Studies on phytase from *Aspergillus niger* NCIM 563:

Production, downstream processing and application

Thesis submitted to AcSIR for the award of the degree of

DOCTOR OF PHILOSOPHY

In Biological Sciences

BY

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Dedicated to

MY PARENTS AND IN-LAWS

Thank you, Mummy and Papa for being examples of love and care to me

Thank you for being an example of hard work and principled living.

Thank you for making our house a home.

Thank you for wholeheartedly and unconditional loving and keeping our family united even though you had a hard time expressing it.

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Thank you for taking care of our son when in need.

I dedicate this doctoral thesis to say a big "THANK YOU" for being my dual parents and to say "I LOVE YOU!"

Table of Contents

CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled

"Studies on phytase from *Aspergillus niger* NCIM 563: Production, downstream processing and application"

submitted by **Mr. Parin C. Shah** to Academy of Scientific and Innovative Research (AcSIR) in fulfilment of the requirements for the award of the Degree of Doctor of Philosophy, embodies original research work under our supervision. We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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DECLARATION

I hereby declare that the work of the thesis entitled

"Studies on phytase from Aspergillus niger NCIM 563: Production, downstream processing and application"

submitted for the degree of Doctor of Philosophy to AcSIR has been carried out by me at NCIM Resource Centre, CSIR-National Chemical Laboratory, Pune 411008, Maharashtra, India, under the supervision of Dr. S. G. Dastager and Dr. J. M. Khire. This work is original and has not been submitted in part or full by me for any other degree or diploma to any other university or institute.

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(Research scholar)

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PARIN C. SHAH

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List of Figures:

List of Tables:

Abstract

The increasing population demands intense agriculture, pressurizing use of fertilizers. Phosphorus (P) is one of the very important and essential element for all life forms along with nitrogen and potassium. The main source of P is phosphate rock, which is mined and majorly used for the production of fertilizers. This is the only source through which it enters in to the ecosystem. Phosphorus, which is added to soil for plant uptake, in turn enters the food chain. While all farmers need access to P, only five countries control around 90% of the world's remaining phosphate rock reservoirs, including China, United States and Morocco (which also controls Western Sahara's reservoirs). No P substitute exists, as its production is limiting and cannot be manufactured, though it can be recovered and reused over and over again. The phosphate rock takes 10-15 million years to cycle naturally, which is a very slow process. Phosphorus doesn't have any gaseous form and has caused a bottleneck in the bio-geochemical cycle. This led the increasing price trend of fertilizers, scarcity of non-renewable P and excessive P loss due to overuse to meet the growing food demand. Over usage of P have increased the threat to the food security along with the ecosystem as it leaches out in the water bodies causing eutrophication. It has been studied and suggested that the peak usage of P reserve will be by 2030 and current high-grade available reserves will be depleted within 2 decades. While the exact timing might be disputed, it is widely accepted that the quality of phosphate rock is decreasing and the cost is increasing.

Increase concerned for P security has urged to strategically identify ways to increase the efficiency in usage of P in agriculture and find as many sustainable ways to reuse it. The majority of P applied to crops as fertilizer, which is banked in the reservoir with organic compounds and minerals in the form of phytic acid. Also, P taken up by plants is stored in seeds in the form of phytic acid, which is acted upon by plant phytases to release P need for its germination. Thus, phytic acid is the potential source of P which needs to be utilized.

Phytic acid acts as an anti-nutrient factor for monogastric animals viz; humans, poultry, etc., as they lack the ability to digest it. Also, being negatively charged it scavenges positively charged cations which are required for growth. This is partially attributed to the wide spreading human nutritional deficiencies of calcium, iron and zinc in developing countries, where the staple foods are of plant origin. It binds to proteins and make them more resistant to proteolytic digestion. Thus inorganic phosphorus is supplemented in diets for poultry, fish and swine to meet their nutritional requirement of phosphorus, after digestion the unutilized phytate phosphorus from plant feed is excreted, becoming an environmental pollutants in areas of intensive animal agriculture; excessive phosphorus in soil runs off in lakes and the seas causing eutrophication and stimulating growth of aquatic organisms that may produce neurotoxins, which are toxic to human health.

Dephosphorylation of phytic acid would release phosphorus that can be utilized for plant growth, thus decreasing the usage of fertilizers. Investigations state that phosphatases have an activity of dephosphorylation but they cannot act on phytic acid. Studies showed that 'phytase', a special class of phosphatase could act on phytic acid sequentially releasing phosphorus.

Phytase (EC 3.1.3.8) (myo-inositol hexakisphosphate phosphohydrolase) 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. Many sources are known to produce phytase viz; plants, animals, fungi and bacteria. Phytase is known since four decades as animal feed enzyme and is widely used in poultry, but still, studies continues to have a phytase with all the characteristics required for the feed enzyme. Most of the commercially available phytase are from *Aspergillus* and *E. coli*. Other than poultry, phytase is studied for application in food, aquaculture and pharma, etc.

There is still a need to have an ideal phytase for poultry application, to show its potential in food and other related applications. In view of its industrial importance, the ultimate objective is to produce this enzyme in bulk at a cost

effective level and establish conditions for its industrial production, rapid downstream processing and formulate the product for its effective application.

Thus, this thesis entitled: 'Studies on phytase from *Aspergillus niger* NCIM 563: production, downstream processing and application', mainly focuses on screening of phytase producing fungus and study the production of phytase under submerged fermentation conditions. The production is enhanced by optimizing the media and is subsequently scaled up followed by cost effective downstream processing. Studies here showed production of potential ideal phytase for poultry application. Among other sectors, application of phytase in food by decreasing phytic acid content is also carried out. Increasing focus on agricultural productivity has in-turn caused over usage of crop protection chemicals *viz;* pesticides. Organophosphorus pesticides are widely used in agriculture which are potential neurotoxins. Degradation of these pesticides on harvested crop which enters the food chain is demonstrated here using phytase.

Thus, the global goal of phosphorus security for food security and environment protection can be met by usage of phytase.

Chapter 1: Introduction

This chapter covers the literature on scope of the study, basic definitions of phytate and phytase enzyme, diversity, classification and sources of phytases, production and downstream processing of the enzyme, applications and recent advances in phytase research. The chapter concludes with the objective and the scope of the thesis.

Chapter 2: Production of phytase by *Aspergillus niger* NCIM 563 under submerged fermentation

This chapter is focused on screening the fungal strains available at NCIM Resource Center for extracellular phytase production in a two-step procedure. The strains were selected depending on the ability to generate zone of clearance around the colony on phytase screening media containing calcium phytate. False positive test was checked by evaluating the phytase production of the positive stains in submerged fermentation in synthetic media. *Aspergillus niger* NCIM

563 was selected in the screening process for further phytase production. Production in synthetic media demonstrated 41.47 IU/mL phytase activity in 15 days using spore inocula. The use of low cost and easily available agricultural waste was taken as a choice for cost effective, green production process. Response surface methodology (RSM), a statistical tool was used for optimization of media to enhance the phytase production. The productivity using rice bran increased from 2.8 IU/mL/day to 6.2 IU/mL/day (68 IU/mL in 11 days), which on optimization increased to 20.6 IU/mL/day (259 IU/mL in 13 days). The variation in phytase production was observed with change in rice bran batch, thus providing inconsistency in production which is not suitable for scaling up the process. Also, the residual rice bran interfered with the downstream processing. Thus, green chickpea flour (GrCf) was used for the first time in phytase production and checked for phytase production and media optimization using RSM. The productivity using GrCf obtained was 7.3 IU/mL/day (66 IU/mL in 9 days). Vegetative inocula was studied for phytase production instead of spore inocula which gave productivity of 14.3 IU/mL/day (86 IU/mL in 6 days). On optimization the productivity increased to 29 IU/mL/day (160 IU/mL in 5.5 days). Thus, use of GrCf containing media gave sustainable source for phytase production and productivity was increased than using inconsistent rice bran by media optimization. Exploiting this observation would be of very high value addition from the phytate feed conversion and also from environment point-of-view.

Chapter 3: Scaling up the submerged phytase production using fermentor for large scale production

On successful media optimization, the production was scaled up to 2 liters volume in 3 liters fermentor sized vessel. Fermentor offers controlled conditions for the production parameters further increasing the productivity and paving way for pilot scale production of the industrially important phytase. Physical parameters like aeration and agitation play a very important role which were optimized by one variable at a time approach. 0.5 vvm aeration gave maximum

production of 159 IU/mL in 5.5 days with initial rpm of 600 using Rushton type agitator. The production level matched the shake flask production was thus scaled up to 10 liters volume in 14 liters fermentor sized vessel maintaining aeration of 0.5 vvm. Maximum activity of 164 IU/mL was obtained in 96 hours (4 days) with initial rpm of 500.

Chapter 4: Downstream processing, formulation and biomass reutilization.

On production in fermentor, downstream processing plays a very important role for any product and process development. It should be cost effective, with minimum steps and less time consuming. It should also be possible to be continuously operated in line with production and can be scale-up. Recently, membrane technology is providing advantages of being used continuously and can be scaled-up with ease of handling and cleaning. Thus, ultrafiltration was considered to be choice for downstream processing of phytase than the multi-step, costly and time consuming chromatographic procedure. Simultaneous purification and concentrating the phytase could be achieved. On centrifugal separation of biomass, various molecular weight membrane cut off were studied which showed that 150 kDa cut off membrane gave very high level of phytase purity along with 2.6 fold concentration and 92 % recovery. On concentrating, the stability studies were carried out at room temperature and at 8 - 10°C using different additives. The phytase showed 100% stability on lyophilization without any additive. The biomass generated after centrifugal separation, was reused for extraction of chitosan which is used in various applications in food, pharma, etc. 100 g biomass generated 50 mg of chitosan which was confirmed using FTIR.

Chapter 5: Application of phytase in food, animal feed and in organophosphorus pesticide degradation.

Phytase is known as animal feed enzyme since four decades, but we still fail to have an ideal phytase having all the characteristic of feed enzyme for poultry application. The phytase needs to be active at gut pH of chicks and should be thermostable for making pellets for chicks. Most of the commercially available phytase are from *Aspergillus* and *E. coli*. They are active at 5.5 and are

not thermostable. Granular enzymes with additives are available which are thermostable but needs multiple dosing to withstand gut pH of 2.0. Current phytase from *A.niger* NCIM 563 is stable over wide range of pH from 2.0 to 9.0 having optimum pH of 2.5. Also, using 3% chitosan or 30% skimmed milk powder gives thermostability at 80°C for 1 min. Studies also showed that it is resistant to gut enzyme pepsin. Thus, the current phytase from *A.niger* NCIM 563 has potential to be used in animal feed requiring less multiple dosage than currently required.

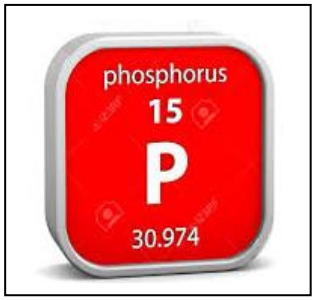
Potential of phytase action in food application is also been studied. Phytic acid in bread can be reduced by using phytase and studies here found that 67% phytic acid content is reduced by usage of phytase.

Growing population demands food thus pressuring agriculture intensification. This in turn increases demand of fertilizers and pesticides. Use of phytase in soil can decrease the demand of fertilizer as phytase can release phosphorus from soil phytic acid. Also, organophosphorus pesticides (OpP) are widely used in agriculture enters our food chain which are harmful to humans and animals. The phosphate group in pesticide imparts toxicity to OpP and its dephosphorylation can reduce its toxicity. The residual OpP on harvest agricultural products entering food chain can be acted upon by phytase which can dephosphorylate OpP, reducing the toxicity and its effect on humans and animals consumption. Studies were carried out in vitro to check the action of phytase on OpP and also on residual OpP present on harvested green chilli. Positive results were obtained using phytase and thus a product can be developed for the same.

Chapter 6: Conclusions and future prospects

A cost effective and green process for phytase production using *Aspergillus niger* NCIM 563 is developed which can be further scaled up for large scale production. Studies on continuous production needs to carried for further cost effectiveness. A formulation with phytase can be developed which can be used

as product in degrading OpP which enters food chain through harvested agricultural products and water.



Chapter 1

Introduction

- General introduction starts with the rationale and significance behind the work, carried out in this thesis followed by an overview of phytase production and application.
- Phosphorus (P) is one of the basic and very important component and energy conduit of life, but its security and sustainable usage is not been effectively thought off.
- Fate of the fertilizers used and usage of phosphorus which is locked in form of phytate.
- Phytase, the only enzyme which can act on phytate by dephosphorylating it. Thus, a boon enzyme and a very crucial process.
- Recent findings on phosphorus and phytase source, production and application of phytase are also overviewed.
- This chapter ends with the need for research and the objectives of the research carried out here.

1. General Introduction

1.1. Phosphorus Importance and Security

Protecting the valuable ecosystem is one of the greatest challenges of the present era. The world's population is exploding and it is expected to reach from 7 billion in 2012 to 9.6 billion in 2050. Thus, with rising environmental risks, food production needs to be increased by 60 to 110 % (Foley et. al. 2005; IAASTD 2008; Tilman et. al. 2011; Pardey et. al. 2014). Agriculture stays at the heart of this challenge, which gives livelihood to 2.5 billion small farmers (FAO 2013). Thus, there is dire need to find ways for sustainable agriculture development which can deliver more food, better ecosystems and improved livelihoods rather than focusing only on productivity (Rockstrom et. al. 2017).

Meeting the demand and supply of food can be addressed by increasing usage of fertilizer nutrients ($N + P_2O_5 + K_2O$). World demand for the total fertilizer nutrients is estimated to grow at 1.8 percent per annum from 2014 to 2018 (200.5 million metric tons) (Fig 1.1). During this period, demand for phosphorus (P), nitrogen (N) and potash (K_2O) is forecasted to grow annually by 2.2, 1.4 and 2.6 percent, respectively. Phosphate fertilizer consumption/demand, includes H_3PO_4 (phosphoric acid) and non-H_3PO_4 based fertilizer. The non-H_3PO_4 fertilizer includes P_2O_5 in single super phosphate, direct application phosphate rock (DAPR) and nitric acid-based phosphate fertilizers, etc.

A rise of 2.1 % from 6.4 % to 8.5 % is expected in the global potential balance of P, which would be from 2.7 million metric tons in 2014 to 3.7 million metric tons in 2018. The overall increase in P_2O_5 demand between 2014 and 2018, would be maximum in Asia (58 %), America (29 %), Europe (9%), Africa (4%) and Oceania (0.5 %) (FAO 2015) (Fig 1.2). Considering high demand, supply for the fertilizer nutrients is going to increase from 46.9 million metric tons in 2014 to 52.2 million metric tons in 2018. IFA states that until 2018, high new capacity units for phosphoric acid are planned for completion (7.3 million metric tons), of which major production unit of 1.8 million metric tons would be located in Morocco, Saudi Arabia (1.5 million metric tons) and China (1.7 million metric tons) (Fig 1.3).

Figure 1.1: *Global fertilizer nutrients demand (FAO 2015)*

Figure 1.2: *Regional and sub-regional shares of world increase in phosphate consumption between 2014 and 2018 (FAO 2015)*

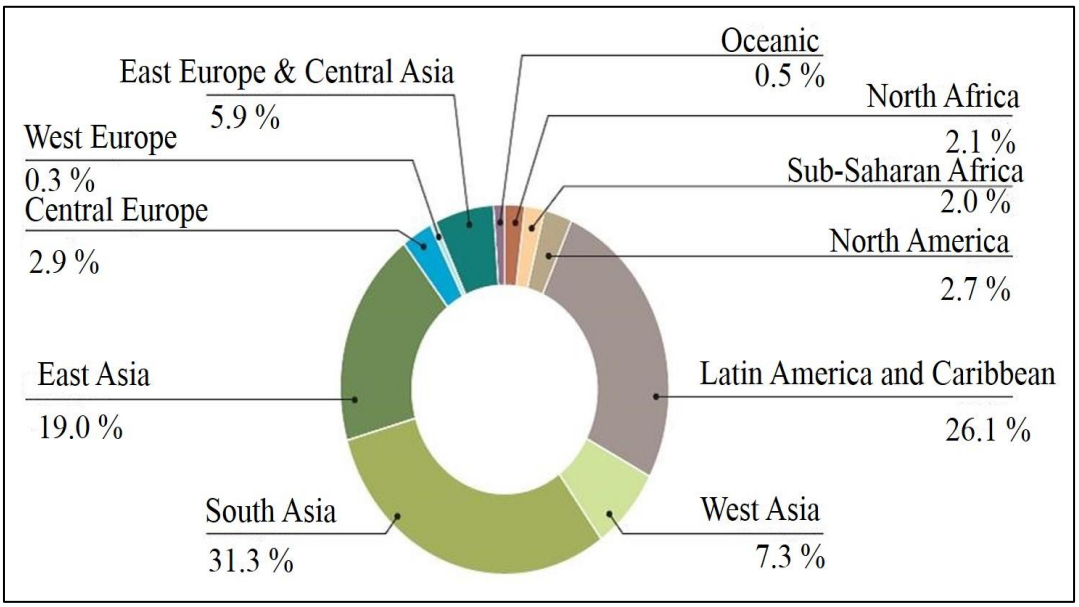
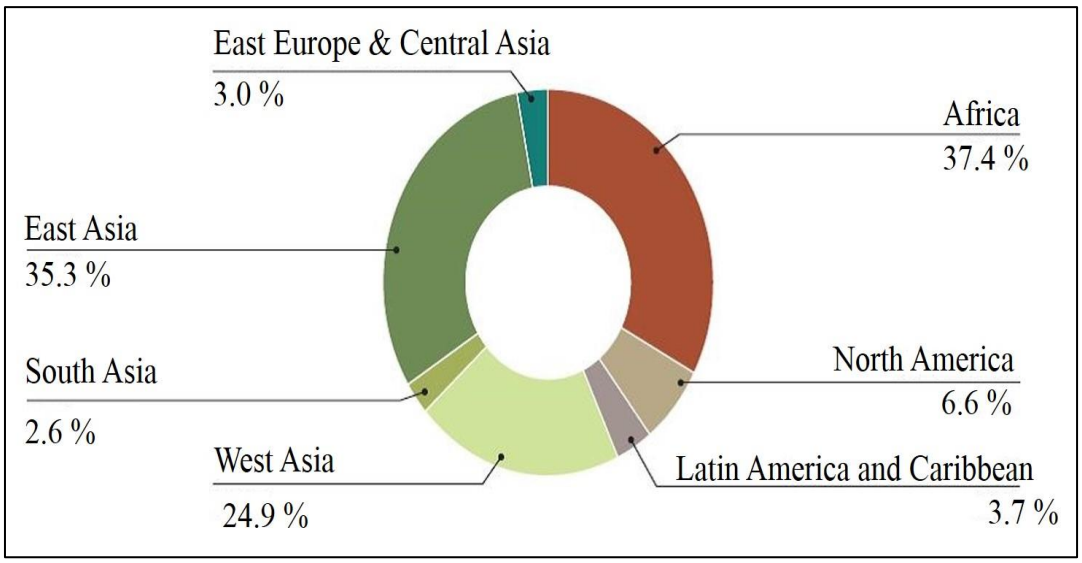


Figure 1.3: *Regional and sub-regional shares of world increase/decrease in phosphoric acid (P_2O_5) supply between 2014 and 2018 (FAO 2015)*



Considering the current rate of usage and the forecast, there is a need to ask a question, 'How long will the reserve's last?' 35 countries have reported 'P' reserves, however, 90 % of the supply is controlled by 5 countries (IFA 2012; Jasinski 2013) (Fig 1.4). Cordell, Drangert et al. (2009) estimated that peak production of current reserves (that is, phosphate rock is known to be economically available for mining and processing) would occur between 2030 and 2040 (Fig 1.5).

Figure 1.4: *Phosphate rock reserves by country, indicating both market concentration and how reported estimates have changed over time (IFA 2012)*

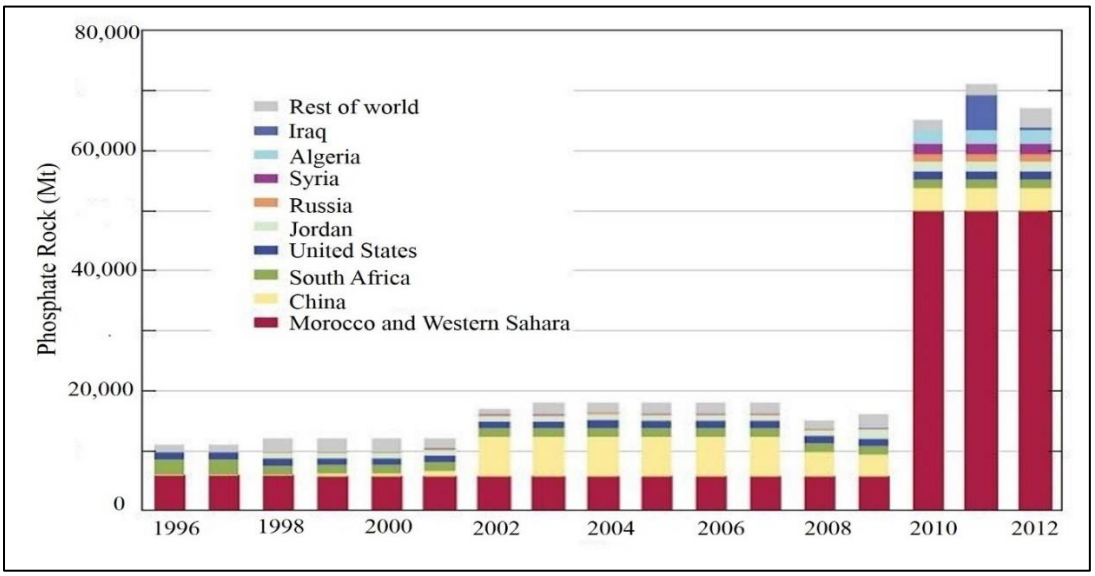
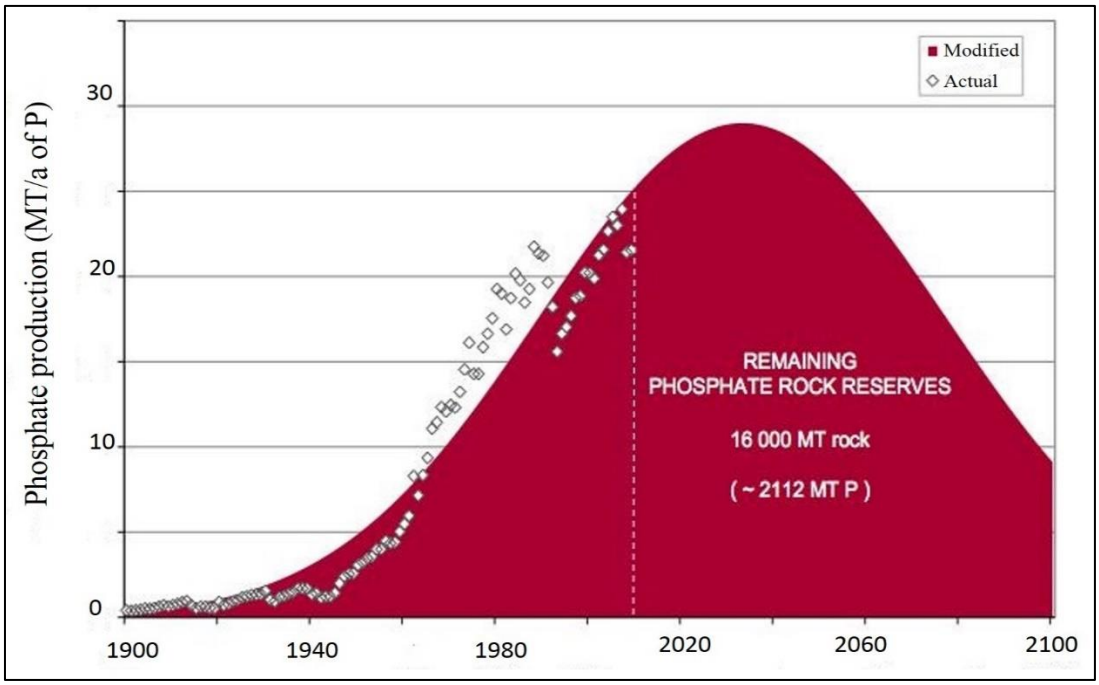


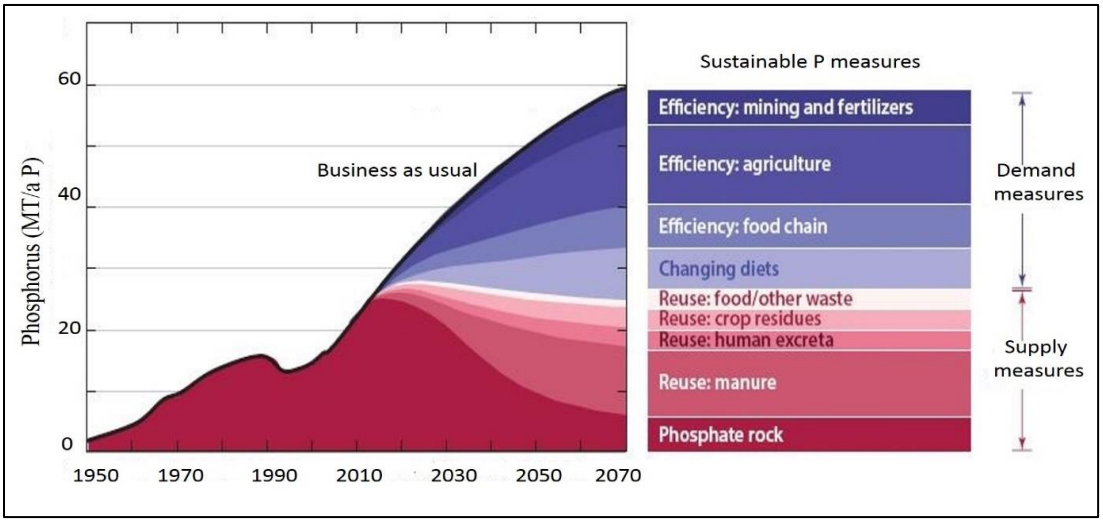
Figure 1.5: *Peak phosphorus curve indicating a peak in production by 2033*



1.2. Phosphorus Sustainability

Uncertainty exists about the timeline of phosphate reserves, and thus, there is a need for sustainable measures to be adopted by all the countries to ensure that all the farmers have both short and long-term supply of sufficient P for growing enough crops to feed the exploding population (Cordell et. al. 2009, 2013) (Fig 1.6). It is been observed that increased efficiency is required in mining and usage of fertilizer in agriculture.

Figure 1.6: *Preferred scenario for sustainable phosphorous management*



Every cell requires P. Plants obtain P from the soil which is present in small amounts in its readily available form. Also, there is no substitute for P in agriculture. If soils are deficient in P, there will be a restriction in food production and thus, P is very vital for future food security. Thus, to increase plant yield, fertilizers are added, over usage of which causes losses to surface water and eutrophication. The biogeochemical cycle of P is very slow and it takes decades for the formation of phosphate rock which is currently widely exploited (Smil 2000) (Fig 1.7).

1.3. Fate of phosphorus fertilizers in soil for agriculture

Phosphorus is found in two different forms in soil: inorganic and organic. $H_2PO_4^-$ and HPO_4^{2-} are the main inorganic forms in the soil, which is used by plants. However, these ions can also absorb onto the surface (or adsorb into) solid matter in the soil, which then becomes unavailable to plants. Between 50 and 80% of P in soil is in the organic form, which is contributed by the breakdown of dead plants etc., as P is found in cell membranes and DNA in living organisms (Fig 1.8).

Figure 1.7: *Phosphorous flow in environment (UNEP Yearbook 2011)*

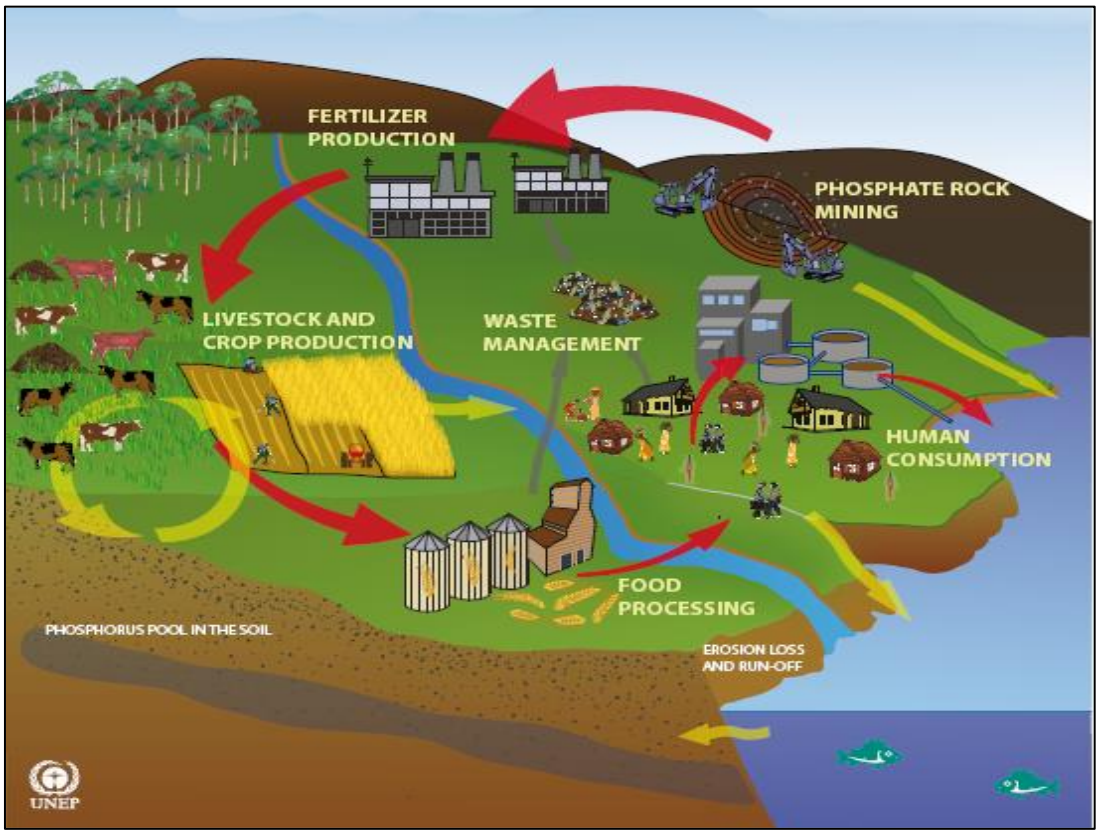
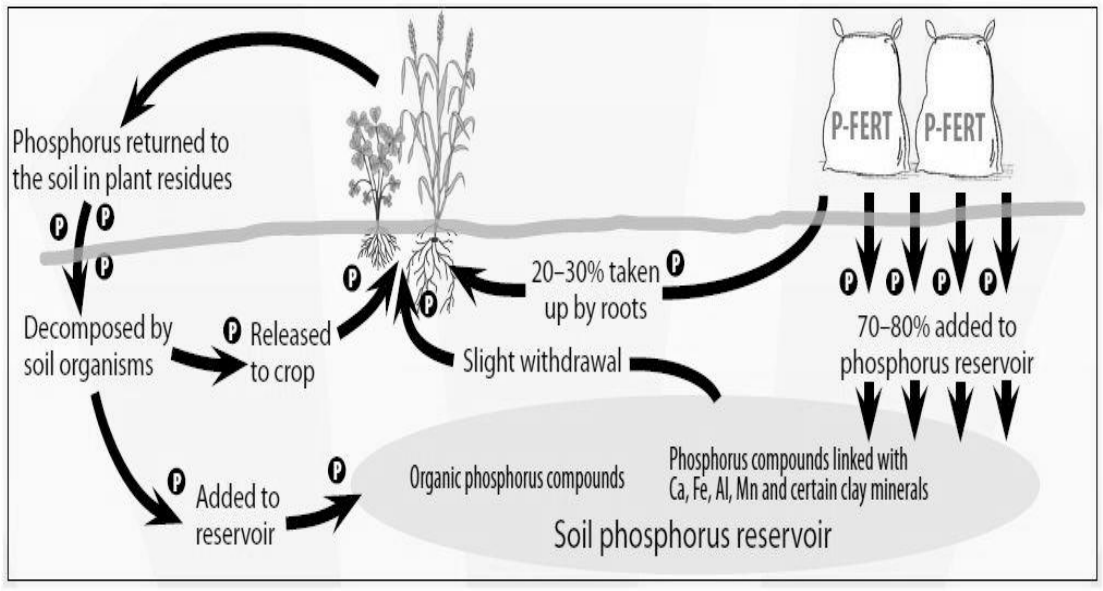


Figure 1.8: *Fate of phosphate fertilizer in soil (Glendinning J S (2000)*



The composition and transformations of organic P compounds in soils have received less attention than inorganic forms. This is partly because inorganic phosphates predominate in temperate arable soils and partly because organic phosphates are even more difficult to study than the inorganic phosphates, which are difficult in extracting without altering them chemically. The organic phosphorus fraction plays a very important role in P economy, as it is major P supplying pool, partly contributing in plant nutrition and in turn the grazing animals. The remaining part is very stable and thus it is not available to plants. The nature of P in ill-defined soil humic materials is largely unknown. Inositol phosphates (inositol hexakisphosphate [phytate]) are the most abundant compounds in soil extracts contributing 50 % of the total organic phosphates content. A number of stereo-isomers have been identified. Only one of these, the myoinositol hexakisphosphate, has been reported in plants so presumably the other isomeric forms are synthesized in the soil by microorganisms. The myo-form appears to be the most abundant isomer in the soil (Dahal 1977; McLaren et. al. 1990, Syers et.al. 1983).

1.4. Phytate (Phytic acid)

The six orthophosphate moieties and 12 coordinated ligands present in the phytate structure allows it to form a complex with metal cations like Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and adsorb to the surface of colloids with several phosphate groups simultaneously (Celi et al., 1999; Martin et al., 2004; Turner et al., 2002a). Among all

soil P compounds, phytate has the lowest bioavailability (Shang et al., 1996) and the strongest affinity to soil particles (Berg and Joern, 2006; Celi and Barberis, 2005; Shang et al., 1992; 1996).

Phytic acid was first described by Posternak in 1903. Its molecular structure was put forth by Anderson in 1914 (Fig 1.9). Trivially it is known as D-*myo*inositol (1, 2, 3, 4, 5, 6) hexakisphosphate, IP6, InsP6 or phytic acid (PA). The prefix – hexakis signifies that the phosphate groups are not internally connected (Johnson and Tate 1969). Phytic acid has a molecular formula of $C_6H_{18}O_{24}P_6$ while its molecular weight is 659.86 g/mol. Salts of phytic acid mostly with magnesium, calcium, sodium and potassium are referred as 'phytate' whereas calcium/ magnesium salts of phytic acid are referred as 'phytin'.

The principal storage form of phosphorus (P) and inositol in cereals, legumes, and oilseeds is in the form of phytic acid [*myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate] (PA) and its salts are the common constituent of plant-derived foods. It gets accumulated during ripening period in seeds, grains and pollen (Loewus 2002; Reddy et. al.1982; Turner et. al. 2002b). Phytic acid contributes around 60–90% of the total phosphorus content in plants (Raboy 2003).

Phytic acid plays an important role during seed germination by serving as a phosphorus and energy storage, a source of cations, a source of *myo*inositol, and also helps in initiating dormancy. Phytic acid is accumulated in the aleurone particles and globoid crystals, respectively, of the cereals and legumes (Reddy et al., 1982). Empson et al. (1991) suggested that the role of phytic acid in seeds is a natural antioxidant during dormancy. Table 1.1 gives an account of phytate content in commonly consumed cereals and grains (Cao et al. 2007).

Monogastric animals (pigs, poultry, fish, and humans) feed maximally on plant-based food and thus are exposed to high concentrations of phytic acid. These animals lack the ability to use P which is in bound form in PA (Singh et al. 2011). The dietary phytate chelates the metal cations required for growth (Iqbal et. al. 1994; Pen et. al 1993). The metal-phytate complex in-turn binds with proteins at various pH affecting its enzymatic activity, solubility and digestibility (Kemme et. al. 1999). Phytic acid also binds to starch directly or indirectly through proteins. Phytic acid interacts with the lipid which forms a major hindrance in deriving energy from lipid sources (Rickard and

Thompson 1997; Leeson 1993). Thus, PA acts as an anti-nutrient molecule (Woyengo and Nyachoti 2013). The excreted animal waste contains a high concentration of PA which enters the soil and is digested by the microorganisms, releasing P. This excess P is washed away in water bodies causing blooms, hypoxia, and death of aqua flora and fauna (Ole et. al. 2002; Vats et. al. 2005).

Figure 1.9: *Structure of phytic acid and its metal complexes*

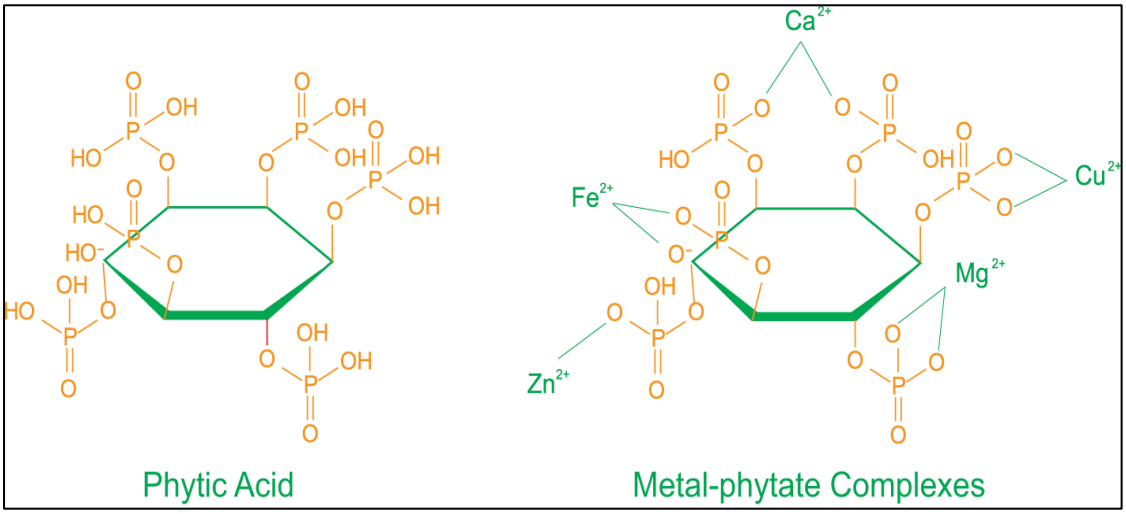


Table 1.1: *Total phosphorus and Phytate P proportion in feed stuff*

	Total phosphorus (g/kg)	**Phytate phosphorus (g/kg)**	**Proportion (%)**
Cereals			
Wheat	3.07	2.19	71.6
Oat	3.60	2.10	59.0
Corn	2.62	1.88	71.6
Barley	3.21	1.96	61.0
Sorghum	3.01	2.18	72.6
Oil seed meal			
Canola	9.72	6.45	66.4

Cotton seed	10.02	7.72	77.1
Corn gluten	4.24	2.67	63.0
Rapeseed	9.60	6.34	66.0
Soybean	6.49	3.88	59.9
By product			
Rice bran	17.82	14.17	79.5
Wheat bran	10.96	8.36	76.3

This PA, which is an environment pollutant, anti-nutrient and important P source molecule needs to be explored towards decrease pollution and increased P supply. The increased supply of P from PA will in-turn reduce the external supply of plant growth phosphate fertilizer and thus, needs to be acted upon by dephosphorylating it.

1.5. Dephosphorylation of Phytic acid

The phosphate group in the PA increases its mineral binding strength. The bioavailability of the essential dietary mineral can be improved by dephosphorylation of PA (Sandberg et. al. 1999).

Hydrolysis of phytate can be carried out at high temperature by autoclaving at 121°C for 1h. Phillippy et. al. (1987) studied the hydrolysis of IP6 (InsP6) and found that at pH 1.0, 2.0, 4.0, 6.0, 8.0, and 10.8, the percentages of InsP6 decomposed were 67.7, 76.8, 89.6, 81.9, 65.8, and 45.1%, respectively. The hydrolysis products were a variety of isomers of InsP1 to InsP5 (myoinositol mono-, bis-, tris-, tetrakis-, and pentakis-phosphate). They also found that in the pH ranges of 1.0 - 10.8, the lower the pH, the more even distribution of inositol phosphate isomers. This is a non-enzymatic process.

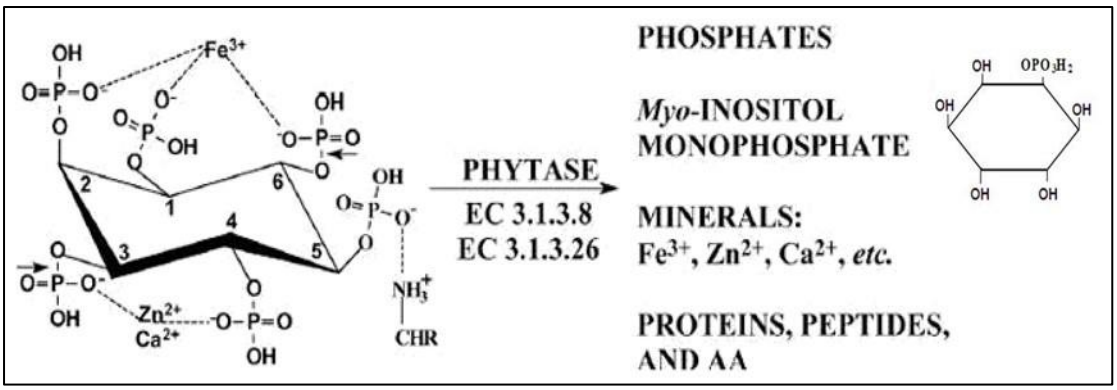
Hydrolytic enzymes are one of the important class of enzymes. Phosphatases are the important group of this class which helps in making inorganic P available to

animals. Thus these can be effectively used for dephosphorylation of PA by action on C-O-P ester bonds (Beck et. al. 1989). The action of phosphatases has been studied in soil and in plant roots (Hayes et.al. 2000; Hubel and Beck 1993; Richardson and Hadobas 1997; Wyss et. al. 1998). Phosphatases are either acid phosphatases or alkaline phosphatases according to their pH optima. Studies have shown that purified acid phosphatases have no action on PA (Konietzny et. al. 1995). Li et. al. (1997) has stated that phytases can be regarded as a special class of acid phosphatases which can act on PA. Thus, phytases are a boon and can be used effectively for the degradation of PA. The action of both acid phosphatases and phytases work in coordination wherein phytases selectively act on PA and the intermediates generated (inositol phosphates) are acted upon by acid phosphatases, thereby accelerating the total dephosphorylation process (Singh and Satyanarayana 2014).

1.6. Phytase

Phytases are a special class of acid phosphatases (myoinositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolases, EC 3.1.3.8 and EC 3.1.3.26) which act by sequential dephosphorylation of PA liberating phosphate and myoinositol intermediates (Wyss et. al. 1999 and Yu et. al. 2012) (Fig 1.10). Identification of phytase for the first time was carried out during the studies on production of various phosphatidyl inositol as intermediates or as end products in rice bran (Suzuki et. al. 1907).

Figure 1.10: *Dephosphorylation of phytate by phytase and possible outcomes*



There are multiple ways in which phytase are classified (Lei et. al. 2007; Lim et. al. 2007).

- Depending on the stereo specificity of phytate hydrolysis, Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) classifies as 3/1-phytase (EC 3.1.3.8), 4/6-phytase (EC 3.1.3.26), and 5-phytase (EC 3.1.3.72).
- Depending upon the source into plant, bacteria, fungi, and yeast phytases.
- Depending upon pH optima into alkaline or acid phytases.
- Depending upon the catalytic mechanism into histidine acid phosphatases (HAP), β-propeller phytase (BPP), protein tyrosine phosphatases (PTP), or purple acid phosphatases (PAP).

The PA from soil is accessed by plant root phytases but they have low hydrolytic activity and is not secreted extracellularly in the rhizosphere. Studies on plant phytases show that many of them are active under acidic conditions and are stable up to 55˚C - 60˚C. For example, a maize and soybean phytase has maximum activity in pH range of 4.5 – 5.0 and stable between 55˚C - 60˚C (Hegeman 2001).

1.7. Microbial sources of phytases

Phytases occur widely among plants, animals, and microorganisms. Microbial sources of phytase are widespread and can be found in soils, aquatic systems, and animals. In the last 15 years, research has indicated that several strains of bacteria, yeast, and fungi can produce high yields of phytase with the application at the industrial scale. These provide a very effective system in dephosphorylating PA in the soil thus promoting plant growth as well as on PA in human food and animal feed (Vats et al. 2005; Singh et al. 2011). These also provide means for commercial production and thus pave the way in utilizing PA and in turn reducing pollution and fertilizer requirement.

Many soil samples were screened for isolation and majority were identified as *Aspergillus niger*, which was the most active group producing phytases (Shieh and Ware 1968). In 1982, Powar and Jagannathan showed that an enzyme that hydrolyzed only phytate was present in culture filtrates of *Bacillus subtilis*. Nayini and Markakis (1984) first reported the extraction of phytase from baker's yeast, *Saccharomyces*

cerevisiae, and performed characterization and purification studies. The first commercial phytase was prepared by fermentation of a genetically modified *A. niger* strain in 1991 by Gist-Brocades and marketed by BASF in Europe under the brand name Natuphos (Haefner et. al. 2005). Ever since the commercial application and the research on phytase developed a symbiotic relationship and became an increasingly important area of interest.

Till today, only a handful of commercial phytase products are available which are produced using submerged fermentation (SmF) process (Haefner et. al. 2005). Depending on the source and/or expression host, phytases can present different biophysical and biochemical properties (Rao et. al. 2009). With this objective in mind, scientists started to purify and express phytase in a wide range of hosts using various production strategies.

- Classical mutagenesis:

The old techniques of mutagenesis using physical and chemical mutagens still seems promising for improving the promising stains and make them commercially viable. Many mutagens have been used for inducing random mutations viz; hydroxyl amine (HA), ethyl methyl sulfonate (EMS), UV, etc. (Baltz 1999). *A. ficuum* NRRL 3135 phytase production was increased 3.3 fold using multiple UV mutations (Marisa et. al. 1994). Harmful spore formation was decreased with increase in phytase production using UV mutation (Gunashree and Venkateswaran 2009).

- Response Surface Methodology:

The phytase production can be increasing by altering the media composition, physical parameters, etc. But, the effect of production is not dependent solely on a component. It is a cumulative effect of each component. Thus, one variable at a time approach (OVAT) cannot be solely used for optimizing the production media. The component interaction effect on phytase production can be studied using response surface methodology (RSM). RSM has been widely used by many for optimizing the media for phytase production as it increases the production and yield as well as decreases the cost (Badamchi 2013; Coban and Demirci 2014). 3.08 fold increase in production (Bhavsar et. al. 2011), 2.9 fold increase in production (Gupta et. al. 2014) was obtained after RSM in SSF with a significant increase in productivity. 5.98 fold increase in production was obtained after RSM in SmF condition (Bhavsar et. al. 2013).

- Cloning and over expression:

This is a recent emerging method that can be used by focusing the application in which it is to be used. The poultry sector currently requires large scale production of the enzyme which would preferably be thermostable and stable over a wide pH range. *A. niger* phytase gene is cloned, characterized and over expressed in *A. niger*, producing 10 fold increased enzyme (van Hartingsveldt 1993). There are many challenges for over expressing phytases in different systems (Table 1.2) (Bhavsar et. al. 2014).

1.8. Need for Research

The first commercial phytase was in 1991, launched as Natuphos, specially aimed to be used as animal feed. Until today there are many bacterial and fungal phytases in the market but there is no ideal phytase in the market (Bhavsar et. al. 2014). Fungal phytases are most explored in poultry feed which are active in pH range of 4.5 to 5.5 but there are very scarce reports on phytase active in pH range of 2.0 – 3.0 which is optimum gut pH of poultry (Table 1.3) (Joshi 2014). The challenges in application vary in different sectors of phytase application as reviewed in Table 1.4 (Bhavsar et. al. 2014).

Table 1.2: *Phytase gene expression*

System	Advantages	Limitations
Fungi	Phytase gene from *A. niger, A. terrus, A. fumigatus* have been expressed for increased production in *A. niger*	Enzyme is less thermostable and thus needs to be engineered
Bacteria	Phytase gene from *A. niger* is expressed in *Streptomyces lividans* for extracellular enzyme and in *E.coli* for intracellular enzyme	Fungal phytase have glycosylation which is a concern to be produced in bacteria
Yeast	Bacterial and fungal phytase have been expressed	Only a few yeasts are able to express
Plants	Fungal phytase has been successfully expressed in tobacco, etc	Less thermostable enzyme produced

Table 1.3: *Commercial phytases available*

Enzyme origin	Expression system	pH optima	Temp optima (°C)	Trade name
A.niger	*A.niger*	2, 5 – 5.5	65	Natuphos
A.niger	*A.niger non – recombinant*	6.0	--	Allzyme
A.niger	*Trichoderma reesei*	2.5	--	Finase
E.coli	*S. pombe* (ATCC 5233)	4.5	55	Phyzyme
E.coli	*P.pastoris*	4.5	--	Quantum
E.coli	*Trichoderma reesei*	--	--	Quantum Blue
E.coli	*P.pastoris*	3.4, 5	58	Optiphos
Peniophora lycii	*A.oryzae*	4 – 4.5	50 - 55	Ronozyme
Citrobacter braakii	*A.oryzae*	--		Ronozyme HiPhos

Table 1.4: *Applications of Phytases*

Application Sector	Use	Enzyme properties	Challenges
Feed industry	Effective P utilization, less P in excretion, increased mineral availability, less DAP required	Active and stable at low pH, low cost of production	Less thermostable
Food Industry	Increased food nutritivity	Active at room temperature	Need to maintain food quality and taste after enzyme action

Pharmacology	Myo - inositol phosphate production used in drugs, cancer research, etc.	Low cost of production	Production of lower myo – inositol phosphate
Aquaculture	Effective P sourcing	Low temp and neutral pH optima enzyme	Need to study different fish species and habitat
Soil remediation	Soil P mobilization for plant growth promotion	Active and stable at wide range of pH and temperature	More effective phytase required

Submerged fermentation (SmF) is widely used method for commercial phytase production as it provides high productivities, ease of operation and scale-up. The process can be easily coupled with the downstream process like centrifugation and filtration. Since the fungal phytase produced is extracellular, the concentrated retentant can be easily mixed with feed for poultry application or formulated in a liquid base for field application.

2. Aims and Objectives

Phytase is proved to be one of the very important industrially produced enzyme. It has been approved to be GRAS (generally recognized as safe) enzyme by FDA (Wodzinski and Ullah, 1996). In view of its industrial importance the ultimate objective was to produce this enzyme in bulk at cost effective level and establish conditions for its industrial production, rapid downstream processing and formulate the product for its effective application using a GRAS cleared organism.

2.1. Objectives of the study:

1. Screening and production of phytase	a. Use of GRAS organism
	b. Ease of handling
	c. Aim for economically viable process
	a. For wide application studies

2. Scale – up production of phytase	b. Prove that the process is viable at industrial scale
3. Studies on downstream processing of the enzyme	a. Obtain high recovery at low cost
4. Studies on applications of phytase	a. Improve its applicability in currently applied areas
	b. Find areas where potential of phytase is untapped
5. Studies on use of biomass generated after fermentation	a. Have a green process by biomass reutilization

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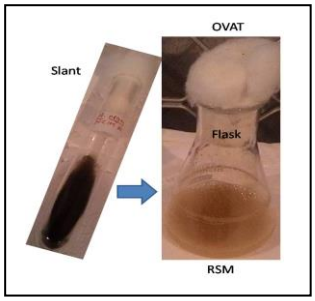


Chapter 2

Production of phytase by *Aspergillus niger* NCIM 563 under submerged fermentation

- *Aspergillus niger* NCIM 563 was chosen was phytase production studies
- Phytase production was studied using agricultural residues where rice bran was considered (68 IU/mL in 11 days using spore inocula)
- Response surface methodology (RSM) was used to enhance the phytase production (259 IU/mL in 13 days using spore inocula)
- Disadvantages with rice bran enforced search for better raw material wherein green chickpea flour was chosen (66 IU/mL in 9 days using spore inocula)
- The production was enhanced using vegetative inocula (86 IU/mL in 6 days) and further by using RSM which gave higher productivity than by using rice bran (160 IU/mL in 5.5 days using vegetative inocula)

1. Introduction

1.1 Phytase

Identification of phytase for the first time was carried out during the studies on hydrolysis of rice bran, which showed production of various phosphatidyl inositol as intermediates or as end products (Suzuki et. al. 1907). *Aspergilli* with phytase production was first demonstrated by Dox and Golden (1911). Plimmer (1913) and Anderson (1914a-c) carried out early studies identifying the organic phosphorus compounds in plant material. The compound was found to be of importance in corn soybean diet and thus many attempts were made to determine its structure. Anderson (1914d) proposed a structure and named it as phytic acid. The structure and all the data for each proposed structures was reviewed and evaluated by Reddy et. al. (1982). Phytic acid was recognized as anti-nutrient for monogastric animals. The commercial production of phytase was first carried out in 1962 at International Minerals and Chemicals in Skokie, Illinois. Over 2000 soil organisms were screened for phytase production by T R Shieh (1968) and found an isolate which can produce more phytase in liquid culture. Kenneth Raper deposited as *Aspergillus ficuum* NRRL 3135 at University of Wisconsin (Wodzinski and Ullah 1996).

Studies for developing strain, media optimization for phytase production was continued by Shieh et. al. (1969), with the aim to replace supplementation of inorganic phosphorus with phytase to monogastric animals. Nelson et. al. (1968) first tested the action of phytase on soybean meal where the phosphorus from phytic acid in soybean is made available to chicks and deposited in their bones. Unfortunately, the low yields of phytase production could not compete with the feeding of inorganic phosphorus.

In 1980's, cloning method for genes was available and Gist-Brocades in Netherlands were working on same organism NRRL 3135, to combat pollution caused by agricultural practices, one of the major issues during that time. The gene for phytase production *phyA* was cloned with amyloglucosidase promoter and leader sequence of NRRL 3135 into *A. niger* CBS 513.88 and increased the yield 1400 times than the parent (Frisvad et. al. 1990, Peterson et. al. 1992). The enzyme was tested as feed enzyme in poultry and swine and was also approved as GRAS for use in food by FDA.

In the USA, phytase has been used as a food additive from 1996 and marketed as Natuphos (Van Gorcom et. al. 1991).

The animal feed formulation industry relies on a few major ingredients viz; cereal grains, legumes and oil seed meal, etc. Cereal grains are a major source of energy whereas legumes and oil seed meal as a protein source. There is always an inclination of using cheapest ingredients to maximize the profits. Conventional feed ingredients are costly and may not be available to all producers at all locations. All over the world, conventional ingredients are being replaced due to the shortage, as they compete with human food supply. With alternative diets, livestock products have poor productivity due to deficiency of nutrients such as amino acids, minerals, energy protein ratio variation or anti – nutritive factors such as non – starch polysaccharides (NSPs), polyphenols or phytic acid (AAF 2013).

Feed enzymes are gaining importance for improving feed conversion and quality. These feed enzymes include carbohydrases for energy as they breakdown indigestible fibers, amylase to handle large concentration of starch, exogenous proteases for enhancing protein digestibility and phytase to help the release of the phytate phosphorus from the feed. The market players have a great opportunity for growth by tapping the untapped regions. The growth of this market is driven by growing customer attention towards good quality feed which should be nutritively balanced. Phytase has thus gained importance as feed enzyme combating the anti-nutritional effects of phytate, decrease environmental pollution, increase the availability of calcium, magnesium, iron and other chelated molecules (Romero 2015).

1.2 Source of phytase

The studies on phytase from *Aspergilli* was started for combating pollution caused during the agricultural practices and to act on phytic acid which is an important source of phosphorus to the monogastric animals and plants. After the entry of Natuphos in the market, many other phytases from fungi, bacteria, etc. were studied.

Different phytases were classified depending on their

- Catalytic activities *viz;* histidine acid phosphatases (HAP), β-propeller phytases (BPP), purple acid phosphatases (PAP), and cysteine phosphatases (CP).
- Stereo specificity of phytate hydrolysis (3 or 6 – phytases)

- pH optima (acid or alkaline)
- Amino acid sequence, reaction mechanism, 3-D conformation and biochemical properties

3-phytases are mainly produced by filamentous fungi and microorganisms and 6-phytases are found mostly in plants (Reddy et. al. 1982). HAP phytases are found mainly in microbes and plants, BPP phytases are found in *Bacillus* and some other bacteria, PAP phytases are found in animals, fungi and plants while CP phytase was found in *Selenomonas ruminantium* (Cheng and Lim 2006; Lim et. al. 2007).

The soil environment is likely to have phytase producing filamentous fungi (Casida 1959) and yeasts (Greenwood and Lewis 1977). Studies showed that the expression of phytase is induced in phosphate deficient conditions (Shieh and Ware 1968). The entry of Natuphos in the market, increased the industrial importance of phytases. Thus, all the commercially available phytases are produced in large volumes and concentration, using recombinant strains. Phytases from fungal as well as the bacterial origin are all expressed majorly in fungal and yeasts systems (Dersjant-Li et. al. 2015).

1.3 Fermentation strategies

Phytase can be produced using submerged fermentation (SmF) as well as under semisolid (Han et al, 1987 a, b) and solid state fermentation processes (Howson and Davis, 1983). The commercial phytases are mainly produced by SmF except for few which are produced by SSF (Dersjant-Li et. al. 2015). Studies have shown that extracellular phytase production is higher in submerged fermentation than in solid state fermentation process (Coban and Demirci 2014). Each process has its pros and cons as compared in Table 2.1 (Mitchell and Lonsane 1992).

Table 2.1: *Comparison of SmF and SSF*

SSF	SmF
Culture medium is not free flowing	Culture medium is always free - flowing
Depth of medium is usually shallow	Medium depth varies with bioreactor
Single water insoluble substrate provides C, N, minerals, and energy	Different water soluble sources of nutrients are used
Gradients in temp, pH, nutrient conc. are common	Nutrients are uniformly distributed throughout the fermentation medium

Inoculum ratio large	Inoculum ratio low
Bacterial and yeast cells adhere to solid and grow	Organism is uniformly distributed
Highly concentrated product	Low concentrated product
Water availability is just sufficient to sustain optimum growth of the culture	Water is abundantly available
Culture system has three phases; solid, liquid and gaseous	Culture system has two phases; liquid and gaseous
Rigorous control of parameters is not required except for heat removal, oxygen supply and moisture	Rigorous control of all the parameters is essential
System may or may not involve agitation	Agitation is essential
Fungal hyphae grow by penetration into the solid substrate particles	Fungal mycelial cells grow as individual filament or in form of pellets

A process needs to be selected which is economically viable and show ease in scaling – up to industrial scale. Industrial production demands uniform and sustainable production process with the same quality of product batch after batch. SSF process seems to have disadvantages. There is no uniformity in the system and lack of agitation, causing variability in product formation. Controlling the parameters e.g. temperature maintenance, etc. is difficult. Supply of oxygen and moisture is also a challenging task which needs to maintain aseptic conditions. Sampling for testing the product formation and growth during the production is a problem. Thus, SmF production process seems to be advantageous over SSF (Lambert et al.1983; Sato & Sudo 1999 and Krishna 2005).

1.4 Production optimization

Considering the natural environment, fungal or yeast phytases would be suitable as a sustainable source of phytase production. As discussed earlier Shieh and Ware (1968) started the preliminary studies on phytase production by screening the organisms on a plate containing calcium phytate. Further, the production media used were malt - yeast extract broth. Different media composition were also tried where corn starch (Sapna and Singh 2013), cane molasses (Singh et. al. 2015), potato dextrose broth (Shivanna and Venkateswaran 2014), dextrin synthetic media (Soni and Khire 2007), etc., were

used for submerged fermentation. For utilizing the agriculture waste and decreasing the production cost, studies using wheat bran (Badamchi et. al. 2013) have also been carried out

The production of extracellular phytase needs to be enhanced for making the process cost effective. There are many strategies used for enhancing the production. Mutagenesis is one of the approaches which used physical mutagens like UV and chemical mutagens like EMS, etc. But this process lack specificity and sensitivity. Chelius and Wodzinski (1994) during the strain improvement studies of *A. niger* NRRL 3135 used UV radiation for isolation of enhanced phytase producing mutant which gave 3.3-fold higher phytase (phyA) than the wild type strain. Transgenic studies using phytase gene from NRRL 3135 are also studied using different systems. In plants, studies using tobacco (Pen et. al. 1993), alfalfa (Gutknecht 1997), wheat (Brinch-Pedersen et al, 2000), canola (Ponstein et al, 2002) and soybean cells (Li et. al. 1997), etc., are carried out but, concern still raised were with respect to thermostability and performance of phytase in adverse conditions. The fungal phytase gene is expressed within fungi. This system actively produces phytases along with other enzymes like proteases, thus increasing the downstream step of purification and the total production cost (Martin et. al. 2003). Very few reports using yeast are available (Rodriguez et al, 2000). The bacterial system is the next most explored system after fungi but its usage is limited as it lacks the post-translational modification and the production of an enzyme with less activity on recombination (Phillipy and Mullaney, 1997).

The concentration of the media components can be optimized for enhanced phytase production. In this method, one of the media components is varied at different concentration keeping all the other components at a constant value. This method is one variable at a time (OVAT) approach. This is a very time consuming and expensive process (Vohra and Satyanarayana 2002). Response surface methodology (RSM) provides a very promising and cost-effective method for enhanced phytase production as it can handle many parameters simultaneously and uses mathematical and statistical tools (Vohra and Satyanarayana 2002; Chadha et. al. 2004). In this method, experiments need to be carefully designed to optimized the response which is the output variable and is dependent on the input variable i.e. the media components and the parameters. Change in output can be studied with the effect of input in series of tests (runs). RSM was originally developed by Box and Draper in 1987, to model the experimental

response. One of the important aspects of RSM is the selection of points/concentration where the response needs to be studied and is called Design of Experiments (DoE). For carrying out DoE, screening experiments are carried out using the media components and production parameters which have either positive, neutral or negative effect on phytase production.

Thus, this chapter aims at selecting a GRAS cleared fungal strain for submerged phytase production. Designing a cost-effective media and optimizing it using RSM for enhanced production.

2. Materials and Methods

2.1. Chemicals

The 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 Sigma, BDH, and Glaxo.

2.2. Agriculture residues and Legume

Various agriculture residues were purchased from the local market. Green chickpea seeds were purchased from the local market and minced in a grinder to obtain green chickpea flour (GrCf).

2.3. Micro-organism

Aspergillus niger NCIM 563, used in the present study was obtained from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory (CSIR-NCL), Pune, India. The stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4^{o}C. Spores from 7 days old PDA slant were harvested for inoculation using the sterile saline solution containing 0.01 % Tween 80.

2.4. Analytical methods

2.4.1. Phytase assay

Phytase activity was measured at 50°C as described earlier (Mandviwala and Khire, 2000). The reaction of 100 µl enzyme with 3mM sodium phytate was carried out at pH 2.5 using 100 mM Glycine-HCl buffer at 50°C for 30 min. The liberated inorganic phosphate as measured by a modification of the ammonium molybdate method (Heinohen and Lathi, 1981). A freshly prepared four ml solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1 M 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 1 µmole phosphorus per minute under standard assay conditions and the enzyme production was expressed as phytase activity (IU/mL). 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 and the specific activity (SpAc) using enzyme activity (IU/mL) and protein concentration (mg/mL) is calculated and expressed as IU/mg.

2.4.3. Sugar content

Total residual reducing sugar concentration was estimated by DNSA method (Miller 1959) using D-glucose as standard obtained from Sigma.

2.5. Media and culture conditions

Preliminary work of strain selection and developing a basal media composition for phytase production was carried out by Mandviwala and Khire (2000) and Soni and Khire (2007). Many organisms were screened from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory (CSIR-NCL), Pune, India, for phytase production using calcium phytate plate. False-positive results obtained due to acid production was confirmed by checking the positive strain for phytase production in submerged shake flask conditions. *Aspergillus niger* NCIM 563 was selected to be the strain of choice. Initial submerged production was carried out using synthetic media which comprised of (per 100mL): Dextrin 5 g; Glucose 2.5 g; $NaNO_3$ 0.86 g; KH_2PO_4 0.004 g; KCl 0.05g; $MgSO_4.7H_2O$ 0.05g; $FeSO_4.7H_2O$ 0.01g, pH 5.5 before sterilization. The media components were selected using one variable at a time approach (OVAT) which gave production of 41.5 IU/mL in 15 days with spore inocula.

In the current thesis, throughout further work on phytase production was carried out using *A. niger* NCIM 563 with the aim for sustainable phytase production in a cost effective manner with respect to time and media components. The strain was found to be non-toxin producer which was tested at Central Avian Research Institute, Bareilly.

3. Experiments, Results and Discussion

3.1. Studies with agriculture residue media

Various agriculture residues such as rice bran, de-oiled rice bran, wheat bran, maize, chickpea, peanut cake (low oil), peanut cake (high oil) and coconut cake were used to study the phytase production. Studies showed the presence of inorganic phosphorus in the suspension of agriculture residue. Initial phosphate concentration of all the agriculture residues available was determined by suspending 1 g of residue in 100 ml distilled water for 30 min, centrifuged to remove supernatant and dried in an oven at 60°C before use. The liberated inorganic phosphate in the supernatant was determined by a modification of the ammonium molybdate method (Heinohen and Lathi, 1981).

3.1.1. Results and Discussion

The results indicate variability in initial inorganic phosphate concentration of various agriculture residues (Table 2.2). Minimum concentration was observed in maize (2.30 mg/g) and maximum in coconut cake (8.0 mg/g).

Table 2.2: *Phosphate concentration in various agriculture residue*

Agriculture residue	Phosphate concentration (mg/g)
Rice bran	2.70 ± 0.14
De-oil rice bran	4.00 ± 0.2
Coconut cake	**8.00 ± 0.4**
Wheat bran	4.02 ± 0.2
Peanut cake (low oil)	4.95 ± 0.25
Peanut cake (high oil)	5.89 ± 0.3
Chickpea	2.80 ± 0.14
Maize	**2.30 ± 0.11**

The values given in the table are the average of three independent experiments with 3 - 5% variation.

Various reports established that the phytase production in submerged and solid state fermentation is affected by the amount of inorganic phosphorus in fermentation medium (Shieh and Ware 1968; Reddy et al. 1982; Dvorakova 1998; Mullaney et al.

2000; Vohra and Satyanarayana 2003). Studies have shown that high concentrations of inorganic phosphate in the culture medium favor the growth of microorganisms and inhibit expression of the genes encoding phytases (Van Hartingsveldt et al. 1993). Thus, studies on phytase production were checked using these agriculture residues which had a varying concentration of inorganic phosphate.

3.2. Phytase production using agriculture residue

The time course of phytase production under submerged fermentation conditions in medium (100 ml in 250 ml flask) according to Shieh and Ware (1968) with few modifications. Soluble starch was replaced with agriculture residue and inorganic phosphorus source was omitted. Thus modified fermentation medium contained (per 100 ml): agriculture residue (1 g); glucose 5 g; $NaNO_3$ 0.86 g; KCl 0.05 g; $MgSO_4.7H_2O$ 0.05 g; $FeSO_4.7H_2O$ 0.01 g, and pH 5.5 before sterilization.

Fermentation medium was inoculated using spore suspension prepared by suspending the spores from 7 days old sporulated slant of *A. niger* NCIM 563 grown on PDA in 10 ml of sterile saline containing 0.01% (v/v) Tween 80. Spores (5×10^7 spores per mL counted using Neubauer chamber) were inoculated to 100 ml media in 250 ml Erlenmeyer flask and incubated at 30°C at 200 rpm. Samples were removed after every 24 h. The biomass was separated by centrifugation and the supernatant was checked for phytase activity.

3.2.1. Results and Discussion

The fungus grew rapidly as indicated in all the agriculture residues with a rapid utilization of glucose. Maximum phytase activity was obtained at around 10 to 11th day (Table 2.3).

Table 2.3: *Time course of phytase production using agriculture residue*

Agriculture residue	Phytase activity (IU/mL) on day				
	4	**7**	**10**	**11**	**12**
Rice bran	**14.5**	**44.0**	**56.2**	**68.0**	**60.5**
De-oil rice bran	3.0	5.2	7.0	0.74	0.5
Coconut cake	2.0	3.0	4.0	3.7	2.5
Wheat bran	1.9	2.6	2.6	2.2	2.0
Peanut cake (low oil)	4.15	7.3	9.3	8.9	5.6

peanut cake (high oil)	3.1	5.2	6.0	6.5	4.2
Chickpea	**14.9**	**40.0**	**59.0**	**58.0**	**53.0**
Maize	**14.7**	**35**	**49**	**53.2**	**49.5**

The values given in the table are the average of three independent experiments with 3 - 5% variation.

Agriculture residues containing less than 4 mg/g inorganic phosphate supported phytase production with maximum activity of 68 IU/ml in rice bran containing medium followed by chickpea and maize (58 and 53.2 IU/ml, respectively). Agriculture residues containing inorganic phosphate more than 4 mg/g supported the growth of the fungus but phytase activity was very low. Earlier studies with the same strain also showed inhibition of phytase production in dextrin-glucose medium containing more than 4 mg/100 ml phosphate (Soni and Khire 2007). Thus soluble inorganic phosphate in the agriculture residue plays very important role in phytase production.

3.3. Effect of treating agriculture residue on phytase production

Treatment of agriculture residues suspended in distilled water with magnesium chloride precipitates the phosphate, so the supernatant becomes free of inorganic phosphate (Sano et al., 1999). This method is useful for the removal of phosphate from liquid medium like diluted molasses, but cannot be used for removal of phosphate from insoluble material like agriculture residue, as precipitate of inorganic phosphate adheres to the agriculture residue. Phosphate concentration was determined in the agriculture residue after a single wash with distilled water for 30 minutes as in experiment no. 1 above. Also, phytase production was determined using washed agriculture residue as in exp. 2 above.

3.3.1. Results and Discussion

Considerable amount of inorganic phosphate was removed when agriculture residues were suspended in distilled water (Table 2.4).

Table 2.4: *Phosphate concentration in various agriculture residue*

Agriculture residue	Phosphate concentration (mg/g)
Rice bran	**0.47 ± 0.02**
De-oil rice bran	1.08 ± 0.05
Wheat bran	0.56 ± 0.03

Peanut cake (low oil)	**2.90 ± 0.14**
Peanut cake (high oil)	2.70 ± 0.13
Chickpea	0.95 ± 0.05
Maize	2.20 ± 0.11
Coconut cake	1.43 ± 0.07

The values given in the table are the average of three independent experiments with 3 - 5% variation.

The phosphate concentration in all the residues decreased below 4 .0 mg/g and thus was expected to support phytase production which failed to do in experiment 2 above due to high phosphate concentration. Pretreatment of agriculture residues with distilled water to remove excess inorganic phosphate has significantly enhanced the phytase activity in case of de-oiled rice bran, wheat bran, peanut cake (low and high oil) and coconut cake (Table 2.5). Maximum increase of 20.3 times in phytase activity was observed in case of wheat bran as compared to de-oiled rice bran, coconut cake, peanut cake high and low oil where in the increase in phytase activity was 6.85, 6.1, 5.3 and 3.0 times, respectively. In case of rice bran and chickpea, the pretreatment with water resulted in 75% decrease in phytase activity which can be co-related to reduction of inorganic phosphate content from 2.7 to 0.47 mg/g in case of rice bran and 2.8 to 0.95 mg/g for chickpea. In case of maize decrease in phytase activity was only 25% as before and after washing the phosphate concentration was nearly same.

Table 2.5: *Time course of phytase production using washed agriculture residue*

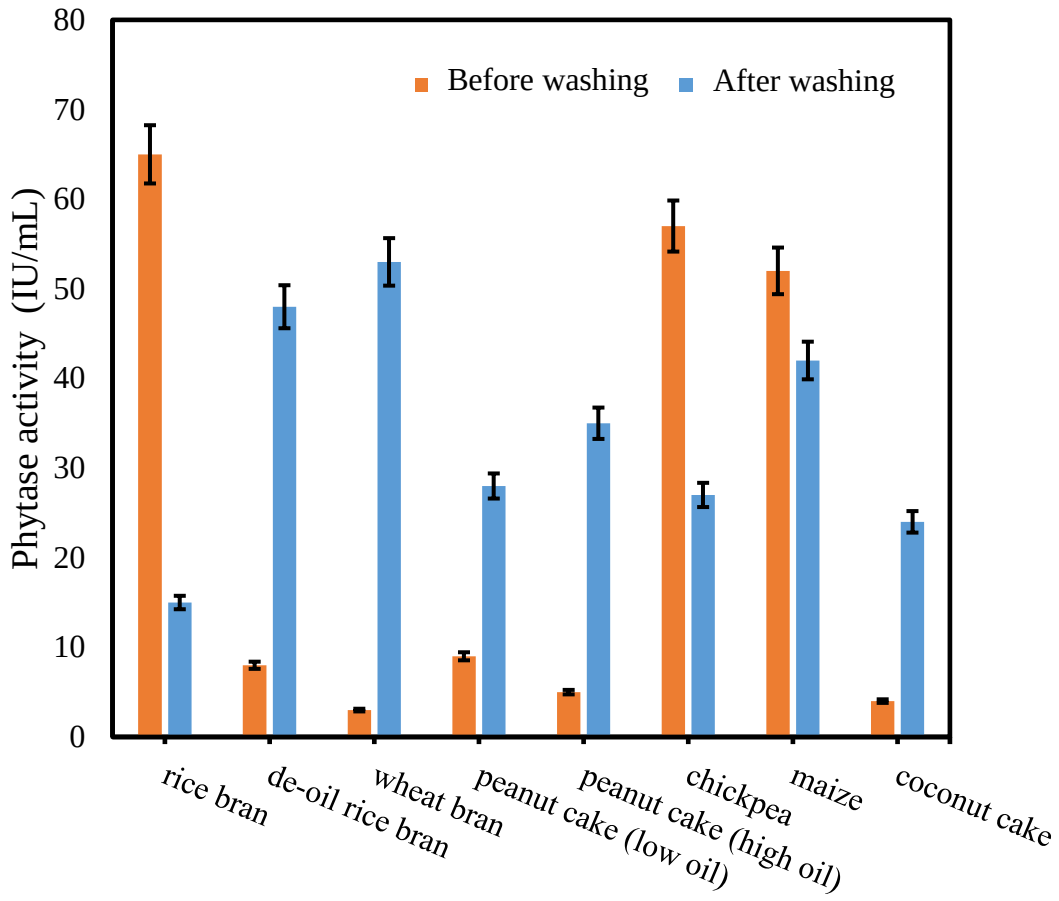
Agriculture residue	**Phytase activity (IU/mL) on day**				
	4	**7**	**10**	**11**	**12**
Rice bran	3.9	9.0	13.6	16.1	14.0
De-oil rice bran	**9.0**	**31.0**	**47.0**	**48.0**	**42.0**
Coconut cake	**6.5**	**12.6**	**23.4**	**24.4**	**20.4**
Wheat bran	**9.0**	**39.3**	**45.4**	**52.9**	**49.2**
Peanut cake (low oil)	**7.5**	**14.0**	**26.6**	**28.5**	**20.6**
Peanut cake (high oil)	**6.6**	**22.0**	**34.2**	**27**	**20.7**
Chickpea	6.0	12.0	18.3	19.4	15.4

Maize	**10.8**	**25.0**	**38.0**	**40.5**	**35.6**

The values given in the table are the average of three independent experiments with 3 - 5% variation.

Washing the agriculture residue incur a cost and the highest phytase production is obtained using unwashed rice bran amongst the residues studied (Fig 2.1). Thus, 1% rice bran media is used to further study the phytase production

Figure 2.1: *Comparison of phytase production in before and after washed agriculture residue*



3.4. Effect of glucose and rice bran concentration on phytase production

OVAT approach was carried out to study phytase production and compare it with the previously studied synthetic media. The phytase production was carried out in submerged fermentation using easily metabolizable sugar, glucose (1 – 9 % w/v), in 1 % rice bran fermentation medium. Also, the effect of phytase production using various

concentration of rice bran were studied and compared with the production in synthetic media.

3.4.1. Results and Discussion

The effect of easily metabolizable sugar, glucose (1 – 9 % w/v) on phytase production, in 1% rice bran fermentation medium indicated a gradual increase in phytase activity with an increase in sugar concentration in the fermentation medium from 1 to 5 %. Maximum activity of 68 IU/ml was produced in medium containing 5 % glucose. Sugar utilization was very rapid and maximum phytase activity was correlated with minimum reducing sugar in fermentation medium (Table 2.6). Easily metabolizable sugar e.g. glucose has been reported to increase phytase production by *A. niger* during submerged and/or solid-state fermentation (Vats and Banerjee 2002; 2004 and Vats et al., 2004).

Table 2.6: *Effect of glucose concentration on phytase production using 1% rice bran*

Production time (days)	**Glucose concentration (%)**									
	1		**3**		**5**		**7**		**9**	
	Activity (IU/mL)	**Residual sugar (%)**	**Activity (IU/mL)**	**Residual sugar (%)**	**Activity (IU/mL)**	**Residual sugar (%)**	**Activity (IU/mL)**	**Residual sugar (%)**	**Activity (IU/mL)**	**Residual sugar (%)**
2	0.94	0.13	1.0	2.2	0.83	4.4	1.06	6.8	1.35	8.9
4	5.7	0.025	12.5	0.073	12.0	1.9	10.4	3.46	8.8	5.82
7	6.0	0.012	21.0	0.02	36.0	0.19	33.0	2.0	30.	4.36
9	7.5	0.014	35.0	0.023	58.0	0.059	58.5	1.31	50.5	2.71
11	7.2	0.019	38.0	0.003	**68.0**	0.019	61.0	0.59	56.0	0.76
12	6.5	0.007	33.0	0.002	58.0	0.011	48.0	0.45	45.0	0.50

The values given in the table are the average of three independent experiments with 3 - 5% variation

The effect of various concentration of rice bran (0.5 to 2%) in fermentation medium containing 5% glucose, indicates that maximum phytase activity was produced in medium containing 1% rice bran i.e. the medium containing 2.7 mg inorganic phosphate per 100 ml medium (Fig 2.2). Increasing the concentration of rice bran above 1% concentration resulted into more inorganic phosphate in the medium which resulted in inhibition of phytase activity.

Earlier studies on phytase production under submerged fermentation medium containing 5% dextrin and 2.5% glucose gave maximum phytase activity of 41.5 IU/ml on the 15th day of production (Soni and Khire 2007). In the present study, medium containing 1% rice bran and 5% glucose gave maximum phytase activity of 68 IU/ml within 11 days (Table 2.7). Thus fermentation time was reduced by four days with the increase in activity by 70%. Thus, the productivity [units of enzyme produced per day (IU/mL/day)] increased from 2.8 IU/mL/day obtained using synthetic media to 6.2 IU/mL/day using rice bran media

Figure 2.2: *Effect of rice bran concentration on phytase production*

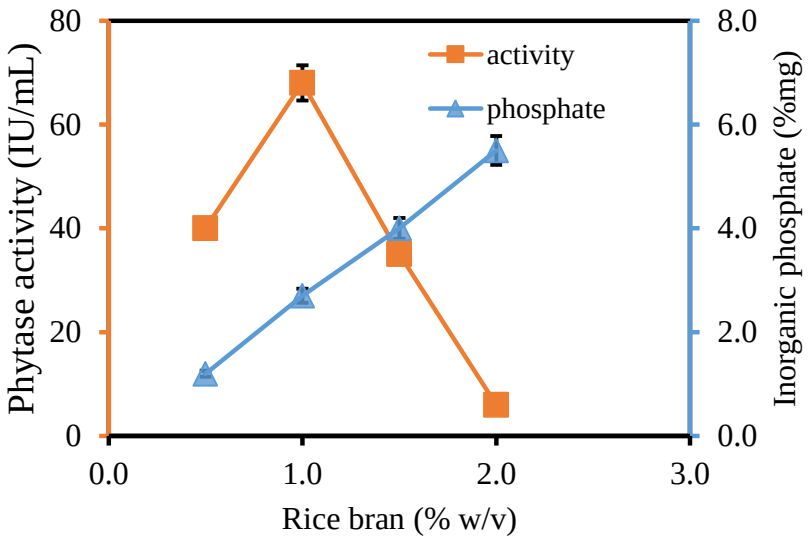


Table 2.7: *Comparison of phytase production in dextrin-glucose and rice bran-glucose medium*

Production time (days)	1% Rice bran and 5% Glucose medium		5 % Dextrin and 2.5% Glucose medium	
	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)
4	12.0	1.9	4.17	5.26
7	36.0	0.19	10.0	4.3
9	58.0	0.05	16.0	3.5
11	68.0	0.019	23.5	2.6
12	62.0	negligible	26.0	2.28
14	59.0	negligible	31.68	1.75
15	51.0	negligible	41.5	1.03
16	50.0	negligible	37.0	0.91

The values given in the table are the average of three independent experiments with 3 - 5% variation

3.5. Studies on enhanced production of phytase using RSM

RSM is a way in which a series of methods are carried out by conducting experiments for exploring optimum operating conditions. The process starts with fitting first order designs (linear equations) and then to the second order designs (quadratic equations). Then the characteristics of the fitted surface are examined to decide further action.

Plackett – Burman design is one of the most common first order design. Carrying out a Plackett – Burman design (PBD) of experiments offers a rapid multifactor way to screen and identify the most significant factors (Plackett and Burman 1946). The design allows two levels for each of the ‘k’ control variables, but requires a much smaller number of experimental runs, especially if ‘k’ is large. It is, therefore, more economical. Its number, ‘n’, of design points is equal to k + 1, which is the same as the number of parameters. The number of design points is equal to the number of parameters to be estimated in the model and in this respect, the design is said to be saturated.

Furthermore, this design is available only when n is a multiple of 4. Therefore, it can be used when the number, 'k', of control variables is equal to 3, 7, 11, 15,.....

In the current experiment, the effect of 11 variables (Xi) viz, 5 basal medium variables and 6 additional variables, likely to influence phytase production were chosen for screening studies using PBD. The PBD is a two-factorial design that can be expected to identify critical chemical and physical parameters required for elevated enzyme production by screening the 'k' variables in 'k+1' experiments (Plackett and Burman 1946). Each independent variable was tested at two levels, a high (+1) level, and a low (−1) level. A set of 12 experiments was thus carried out to determine phytase production as per the PBD of experiments (Table 2.8).

Table 2.8: *PBD trials for phytase production*

Run No.	A	B	C	D	E	F	G	H	J	K	L	Phytase production
	Glucose	Rice bran	$NaNO_3$	$MgSO_4$	$FeSO_4$	KCl	Incubation period	Inoculum level	Tween 80	$MnSO_4$	Dextrin	
	(g %)	(g %)	(g %)	(g %)	(g %)	(g %)	(days)	(spores/mL)	(g %)	(g %)	(g %)	IU/mL
1	3	0.8	1.1	0.04	0.04	0.1	4	10^9	0.015	0.02	0.5	8 ± 0.4
2	**8**	**1.5**	**1.1**	**0.04**	**0.01**	**0.04**	**13**	**10^5**	**0.015**	**0.02**	**0.5**	**100 ± 5**
3	8	1.5	0.7	0.1	0.04	0.1	4	10^5	0.012	0.02	0.5	9 ± 0.45
4	8	0.8	1.1	0.1	0.01	0.1	13	10^9	0.012	0.005	0.5	60 ± 3
5	**3**	**0.8**	**0.7**	**0.04**	**0.01**	**0.04**	**4**	**10^5**	**0.012**	**0.005**	**0.5**	**4 ± 0.2**
6	8	0.8	0.7	0.04	0.04	0.04	13	10^9	0.012	0.02	1.0	86 ± 4.3
7	8	1.5	0.7	0.04	0.01	0.1	4	10^9	0.015	0.005	1.0	5 ± 0.25
8	8	0.8	1.1	0.1	0.04	0.04	4	10^5	0.015	0.005	1.0	10 ± 0.5
9	3	1.5	1.1	0.04	0.04	0.1	13	10^5	0.012	0.005	1.0	48 ± 2.4
10	3	1.5	0.7	0.1	0.04	0.04	13	10^9	0.015	0.005	0.5	36 ± 1.8
11	3	0.8	0.7	0.1	0.01	0.1	13	10^5	0.015	0.02	1.0	43 ± 2.1
12	3	1.5	1.1	0.1	0.01	0.04	4	10^9	0.012	0.02	1.0	6 ± 0.3

The values given in the table are the average of three independent experiments with 3 - 5% variation

The complete matrix for screening was designed using a standard Plackett–Burman orthogonal array constructed using Design Expert (DE) software Version 7.1.2, Stat-Ease, Minneapolis, MN, USA. The effect E (Xi) of the tested variable Xi was determined by:

$$E(X_i) = 2[\sum R(H) - \sum R(L)] / N \quad (1)$$

where, N denotes the number of trials with R (H) and R (L) representing a response obtained at either the high or low level, respectively, for the variable (Xi) from an experiment. Note that R (H) and R (L) are summed to obtain the average response at the high/low level, respectively, and their difference calculated to estimate the effect E (Xi). The variables with higher percent contributions were considered to influence phytase activity and chosen for further optimization studies. The percent contribution was obtained by taking the E (Xi) of each variable and dividing it by total sum of squares of E (Xi) for all the variables.

3.5.1. Results and Discussion

The potential effect of 11 variables on phytase production were evaluated using PBD. The PBD design matrix for experimental design of the selected variables is shown in Table 2.8 along with the obtained responses on phytase production. Maximum phytase production of 100 U/mL was observed in trial number 2. The variables and their effect E (Xi), as calculated by Eq. (1) and percent contribution (Table 2.9) show that phytase production is significantly affected by glucose, $MgSO_4$, KCl, incubation period, and $MnSO_4$.

Table 2.9: *Variables contribution for phytase production*

Code	**A**	B	C	**D**	E	**F**	**G**	H	J	**K**	L
Variable	**Glucose**	Rice bran	$NaNO_3$	**$MgSO_4$**	$FeSO_4$	**KCl**	**Incubation period**	Inoculum level	Tween 80	**$MnSO_4$**	Dextrin
% Contribution	**10.48**	0.026	1.53	**5.03**	0.27	**3.12**	**73.77**	0.087	0.11	**5.31**	0.27
Effect x 10^3	**20.85**	-1.04	7.96	**-14.45**	-3.34	**-11**	**55.34**	-1.89	-2.12	**14.84**	-3.36

These factors account for 97.8% of the total contribution while the remaining variables ($NaNO_3$, $FeSO_4$, inoculum level, Tween 80, and dextrin) account for only 2.2%. The variables identified by PBD to be insignificant and are therefore maintained at mean levels in further optimization studies.

After first order design, second order designs are applied for further optimization. Central Composite Design (CCD), is one of the most popularly used second order designs. CCD was first introduced by Box and Wilson in 1951. This design consists of the following three portions:

1. A complete (or a fraction of) 2^k factorial design whose factors' levels are coded as −1, 1. This is called the factorial portion.
2. An axial portion consisting of 2^k points arranged so that two points are chosen on the axis of each control variable at a distance of α from the design center (chosen as the point at the origin of the coordinates system).
3. n_0 center points.

The five medium components (Table 2.10) identified by PBD, as significant factors, for phytase production were further optimized by CCD.

Table 2.10: *Selected variables and their assigned levels by CCD*

Code	A	B	C	D	E
Variable	Glucose	$MgSO_4$	Incubation period	KCl	$MnSO_4$
(-) α	1.8	0.025	1.8	0.025	0.001
(-) 1	3.0	0.04	4.0	0.040	0.005
0	5.5	0.07	8.5	0.070	0.012
(+) 1	8.0	0.10	13.0	0.100	0.02
(+) α	9.2	0.11	15.2	0.110	0.024

The CCD design matrix and levels of the variables comprising and the obtained response values are shown in Table 2.11.

Table 2.11: *CCD experimental design and results*

Trial number	Factor level					Phytase production (IU/mL)
	A Glucose	**B $MgSO_4$**	**C Incubation period**	**D KCl**	**E $MnSO_4$**	
1	+ α	0	0	0	0	97.00
2	-1	1	1	1	-1	37.00
3	0	0	0	1	0	97.74
4	-1	1	-1	1	1	32.34
5	0	0	0	0	0	114.48
6	1	1	-1	1	-1	176.91
7	-1	1	1	-1	1	17.04
8	-1	-1	-1	1	1	33.90
9	-1	-1	-1	-1	-1	17.21
10	1	-1	1	1	-1	192.40
11	1	-1	-1	-1	-1	29.54
12	**0**	**0**	**0**	**- α**	**0**	**1.50**
13	0	0	0	0	+ α	97.17
14	-1	-1	1	1	-1	26.27
15	1	-1	-1	1	1	89.20
16	1	-1	-1	1	1	199.90
17	-1	-1	-1	1	-1	34.05
18	0	0	0	0	0	91.04

19	0	0	0	+ α	0	52.50
20	-1	1	1	1	1	27.35
21	0	+ α	0	0	0	130.28
22	1	-1	1	-1	-1	27.92
23	0	0	+ α	0	0	77.50
24	1	1	1	-1	-1	25.17
25	1	-1	1	-1	1	28.20
26	1	1	1	-1	1	27.91
27	0	0	- α	0	0	80.95
28	0	0	0	0	0	82.25
29	-1	-1	1	-1	1	23.70
30	0	0	0	0	0	93.06
31	-1	1	1	-1	-1	13.34
32	1	1	-1	-1	-1	30.05
33	1	-1	-1	1	-1	241.29
34	1	-1	-1	-1	-1	29.61
35	-1	1	-1	-1	-1	19.20
36	-1	-1	-1	-1	1	19.01
37	1	1	-1	-1	1	25.94
38	0	0	0	0	- α	95.26
39	**1**	**1**	**1**	**1**	**-1**	**259.80**

40	-1	-1	1	1	1	26.05
41	0	0	0	0	0	103.75
42	1	-1	1	1	1	247.31
43	0	- α	0	0	0	95.77
44	1	1	1	1	1	248.50
45	-1	-1	1	-1	-1	20.33
46	- α	0	0	0	0	9.98
47	1	1	-1	1	1	236.13
48	0	0	0	0	0	99.08
49	-1	1	-1	1	-1	30.55
50	-1	1	-1	-1	1	14.91

The values given in the table are the average of three independent experiments with 3 - 5% variation

It may be observed that trial number 39 showed the highest phytase activity (259.8 ± 13 U/mL) while trial number 12 showed the lowest phytase activity (1.5 ± 0.07 U/mL). The wide range of activity shows the sensitivity of the process to experimental conditions and the need for process optimization. The obtained results were fitted to a polynomial equation to three significant digits:

$$y = 91700 + 48300A + 48100D + 46000AD – 10900A^2 + 15600B^2 – 22700D^2 \quad (2)$$

Here y is the predicted response, 91700 is the intercept with variables A, B, and D corresponding to glucose, $MgSO_4$, and KCl. It may be seen that an interaction term AD is present in the above model. Non-significant variable and interaction terms were excluded by systematically carrying out ANOVA analysis to obtain the above reduced but improved model description Eq. (2).

The best linear fit regression model obtained using the significant PBD variables gave a model F value 106.3 by ANOVA (Table 2.12) suggesting a very high significance of regression.

Table 2.12: *ANOVA for the reduced quadratic model*

Source	Sum of squares	Degree of freedom	Mean square	F value	p value Prob > F
Model	2.4*10^11	6	4.1*10^11	106.3	<0.0001
A	8.5*10^10	1	8.5*10^10	219.0	<0.0001
D	8.4*10^10	1	8.4*10^10	217.2	<0.0001
AD	6.8*10^10	1	6.8*10^10	174.5	<0.0001
A^2	1.5*10^9	1	1.5*10^9	3.9	0.056
B^2	3.0*10^9	1	3.0*10^9	8.0	0.0072
D^2	6.5*10^9	1	6.5*10^9	16.8	0.0002
Residual	1.6*10^10	43	3.8*10^8		
lack of fit	1.6*10^10	36	4.4*10^8	5.0	0.016
Pure error	6.2*10^8	7	8.9*10^7		
Cor total	2.6*10^11	49			
Coefficient of determination (R^2) 0.94; correlation coefficient (adjusted R^2) 0.93; predicted R^2 0.87; adequate precision 33.5					

This implies that there is only a 0.01% chance that this "Model F-Value" could occur due to noise. The coefficient of determination of (R^2) 0.94 suggests that 94% of the variability is explained by the model. The predicted R^2 value of 0.87 suggests a strong agreement between observed and model predicted value. Adequate precision measures signal to noise ratio and a ratio greater than 4 is desirable. As per the interactive term in the equation 2, the 3D response surface plot (Fig 2.3) shows the positive effect of the interaction between glucose and KCl for phytase production. An increase in phytase production is seen as the concentration of these variables is increased towards the +1 level.

The model was validated with time course production of phytase using RSM optimized media which comprised of glucose (8 %), rice bran (1.5 %), $NaNO_3$ (1.1 %),

$MgSO_4$ (0.1 %), $FeSO_4$ (0.01 %), KCl (0.1 %), inoculum level (1x10^5 spores/mL), tween 80 (0.005 %), $MnSO_4$ (0.02 %), dextrin (0.5 %). 268 IU/mL phytase activity was obtained in 13 days of production (Fig 2.4).

Thus the productivity of phytase production increased from 6.2 IU/mL/day to 20.6 IU/mL/day after optimization using RSM.

Figure 2.3: *3D graph showing interaction of glucose and KCl on phytase production*

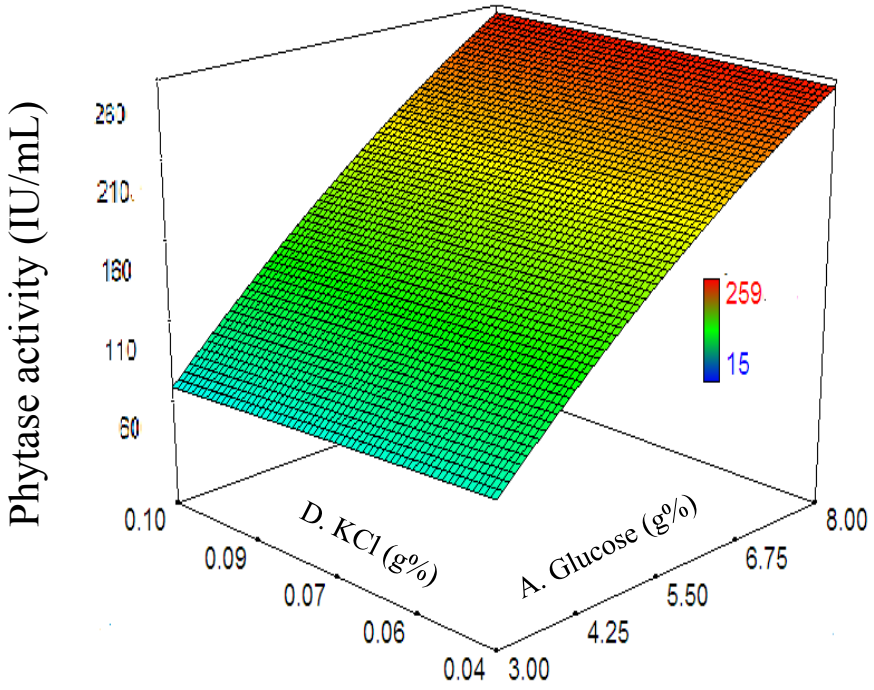
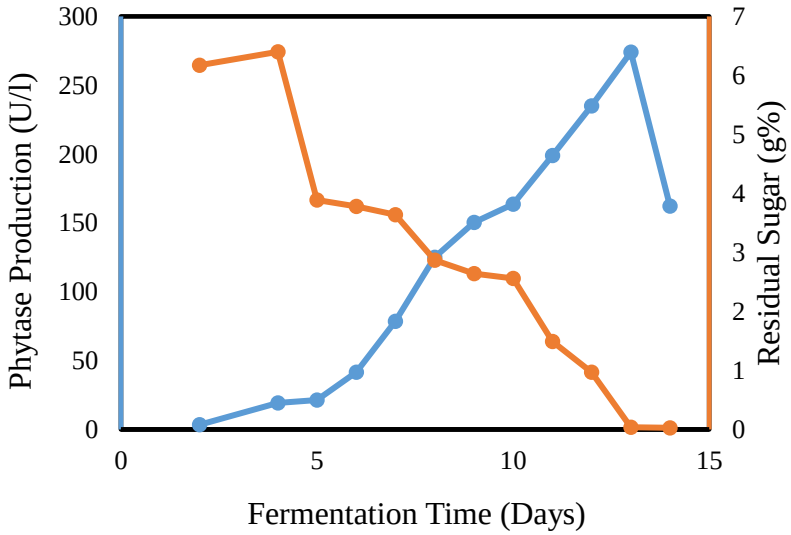


Figure 2.4: *Validation of phytase production using RSM optimized media*



3.6. Summary of experiments carried out:

Present studies on phytase production under submerged fermentation conditions by *A. niger* NCIM 563, indicated that pretreatment of agriculture residues with distilled water was useful when initial inorganic phosphate content of the residue was above 4 mg/g, which otherwise inhibit phytase production. There was a substantial increase in phytase activity when this excess phosphate was removed by washing. Maximum increase (20.3 times) in phytase activity was observed in the case of wheat bran. However pretreatment was not useful in case of rice bran where initial inorganic phosphate content was 2.7 mg/g which was reduced to 0.47 mg/g after washing the residue with distilled water which resulted in decrease in phytase activity from 68 IU/ml (before washing) to 16.1 IU/ml (after washing). Similarly, there was an increase in productivity and reduction in fermentation time when agriculture residue was used instead of synthetic media in submerged fermentation. Maximum phytase activity of 68 IU/ml was produced on the 11^{th} day of fermentation compared to 41.5 IU/ml activity on the 15^{th} day of submerged fermentation using 5% dextrin and 2.5% glucose. Similarly, the cost of any agriculture residue is much cheaper than dextrin. Enhancing the phytase production using RSM is the next step towards developing a cost-effective production process.

The productivity of any fungal fermentation is affected by process parameters and media composition and therefore the present investigation was performed to statistically optimize the medium components for the production of the phytase from *A. niger* using PBD and CCD methodologies. All significant variables involved in phytase production were evaluated by PBD because it can test a large number of variables while avoiding the loss of any essential information in subsequent optimization studies. Based on analysis of PBD, five key variables, viz., glucose, $MgSO_4$, KCl, incubation period, and $MnSO_4$, were found to affect the phytase production by *A. niger*. This was followed by use of a multifactorial response surface approach employing CCD, an effective design strategy, for studying the effects of key variables and their mutual interactions. This study resulted in an overall 3.32 fold enhancement in phytase activity of parent strain.

3.7. Observations after the experiments

- ✓ *Aspergillus niger* NCIM 563 is safe and non-toxin producer
- ✓ Rice bran was easily available in large quantity for phytase production

- ✓ Varying concentration of phosphate was observed per batch of rice bran procured and thus needed pre-treatment as per the phosphate concentration
- ✓ Inconsistency in phytase production was observed with change in rice bran batch
- ✓ Minute particles of bran failed to settle after centrifugal separation of biomass
- ✓ Additional filtration step was required to get clear enzyme supernatant which increased downstream process cost
- ✓ Mixture of fungal biomass and rice bran was obtained after its centrifugal separation which interfered its further usage
- ✓ Time required for maximum production was too long thus needed further optimization
- ✓ Selecting the variables for optimization is very crucial viz; incubation time should not be considered as variable

All the results from experiment 3.1 to 3.4 are published in:
Kavita Bhavsar, Parin Shah, Sarvesh Kumar Soni, Jayant Khire (2008): Influence of pre-treatment of agriculture residues on phytase production by *Aspergillus niger* NCIM 563, under submerged fermentation conditions. African Journal of Biotechnology, 7(8): 1101-06.

All the results in experiment no. 3.5 is published in:
K. Bhavsar, P. Gujar, P. Shah, V. Ravi Kumar, J. M. Khire (2013): Combinatorial approach of statistical optimization and mutagenesis for improved production of acidic phytase by *Aspergillus niger* NCIM 563 under submerged fermentation condition. Applied Microbiology and Biotechnology, 97(2):673-9

3.8. Further optimization

Agricultural residues including rice bran, wheat bran, groundnut oil cake, corn starch, etc., are widely used as substrates for phytase production (Bhavsar et al. 2008; Alves et al. 2016; Buddhiwant et al. 2015). However, the cost of production increases with use of agricultural residue as it needs pre-treatment (Bhavsar et al. 2008; Rani et

al. 2014). The need for cost-effective sustainable production requires an alternative substrate for phytase production. The chemical composition of rice bran as per literature states presence of 11 – 13 % protein, 11 – 22 % fats, 10 – 14 % fibre, and other components like cellulose, reducing sugar, ash, etc. (Augustine and Roy 1981). In this context, the use of protein, fibre, and carbohydrate rich legume flour as substrate similar to rice bran composition has advantages for sustainable low-cost phytase production as the pre-treatment step can be avoided (Maheri-Sis et. al. 2008; Jukanti et. al. 2012). India is the largest producers of chickpea (*Cicer arietinum*) (7.17 metric tons in 2014–2015) but around 20% of the cultivated chickpea seeds are rejected due to non-uniform growth, color and damage during harvesting and post-harvesting process (Torres-Fuentes et al. 2011). These rejected green seeds are, however, rich in protein, carbohydrate, lipids and major dietary minerals such as calcium, phosphorus, magnesium, iron and potassium (Christodoulou et al. 2006) and have been used in poultry diet (Garsen et al. 2007). Also, in Table 2.3 above, the second highest phytase production is obtained using chickpea agricultural waste. For the above reasons, in the present study, in view of its availability as agricultural waste, we used green chickpea flour (GrCf) as the substrate of choice for producing and maximizing phytase production which is the first report.

In the present study for increasing the production of extracellular phytase from GrCf using *A. niger*, growth media optimization was undertaken using shake flasks under submerged fermentation condition (SmF) by employing an effective hybrid strategy that involves carrying out statistical sets of experiments. In this approach, we initially aim at identifying the significant factors influencing phytase production by implementing a Plackett–Burman Design (PBD) of experiments (Plackett and Burman 1946). Subsequently, the second set of experiments can then be conducted to further optimize the levels of the significant factors that maximize phytase production by application of a more rigorous experimental design such as the Box-Behnken design (BBD) (Box et al. 1978; Khuri and Cornell 1987). BBD was developed by Box and Behnken in 1960, which has three levels for each variable studied. Thus, this design approach is economical as it has less levels (-1, 0 +1) than CCD which has five levels for each variable ($-\alpha$, -1, 0 +1, $+\alpha$). Hence, BBD is more popular in industrial research.

Obtaining positive results would then suggest scale-up studies with 2 and 10-L fermenters so as to confirm maintenance or obtain improvements in the production levels for process feasibility.

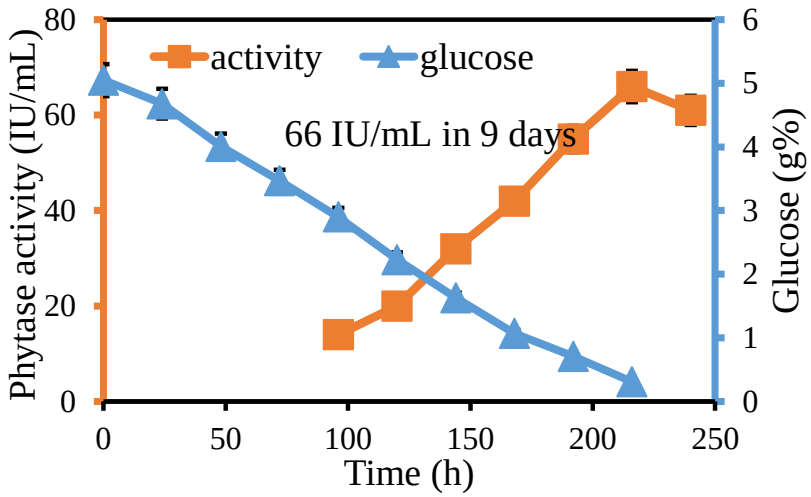
3.9. Phytase production using green chickpea flour media

A time course of the phytase production was studied using green chickpea flour media (pH 5.5) in triplicates. 100 mL media comprising of (g%): 1.0 GrCf; 5.0 glucose; 0.86 $NaNO_3$; 0.05 KCl; 0.05 $MgSO_4 \cdot 7H_2O$; 0.01 $FeSO_4 \cdot 7H_2O$ was dispensed in 250 mL Erlenmeyer flask and sterilized by autoclaving at 121 °C for 20 min. Spores from 7 days old PDA slant were gently scraped using sterile wire loop with 25 mL sterile saline solution containing 0.01% Tween 80. The spore suspension was collected in a sterile tube and homogenized by vortexing for 1 min. 1×10^7 spores (using Neubauer chamber) was used as spore inoculum (Sp-I) for inoculating the basal media and incubated at 28 °C at 170 rpm under aerated culture conditions.

3.9.1. Results and Discussion

The phytase production was tested using green chickpea flour media, which showed, a slow and gradual increase in phytase production using Sp-I, with the maximum activity of 66 ± 3.3 IU/mL on the 9th day, (i.e., 216 h) (Fig 2.5).

Figure 2.5: *Phytase production using green chickpea flour media*



The glucose concentration also showed a gradual depletion, with complete utilization by 10th day (i.e., 240 h). Thus, the productivity [units of enzyme produced per day (IU/mL/day)] obtained using Sp-I was 7.3 IU/mL/day which is better than that obtained by unoptimized rice bran media which was 6.2 IU/mL/day.

3.10. Studies on effect of inoculum types on phytase production

Inoculum is one of the important parameters which influences the fungal morphology and phytase productivity. Studies on phytase production using different types of inoculum (spore or vegetative) were carried out using *A. niger* (Papagianni et

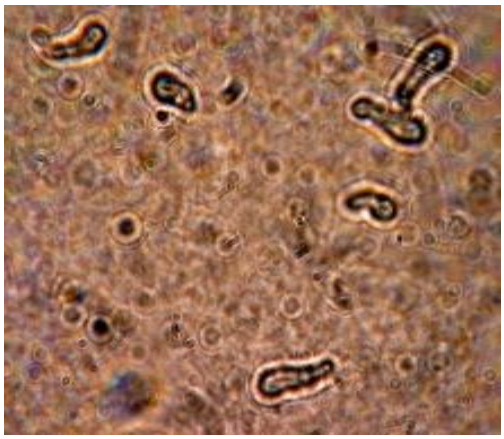
al., 2001). A similar strategy for protease production was carried out using *A. niger* (Papagianni and Moo-Young 2002). Increased production of enzyme was obtained using vegetative inoculum.

In the present experiment, vegetative inoculum (Ve-I) was prepared by inoculating 5 mL green chickpea flour media with 1×10^7 spores under same aerated culture conditions for 10 h which was used for inoculating 95 mL production media. The samples were withdrawn every 24 h and centrifuged for biomass separation. The supernatant was checked for total residual glucose and phytase activity.

3.10.1. Results and Discussion

Vegetative germination of spores was observed every hour and it was observed that the spore germination started in 8 hours and maximum germination was observed in 10 hours and thus was decided to inoculate the production media (Fig 2.6).

Figure 2.6: *Germination of spores of A. niger NCIM 563*

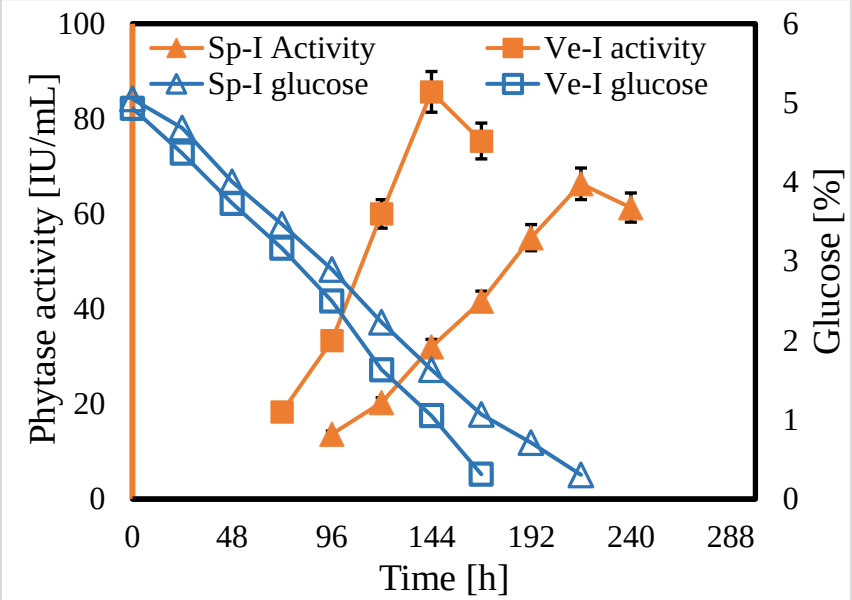


Using Ve-I provided higher phytase activity of 86 ± 4.3 IU/mL in a lower production time of 6 days (i.e., 144 h) with glucose depletion in 7 days (i.e., 168 h) (Fig 2.7). Thus, the productivity [units of enzyme produced per day (IU/mL/day)] increased from 7.3 IU/mL/day obtained using Sp-I to 14.3 IU/mL/day using Ve-I. Thus, the type of inoculum has marked effect on the production of phytase and all further studies were therefore carried out using Ve-I.

The literature states that for the fermentation process, the majority of the organisms used belongs to filamentous fungi and *Streptomyces*. Their sporulation capability has made it advantageous to use for the production. The use of spore directly for production would avoid the cost of installation and operation of the seed tanks. However, the

production time would be higher than the usage of vegetative cells, allowing less number of fermentations to be carried out per year. Development and maintenance of vegetative cells would incur labor cost which would be high, but part of the cost can be recovered. The type of inocula to be used depends on the scale of fermentation operations (Project No: RE-02091011297).

Figure 2.7: *Comparison of phytase production using spore and vegetative inocula*



3.11: Media optimization using RSM for enhanced phytase production

Carrying out a PBD experiments offers a rapid multifactor way to screen and identify the most significant factors (Plackett and Burman 1946). The potential effect of 10 variables (Table 2.13) on phytase production, were evaluated in 12 PBD runs at two levels, low level (−) and high level (+). The complete PBD matrix for screening was designed using a standard Plackett–Burman orthogonal array constructed using Design Expert Software (DES) Version 7.1.2, Stat-Ease, Minneapolis, MN, USA. The response values of phytase produced in IU/mL were analyzed to obtain a best-fit linear mathematical model. Subsequently, a BBD of experiments was generated by DES and studies at three different levels −1, 0, +1 (Table 2.15) were carried out to further optimize enzyme production levels with respect to the major factors identified by PBD. The less significant factors were maintained at the average of the high and low levels used in the PBD study. A best-fit model for enzyme production was further studied by ANOVA to test for its statistical significance.

***Table 2.13**: Selected variables for PBD and their assigned levels for phytase production*

S. No.	Code	Variables	Low level (-)	High level (+)
1	A	Glucose (g)	3.00	5.00
2	B	$NaNO_3$ (g)	0.30	0.80
3	C	$MgSO_4.7H_2O$(g)	0.04	0.10
4	D	KCl (g)	0.04	0.10
5	E	$FeSO_4.7H_2O$ (g)	0.01	0.02
6	F	Tween 80 (µl)	10.00	20.00
7	G	GrCf* (g)	0.75	1.25
8	H	Dextrin (g)	0.20	0.50
9	J	$MnSO_4 \cdot H_2O$ (g)	0.005	0.020
10	K	$CaCl_2 \cdot 2H_2O$ (g)	0.10	0.30

*GrCf – green chickpea flour

3.11.1. Results and Discussion

For the 12 PBD runs the experimentally obtained maximum response values of phytase activity (IU/mL) obtained on the 6th day (i.e., 144 h) are reported in Table 2.14.

Table 2.14:** PBD for the selected variables along with the experimentally obtained mean phytase production response values

Run No.	A	**B**	C	D	E	F	**G**	H	**J**	**K**	**Phytase activity**
	Glucose	**$NaNO_3$**	$MgSO_4$	KCl	$FeSO_4$	Tween 80	**GrCf**	Dextrin	**$MnSO_4$**	**$CaCl_2$**	
	(g %)	(g %)	(g %)	(g %)	(g %)	(%)	(g %)	(g %)	(g %)	(g %)	IU/mL
1	5.0	0.3	0.1	0.1	0.02	0.01	0.75	0.2	0.02	0.1	93±4.6
2	3.0	0.3	0.04	0.04	0.01	0.01	0.75	0.2	0.005	0.1	109±5.4
3	3.0	0.8	0.04	0.1	0.02	0.01	1.25	0.5	0.02	0.1	84±4.2
4	3.0	0.3	0.04	0.1	0.01	0.02	1.25	0.2	0.02	0.3	89±4.5
5	3.0	0.3	0.1	0.04	0.02	0.02	0.75	0.5	0.02	0.3	33±1.7
6	5.0	0.8	0.04	0.04	0.01	0.02	0.75	0.5	0.02	0.1	61±3
7	3.0	0.8	0.1	0.04	0.02	0.02	1.25	0.2	0.005	0.1	121±6
8	5.0	0.8	0.1	0.04	0.01	0.01	1.25	0.2	0.02	0.3	52±2.6
9	5.0	0.3	0.04	0.04	0.02	0.01	1.25	0.5	0.005	0.3	76±3.8

10	**5.0**	**0.3**	**0.1**	**0.1**	**0.01**	**0.02**	**1.25**	**0.5**	**0.005**	**0.1**	**132**±6.6
11	5.0	0.8	0.04	0.1	0.02	0.02	0.75	0.2	0.005	0.3	53±2.7
12	3.0	0.8	0.1	0.1	0.01	0.01	0.75	0.5	0.005	0.3	69±3.5
% Contribution	1.24	**7.27**	0.67	3.97	2.32	0.03	**15.89**	3.3	**18.82**	**44.6**	
Effect	-6.33	**-15.33**	4.67	11.33	-8.67	1	**22.67**	-10.33	**-24.67**	**-38**	

*GrCf – green chickpea flour

** Experiments were carried out in triplicate along with the type of error analyses (i.e., mean ± SD).

Run number 10 showed a maximum phytase activity of 132 ± 6.6 IU/mL. Regression analysis of the response values obtained for the PBD runs yielded a best fit linear model, viz.,

$$\text{Phytase activity} = 111.09 - 30.67 \times [NaNO_3] + 45.33 \times [\text{GrCf}] - 1644.44 \times [MnSO_4 \cdot H_2O] - 190.0 \times [CaCl_2 \cdot 2H_2O] \quad (3)$$

The suitability of the model was further corroborated by ANOVA tests. Thus, the obtained model F-value of 11.35 implies the model is significant and that there is only a 0.35% chance that the model F-value could occur due to noise. The values of Prob > F was less than 0.05, for the four variables showing their significance. The coefficient of determination R^2 = 0.87 provided a satisfactory measure of the variability in the observed response that could be explained by the model. The Pred R^2 of 0.61 is in reasonable agreement with Adj-R^2 of 0.79. The adeq precision, a measure of the signal-to-noise ratio, is found to have a high value of 9.72 and this indicated the signal strength to be strong. All the above tests confirm that the model Eq. (3) can be used to navigate the design space. In fact, using the above model, it was found that, the above four factors accounted for 86.68% of the total contribution to the estimates of the response values. The remaining variables then accounted for only 13.32% and thus PBD identified them to be less significant. The ANOVA results were complemented by the fact that four out of the 10 factors studied in the PBD, namely, $NaNO_3$, GrCf, $MnSO_4 \cdot H_2O$, and $CaCl_2 \cdot 2H_2O$ were significant on comparing their t-values using a Pareto chart (Fig 2.8).

The maximum contributory factors identified by PBD for phytase production were further optimized by a BBD of experiments (29 runs) generated by DES employing three chosen levels for each variable (Table 2.15).

The optimization studies using BBD for the four significant variables showed remarkably improved phytase activity (160 ± 8.0 IU/ mL) in run number 25 (Table 2.16). The wide variation in activity reported in all the runs bring out the process sensitivity to the experimentally chosen conditions and shows the usefulness of having carried out this systematic optimization study.

Figure 2.8: *Pareto chart showing positive and negative effect of significant factors by PBD analysis*

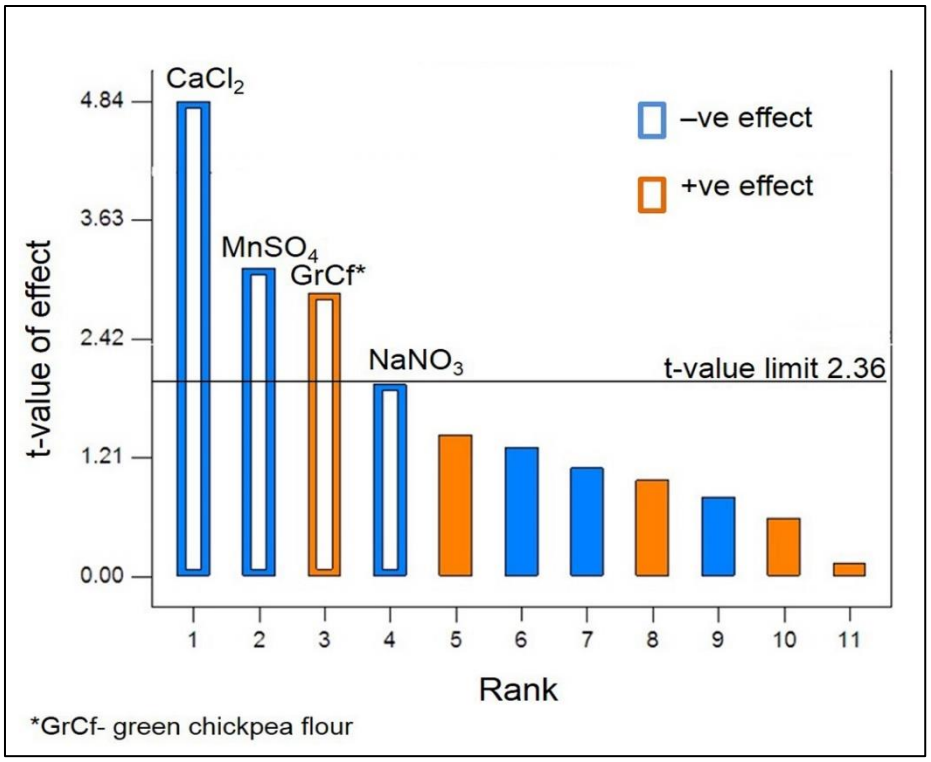


Table 2.15: *Selected variables for BBD and their assigned levels for phytase production*

Variables	Variable code	Units	Levels		
			-1	0	1
$NaNO_3$	A	g	0.200	0.400	0.600
$MnSO_4 \cdot H_2O$	B	g	0.005	0.013	0.020
$CaCl_2 \cdot 2H_2O$	C	g	0.050	1.000	0.150
GrCf*	D	g	1.000	1.500	2.000

*GrCf – green chickpea flour

Table 2.16: *BBD for the selected variables and mean phytase production response values***

Run no.	A $NaNO_3$ g%	B $MnSO_4 \cdot H_2O$ g%	C $CaCl_2 \cdot 2H_2O$ g%	D GrCf* g%	Phytase activity (IU/mL)
1	0.4	0.005	0.05	1.5	82±4.1
2	0.6	0.013	0.05	1.5	67±3.3
3	0.4	0.013	0.05	1.0	116±5.8
4	0.4	0.013	0.15	1.0	114±5.7
5	0.4	0.020	0.05	1.5	90±4.5
6	0.6	0.013	0.15	1.5	70±3.5
7	0.4	0.020	0.10	2.0	5±0.3
8	0.4	0.020	0.15	1.5	85±4.3
9	0.4	0.013	0.10	1.5	53±2.7
10	0.6	0.013	0.10	1.0	123±6.2
11	0.4	0.013	0.05	2.0	6±0.3
12	0.6	0.013	0.10	2.0	7±0.4
13	0.4	0.013	0.10	1.5	70±3.5
14	0.2	0.013	0.15	1.5	92±4.6
15	0.6	0.020	0.10	1.5	125±6.3
16	0.4	0.013	0.10	1.5	117±5.9
17	0.6	0.005	0.10	1.5	92±4.6
18	0.4	0.005	0.10	2.0	7±0.4
19	0.2	0.013	0.05	1.5	95±4.8
20	0.4	0.013	0.10	1.5	112±5.6
21	0.4	0.005	0.10	1.0	142±7.1
22	0.2	0.013	0.10	1.0	127±6.4
23	0.4	0.013	0.10	1.5	155±7.8
24	0.2	0.020	0.10	1.5	140±7
25	**0.4**	**0.020**	**0.10**	**1.0**	**160±8**
26	0.2	0.005	0.10	1.5	134±6.7
27	0.4	0.005	0.15	1.5	56±2.8
28	04	0.013	0.15	2.0	22±1.1
29	0.2	0.013	0.10	2.0	14±0.7

*GrCf – green chickpea flour

** Experiments were carried out in triplicate along with the type of error analyses (i.e., mean ± SD).

The response data of BBD runs was regressed successfully using actual factors and interestingly showed linear dependency without interacting terms, namely,

$$\text{Phytase activity} = 286.74 - 49.17 \times [NaNO_3] - 120.83 \times [\text{GrCf}] \quad (4)$$

The ANOVA analysis (Table 2.17) of the above model satisfied the statistical tests with an obtained model F-value of 27.95 implying that the model is significant with only a 0.01% chance that the model F-value could arise due to noise.

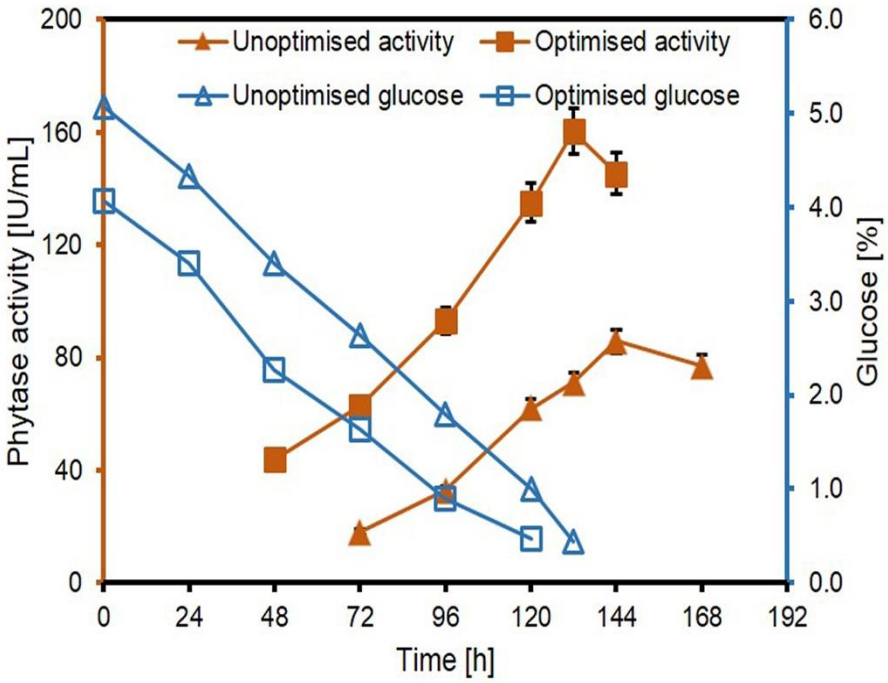
Table 2.17: *ANOVA for the reduced quadratic model*

Source	Sum of squares	Degree of freedom	Mean square	F value	p value Prob > F
Model	44962.42	2	22481.21	27.95	<0.0001
A	1160.33	1	1160.33	1.44	0.2406
D	43802.08	1	43802.03	54.45	<0.0001
Residual	20915.72	26	804.45		
Lack of fit	13568.92	22	616.77	0.34	0.9583
Pure error	7346.80	4	1836.70		
Cor total	65878.14	28			
Coefficient of determination (R^2) 0.68; correlation coefficient (adjusted R^2) 0.66; predicted R^2 0.62; adequate precision 15.4					

The value of correlation coefficient (Pred R^2 = 0.62) for phytase production suggested a good agreement between the observed and model predicted response values. The coefficient of determination (R^2 = 0.68), suggests that 68% of the variability in the data was explained by Eq. 4. The obtained signal-to-noise ratio value of 15.04 brought out the presence of an adequate signal. The final formulation of ten variables in the range studied in PBD and BBD showed that the highest activity of 160 ± 8.0 IU/mL was obtained in 132 h with 100 mL media comprising of (g%): 4.0 glucose; 0.4 $NaNO_3$; 0.075 $MgSO_4 \cdot 7H_2O$; 0.075 KCl; 0.015 $FeSO_4 \cdot 7H_2O$; 0.015 Tween 80; 1.0 GrCf; 0.35 dextrin; 0.02 $MnSO_4 \cdot H_2O$ and 0.1 $CaCl_2 \cdot 2H_2O$.

A time course phytase production using optimized media was carried out, to validate the formulation. The results obtained after showed that, a 1.86 fold enhancement in phytase activity from 86 ± 4.3 IU/mL to 160 ± 8.0 IU/mL with glucose completely utilized in 5.5 days (i.e., 132 h) was achieved by adopting the outlined hybrid media optimization approach (Fig. 2.9). Thus the productivity increased from 14.3 IU/mL/day to 29 IU/mL/day.

Figure 2.9: *Validation of phytase production using RSM optimized media and comparing with the un-optimized media*



The effect of varying the phosphate concentration by addition of sodium phytate and KH_2PO_4 using the optimized media formulation with 1% GrCf was thus studied. A decrease in phytase production to 79 IU/mL on an addition of sodium phytate (0.004 g %) and to 151 IU/mL on an addition of KH_2PO_4 (0.002 g %). This may be compared to phytase activity of 160 IU/mL obtained using the optimized media with 1% GrCf and suggests that it optimally provides the requirements of phosphate.

All the results of experiment no. 3.9 to 3.11 are published in:
Parin C. Shah, V. Ravi Kumar, Syed G. Dastager and Jayant M. Khire (2017): Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides. AMB Express, 7:66. Doi 10.1186/s13568-017-0370-9

4. Conclusions

Phytase is widely used to act on phytic acid for the aim of increasing the bioavailability of phosphorus, proteins and essential minerals in animal diets. It is commercially produced by employing a submerged fermentation process using spore inoculum (Coban and Demirci 2014; Krishna and Nokes 2001). We considered it desirable to carry out studies that use vegetative inoculum for phytase production over spore inoculum. The results obtained suggest that phytase production may, in fact, be enhanced using vegetative inoculum. This result is of considerable significance. Similar results of enhanced phytase production have been observed using vegetative inoculum in solid state fermentation (Krishna and Nokes 2001). Our studies also showed that the use of GrCf as a substrate for phytase production with *A. niger* NCIM 563 gives activity and productivity higher than earlier reports under submerged fermentation conditions using different strains and substrates (Table 2.18).

Table 2.18: *Characteristics of phytases from Aspergillus genera under SmF*

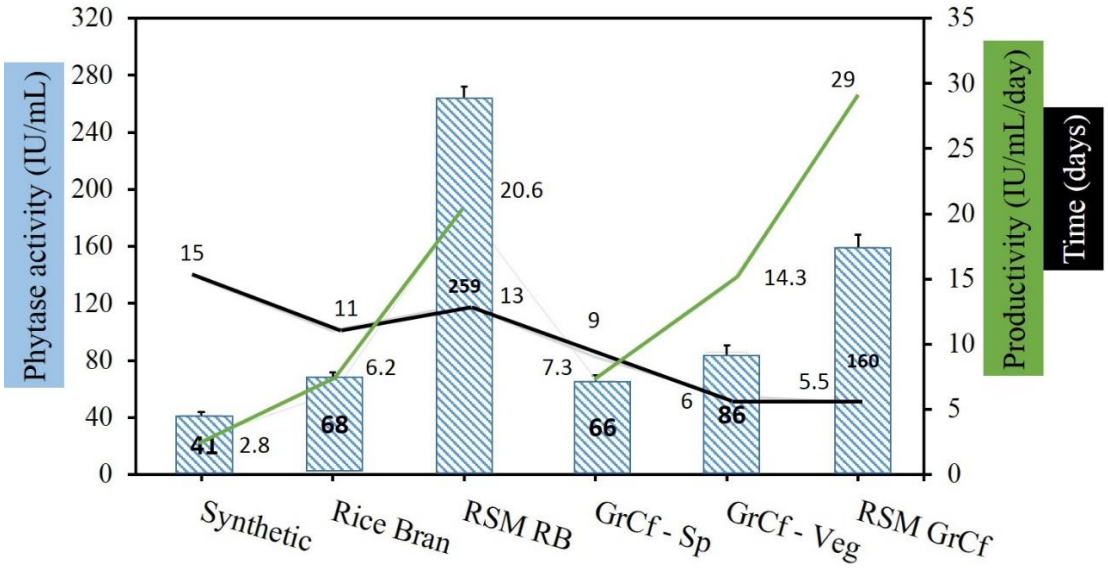
Microbial Strain	Media	Fold Increase after RSM	pH	Temp (°C)	Phytase Activity (IU/ml)	Productivity (IU/ml/day)	Reference
A. fumigatus NF 191	PSM RSM	1.3			101.79	25.4	Gangoliya S S et. al. 2015
A. oryzae SBS 50 (GRAS)	Starch (OVAT)	5.4	5	35	15.7	3.9	Sapna and B. Singh 2013
A. niger NCIM 563	Dextrin		2.5	55	41.47	2.96	Soni and Khire 2007
A. niger NCIM 563	Dextrin		5	55	10.71	0.77	Soni and Khire 2007
A. niger SBS 49	Cane molasses media (OVAT)	23.14	3	50	14.28	7.1	Singh, Kumari et. al. 2015

A. niger CFR 335	Potato dextrose broth				9.6	1.92	Shivanna and Venkateswaran 2014
A. ficuum SGA 01	Potato dextrose broth				8.2	1.64	Shivanna and Venkateswaran 2014
A. ficuum NRRL 3135	MRS medium				2.27	0.45	Coban and Demirci 2014
A. heteromorphus MTCC 10685	Phytase screening medium (RSM)	1.75	6	30	24.88	5	Suman Lata et. al. 2013
A. niger ATCC 9142	corn starch medium		5	65			Casey and Walsh 2003
***A. niger* NCIM 563**	**Rice bran RSM**	**3.74**	**2.5**	**50**	**268**	**20.6**	**Current work**
***A. niger* NCIM 563**	**Green chickpea flour RSM**	**2.42**	**2.5**	**50**	**160**	**29**	**Current work**

Statistical experimentation for media optimization provides a time-saving approach for enhancing phytase production (Bhavsar et al. 2013) and provides the base experimental conditions maintaining/improving the productivity for scale-up. Our results with this approach showed that the phytase productivity obtained using rice bran (6.2 IU/mL/day) to 20.6 IU/mL/day which further improved to 29 IU/mL/day using green chickpea flour (Fig 2.10).

The increase in productivity with the reduction in production time under shake flask conditions may be due to the effective composition and concentration of the media components etc. This observation further supports the biotech potential of the present phytase production process. In conclusion, the current process can be further studied towards scale – up production using fermentor, to have a large volume of phytase to further study its application.

Figure 2.10: *Comparison of productivities using rice bran and green chickpea flour media*



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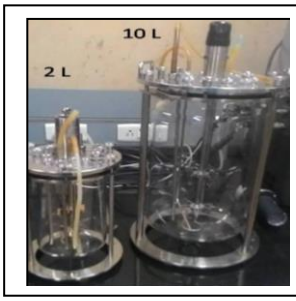


Chapter 3

Scaling up the submerged phytase production using fermentor for large scale production

- Fermentor offers controlled conditions for the production
- It further increases the productivity and pave way for pilot scale production of the industrially important phytase.
- Scale-up studies started with 2 liters phytase production in 3 liters fermentor sized vessel
- Aeration and agitation were optimized for maximum production of phytase
- 0.5 vvm aeration gave maximum production of 159 IU/mL in 5.5 days with initial rpm of 600 using Rushton type agitator
- Further scaling up to 10 liters volume in 14 liters fermentor sized vessel maintaining aeration of 0.5 vvm was studied
- Maximum activity of 164 IU/mL was obtained in 96 hours (4 days) with initial rpm of 500

1. Introduction

Biotechnology has developed rapidly which has influenced many sectors like agriculture, food, fine chemicals, and pharmaceuticals, etc. Industrial processes thus gained importance and need for efficient process arises. Scale-up studies play a very important role in collecting data of bench and pilot scale plants for understanding the process extensively. However, working with microbial culture makes it difficult to generate the data with high reproducibility.

Enzymes have been traditionally used for multiple applications like food, textile, etc., which are of microbial origin and produced by fermentation technology. But, this industrial process didn't seem to start until late 19^{th} century. Large-scale microbial fermentations started to develop from 1930 until 1965, which took pace in production of industrial amylase and proteinase. In 1978, Willy Kunhe, a German chemist coined the term "enzyme" for describing continuous catalysis with cell-free extract, which were the chemical entities from yeast (Godfrey 1997). There are very few reports on scale-up studies for phytase production. Submerged phytase production studies were carried out using 2-L fermentors (Coban and Demirci 2014; 2015), 7-L fermentor (Vats et.al. 2004) and 14-L fermentor (Shah et. al. 2009) scales.

1.1. Scope

A fermentation process starts as a research and development (R&D) project in a laboratory, which is then thought off to be operated for commercial production. This is a very important activity wherein the microorganisms play a very critical role. Microbial fermentation process has become a critical activity to produce industrially important products. Process scale-up using fermentors is a rigorous activity which involves intense R&D and considers many aspects to make it a successful technology.

The process scale-up involves high economic constraints and thus defining the project goals and timeline is very crucial in terms of annual output as per the market demand, productivity of the process, yield as per the unit operations selected, total cycle time and cost of operations and product. Each of this section needs to be optimized for maximum output. There may be other targets to be considered as the process is scaled-up from the laboratory to a pilot scale and further up to production plant (Yang 2010). This chapter talks over goals for microbial fermentation scale-up and plans to execute them.

1.2. Fermentor

The overall success of the process during scale-up studies depends on the effective understanding of the process parameters, inoculum development, etc. Since this is an important industrial technology for commercial production of life-saving and day to day needed products. Many fermentation processes are studied to understand the scale-up process and make it more cost effective and consistent. The entire process is divided into three steps: upstream, fermentation in fermentor and downstream and operated in batch, fed-batch, and continuous mode. Around 10 to 50% of the operating and fixed cost is taken up by the fermentation step, which depends on the product, its concentration, and purity.

Fermentor provides various facilities which help in making the process viable for large scale production. It provides:

- Aseptic operation for a number of days
- Facility for agitation and aeration
- Minimum power consumption
- Control of Process parameters
- Sampling facility
- Minimum evaporation losses
- Simple operation, harvesting, cleaning, and maintenance
- Suitability to variety of processes
- Smooth internal surfaces
- Geometric similarity to pilot plant
- Service provisions: steam, drainage, electricity, water, compressed air

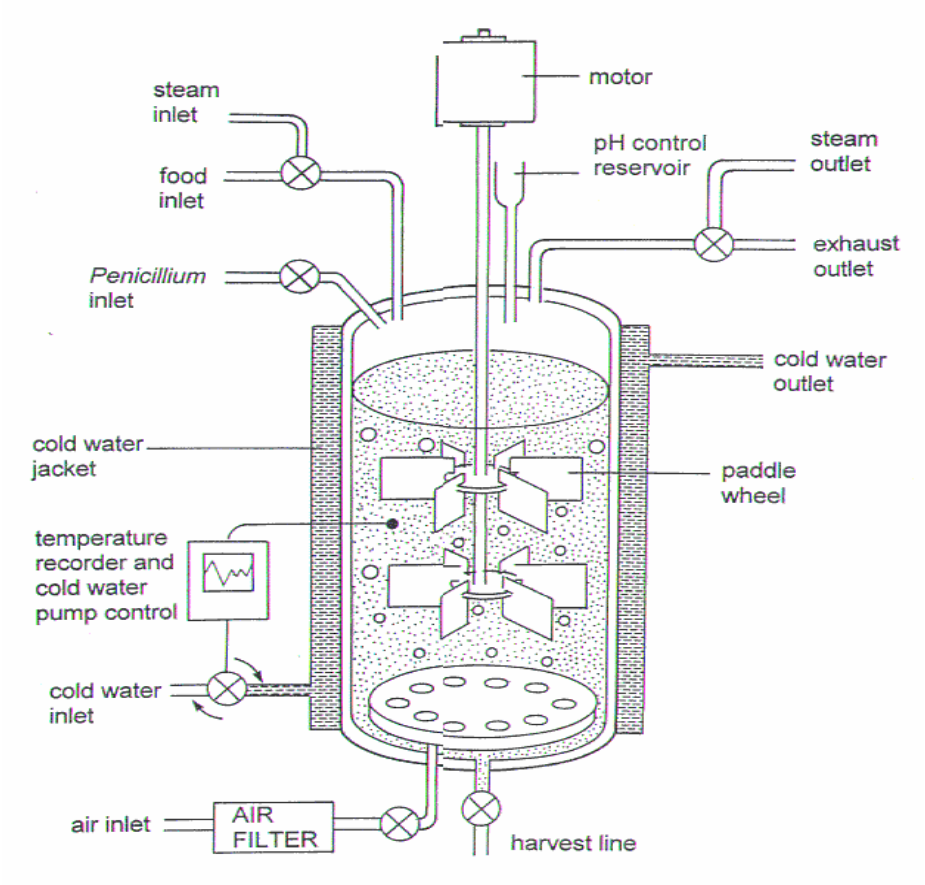
There is no universal fermentor, but it should be designed such to meet the facilities. An ideal fermentor is depicted in Fig 3.1. The body for bench-scale production is constructed of glass with stainless steel top plate to make it airtight. Baffles are provided to avoid vortex formation and effective mixing of the broth. Air sparger is provided for an effective supply of air/oxygen in the system. For increasing the dissolved oxygen level, effective agitation system is provided. This agitation system also helps in an effective mixing of broth and organism making the system homogenous. Agitator are of four classes namely open turbine, disc turbine, vaned disc and marine impeller. Rushton disc turbine impeller is widely used for the microbial fermentation process. Different valves and seals/rings are provided for the effective functioning of the system. There are many types of fermentors viz; stirred tank, airlift, fluidized bed, packed bed,

bubble column, etc. Stirred tank type is of choice for industrial scale production (Fig 3.1).

There are many controlling and monitoring system in the fermentation system, which are an integral part of the high-quality fermentor.

- Temperature is controlled in the system as heat is generated due to microbial activity, using heating jackets externally or heating coils internally. Three types of measuring devices exist *viz;* non-electrical, electrical and radiation type.

Figure 3.1: *An ideal fermentor*



- Gas flow is measured using rotameter and thermal mass flow meter and placed at upstream of the system
- Liquid flow rate is measured using rotameter and controlled using a syringe or peristaltic pump
- Dissolved oxygen is measured as pO_2 using polarographic electrode and many other types of electrodes
- pH is measured using glass or combined electrode.

- ❖ Foam sensors are also available which can be controlled using different chemicals like silicone oil, etc.

1.3.Process scale-up

There are multiple steps which needs to be considered during the scale-up fermentation process which are explained by many researchers in detailed by Yang (2008, 2010); Schmidt (2005); Musoni et. al. (2015); Yang and Cui (2004); Berge et. al. (2008), Garcia-Ochoa and Gomez (2009).

1.3.1. Microbial bank

Scaling-up the fermentation process needs a stable source of microorganism for ensuring consistent production at predicted level over a long term. A common practice followed is by the maintenance of working culture and master culture, the latter being aimed at preservation for an extensive period. Usually the master cultured are lyophilized or stored under -70°C liquid nitrogen or -80°C freezer. The unit size of the storage vial needs to be designed depending on the production inoculation needs it should be regularly checked for stability and quality.

1.3.2. Inoculum seed development

The traditional seed development includes the use of stored vial which undergoes thawing and its inoculation into tube broth. The purpose of this is to develop the cells for inoculation in the production fermentor. The media used for the seed development from the vial is selected to support growth and not product formation. On seed development, the organism is maintained on a simple media which is then used for inoculum development.

1.3.3. Scale-up parameters

Demonstration of production at large scale is the purpose of scale-up, which is studied in detailed at shake flask level. Most of the parameters at shake flask level are quantified at fermentor scale. Thus, the operational parameters and their ranges can be set and the process can be proven with multiple runs to show the consistency and significance. Agitation is the only non-linear parameter which needs to be studied and based on the mixing time of the media and maintaining effective oxygen levels.

Linear scale-up parameters which need to be studied are:

1.3.3.1. pH:

This is one of the important parameters which is either controlled or monitored during the production process. Automated control systems are available for controlled pH operations which use pumps, valves, etc.

1.3.3.2.Temperature:

This is a very critical parameter for maximum production, which is maintained by effective agitation/mixing and heat transfer rate. Challenges arise where there is limiting cooling/heating capacity with effective sensing mechanism and control.

1.3.3.3. Dissolved oxygen (DO) level:

This parameter is also maintained for consistent oxygen availability to the organism. Minimum DO levels are defined for a process for effective oxygen availability. Automated DO control mechanisms are available which can be maintained by air flow rate, agitation rate, and pressure.

1.3.3.4. Air flow rate:

This is calculated based on the preliminary studies for providing a constant air flow rate and determining the production level. It is calculated by diving the constant air flow per fermentation broth volume and is expressed as vvm. VVM is the volume of air flow per min per volume of fermentor broth. For e.g. 2-liters fermentor broth is supplied with 1 liter of air per min is said to have air flow rate of ½ = 0.5 vvm. The airflow rate is measured using a mass flow device, which is calibrated depending upon the atmospheric pressure at the point of action.

1.3.3.5. Final working volume in fermentor:

Usually, 70 – 80 % working volume of the total vessel volume is considered effective for production. This can be varied depending on potential foaming and keeping the media in well-mixed condition

Agitation, air flow rate and temperature control are the three most important parameters which provide an effective and ambient condition for production formation. These are the three parameters which need to be optimized at each level of scale-up.

1.3.4. Process evaluation:

The success of the process depends on the yield obtained on scale-up which needs to be similar to flask level studies. The trend of production and metabolite profiles at different scale should be similar and comparable to flask level. The

process from inoculum development to production needs to be simple and easily executable by the team in the production line. Also, the product quality should be examined at regular intervals to assess the production in progress.

1.3.5. Product formation and storage

The intermediate product formation at the end of fermentation needs to be studied so as to have less product degradation in the fermented broth. This is dependent on the product form and place of production i.e. intracellular or extracellular. Cooling the fermentor can slow down the product loss but this is possible only for fermentor which is of less volume up to 10-liters.

1.3.6. Washing and cleaning fermentor

This the least liked activity but a very crucial step for the success of fermentor batches. This helps in reducing contamination in subsequent batches of fermentation. The person carrying out this activity needs to know how to dismantle and mantle it again after washing and cleaning. The hotspots of microorganism's presence need to be identified and cleaned within the fermentor with utmost care. Usage of effective chemicals needs to be applied for effective cleaning and depending on the fermentor material in use. Thus, a fermentor needs to be cleaned immediately after the run so as to save on time and cost.

2. Fermentor scale production

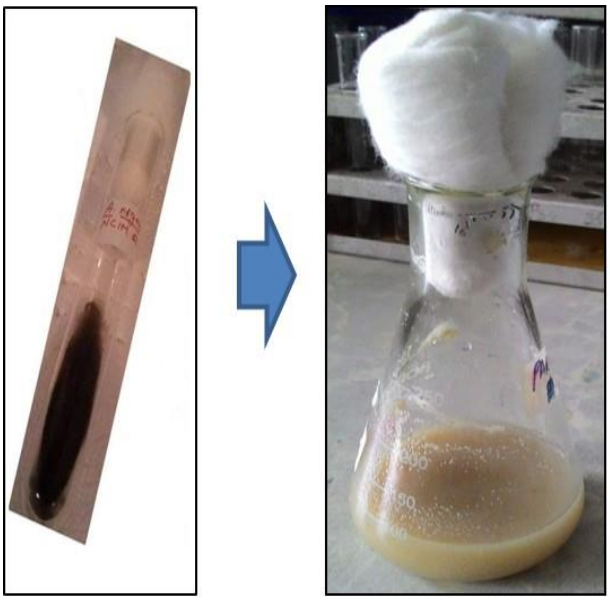
2.1. Inoculum preparation

Aspergillus niger NCIM 563, used in the present study is stored for long term as a lyophilized vial. The vials are revived in potato dextrose broth and then maintained as the stock cultures on Potato Dextrose Agar (PDA) slants and stored at 4^{o}C. Spores from 7 days old freshly prepared PDA slant (Fig 3.2) were harvested for inoculation using a sterile saline solution containing 0.01 % Tween 80.

Inoculum seed development for laboratory scale production is carried using the production media which was optimized by RSM. The media comprised of (g %): 4.0 glucose; 0.4 $NaNO_3$; 0.075 $MgSO_4 \cdot 7H_2O$; 0.075 KCl; 0.015 $FeSO_4 \cdot 7H_2O$; 0.015 Tween 80; 1.0 GrCf; 0.35 dextrin; 0.02 $MnSO_4 \cdot H_2O$ and 0.1 $CaCl_2 \cdot 2H_2O$. Harvested spores (1 x 10^7 spores/mL using Neubauer chamber) are inoculated in 5 mL media and

incubated at 28°C at 200 rpm for 10 h, which were then used to inoculate 95 mL production media. It was incubated at 28°C at 200 rpm for 36 h (Fig 3.2).

Figure 3.2: *Spores developed on PDA slant and vegetative inocula*



2.2. Phytase production at 3-L fermentor scale

3-L fermentor (New Brunswick – NBS – BioFlow 110) with working volume of 2-L was used to study the submerged extracellular phytase production. 180 mL of 36 h vegetative inocula was used to inoculate 1.8-L production media. The fermentor system was equipped with dissolved oxygen (DO) probe (polarographic electrode – Mettler-Toledo), pH electrode and Rushton-type impeller fitted with six blades. Four peristaltic pumps were plugged for acid, base, antifoam and feed mode. Agitation was controlled by PID. The aeration system was an air inlet through a sparger with air-flow meter and filter. Silicone oil was used as antifoam agent. The initial agitation was set at 250 rpm and DO was maintained at 100 %. The effect of aeration and pH on phytase production. Fermentation was carried out with sampling at regular intervals. The arrangement of fermentor is seen as in Fig 3.3.

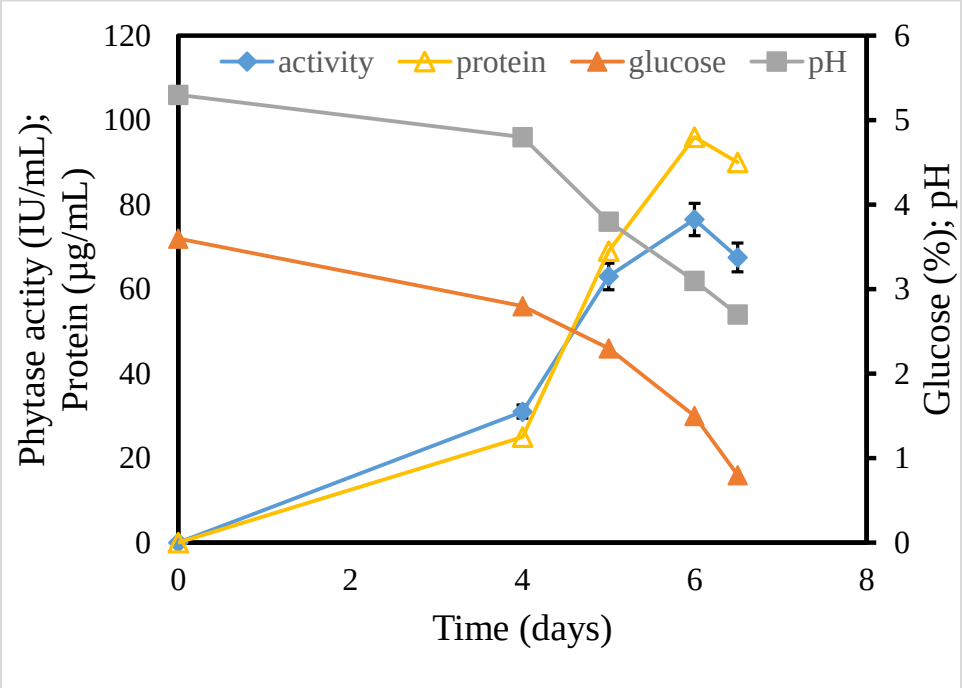
Figure 3.3: *Three liters fermentor in working condition*

2.2.1. Studies at 3-L scale

Multiple runs at 3-L scale were carried out to understand the process of phytase production. The conditions set prior to studies conducted were as follows: temperature 28°C, agitation starting at 250 rpm, 0.2 vvm air flow rate and maximum DO set at 100%. Regular sampling was carried out to calculate phytase activity and glucose depletion rate. It was observed (Fig 3.4) from the preliminary runs that, pH gradually decreased from initial pH of 5.5 to 2.7. Glucose depletion rate was gradual, with a gradual increase in phytase production. The agitation rate was increased gradually to maintain minimum DO of 20%. But the phytase production was far too less than that obtained in shake flask experiments. Thus optimization of parameters was required to enhance the production.

Studies using different airflow rate was studied viz; 0.2 and 0.5 vvm. The increase in phytase production was observed at 0.5 vvm. For the present studies, we chose to keep the aeration rate constant at 0.5 vvm while varying the initial agitation speeds (400, 500, 600 rpm).

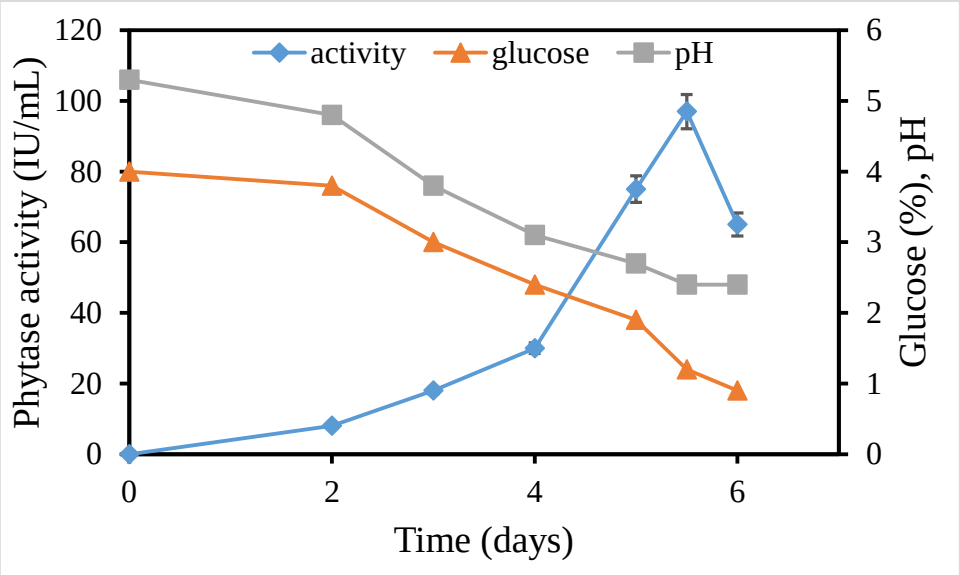
Figure 3.4: *Preliminary phytase production at two liters working volume scale*



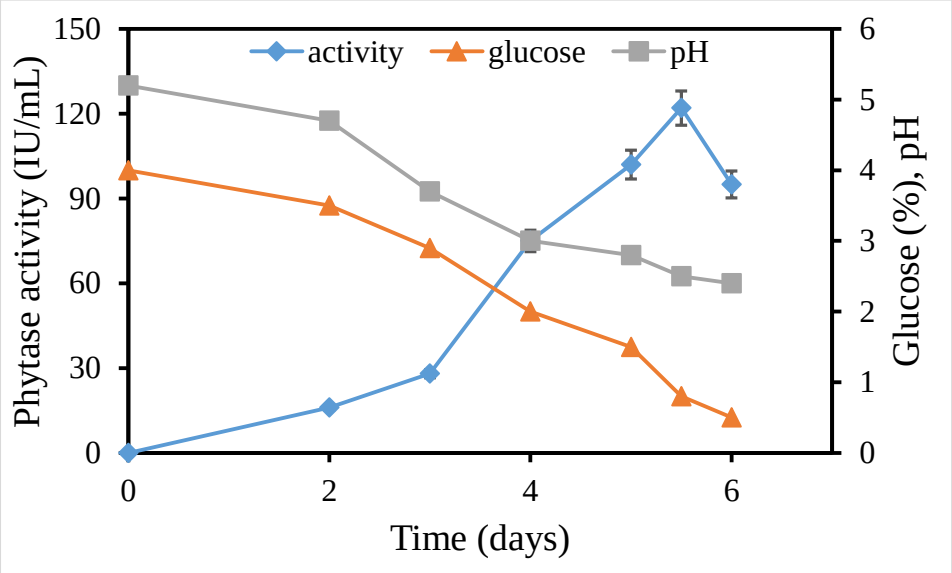
The effect of initial agitation rate was also studied which showed a significant increase in phytase production (Fig 3.5 a-c). The behavior in time of DO and pH were simultaneously monitored. For the 400 rpm run, a decrease in DO was observed till 36 h. 50% DO was maintained by a gradual increase in rpm and it was thus maintained at the higher rpm. The pH of the media gradually decreased from 5.0 to 2.3. For the 2-L batch study with an initial rpm of 400, the maximum activity of 97 ± 4.8 IU/mL was obtained in 132 h. Phytase production of 122 ± 6.1 IU/mL and 158 ± 7.9 IU/mL was achieved at initial rpm of 500 and 600 respectively, in 132 h. Thus, phytase production was successfully scaled up to 2-L production from the shake flask experiment. Successful production at 2-L working volume, paved way to study production at 10-Lworking volume to bring out the process biotech potential.

Figure 3.5: *Effect of agitation on phytase production*

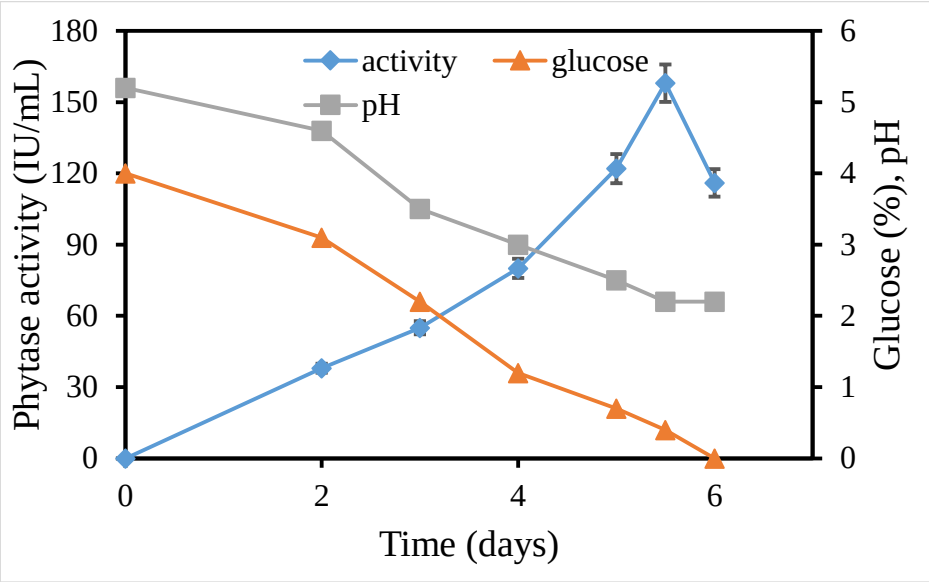
a) 400 rpm



b) 500 rpm



c) 600 rpm



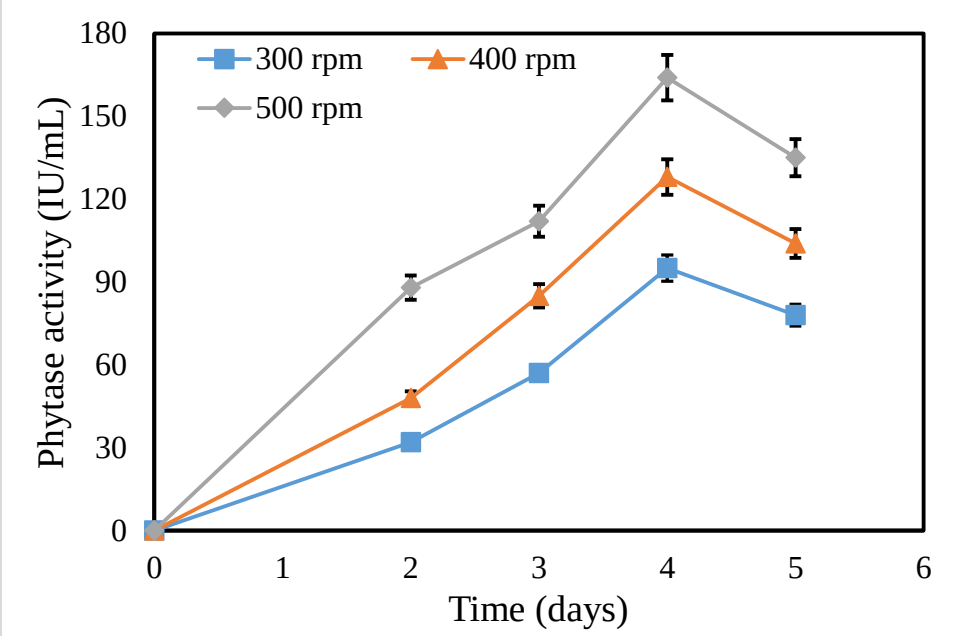
2.3. Studies at 14-L scale

Multiple runs at 14-L scale with working volume of 10-L were carried out to understand the process of phytase production at this scale (Fig 3.6).

The fermentor containing 9-L medium that was earlier sterilized in situ was inoculated with 1-L 36 h old Ve-I. The conditions set prior to studies conducted were as follows: temperature 28°C, agitation starting at 300 rpm, 0.5 vvm air flow rate and maximum DO set at 100%. Regular sampling was carried out to calculate phytase activity and glucose depletion rate. It was observed from the preliminary runs that, pH gradually decreased from initial pH of 5.5 to 2.0 as observed at 2-L scale, but at a faster rate. Glucose depletion rate was also gradual, with a gradual increase in phytase production and agitation rate to maintain minimum DO of 20%. But the phytase production was far too less than that obtained in shake flask and 2-L scale experiments. Thus optimization of parameters was required to enhance the production. Similar to studies in the 2-L scale and varying the initial agitation speed (400, 500 and 600 rpm), the DO and pH were monitored in 10-L scale. DO and pH pattern was again observed, but with higher decreasing rates than in the 2-L scale (Fig 3.7).

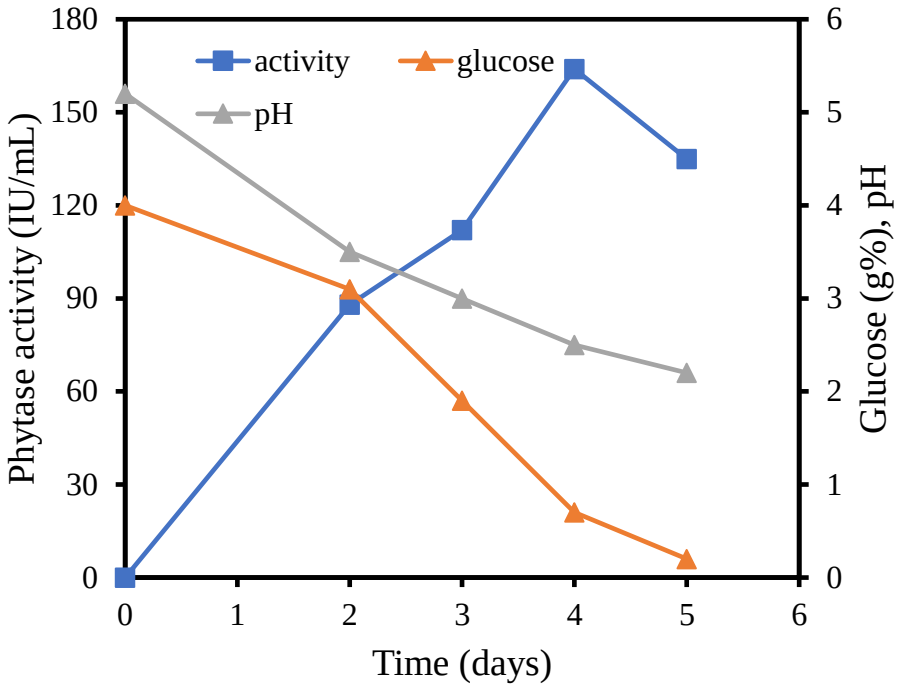
Figure 3.6: *Fourteen liters fermentor in working condition*

Figure 3.7: *Effect of agitation on phytase production at ten liters working volume scale*



Remarkably, the maximum phytase activity of 164 ± 8.2 IU/mL was maintained but it is important to note that the maximum activity was obtained in a much shorter time of 96 h at 500 rpm (Fig 3.8).

Figure 3.8: *Maximum phytase production at ten liters scale*



Our results with this approach showed that the phytase productivity obtained in a 10-L fermentor working volume (41.0 IU/mL/day) was improved by a factor of 2.87 and 1.41 times from shake flask (14.3 IU/mL/day) and 2-L scale (29 IU/mL/day) experiments, respectively. The increase in productivity with a reduction in production time at 10-L fermenter scale may be due to the better maintenance of fermentation parameters viz., agitation, temperature, aeration, etc.

This observation further supports the biotech potential of the present phytase production process. Studies of submerged phytase production using *S. thermophile* in cane molasses medium showed that the productivity obtained in the 10-L working volume fermenter (5.2 IU/mL/day) was improved by a factor of 2.2 times from shake flask (2.5 IU/mL/day) experiments and having optimum activity at pH 5.5 and 45 °C (Singh and Satyanarayana 2008). A similar trend of increased productivity by 1.5 times for glucoamylase production was observed by Kumar et al. (2007).

2.4. Fermentor termination

The biomass on fermentor termination is allowed to settle down and passed on for centrifugation for its separation from fermentor broth which has the enzyme phytase (Fig 3.9). Approximately 1.5-L and 8-L fermentor broth is obtained from 2-L and 10-L working volume fermentor on harvesting.

Figure 3.9: *Centrifugation for biomass separation after production in fermentor*

The enzyme supernatant is processed immediately or stored at 4°C until further processing. Thus, the present study shows an efficient process for phytase production and provide need to develop an efficient downstream process.

All the results of experiments mentioned in this chapter are published in:
Parin C. Shah, V. Ravi Kumar, Syed G. Dastager and Jayant M. Khire (2017): Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides. AMB Express, 7:66. Doi 10.1186/s13568-017-0370-9

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Chapter 4

Downstream processing, formulation and biomass reutilization

- Downstream process (DSP) is important in product and process development and should be cost effective and easily scalable.
- Membrane technology has been effectively developed for DSP and applied here to obtained 92% phytase recovery
- Simultaneous concentration and purification was possible using ultrafiltration
- Formulation studies designed depending on the application and characteristics of phytase
- Biomass generated as byproduct of fermentation is rich source of chitin
- Chitin can be converted to chitosan having important application in pharmaceuticals and is thus performed here.
- Thus, a cost effective green process is developed for phytase DSP and formulation with biomass reutilization

1. Introduction

1.1. Downstream processing

Manufacturing of biologically important product is divided into two stages. The first stage (upstream) is the microbial production of a target compound, which is then extracted and purified in the second stage (downstream). Thus, the initial stage is based on the cell density and division rate while the stage later is no longer dependent on biological parameters. Thus, the fate of the product in downstream process (DSP) is solely dependent on chemical and physical parameters (Ersson et.al. 2011)

The DSP is not a single step process as the complex product is formed after fermentation. The desired compound needs to be separated selectively from the complex entity. Thus, DSP involves multiple steps like harvesting, clarification, capturing, and polishing, which makes the product pure and homogenous after each step using more than 40 – 60 % of the total product development cost. Laboratory studies of DSP are of low cost due to small scale, but additional challenges arise as the process is scaled-up. So, large scale process needs to be of minimal steps and it would be advantageous to consider this point during lab scale studies. Also, strategies should be developed during DSP with respect to the product formation viz; intra or extracellular, size, solubility, etc., so as to speed up the DSP and get maximum purified product with high recovery (Ersson et.al. 2011).

Harvesting involves separation of material with desired product from the complex material generated after the production process. For intracellular desired product, biomass is separated from the broth, latter being discarded and vice versa for extracellular products (Shukla and Kandula 2009). Clarification is mostly integrated with harvesting to remove bulk impurities generated mostly after cell disruption for an intracellular product (Su et.al. 2011). Depending on the need for the level of purification, the desired product can be locked using ligand in the chromatographic procedure (Vunnum et.al. 2009) which is then released off or a reverse process where contaminants are separated by binding them to ligands and product is allowed to pass through the column. The second way described is called polishing (Ghose et.al. 2009).

Centrifugation and microfiltration are widely used clarification process.

1.2. Centrifugation:

It is a unit operation mechanical process utilizing centrifugal force applied for separation of cell debris and precipitates. Dissolved macromolecules can also be separated by ultracentrifugation, which is an expensive process on scaling up and hence

ultrafiltration is used for large scale operations. Centrifugation fails to remove small particles and thus is used in combination with filtration system (Middleberg 2000)

1.3. Microfiltration:

This process operates at low temperature and pressure, with pore size ranging from 0.1 – 10 µm requiring no mechanical process. The crude fermentation broth is forced through a semipermeable membrane with a certain molecular weight cut off range such that desired molecule is retained as retentant and undesired molecules pass through the membrane as filtrate/permeate. It is used upstream of the chromatographic process as a prefilter to avoid fouling of column (Walter et.al. 2011).

1.4. Aqueous two-phase extraction

Liquid-liquid partitioning using the principle of differential solubility is applied with the use of aqueous polymers and salt solution. This is a two-phase partitioning process where cell debris separate in an aqueous phase, impurities and nucleic acid at interphase which is removed by centrifugation and desired protein tend to be in polymer phase. This process gives high recovery of the product (Banik et.al. 2003).

1.5. Chromatographic process

This is also a process where components in the mixture are separated by two phase having differential affinities. This process considers the physical and chemical properties of the biological entity. This a very effective process at laboratory scale studies. At the industrial scale, a technique is suitable only which can be scalable. The major disadvantage of this process is cleaning and validation. This process is time consuming and expensive (Janson 2011; Gottschalk 2008).

1.6. Ultrafiltration (UF)

As described in 1.1.2. above, microfiltration is widely used as clarification process. UF is a modern protein purification process which can be used as a final step in the formulation of protein. Two modes of filtration are considered viz; dead end filtration (normal flow filtration), where the feed stream passes on to the filter perpendicularly and cause blockage as the process progresses. The second mode is tangential flow filtration (TFF) (cross-flow filtration), where the feed stream passes parallel to the membrane causing fewer chances of blockage. TFF is widely used industrial process working in pore size range of 10 – 100 nm (Walter et. al. 2011; Zydney and Kuriyel 2000). Conventional TFF needs usage of UF skid, large pump and recirculation tank, which requires space. A new technology of single-pass TFF (SPTFF) is emerging where recirculation doesn't need loop and tank (De Los Reyes 2008).

Few process operations are key to cost effective optimal process which should be ordered as consecutive separation steps exploiting different principles of separation. However optimizing individual step would decrease overall production efficiency. Modeling techniques can be used for designing and developing a robust efficient and simple process to overcome existing bottlenecks. Validation of DSP is also a crucial step after usage of membrane-based system (Michaels 1991; Millipore 1990) or chromatographic procedure (PDA 1992). Kuwahara and Chuan (1995) has extensively published a review on process validation.

The downstream process using chromatographic methods has multiple steps resulting in low desired purified product recovery and difficult for handling large volumes (Monteiro et.al. 2015; Shimizu 1992). The cost of substrate and downstream process cause hindrance in commercial production of phytases (Shivange et.al. 2012). Thus, there is a need to have minimum step downstream processing with maximum recovery.

1.2. Formulation

Once the enzyme is concentrated and purified to the desired level depending on the application, with maximum recovery using an appropriate DSP method/s, the next most important objective is the stability of the enzyme. It needs to be retained for a prolonged period to develop as a product. The freshly prepared enzyme will have a very high overall activity which decreases as time passes. Thus, to sell an industrial product, the enzyme should have a storage life that is guaranteed.

Thus, a manufacturer uses all types of substances to maintain a high stability of enzyme which is confidential and which gives a competitive edge over other products. Thus, enzyme formulation is very crucial and skilled activity which provides maximum revenue from the product. The three-dimensional structure of the enzyme needs to be maintained to prevent itself from denaturation. This can be maintained using additives or by immobilizing the enzyme which protects it and helps in its slow release at the site of action or in the vicinity. Thus, formulation product can be developed in solid or liquid form (Chaplin 2014).

The majority of the commercially available phytases are in liquid form and few in solid form (Menezes-Blackburn et. al. 2015). As per the need phytase is available as granules, spray dried powder, coated granules, immobilized enzymes and liquid formulations with additives to avoid microbial contamination and physical stability

(Novozymes 2017). Depending upon the application, the qualities are imparted to the enzyme based product like thermostability, etc.

1.3. Biomass utilization

Aspergillus niger is widely used for citric acid and various industrial enzyme production (Usami 1978; Pyc et.al. 2003). It has a well-evolved enzymatic system and thus can utilize various substrates. After the citric acid production, the biomass is generated in large quantities as a byproduct and thus utilizing it would be of great worth. Thus, cell wall analysis has been carried out by Johnston (1965), so as to design ways to utilize it.

The fungal biomass can be utilized for production of chitin which is second abundant bio-polymer in nature after cellulose and found in the exoskeleton of invertebrates and cell walls of fungi (Lower 1984; Tan 1996). Chitin can be used for the production of chitosan which is deacetylate form of chitin and glucosamine which is a monomer of chitosan. Commercially, chitin, chitosan, and glucosamine is produced using crab shells and shrimp from crustaceans (Khora and Yong 2003). Chitosan has found various applications in pharmaceuticals and food sector (Majeti and Kumar 2000). The biomass has been applied in various sectors like dyes removal in wastewater and trace element bio-sorption reviewed by Shahlaeia and Pourhossein (2013).

Thus, a cost effective, minimum step DSP, generating biomass and fermented broth containing phytase can be completely utilized making the process greener.

2. Downstream processing of phytase from *A.niger* NCIM 563

After a successful production of phytase at 10-L fermentor scale and separation of biomass after terminating the production process by centrifugation, generates fermented broth containing extracellular phytase. This broth needs to be processed such that, handling becomes easy and maximally stabilized with effectively produced activity. Centrifugal separation of biomass fails to settle fine particles which need to be removed for further processing of the enzyme.

Microfiltration plays a very important role which helps in easy removal of these fine particles without mechanical process. It operates on pressure phenomenon. The filtration capacity is expressed as flux which is the volume of the permeate passing through the membrane area per unit time ($L/m^2/h$). The driving force for the flux is the difference in pressure between feed and permeate sides and is known as transmembrane pressure. Viscosity and hydraulic resistance decrease flux (Walter et. al. 2011).

2.1. Microfiltration (MF) of phytase

The fermented broth (200 mL) containing phytase (150 IU/mL; specific activity 50 IU/mg) was passed through ultrafiltration process using 10 kDa cut-off membrane (PALL - Minimate TFF cassette system). This operation removes all the fine particles and salts from the broth along with excess water. For effective removal, the concentrated retentate (20 mL) was diluted with demineralized water which was concentrated again. Polyether sulfone membrane is used in the cassette, with an effective filtration area of 50 cm^2. The feed flow rate is 2 mL/min at a flux of 50 $L/m^2/h$ and maximum operating pressure of 2 psi. The concentration factor was calculated as the ratio of feed volume at the beginning of the operation V_{feed} and retentant volume V_{ret}.

2.1.1. Results and Discussion

The volume containing phytase was concentrated 1.5-fold by MF (Table 4.1).

Table 4.1: *Feed and retentant phytase activity and specific activity after MF (10kDa)*

Sample	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Recovery (%)	Fold purification
Crude	30,000	550	54.5	--	1
UF concentrated	28,500	349	81.75	95	1.5

The volumetric concentration factor was 10-fold. No phytase activity was detected in permeate and decrease in the activity may be due to shear stress. After the

clarification step, phytase is further purified, so as to have more concentrated product requiring less dosage of application and small unit size for storage.

2.2. Partial purification of phytase

2.2.1. Chromatography

The chromatographic procedure has been used widely at industrial scale for protein purification. However, this is a costly and time-consuming step (Soni et.al. 2010). Phytase after microfiltration is purified and concentrated using a multistep chromatographic procedure.

Step 1 - Crude enzyme obtained after separating the biomass by centrifugation and microfiltration was subjected to ammonium sulphate precipitation (95% saturation) with constant stirring. The precipitate was collected by centrifugation (5000 × g, 20 min) and dissolved in minimum volume of 50 mM sodium acetate buffer, pH 5.5.

Step 2 - The enzyme was desalted by passing it through Sephadex G-25 column and fractions were estimated for phytase activity and dialyzed overnight at 4°C against the same buffer.

Step 3 - The dialyzed solution was subjected to hydrophobic column chromatography using Phenyl - Sepharose CL-4B (30 ml bed volume), previously equilibrated with 30% ammonium sulphate in 20 mM acetate buffer, pH 2.5. The column was washed thoroughly with 20-bed volumes of the above buffer and eluted with a 120 ml linear decreasing gradient of ammonium sulphate (30–0%) with a flow rate of 20 ml/h and approximately 3 ml fractions were collected. Fractions showing activity at pH 2.5 were pooled and estimated for phytase activity.

Step 4- The fractions were pooled and loaded on a Sephacryl S-200 gel filtration column with a flow rate of 12 ml/h and 2 ml fractions were collected. All the purification procedures were carried out at 4°C.

2.2.1.1. Results and Discussion

There are multiple steps using a chromatographic procedure. Loss occurs at each step and needs standardization and validation. Also, it is very difficult to maintain the operating conditions. The bottleneck of this method is handling large volumes of phytase broth. This method of purification can be applied when a very high purity of phytase is required. This method would be beneficial for application of phytase in pharmaceuticals. An alternative, less time consuming, cost effective method need to be designed which can give high recovery of an enzyme for its application in animal feed,

food, etc. where partially purified phytase can be used. Table 4.2 shows the specific activity, phytase recovery, yield and fold purification after using chromatographic steps.

Table 4.2: *Purification chart using chromatography*

Purification Steps	Total Activity (Units)	Total protein (mg)	Specific Activity (Units /mg)	Purification (fold)	Recovery (%)
Crude culture filtrate	50350	240	210	1	100
Phenyl Sepharose CL – 4B (5%)	30986	19	1631	7.77	61.5
Sephacryl S - 200	14800	5.1	2902	13.82	29

2.2.2. Ultrafiltration (UF) for phytase purification

Aqueous two-phase system (ATPS) method has been recently developed for protein purification and concentration (Bhavsar et. al. 2012). It gives very high recovery (but it involves a repeated separation operation strategy that uses hazardous polyethylene glycol together with salt (Goja et. al. 2013). Thus, in the current study use of ultrafiltration was considered for the first time for protein purification.

Ultrafiltration is an attractive technique for enzyme separation especially because of the high separation factor achieved with cost effectiveness (Sariyska et al. 2014; Wu et al. 2007). In UF by selecting a membrane with a suitable enzyme molecular weight cut-off, only undesirable impurities (mainly lower molecular weight solutes) are allowed to pass through while concentrating the enzyme in the solution. Also, the UF separation process offers important benefits by allowing operation in a continuous fashion and realize high throughputs with superior product quality.

Studies on phytase from *A.niger* NCIM 563 by Soni et. al. (2010), showed that it is a bulky molecule with high molecular weight of 264 kDa. Thus, using multiple molecular weight cut-off membrane can help in getting purified product. Thus, studies of phytase purification and concentration were carried out using 50 kDa, 100 kDa, 150 kDa cut-off, with the aim to get maximum recovery of phytase with high molecular weight cut off membrane, so as to remove maximum impurities. Samples were withdrawn after UF and were checked for phytase activity and total protein.

2.2.2.1. Results and Discussion

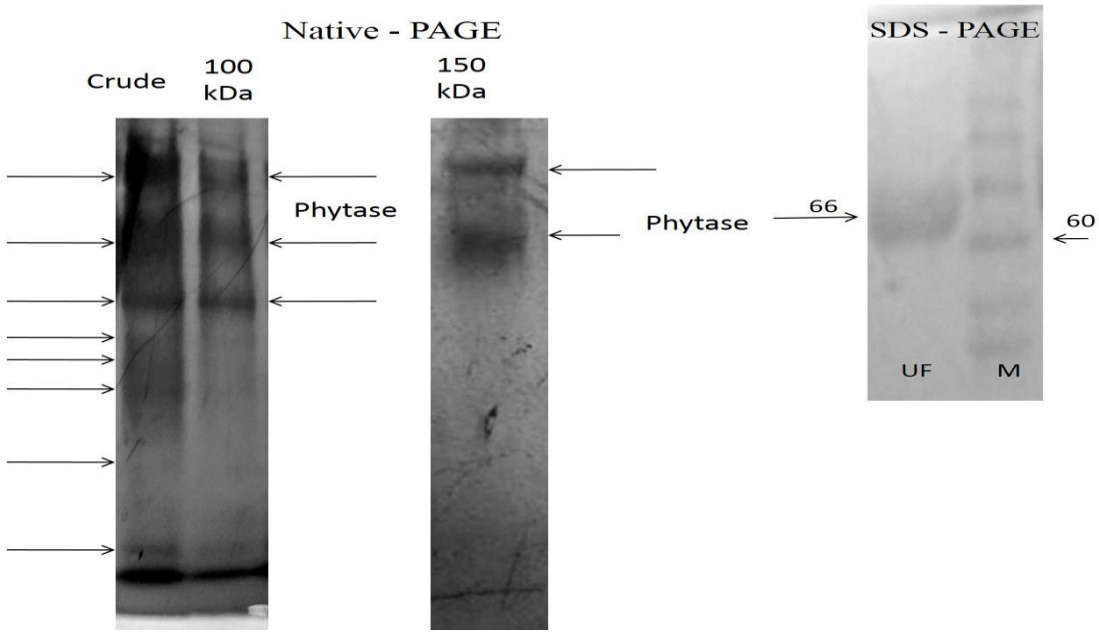
The aim after the production using fermentor was to have an efficient and easily scalable process for downstream operation than chromatography, which can handle large volumes of broth. UF has proved to be time and cost saving process than chromatography and safe/green and high recovery process than ATPS. High recovery of phytase (92 %) is obtained using 150 kDa cut-off membrane (Table 4.3). This method of down streaming can be easily made in line process, which can be used continuously and can handle large volumes of broth.

Table 4.3: *Purification chart using UF (150kDa)*

Purification Steps	Total Activity (Units)	Total protein (mg)	Specific Activity (Units /mg)	Fold Purification	Recovery (%)
Crude culture filtrate	50350	950	53	1	100
After UF	46624	338	138	2.6	92

An additional advantage of using UF, is it can simultaneously concentrate and partially purify the enzyme, with no pre-treatment and in a single step as confirmed by SDS – PAGE (Fig. 4.1). This seems to be a very cost saving method for large scale operations.

Figure 4.1: SDS – PAGE of phytase after ultrafiltration



The other known methods studied for the recovery and purification of phytase from different sources are compiled by Bhavsar et. al. (2012). The chromatography method is seen to be less suitable for downstream processing of phytase and improvements are especially needed with respect to yield, purity and energy consumption. In comparison, the current method of UF seems to be a simple and efficient process and can be further improved.

The majority of reports use chromatography and ATPS method for enzyme purification. This is the first study, where high molecular weight cut off UF can be directly used for protein concentration and partial purification for large volumes of broth. Thus, in a single step using UF, maximum phytase can be recovered which will be advantageous for the application.

3. Formulation of phytase

After DSP is the formulation step, wherein the phytase needs to be stored in active conditions.

3.1. Carrier effect:

To evaluate the stability of phytase in powder form, the concentrated enzyme was mixed with various carriers, dried to obtain free flowing material and activity was monitored. Thus, 4 ml concentrated enzyme (232 IU) was mixed with 10 g sterilized carrier like wheat bran, rice bran, calcium carbonate and silica and dried at 50°C for 2 h (moisture content < 4 %). Samples were removed after various time intervals and evaluated for phytase activity.

3.1.1. Results and Discussion

Among various carriers evaluated wheat bran and rice bran were found to retain total phytase activity in dry free flowing form. However, calcium carbonate and silica gel powders were found to loose phytase activity very rapidly (Table 4.4).

Table 4.4: *Effect of carrier on phytase*

Carrier	**Residual phytase activity (%)**		
	0h	**7th day**	**15th day**

Wheat bran	100	100 ± 3.0	100 ± 3.0
Rice bran	100	81 ± 3.0	68 ± 4.0
Calcium carbonate	100	32 ± 4.0	27 ± 4.0
Silica	97	11 ± 5.0	7 ± 5.0

Thus concentrated liquid phytase solution can be mixed with either wheat bran or rice bran and dried at 50°C for 2 h to remove the moisture. This method is more suitable and cost effective than the existing commercial phytase which is generally available in granular or powder form.

3.2. Additive effect:

Phytase is applied in mash feed and pelleted feed. So stability of phytase enzyme in liquid form was evaluated supplemented with various additives like solvents, antibiotics, formalin, glycine and sodium chloride for different time intervals at room temperature and 8–10°C. Effect of various additives in the liquid enzyme indicates that enzyme preparation can be stabilized in liquid form before mixing into carrier for its easy application.

3.2.1. Results and Discussion

Phytase retains total activity without any contamination in presence of 50% methanol, ethanol, chloroform and acetone at room temperature and at 8–10°C (Fig. 4.2 - a, b). Effect of various reagents indicates that fermented broth retains its total activity at room temperature by the addition of penicillin and bavistin (0.1%), formalin (0.2%), sodium chloride (10%) and glycine (1 M) (Fig. 4.2 - a). Its, stability can be extended up to 15 days by storage at 8–10°C using the same additives (Fig. 4.2 – b). Use of antimicrobial agents and sodium chloride has been reported to avoid contamination in fermented broth and stabilization of alkaline protease activity from *Conidiobolous coronatus* (Laxman et. al. 2005).

Figure 4.2 – a: *Stability of phytase at room temperature*

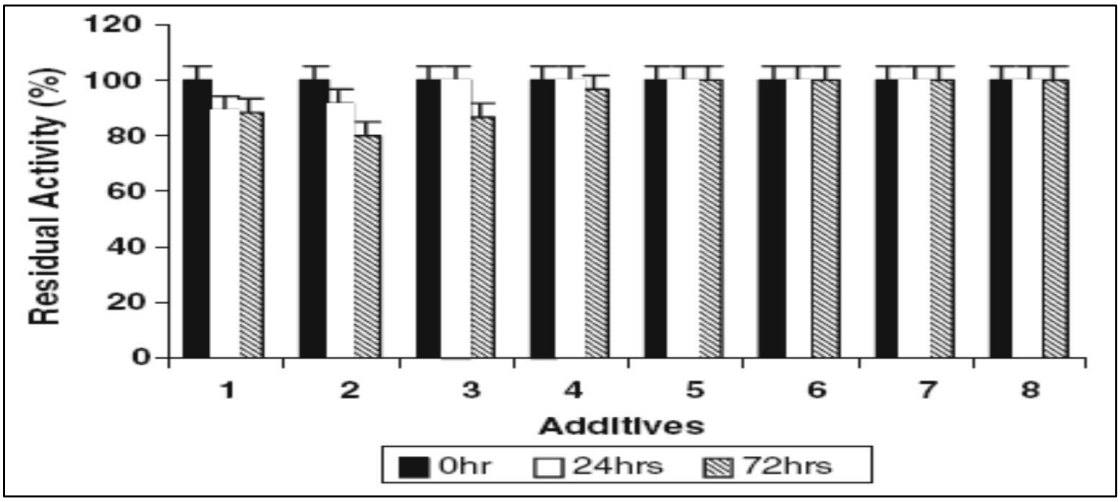
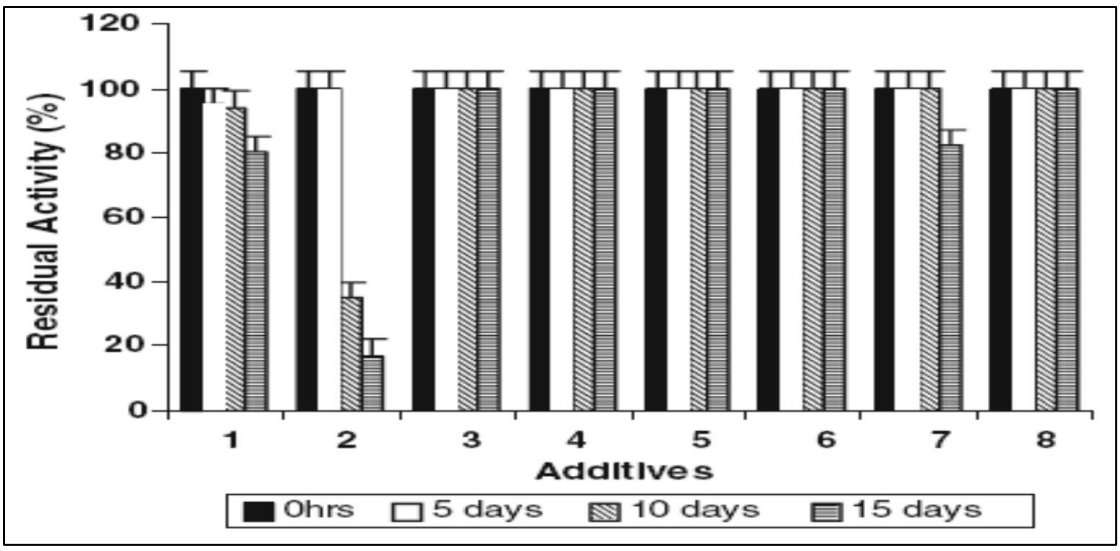


Figure 4.2– b: *Stability of phytase at 8 - 10°C*



1. Chloroform (50%), 2. Acetone (50%), 3. Methanol (50%), 4. Ethanol (50%),
5. Bavistin (0.1%) 6. Penicillin (0.1%), 7. Formalin (0.2%), 8. NaCl (10%)

Lyophilization was also carried out which showed 100% activity retained. Thus phytase can be effectively stored maintaining its activity.

4. Fungal biomass utilization

The washed mycelia (free from growth medium) from SmF were dried at 50°C until constant weight. 100 g of dried mycelia were autoclaved at 121°C for 40 min after homogenizing in a blender with 1 N NaOH solution, (1:30 w/v) (alkali treatment). The alkali insoluble material (AIM) fractions were recovered after centrifugation at 6000

rpm, washed with distilled water several times and centrifuged until a neutral condition was obtained (pH 7). The AIMs were dried in an oven at 40°C. Dried AIM was treated with acetic acid 2 % (v/v) as a chitosan solvent under reflux conditions for 8 hrs at 95°C (1:30 w/v). Afterward, by centrifugation, the acid insoluble fraction was precipitated at 6000 rpm for 15-20 min and the supernatant containing the chitosan was isolated. To precipitate fungal chitosan, the pH was adjusted with a 2 N NaOH solution, and the flocculated chitosan was centrifuged at 6000 rpm for 15 min. The isolated chitosan was washed four to five times with distilled water to neutralize. At the same time, ethanol (96 %) was employed to rinse the chitosan, which was then dried in a vacuum oven dryer at 60°C (Niederhofer and Muller 2004; Guha et. al. 2005; Nyein et. al. 2006) The IR spectrum of chitosan was carried out using the KBr disc method in a FTIR spectrophotometer.

4.1. Results and Discussion

The yield of the chitosan production from *A. niger* NCIM 563 was 50 mg per 100 gm of biomass (Fig 4.3). The chitosan was confirmed by FTIR (Fig 4.4). The spectrum is taken in the range from 4000 to 500 cm^{-1}. The extracted chitosan is shown in red spectra and standard chitosan from Sigma is shown in blue colour. The extracted chitosan has a characteristic band for chitosan (Wanule et. al. 2014).

The spectra showing a peak at 3460 cm^{-1} indicate symmetric stretching vibration of OH and amine. The absorption peak at 2923.62 cm^{-1} indicates the presence of CH stretch. The absorption band at 1621.20 was due to C=O stretching (amide I), 1419.09 is C-H deformation vibration and 1057.38 is the peak for C-O stretching. The peak at 870.13 is a ring stretching a characteristic bond for β-1, 4 glycosidic linkage. Thus, confirming the presence of chitosan.

Thus, a greener process is developed wherein the enzyme along with the biomass generated as the byproduct of fermentation is utilized for production of value added product.

Figure 4.3: *Extracted chitosan from biomass*

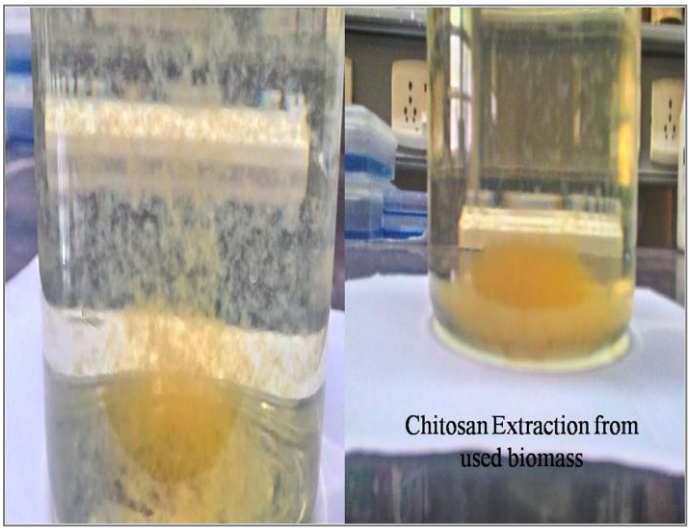
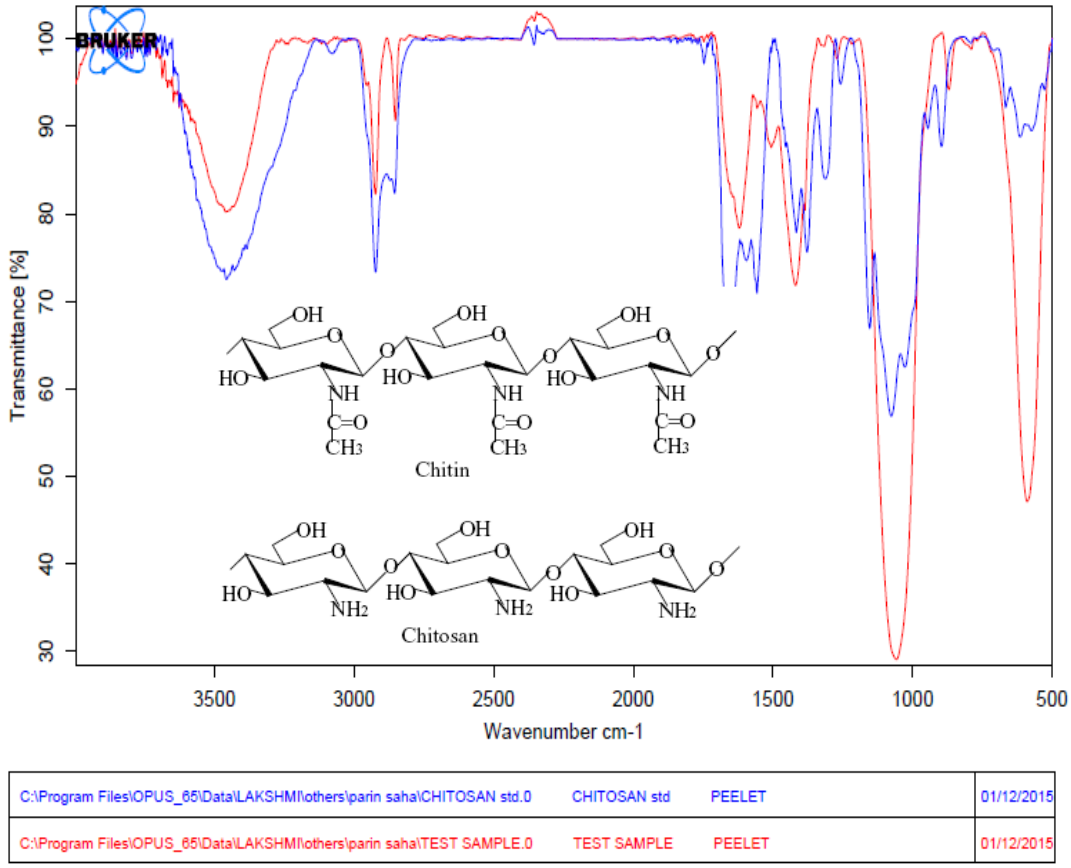


Figure 4.4: *FTIR analysis of the extracted chitosan against standard chitosan*



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Part of the results of experiments mentioned in this chapter are published in:

P. Shah, K. Bhavsar, S. K. Soni, J. M. Khire (2009): Strain improvement and up scaling of phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. Journal of Industrial Microbiology and Biotechnology, 36:373 - 80

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Chapter 5

Application of phytase in food, animal feed and in pesticide degradation.

- Phytase is known as animal feed enzyme from more than six decades
- A single enzyme doesn't have all required characteristics and thus no ideal phytase is commercially available for poultry application
- Current studies on phytase prove potential towards ideal phytase
- Few reports available for phytase application in human food but no product available in market
- 67% reduction in phytic acid is obtained by application studies in bread making
- Application studies for organophosphorus pesticide degradation is carried out to widen the application horizon
- Current studies show potential degradation of chlorpyrifos in vitro as well as on harvested green chilli.
- Also, phytase action showed positive result in degradation of monocrotophos and methyl parathion

1. Introduction

The research on phytase started in 1907 spanning many years until it was commercialized in 1991 (Vats et. al. 2005). In spite of having a valuable advantage, phytase was not widely used due to its high production cost. Awareness about pollution and increasing cost of fertilizer demanded industrial phytase production which increased and reached to $350 million company in 2013. The feed enzyme market including phytase, carbohydrases, etc is expected to reach at $1,193 million company in 2018. This growth is increasing at the rate of 7.3 % every year (Lie et. al. 2013; PR web 2013)

Many reports are available for the first commercial recombinant phytase production and its application (Haefner et. al. 2005, Simons et al. 1990, Jongbloed et al. 1992). The major sector in which the application of phytase used was in animal feed as an excess of inorganic (rock) phosphates had been utilized. Di-calcium phosphate and mono-calcium phosphate are widely used as the source of inorganic form of phosphate. Their cost went on increasing gradually at an alarming rate after 2007. Along with these sources, cheap sources of phosphorus were also used like meat and bone meal, which were banned due to cross-species transfer of diseases (Lei et. al. 2013).

1.1. Application in animal feeds

Monogastric animals (pigs, poultry, fish, and humans) feed maximally on plant - based food and thus are exposed to high concentrations of phytic acid. These animals lack the ability to use phosphorus which is in bound form in phytic acid (Singh et al. 2011). The dietary phytate chelates the metal cations required for growth (Iqbal et. al. 1994; Pen et. al 1993). The metal-phytate complex in-turn binds with proteins at various pH affecting its enzymatic activity, solubility and digestibility (Kemme et. al. 1999). Phytic acid also binds to starch directly or indirectly through proteins. It also interacts with the lipid which forms a major hindrance in deriving energy from lipid sources (Rickard and Thompson 1997; Leeson 1993). Thus, phytic acid acts as an anti-nutrient molecule (Woyengo and Nyachoti 2013). Thus, major research on phytase is carried out on effective use of phytase for maximum utilization of phytic acid.

1.1.1. Poultry feed Application

The usage of inorganic phosphates in the diet of poultry (broiler chickens) cause passage into excreta and in-turn into water bodies causing pollution. This indulged into heavy financial penalties to the poultry units (Chesson 1993). The application of phytase reduced pollution and thus was widely accepted as feed enzyme (Bedford 2003). Effective application and advantages of phytase to the poultry can be judged by its application in feed without the usage of inorganic phosphorus. Studies on poultry by Simons et. al. (1990) and Cabahug et. al. (1999) showed the benefits of phytase application. The pH variability in the digestive track of poultry demands phytase having broad pH stability. The pH in the crop region ranges from 5.2 to 5.8. The proventriculus is a highly acidic region having pH of 2.5 to 2.8. Then there is a small intestine region where least action of phytase is observed due to the presence of strongly acting proteases (Dersjant et. al. 2015). The research on poultry feed for layers (egg laying) is not much focused because non- addition of phytase in their feed doesn't affect egg quality (Selle and Ravindran 2007). Thus, there is a need to have phytase with acidic pH optima for it application in poultry feed.

1.1.2. Swine feed Application

Similar to poultry, there is pH variability seen in the digestive track of swines. The empty stomach of swine has pH of 2.0 to 2.5, which increases to pH 5.0 on feed and remains stable at pH 4.0 after the feed for 2 to 5 hours. The small intestine also showed a wide range of pH from 3.5 to 5.5. The studies by Yi and Kornegay (1996) reported the maximum action of phytase in the stomach region of the swine. The supplementation of phytase in the swine diet helped in increasing the bone phosphorus, calcium and ash (Mroz et. al. 1994). This sector demands phytase with broad pH stability from 2.0 to 5.5.

1.1.3. Aqua feed Application

The usage of phytase played a very important role in aqua feeds, as the excess of inorganic phosphorus added in the diet of aqua feeds, enter directly into the water bodies through excretal route causing intensive pollution. Fishes have varied pH range and thus are classified as gastric fish and agastric fishes. The pH in the digestive track of agastric fishes ranges from 6.8 to 7.3 and is acidic in gastric fishes (Ji. 1999). Thus, phytase having optimal pH action in the neutral range would be beneficial here. Also,

a lower optimum temperature is the requirement for application of phytase in aqua feeds.

1.2. Human food Application

Studies showed that phytic acid in the plant-based food forms complex with essential metal ions like calcium, magnesium, iron, etc. under normal acidic conditions of the stomach (pH 3.0 to 6.8), making it unavailable for human nutrition (Costello et. al. 1976). Much of the phytic acid is been degraded during the food preparation stages like steaming, germination, etc. Still, there seems need to use phytase in human food for better absorption of minerals. There are several reports where food improvement/fortification is observed viz; iron absorption increased by usage of phytase in wheat flour (Sandberg et. al. 1996), improved bread characteristics and nutrition level (Haros et. al. 2001); soy milk dephytinization (Usahashree et. al. 2012); etc. Thus, phytase has shown applicability in human nutrition as in animal feed nutrition, however, lacks its acceptance due to recombinant nature of the enzyme. Thus, this sector demands native phytase stable over wide range of pH (3.0 to 6.8).

1.3. Application in Agriculture

The introductory chapter explains about the fate of phosphorus in the agriculture soil wherein maximum of phosphorus is blocked in organic form as phytic acid. Thus, phytase application to combat phytic acid presence can be beneficial in plant growth promotion. There are various reports were phytase has improved plant growth viz; in maize (Findenegg and Nelemans 1993), wheat (Singh and Satyanarayana 2010). Gujar et. al. (2013) performed experiments to show advantageous effects of phytase over commercial usage of fertilizers. Studies are also carried out where phytase is expressed in plants for increased benefits. Transgenic rice and wheat were produced showing increased growth due to enhanced bioavailability of phosphate and iron (Lucca et. al. 2002).

There are many more applications where phytase has shown its potentials but not been explored on a mass scale. Table 5.1 summarizes the potential application of phytase and challenges for its usage on reviewing by Bhavsar et. al. (2014).

Table 5.1: *Potential applications of phytase*

Application	Role and effect	Properties	Challenges
Feed Industry	Effective usage of phosphorus, decreased excretion of phosphorus, cheap substitute of phosphorus	Low cost of production, active in the stomach, stable during processing and storage,	Lack few desirable properties like thermostability, etc.
Food Industry	Increased bioavailability od essential metals, improved food processing	--	Maintaining food taste and health benefits
Myo-inositol phosphate	The intermediates are used in pharma for preparing drugs of cell signaling, enzyme stabilizers, etc.	--	Developing process to produce selective intermediate showing maximum effect
Aquaculture	Substitute expensive protein source, maintains phosphorus levels in water	Phytase active at low temperature and broad pH optima is required	Effect of phytase action on various biochemical systems needs to be investigated
Soil amendment	Plant growth stimulator, help in mobilization phosphorus from unavailable to available form	Broad pH optima and stability with high catalytic activity is required	Less requirement of phytase for excess supplement should be decreased

Hydroxyapatite formation	Simple bio catalytic process	Cost effective as compared to commercial process	Develop product for mass application
Nanoparticles	Prepared using phytase and ionic liquid	Effectively used as biocatalyst and for drug delivery	More application studies required for biomedical applications

2. Quest for effective phytase

Considering the major application sectors of phytase in animal feed and then in agriculture and human nutrition, there is a demand to have phytase based product in the market. There are only handful of phytase products in the market (Table 5.2) (Joshi 2014).

Table 5.2: *Commercial phytases in market*

Enzyme origin	Expression system	pH optima	Temp optima (^{O}C)	Trade name
A.niger	*A.niger*	2, 5 – 5.5	65	Natuphos
A.niger	*A.niger* (non – recombinant)	6.0	--	Allzyme
A.niger	*Trichoderma reesei*	2.5	--	Finase
E.coli	*S. pombe* (ATCC 5233)	4.5	55	Phyzyme
E.coli	*P. pastoris*	4.5	--	Quantum
E.coli	*Trichoderma reesei*	--	--	Quantum Blue
E.coli	*P. pastoris*	3.4, 5	58	Optiphos
Peniophora lycii	*A. oryzae*	4 – 4.5	50 - 55	Ronozyme

Citrobacter braakii	*A. oryzae*	--		Ronozyme HiPhos

The demand from a sector needs to be considered and should search for the phytase with required characteristics. Most of the bacterial phytase have pH optima in neutral to alkaline range, and dependent on calcium for its action (Kerovuo et. al. 1998). The fungal phytase have pH optima in the acidic range, glycosylated and are have less proteolytic resistance than bacterial phytase (Rodriguez et al, 1999; Igbasan et al, 2000; Wyss et al, 1999) (Table 5.3). The majority of phytase have an optimum temperature range of 40 - 60°C, wherein *A. fumigatus* and *Schwanniomyces castellii* are most thermotolerant phytases reported (Segueilha et. al. 1992). *A. niger* (p*hy A*), which is most exploited for commercial phytase has two pH optima at 2.5 and 5.0 and optima temperature range of 55 - 60°C (Han et al, 1999). *E.coli* phytase (*app A*) is found to be more superior to fungal phytase (Wyss et al, 1999; Augspurger et al, 2003). Many more phytases from different sources are reported and summarized in Table 5.3.

Table 5.3: *Overview of phytases*

Phytase source	**Mol. Wt. (kDa)**	**Temp. Opt. (°C)**	**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	0.019	-	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	-	7.4-8.0	-
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-	0.25	-	-

P. spartinae	-	75-80	4.5- 5.0	0.135	-	-
Bacteria						
Aerobacter aerogenes	-	25	4.0-5.0	0.135	-	-
Bacillus sp DS 11	-	70	7.0	0.55	5.3	P
B. subtilis	38	60	6.0-6.5	-	-	-
B. subtilis	43	55	7.0-7.5	-	6.5	P
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

As per the major application sectors, it is very difficult to have a single native enzyme catering to all the sectors. For animal feed application, a single enzyme should be produced from in native form and should fulfill certain characters;

- High activity al low pH
- High affinity for phytic acid
- High speed to release phosphorus from phytic acid
- Highly thermostable
- Scientific proof of action *in vivo*

An enzyme meeting all the above criteria seems to be an ideal phytase and this is possible as research still continues on it.

3. Test of phytase from *A.niger* NCIM 563 to be an 'Ideal Enzyme'

Sequential experiments were carried out to meet the criteria for ideal phytase in the animal feed sector.

3.1. pH stability of phytase

The influence of pH on phytase activity was determined by assaying in the pH range of 1.5 and 9.0 using 100 mM buffers: glycine - HCl (pH 2.0 - 3.0), sodium acetate (pH 4.0 - 6.0), Tris - HCl (pH 7.0 - 8.0), and glycine - NaOH (pH 9.0) at 50 °C. The pH stability using the same buffer solutions was also determined by pre-incubating enzyme samples at 35 °C for the period of 12 h considering phytase activity at zero time as 100%. The stability of phytase was also checked at gastric conditions of poultry. One gram soybean meal was dissolved in 9 mL of simulated gastric fluid (250 mM glycine - HCl containing 2.0 mg/mL NaCl and 3.2 mg/mL of pepsin) and the pH was adjusted over a range from 1.5 to 6.5 using HCl and NaOH as required. The solutions were incubated at 37 °C for 30 mins, as the poultry gut temperature varies from 37 – 39 °C (Lei and Stahl 2001). 40 IU of phytase was added to the solution and incubated at 37 °C for 60 mins. The released phosphorus was determined as described in phytase assay (Chapter 3).

3.1.1. Results and Discussion

The conditions for optimum activity and stability of the phytase was assessed by carrying out studies with respect to pH. Studies with varying pH interestingly showed that the enzyme showed high activity at a low pH value of 2.5 (Fig 5.1). Considering the activity at pH 2.5 to be 100%, only 50 and 10% activity was observed at pH 4.5 and 7.0, respectively. It may be noted that the phytase showed overall broad pH stability from pH 2.5–9 (Fig 5.1).

Experiments also showed that the enzyme activity was retained for 12 h in the pH range studied. The pH and temperature stability profile was also determined under poultry gut conditions as described in methods. High efficacy of phosphate release was shown by the phytase in simulated gastric fluid in pH ranging from 2.0 to 4.5 (Fig 5.2).

Figure 5.1: *pH optima and stability of phytase*

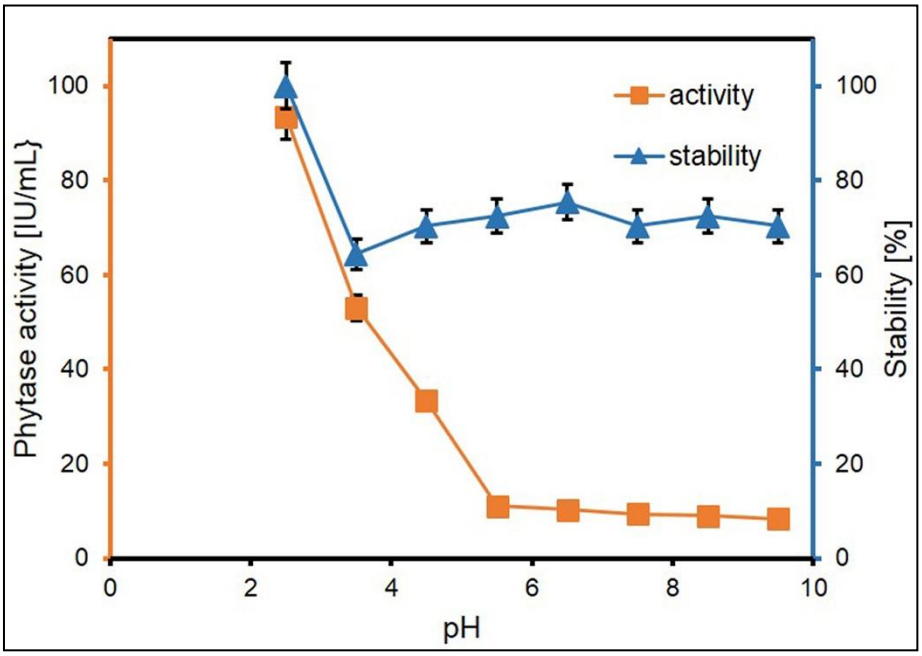
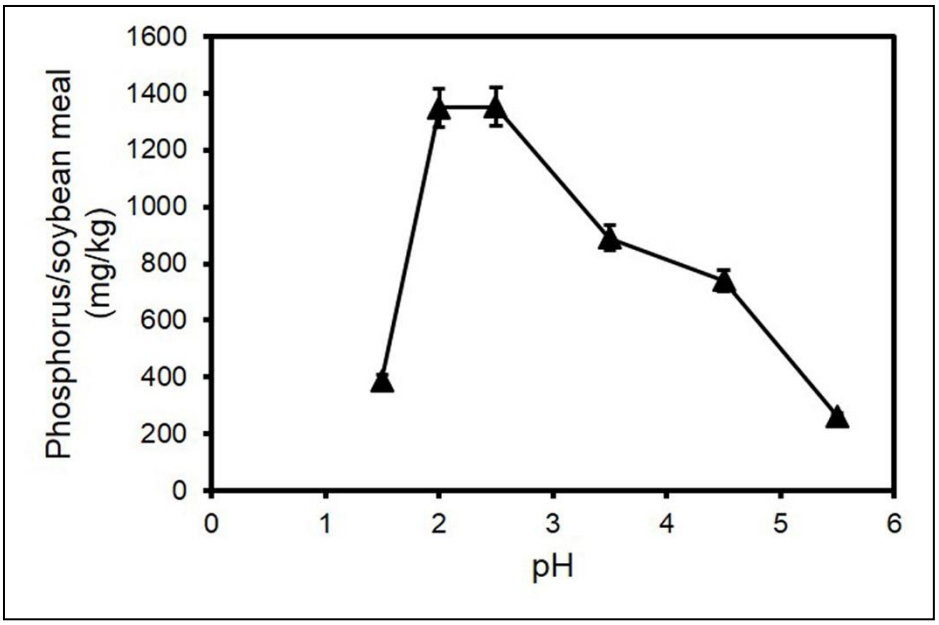


Figure 5.2: *Stability studies of phytase under simulated gastric conditions*



The results of pH studies provide confirmation about the suitability of phytase for application in poultry, swine, fish and human diets which need enzyme action at acidic pH. The characteristics of the current phytase produced outstand from other reported enzymes as in Table 5.3.

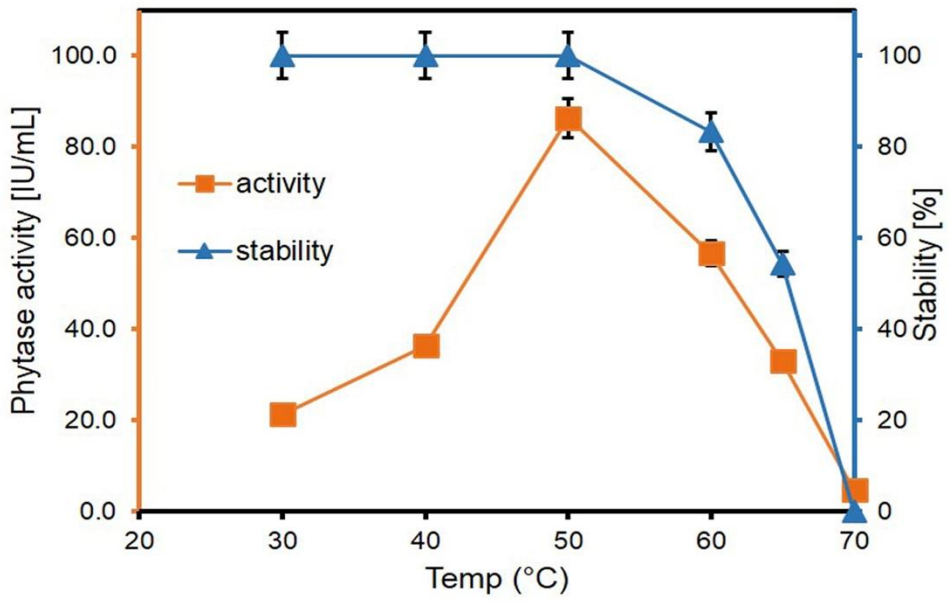
3.2.Temperature stability of phytase

The studies on optimum temperature were carried out in the temperature range of 30–70 °C, while the temperature stability was determined by incubating the enzyme samples over the above temperature range for a period of 1 h on comparing with the control without incubation.

3.2.1. Results and Discussion

The conditions for optimum activity and stability of the phytase was assessed by carrying out studies with respect to temperature. Optimum temperature studies ranged from 30 to 70°C. It was observed that the optimum temperature for maximum phytase activity is 50°C (Fig 5.3). On considering the activity at 50 °C to be 100%, we observe that at 35°C, the activity reduces to 30% of maximum activity and at 60°C to 60% of maximum activity. Temperature stability studies were then carried out, which showed 100% stability in 1 h from 30 to 50 °C (Fig 5.3) while 20% activity was reduced at 60 °C, 50% at 65 °C and 100% at 70 °C.

Figure 5.3: *Temperature optima and stability of phytase*



Thus, considering the results, it seems that the enzyme to be made industrially viable as a product, needs to have higher temperature stability. There are ways to increase it is by use of thermotolerant enzymes, recombination, protein engineering and thermoprotective coating.

3.3.Thermostability studies of phytase

The usage of external agents for imparting thermoprotectiveness to phytase seems to be of great interest (Phillippy 2001). The process for animal feed preparation has a step of pelletization, where in the feed enzymes are exposed to high temperature of 95°C, for a very short period. Different additives were added to phytase solution viz; mannitol, glycine etc. and checked for thermostability at a higher temperature (70 to 90°), considering the sample without additive as a control.

3.3.1. Results and Discussion

A variety of thermos-stabilizers were tried to obtain the desired phytase product viz; mannitol and sorbitol (25%), carboxymethyl cellulose (low and high viscosity), PEG 4000 and 6000 (2%), Betaine HCl and glycine (2%), ethanol (5 and 10%), arginine and sodium common salt (2%), skimmed milk (10 to 30 %). It was observed that 80% activity of phytase was retained at 80°C for 1 min, by usage of 30% skimmed milk.

Chitosan which was extracted from fungal biomass after phytase production as described in the previous chapter was also tried for imparting thermostability to phytase. 3% chitosan provided 80% retention in the activity of phytase at 80°C for 1 min.

Further, increase in thermostability can be achieved by usage of thermoprotective coating adopted by a research group in Danisco. This is a patented technology, developing heat tolerant phytase granules imparting stability up to 95°C (Dupont: www.dupont.com).

Thus, phytase from *A.niger* NCIM 563, has gained the thermostability property, paving a way towards ideal enzyme characteristics. Further, consideration for making the current phytase as an ideal enzyme can be made with the results obtained from the studies done by Soni et. al. (2007; 2010). It reports that the current phytase produced has a high affinity for phytic acid making it much stronger to be an ideal phytase.

4. Application of phytase in human food

Phytase produced here using *A. niger* NCIM 563, shows multiple characteristics which suit the cadre of an ideal enzyme. Being the sole enzyme to act on phytic acid, the enzyme action can be used to cater the sector where there is a hindrance due to phytic acid. One such sector which has a very high impact on life of humans and to

some extent animals is food. The food products which has phytic acid content may affect the growth and thus can be acted upon by phytase. Whole-grain based food products have increasing demand and studies show that phytic acid content in them reduced the quality of the food products. Thus, application of phytase for increasing the nutritional value of the food products have been reported (Haros et al. 2001; Park et al. 2011). Also, FSSAI, which is the food safety controlling body in India, has conveyed all the leading brands of wheat flour supplier to fortify it with iron, vitamins, etc. (Food safety 2017). Phytase thus, can play a very important role in wheat fortification. Phytase produced here was thus used to check its fortification property and thus can be part of flour fortification.

Much of the research carried out is focused on negative aspects of phytic acid on human health. However, studies showed positive and beneficial effects of phytic acid. It has been a good antioxidant and an anticancer agent. Burgess and Gao (2002) showed antioxidant property of phytic acid by chelating iron. Evidence in developing countries relying majorly on plant-based foods show beneficial effects of phytic acid. It has proved helpful in diabetes, arteriosclerosis, etc. (Kumar et. al. 2010).

4.1. Application of phytase by degradation of phytic acid in bread

Awareness in food quality has increased usage of whole wheat flour for preparing healthy products. Whole wheat bread is widely used and is thus used to study the action of phytase. Wheat flour and other ingredients required for preparing the bread was purchased form the local market. The bread dough formula for 100g consisted of wheat flour (100g), dry yeast (3.0 g), salt (2.0 g), sugar (2.0 g) and water up to optimum absorption. The ingredients were mixed properly and treated with phytase (500U/100g). The same procedure was followed for negative control wherein no phytase treatment was given. After mixing, the dough was kneaded, rested (10 min) and divided multiple times. The dough was mechanical shaped, proofed (at 35-40°C for 2 h or up to optimum volume increment) and baked (150 ºC, 45min). Before and after proofing the dough, samples were withdrawn to determine the reduction in the phytic acid content of the wheat flour. After baking, loaves were cooled for 2 hrs at room temperature.

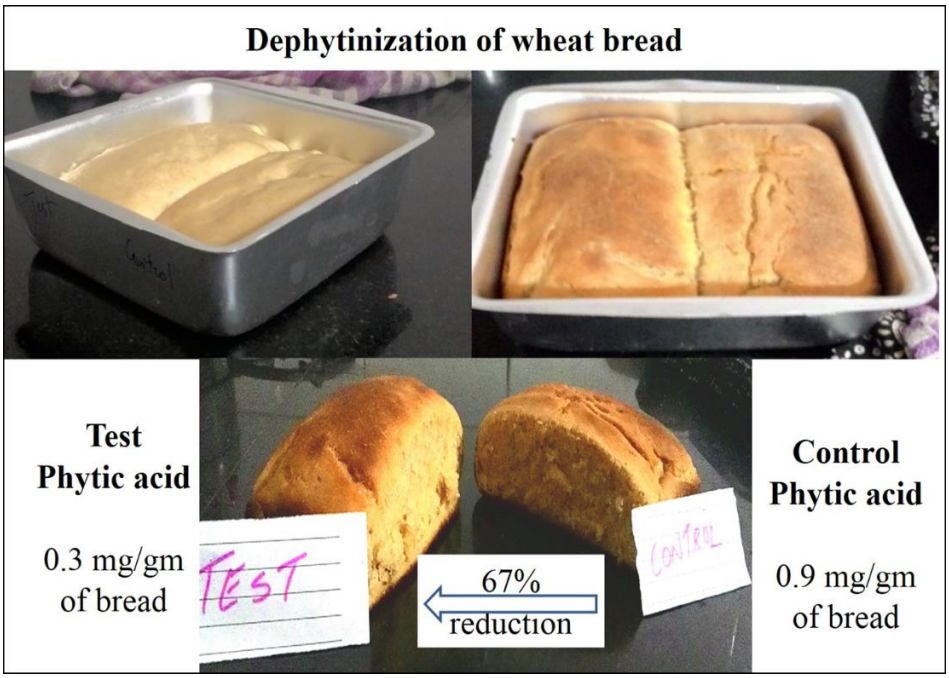
4.1.1. Results and Discussion

The samples which were withdrawn before and after proofing the dough are checked for the phytic acid content in it. The maximum action of phytase takes place

during the proofing stage. The conditions of proofing (35-40°C for 2 hrs), seems to optimal for the action of phytase as per characteristics of phytase obtained in the previous section. Indigenous phytase activity in the wheat flour and dry yeast was checked and found to be non-significant (data not shown). Thus, the results obtained here is mainly due to the action of phytase. Fig. 5.4 shows the action of phytase to be beneficial in the bread making process. The bread with phytase treatment (test), show more leavening that the phytase untreated bread. 67 % reduction in the phytic acid content was obtained after the phytase action i.e. from 0.9 mg/g of bread to 0.3 mg/g of bread. The produced bread was consumed and found to be safe without any change in the taste. Thus, the action of phytase has reduced more than 50 % of phytic acid, reducing the anti-nutritional content in bread. The presence of less concentration of phytic acid would be beneficial to human health.

The phytase produced here is from a safe organism which doesn't produce any toxin and is widely used for feed application. The use of phytase is thus a safe option to reduce phytic acid content in food. Application of phytase in animal feed and human food is met, making it more ideal for usage in multiple sectors.

Figure 5.4: *Action of phytase on wheat flour used for bread making*



5. Potential application of phytase degradation of organophosphorus pesticides

Over the past century, an increase in demand for food grains and vegetables has led to an extensive use of manmade pesticides in agriculture. In fact, crop protection in India is known to annually use nearly 40,000 metric tons of pesticides. Organophosphorus pesticides (OpP), are widely used in agriculture for controlling variety of sucking, chewing and boring insects, spider mites, aphides, and pests. In particular, OpP cannot be easily removed by washing and rinsing with tap water (Vendan 2016) and this leads to bioaccumulation in the food chain.

Organophosphorus pesticides are esters of phosphoric acid, which include aliphatic, phenyl and heterocyclic derivatives (Baishya and Sarma 2015), which are known to be potent irreversible acetyl-choline esterase (AChE) inhibitors by phosphorylation of the serine residue at the enzyme active site (Fig 5.5 a, b) (Santos et. al. 2007). This leads to adverse effects on the nervous system of exposed animals including humans (Mileson et al. 1998), and there exists a dire need to degrade OpP post-harvest, so as to prevent their entry into the food chain. The currently used physicochemical processes for OpP remediation include incineration or disposal in landfills that are expensive, non-ecofriendly and the process is often incomplete leading to the formation of toxic intermediates (Debarati et al. 2005).

Figure 5.5 – a: *Action of OpP on acetyl-choline esterase*

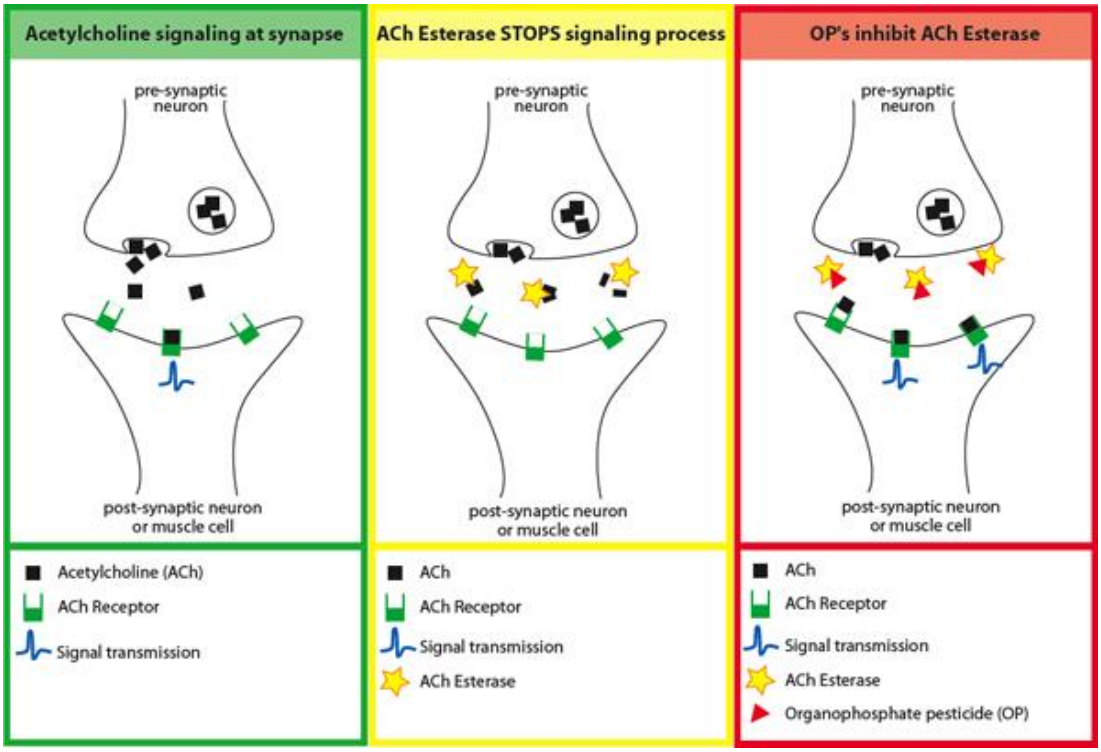
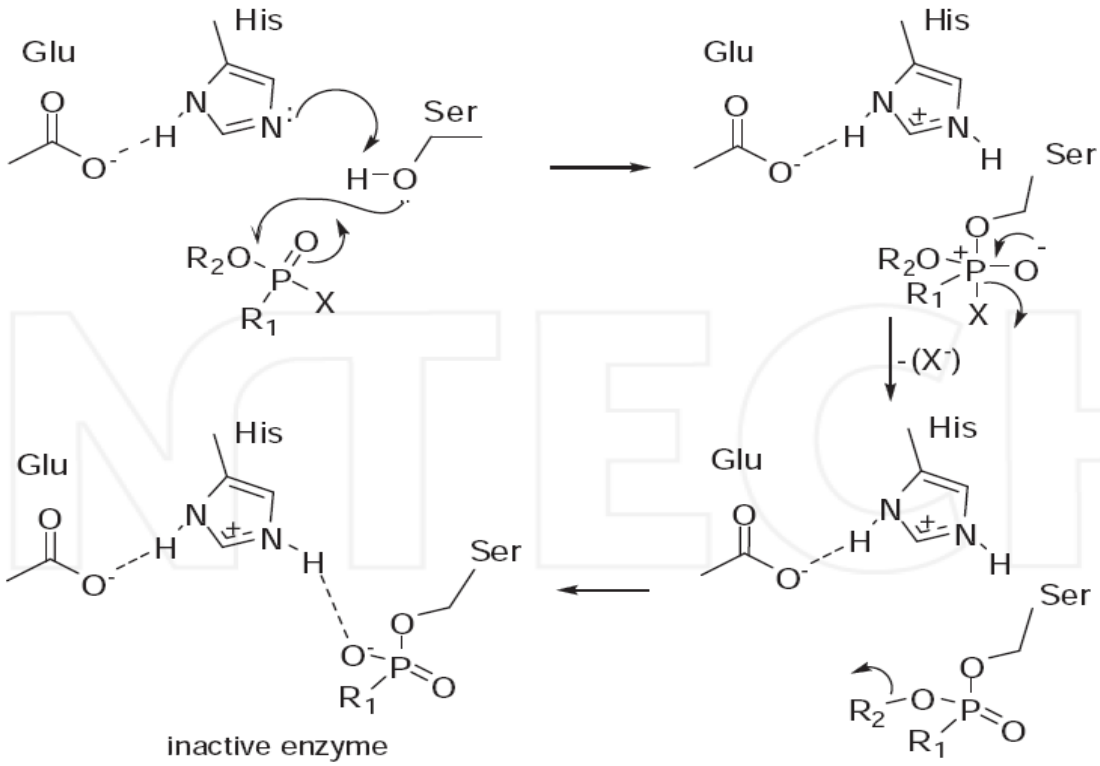


Figure 5.5 – b: *Action of OpP on acetyl-choline esterase active site using phosphorus*



Alternatively, the use of whole cell microorganisms is advantageous because it offers a safe, economic and eco-friendly green option (Rayu et al. 2012; Sutherland et

al. 2004). The factors that impact bioremediation are mainly the availability of organic sources for microbial growth, optimal pH, bioavailability of inhibitory substrates and the satisfaction of regulatory norms for release of microbes into the environment (Boopathy 2000), which can be addressed by using cell-free microbial enzymes that can act on diverse pollutants (Scott et al. 2011). Most widely studied cell-free enzymes for OpP degradation are from bacteria, viz., organophosphorus hydrolase (EC 8.1.3.1) (Gao et. al. 2012), phosphotriesterase (EC 3.1.8.1) (Chino-Flores et al. 2012) and organophosphorus acid hydrolase (EC 3.1.8.1) (Theriot and Grunden 2011). There are few reports where, fungi have been studied for OpP degradation by cleaving the phosphate group (John and Shaike 2015; Wyss et al. 1999).

Phytase or myo-inositol hexakisphosphate phosphohydrolases (EC 3.1.3.8) produced currently from *Aspergillus niger* NCIM 563, is a good example of a hydrolytic enzyme that can release inorganic phosphorus by the degradation of phytic acid. Current progress on phytase research is focused on phytic acid degradation for major animal feed supplementation, plant growth promotion and human nutrition (Dersjant-Li et al. 2015; Kumar et al. 2010). The GRAS cleared phytase enzyme has not been studied for its potential for biodegradation of toxic pesticides by phosphorous release and is therefore studied here. A positive result would bring out a novel and useful enzymatic application of phytase for organophosphorus pesticide detoxification.

5.1. Chemicals

3, 5, 6-trichloro-2-pyridinol (TCP) and diethyl thiophosphate (DETP) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Acetonitrile (ACN) of HPLC grade was purchased from Merck. All other chemicals used were of analytical grade. Chlorpyrifos (CPyF) (Dursban 2E©, 20%), monocrotophos (MCP) (36%) and methyl parathion (MP) (50%), harvested green chilli were purchased from a local market.

5.2. Analytical methods

The analysis of OpP's were carried out using HPLC (Dionex-ASI 100, auto sampler series) with reverse-phase column (C18 −4.6 × 250 mm, Waters) maintained at 40°C. The mobile phase used was mixture of acetonitrile: water (70:30 v/v), with flow rate of 0.5 mL/min. The detection was done at 230 nm and an injection volume was 50 µL. Standard solutions of OpP was transferred in vials to reach final concentrations in the range of 100–1000 ppm using mobile phase. The analysis of CPyF and its degraded

products on green chilly were carried out using LC–MS (Waters-Xevo TQD-USA) with reverse phase C18 column (Acquity—UTLC BEH—2.1 × 100 mm) maintained at 45 °C. The mobile phase used was mixture of acetonitrile: water (70:30 v/v), with flow rate of 0.3 mL/min. An injection volume of sample was 5 μL. Standard solutions of each were transferred to vials to reach a concentration in the range of 0.2–1 ppm using mobile phase.

5.3. Biodegradation of OpP using phytase

The aim of this experiment is to check the potential of phytase in degradation of OpP. Multiple OpP are considered here which had different phosphorus bonds, to show that phytase has a wide range of action.

The potential of extra-cellular phytase, produced in optimized chickpea flour media, was studied for biodegradation of CPyF, a water insoluble organophosphate. 1 mL stock solution of CPyF (10,000 ppm) was incubated with 100 μL mycelial free phytase (100 IU; specific activity 53 IU/mg) for 2 h at 35 °C, pH 7 as well as its optimum conditions (50 °C, pH 2.5). The selection of a high concentration of CPyF was employed for sensing and quantifying the degradation metabolites. The treated sample was diluted 10 times with mobile phase and the amount of residual CPyF was monitored using HPLC as mentioned in analytical methods. For other OpP's (MCP and MP), studies were carried out in a similar way.

5.3.1. Results and Discussion

The ability of crude phytase, (100 IU) produced using optimized chickpea flour media, to degrade CPyF was studied both under normal conditions (35°C, pH 7.0) as well as under optimum enzyme conditions (50°C, pH 2.5). HPLC analyses, showed a single major peak for the sample containing only CPyF (control sample) at 35 °C, pH 7.0 (Fig. 5.6a) as well as at 50 °C, pH 2.5 (Fig. 5.6c) and having a retention time of 3.62 min with a relative area of 97%. On the other hand, for a phytase treated sample at 35 °C, pH 7.0, multiple peaks were observed. Notably, it was observed that there was a decrease in relative area by 72% at the retention time of CPyF (Fig. 5.6b). A similar study with phytase at 50 °C, pH 2.5, the relative peak area seen at a retention time of CPyF showed an even higher decrease in relative area by 91% (Fig. 5.6d).

The positive result for CPyF suggested degradation studies with other OpP's, namely, MCP, and MP by phytase would be useful. HPLC analysis with MCP having a retention time of 5.5 min for the control sample (Fig 5.7a) at 35 °C, pH 7.0 showed

that a higher unit of phytase (250 IU) to obtain 53% degradation in 4 h (Fig 5.7b) when compared to CPyF (phytase 100 IU, 72% degradation) (Fig. 5.6) in 2 h. Better degradation results with MP (HPLC retention time of 9.5 min for the control sample (Fig 5.7c) in comparison with MCP were obtained by a higher decrease in peak area (77%) on treatment with phytase (250 IU) (Fig 5.7d) in 4 h. Thus, all the above results with CPyF, MCP and MP corroborate the finding that phytase has the ability to effectively act on OpP and degrade them.

Figure 5.6: *Degradation of chlorpyrifos using phytase*

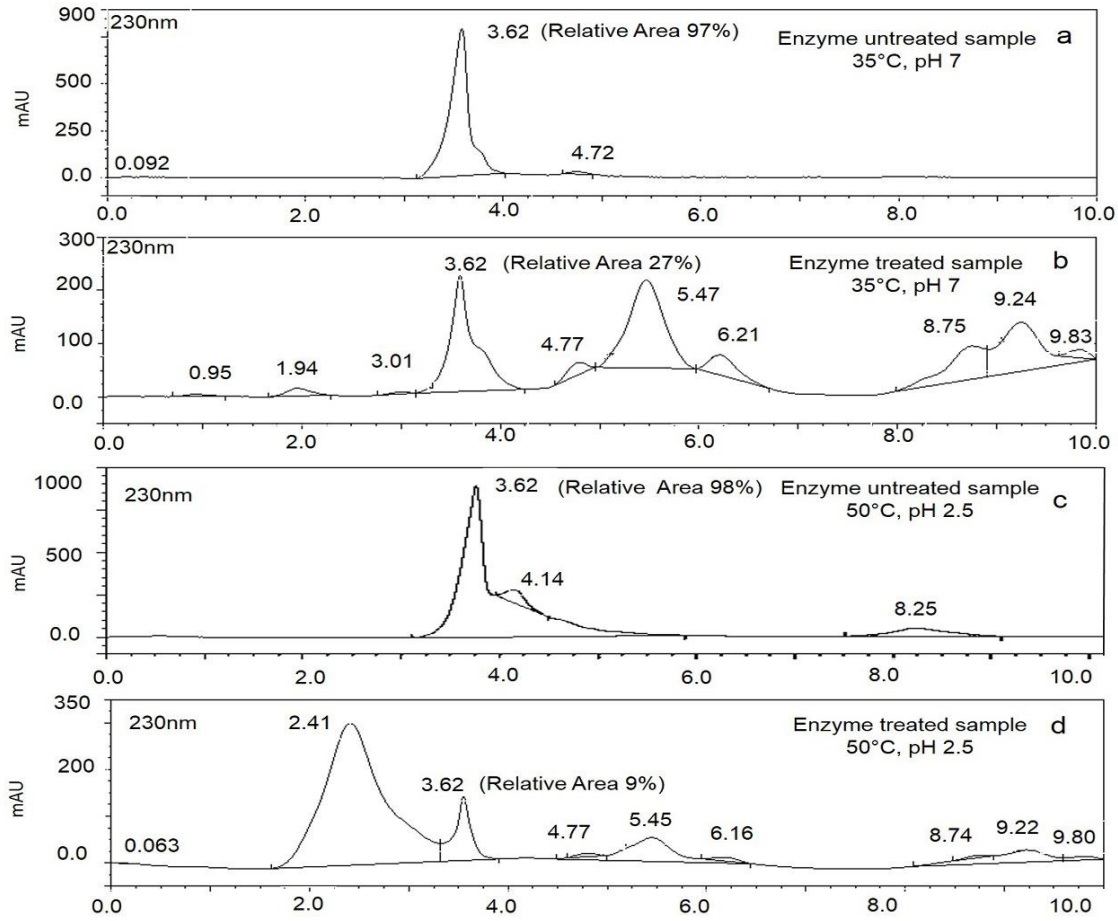
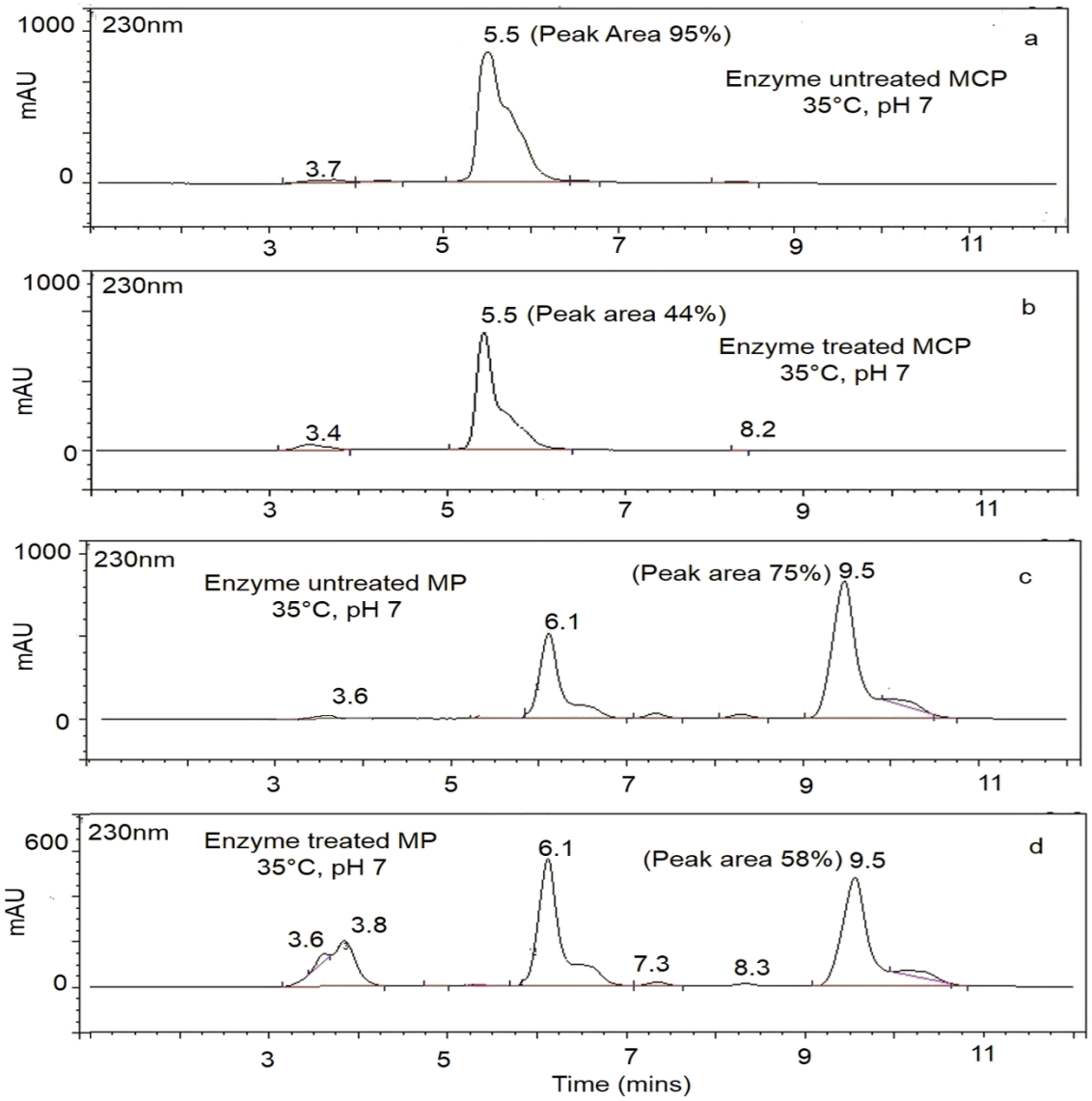


Figure 5.7: *Degradation of monocrotophos (MCP) and methyl parathion (MP) using phytase*



5.4. Application of phytase on harvested green chilli (*Capsicum annuum L*)

Phytase shows dephosphorylation action by breaking the phospho-ester bond to release phosphate from the substrate (Joshi 2014). As per food safety and standards authority of India (FSSAI), limit of CPyF on vegetables is 0.2 ppm (FSSAI notification 2011). Detoxification of

CPyF is achieved by cleaving the phospho-ester bond, generating 3, 5, 6-trichloro-2-pyridinol (TCP) as the major product along with diethyl thiophosphate (DETP) (Chen et al. 2012; Hanley et al. 2000; Bicker et al. 2005), which are both water soluble. Human studies show that, both the metabolites are considered as urinary markers of CPyF

exposure and are easily excreted through urine within 12 h. Studies on rat show that, TCP and DETP are the predominant urinary metabolites of CPyF catabolism (Bicker et al. 2005). In the present study, the applicability of phytase applied on post-harvest chillies to degrade water insoluble CPyF was therefore studied with respect to the formation of TCP and DETP water-soluble degradation products.

The potential of phytase to degrade CPyF on harvested green chilly was studied at 35 °C, pH 7.0. Green chillies (250 g), obtained from the local market was sprayed with CPyF (20 ppm) and dried. One part of the chillies (test) was treated with crude mycelial—free phytase (80 IU) at 35 °C and pH 7.0 for 2 h keeping the second part untreated (control). Both the parts were separately cut into small pieces and homogenized with a household mill (equipped with stainless steel knives). 10 mL of acetonitrile was added to 10 ± 0.1 g sample and vortexed for 1 min to which 10 g sodium sulfate was added and homogenized at 15,000 rpm for 1 min. The samples were centrifuged at 5000 rpm for 5 min and 5 mL of the supernatant were transferred to a 15 mL PTFE tube. 10 mg graphitized carbon black and 25 mg primary secondary amine were added to remove colored impurities. The extract was shaken using a vortex mixer for 30 s and centrifuged at 10,000 rpm for 5 min. 2 mL of the supernatant was used to analyze the presence of CPyF and its degraded metabolites using LC–MS as mentioned in analytical methods.

5.4.1. Results and Discussion

LC–MS analysis of standard CPyF showed that, it eluted with a retention time (RT) of 5.56 min and m/z of 349.90 while DETP and TCP eluted at 0.72 and 3.48 min with m/z values of 169.17 and 198, respectively. In phytase untreated sample (control), a single peak was detected after LC–MS analysis with a RT of 5.56 min and m/z of 349.90 indicating it to be CPyF (Fig. 5.8a). In phytase treated sample, 3 peaks (Fig. 5.8b–d) were observed at RT of 0.72, 3.48 and 5.56 min. MS analysis of these peaks showed m/z values of 169.17, 198 and 349.90, respectively. On comparing with the standards, the 3 peaks were ascertained to be DETP, TCP, and CPyF, respectively.

The percent degradation of CPyF using phytase from *A. niger* NCIM 563 can vary depending on pH and temperature prevailing at the field. Peak area analysis shows 8% degradation of CPyF at 35 °C and pH 7.0 in 2 h using phytase (80 IU). To increase the degradation, higher units of phytase or reaction time may thus be required. On using higher units of phytase (250 IU) to degrade CPyF on green chilli our results, in fact, showed that 90% degradation was possible in 12 h (Fig 5.9 a, b). It is reported that TCP

is not fetotoxic and teratoxic in either rat or rabbits at dosage levels of 100 ppm. TCP is shown to have moderate toxicity to salmonoids at a LD50 value of 1.8 ppm (Marino et al. 1999). Studies of TCP showed that a minimum concentration of 0.6 ppm when exposed for 24 h is toxic during the multiple developmental stages of zebra fish (Suvarchala and Philip 2016). Our observation is that phytase can degrade CPyF present on raw agricultural products. Thus, development of a potential new way that prevents toxic OpP from entering the food chain by forming easily removable metabolites could become possible.

Phytase production using *A.niger* NCIM 563, exhibits application in animal feed and human food. It has also shown an application in degradation of organophosphorus pesticides which in turn can help the agriculture sector to make the soil pesticide free. Thus, phytase shows tremendous potential in improving the health of living forms as well protecting our planet "Earth", from pollution and making it a better place to live in.

Part of the results of experiments are published in:

Parin C. Shah, V. Ravi Kumar, Syed G. Dastager and Jayant M. Khire (2017): Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides. AMB Express, 7:66. Doi 10.1186/s13568-017-0370-9

Figure 5.8: *Action of phytase on CPyF present on harvested green chillies*

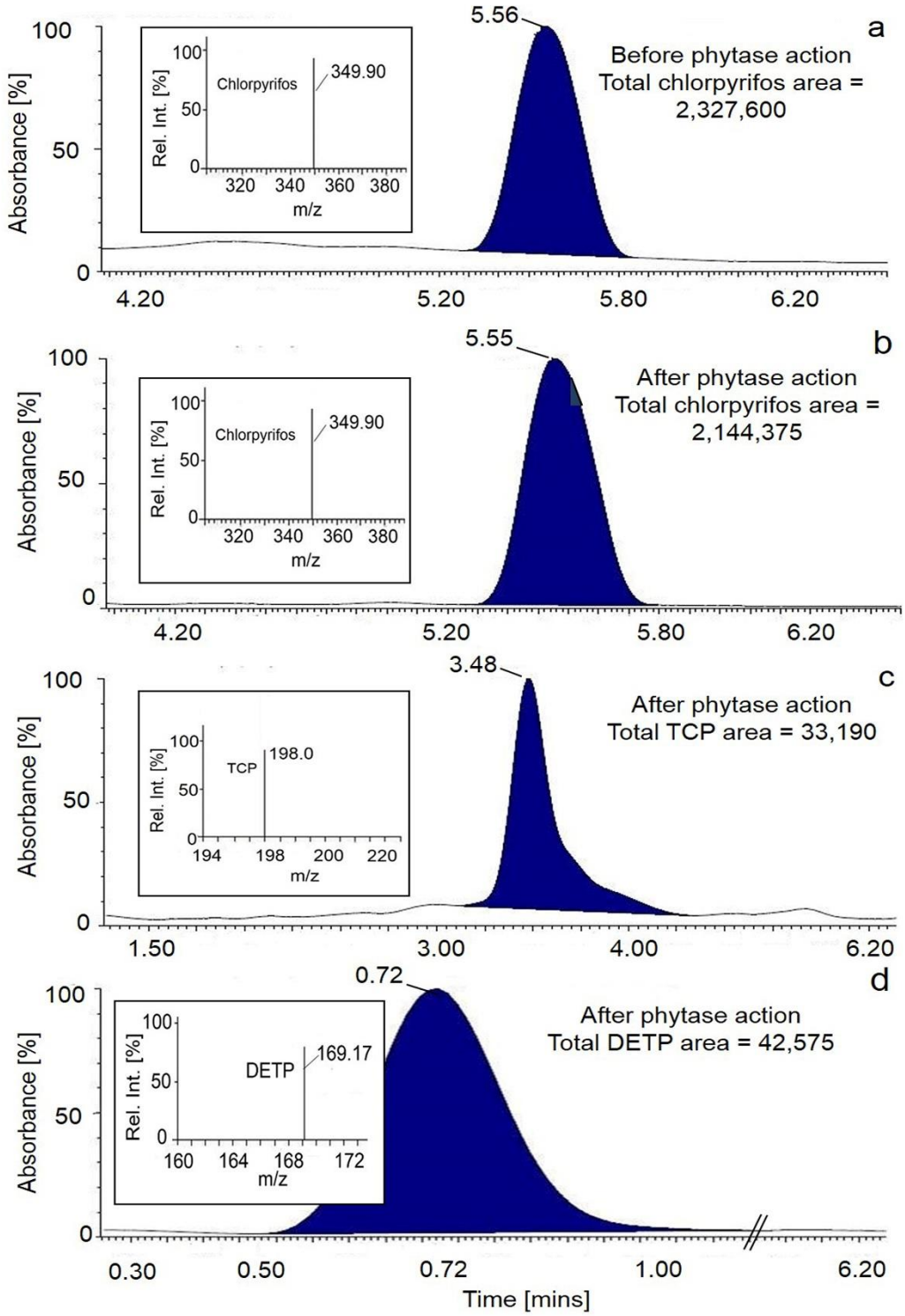
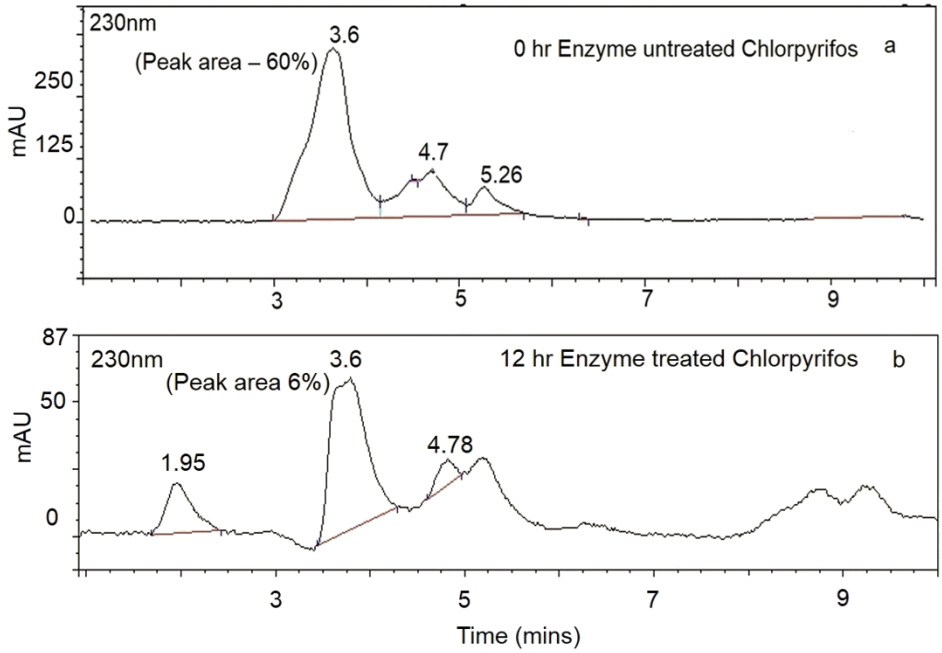


Figure 5.9: *Enhanced degradation of CPyF using higher phytase units*



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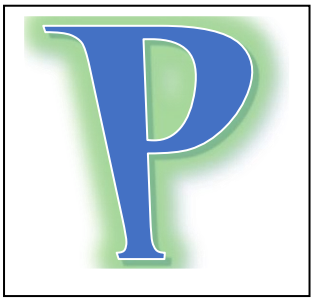


Chapter 6

Discussions and future prospects

This chapter summarizes the salient features of the work presented in the thesis and emphasizes on possible future potential developments in the research area of phytase.

1. General discussion

The current scenario is focused on conserving water and energy. However, the demand for conserving phosphorus towards meeting the global food security is increasing. Not much research is being carried out on conserving one of the fundamental building blocks of life i.e. elemental phosphorus, which is also essential for food production (Cordell et. al. 2009).

There is some common misunderstanding about phosphorus security, due to lack of data and research, etc. (Cordell 2010). According to Warren (2008), phosphorus is not running out as the law of mass conservation exists, nor there is scarcity or shortage of phosphorus, which is used interchangeably in the scientific literature. The shortage or scarcity mentioned is related to phosphate rock, which is the only source of phosphate reserves. There also arise misunderstanding in the usage of terms like 'phosphorus', 'phosphate rock', 'phosphate', 'phosphate reserves', and are importantly non-interchangeable. There is no substitute for the elemental phosphorus in the growth of all living organisms and is a renewable source of phosphorus which is cycled between dead to living organisms and plant to the soil at a very slow speed taking days to years (Johnston A E (2000).

However, there is a substitute for phosphate rock as the source of fertilizer and other industrial application and which is non-renewable source cycling between lithosphere and hydrosphere at the slowest speed taking millions of years (Gilbert N (2009).

There are uncertainty and lack of consensus about phosphate reserves timeline. The gap between demand and supply will widen along with increasing environmental and economic costs, which will keep on increasing. There will be fluctuations in food production system, its availability and price volatility. Thus, the countries need to ensure smooth supply of phosphate (for both short and long terms) to farmers for sufficient food production to meet the growing population (Warren 2008). In the current century, other than diffusion of phosphorus in water bodies through agriculture, industries growing at tremendous rate have also contributed phosphorus in water bodies by using phosphorus-based detergents. There arise a gap of inadequate waste water treatment facilities which has also contributed phosphorus drainage in water bodies (Seitzinger et. al. 2010). An integrated approach needs to be developed, wherein

phosphorus needs to extracted from renewable sources like food waste, crop residues, excreta, etc., and increase efficiency in the agricultural system of usage of fertilizers, etc. (Warren 2008).

The fate of phosphorus added as fertilizer in soil is clearly understood, wherein the maximum of phosphorus is fixed in clay which has a high concentration of aluminium than iron and calcium. In the initial stages, phosphorus is present as aluminium and calcium phosphate and as time passes, both these forms change into less soluble iron phosphate form. This process speeds up with increased moisture content in soil (Change and Chu 1961). Further, the bound phosphorus is held with the organic content in soil forming phytate, which is phytic acid bound to metal ions. Phosphatases cannot act on phytic acid and phytase, which is the sole enzyme acting on it, thus play a very important role in providing the renewable source of phosphorus. This would release the burden on usage of fertilizer as well as decrease the pollution protecting the environment. The plant residue, as well as the cereals and legumes, contain phytic acid which is storage form of phosphorus in plants, enters the food chain of animals as well as humans, which they cannot digest it and in turn acting as an anti-nutrient factor (Cowieson et.al. 2016).

Much of the cereal and legume-based diet is utilized in poultry, swine and in aqua feed, which contains a high concentration of phytic acid. All these monogastric animals lack the ability to digest phytic acid and phytase thus plays a very important role in decreasing the anti-nutrient factor concentration and in turn increase the supply of phosphorus to them which is a very essential element for growth and development (Cowieson et.al. 2016). There is no ideal phytase available in the market which can cater all the sectors of animal feed, nor which has all the characteristics to cater a single sector. Thus, research on phytase is still on, to search for an ideal phytase. Studies are going on to increase its thermostability, catalytic efficiency and pepsin resistance, etc. (Niu et. al. 2017).

Along with increased usage of fertilizers for intense agriculture, usage of plant protection chemicals (pesticides) has also increased at an alarming rate. These too are phosphorus-based compounds, putting further load on the requirement of phosphorus. Much of the organophosphorus pesticides remain unused in the soils and are not easily degraded in soil. These needs to be treated and can be tried to be used as a source of phosphorus. Also, part of organophosphorus pesticides enters our food chain and which is hazardous acting on our nervous system (acetyl-choline esterase enzyme) causing

paralysis. Our gut microbes have shown the ability to degrade them causing intestinal dysfunction. Studies have shown that these pesticides induce glucose intolerance in the exposed persons causing diabetes (Velmurugan et. al. 2017). A similar inference is also been drawn with studies on rodents, which has prompted increased prevalence of diabetes in Indian villagers (farmers), who are regularly exposed to pesticides (Evangelou et. al. 2016). Thus, there is a need to control the usage of these pesticides or cater them by some means to reduce their effects

2. Current work inference

For sustainable production, easy acceptability and after detailed literature search, it can be concluded that phytase from a fungal source and native nature would be suitable. Extracellular phytase produced here using *A.niger* NCIM 563, under submerged fermentation conditions, suffice this need.

Also, phytase from *A. niger* NCIM 563 is stable over wide range of pH and temperature having optimal pH of 2.5 and temperature of 50°C. It is active in simulated gastric fluid and can be made thermos-stable using skimmed milk or chitosan. Thus, this seems to be an ideal phytase for application in animal feed.

A complete process for sustainable production is developed here with protein and other nutritive source of raw material like chickpea flour. It has been showed that consistent phytase production can be obtained using chickpea sourced from a different location. This, sustainability was not observed on using the agriculture residue wherein the production varied with a change in the residue batch.

The current production of phytase (160 IU/mL in 5.5 days with the productivity of 29 IU/mL/day), using *A.niger* NCIM 563, is found to be the highest than many of the reported *Aspergillus* strains (Table 6.1).

Table 6.1: *Characteristics of phytases from Aspergillus genera under SmF*

Microbial Strain	Media	Fold Increase after RSM	pH	Temp (ºC)	Activity (IU/ml)	Productivity (IU/ml/day)	Enzyme property	Purification	Recovery (%)
Gangoliya S S et. al 2015									
A. fumigatus NF 191	PSM RSM	1.3			101.79	25.4	--	--	--
Sapna and Singh 2013									
A. oryzae SBS 50 (GRAS)	starch (OVAT)	5.4	5	35	15.7	3.9	pepsin and trypsin resistant	--	--
Soni and Khire JM 2007									
A. niger NCIM 563	Dextrin	--	2.5	55	41.47	2.96	--	CC	30
A. niger NCIM 563	Dextrin	--	5	55	10.71	0.77	--	CC	26
Singh et. al. 2015									
A. niger SBS 49	Cane molasses media (OVAT)	23.14	3	50	14.28	7.1	--	--	--
Gunashree and Govindarajulu 2014									
A. niger CFR 335	Potato dextrose broth	--	--	--	9.6	1.92	--	--	--
A. ficuum SGA 01	Potato dextrose broth	--	--	--	8.2	1.64	--	--	--
Coban et. al. 2014									

A. ficuum NRRL 3135	MRS medium	--	--	--	2.27	0.45	--	--	--
Suman et. al. 2013									
A. heteromorphus MTCC 10685	Phytase screening medium (RSM)	1.75	6	30	24.88	5	--	--	--
Casey and Walsh 2003									
A. niger ATCC 9142	corn starch medium	--	5	65	--	--	--	UF, CC, CF	26
Current Work									
A. niger NCIM 563	**Rice bran RSM**	**3.74**	**2.5**	**50**	**268**	**20.6**	**pepsin resistant**	**--**	**--**
A. niger NCIM 563	**Chickpea flour RSM**	**2.4**	**2.5**	**50**	**160**	**29**	**pepsin resistant**	**UF**	**92**

-- No information available

The productivity is higher than many of the expressed phytase produced by bacterial and fungal strains (Table 6.2). The *E. coli* strains which produce phytase have optimum activity at pH 5.5 whereas the currently produced enzyme using *A. niger* NCIM 563 is active at pH 2.5 and is native in origin, which is beneficial characteristic for animal feed application. This would also require less dosage compared to phytase having maximum activity at pH 5.5. Being native in origin, it would be more acceptable than a genetically modified product.

Table 6.2: *Comparison of heterologous expression of phytases with current native phytase*

Phytase source	Production strain	Phytase activity (IU/mL)	Phytase productivity (IU/L/h)	References
B. amyloliquefaciens	*B. subtilis*	2	167	Kim et. al. 1999
B. licheniformis	*B. subtilis*	28		Tye et. al. 2002
E. coli	*S. 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. 2000
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
A. terreus	*H. polymorpha*	4.5	15	Mayer et. al. 1999

Consensus	*H. polymorpha*	13.5	46	
***A.niger* NCIM 563**	**native**	**160**	**1212**	**Current work**

Further, the phytase production is scaled up to 10 L working volume using fermentor, with a single step simultaneous concentrating and purifying downstream process, giving purified enzyme with a maximum yield of 92 %. This, will provide ease in handling and reduce the dosage required, which in turn reduce the cost of application.

Studies showed the ease of storage and formulating concentrated phytase in different forms *viz;* solid and liquid providing means to cater different sectors as per its requirements.

The centrifugally separated fungal biomass generated after the fermentation process is utilized effectively for the production of an industrially important product i.e. chitosan, thus increasing the value of the process and making the process greener/ eco-friendly.

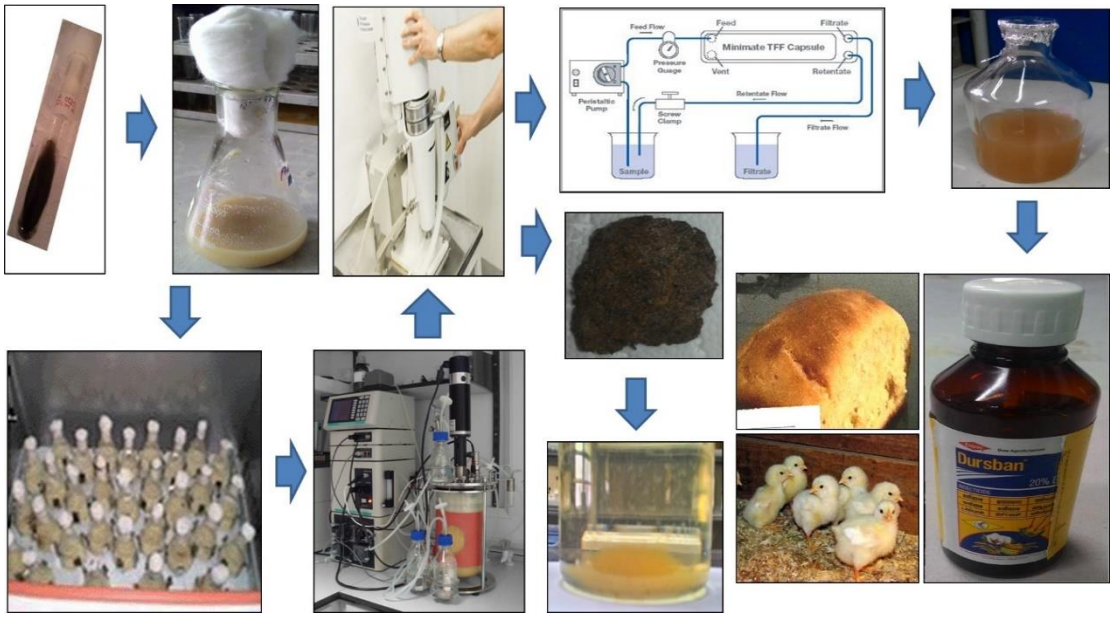
Along with the potential of current phytase in the application of animal feed, it has also shown its potential in degradation of phytic acid in wheat flour. This study has also shown that, the same phytase can be used effectively in bread making process. Being non-toxic, it finds tremendous application in the food sector.

The current study has also shown the use of phytase in degrading the organophosphorus pesticides which are entering into the food chain. Phytase reduces the hazardous effects of pesticides on the health of animals and humans which are caused by entering the food chain.

Phytase, produced here using *A.niger* NCIM 563, seems to be a very important enzyme, which can cater agriculture, food, animal feed and pesticide sectors, which are important for the sustainability of life.

A brief graphical summary is presented here which proves its tremendous potential for commercialization. (Fig 6.1).

Figure 6.1: *Graphical summary of the thesis*



3. Scope for future work

✓ The current method for grading the phytase for commercial application is done at pH 5.5 and at 37°C. But the market demands phytase active at acidic pH. Thus, a new evaluation system should be developed which will be a standard system to evaluate the phytase at acidic pH and temperature optima in the range spanning the application sectors *viz;* poultry, swine, fist, etc.

✓ The study carried out here is a batch process for phytase production. Studies on continuous production would be beneficial to have a continuous production. The current downstream process applied here can easily be made continuous and scaled up for handling larger volume. Thus, continuous production of industrially important phytase can be achieved.

✓ Further scale-up studies needs to be carried to match the industrial need of production. There are many challenges during the scale-up process and they need to be studied for successful and sustainable large-scale production.

✓ The phytase produced here should be applied in poultry feed to match with commercially applied phytase. Also, study the palletization procedure for making a final product for poultry. The commercially available patented technology for thermos-stability for getting granules can be applied and studied, along with our process and compare both the process.

- ✓ A phytase based product need to be developed and in bakery industry and check for its effect on product taste, texture, etc.
- ✓ A phytase based product can also be developed for washing the fruits and vegetables containing trace concentration of organophosphorus pesticides.
- ✓ A product can also be developed to wash hands like hand wash, soap bar, etc., on exposure to pesticides especially for farmers who are directly exposed to them.
- ✓ A phytase immobilized product can be developed to treat the pesticide contaminated water from well, which is used to drink and cook food. The same principle of water treatment can be further utilized to treat the waste water of the pesticide producing company.
- ✓ Phytase has been extensively studied by many researchers and proved beneficial in plant growth promotion. It is applied in the soil to show its effect. Along with this property, phytase in the present study has been proved to be effective in degrading the organophosphorus pesticide. Thus, the soil can be made more productive by degrading the pesticide after the crop is harvested. The excess concentration of pesticide in the soil can be brought to the effective concentration.
- ✓ The action of phytase is studied on three organophosphorus pesticides and can be taken forward to treat many and more pesticides in the same category.
- ✓ A standardized protocol can be developed using phytase for generation of selective myo-inositol phosphate derivative, which has wide applications in pharmaceutical industries.

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Book Chapter

- 2013: Phytase isozymes from *Aspergillus niger*. BIOTECHNOLOGY: Beyond Borders

Conferences and poster presentations

- 2013 – Indo – Mexico Workshop – Production of Acidic Phytase from *Aspergillus niger* NCIM 563 and its mutant
- 2015 – AMI - Submerged production of phytase using *Aspergillus niger* NCIM 563 with high recovery – a greener approach

Poster presentations on National Science Day at CSIR – NCL

- 2014 – Roadway towards production of novel acidic phytase from *Aspergillus niger* NCIM 563

- 2015 – Process optimization for submerged phytase production using *Aspergillus niger* NCIM 563
- 2016 – Phytase production by *A. niger* NCIM 563 under submerged fermentation conditions: Up-scaling, efficient down streaming and novel application in pesticide degradation
- 2017 – Phytase as a versatile enzyme in food, feed and agriculture - An Integrated Approach

ANNEXURE

Publications

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Full Length Research Paper

Influence of pretreatment of agriculture residues on phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions

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The extracellular phytase production by *Aspergillus niger* NCIM 563 was evaluated in medium containing various agriculture residues under submerged fermentation conditions. Phytase production was affected by inorganic phosphate content of agriculture residues which ranged from 2.8 to 8 mg/g. The agriculture residues containing less than 4 mg/g inorganic phosphate supported phytase production with maximum activity of 68 IU/ml in medium containing 1% rice bran on 11th day of fermentation. Addition of glucose up to 5% in fermentation medium containing 1% rice bran, enhanced phytase production. Pretreatment of agriculture residues with water to remove excess inorganic phosphate has significantly enhanced the phytase activity in case of de-oiled rice bran, wheat bran, peanut cake (low and high oil) and coconut cake. Maximum increase of 20.3 times in phytase activity was observed in case of wheat bran as compared to de-oiled rice bran, coconut cake, peanut cake high and low oil in which the increase in phytase activity was 6.85, 6.1, 5.3 and 3.0 times, respectively. Maximum phytase activity of 68 IU/ml was produced on the 11th day of fermentation compared to earlier reported 41.47 IU/ml phytase activity on the 15th day of submerged fermentation using 5% dextrin and 2.5% glucose, thus increasing productivity.

Key words: Phytase, *Aspergillus niger*, submerged fermentation, phytate degradation, poultry feed supplement, agriculture residue.

INTRODUCTION

Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) is a major storage form of phosphorus and the source of inositol in plant seeds (Reddy et al., 1982; Dvorakova 1998). Phytases (EC 3.1.3.8 for 3-phytase and EC 3.1.3.26 for 6-phytase) hydrolyses phytic acid to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates (Mullaney et al., 2000). Since phytase is either absent or present at a very low level in the gastrointestinal tract of monogastric animals (Jongbloed et al., 1992; Maenz and Classen, 1998; Selle and Ravindran, 2007) dietary phytate is not digested in the intestine and consequently accumulates in fecal materials. Phytate thus contributes to phosphorus pollution in areas of intensive animal production. Due to its strong chelating property phytic acid is regarded as an anti-nutritive factor because it forms insoluble complexes with nutritionally important minerals such as calcium, zinc, magnesium and iron, decreasing their bioavailability (Erdman and Poneros-Schneier, 1989; Fox and Tao, 1989). Phytase has been used as feed supplement to improve phosphorus nutrition and reduce phosphorus in excretory products of animals (Ravindran et al., 2001).

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

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ultimate objective is to produce this enzyme at cost effective level and establish conditions for its industrial application. In the present communication we report enhanced phytase production under submerged fermentation conditions using pretreated agriculture residues to remove excess inorganic phosphate which otherwise inhibit phytase production. We report a simple pretreatment of agriculture residue for phytase production and for enhanced phytase activity. Earlier we have reported phytase production by solid-state fermentation (SSF) of agriculture residue using *A. niger* NCIM 563 which was highly active at pH 5.0 (Mandviwala and Khire, 2000) and process for preparation of acidic phytase using dextrin-glucose medium under submerged fermentation condition (Soni and Khire, 2005; Soni and Khire, 2007).

MATERIALS AND METHOD

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 Sigma, BDH and Glaxo.

Fungal strain

A. niger NCIM 563 was used in the present study from NCIM Resource Center, Pune, India. The stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C. Spores for inoculation were obtained by culturing the strain at 30°C on PDA slants for 7 days, followed by washing with 10 ml sterile distilled water containing 0.01% Tween 80.

Agriculture residues

Various agriculture residues such as rice bran, de-oiled rice bran, wheat bran, maize, chickpea, peanut cake (low oil), peanut cake (high oil) and coconut cake were purchased from a local market.

Inorganic phosphate content of agriculture residue

Initial inorganic phosphate content of each agriculture residue was determined by suspending 1 g of residue in 100 ml distilled water for 30 min, centrifuged to remove supernatant and dried in oven at 60°C before use. The liberated inorganic phosphate in the supernatant was determined by a modification of the ammonium molybdate method (Heinohen and Lathi, 1981).

Reduction of inorganic phosphate from agriculture residues

For reduction of inorganic phosphate content residues were treated by two methods. In the first method (Sano et al., 1999) 10 g agriculture residue was suspended in 100 ml of 10 mM $MgCl_2$ pH 8.5 (with 2M KOH) and kept at 4°C for 24 h. The supernatant was removed by centrifugation at 8000 g for 15 min and residue was dried in oven before use. In second method 1 g agriculture residue was suspended in 100 ml distilled water. Supernatant was removed by centrifugation and residue was oven dried before use and checked for phosphate content (Heinohen and Lathi, 1981).

Medium and culture conditions

Fermentation medium for phytase production was according to Shieh and Ware (1968) with few modifications. Soluble starch was replaced with agriculture residue and inorganic phosphorus source was omitted. Thus modified fermentation medium contained (per 100 ml): rice bran 1 g; glucose 5 g; $NaNO_3$ 0.86 g; KCl 0.05 g; $MgSO_4.7H_2O$ 0.05 g; $FeSO_4.7H_2O$ 0.01 g, and pH 5.5 before sterilization.

Fermentation medium (100 ml in 250 ml Erlenmeyer flask) was inoculated with 1% (v/v) of spore suspension (5 x 10^7 spores per ml) prepared by suspending the spores from 7 day old sporulated slant of *A. niger* NCIM 563 grown on PDA in 10 ml of sterile distilled water containing 0.01% (v/v) Tween 80 and incubated at 30°C at 200 rpm. Samples were removed after every 24 h and checked for pH, growth, total residual reducing sugar, extra cellular protein and phytase activity. Various agriculture residues and glucose conc. were used in the fermentation medium to study their effect on production of phytase.

Phytase assay

Phytase activity was measured at 50°C as described earlier (Mandviwala and Khire, 2000). 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 (Heinohen and Lathi, 1981). A freshly prepared four ml solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1 M 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 1 µmole phosphorus per minute under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3 - 5% variability was observed.

Protein estimation

Protein concentration in the culture filtrate was determined by the method of Lowry et al. (1951), using Bovine serum albumin as a standard. The biomass was measured after drying at 105°C for 24 h.

Sugar content

Total residual reducing sugar concentration was estimated by DNSA method (Miller 1959) and HPLC system (Dionex India Limited, Mumbai, India) equipped with UV- or RI-detectors. An ion exclusion column (Aminex HPX-87H; Bio-Rad, Hercules, CA, USA) was used at a temperature of 38°C with 8 mM H_2SO_4 as a mobile phase at a flow rate of 6 ml/min.

RESULTS AND DISCUSSION

During the past decade, the addition of microbial phytase in poultry diets has increased remarkably with substantial documented evidence of its role in release of phytate-bound phosphorus and reduction of otherwise undigested phosphorus in excreta (Selle and Ravindran, 2007). Mondal et al. (2007) has shown that supplementation of microbial phytase in soybean meal based broiler diets

Table 1. Initial phosphate content in various agriculture residues.

Agriculture residue	Phosphate concentration (mg/g)	
	Before treatment	After treatment
Rice bran	2.7	0.47
De – oiled rice bran	4.0	1.08
Wheat bran	4.02	0.56
Peanut cake (low oil)	4.95	2.9
Peanut cake (high oil)	5.89	2.7
Chickpea	2.8	0.95
Maize	2.3	2.2
Coconut cake	8.0	1.43

The values given in the table are the average of three independent experiments with 3 - 5% variation.

containing low phosphorus has increased the retention of Ca and P with compensation of the untoward effect of low phosphorus levels from the diet. Similarly, Panda et al. (2007) has also shown that supplementation of microbial phytase to low non phytate phosphorus diets for broiler chickens improved bone mineralization and retention of nutrients. Only the cost of microbial phytase production should be economical to justify its application in poultry feed. In the present communication we report simple pretreatment of agriculture residues for increased phytase activity under submerged fermentation conditions.

Initial phosphate concentration of various agriculture residues and effect of pretreatment

Preliminary results indicate that initial inorganic phosphate concentration of various agriculture residues ranges from 2.30 mg/g to 8.0 mg/g (Table 1). Treatment of agriculture residues suspended in distilled water with magnesium chloride precipitates the phosphate**,** so the supernatant becomes free of inorganic phosphate. This method is useful for the removal of phosphate from liquid medium like diluted molasses (Sano et al., 1999) but can not be used for removal of phosphate from insoluble material like agriculture residue, as precipitate of inorganic phosphate adheres to the agriculture residue. On the other hand, considerable amount of inorganic phosphate was removed when agriculture residues were suspended in distilled water (Table 1). *A. niger* NCIM 563 grew well and produced phytase in modified basal medium (100 ml in 250 ml flask) containing 1 g rice bran and 5.0 g glucose as carbon source.

Time course of phytase production with untreated agriculture residue

The time course of phytase production under submerged fermentation conditions in medium (100 ml in 250 ml flask) containing 1 g agriculture residue and 5.0 g glucose and inorganic salts is shown in Table 2a. The fungus grew rapidly as indicated in all the agriculture residues with rapid utilization of glucose. Maximum phytase activity was secreted at around 10 to 11th day. Agriculture residues containing less than 4 mg/g inorganic phosphate supported phytase production with maximum activity of 68 IU/ml in rice bran containing medium followed by chickpea and maize (58 and 53.2 IU/ml, respectively). Agriculture residues containing inorganic phosphate more than 4 mg/g supported the growth of the fungus but phytase activity was very low. Earlier we have reported inhibition of phytase secretion in medium containing more than 4 mg/100 ml medium in dextrin-glucose medium. Various reports established that the phytase production in submerged and solid state fermentation is affected by the amount of inorganic phosphorus in fermentation medium (Shieh and Ware, 1968; Reddy et al., 1982; Dvorakova 1998; Mullaney et al., 2000; Vohra and Satyanarayana, 2003). High levels of inorganic phosphorus repress the biosynthesis of phytase. Thus soluble inorganic phosphate in the agriculture residue plays very important role in phytase secretion.

Time course of phytase production with pretreated agriculture residue

Time course of phytase production in medium (100 ml in 250 ml flask) containing 1 g pretreated agriculture residue and 5.0 g glucose and inorganic salts is shown in Table 2b. Pretreatment of agriculture residues with water to remove excess inorganic phosphate has significantly enhanced the phytase activity in case of de-oiled rice bran, wheat bran, peanut cake (low and high oil) and coconut cake. Maximum increase of 20.3 times in phytase activity was observed in case of wheat bran as compared to de-oiled rice bran, coconut cake, peanut cake high and low oil where in the increase in phytase activity was 6.85, 6.1, 5.3 and 3.0 times, respectively. In case of rice bran and chickpea, the pretreatment with water resulted in 75% decrease in phytase activity which can be co-related to reduction of inorganic phosphate content from 2.7 to 0.47 mg/g in case of rice bran and 2.8 to 0.95 mg/g for chickpea. In case of maize decrease in phytase activity was only 25% (Figure 1).

Effect of glucose on phytase production

The effect of easily metabolizable sugar, glucose (1 - 9% w/v) on phytase production, in 1% rice bran fermentation medium indicates that gradual increase in phytase activity was observed when sugar concentration in the fermentation medium was increased from 1 to 5%. Maximum activity of 68 IU/ml was produced in medium containing 5% glucose. Sugar utilization was very rapid

Table 2a. Time course of phytase production using agriculture residues before pretreatment.

Agriculture residues	Phytase activity (IU/ml) on day				
	4	7	10	11	12
Rice bran	14.5	44	56.2	68	60.5
De-oiled rice bran	3.0	5.2	7.0	0.74	0.50
Wheat bran	1.9	2.6	2.6	2.2	2.0
Peanut cake (low oil)	4.15	7.3	9.3	8.9	5.6
Peanut cake (high oil)	3.1	5.2	6.0	6.5	4.2
Chickpea	14.9	40	59	58	53
Maize	14.7	35	49	53.2	49.5
Coconut cake	2.0	3.0	4.0	3.7	2.5

The values given in the table are the average of three independent experiments with 3 - 5% variation.

Table 2b. Time course of phytase production using agriculture residues after pretreatment.

Agriculture residues	Phytase activity (IU/ml) on day				
	4	7	10	11	12
Rice bran	3.9	9.0	13.6	16.1	14.0
De-oiled rice bran	9.0	31.0	47.0	48.0	42.0
Wheat bran	9.0	39.3	45.4	52.9	49.2
Peanut cake (low oil)	7.5	14.0	26.6	28.5	20.6
Peanut cake (high oil)	6.6	22.0	34.2	27.8	20.7
Chickpea	6.0	12.0	18.3	19.4	15.4
Maize	10.8	25.0	38.0	40.5	35.6
Coconut cake	6.5	12.6	23.4	24.4	20.4

The values given in the table are the average of three independent experiments with 3 - 5% variation.

Table 3. Phytase production at different glucose concentration using 1% rice bran.

Days	Glucose concentration in fermentation medium									
	1%		3%		5%		7%		9%	
	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)
2	0.94	0.13	1.0	2.2	0.83	4.4	1.06	6.8	1.35	8.9
4	5.7	0.025	12.5	0.073	12.0	1.9	10.4	3.46	8.8	5.82
7	6.0	0.012	21.0	0.02	36.0	0.19	33.0	2.0	30.0	4.36
9	7.5	0.014	35.0	0.023	58.0	0.059	58.5	1.31	50.5	2.71
11	7.2	0.019	38.0	0.003	68.0	0.019	61.0	0.59	56.0	0.76
12	6.5	0.007	33.0	0.002	58.0	0.011	48.0	0.45	45.0	0.50

The values given in the table are the average of three independent experiments with 3 - 5% variation.

and maximum phytase activity was correlated with minimum reducing sugar in fermentation medium (Table 3). Easily metabolizable sugar e.g. glucose has been reported to increase phytase production by *A. niger* during submerged and/or solid-state fermentation (Vats and Banerjee, 2002; Vats and Banerjee, 2004; Vats et al., 2004).

Effect of rice bran concentration production of phytase

The effect of various concentration of rice bran (0.5 to 2%) in fermentation medium containing 5% glucose, indicates that maximum phytase activity was produced in

Table 4. Comparison of phytase activity in Dextrin-glucose and rice bran-glucose medium.

Fermentation time (days)	1% Rice bran and 5% Glucose medium		5 % Dextrin and 2.5% Glucose medium	
	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)
4	12	1.9	4.17	5.26
7	36	0.19	10	4.3
9	58	0.05	16	3.5
11	68	0.019	23.5	2.6
12	62	0.018	26	2.28
14	59	0.010	31.68	1.75
15	51	0.009	41.47	1.03
16	50	0.009	37	0.91

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

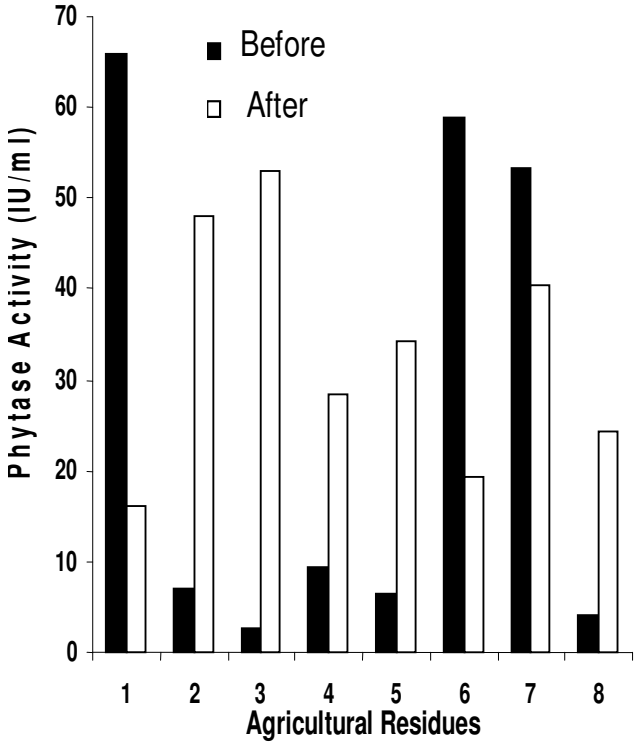

Figure 1. Comparison of phytase activity before and after pretreatment of agriculture residue.
1 = Rice bran; 2 = de =oil rice bran; 3 = wheat Bran; 4 = peanut cake (low oil); 5 = peanut cake (high oil); 6 = chickpea; 7 = Maize; and 8 = coconut cake

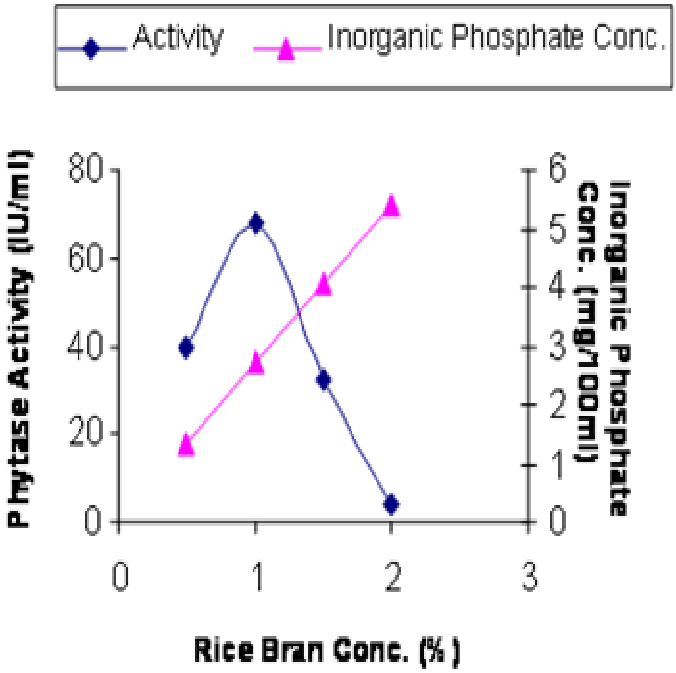

Figure 2. Effect of various concentration of rice bran on phytase production

medium containing 1% rice bran i.e. the medium containing 2.7 mg inorganic phosphate per 100 ml medium (Figure 2). Increasing the concentration of rice bran above 1% concentration resulted into more inorganic phosphate in the medium which resulted in inhibition of phytase activity. Similarly, medium containing 0.5% rice bran resulted in less phytase activity due to insufficient inorganic phosphate in the medium.

Comparison of phytase production in dextrin-glucose and rice bran-glucose medium

Earlier we have reported phytase production under submerged fermentation medium containing 5% dextrin and 2.5% glucose, in which maximum phytase activity of 41.47 IU/ml was produced on 15th day of fermentation medium (Soni and Khire, 2005, 2007). In the present work medium containing 1% rice bran and 5% glucose gave maximum phytase activity of 68 IU/ml was produced within 11th day only (Table 4). Thus fermentation time was reduced by four days with increase in activity by 70%.

Conclusion

Present studies on phytase production under submerged fermentation conditions by *A. niger* NCIM 563, indicates that pretreatment of agriculture residues with distilled water was useful when initial inorganic phosphate content of the residue was above 4 mg/g, which otherwise inhibit phytase production. There was substantial increase in phytase activity when this excess phosphate was removed by pretreatment. Maximum increase (20.3 times) in phytase activity was observed in case of wheat bran. However pretreatment was not useful in case of rice bran where initial inorganic phosphate content was 2.7 mg/g which was reduced to 0.47 mg/g after washing the residue with distilled water which resulted in decrease in phytase activity from 68 IU/ml (before treatment) to 16.1 IU/ml (after treatment). Similarly there was increase in productivity and reduction in fermentation time when agriculture residue was used instead of dextrin in submerged fermentation. Maximum phytase activity of 68 IU/ml was produced on the 11th day of fermentation compared to 41.47 IU/ml activity on the 15th day of submerged fermentation using 5% dextrin and 2.5% glucose. Similarly, cost of any agriculture residue is much cheaper than dextrin. Further experiments in up scaling the process at 10 L fermenter scale and application of phytase in poultry feed are in progress.

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ORIGINAL PAPER

Strain improvement and up scaling of phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions

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Abstract Combination of physical and chemical mutagenesis was used to isolate hyper secretory strains of *Aspergillus niger* NCIM 563 for phytase production. Phytase activity of mutant N-1 and N-79 was about 17 and 47% higher than the parent strain. In shake flask the productivity of phytase in parent, mutant N-1 and N-79 was 6,181, 7,619 and 9,523 IU/L per day, respectively. Up scaling of the fermentation from shake flask to 3 and 14 L New Brunswick fermenter was studied. After optimizing various fermentation parameters like aeration, agitation and carbon source in fermentation medium the fermentation time to achieve highest phytase activity was reduced considerably from 14 days in shake flask to 8 days in 14 L fermenter. Highest phytase activity of 80 IU/ml was obtained in 1% rice bran–3.5% glucose containing medium with aeration 0.2 vvm and agitation 550 rpm at room temperature on 8th day of fermentation. Addition of either bavistin (0.1%), penicillin (0.1%), formalin (0.2%) and sodium chloride (10%) in fermented broth were effective in retaining 100% phytase activity for 8 days at room temperature while these reagents along with methanol (50%) and ethanol (50%) confer 100% stability of phytase activity at 4°C till 20 days. Among various carriers used for application of phytase in feed, wheat bran and rice bran were superior to silica and calcium carbonate. Thermo stabilization studies indicate 100% protection of phytase activity in presence of 12% skim milk at 70°C, which will be useful for its spray drying.

Keywords Phytase · *Aspergillus niger* · Submerged fermentation · Phytate degradation · Poultry feed supplement · Agriculture residue

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Introduction

Phytic acid (myo-inositol hexakisphosphate) is the major storage form of phosphorus in cereals and leguminous plants [21], a form that is not readily assimilated by animals. Monogastric animals, especially swine and poultry, lack sufficient amount of intrinsic phytases to hydrolyze the phytic acid complexes in feed [3] which results in release of undigested phytate phosphorus in feces and urine resulting in severe pollution of water resources [19]. Moreover phytic acid has antinutritive properties as it complexes with proteins and nutritionally important metals such as calcium, zinc, magnesium and iron, decreasing their bioavailability [20]. Phytic acid is also known to inhibit a number of nutritionally important enzymes in vivo [9]. Phytases (myo-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 and 3.1.3.26) belongs to a subclass of histidine acid phosphatases [18] and catalyzes the hydrolytic degradation of myo-inositol hexakisphosphate (Ins P6) to free inorganic phosphate (Pi), to yield lower myo-inositol phosphate esters (Ins P5–P1) and, in some cases, free myo-inositol, making phosphorus available for bioabsorption [12]. Addition of phytase to poultry feed increases the bioavailability of phosphorus from feed and also reduces the amount of phosphorus in manure [27].

Phytase is present in plants, animal tissues and also produced by large number of bacteria, yeasts and fungi [4, 24, 26]. Among fungi strains of *Aspergillus niger* (syn. *A. ficuum*) produce large amounts of extracellular phytase [5] and show more acid tolerance than bacteria and yeasts [13]. In view of its industrial importance the ultimate objective is to produce this enzyme at cost effective level by hyper secretory strains along with effective down streaming and formulation for its industrial application. In the present communication, we report enhanced phytase production by

mutant strain N-1 and N-79 as compared to parent strain. The process has also been up scaled from shake flask to 3 and subsequent 14 L New Brunswick fermenter. Stabilization of phytase at room temperature and higher temperature was studied for its application in mash feed and pelleted feed. Earlier, we have reported phytase production under solid-state fermentation (SSF) [16] and submerged fermentation using dextrin–glucose medium [22, 23] and various agriculture residues [2].

Materials and methods

Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louis, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including Sigma, BDH and Glaxo. Agriculture residues like wheat bran and rice bran were purchased from local market.

Fungal strain

The strain *A. niger* NCIM 563 used throughout the present study was from NCIM Resource Center, Pune, India. It was maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C.

Isolation of mutant

The spores from 7 days old slant of *A. niger* NCIM 563 grown on PDA were collected by scraping in 10 ml sterile saline containing 0.01% Tween 80. After addition 0.2% (v/v) ethyl methyl sulfonate (EMS), the suspension was incubated at room temperature for 24 h followed by UV-irradiation, which resulted in 99% killing of spores after exposure for 4 min. Mutants were selected by spreading UV mutated spores on slightly modified phytase screening medium (PSM) agar plates [11] containing 0.5% calcium phytate and 0.05% $NaNO_3$ and selection of colony with enhanced zone of hydrolysis by phytase.

Medium and culture conditions

Fermentation medium for phytase production was according to Bhavsar et al. [2]. Thus modified fermentation medium 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 (5×10^7 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.

Fermenter studies

After optimizing the production of phytase under shake flask conditions it was further optimized in 3 and 14 L (New Brunswick, USA) fermenter with working volume of 1.8 and 10 L, respectively. The medium and production conditions were selected on the basis of studies carried out in shake flask level. Thus, medium containing 1% rice bran–5% glucose supplemented with inorganic salts was used. The fermenter was run with aeration of 0.2 vvm and agitation (300–500 rpm), respectively (Fig. 3). Inoculum was prepared by inoculating three 250 ml flasks containing 100 ml of sterile agriculture residue containing medium with 5×10^7 spores/ml and incubating at 30°C at 200 rpm for 72 h. The fermenter containing 1.8 L medium was in situ sterilized and then inoculated aseptically. The fermenter was equipped with different controls such as pH, temperature, dissolved oxygen, agitation and antifoam. Samples were withdrawn at regular time intervals and analyzed for protein content, residual sugar and phytase activity. The process was further up scaled to 14 L fermenter using 1% rice bran and 4% glucose supplemented with salts with aeration and agitation at 0.2 vvm and 400 rpm, respectively.

Phytase assay

Phytase activity was measured at 50°C as described earlier [16]. 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 [10]. 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. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 µmol phosphorus/min under standard assay conditions.

Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3–5% variability was observed.

Protein estimation

Protein concentration in the culture filtrate was determined by the method of Lowry et al. [15] using Bovine serum albumin as a standard.

Sugar content

Total residual reducing sugar concentration was estimated by DNSA method [17] and HPLC system (Dionex India Limited, Mumbai, India) equipped with UV- or RI-detectors. An ion exclusion column (Aminex HPX-87H; Bio-Rad, Hercules, CA, USA) was used at a temperature of 38°C with 8 mM H_2SO_4 as a mobile phase at a flow rate of 6 ml/min.

Stability of phytase in presence of various additives

To determine the stability of phytase in presence of various additives the fermented broth was supplemented with various additives like solvents, antibiotics, formalin, glycine and sodium chloride for different time intervals at room temperature and 8–10°C and residual activity was evaluated.

Release of inorganic phosphorus from poultry feed ingredients

To study effect of phytase in removal of phytates from poultry feed ingredients 1 g of each agriculture residue was suspended in 10 ml glycine–HCl buffer (100 mM pH 2.5). To this 1 ml of phytase enzyme (272 IU) is added followed by incubation at 39°C for 2 h to simulate the condition of poultry stomach. The samples were removed after various time intervals up to 2 h and checked for the release of inorganic phosphate from agriculture residue by action of enzyme using ammonium molybdate method [10]. At the same time initial phosphorus of agriculture residue was also checked.

Formulation of phytase in powder form

To evaluate the stability of phytase in powder form the concentrated enzyme was mixed with various carriers, dried to obtain free flowing material and activity was monitored. Thus 4 ml concentrated enzyme (232 IU) was mixed with 10 g sterilized carrier like wheat bran, rice bran, calcium carbonate and silica and dried at 50°C for 2 h (moisture content <4%). Samples were removed after various time intervals and evaluated for phytase activity.

Results and discussions

Isolation of mutants

More than 1,200 mutants were isolated on plates containing 0.5% calcium phytate. Mutants were selected on the basis of small compact colony with large zone of hydrolysis on calcium phytate plate as compared to parent strain. All the positive mutants were quantified for phytase production using rice bran–glucose–salt medium in shake flask condition. Mutants N-1 and N-79 were found to be superior to parent strain as they produce 80 and 100 IU/ml phytase activities on 10.5th day as compared to 68 IU/ml of phytase activity by parent on 11th day. There was difference in amount of protein secretion in these strains (Table 1). Native and SDS-gel electrophoresis of fermented broth of parent N-1 and N-79 show significant difference in protein pattern suggesting their unique nature (Fig. 1). Similar observation was reported by Chelius and Wodzinski [5] during strain improvement of *A. niger* for phytase production using combination of UV radiation and resistance to hygromycin B in which mutant 2DE was found to produce 3.3-fold increase in phytase activity with protein concentration in fermented broth 0.530 and 0.171 μg/μl in parent and mutant 2DE, respectively. In the present studies mutant (N-79) show 54% increase in productivity of phytase in rice bran-glucose-salt fermentation medium.

Up scaling of phytase production (fermenter studies)

After optimizing the production of phytase under shake flask conditions it was further optimized in 3 and 14 L (New Brunswick, USA) fermenter with working volume of 1.8 and 10 L, respectively.

Three-liter fermenter

The production pattern of phytase by *A. niger* NCIM 563 studied using a laboratory scale fermenter (New Brunswick, USA) of 3 L capacity with a working volume of 1.8 L. The fermenter was run with aeration of 0.2 vvm and agitation (300–500 rpm), respectively. Samples were withdrawn at regular time intervals and analyzed for protein content,

Table 1 Phytase production by parent and mutants in rice bran–glucose medium

Culture	Protein (mg/ml)	Activity (IU/ml)	Sp. activity (IU/mg)	Productivity (IU/L/d)
Parent	0.201	68 ± 3.4	338	6,181
Mutant N-1	0.146	80 ± 3.2	548	7,619
Mutant N-79	0.119	100 ± 3.0	840	9,523

The culture was grown under submerged fermentation condition in medium containing rice bran–glucose–salts medium at 30°C with shaking (200 rpm) as described in "Material and methods"

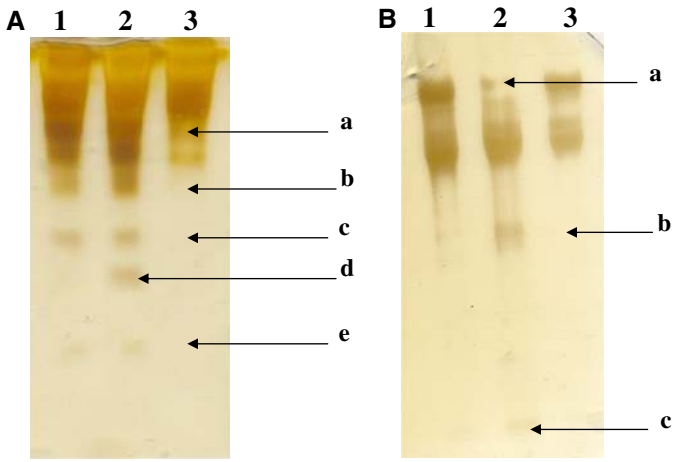

Fig. 1 **a** Comparison of protein profile of extracellular culture filtrate of parent and mutants on native gel electrophoresis (8%). *Lane 1* parent, *lane 2* mutant N-1, *lane 3* mutant N-79. *Arrows a, b, c* and *e* show the difference in proteins of parent *A. niger* NCIM 563 and mutant N-79 and *arrow d* shows the difference of parent and mutant N1. **b** Comparative protein profile of extra cellular culture filtrate of parent and mutants on SDS-PAGE. *Lane 1* parent, *lane 2* mutant N-1, *lane 3* mutant N-79. *Arrows a* and *c* show the difference in protein of parent *A. niger* NCIM 563 and mutant N1 and *arrow b* shows the difference of mutant N79 with parent *A. niger* NCIM 563

residual sugar and phytase activity. The pH of the fermenter was not controlled but was monitored. Initial pH of the medium was around 5.5, which decreased to pH 1.8–2.0 at the end of fermentation. Controlling the pH of the fermenter to 5.5 or at acidic side does not significantly increase or decrease the phytase activity (Data not shown). According to Vats et al. [25] phytase production by *A. niger* van Teighem in laboratory scale fermenter was influence by the pH of fermentation medium. They reported maximum of 141 nKat/ml phytase activity when the medium pH was maintained at pH 2.5 as compared to 17 nKat/ml units at controlled pH 5.5. Effect of various agitation speeds on phytase production indicated that maximum phytase activity of 41 IU/ml (680 nKat/ml) was obtained on 7–8th day at 400 rpm (Fig. 2). Similarly effect of various concentration of glucose in fermentation medium indicates that maximum phytase activity of 51 IU/ml (846 nKat/ml) was produced on 8th day of fermentation when glucose concentration in the medium was 4% (Fig. 3).

Fourteen-liter fermenter

From 3 L fermenter the process was further up scaled to 14 L fermenter using 1% rice bran and glucose (3.5–5%) supplemented with salts with aeration and agitation at 0.2 vvm and 400 rpm, respectively. Maximum phytase activity of 68 IU/ml (1,128 nKat/ml) and 66 IU/ml (1,095 nKat/ml) was obtained at glucose concentration (4 and 3.5%), respectively on the 10th day of fermentation (Fig. 4). Further optimization of agitation speed (400–550 rpm) in 1% rice bran–3.5% glucose–salt medium indicate that maximum phytase activity of 80 IU/ml (1,328 nKat/ml) was obtained on 10th day of fermentation at 550 rpm (Fig. 5). However, in case of *A. niger* van Teighem phytase production in 7 L fermenter was significantly influenced by higher agitation rate. A maximum of 203 nKat/ml phytase units were achieved at 300 rpm as compared to 91 nKat/ml at 500 rpm [25].

Stability of phytase in liquid and solid conditions

Phytase is applied in mash feed and pelleted feed. So stability of phytase enzyme in liquid form was evaluated. Effect of various additives in the liquid enzyme indicates that enzyme preparation can be stabilized in liquid form before mixing into carrier for its easy application. Phytase retains total activity without any contamination in presence of 50% methanol, ethanol, chloroform and acetone at room temperature and 8–10°C (Fig. 6a, b). Effect of various reagents indicates that fermented broth retains its total activity at room temperature by addition of penicillin and bavistin (0.1%), formalin (0.2%), sodium chloride (10%) and glycine (1 M) (Fig. 6a). Sugar alcohols were not effective in increasing the thermostability of phytase (Data not shown), even though there are several reports of increased thermo stability of various enzymes as in case of xylanase from alkalothermophilic *Thermomonospora* sp. [7] and protease from *Bacillus cereus* BG1 [8]. Addition of skimmed milk (8–12%) to phytase enzyme solution was found useful to retain the phytase activity at 70°C (Table 2). Above 70°C skimmed milk was not effective in retaining the thermo stability. Addition of 6–12% milk powder, a source of casein and calcium ions, was found to retain 76–82% of lipase activity when added to fermented broth before spray drying [1, 6]. Use of antimicrobial agents and sodium chloride has been reported to avoid contamination in fermented broth

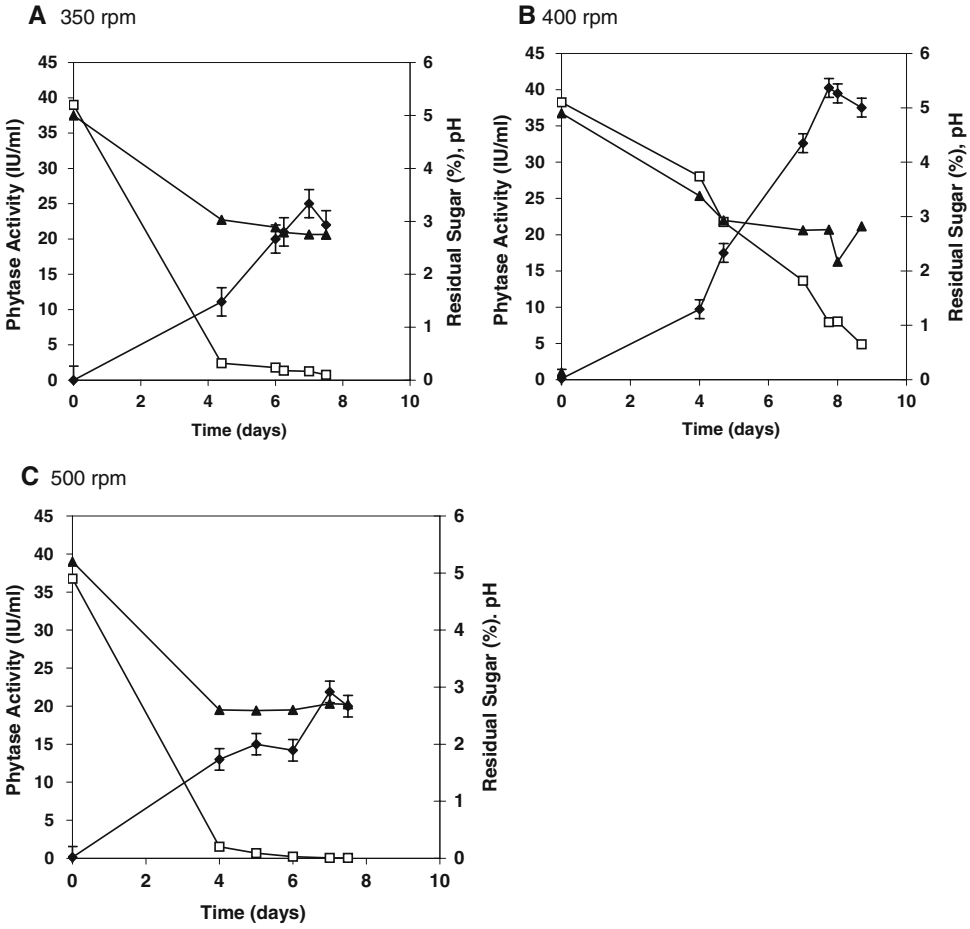

Fig. 2 Effect of agitation on phytase production in 3 L fermenter. **a** 350 rpm, **b** 400 rpm, **c** 500 rpm. *Filled square* phytase activity, *filled triangle* pH, *open square* residual sugar

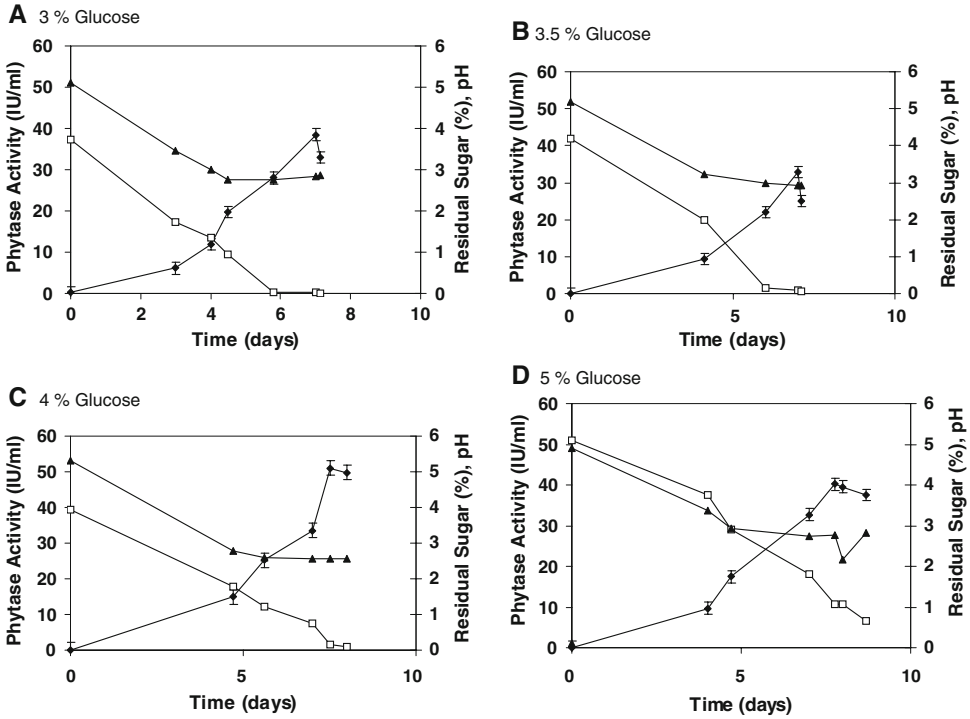

Fig. 3 Effect of glucose concentration on phytase production in 3 L fermenter. **a** 3%, **b** 3.5%, **c** 4% and **d** 5%. *Filled square* phytase activity, *filled triangle* pH, *open square* residual sugar

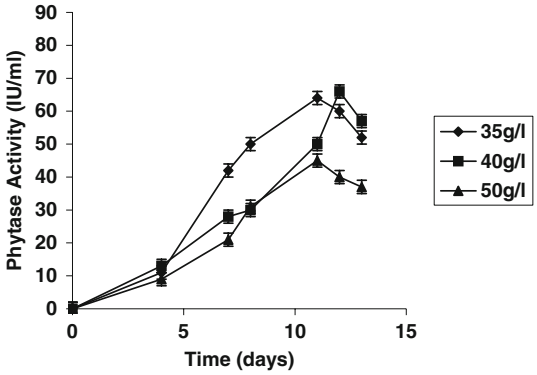

Fig. 4 Effect of glucose concentration on production of phytase in 14 L fermenter. *Filled diamond* 35 g/L, *filled square* 40 g/L, *filled triangle* 50 g/L

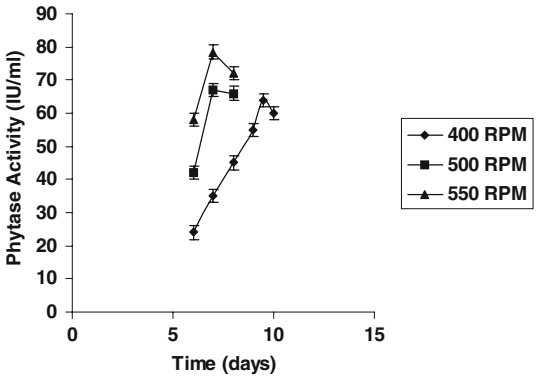

Fig. 5 Effect of agitation speed on phytase production in 14 L fermenter. *Filled diamond* 400 rpm, *filled square* 500 rpm, *filled triangle* 550 rpm

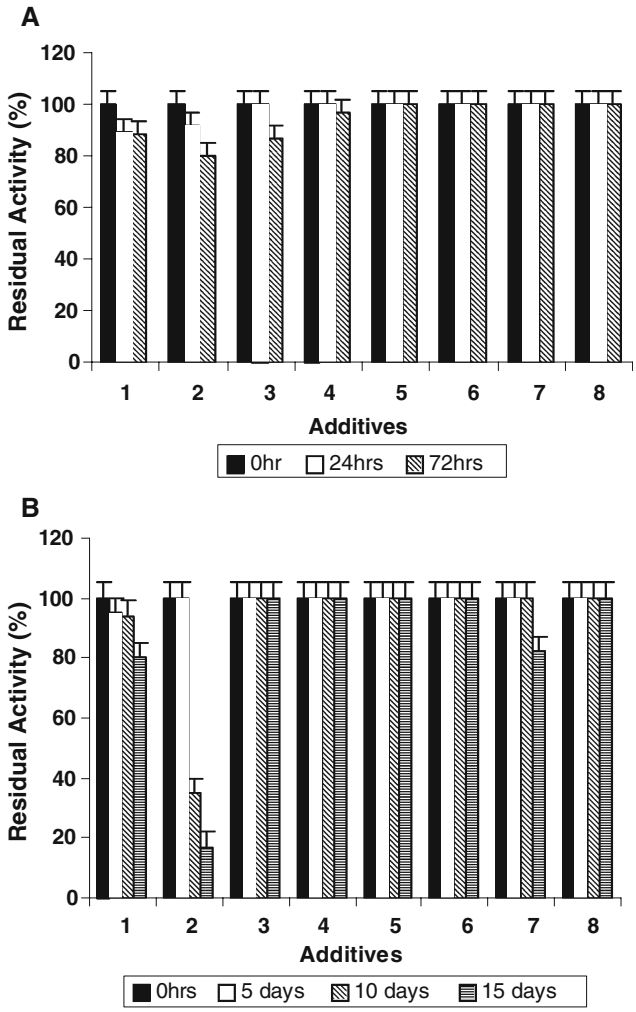

Fig. 6 **a** Effect of various additives on stability of fermented broth (phytase) at room temperature. **b** Effect of various additives on stability of fermented broth (phytase) at 4ºC. 1-Chloroform (50%), 2-acetone (50%), 3-methanol (50%), 4-ethanol (50%), 5-bavistin (0.1%), 6-penicillin (0.1%), formalin (0.2%), NaCl (10%)

and stabilization of alkaline protease activity from *Conidiobolous coronatus* [14].

Release of inorganic phosphorus from poultry feed ingredients

In poultry, the feed supplied is generally digested within 2 h so to determine efficacy of phytase in poultry feed various feed ingredients were treated with phytase at 39°C for 2 h to simulate the condition of poultry stomach and the amount of Pi released was evaluated. From most of the feed ingredients 4.46–12.9 mg/g inorganic phosphorus was found to be released (Table 3).

Bioformulation for application in poultry feed

Among various carriers evaluated wheat bran and rice bran were found to retain total phytase activity in dry free flowing form. However, calcium carbonate and silica gel powders were found to loose phytase activity very rapidly (Table 4). Thus concentrated liquid phytase solution can be mixed with either wheat bran or rice bran and dried at 50°C for 2 h to remove the moisture and can be subsequently mixed with poultry feed as source of phytase. This method is more suitable and cost effective than the existing commercial phytase which is generally available in granular or powder form.

Conclusions

Earlier we have reported phytase production by *A. niger* NCIM 563 under solid-state and submerged fermentation conditions [2, 16, 23]. In the present studies we have successfully isolated hyper secretary mutants (N-1 and N-79) by combination of physical (UV) and chemical (EMS) mutagenesis with increase in productivity of parent strain from 6,181 IU/L per day (1,02,604 nKat/L per day) to mutant strain (N-79) to 9,523 IU/L/d (1,58,081 nKat/L/d).

Table 2 Effect of skim milk on stability of phytase at higher temperature

Skimmed milk powder (%)	Residual phytase activity (%) after 3 min treatment at	
	70°C	80ºC
Control	0	0
3	38 ± 5.0	0
6	58 ± 4.8	0
7	64 ± 4.2	0
8	93 ± 3.6	0
9	99 ± 3.25	0
12	100 ± 3.0	0

Various concentration of skim milk powder was mixed with phytase solution and subjected to 70 and 80ºC for 3 min. It was cooled immediately in ice and residual phytase activity was determined as described in "Materials and methods"

Table 3 Effect of phytase on release of Pi from agriculture residues

Agriculture residue (1 g)	Initial phosphorus (mg)	Phosphorus released after phytase action (mg)
Wheat bran	4.02	7.01
Rice bran	2.7	4.85
De-oiled rice bran	4.0	7.2
Coconut cake	8.0	12.9
Maize	2.3	4.46
Chickpea	2.8	5.12
Soybean	1.7	5.6

Agriculture residue (1 g) was mixed in 10 ml glycine–HCl buffer (100 mM, pH 2.5) and treated with phytase enzyme (272 units) at 39ºC for 2 h. Inorganic phosphorus of the residues was measured before and after phytase treatment as described in "Materials and methods"

Table 4 Effect of carrier on stability of phytase

Carrier	Residual phytase activity (%)		
	0 h	7th day	15th day
Wheat bran	100	100 ± 3.0	100 ± 3.0
Rice bran	100	81 ± 3.0	68 ± 4.0
Calcium carbonate	100	32 ± 4.0	27 ± 4.0
Silica	97	11 ± 5.0	7 ± 5.0

10 g carrier was sterilized for 1 h in Erlenmeyer flask. Four ml concentration enzyme (232 units phytase activity) was mixed with the carrier and dried at 50ºC for 2 h as described in "Materials and methods". Samples were removed after various time intervals and checked for residual phytase activity

Even though UV mutagenesis has been reported by Chelius [5] the chemical mutagenesis by ethyl methyl sulfonate (EMS) has not reported so far for isolation of hyper secretary mutant for phytase production. We have up scaled the fermentation from shake flask to 3 L and subsequently to 14 L fermenter with reduction in fermentation time from 13 to 14 days to shake flask to 7–8 days in 14 L fermenter thus increasing the overall productivity by 54%. Studies on preservation of fermented broth for further down streaming indicate that antimicrobial agents like penicillin and bavistin along with formalin and sodium chloride could be used to stabilize the phytase activity. Thermo stabilization studies indicate100% protection of phytase activity in presence of 12% skim milk at 70°C, which will be useful for its spray drying to obtain phytase enzyme in powder form. Similarly formulation studies indicate that concentrated enzyme can be mixed with agriculture residues like wheat bran or rice bran which may be useful to supply the enzyme in form of free flowing powder. Further work on up scaling the process to 100 L fermenter with hyper secretary mutant N-79 is in progress.

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Combinatorial approach of statistical optimization and mutagenesis for improved production of acidic phytase by *Aspergillus niger* NCIM 563 under submerged fermentation condition

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Abstract Combination of statistical optimization and mutagenesis to isolate hypersecretory strains is studied to maximize phytase production from *Aspergillus niger* NCIM 563 under submerged fermentation. The overall results obtained show a remarkable 5.98-fold improvement in phytase production rates when compared to that using basal medium. Optimization of culture conditions from parent strain is studied first by the Plackett–Burman technique to evaluate the effects of 11 variables for phytase production. The results showed that glucose, $MgSO_4$, KCl, incubation period, and $MnSO_4$ are the most significant variables affecting enzyme production. Further optimization in these variables, using a central composite design technique, resulted in 3.74-fold increase in the yield of phytase production to 254,500 U/l when compared with the activity observed with basal media (68,000 U/l) in shake flask. Our experiments show that the phytase from *A. niger* NCIM 563 exhibits desirable activity in simulated gastric fluid conditions with low pH and also improved thermostability when compared to commercial phytase. The improved yield demonstrates the potential applicability of phytase enzyme as a source of phytase supplement for phosphorus nutrition and environmental protection in animal feed industry. Physical and chemical mutagenesis experiments were carried out in parallel to isolate hypersecretory mutants that could possibly further enhance the enzyme production. Using optimized media conditions of the parent strain, our results show that mutant strain *A. niger* NCIM 1359 increased the phytase activity by another 1.6-fold to 407,200 U/l.

Keywords Phytase · *Aspergillus niger* · Mutagenesis · Statistical optimization · Animal feed

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Introduction

Phosphorus is a nonrenewable and the third most expensive nutrient in poultry production, after energy and protein. Although, plants store phosphorus in the form of phytate (inositol 6-phosphate), it is largely unavailable to monogastric animals due to lack of adequate levels of phytase. Therefore, inorganic phosphorous supplement of animal feeds is still the method of choice (Dvorakova 1998). Phytate acts as an antinutritional factor since it causes mineral deficiency by chelating metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+} (Mitchell et al. 1997). The unutilized phytate and phosphorus supplements are excreted by animals and this creates global ecological problems (Mandviwala and Khire 2000). The use of phytase in animal feed will overcome the antinutritional effects of phytate, decrease environmental pollution, increase mineral bioavailability, and abolish the addition of inorganic phosphate in animal feed (Reddy et al. 1982).

The available phytase preparations used as feed additives are of fungal origin, produced by recombinant strains under

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submerged fermentation condition, and have shortcomings especially with regard to obtaining diluted product, sensitivity to heat, inactivation under low pH conditions present in the stomach of animals (Afinah et al. 2010), and high product recovery costs. These disadvantages need to be alleviated while at the same time producing phytase with high yield and purity. Micro-organisms produce low levels of phytase and it would be beneficial if these production rates be improved with desirable process features by employing statistical techniques and strain improvement programs.

A one variable approach at a time (OVAT) was used in our preliminary studies (Bhavsar et al. 2008) for development of production medium for phytase production at low pH. Also, we have reported high levels of phytase (optimum pH 5.0 by solid state fermentation using statistical techniques that involve a combination of Plackett–Burman (PBD) and a Box–Behnken design (Bhavsar et al. 2011). Despite the importance of phytase in poultry feed, there are very few reports on its enhanced production by statistical optimization and mutagenesis due to its low expression (Bogar et al. 2003; Chadha et al. 2004; Tanyildizi et al. 2005; Vohra and Satyanarayana 2002). The present investigation therefore aims at achieving multifold improvement in phytase production using *Aspergillus niger* NCIM 563 under submerged fermentation (SmF) by statistical optimization along with mutagenesis studies.

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 (Parekh et al. 2000). Mutagenic procedures can be carried out in terms of type of mutagen and dose to obtain mutant types that may be screened for improved activity. Primary screening process studies for selection of mutant on phytase screening media showed lack of specificity and sensitivity to discriminate between phytase and acid-phosphatase activity. The time-consuming statistical optimization and mutagenesis experiments were therefore carried out in parallel because it may be reasonably expected that promising mutant strains may also be benefited from optimized media formulation obtained with the parent strain. Experiments with mutant strains were therefore carried out on the basis of this conjecture and the results obtained validate the employed approach.

Materials and methods

Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade. Rice bran was purchased from the local market.

Microorganism, culture media, and enzyme production

A. niger NCIM 563, used in the present study, was obtained from the 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 °C. Spores for inoculation were obtained by culturing the strain at 30 °C on a PDA slant for 7 days, followed by washing with 10 ml sterile saline containing 0.01% Tween 80.

The basal fermentation medium according to Bhavsar et al. 2008 contained rice bran, glucose, $NaNO_3$, $MgSO_4.7H_2O$, KCl, and $FeSO_4.7H_2O$. The fermentation medium for optimization via statistical design of experiments included additional components, namely, $MnSO_4$, dextrin, and Tween 80 at various concentrations as required by the experimental design. The fermentation medium pH 5.5 before sterilization (100 ml in 250 ml Erlenmeyer flask) was sterilized by autoclaving at 121 °C for 15 min. On cooling the fermentation medium was inoculated with desired spore suspension and the incubation time adjusted as per the experimental design. Flasks were incubated at 30 °C at 200 rpm and samples removed after every 24 h. Enzyme production was expressed as enzyme activity U/ml.

Analytical methods

Phytase activity was measured using 100 mM Glycine–HCl buffer, pH 2.5 at 50 °C for 30 min as described earlier (Soni and Khire 2007). One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 μmol phosphorus per minute under standard assay conditions. Total residual reducing sugar concentration was estimated by DNSA method (Miller 1959). Protein concentration in the culture filtrate was determined using bovine serum albumin as a standard (Lowry et al. 1951).

Partial purification of enzyme

After fermentation, the 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 minimum volume of 100 mM Glycine–HCl buffer, pH 2.5 and the salt was removed by passing through Sephadex G-25 column. Active fractions were concentrated through YM-30 membrane (Millipore) and used for phytase activity measurement.

Release of phosphorus from soybean in simulated gastric fluid

One gram soybean meal was dissolved in 9 ml simulated gastric fluid (SGF; 250 mM Glycine–HCl containing 2.0 mg/ml NaCl and 3.2 mg/ml pepsin) and the pH was adjusted with HCl or NaOH to 1.5, 2.0, 2.5, 3.5, 5.5, or 6.5 as required. The solutions were incubated with agitation at 37 °C for 30 min, and the pH again adjusted to the corresponding values. One milliliter partially purified enzyme was then added to the solutions and incubated by agitation at 37 °C for 60 min. The amount of released phosphorus was determined as described in analytical methods.

Thermo-stabilization of phytase

Temperature stability profile (80 °C over a 5-min time course in 100 mM Glycine–HCl buffer pH 2.5) of partially purified phytase was determined in presence of 10% poly vinyl pyrrolidone (PVP). The residual phytase activity was determined by modified ammonium molybdate method as described in analytical methods.

PBD studies for screening of culture variables

The effect of 11 variables (X_i) viz, 5 basal medium variables and 6 additional variables, likely to influence phytase production were chosen for screening studies using PBD. The PBD is a two-factorial design that can be expected to identify critical chemical and physical parameters required for elevated enzyme production by screening the N variables in $N+1$ experiments (Plackett and Burman 1946). Each independent variable was tested at two levels, a high (+1) level and a low (−1) level. A set of 12 experiments was thus carried out to determine phytase production as per the PBD of experiments (Table S1). The complete matrix for screening was designed using a standard Plackett–Burman orthogonal array constructed using Design Expert (DE) software Version 7.1.2, Stat-Ease, Minneapolis, MN, USA.

The effect E (X_i) of the tested variable X_i was determined by:

$$E(X_i) = \frac{2[\sum R(H) - \sum R(L)]}{N} \quad (1)$$

where, N denotes the number of trials with R (H) and R (L) representing a response obtained at either the high or low level, respectively, for the variable (X_i) from an experiment. Note that R (H) and R (L) are summed to obtain the average response at the high/low level, respectively, and their difference calculated to estimate the effect E (X_i). The variables with higher percent contributions were considered to influence phytase activity and chosen for further optimization studies. The percent contribution was obtained by taking the E (X_i) of each variable and dividing it by total sum of squares of E (X_i) for all the variables (Table S2).

Central composite design studies with chosen variables

Based on the results of the PBD, a central composite design (CCD) of experiments was carried out to arrive at optimized levels of the chosen variables that further maximize phytase production. According to CCD, the total number of experiments is 2^k+2k+CP, where k is the number of independent variables and CP is the number of repetitions of the experiments at the center point. In order to ensure the design is rotatable, star points were set at ±(α) value at 1.459. This was used to develop a mathematical correlation between five variables on production of phytase. Each variable was studied at five levels, i.e., $-\alpha$, −1, 0, +1, and $+\alpha$ as shown in Table 1. A matrix of 50 runs with 5 variables was generated using DE software to generate the response surface plot (Table S3). The optimum values of the variables and the behavior of the system were studied using the quadratic equation model. The quality for the fit of the second order equation model was expressed by the coefficient of determination R^2, and its statistical significance was determined by F test. To validate the response surface model, the maximum value was confirmed according to the optimum conditions predicted by the model. All experiments are carried out in triplicates and their mean values are presented.

Isolation of *A. niger* mutant

The spore suspension (10^7/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 samples were suitably plated on PDA plates. The colony forming unit per milliliter (CFU/ml) was used then to calculate the 99% kill time. Mutants obtained were selected by spreading the chemically treated spore suspension on phytase screening medium (PSM) agar plates containing 0.1% calcium phytate and 0.05% $NaNO_3$ and the selection of colony was dependent on observation of an enhanced zone of hydrolysis. Mutants were confirmed for phytase production using optimized medium under SmF and samples were removed periodically and checked for enzyme production as described earlier.

Results

Phytase production in SmF using basal medium

Our preliminary study on the effect of different process parameters on phytase production was carried using a classical OVAT approach. Among the various agriculture

Table 1 Selected variables and their assigned levels by CCD

Variable code	Variable with designate	Coded level and uncoded level				
		$-\alpha$	−1	0	+1	$+\alpha$
A	Glucose (g %)	1.762	3.0	5.50	8.0	9.238
B	$MgSO_4 \cdot 7H_2O$ (g %)	0.025	0.04	0.07	0.1	0.115
C	Incubation period (days)	1.77	4	8.5	13	15.23
D	KCl (g %)	0.025	0.04	0.07	0.1	0.115
E	$MnSO_4$ (g %)	0.001	0.005	0.012	0.02	0.024

residues tested, rice bran showed maximal phytase activity. Optimization by OVAT showed that *A. niger* NCIM 563 produced best phytase activity (68,000 U/l) at low pH 2.5 on the 11th day under SmF conditions in basal medium. The basal fermentation medium (100 ml in 250 ml Erlenmeyer flask) consisted of 1 g rice bran, 5 g glucose, 0.86 g $NaNO_3$, 0.05 g KCl, 0.05 g $MgSO_4$, and 0.01 g $FeSO_4$ with inoculum level 1 × E+07 spores/ml. Other components, viz., dextrin, $MnSO_4$, and surfactant (Tween 80), also have a positive effect on phytase production (Bhavsar et al. 2008) and were thus included in the further studies for optimization of media formulation.

Screening of important culture variables

The potential effect of 11 variables on phytase production (glucose, rice bran, $NaNO_3$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, KCl, incubation period, inoculum level, Tween 80, $MnSO_4$, and dextrin) were evaluated using PBD. The PBD design matrix for experimental design of the selected variables is shown in Table S1 along with the obtained responses on phytase production. Maximum phytase production of 99,900 U/l was observed in trial number 2. The variables and their effect E (X_i), as calculated by Eq. (1) and percent contribution (Table S2) show that phytase production is significantly affected by glucose, $MgSO_4$, KCl, incubation period, and $MnSO_4$. These factors account for 97.8% of the total contribution while the remaining variables ($NaNO_3$, $FeSO_4$, inoculum level, Tween 80, and dextrin) account for only 2.2%. The variables identified by PBD to be insignificant and are therefore maintained at mean levels in further optimization studies. The best linear fit regression model obtained using the significant PBD variables gave a model *F* value 108.4 by ANOVA. This implies that there is only a 0.01% chance that this "Model F-Value" could occur due to noise. The coefficient of determination is R^2=0.98 and provides a measure of how much of the variability in the observed response values can be explained by the analysis.

Optimization of the screened variables

The five medium components (Table 1) identified by PBD, as significant factors, for phytase production were further optimized by CCD. The CCD design matrix and levels of the variables comprising and the obtained response values are shown in Table S3. It may be seen that trial number 39 showed the highest phytase activity (259,800 U/l) while trial number 12 showed the lowest phytase activity (2,100 U/l). The wide range in activity shows the sensitivity of the process to experimental conditions and the need for process optimization. The obtained results were fitted to polynomial equation to three significant digits:

$$\hat{y} = 91700 + 48300A + 48100D + 46000AD - 10900A^2 + 15600B^2 - 22700D^2 \quad (2)$$

where $\hat{y}$ is the predicted response, 91700 is the intercept with variables *A*, *B*, and *D* are corresponding to glucose, $MgSO_4$, and KCl. It may be seen that an interaction term *AD* is present in the above model. Non-significant variable and interaction terms were excluded by systematically carrying out ANOVA analysis to obtain the above reduced but improved model description Eq. (2). The Fisher *F* test (Table 2) for the model gave a very low probability value (*P* model> *F*=0.0001) and this favorably suggests a very high significance of the regression model. The coefficient of determination (R^2) is 0·937 for phytase production, suggesting that 93·7% of the variability is explained in the model. The adjusted R^2 (0.93) is also very high and corroborates the significance of the model. The value of correlation coefficient (predicted R^2) for phytase production is 0·87 and suggests a strong agreement between the observed and model predicted values of phytase production. Adequate precision measures signal to noise ratio and a ratio greater than four is desirable. The obtained value of adequate precision (= 33.5) for phytase production indicates that the model Eq. (2) can be used to navigate the design space. The model *F* value of 106.3 implies there is only a 0.01% chance that this model *F* value occurs due to noise. Values of $p>F$ (<0.05) indicate that the model terms *A*, *D*, *AD*, B^2, and D^2 in Eq. (2) are significant. The values of $p>F$ did not satisfy this criteria for the other terms.

Validation of model

To validate the model, a time course for phytase production was carried out under conditions predicted by RSM as

Table 2 ANOVA study for the reduced quadratic model[a]

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*p* value Prob > *F*
Model	2.4E+11	6	4.1E+10	106.3	<0.0001
A	8.5E+10	1	8.5E+10	219.0	<0.0001
D	8.4E+10	1	8.4E+10	217.2	<0.0001
AD	6.8E+10	1	6.8E+10	174.5	<0.0001
A^2	1.5E+09	1	1.5E+09	3.9	0.056
B^2	3.0E+09	1	3.0E+09	8.0	0.0072
D^2	6.5E+09	1	6.5E+09	16.8	0.0002
Residual	1.6E+10	43	3.8E+08		
Lack of fit	1.6E+10	36	4.4E+08	5.0	0.016
Pure error	6.2E+08	7	8.9E+07		
Cor total	2.6E+11	49			

Coefficient of determination (R^2) 0.94; correlation coefficient (adjusted R^2) 0.93; predicted R^2 0.87; adequate precision 33.5

[a]Eq. (2)

shown in Fig 1. The experimental response for phytase production was 254,500 U/l which is very close to the predicted response. A sharp decrease in phytase activity after 13th day is observed due to depletion of glucose in fermentation medium. The 3D response surface plot (Fig S1) shows the positive effect of the interaction between glucose and KCl for phytase production. An increase in phytase production is seen as the concentration of these variables is increased towards the +1 level. Overall, optimum values of the tested variables were obtained as 8% glucose, 0.1% $MgSO_4$, 0.1% KCl, and 0.005% $MnSO_4$ with 13 days of incubation period.

Release of phosphorus from soybean in simulated gastric fluid

Partially purified phytase exhibited high efficacy in phytate hydrolysis at different pH releasing 400, 1340, 1342, 800.7, 240, and 882 mg inorganic phosphorus/kg soybean meal at pH 1.5, 2.0, 2.5, 3.5, 5.5, and 6.5, respectively, under simulated gastric conditions.

Thermo-stabilization of phytase

The phytase thermostability was improved at 80 °C in presence of PVP which imparts 100% protection to enzyme up to 1 min (Fig S2). This thermostability profile is superior when compared to Allzyme and Natuphos (commercial phytase) that show only 50% and 75% residual activity (Casey and Walsh 2003).

Isolation of phytase producing *A. niger* mutant

Mutants were selected on the basis of small compact colony with large zone of hydrolysis on calcium phytate plate as compared to parent strain. The mutants *A. niger* NCIM 1359

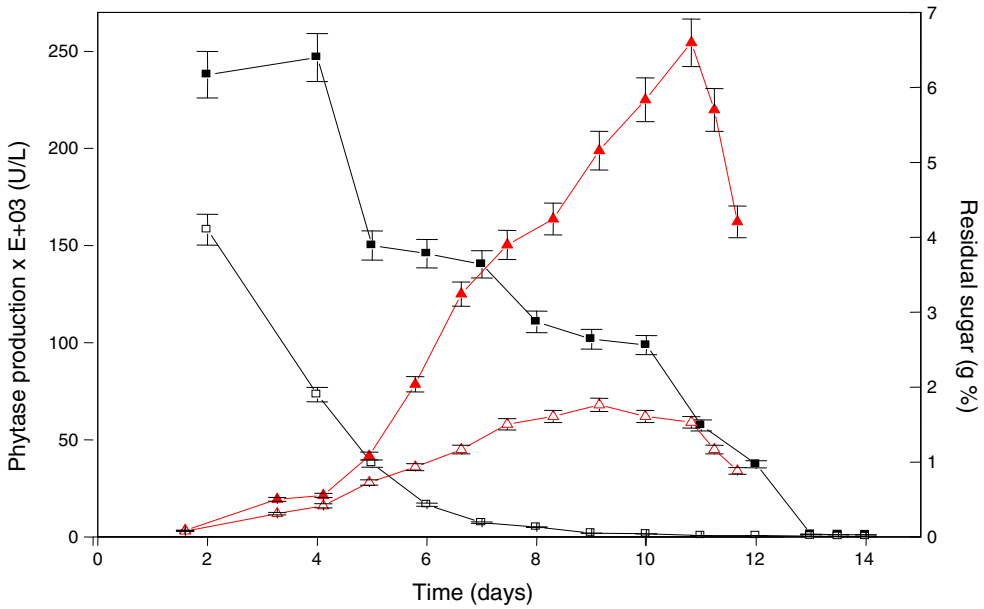

Fig. 1 Time course of phytase production under SmF by *A. niger* NCIM 563 using OVAT and RSM optimized media. *Triangle* phytase production (OVAT), *filled triangle* phytase production (RSM), *square* residual sugar (g %; OVAT), *filled square* residual sugar (g %; RSM)

and 1360 also exhibited 407,200 and 389,700 U/l phytase activity on 13th and 10th day, respectively using RSM optimized media. A remarkable difference among these two mutants strains was the delay in the sporulation time of the mutant strains (7th day) as compared to parent (4th day). The morphological patterns of mutant strains such as mycelial morphology, sporangium shape, and sporangium size were evaluated by using microscopy (Fig. 2).

Discussion

Although a broad range of microorganisms can produce phytase, their expression levels of phytase are too low for economic considerations. Productivity of any fungal fermentation is affected by process parameters and media composition and therefore the present investigation was performed to statistically optimize the medium components for the production of the phytase from *A. niger* using PBD and CCD methodologies. All significant variables involved in phytase production were evaluated by PBD because it can test a large number of variables while avoiding the loss of any essential information in subsequent optimization studies. Based on analysis of PBD, five key variables, viz., glucose, $MgSO_4$, KCl, incubation period, and $MnSO_4$, were found to affect the phytase production by *A. niger*. This was followed by use of a multifactorial response surface approach employing CCD, an effective design strategy, for studying the effects of key variables and their mutual interactions. This study resulted in an overall 3.74-fold enhancement in phytase activity of parent strain which is 274% higher than that observed with the basal medium.

Phytase from *A. niger* NCIM 563 is more resistant to pepsin and therefore releases more inorganic phosphorus from soybean meal under emulated gastric conditions over a much broader pH range (1.5–6.5) as compared to *Yersinia rhodei* and commercial phytase from *A. niger* (Huoqing et al. 2008). This brings out a significant advantage of *A. niger* over other phytases as it functions well under gastric conditions.

Liquid and dry enzyme formulations are used on a commercial scale by the feed industry. We have earlier reported the liquid formulation specifically used in mash feed, which can be added to the feed after pelleting (Shah et al. 2009). This is done to avoid heat inactivation of the phytase that may occur due to temperature of pelleting process (60–95 °C). However, this technique has certain disadvantages such as difficulties in mixing (due to small enzyme amounts) leading to the heterogeneous distribution of enzyme in feed. In addition, specialized equipment is needed to add liquids to the feed after pelleting and this is not currently available at most feed mills due to the extra cost involved. Thermostability is considered an important and useful criterion for industrial application of phytase. As shown, enzyme from *A. niger* NCIM 563 is more heat stable and hence should have great potential use in pelleted feed than available commercial preparations.

In parallel with media optimization studies, mutagenesis studies were conducted, using hydroxyl amine, for obtaining a hypersecretory strain. The selection of mutants was based on enhanced hydrolysis zone on PSM plates. 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 production (407,200 U/l) and improved the yield 1.6 times

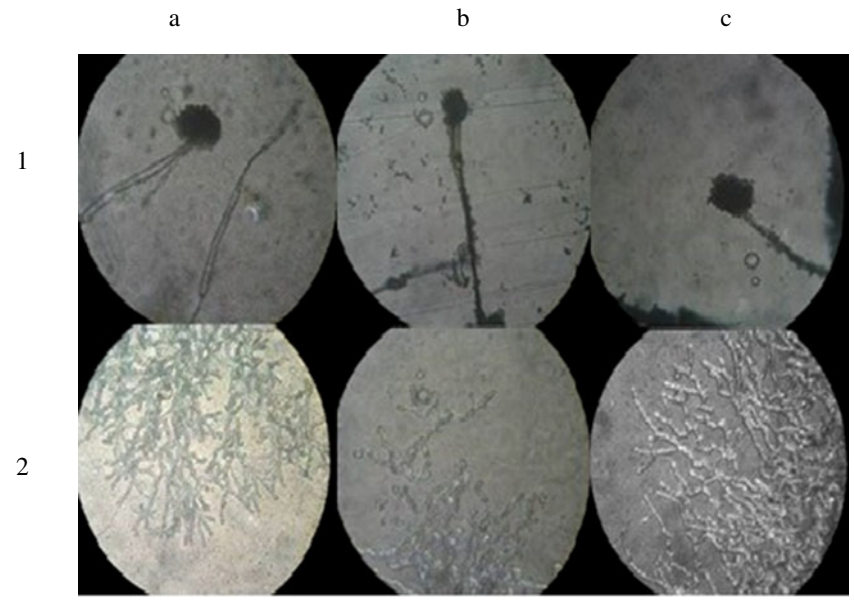

Fig. 2 Morphological variations amongst parent and mutant strains. *1* Sporangium shape. *2* Mycelial morphology. *a A. niger* NCIM 563, *b A. niger* NCIM 1359, *c A. niger* NCIM 1360

as compared to production by parent strain. 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.

Strain improvement is associated with disadvantages, such as low mutation frequency in the desired gene. Even if mutants with the desired phenotype are isolated, there is no guarantee that the mutation has occurred in the gene of interest. These shortcomings need to be alleviated and with the recent development of gene-cloning and sequencing techniques we can now locate whether mutation has occurred in gene by a single base change of DNA (an insertion or a deletion). 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 combined results of statistical optimization and mutagenesis show a remarkable 6-fold improvement in phytase production rates to 407,200 U/l and suggest its potential for industrial application. Also, *A. niger* outperforms the phytase production rates by an exceptional 32-fold increase in comparison to other organisms, viz., *Sporotrichum thermophile* with 10,100 (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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Supplementary data belonging to the article:

Combinatorial approach of statistical optimization and mutagenesis for improved production of acidic phytase by *Aspergillus niger* NCIM 563 under submerged fermentation condition

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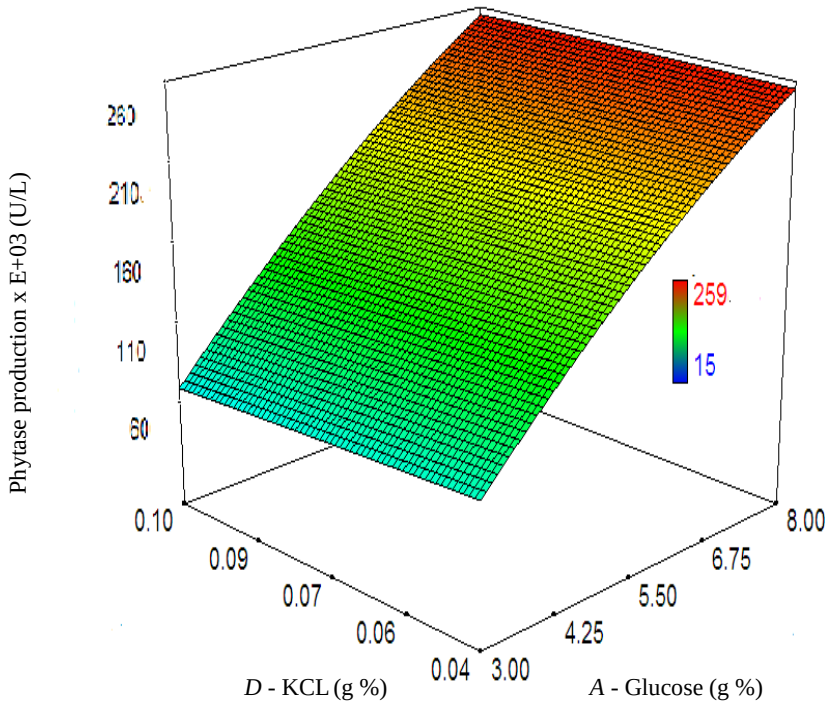

Fig S1: Three-dimensional graph showing the interaction of glucose and potassium chloride on phytase production *by A. niger* NCIM 563 in SmF.

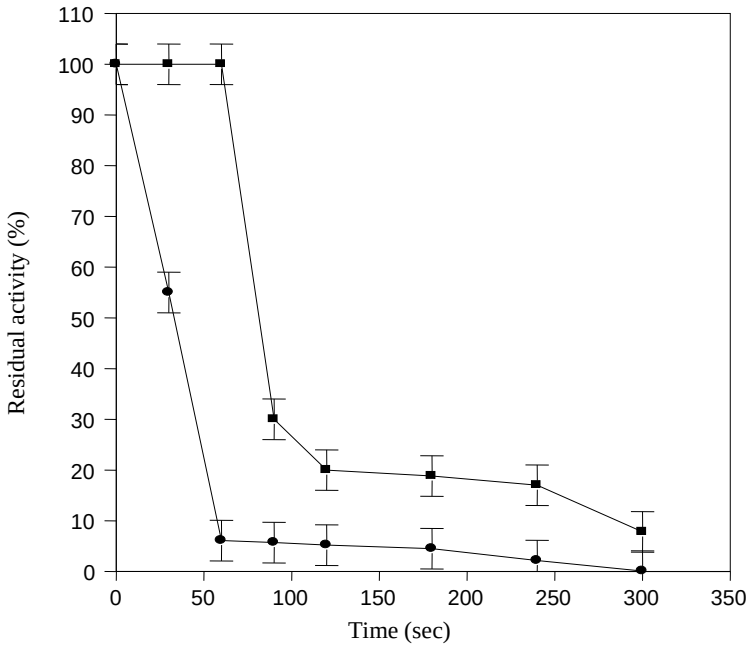

Fig S2: Thermostability profile of phytase and phytase-PVP following heating to 80ºC over the indicated time course.

—■— Phytase; —●— Phytase + PVP

Table S1: The variables, levels, PBD matrix and corresponding values of phytase production

Trial number	Factor level											Phytase production x E+03 (U/L)
	A Glucose (g %)	***B*** Rice bran (g %)	***C*** $NaNO_3$ (g %)	***D*** $MgSO_4$ (g %)	***E*** $FeSO_4$ (g %)	***F*** KCl (g %)	***G*** Incubation Period (day)	***H*** Inoculum level (spores/ml)	***J*** Tween 80 (g %)	***K*** $MnSO_4$ (g %)	***L*** Dextrin (g %)	
1	-1(3)	-1(0.8)	1(1.1)	-1(0.04)	1(0.04)	1(0.1)	-1(4)	$1(10^9)$	1(0.015)	1(0.02)	-1(0.5)	8.14
2	1(8)	1(1.5)	1(1.1)	-1(0.04)	-1(0.01)	-1(0.04)	1(13)	$-1(10^5)$	1(0.015)	1(0.02)	-1(0.5)	99.91
3	1(8)	1(1.5)	-1(0.7)	1(0.1)	1(0.04)	1(0.1)	-1(4)	$-1(10^5)$	-1(0.012)	1(0.02)	-1(0.5)	9.55
4	1(8)	-1(0.8)	1(1.1)	1(0.1)	-1(0.01)	1(0.1)	1(13)	$1(10^9)$	-1(0.012)	-1(0.005)	-1(0.5)	60.50
5	-1(3)	-1(0.8)	-1(0.7)	-1(0.04)	-1(0.01)	-1(0.04)	-1(4)	$-1(10^5)$	-1(0.012)	-1(0.005)	-1(0.5)	4.06
6	1(8)	-1(0.8)	-1(0.7)	-1(0.04)	1(0.04)	-1(0.04)	1(13)	$1(10^9)$	-1(0.012)	1(0.02)	1(1.0)	86.50
7	1(8)	1(1.5)	-1(0.7)	-1(0.04)	-1(0.01)	1(0.1)	-1(4)	$1(10^9)$	1(0.015)	-1(0.005)	1(1.0)	5.12
8	1(8)	-1(0.8)	1(1.1)	1(0.1)	1(0.04)	-1(0.04)	-1(4)	$-1(10^5)$	1(0.015)	-1(0.005)	1(1.0)	9.60
9	-1(3)	1(1.5)	1(1.1)	-1(0.04)	1(0.04)	1(0.1)	1(13)	$-1(10^5)$	-1(0.012)	-1(0.005)	1(1.0)	48.25
10	-1(3)	1(1.5)	-1(0.7)	1(0.1)	1(0.04)	-1(0.04)	1(13)	$1(10^9)$	1(0.015)	-1(0.005)	-1(0.5)	36.55
11	-1(3)	-1(0.8)	-1(0.7)	1(0.1)	-1(0.01)	1(0.1)	1(13)	$-1(10^5)$	1(0.015)	1(0.02)	1(1.0)	42.93
12	-1(3)	1(1.5)	1(1.1)	1(0.1)	-1(0.01)	-1(0.04)	-1(4)	$1(10^9)$	-1(0.012)	1(0.02)	1(1.0)	6.11

The values given are the average of three independent experiments.

Table S2: Effects of PBD

Code	Variable	Effect* x E+03	% Contribution
A	Glucose	20.85	10.48
B	Rice Bran	-1.04	0.026
C	$NaNO_3$	7.96	1.53
D	$MgSO_4 .7H_2O$	-14.45	5.03
E	$FeSO_4 .7H_2O$	-3.34	0.27
F	KCl	-11.37	3.12
G	Incubation period	55.34	73.77
H	Inoculum level	-1.89	0.087
J	Tween 80	-2.12	0.11
K	$MnSO_4$	14.84	5.31
L	Dextrin	-3.36	0.27

*Eqn 1

Table S3: The CCD experimental design: the level of variables and corresponding results

Trial number	Factor level					Phytase production x E+03 (U/L)
	A **Glucose**	***B*** **$MgSO_4$**	***C*** **Incubation period**	***D*** **KCl**	***E*** **$MnSO_4$**	
1	+ α	0	0	0	0	97.00
2	-1	1	1	1	-1	37.00
3	0	0	0	1	0	97.74
4	-1	1	-1	1	1	32.34
5	0	0	0	0	0	114.48
6	1	1	-1	1	-1	176.91
7	-1	1	1	-1	1	17.04
8	-1	-1	-1	1	1	33.90
9	-1	-1	-1	-1	-1	17.21
10	1	-1	1	1	-1	192.40
11	1	-1	-1	-1	-1	29.54
12	0	0	0	- α	0	1.50
13	0	0	0	0	+ α	97.17
14	-1	-1	1	1	-1	26.27
15	1	-1	-1	1	1	89.20
16	1	-1	-1	1	1	199.90
17	-1	-1	-1	1	-1	34.05
18	0	0	0	0	0	91.04
19	0	0	0	+ α	0	52.50
20	-1	1	1	1	1	27.35
21	0	+ α	0	0	0	130.28
22	1	-1	1	-1	-1	27.92
23	0	0	+ α	0	0	77.50
24	1	1	1	-1	-1	25.17

25	1	-1	1	-1	1	28.20
26	1	1	1	-1	1	27.91
27	0	0	- α	0	0	80.95
28	0	0	0	0	0	82.25
29	-1	-1	1	-1	1	23.70
30	0	0	0	0	0	93.06
31	-1	1	1	-1	-1	13.34
32	1	1	-1	-1	-1	30.05
33	1	-1	-1	1	-1	241.29
34	1	-1	-1	-1	-1	29.61
35	-1	1	-1	-1	-1	19.20
36	-1	-1	-1	-1	1	19.01
37	1	1	-1	-1	1	25.94
38	0	0	0	0	- α	95.26
39	1	1	1	1	-1	259.80
40	-1	-1	1	1	1	26.05
41	0	0	0	0	0	103.75
42	1	-1	1	1	1	247.31
43	0	- α	0	0	0	95.77
44	1	1	1	1	1	248.50
45	-1	-1	1	-1	-1	20.33
46	- α	0	0	0	0	9.98
47	1	1	-1	1	1	236.13
48	0	0	0	0	0	99.08
49	-1	1	-1	1	-1	30.55
50	-1	1	-1	-1	1	14.91

The values given are the average of three independent experiments.

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ORIGINAL ARTICLE

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Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides

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Abstract

The production of phytase using *Aspergillus niger* NCIM 563 under submerged fermentation conditions was studied using protein rich chickpea flour as substrate. Employing a hybrid statistical media optimization strategy of Plackett–Burman and Box-Behnken experimental designs in shake-flasks gave an increased phytase activity from an initial 66 IU/mL in 216 h to 160 IU/mL in a reduced time of 132 h. Productivity, thus increased by 3.97 times from 7.3 to 29 IU/mL/day. Using the optimized media, the production was successfully scaled-up further and improved up to 164 IU/mL in 96 h by studies carried out employing 2 and 10-L fermenters. The enzyme supernatant was recovered using centrifugal separation of biomass and the stability of the produced phytase was tested for animal feed applications under gastric conditions. In vitro degradation studies of water soluble monocrotophos, methyl parathion and water insoluble chlorpyrifos, pesticides used extensively in agriculture was carried out. It was observed by HPLC analysis that phytase could degrade 72% of chlorpyrifos at pH 7.0, 35 °C. Comparable results were obtained with monocrotophos and methyl parathion. With chlorpyrifos at higher temperature 50 °C as much as 91% degradation could be obtained. The degradation of chlorpyrifos was further validated by spraying phytase on harvested green chilli (*Capsicum annuum* L) under normal conditions of pH 7.0, 35 °C and the degradation products obtained analyzed by LCMS. Thus, the present study brings out a potentially novel application of phytase for biodegradation of organophosphorus pesticides.

Keywords: *Aspergillus niger*, Green chickpea flour, Fermenter scale, Organophosphorus pesticide, Phytase, Submerged fermentation

Introduction

Over the past century, an increase in demand of food grains and vegetables has led to an extensive use of man-made pesticides in agriculture. In fact, crop protection in India is known to annually use nearly 40,000 metric tons of pesticides. Organophosphorus pesticides (OpP), are widely used in agriculture for controlling variety of sucking, chewing and boring insects, spider mites, aphides and pests. In particular, OpP cannot be easily removed by washing and rinsing with tap water (Vendan 2016) and this leads to bioaccumulation in the food chain.

Organophosphorus pesticides are esters of phosphoric acid, which include aliphatic, phenyl and heterocyclic derivatives (Baishya and Sarma 2015), which are known to be potent irreversible acetylcholinesterase (AChE) inhibitors by phosphorylation of the serine residue at the enzyme active site. This leads to adverse effects on the nervous system of exposed animals including humans (Mileson et al. 1998), and there exists a dire need to degrade OpP post-harvest, so as to prevent their entry into the food chain.

The currently used physicochemical processes for OpP remediation include incineration or disposal in landfills that are expensive, non-ecofriendly and the process is often incomplete leading to formation of toxic intermediates (Debarati et al. 2005). Alternatively, the use of whole-cell microorganisms is advantageous because it offers a

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safe, economic and eco-friendly green option (Rayu et al. 2012; Sutherland et al. 2004). The factors that impact bioremediation are mainly the availability of organic sources for microbial growth, optimal pH, bioavailability of inhibitory substrates and the satisfaction of regulatory norms for release of microbes into the environment (Boopathy 2000), which can be addressed by using cell-free microbial enzymes that can act on diverse pollutants (Scott et al. 2011).

Most widely studied cell-free enzymes for OpP degradation are from bacteria, viz., organophosphorus hydrolase (EC 8.1.3.1) (Gao et al. 2012), phosphotriesterase (EC 3.1.8.1) (Chino-Flores et al. 2012) and organophosphorus acid hydrolase (EC 3.1.8.1) (Theriot and Grunden 2011). There are few reports where, fungi have been studied for OpP degradation by cleaving the phosphate group (John and Shaike 2015; Wyss et al. 1999). Phytase (PYT) or myo-inositol hexakisphosphate phosphohydrolase (EC 3.1.3.8) from *Aspergillus niger* NCIM 563, is a good example of a hydrolytic enzyme that can release inorganic phosphorus by the degradation of phytic acid (Bhavsar et al. 2008). Current progress on PYT research is focused on phytic acid degradation for major animal feed supplementation, plant growth promotion and human nutrition (Dersjant-Li et al. 2015; Kumar et al. 2010). The GRAS cleared PYT enzyme has not been studied for its potential for biodegradation of toxic pesticides by phosphorous release and is therefore studied here. A positive result would bring out a novel and useful enzymatic application of PYT for pesticide detoxification.

Agricultural residues including rice bran, wheat bran, groundnut oil cake, corn starch, etc., are widely used as substrates for PYT production (Bhavsar et al. 2008; Alves et al. 2016; Buddhiwant et al. 2015). However, the cost of production increases with use of agricultural residue as it needs pre-treatment (Bhavsar et al. 2008; Rani et al. 2014). The need for cost effective sustainable production requires alternative substrate for PYT production. In this context, the use of protein-rich legume flour as substrate has advantages for sustainable low cost PYT production because it can avoid the pre-treatment step. India is the largest producers of chickpea (*Cicer arietinum*) (7.17 metric tons in 2014–2015) but around 20% of the cultivated chickpea seeds are rejected due to non-uniform growth, color and damage during harvesting and post-harvesting process (Torres-Fuentes et al. 2011). These rejected green seeds are however, rich in protein, carbohydrate, lipids and major dietary minerals such as calcium, phosphorus, magnesium, iron and potassium (Christodoulou et al. 2006) and has been used in poultry diet (Garsen et al. 2007). For the above reasons, in the present study, in view of its availability as agricultural waste, we used green chickpea flour (GrCf) as the substrate of choice for producing and maximizing PYT production. The increased production of PYT would facilitate studying for animal feed applications and as discussed earlier in conducting studies related to dephosphorylation potential of OpP by this extracellular enzyme.

In the present study for increasing the production of extracellular PYT from GrCf using *A. niger*, growth media optimization was envisaged using shake flasks under submerged fermentation condition (SmF) by employing an effective hybrid strategy that involves carrying out statistical sets of experiments (Shah et al. 2009). In this approach, we initially aim at identifying the significant factors influencing PYT production by implementing a Plackett–Burman Design (PBD) of experiments (Plackett and Burman 1956). Subsequently, a second set of experiments can then be conducted to further optimize the levels of the significant factors that maximize PYT production by application of a more rigorous experimental design such as the Box Behnken design (BBD) (Box et al. 1978; Khuri and Cornell 1987). Obtaining positive results would then suggest scale-up studies with 2 and 10-L fermenters so as to confirm maintenance or obtain improvements in the production levels for process feasibility. This would also require assessing the suitability and stability of the PYT produced by GrCf so that animal feed applications can become possible. As discussed above, from a novel application point of view, it would be interesting to study and test the enzyme effectiveness for in vitro pesticide degradation using for example a commercially available OpP such as water insoluble chlorpyrifos (CPyF) (Dursban 2E©) and water soluble monocrotophos (MCP) and methyl parathion (MP). Again, a positive result would suggest carrying out studies with a test system such as post-harvest fresh green chillies (*Capsicum annum* L) treated with CPyF. The results obtained by carrying out suitable studies for the above objectives and plan are discussed in this work.

Materials and methods

Chemicals

Phytic acid sodium salt, 3, 5, 6-trichloro-2-pyridinol (TCP) and diethyl thiophosphate (DETP) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Acetonitrile (ACN) of HPLC grade was purchased from Merck. All other chemicals used were of analytical grade. CPyF (Dursban 2E©, 20%), MCP (36%) and MP (50%), harvested green chilli, soybean meal and seeds of green chickpea were purchased from a local market. The seeds were minced in a grinder to obtain green chickpea flour (GrCf).

Microorganism and production in basal media

Aspergillus niger NCIM 563, used in the present study was obtained from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory (CSIR-NCL), Pune, India, which was maintained on potato dextrose agar (PDA) slants and stored at 4 °C. A time course of the PYT production was studied using the basal media (pH 5.5) in triplicates. 100 mL media comprising of (g%): 1.0 GrCf; 5.0 glucose; 0.86 $NaNO_3$; 0.05 KCl; 0.05 $MgSO_4{\cdot}7H_2O$; 0.01 $FeSO_4{\cdot}7H_2O$ was dispensed in 250 mL Erlenmeyer flask and sterilized by autoclaving at 121 °C for 20 min. Spores from 7 days old PDA slant were gently scraped using sterile wire loop with 25 mL sterile saline solution containing 0.01% Tween 80. The spore suspension was collected in sterile tube and homogenized by vortexing for 1 min. 1×10^7 spores (using Neubaur chamber) was used as spore inoculum (Sp-I) for inoculating the basal media and incubated at 28 °C at 170 rpm under aerated culture conditions. Vegetative inoculum (Ve-I) was prepared by inoculating 5 mL basal media with 1×10^7 spores under same aerated culture conditions for 10 h which was used for inoculating 95 mL basal media. The samples were withdrawn every 24 h and centrifuged. The supernatant was checked for total residual glucose and PYT activity.

PYT assay

The PYT analysis solution consisting of 3 mM sodium phytate with 100 mM glycine–HCl buffer (pH 2.5) and 100 µL of liquid enzyme extract solution was incubated for 30 min at 50 °C. The liberated inorganic phosphate was measured by the ammonium molybdate method (Heinohen and Lathi 1981). A freshly prepared solution of acetone, 5 N H_2SO_4, 10 mM ammonium molybdate (2:1:1, v/v) and 400 µL citric acid (1 M) was added to the enzyme-substrate solution and absorbance was measured at 370 nm against blank consisting of buffer and substrate. One unit of PYT activity (IU) was expressed as the amount of enzyme that liberates 1 µmol phosphorus/min/mL under standard assay conditions while enzyme production was expressed as PYT activity IU/mL.

Stability studies

The influence of pH and temperature on PYT activity was determined by assaying in the pH range of 1.5 and 9.0 using 100 mM buffers: glycine–HCl (pH 2.0–3.0), sodium acetate (pH 4.0–6.0), Tris–HCl (pH 7.0–8.0), and glycine–NaOH (pH 9.0) at 50 °C. The pH stability using the same buffer solutions was also determined by pre-incubating enzyme samples at 35 °C for the period of 12 h considering PYT activity at zero time as 100%. The studies on optimum temperature were carried out in the temperature range of 30–70 °C, while the temperature stability was determined by incubating the enzyme samples over the above temperature range for a period of 1 h on comparing with the control without incubation. The stability of PYT was also checked at gastric conditions of poultry. One gram soybean meal was dissolved in 9 mL of simulated gastric fluid (250 mM glycine–HCl containing 2.0 mg/mL NaCl and 3.2 mg/mL of pepsin) and the pH was adjusted over a range from 1.5 to 6.5 using HCl and NaOH as required. The solutions were incubated at 37 °C for 30 min, as the poultry gut temperature varies from 37–39 °C (Lei and Stahl 2001). 40 IU of PYT was added to the solution and incubated at 37 °C for 60 min. The released phosphorus was determined as described in PYT assay.

Biodegradation of OpP using PYT

The potential of extra-cellular PYT, produced in basal media, was studied for biodegradation of CPyF, a water insoluble organophosphate. 1 mL stock solution of CPyF (10,000 ppm) was incubated with 100 µL mycelial free PYT (100 IU; specific activity 53 IU/mg) for 2 h at 35 °C, pH 7 as well as its optimum conditions (50 °C, pH 2.5). The selection of a high concentration of CPyF was employed for sensing and quantifying the degradation metabolites. The treated sample was diluted 10 times with mobile phase and the amount of residual CPyF was monitored using HPLC as mentioned in analytical methods. For other OpPs (MCP and MP), studies were carried out in similar way.

Analytical methods

Concentration of the protein (mg) was determined using Lowry method with bovine serum albumin as standard (Lowry et al. 1951) and specific activity (IU/mg) using enzyme units and protein concentration were calculated. Di-nitro salicylic acid (DNSA) method (Miller 1959) was used to calculate total residual reducing sugar concentration.

The analysis of OpPs were carried out using HPLC (Dionex-ASI 100, auto sampler series) with reverse-phase column (C18 −4.6 × 250 mm, Waters) maintained at 40 °C. The mobile phase used was mixture of ACN: water (70:30 v/v), with flow rate of 0.5 mL/min. The detection was done at 230 nm and injection volume was 50 µL. Standard solutions of OpP was transferred in vials to reach final concentrations in range of 100–1000 ppm using mobile phase. The analysis of CPyF and its degraded products on green chilly were carried out using LC–MS (Waters-Xevo TQD-USA) with reverse phase C18 column (Acquity—UTLC BEH—2.1 × 100 mm) maintained at 45 °C. The mobile phase used was mixture of ACN: water (70:30 v/v), with flow rate of 0.3 mL/min. Injection volume of sample was 5 µL. Standard solutions of each was transferred in vials to reach concentration in range of 0.2–1 ppm using mobile phase.

Application of PYT on harvested green chilli (*Capsicum annuum* L)

The potential of PYT to degrade CPyF on harvested green chilly was studied at 35 °C, pH 7.0. Green chillies (250 g), obtained from local market was sprayed with CPyF (20 ppm) and dried. One part of the chillies (test) was treated with crude mycelial—free PYT (80 IU) at 35 °C and pH 7.0 for 2 h keeping the second part untreated (control). Both the parts were separately cut into small pieces and homogenized with a household mill (equipped with stainless steel knives). 10 mL of ACN was added to 10 ± 0.1 g sample and vortexed for 1 min to which 10 g sodium sulfate was added and homogenized at 15,000 rpm for 1 min. The samples were centrifuged at 5000 rpm for 5 min and 5 mL of the supernatant were transferred to a 15 mL PTFE tube. 10 mg graphitized carbon black and 25 mg primary secondary amine were added to remove colored impurities. The extract was shaken using a vortex mixer for 30 s and centrifuged at 10,000 rpm for 5 min. 2 mL of the supernatant was used to analyze the presence of CPyF and its degraded metabolites using LC–MS as mentioned in analytical methods.

Media optimization in shake flasks

Carrying out a PBD of experiments offers a rapid multifactor way to screen and identify the most significant factors (Plackett and Burman 1956). The potential effect of 10 variables (Additional file 1: Table S1) on PYT production, were evaluated in 12 PBD runs at two levels, low level (−) and high level (+). The choice of the above variables was made based on reports available for enhanced PYT production by solid state fermentation (Bhavsar et al. 2013). The complete PBD matrix for screening was designed using a standard Plackett–Burman orthogonal array constructed using Design Expert Software (DES) Version 7.1.2, Stat-Ease, Minneapolis, MN, USA. The response values of PYT produced in IU/mL were analyzed to obtain a best-fit linear mathematical model that could be further analyzed by ANOVA for acceptability. Subsequently, a BBD of experiments was generated by DES and studies at three different levels −1, 0, +1 (Additional file 1: Table S2) were carried out to further optimize enzyme production levels with respect to the major factors identified by PBD. The less significant factors were maintained at the average of the high and low levels used in the PBD study. A best-fit model for enzyme production was further studied by ANOVA to test for its statistical significance.

Fermenter scale production

Scale-up studies in a batch fermenter (New Brunswick BioFlo 110) with 2-L media was carried out on the basis of optimized media formulation. Successive fermentation batches at different rpm (400, 500 and 600 rpm) were performed with constant aeration of 0.5 vvm and temperature of 28 °C. The fermenter containing 1.8-L medium that was earlier sterilized in situ was inoculated with 36 h old Ve-I (200 mL). The dissolved oxygen (DO) was measured using Mettler Toledo oxygen probe. Samples from the fermenter were withdrawn at regular intervals and analyzed for PYT activity and residual glucose on biomass separation. The process was further scaled up using a 10-L capacity fermenter with 1-L, 36 h old Ve-I.

Results

PYT production in basal media

The PYT production was tested using two types of inoculum; spore (Sp-I) and vegetative (Ve-I). The results showed, slow and gradual increase in PYT production using Sp-I, with the maximum activity of 66 ± 3.3 IU/mL on 9th day, (i.e., 216 h) (Fig. 1). The glucose concentration also showed a gradual depletion, with complete utilization by 10th day (i.e., 240 h). In fact, using Ve-I, higher PYT activity of 86 ± 4.3 IU/mL was observed in a lower production time of 6 days (i.e., 144 h) with glucose depletion in 7 days (i.e., 168 h). Thus, the productivity [units of enzyme produced per day (IU/mL/day)] increased from 7.3 IU/mL/day obtained using Sp-I to 14.3 IU/mL/day using Ve-I. Thus, the type of inoculum has marked effect on the production of PYT and all further studies were therefore carried out using Ve-I.

Stability studies of PYT

The conditions for optimum activity and stability of the PYT was assessed by carrying out studies with respect to temperature and pH. Optimum temperature studies ranged from 30 to 70 °C. It was observed that, the optimum temperature for maximum PYT activity is 50 °C

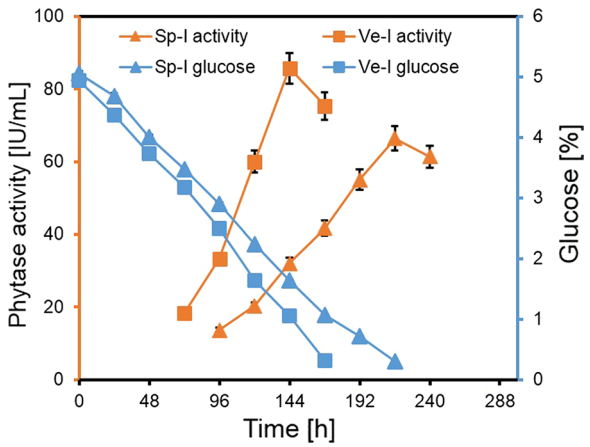

Fig. 1 Comparison of phytase production using spore inocula (Sp-I) and vegetative inocula (Ve-I). Experiments were carried out in triplicate (mean ± SD)

(Additional file 1: Figure S1). On considering the activity at 50 °C to be 100%, we observe that at 35 °C, the activity reduces to 30% of maximum activity and at 60 °C to 60% of maximum activity. Temperature stability studies were then carried out, which showed 100% stability in 1 h from 30 to 50 °C (Additional file 1: Figure S1) while 20% activity was reduced at 60 °C, 50% at 65 °C and 100% at 70 °C. Studies with varying pH interestingly showed that the enzyme showed high activity at a low pH value of 2.5 (Additional file 1: Figure S2). Considering the activity at pH 2.5 to be 100%, only 50 and 10% activity was observed at pH 4.5 and 7.0, respectively. It may be noted that the PYT showed overall broad pH stability from pH 2.5–9 (Additional file 1: Figure S2). Experiments showed that the enzyme activity was retained for 12 h in the pH range studied. The pH and temperature stability profile was also determined under poultry gut conditions as described in methods. High efficacy of phosphate release was shown by the PYT in simulated gastric fluid in pH ranging from 2.0 to 4.5 (Additional file 1: Figure S3).

Biodegradation of OpP using PYT

The ability of crude PYT, (100 IU) produced using basal media, to degrade CPyF was studied both under normal conditions (35 °C, pH 7.0) as well as under optimum enzyme conditions (50 °C, pH 2.5). HPLC analyses, showed a single major peak for the sample containing only CPyF (control sample) at 35 °C, pH 7.0 (Fig. 2a) as well as at 50 °C, pH 2.5 (Fig. 2c) and having a retention time of 3.62 min with a relative area of 97%. On the other hand, for a PYT treated sample at 35 °C, pH 7.0, multiple peaks were observed. Notably, it was observed that there was decrease in relative area by 72% at the retention time of CPyF (Fig. 2b). A similar study with PYT at 50 °C, pH 2.5, the relative peak area seen at retention time of CPyF showed an even higher decrease in relative area by 91%

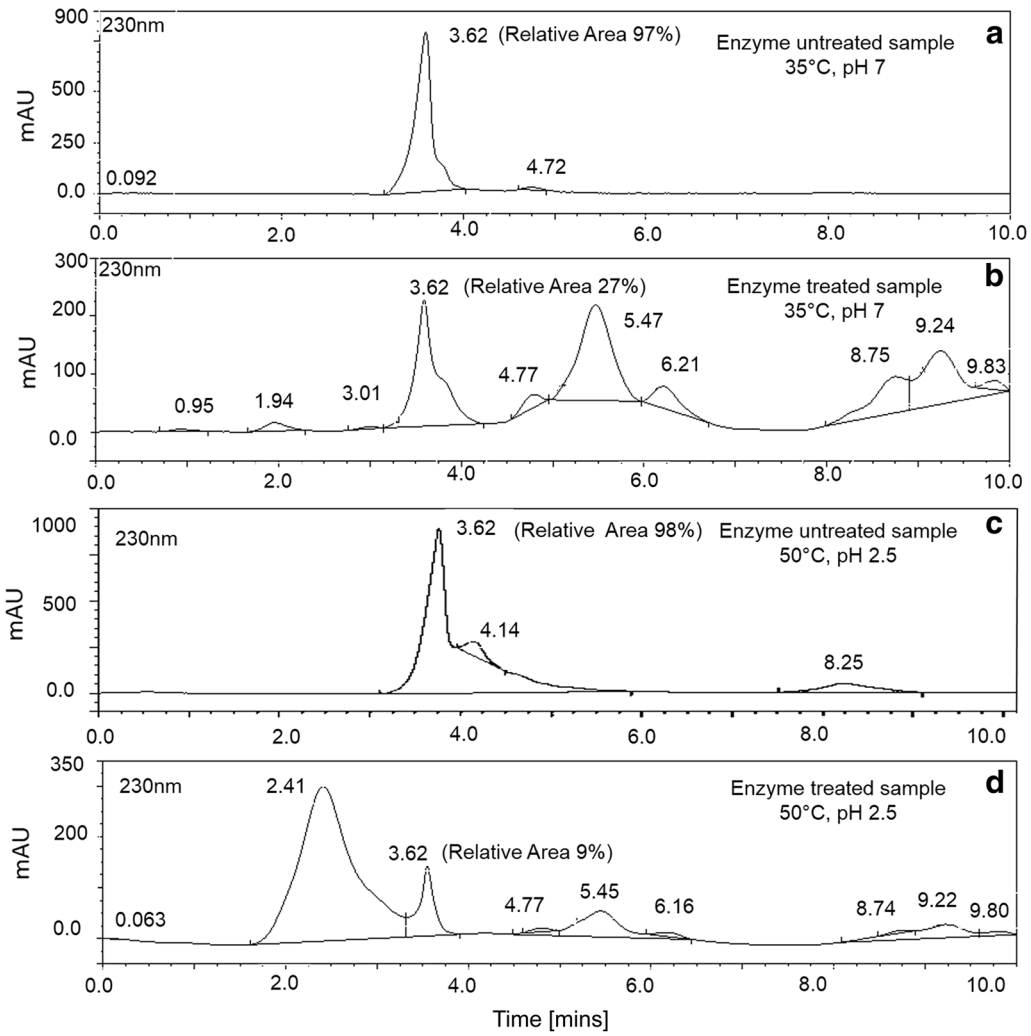

Fig. 2 Reduction of CPyF using phytase at different conditions **a**, **c**. CPyF before phytase action, **b**, **d**. CPyF after phytase action

(Fig. 2d). The positive result for CPyF suggested degradation studies with other OpPs, namely, MCP and MP by PYT would be useful. HPLC analysis with MCP having a retention time of 5.5 min for the control sample (Additional file 1: Figure S4a) at 35 °C, pH 7.0 showed that a higher unit of PYT (250 IU) to obtain 53% degradation in 4 h (Additional file 1: Figure S4b) when compared to CPyF (PYT 100 IU, 72% degradation) (Fig. 2) in 2 h. Better degradation results with MP (HPLC retention time of 9.5 min for the control sample, Additional file 1: Figure S4c) in comparison with MCP were obtained by a higher decrease in peak area (77%) on treatment with PYT (250 IU) (Additional file 1: Figure S4d) in 4 h. Thus, all the above results with CPyF, MCP and MP corroborate the finding that PYT has the ability to effectively act on OpP and degrade them.

Application of PYT on harvested green chilli (*Capsicum annuum* L)

Phytase shows dephosphorylation action by breaking the phospho-ester bond to release phosphate from substrate (Joshi 2014). As per food safety and standards authority of India (FSSAI), limit of CPyF on vegetables is 0.2 ppm (FSSAI notification 2011). Detoxification of CPyF is achieved by cleaving the phospho-ester bond, generating TCP as the major product along with DETP (Chen et al. 2012; Hanley et al. 2000; Bicker et al. 2005), which are both water soluble. Human studies show that, both the metabolites are considered as urinary markers of CPyF exposure and are easily excreted through urine within 12 h. Studies on rat show that, TCP and DETP are the predominant urinary metabolites of CPyF catabolism (Bicker et al. 2005). In the present study, the applicability of PYT applied on post-harvest chillies to degrade water insoluble CPyF was therefore studied with respect to the formation of TCP and DETP water soluble degradation products.

LC–MS analysis of standard CPyF showed that it eluted with a retention time (RT) of 5.56 min and *m/z* of 349.90 while DETP and TCP eluted at 0.72 and 3.48 min with *m/z* values of 169.17 and 198, respectively. In PYT untreated sample (control), a single peak was detected after LC–MS analysis with a RT of 5.56 min and *m/z* of 349.90 indicating it to be CPyF (Fig. 3a). In PYT treated sample, 3 peaks (Fig. 3b–d) were observed at RT of 0.72, 3.48 and 5.56 min. MS analysis of these peaks showed *m/z* values of 169.17, 198 and 349.90, respectively. On comparing with the standards, the 3 peaks were ascertained to be DETP, TCP and CPyF, respectively. The percent degradation of CPyF using PYT from *A. niger* NCIM 563 can vary depending on pH and temperature prevailing at the field. Peak area analysis shows 8% degradation of CPyF at 35 °C and pH 7.0 in 2 h using PYT (80 IU). To increase the degradation, higher units of phytase or reaction time may thus be required. On using higher units of PYT (250 IU) to degrade CPyF on green chilli our results in fact showed that 90% degradation was possible in 12 h (Additional file 1: Figure S5a, b). It is reported that TCP is not fetotoxic and teratoxic in either rat or rabbits at dosage levels of 100 ppm. TCP is shown to have moderate toxicity to salmonoids at LD_{50} value of 1.8 ppm (Marino et al. 1999). Studies of TCP showed that a minimum concentration of 0.6 ppm when exposed for 24 h is toxic during the multiple developmental stages of zebra fish (Suvarchala and Philip 2016). Our observation is that PYT can degrade CPyF present on raw agricultural products. Thus, development of a potential new way that prevents toxic OpP from entering the food chain by forming easily removable metabolites could become possible.

Media optimization for enhanced PYT production in shake flask

For the 12 PBD runs the experimentally obtained maximum response values of PYT activity (IU/mL) obtained on the 6th day (i.e., 144 h) are reported in Additional file 1: Table S3. Run number 10 showed a maximum PYT production value of 132 ± 6.6 IU/mL. Regression analysis of the response values obtained for the PBD runs yielded a best fit linear model, viz.,

$$\begin{aligned}\text{Phytase activity } &= 111.09 - 30.67 \times [NaNO_3] \\ &\quad + 45.33 \times [\text{GrCf}] \\ &\quad -1644.44 \times [MnSO_4 \cdot H_2O] \\ &\quad -190.0 \times [CaCl_2 \cdot 2H_2O]\end{aligned} \quad (1)$$

The suitability of the model was further corroborated by ANOVA tests. Thus, the obtained model F-value of 11.35 implies the model is significant and that there is only a 0.35% chance that the model F-value could occur due to noise. The values of Prob > F was less than 0.05, for the four variables showing their significance. The coefficient of determination $R^2 = 0.87$ provided a satisfactory measure for the variability in the observed response that could be explained by the model. The Pred R^2 of 0.61 is in reasonable agreement with Adj-R^2 of 0.79. The adeq precision, a measure of the signal-to-noise ratio, is found to have a high value of 9.72 and this indicated the signal strength to be strong. All the above tests confirm that the model Eq. (1) can be used to navigate the design space. In fact, using the above model, it was found that, the above four factors accounted for 86.68% of the total contribution to the estimates of the response values. The remaining variables then accounted for only 13.32% and thus PBD identified them to be less significant. The ANOVA results were complemented by the fact that four out of the 10 factors studied in the PBD, namely, $NaNO_3$, GrCf, $MnSO_4 \cdot H_2O$

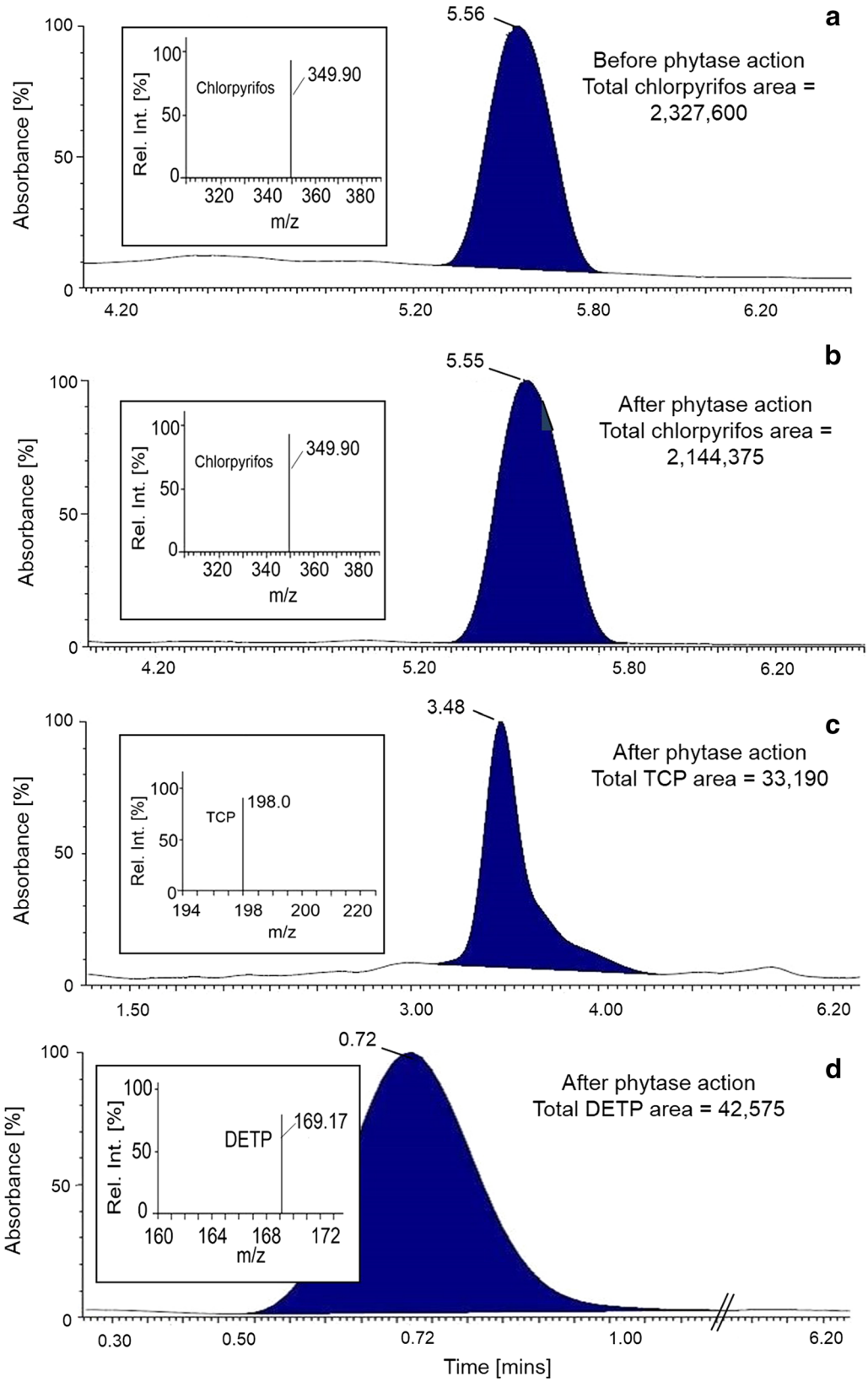

Fig. 3 Analysis of CPyF and its degraded metabolites using LC–MS. **a** CPyF on green chilli before phytase action. **b** CPyF on green chilli after phytase action. **c** Release of TCP after phytase action. **d** Release of DETP after phytase action

and $CaCl_2{\cdot}2H_2O$ were significant on comparing their t-values using a Pareto chart (Additional file 1: Figure S6).

The maximum contributory factors identified by PBD for PYT production were further optimized by a BBD of experiments (29 runs) generated by DES employing three chosen levels for each variable. Additional file 1: Table S4 gives the BBD for the four significant variables along with the experimentally obtained PYT activity. We observe that the optimization studies showed that run number 25 remarkably improved the PYT activity (160 ± 8.0 IU/mL). The wide variation in activity reported in all the runs bring out the process sensitivity to the experimentally chosen conditions and shows the usefulness of having carried out this systematic optimization study. The response data of BBD runs was regressed successfully using actual factors and interestingly showed linear dependency without interacting terms, namely,

$$\text{Phytase activity} = 286.74 - 49.17 \times [NaNO_3] - 120.83 \times [\text{GrCf}] \quad (2)$$

The ANOVA analysis of the above model satisfied the statistical tests with an obtained model F-value of 27.95 implying that, the model is significant with only a 0.01% chance that the model F-value could arise due to noise. The value of correlation coefficient (Pred $R^2 = 0.62$) for PYT production suggested a good agreement between the observed and model predicted response values. The coefficient of determination ($R^2 = 0.68$), suggests that 68% of the variability in the data was explained by Eq. 2. The obtained signal-to-noise ratio value of 15.04 brought out the presence of an adequate signal.

The final formulation of ten variables in the range studied in PBD and BBD showed that the highest activity of 160 ± 8.0 IU/mL was obtained in 132 h with 100 mL media comprising of (g%): 4.0 glucose; 0.4 $NaNO_3$; 0.075 $MgSO_4{\cdot}7H_2O$; 0.075 KCl; 0.015 $FeSO_4{\cdot}7H_2O$; 0.015 Tween 80; 1.0 GrCf; 0.35 dextrin; 0.02 $MnSO_4{\cdot}H_2O$ and 0.1 $CaCl_2{\cdot}2H_2O$. To validate the formulation, a time course experiment for PYT production was carried out using this optimized condition. The results obtained after showed that a 1.86 fold enhancement in PYT activity from 86 ± 4.3 IU/mL to 160 ± 8.0 IU/mL with glucose completely utilized in 5.5 days (i.e., 132 h) was achieved by adopting the outlined hybrid media optimization approach (Fig. 4). The effect of varying the phosphate concentration by addition of sodium phytate and KH_2PO_4 using the optimized media formulation with 1% GrCf was thus studied. Results showed that the PYT activity decreased to 79 IU/mL on addition of sodium phytate (0.004 g%) and to 151 IU/mL on addition of KH_2PO_4 (0.002 g%). This may be compared to PYT activity of 160 IU/mL obtained using the optimized media with 1% GrCf and suggests that it optimally provides the requirements of phosphate.

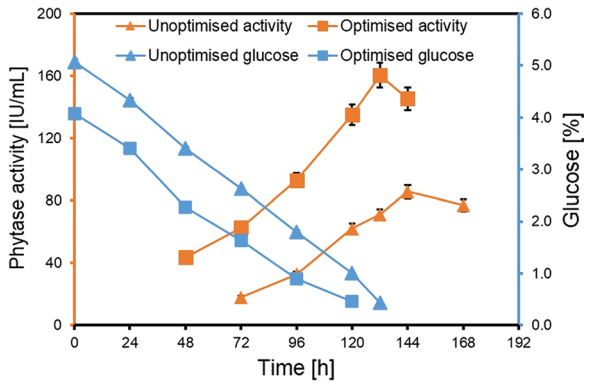

Fig. 4 Validation of phytase production by using unoptimized and optimized media. Experiments were carried out in triplicate (mean ± SD)

Scale-up studies of PYT production in batch fermenters

The optimized media formulation was studied in scaled-up larger volume batches using fermenters to ensure maintenance of the PYT production throughput. The results of fermentation studies carried out with 2 and 10-L media scaled-up volume batches are discussed below. It is expected that when operating with larger volumes, the dissolved oxygen (DO) is an important factor to consider and this would depend on both the aeration rate and the agitation speed. For the present studies, we chose to keep the aeration rate constant at 0.5 vvm while varying the initial agitation speeds (400, 500, 600 rpm). The behavior in time of DO and pH were simultaneously monitored. For the 400 rpm run, a decrease in DO was observed till 36 h. 50% DO was maintained by gradual increase in rpm and it was thus maintained at the higher rpm. The pH of the media gradually decreased from 5.0 to 2.3. For the 2-L batch study with an initial rpm of 400, the maximum activity of 97 ± 4.8 IU/mL was obtained in 132 h. PYT production of 122 ± 6.1 IU/mL and 158 ± 7.9 IU/mL was achieved at initial rpm of 500 and 600 respectively, in 132 h. Thus, PYT production was successfully scaled up to 2-L production from the shake flask experiment (158 ± 7.9 IU/mL at 600 rpm) (Fig. 5a).

Successful production at 2-L scale, paved way to studying the feasibility of enzyme production in a 10-L volume fermenter to bring out the process biotech potential. Similar to studies in the 2-L scale and varying the initial agitation speed (400, 500 and 600 rpm), the DO and pH were monitored in 10-L scale. DO and pH pattern were again observed, but with higher decreasing rates than in the 2-L scale. Remarkably, the maximum PYT activity of 164 ± 8.2 IU/mL was maintained but it is important to note that the maximum activity was obtained in a much shorter time of 96 h at 500 rpm (Fig. 5b).

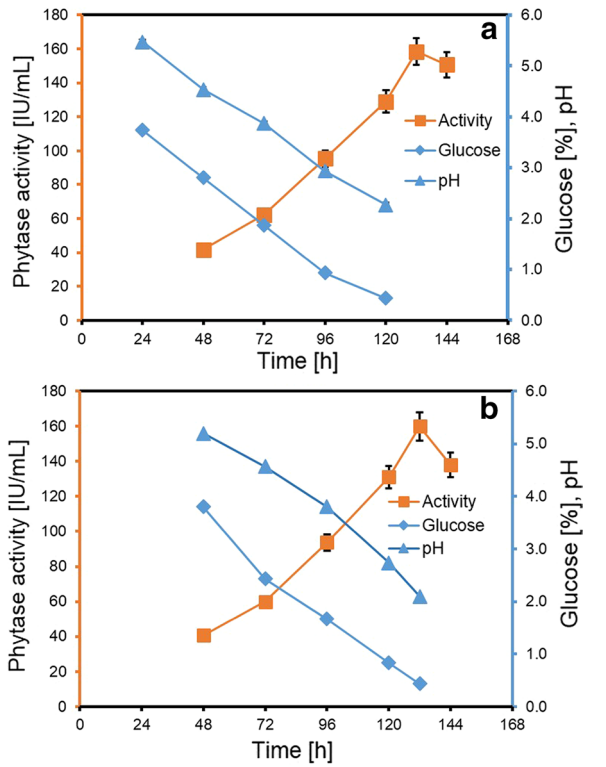

Fig. 5 Fermenter scale production **a** 2-L (at 600 rpm) and **b** 10-L (at 500 rpm) depicting phytase activity, pH and glucose concentration. Experiments were carried out in triplicate (mean ± SD)

Discussion

Phytase is widely used to act on phytic acid for the aim of increasing the bioavailability of phosphorus, proteins and essential minerals in animal diets. It is commercially produced by employing a submerged fermentation process using spore inoculum (Coban and Demirci 2014; Krishna and Nokes 2001). We considered it desirable to carry out studies that use vegetative inoculum for PYT production over spore inoculum that is commonly employed for PYT production. Results obtained suggest that PYT production may in fact be enhanced using vegetative inoculum. This result is of considerable significance. Similar results of enhanced PYT production have been observed using vegetative inoculum in solid state fermentation (Krishna and Nokes 2001). Our studies also show that the use of GrCf as substrate for PYT production with *A. niger* NCIM 563 gives activity and productivity higher than earlier reports under submerged fermentation conditions using different strains and substrates (Table 1). Our observation is that the PYT produced using GrCf is active and highly stable over wide range of temperature and pH that simulates gastric conditions and brings out its superiority for use in animal feed applications. A novel application of PYT in degrading CPyF, MCP and MP is also discussed here to bring out its potential as an agent for pesticide degradation. It needs to be noted that FDA has approved *A. niger* as a generally-recognized-as-safe (GRAS) organism (Schuster et al. 2002). Together with the laboratory study and analysis report obtained from Central Avian Research Institute, Bareilly (UP), India confirms that *A. niger* NCIM 563 is a non-mycotoxin producer and the PYT obtained can thus be advantageously exploited.

Statistical experimentation for media optimization provides a time saving approach for enhancing PYT production (Bhavsar et al. 2013) and provides the base experimental conditions maintaining/improving the productivity for scale-up. Our results with this approach showed that the PYT productivity obtained in a 10-L fermenter working volume (41.0 IU/mL/day) was improved by a factor of 2.87 and 1.41 times from shake flask (14.3 IU/mL/day) and 2-L scale (29 IU/mL/day) experiments, respectively. The increase in productivity

Table 1 Comparing phytase productivity of *A. niger* NCIM 563 with other *Aspergillus* species

Microbial strain	Media	Optimum pH	Optimum temp (°C)	PYT activity (IU/mL)	Productivity (IU/mL/day)	Reference
A. niger NCIM 563	GrCf[a] (optimized)	2.5	50	160.0	29.00	Present work
A. fumigatus NF 191	PSM (optimized)	(–)	(–)	101.79	25.40	Gangoliya et al. (2015)
A. oryzae SBS 50	Starch	5.0	35	15.70	3.90	Sapna (2013)
A. niger CFR 335	Potato dextrose broth	(–)	(–)	9.60	1.92	Shivanna and Venkateswaran (2014)
A. ficuum SGA 01	Potato dextrose broth	(–)	(–)	8.20	1.64	Howson and Davis (1983)
A. ficuum NRRL 3135	MRS medium	(–)	(–)	2.27	0.45	Howson and Davis (1983)
A. heteromorphus MTCC 10685	Phytase screening medium (optimized)	6.0	30	24.88	5.00	Lata et al. (2013)

(–) No data reported

[a] GrCf-green chickpea flour

with reduction in production time at 10-L fermenter scale may be due to the better maintenance of fermentation parameters viz., agitation, temperature, aeration, etc. This observation further supports the biotech potential of the present PYT production process. Studies of submerged PYT production using *S. thermophile* in cane molasses medium showed that the productivity obtained in the 10-L working volume fermenter (5.2 IU/mL/day) was improved by a factor of 2.2 times from shake flask (2.5 IU/mL/day) experiments and having optimum activity at pH 5.5 and 45 °C (Singh and Satyanarayana 2008). Similar trend of increased productivity by 1.5 times for glucoamylase production was observed by Kumar et al. (2007).

In conclusion, the present study shows an efficient process of producing PYT, which has wide applications prospects in animal feed and agriculture. Our studies obtained high yields of PYT from *A. niger* NCIM 563 using GrCf. The PYT from *A. niger* was found to be stable over wide range of temperature and pH and has thus shown the necessary potential for use as animal feed supplement as well as on crops for field applications. The PYT from *A. niger* NCIM 563 beneficially shows biodegradation of CPyF when tested on green chillies. Studies show biodegradation of CPyF is maximum at conditions chosen close to the enzyme optimum conditions for high PYT activity (50 °C and pH 2.5). These findings thus bring out an interesting and new applications for PYT produced from GrCf and *A. niger* NCIM 563. Although, the above optimal conditions do not correspond to field, they do justify the need to carry out studies that would improve the efficacy of PYT for biodegradation of OpP compounds.

Additional files

Additional file 1. Additional figures and tables.

Additional file 2. Additional data.

Abbreviations

BBD: Box-Behnken design; CPyF: chlorpyrifos; GrCf: green chickpea flour; MCP: monocrotophos; MP: methyl parathion; OpP: organophosphorus pesticide; PYT: phytase; PBD: Plackett–Burman design.

Authors' contributions

PS carried out the experiments. JM participated in the design of the study, supervised the research work and reviewed the manuscript. VR designed the statistical optimization studies, drafted and edited the original manuscript. SD supervised the research work, administered the project and funding. All authors have read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

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Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides

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Fig. S1 Studies on optimum temperature and stability of PYT at different temperature

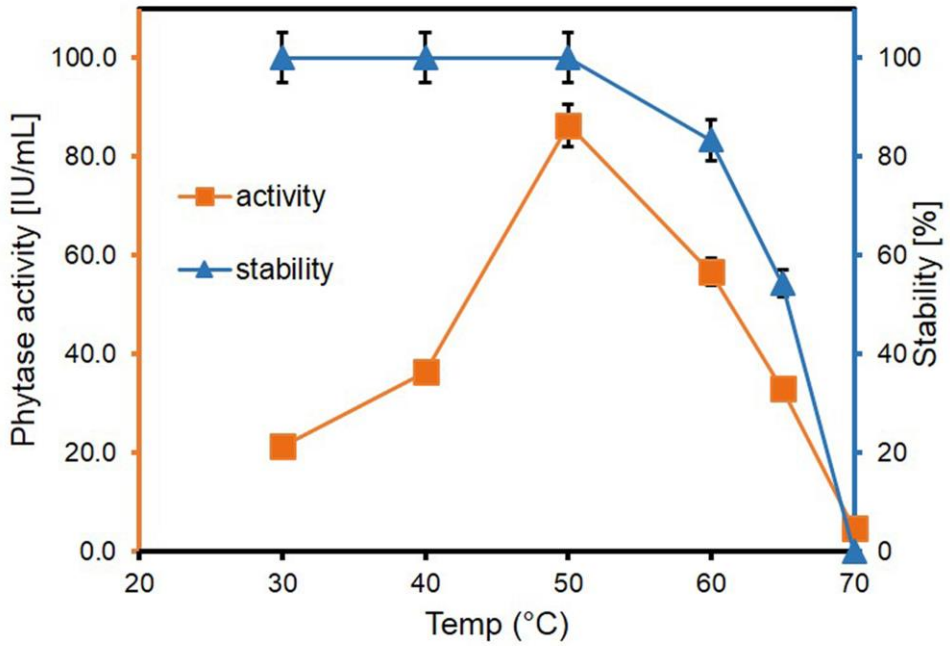


Fig. S2 Studies on optimum pH and stability of PYT at different pH

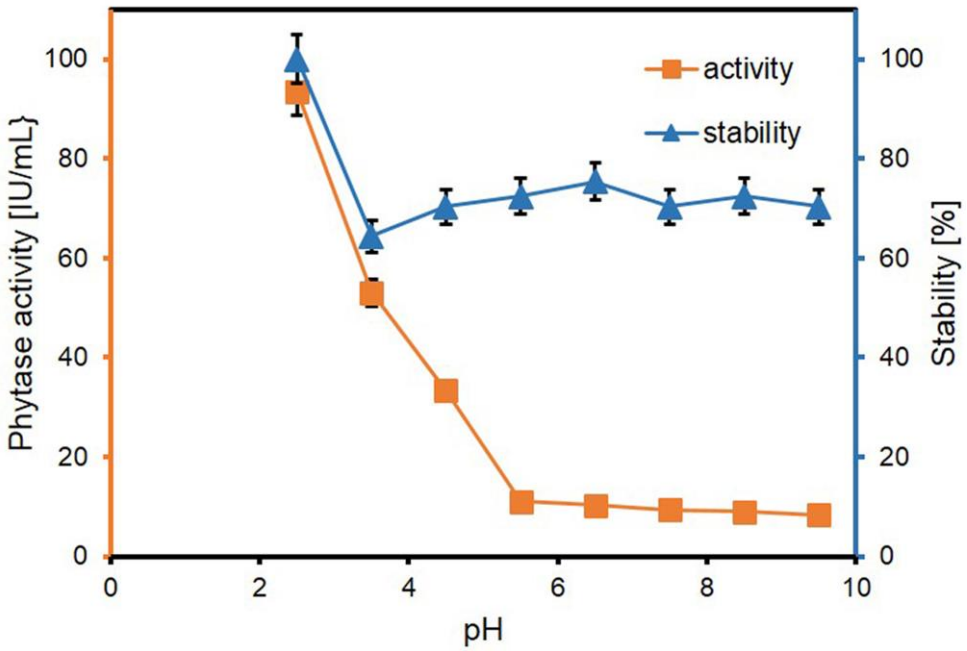


Fig. S3 Efficacy of PYT in stimulated gastric fluid at different pH

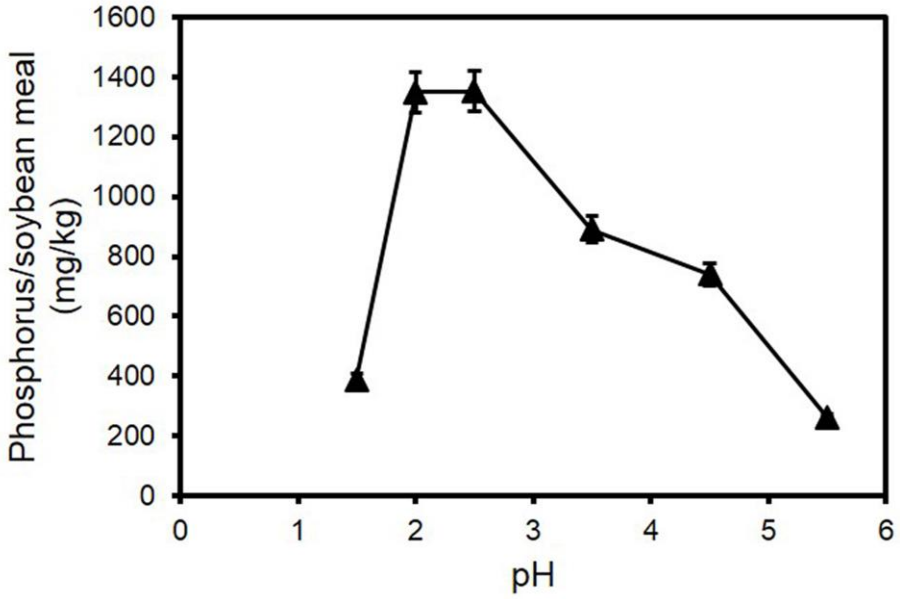


Fig. S4 Degradation of OpP: (a, b) MCP and (c, d) MP using PYT

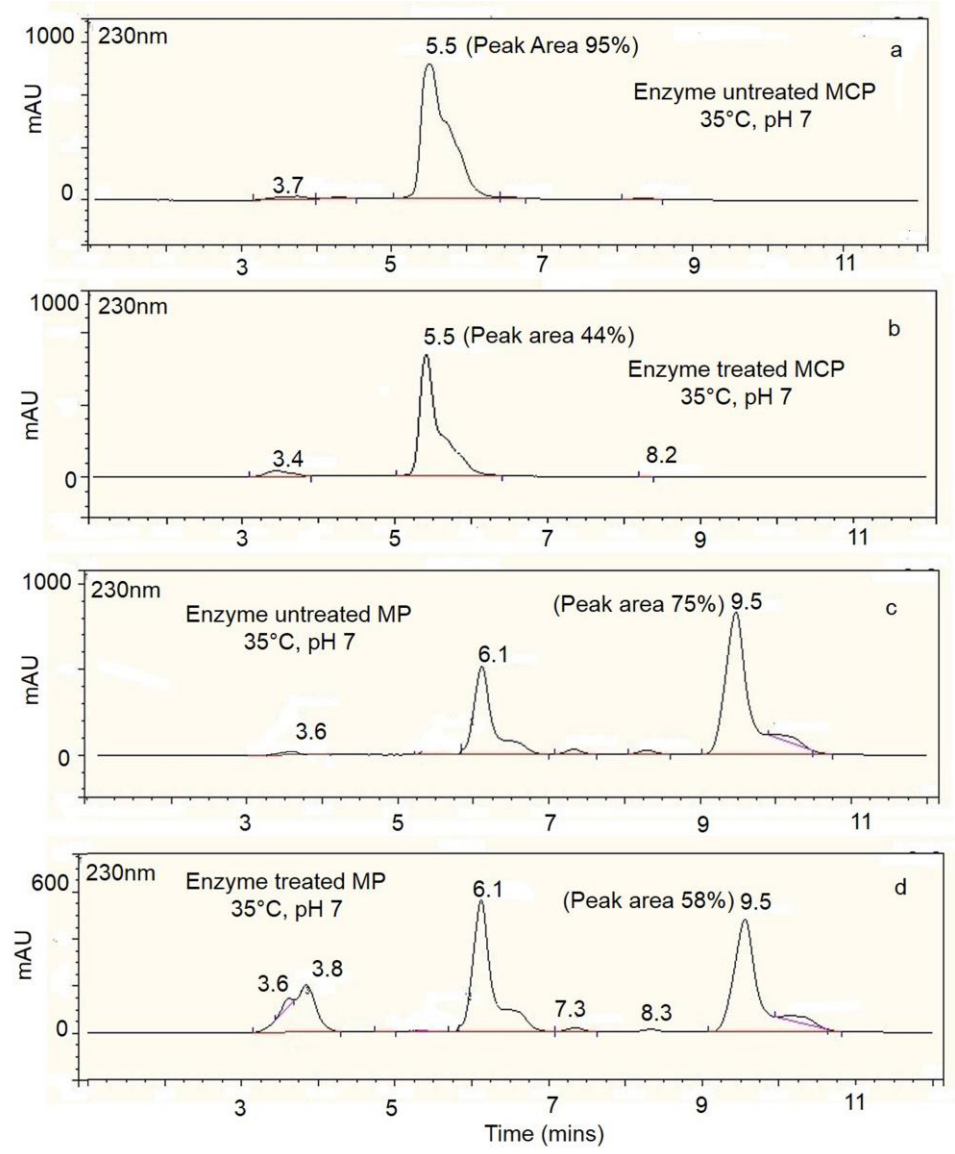


Fig. S5 Degradation of CPyF on harvested green chilli (a) control; (b) using 250 IU PYT

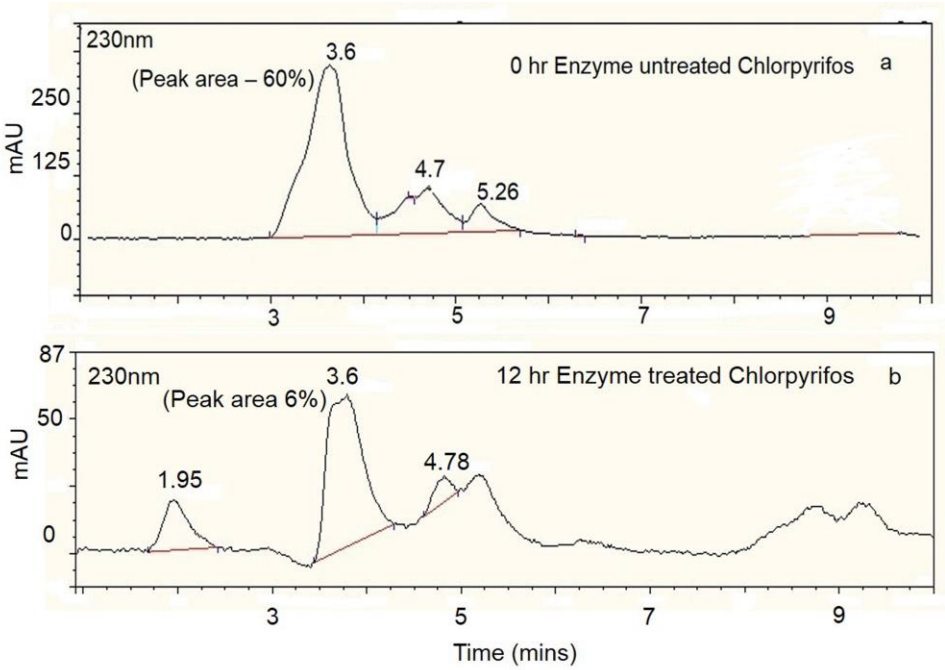
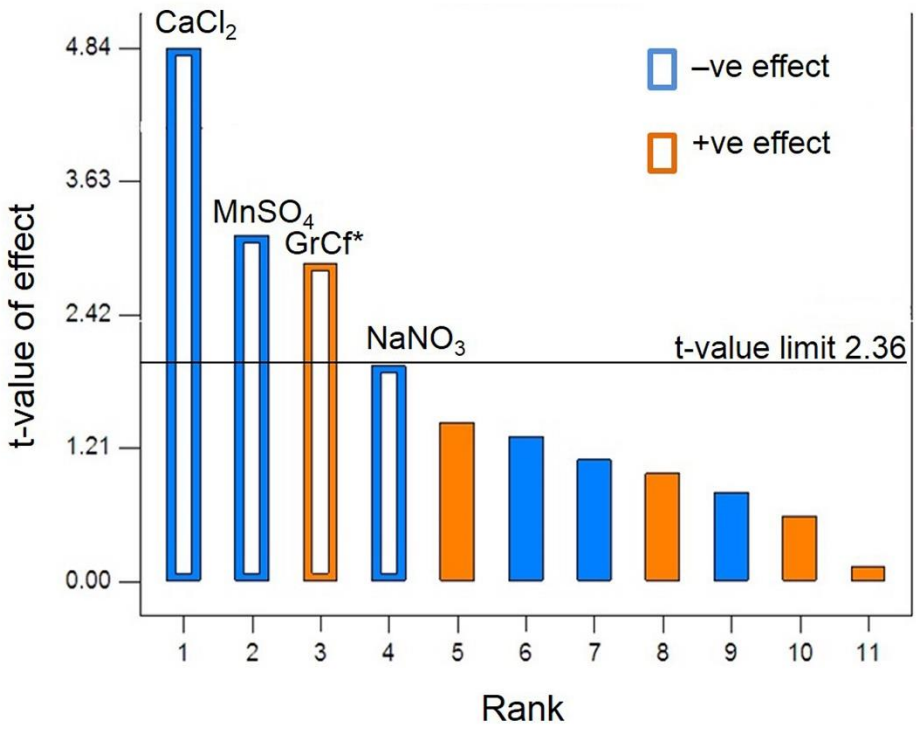


Fig. S6 Pareto chart showing positive and negative effect of significant factors by PBD analysis



*GrCf- green chickpea flour

Table S1 Selected variables for PBD and their assigned levels for studying PYT production in 100 mL media

S. No.	Code	Variables	Low level (-)	High level (+)
1	A	Glucose (g)	3.00	5.00
2	B	$NaNO_3$ (g)	0.30	0.80
3	C	$MgSO_4 .7H_2O$(g)	0.04	0.10
4	D	KCl (g)	0.04	0.10
5	E	$FeSO_4.7H_2O$ (g)	0.01	0.02
6	F	Tween 80 (µl)	10.00	20.00
7	G	GrCf* (g)	0.75	1.25
8	H	Dextrin (g)	0.20	0.50
9	J	$MnSO_4 \cdot H_2O$ (g)	0.005	0.020
10	K	$CaCl_2 \cdot 2H_2O$ (g)	0.10	0.30

*GrCf – green chickpea flour

Table S2 Selected variables for BBD and their assigned levels for studying PYT production in 100 mL media

Variables	Variable code	Units	Levels		
			-1	0	1
$NaNO_3$	A	g	0.200	0.400	0.600
$MnSO_4 \cdot H_2O$	B	g	0.005	0.013	0.020
$CaCl_2 \cdot 2H_2O$	C	g	0.050	1.000	0.150
GrCf*	D	g	1.000	1.500	2.000

*GrCf – green chickpea flour

Table S3 PBD for the selected variables along with the experimentally obtained mean PYT production response values**

Run no.	A Glucose g%	B $NaNO_3$ g%	C $MgSO_4 \cdot 7H_2O$ g%	D KCl g%	E $FeSO_4 \cdot 7H_2O$ g%	F Tween 80 %	G GrCf* g%	H Dextrin g%	J $MnSO_4 \cdot H_2O$ g%	K $CaCl_2 \cdot 2H_2O$ g%	PYT activity (IU/mL)
1	5.0	0.3	0.1	0.1	0.02	0.01	0.75	0.2	0.02	0.1	93±4.6
2	3.0	0.3	0.04	0.04	0.01	0.01	0.75	0.2	0.005	0.1	109±5.4
3	3.0	0.8	0.04	0.1	0.02	0.01	1.25	0.5	0.02	0.1	84±4.2
4	3.0	0.3	0.04	0.1	0.01	0.02	1.25	0.2	0.02	0.3	89±4.5
5	3.0	0.3	0.1	0.04	0.02	0.02	0.75	.5	0.02	0.3	33±1.7
6	5.0	0.8	0.04	0.04	0.01	0.02	0.75	0.5	0.02	0.1	61±3
7	3.0	0.8	0.1	0.04	0.02	0.02	1.25	0.2	0.005	0.1	121±6
8	5.0	0.8	0.1	0.04	0.01	0.01	1.25	0.2	0.02	0.3	52±2.6
9	5.0	0.3	0.04	0.04	0.02	0.01	1.25	0.5	0.005	0.3	76±3.8
10	**5.0**	**0.3**	**0.1**	**0.1**	**0.01**	**0.02**	**1.25**	**0.5**	**0.005**	**0.1**	**132**±6.6
11	5.0	0.8	0.04	0.1	0.02	0.02	0.75	0.2	0.005	0.3	53±2.7
12	3.0	0.8	0.1	0.1	0.01	0.01	0.75	0.5	0.005	0.3	69±3.5

*GrCf – green chickpea flour

** Experiments were carried out in triplicate along with the type of error analyses (i.e., mean ± SD).

Table S4 BBD for the selected variables along with the experimentally obtained mean PYT production response values**

Run no.	A $NaNO_3$ g%	B $MnSO_4 \cdot H_2O$ g%	C $CaCl_2 \cdot 2H_2O$ g%	D GrCf* g%	PYT activity (IU/mL)
1	0.4	0.005	0.05	1.5	82±4.1
2	0.6	0.013	0.05	1.5	67±3.3
3	0.4	0.013	0.05	1.0	116±5.8
4	0.4	0.013	0.15	1.0	114±5.7
5	0.4	0.020	0.05	1.5	90±4.5
6	0.6	0.013	0.15	1.5	70±3.5
7	0.4	0.020	0.10	2.0	5±0.3
8	0.4	0.020	0.15	1.5	85±4.3
9	0.4	0.013	0.10	1.5	53±2.7
10	0.6	0.013	0.10	1.0	123±6.2
11	0.4	0.013	0.05	2.0	6±0.3
12	0.6	0.013	0.10	2.0	7±0.4
13	0.4	0.013	0.10	1.5	70±3.5
14	0.2	0.013	0.15	1.5	92±4.6
15	0.6	0.020	0.10	1.5	125±6.3
16	0.4	0.013	0.10	1.5	117±5.9
17	0.6	0.005	0.10	1.5	92±4.6
18	0.4	0.005	0.10	2.0	7±0.4
19	0.2	0.013	0.05	1.5	95±4.8
20	0.4	0.013	0.10	1.5	112±5.6
21	0.4	0.005	0.10	1.0	142±7.1
22	0.2	0.013	0.10	1.0	127±6.4
23	0.4	0.013	0.10	1.5	155±7.8
24	0.2	0.020	0.10	1.5	140±7
25	**0.4**	**0.020**	**0.10**	**1.0**	**160±8**
26	0.2	0.005	0.10	1.5	134±6.7
27	0.4	0.005	0.15	1.5	56±2.8
28	04	0.013	0.15	2.0	22±1.1
29	0.2	0.013	0.10	2.0	14±0.7

*GrCf – green chickpea flour

** Experiments were carried out in triplicate along with the type of error analyses (i.e., mean ± SD).