauf and Rimbach, 1997). Phosphorus deficiency in soils is a major constraint for agricultural production worldwide. A large proportion of endogenous soil phosphorus occurs in organic forms, of which derivatives of inositol phosphates constitute a major fraction (Richardson et al, 2005). Plants fail to utilize the insoluble phytates directly, and therefore these phytates should be dephosphorylated by phytases before assimilation. This study emphasizes the potential of *A. niger* phytase for improving plant phosphorus acquisition and phytoremediation for plant growth promotion.

The fact that the experiments were carried out in farm soil suggests a more promiscuous approach for use of phytase as both a plant growth promoter and as a soil amendment. These studies yield additional insights into the physiological roles of phytase in agriculture. The eco-friendly potential of phytase to utilize phosphorus from phytic acid in soil is

evident, which further limits the addition of more phosphorus than is needed, thereby lessening fertilizer costs and environmental pollution.

Soy based products are nutritional banks and have a tremendous value in infant foods. These are also prominently used to ensure adequate nutrition in children with lactose intolerance. But since the phytate content of soy is high, mineral absorption in these foods is limited. Use of phytase to overcome this hindrance was the objective of this study. One molecule of phytate binds to six molecules of ferric ion thus phytate proves to be a strong inhibitor at relatively low concentrations (Hurrel et al, 2003). Thus for phytase supplementation to be effective about 80-90 % of phytate must be degraded (Hurrel et al, 2004). Hurrel et al (2004) iron absorption increased two fold when phytate in food was degraded by 90 %. Similarly, Lei et al (2013) predicted that a 50 % (improvable to 60-80 %) decrease in fetal phosphorus excretion could be achieved if proper dosage of phytase was supplemented. In our experiment, we could achieve 82.8 % decrease in phytic acid content in soybean flour while a 91.9 % decrease in soy based infant food which is comparable in a positive way. Moreover, phytase supplementations also led to increasing mineral availability from both soybean flour and infant foods which is indicative of its promising application in infant food industry. Altogether, supplementation of phytase is a very promiscuous economical and eco-friendly approach which can be exploited at a commercial scale.

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CHAPTER 7 GENERAL DISCUSSION AND CONCLUSIONS

General Discussion

Though researchers have been interested in phytases for over a century due to their multifarious applications in food and feed industry, these enzymes have recently emerged as key enzymes in expanding white biotechnology. A lot of time and money is spent on research involving screening and selection of phytase producing organisms. Much R & D efforts have been made to improve the yields of phytase which include the mutation, cloning and expression in suitable host organisms and also optimization studies. The main area of phytase application has been food; however they are not yet employed in food processing applications in spite of the large amount of literature regarding food quality improvement using phytase being available.

Highlights of the Research work

- The approach of combinatorial mutagenesis was found to be effective for isolation of hypersecretory mutants of phytase.
- This is the 1st report where in Hydroxylamine is used as a mutagen for phytase overproduction.
- It is also to be noted that the high phytase activity exhibited by mutants of *A. niger* NCIM 563 is significantly superior when compared other organisms. Enhanced production of acidic phytase might provide a significant boost to exploit economic feasibility and commercial scale viability.

- Considering the importance of costing at industrial level, use of Soy bean flour as a futuristic substrate for phytase production looks promising. Further insights into industrial applicability could be obtained after production studies at larger scale.

- Significant differences were observed in activities of CMCase, xylanase, β-glucosidase, acid phosphatase and amylase among the parent and the mutant *A. niger* strains in the present study. In addition to high-phytase activity, the mutant strains showed increased xylanase activity compared to the parental strain. With the secretion of enzymes with cellulolytic, phytase and xylanase activities, these mutants exhibited a potential for being industrial sources of multi-enzyme blends to hydrolyze both phytic acid and non-starch carbohydrates in animal feed.

- Scanning electron microscopy revealed noteworthy alterations in morphology of mutant strains compared to *A. niger* NCIM 563. Previous studies have suggested the role of morphological parameters such as pellicle size in increasing enzyme production; comparable to the observations recorded in this research work.

- Phytase was successfully purified from *A. niger* NCIM 1359 and NCIM 1360 with specific activity of 815 and 905 U/mg with 33.86 and 34.01 fold purification respectively.

- MALDI TOF effectively predicted the molecular mass of phytase subunit to be 66 kDa from both the parent and mutants strains. It could not detect any variation in the polypeptides of the mutant strains as compared to the parental strain; indicating absence of mutations in the phytase polypeptide. However, the occurrence of

mutations in regulatory regions of the phytase gene cannot be neglected.

- The severe mutagenesis conditions using both chemical and physical mutagens possibly resulted in higher levels of polymorphism among parent and mutant strains as successfully detected by both ISSR and SRAP marker systems.
- Both the marker systems indicated higher similarity of NCIM 1360 to the parental strain compared to NCIM 1359.
- Combining ISSR and SRAP analysis was an effective and reliable method for assessing polymorphism among the parent and the mutant strains which can be attributed to the difference in the target genomic regions for the two marker systems; ISSR marker amplification are located in the nucleotide repeat containing regions, while SRAP aims the coding regions.
- Gene sequences from all the three strains were found to be identical. There is a probability that the promoter region of the 3-Phytase B gene might harbor mutations leading to higher expression of the gene in mutant strains. However this hypothesis needs to be evaluated to know the genetic basis of phytase hyper secretion by both the mutant strains.
- The noteworthy potential of *A. niger* phytase for improving plant phosphorus acquisition and phyto-remediation for plant growth promotion is evident through these studies.
- These studies also yield additional insights into the physiological roles of phytase in agriculture and are relevant to current efforts to engineer Pi-efficient transgenic crops that can utilize the

phosphorus already in the phytic acid, without adding more P than is needed, thereby lessening costs and pollution.

- The ability of phytase to improve mineral mobilization and dephytinization of in soy based foods (soy flour and infant food) suggests its potential application in food processing and feed industry (Functional foods).

Scope for Future Work

- Studies on up-scaling for phytase production from mutant strains at 14 L and consequently 100 L fermentor could be undertaken for commercialization of phytase.
- The cocktail of enzymes from the above said strains could be exploited at animal trial level in order to assess the applicability in animal feed as a multi enzyme blend supplement.
- Since gene level variation among parent and mutant strain was found to be absent, sequence analysis of regulatory regions in phytase genes from all three strains can be undertaken so as to pin point the site of mutagenesis responsible for hyper secretion of phytase in mutant strains.
- Field trials of phytase as a plant growth promoter could be taken up to achieve a scaled up version of the laboratory experiments, and make realize the commercial feasibility of phytase application.
- In accordance with the laboratory experimental observations of the potential of phytase to enhance mineral bioavailability it is suggested that more *in vitro* experiments are needed to be undertaken to develop phytase as an infant food supplement.

LIST OF PUBLICATIONS

Thesis

1. Bhavsar KP, **Gujar PD**, Shah P, Ravi Kumar V & Khire JM (2013) Combinatorial approach of statistical optimization and mutagenesis for improved production of acidic phytase by *Aspergillus niger* NCIM 563 under submerged fermentation condition. Appl Microbiol Biotechnol 97(2):673-679.

2. **Gujar PD**, Bhavsar KP, Khire JM (2013) Effect of phytase from *Aspergillus niger* on plant growth and mineral assimilation in wheat (*Triticum aestivum* Linn.) and its potential for use as a soil amendment. J Sci Food Agric. 93(9):2242-2247.

3. **Gujar PD**, Dixit RP, Kadoo NY, Gupta VS and Khire JM. Genetic, morphological and physiological profiling of *Aspergillus niger* NCIM 563 and its mutants with high phytase activity (Manuscript completed).

4. **Gujar PD**, Kapil Kumar, Kumar Raja P and Khire JM. Production of phytase by *Aspergillus niger* NCIM 563 and its mutants in soy bean flour medium and its application in phytic acid degradation and mineral release from soy bean flour and infant foods (Manuscript completed).

Other:

5. Pable A, **Gujar P** and Khire JM. (2013). Selection of phytase producing yeast strains for improved mineral mobilization and dephytinization of chickpea flour. J Food Biochem doi: 10.1111/jfbc.12020

LIST OF PATENTS:

1. Mutants of *Aspergillus niger* NCIM 563 overproducing phytase (IND, US & UK) (PROVISIONAL).
2. Gene sequences of phytase from *Aspergillus niger* NCIM 563, NCIM 1359 and NCIM 1360 (US & UK) (PROVISIONAL).

ANNEXURE

Chapter 4

Differential Enzyme production T test

	563 vs 1359	1359 vs 1360	563 vs 1360
CMCase			
t Stat	46.6	42.44191	10.73313
P(T<=t) two-tail	1.27E-06	1.842E-06	0.000427
Xylanase			
t Stat	153.652	115.75	587.165
P(T<=t) two-tail	1.08E-08	3.341E-08	5.05E-11
B-glucosidase			
t Stat	17.04113	61.415651	342.2782
P(T<=t) two-tail	6.95E-05	4.21E-07	4.37E-10
Acid phosphatase			
t Stat	269.19	7.080059	542.1129
P(T<=t) two-tail	1.14E-09	0.0021006	6.95E-11
Amylase			
t Stat	158.1139	19.99187	92.44821
P(T<=t) two-tail	9.6E-09	3.694E-05	8.21E-08
Phytase			
t Stat	157.21	37.952432	17.4405
P(T<=t) two-tail	9.82E-09	2.879E-06	6.35E-05

T test for SEM measurement data

	Conidiospore Diameter		
Culture	563 vs 1359	563 vs 1359	563 vs 1359
t Stat	7.337458	11.36194	11.73419
P(T<=t) two-tail	0.001837	0.000342	0.000302
	Conidiophore Width		
Culture	563 vs 1359	563 vs 1359	563 vs 1359
t Stat	9.04519190	29.117	9.89259876
P(T<=t) two-tail	0.01200300	0.0012	0.01006431

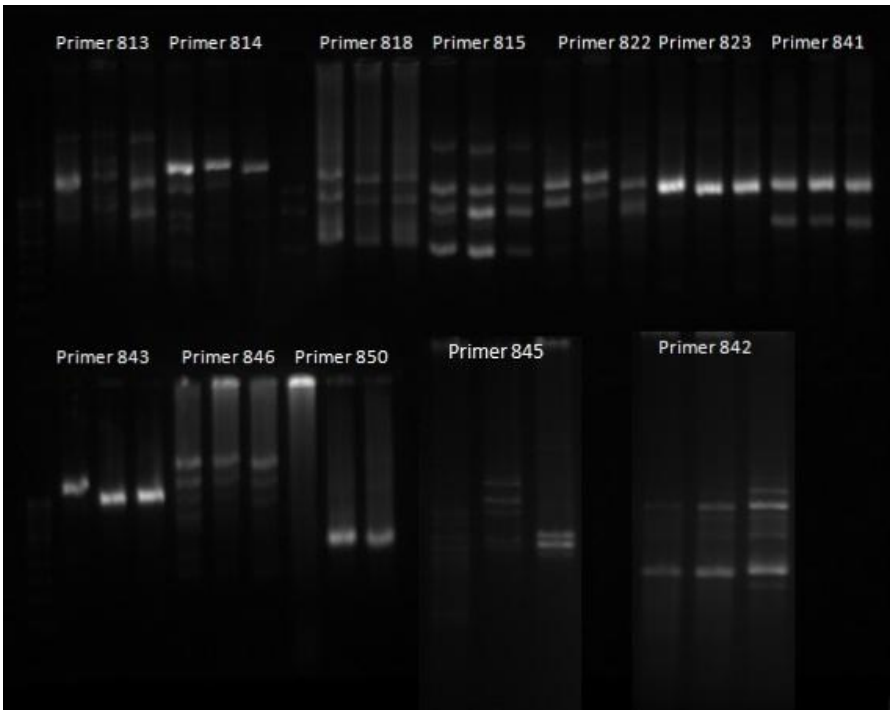
Chapter 5

SRAP Primer Sequences

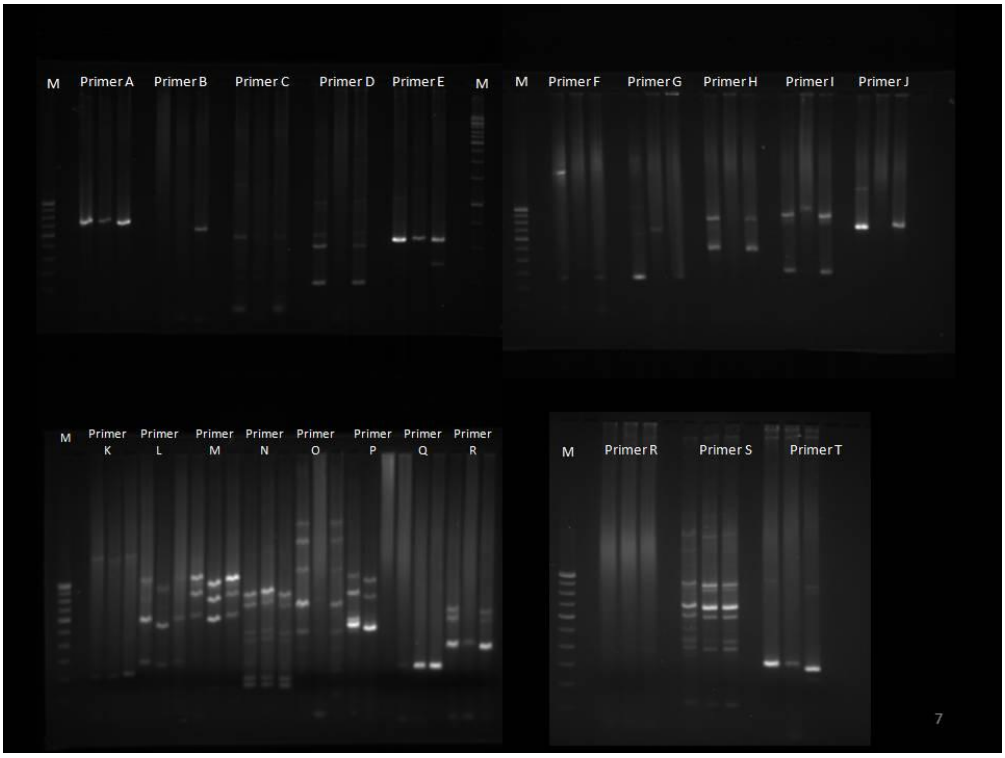
Name of Primer	Sequence 5'-3'	Name of Primer	Sequence 5'-3'
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCAAACCGGTAG	Em6	GACTGCGTACGAATTGCA
Me7	TGAGTCCAAACCGGTTG	Em7	GACTGCGTACGAATTATG
Me8	TGAGTCCAAACCGGTGT	Em8	GACTGCGTACGAATTAGC
Me9	TGAGTCCAAACCGGTCA	Em9	GACTGCGTACGAATTACG
Me10	TGAGTCCAAACCGGATG	Em10	GACTGCGTACGAATTTAG
Me11	TGAGTCCAAACCGGACA	Em11	GACTGCGTACGAATTTCG
Me12	TGAGTCCAAACCGGGAT	Em12	GACTGCGTACGAATTGTC
Me13	TGAGTCCAAACCGGTAA	Em13	GACTGCGTACGAATTGGT
Me14	TGAGTCCAAACCGGGCT	Em14	GACTGCGTACGAATTCAG
Me15	TGAGTCCAAACCGGTGC	Em15	GACTGCGTACGAATTCTG
Me16	TGAGTCCAAACCGGAGG	Em16	GACTGCGTACGAATTCGG
Me17	AGCGAGCAAGCCGGTGG	Em17	GACTGCGTACGAATTCCA
Me18	GAGCGTCGAACCGGATG	Em18	GACTGCGTACGAATTGAT
Me19	CAAATGTGAACCGGATA	Em19	TGTGGTCCGCAAATTTAG
Me20	GAGTATCAACCCGGATT	Em20	AGGCGGTTGTCAATTGAC
Me21	GTACATAGAACCGGAGT	Me24	GACCAGTAAACCGGATG
Me22	TACGACGAATCCGGACT	Me25	CAGGACTAAACCGGATA
Me23	CACAGTCATGCCGGAAT		

Gel images

ISSR



SRAP



Enzyme sequencing

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

Resulting peptides were delivered via nanoLC to the Q-ToF2 for tandem MS analysis. An automated experiment (DDA = data dependent acquisition) was run where selected peptides automatically enter MSMS for fragmentation.

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

phytase B (Phytase I)

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

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

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

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

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
10	403.7043	805.3940	805.3970	-0.0030	0	41	7.3	1		R.YPSPSAGK.S
11	403.7052	805.3958	805.3970	-0.0012	0	(41)	8.1	1		R.YPSPSAGK.S
23	471.2248	940.4350	940.4403	-0.0052	0	45	2.3	4		K.QFSQEFR.D
26	618.7672	1235.5198	1235.5319	-0.0121	0	36	20	1		K.HYGGNGPYSER.V
28	429.2082	1284.6028	1284.6211	-0.0183	1	54	0.32	1		R.HGERYPSPSAGK.S
29	676.7743	1351.5340	1351.5384	-0.0044	0	33	31	1	U	R.SSPIACQEGAAMD.- + Carbamidomethyl (C); Oxidation (M)
40	851.4106	1700.8066	1700.8114	-0.0047	0	(103)	3.1e-06	1	U	R.DPPTGCEVDQVIMIK.R + Carbamidomethyl (C)
43	859.4066	1716.7986	1716.8063	-0.0076	0	128	1.1e-08	1	U	R.DPPTGCEVDQVIMIK.R + Carbamidomethyl (C); Oxidation (M)
50	885.9133	1769.8120	1769.8254	-0.0134	0	(24)	2.2e+02	1		R.LSCQATALSDEGTYVR.L + Carbamidomethyl (C)
51	885.9187	1769.8228	1769.8254	-0.0026	0	117	1.3e-07	1		R.LSCQATALSDEGTYVR.L + Carbamidomethyl (C)
52	885.9203	1769.8260	1769.8254	0.0006	0	(50)	0.66	1		R.LSCQATALSDEGTYVR.L + Carbamidomethyl (C)
53	885.9206	1769.8266	1769.8254	0.0012	0	(44)	2.4	1		R.LSCQATALSDEGTYVR.L + Carbamidomethyl (C)
57	758.6859	2273.0359	2273.0542	-0.0183	0	(12)	3.1e+03	1	U	R.YGHLWDGETVVPFFSSGYGR.V
59	758.6878	2273.0416	2273.0542	-0.0126	0	(83)	0.00026	1	U	R.YGHLWDGETVVPFFSSGYGR.V
60	758.6881	2273.0425	2273.0542	-0.0117	0	(53)	0.23	1	U	R.YGHLWDGETVVPFFSSGYGR.V
61	758.6893	2273.0461	2273.0542	-0.0081	0	(10)	4.7e+03	1	U	R.YGHLWDGETVVPFFSSGYGR.V
62	1137.5370	2273.0594	2273.0542	0.0052	0	(19)	6.5e+02	1	U	R.YGHLWDGETVVPFFSSGYGR.V

☑	63	1137.5381	2273.0616	2273.0542	0.0074	0	96	1.2e-05	1	U	R.YGHLWDGETVVPFFSSGYGR.V
☑	77	809.7274	2426.1604	2426.2343	-0.0739	0	119	6.2e-08	1	U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	79	809.7424	2426.2054	2426.2343	-0.0289	0	(23)	2.4e+02	1	U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	80	1214.1191	2426.2236	2426.2343	-0.0106	0	(117)	9e-08	1	U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	81	809.7496	2426.2270	2426.2343	-0.0073	0	(51)	0.35	1	U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	82	809.7505	2426.2297	2426.2343	-0.0046	0	(72)	0.0027	1	U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	83	809.7507	2426.2303	2426.2343	-0.0040	0	(11)	3.8e+03	1	U	R.VAFGNPYSTGNIVPQGGHLTIER.L
☑	85	809.7511	2426.2315	2426.2343	-0.0028	0	(92)	2.8e-05	1	U	R.VAFGNPYSTGNIVPQGGHLTIER.L

Proteins matching a subset of these peptides:

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

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

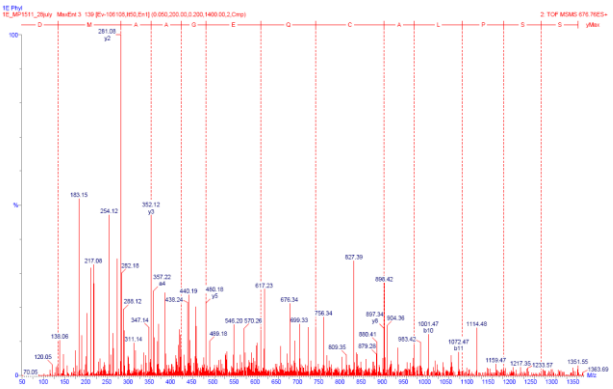
Sequence Coverage: **24%**

Matched peptides shown in **Bold Red**

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

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

In this the sequence I obtained was SSPIACQEGAAMD.



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

P34754	SSPIACQEGDAMD	479
P34755	SSPIACQEGDAMD	479
ABG88857	SSPIACQEGDAMD	463
1QFX_A	SSPIACQEGDAMD	460
EHA18670	SSPIACQEG**A**AMD	479
ABS83554	SSPIACQEGDAMD	479
ABS83553	SSPIACQEGDAMD	479
ABQ82276	SSPIACQEGDAMD	479
XP_001393206	SSPIACQEG**A**AMD	479
ABC39588	SSPIACQEGDAMD	479
ADF49635	SSPIACQEGDAMD	460
ABS29445	SSPIACQEGDAMD	459

Notes on interpreting your *de novo* sequence data

Manual sequencing by ESI tandem MS with low energy collision induced dissociation (CID), even with the assistance of specialized software (such as Waters/Micromass PepSeq and MasSeq which is what we use), is often open to interpretation of the mass spectra produced. Sometimes the spectrum is clean and the results clear, but in other cases some of the residues are not easy to call unambiguously. Extra information can assist in the interpretation of the resultant spectra and amino acid sequences. However, it is also important to back up the information by examination of any similar protein sequences and re-check the assignments made. Here are some notes to assist in your understanding and interpretation of the de novo sequence information generated using the Q-ToF2.

On the spectrum section of the BioLynx Peptide Sequencing report, where 2 residues appear "joined together" rather than spaced out separately with a line down to the x-axis denoting the position of the allocated ion, the residues

can be in either order. Similarly, in any text document provided by me (SL) if two or more adjacent residues are underlined, it means they can be in any order.

Leucine (L) and Isoleucine (I) are isobaric (both have a mono-isotopic mass value of 113.08406) and they cannot be differentiated in this type of analysis. Therefore, when you see I or L called in a *de novo* sequence, the residue can be either I or L. Often an alignment with related protein sequences helps to call the residue more definitively, or examination of nucleic acid sequence, where available.

Similarly, glutamine (Q) and lysine (K) differ by only 0.036 u.
Also, phenylalanine (F) and oxidized methionine (M^{SO}) differ by only 0.033 u.
It can be difficult to differentiate between these pairs of residues, so for example, if you see an F called, it could be an oxidised methionine residue, and vice versa.

Common derivatives used for Cys include iodoacetamide, iodoacetic acid and vinyl pyridine - all result in unique amino acid residue masses (159, 160, and 208, respectively). We routinely use iodoacetamide during processing, resulting in the modification of cysteine with a mass of 160 (denoted C^{AM} or CAM)

If there are two or more consecutive glycine residues in a sequence, there is often no abundant ion from cleavages between them.
Similarly, sometimes where Gly-Ala appear consecutively there is no abundant ion from cleavages between them.
Gly-Gly is isomeric to asparagine (Asn) (N).
Gly-Ala is isomeric to glutamine (Gln) (Q) and isobaric to lysine (Lys) (K).
It's therefore possible to assign the mass between two sequence ions as Asn or Gln/Lys, instead of Gly-Gly or Gly-Ala, respectively.

Losses of water (18) ammonia (17) and carbon monoxide (28) from the sequence fragment ions are often present.

The amino acid Pro is associated with very abundant y-type fragment ions. Therefore in C-terminal fragment ions, the y ion that results from cleavage adjacent to Pro is often easily identifiable because of its intensity.

To obtain the entire sequence of any peptide from tandem MS data it is necessary to obtain fragmentation at every peptide bond, a situation that often does not occur. It is common to not find any fragments resulting from cleavage between the first and second amino acids (in which case only the combined mass of the 2 terminal amino acids can be determined). Also, cleavages on the C-terminal side of Proline are often absent or of low intensity, which can preclude determination of the entire sequence.

Susan Liddell, Nottingham University

Designing primers for sequencing

Primers for full length genomic sequence

>gi|145241358:2629251-2631086 Aspergillus niger CBS 513.88 supercontig An08

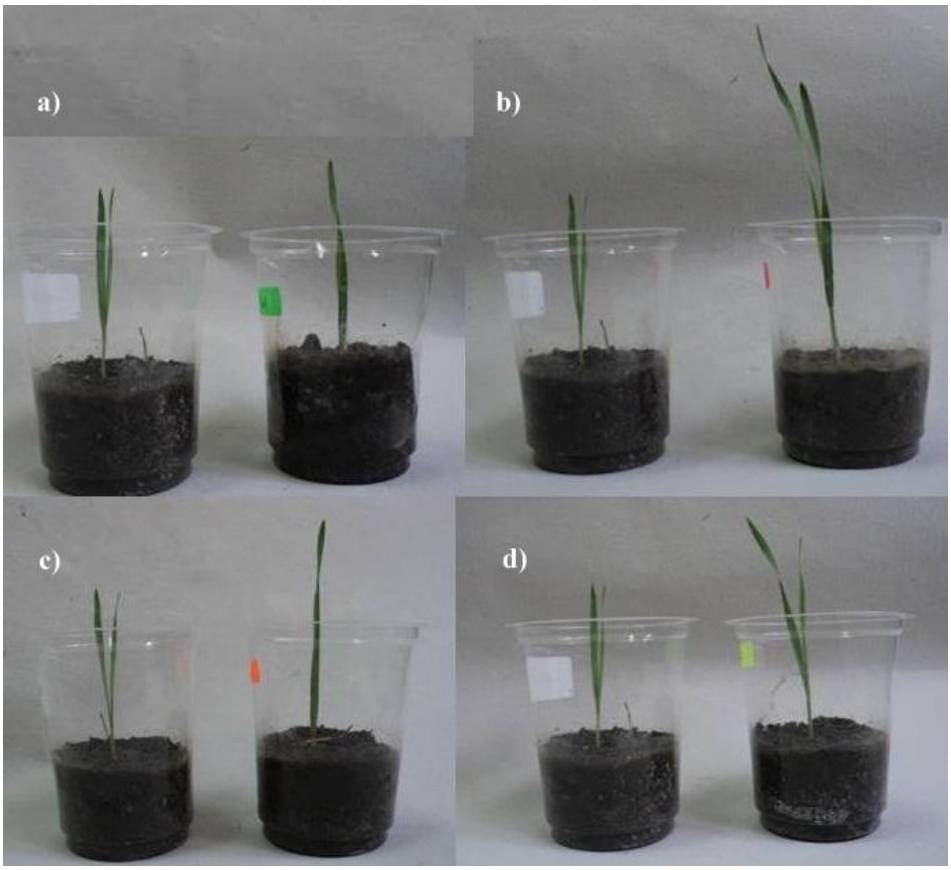
CATCTCTAAGCCTCGAAGCATTTCCTCCCGTTTCCGTCAATAGTAACTCCAGA**ATG**CCTCGCACTTCTCT
CCTCACCCTGGCCTGTGCCCTGGCCACGGGCGCATCCGCTTTCTCCTACGGCGCTGCCATTCCCCAGTCC
ACCCAGGAAAAGCAGTTCTCTCAGGAGTTCCGCGATGGCTACAGCATCCTGAAGCACTACGGTGGTAACG
GACCCTACTCTGAGCGGGTGTCGTATGGAATCGCCCGCGATCCCCCGACCGGCTGCGAGGTCGATCAAGT
CATCATGATCAAGCGTCACGGCGAGCGCTACCCTTCCCCTTCCGCCGGCAAGAGCATTGAGGAGGCTCTA
GCCAAGGTCTACAGCATCAACACCACCGAATACAAGGGTGACCTGGCCTTCCTTAACGACTGGACCTACT
ACGTCCCCAACGAGTGCTATTACAATGCCGAGACCACCAGCGGTCCCTACGCCGGTCTGCTGGATGCTTA
CAACCATGGCAACGAGTACAAGGCCCGCTACGGCCACCTTTGGGACGGCGAGACAGTCGTGCCCTTCTTC
TCCAGTGGCTACGGACGGGTCATCAACACGGCCCGCAAGTTCGGCGAGGGTTTCTTTGGCTACAACTACT
CCACCAACGCGGCCCTCAACATCATCTCCGAGTCCGAGGTCATGGGTGCCGACAGCCTGACTCCCACCTG
TGACACCGACAACGACCAGACCACCTGCGACAACCTGACCAACCAGCTGCCCCAGTTCAAGATTGCTGCT
GCCCGTCTCAACTCGCAAAACCCTGGCATGAACCTCACTGCATCCGATGTCTACAACCTGATGGGTATGT
GACACTCTCTGTGGCATCTGATAATCTTCGAGTCCAACGGAAGCTGACAATGTATCAGTAATGGCCTCCT
TTGAGCTTAATGCTCGTCCCTTCTCCAACTGGATCAACGCCTTCACCCAAGACGAATGGGTCAGCTTCGG
CTACGTTGAGGATCTAAACTACTACTACTGCGCAGGGTGAGATTATTCCTTCCTTCCTCAAATTAAACCT
GGATCGGCAACTAAATGATCAACACAGTCCCGGTGACAAGAACATGGCCGCTGTGGGCGCTGTCTATGCC
AATGCCAGTCTCACTCTCCTGAATCAAGGACCCAAGGAAGCCGGCTCCTTGTTCTTCAACTTGTACGTCT
CACGCAGACTCACCCCTCCTGTCAAAGAAACCCTTCACTAACATACTACAGCGCCCACGACACCAACATC
ACCCCCATCCTCGCCGCCCTGGGCGTCCTCATCCCCACCGAAGACCTGCCCCTGGACCGCGTCGCCTTCG
GCAACCCCTACTCGACCGGCAACATCGTGCCCCAGGGTGGCCACCTGACCATCGAGCGCCTCAGCTGCCA
GGCCACCGCTCTATCGGACGAGGGCACCTACGTGCGTCTGGTACTGAACGAGGCCGTGCTCCCCTTCAAC
GACTGCACCTCCGGACCAGGCTACTCCTGCCCTCTGGCCAACTACACTGCCATCCTGAACAAGGACCTGC
CCGACTACACGACCACCTGCAACGTGTCTGCATCCTACCCGCAGCACCTGAGCTTCTGGTGGAATTACAA
CACCACTACAGAGCTGAACTACCGCTCTAGCCCGATTGCTTGCCAGGAGGGTGCTGCTATGGAC**TAA**ATG
GAGAGAGATGAGACAGAAGTACTTGGGTGATGATTGGATATTCATGTTTGATTGGTGTAGCGATCACCGT
GTTATTAAAAGTCTTGTATAGTCATGCGGTGAATGTAAATAATAGTAATGATAGCAATGATGCAAATTGG
AATTCTTTTCTCTAAA

Primer sequences

Name	Sequence	Length (b)	Tm (°C)	Amplicon length (bp)
Full length clone				
PFL-F	GCCTCGAAGCATTTCCTCCCGT	22	59.22	1740
PFL-R	CGGTGATCGCTACACCAATCAAACA	25	57.89	
Primers amplifying overlapping regions for sequencing				
PSA-F	TCCTCCGTCCTCGCTTCCGC	20	60.66	716
PSA-R	ACGACTGTCTCGCCGTCCCA	20	59.90	
PSB-F	CCTTCCGCCGGCAAGAGCAT	20	59.76	703
PSB-R	TCACCCTGCGCAGTAGTAGTAGTT	24	57.15	
PSC-F	TCGCAAAACCCTGGCATGAACC	22	58.27	710
PSC-R	AGCCTGGTCCGGAGGTGCAG	20	60.53	
PSD-F	CCTGGGCGTCCTCATCCCCA	20	59.97	692
PSD-R	AGGCAGATGCGACCGTGGAG	20	58.87	

Chapter 6

Pot experiment



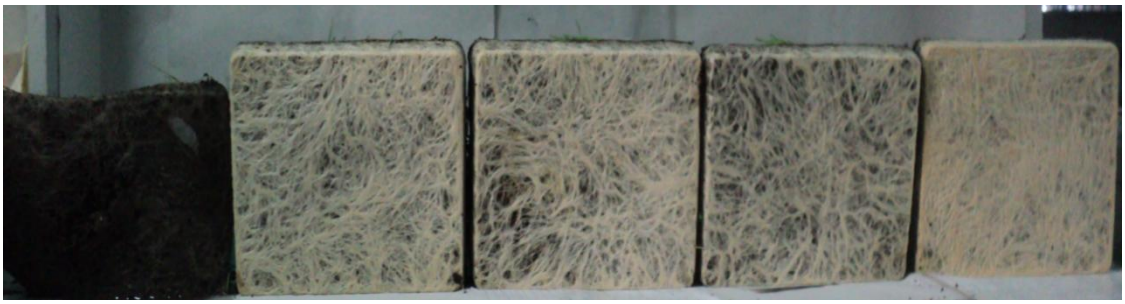
a) Control vs. AW (1.32 mg sodium phytate+ 6 IU phytase)

b) Control vs. BW (1.32 mg sodium phytate+ 12 IU phytase)

c) Control vs. CW (3.3 mg sodium phytate+ 6 IU phytase)

d) Control vs. DW (3.3 mg sodium phytate+12 IU phytase)

Rooting



An interesting observation recorded was about the differential rooting depicted by various combinations of the chemical fertilizers and the enzyme. While DAP showed intense rooting. Enzyme combination showed much better rooting than the other chemical fertilizers.