Phytase production under solid state fermentation and its application in Feed and Food

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BY

PRIYANKA G. BUDDHIWANT

(10BB11A2605)

RESEARCH SUPERVISOR:

DR. MAHESH S. DHARNE

RESEARCH CO SUPERVISOR:

DR. JAYANT M. KHIRE

NCIM RESOURCE CENTRE

BIOCHEMICAL SCIENCES DIVISION

CSIR– NATIONAL CHEMICAL LABORATORY,

PUNE 411 008, INDIA

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis **entitled "Phytase production under solid state fermentation and its applications in feed and food"** submitted by **Ms. Priyanka Buddhiwant** 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.

Ms. Priyanka Buddhiwant

(Student)

Dr. Mahesh Dharne

(Supervisor)

Dr. Jayant Khire

(Co-Supervisor)

DECLARATION

I hereby declare that the work of the thesis entitled "Phytase production under solid state fermentation and its applications in feed and food" 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. M. S. Dharne 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.

Priyanka.

Ms. Priyanka G. Buddhiwant

(Research scholar)

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Abbreviations:

°C	degree centigrade
µl	microliter
Å	Angstrom
ADP	Adenosine di phosphate
AMP	Adenosine mono phosphate
ANOVA	Analysis of variance
ATP	Adenosine tri phosphate
ATPE	Aqueous two phase extraction
BBD	Box Behnken Design
BPP	β propeller phytase
CCD	Central Composite design
CP	Cysteine Phosphatase
DCP	Dicalcium phosphate
EDTA	Ethylene diamine tetraacetic acid
FTU	Phytase (fytase) unit
g, mg, µg, ng	gram, milligram, microgram, nanogram
Gds	gram of dry substrate
GI	Gastrointestinal
GOC	Groundnut oilcake
GRAS	Generally regarded as safe
h	Hour
HAP	Histidine acid phosphatase
HAPhy	Histidine acid phytase
HCl	Hydrochloric acid
ICP AES	Inductively coupled plasma atomic emission spectroscopy
IP1, IP2, IP3,IP4,IP5	Inositol mono-, bis-, tris-, tetrakis-, pentakis-phosphate
IP6	Phytic acid
IU	International Unit
kDa	Kilodalton
L, mL, µL	liter, milliliter, microliter
M, mM, µM	molar, millimolar, micromolar
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time Of Flight

MCP	Mono calcium phosphate
MW	Molecular Weight
NCIM	National Collection of Industrial Microorganisms
OVAT	One Variable Approach
P	Phosphorus
PA	Phytic acid
PAGE	Polarcylamide gel electrophoresis
PAP	Purple acid phosphatases
PBD	Placket Burman Design
PDA	Potato Dextrose Agar
PSF	Poly styrene foam
PUF	Poly urethane foam
RP HPLC	Reverse phase high performance liquid chromatography
RSM	Response surface methodology
SBF	Soybean flour
SEM	Scanning electron microscope
SmF	Submerged fermentation
SSF	Solid state fermentation
TCP	Tri calcium phosphate
Wt	Weight

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Abstract:

Phytase, one of the important feed enzymes is used in monogastric animal feeds like poultry, swan and fish. Phytase breaks down the phosphorus containing antinutrient phytic acid and releases phosphates. Phytic acid is the intrinsic part of plant seeds, a storage house of phosphorus. Although the plant based food is rich in phytic acid, phosphorus requirement of the animals are often fulfilled by P supplements in the feeds. The bound p in phytic acid cannot be utilized by monogastric animals because of lack of gut phytase activity. Moreover the undigested phytic acid causes the phosphorus pollution in the area of extensive animal farming. Addition of phytase in feed is the feasible approach to solve the problem of phytic acid utilization. Apart from this the highly negatively charged phytate has a chelating ability and binds to mineral ions and proteins hence termed as antinutrient. Thus phytase addition in feed has dual benefit of P release and nutritional ad.

Phytase has been reported in different micro-organisms however for commercial purpose the fungal phytases are preferred as they are more stable and can be produced in large quantity. There are various commercial phytases available in the market. These are primarily produced by submerged fermentation of recombinant strains which makes them costly to be used widely. To be economical the production and down streaming cost of an product should be less.

Our lab has reported the high phytase producing strain of *Aspergillus niger* NCIM 563. Enzyme production was optimised on submerged and solid state fermentation and enzyme has been characterized.

Our objective was to minimise the cost of production and maximise the enzyme production for poultry application purpose. Utilization of cheap raw substrate and formulating a simple process is the key for cost reduction. We selected ground nut oil cake , a waste product of oil

industry. Oil cakes are rich in proteins and are generally used in animal feeds. We chosen solid state fermentation as the best method for production and optimized the process by response surface methodology, a statistical approach. By minimum downstream processing the enzyme was made ready to use as poultry feed supplement. The efficiency of our enzyme was checked in vitro and in field study on poultry birds.

Further the phytase has application in cereal based human food also. For this intention we have produced enzyme on poly urethane based optimized media to get a cleaner and purer enzyme. The enzyme is then applied during whole wheat and multigrain bread making. The final product was then nutritionally analyzed for phytic acid and minerals.

The whole work has been divided into four chapters and final section concludes and discuss the future possibilities.

Chapter 1: **Introduction: Fungal phytases**

The first chapter of thesis provides a general introduction to the phytase enzyme, its reaction mechanism and types. We have also outlined the history of phytase from lab to market. Source of phytase and accordingly its properties are also mentioned. we have compared the different fermentation methods and justified the selection of SSF as a method of choice. Varied applications of phytase have also been explained. At the end we have described the basic work done in our lab and objectives of the presented work.

Chapter 2: **Phytase production optimization under solid state fermentation**

This chapter is focused on optimization of phytase production by using solid state fermentation (SSF) technique. SSF is a low cost, higher production method and provide preferable conditions for fungal growth. The use of agro-industrial waste for phytase production using solid state fermentation (SSF) is of particular interest due to availability,

low cost and an eco-friendly process. Our studies deals with use of ground nut oil cake and response surface methodology for phytase production employing SSF. Placket–Burman design (PBD) was used to evaluate the effect of 11 variables and it was found that production was primarily influenced by four variables namely glucose, dextrin, distilled water, and $MgSO_4.7H_2O$. Further optimization of selected variables using Box-Behnken design (BBD) leads to about 37 fold increase in enzyme production from 15 IU/gds to 550 IU/gds. A comparison with available reports of phytase production proves that this the highest phytase production reported with SSF. The existing SSF processes for phytase production are facing engineering challenges due to low productivity. Hence high yield of phytase overcome the above limitations show the potential of oilcakes using SSF.

Chapter 3: **In vitro and in field poultry study of efficacy of phytase**

This chapter is concerned with application of phytase in poultry feed and its efficiency in releasing P and minerals in vitro and in vivo. The koji obtained after SSF is dried with stabilizer and made in a powder form. The dried koji was analysed for toxins at CARI. U.P. The enzyme in dried form is checked for activity, pH stability and shelf life at room temperature. The ability of enzyme to dephytinize the soya meal under simulated gastric condition and to release minerals is studied. Positive results guide us towards in field poultry study. Poultry feed is designed including our enzyme (powder form) and a 42 days trial was conducted on broilers. Results indicated that the enzyme was active in poultry gut and was able to replace up to 0.2% of P in feed. Thus with a minimum downstream processing we were able to prepare a low cost feed supplement with high phytase activity and efficiency in poultry feed.

Chapter 4: **Phytase Production on Poly-Urethane foam and its application in bread making**

Solid state fermentation (SSF) is a preferable method for fungal fermentation as it provides a solid support for good mycelial growth. Agricultural residues can be utilized for this purpose. These residues besides providing support also act as nutrient media however; there is a problem of uniformity with this support. These residues can be replaced with inert solid supports like polymeric urethane and styrene foam (PUF, PSF). PUF is particularly reported for SSF as it is porous, cheap, reusable and less complicated product recovery is an added advantage. In our study we have selected PUF as a reliable solid support for phytase producing fungus *A.niger* NCIM 563. Total phytase production found to be 3600 U on 4^{th} day of fermentation with 1g PUF and 25ml liquid media. After three successive cycle of fermentation, production reached to 5000U. This is the first report of this much high yield of phytase on PUF support. The liquid media used was based on the optimised media as obtained in chapter 2.The enzyme obtained from PUF fermentation was with less impurity and therefore easy to purify. It was purified in a single step ultra filtration method. In the present study we have applied this Phytase during bread making process. Phytase breaks down the phytic acid of flour which leads to dephytinization and augment soluble mineral content of bread up to 90%. Purified enzyme has broad range of applications in human food products.

Conclusion and future prospective

This chapter provides an account of the whole study and highlights the significant findings of the present work.

Chapter 1

Introduction: Fungal Phytases

Summary: Phytases are phospho-hydrolytic enzymes that catalyze the stepwise removal of utilizable phosphate from phytate. This enzyme has been studied intensively because of the need for reducing phytate content in animal feed and food for human consumption and combating phosphorus pollution. Although phytase is reported in various groups of microbes, plants and animals, microbial sources are promising among them. Higher thermal and pH stability makes fungal phytase a suitable choice as feed additive. Phytases have been produced by fungi in solid-state fermentation (SSF) and submerged fermentation (SmF). The fungal phytases are high molecular weight proteins ranging from 35 to 500 kDa. They are optimally active within pH and temperature ranges between 4.5 and 6.0, and 45 and 70 °C respectively. This chapter describes some basics of phytases, studies concerning the production, biochemical properties of fungal phytase, progress of fungal phytase from laboratory to industrial level, potential applications and future demand of phytase in promoting animal agriculture, human health, and ecological sustainability.

General Introduction:

During the last 25 years, phytases have attracted considerable interest from both scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology due to the diverse applications. Phytase is a phosphohydrolytic enzyme which acts on the substrate Phytic acid.

1. Phytic acid:

Phytic acid [*myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate] is the principal storage form of phosphorus (P) and inositol in cereals, legumes, and oilseeds. Phytic acid and its salts are common constituent of plant-derived foods. It represents around 60–90% of the total phosphorus content in plants [Raboy 2003]. It has several physiological roles and also affects the functional and nutritional properties of food ingredients. It accumulates in seeds and grains during ripening along with other storage substances such as starch and lipids. In cereals and legumes, phytic acid accumulates in the aleurone particles and globoid crystals, respectively [Reddy et al., 1982]. Phytic acid in seeds and grains serves as a phosphorus store, an energy store, a source of cations, a source of *myo*-inositol, and also helps in initiating dormancy. It may also serve several other unknown functions in seeds. Empson et al.[1991] suggested that the role of phytic acid in seeds is a natural antioxidant during dormancy. Table 1 gives an account of phytate content in commonly consumed cereals and grains. [Cao et al. 2007]

In spite of such large percentage , the bound form of phosphorus is largely unavailable to non-ruminants animals such as poultry, fish and humans due to very low or no activity of phytate degrading enzymes *i.e*. phytase (myo-inositol hexakisphosphate phosphohydrolase) in their digestive tracts [Singh et al. 2011]. Thus they require an additional source of phosphorus to avoid nutritional deficiency. Apart from these, phytic acid being a polyanionic

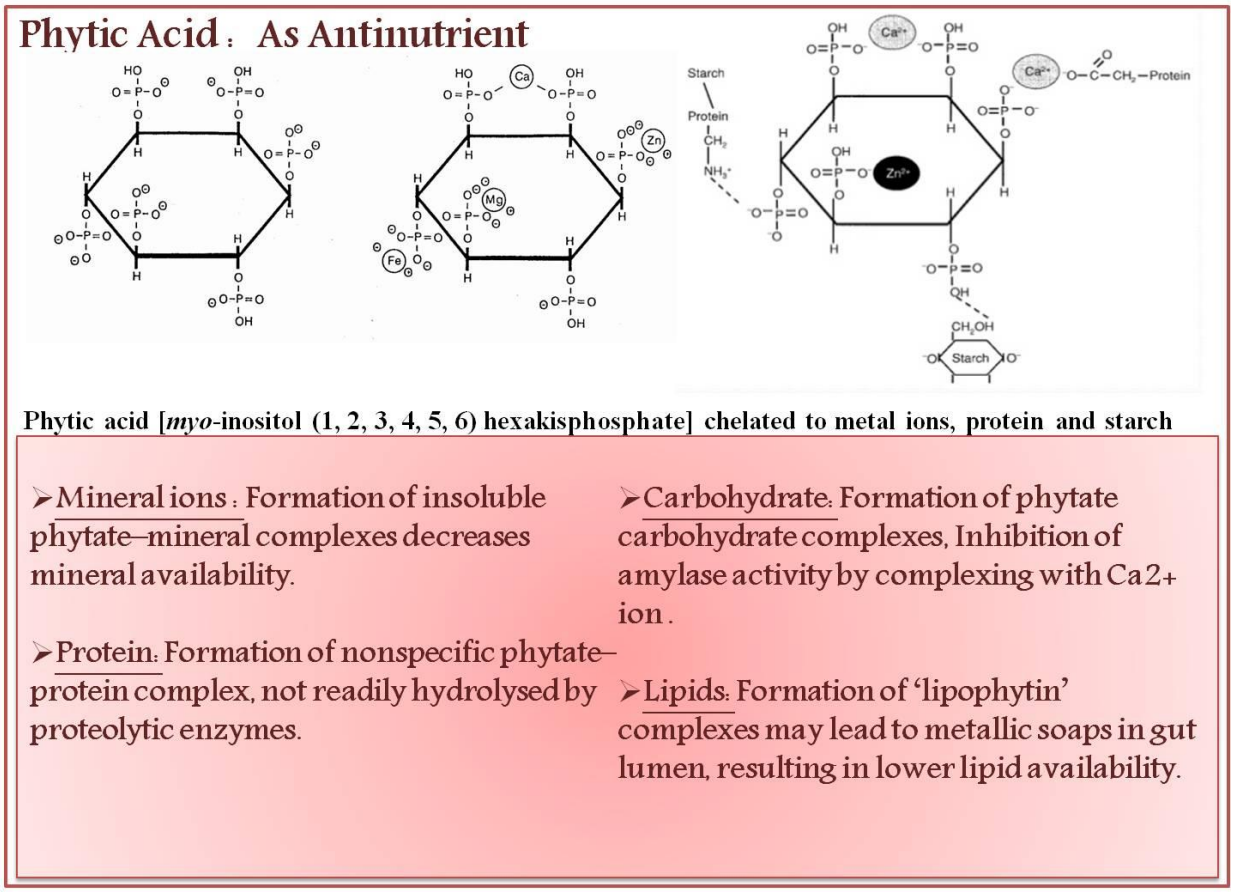
chelating agent, form complexes with various metal cations like Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and with proteins under both acidic and alkaline pH conditions which affect protein structure, resulting in decrease in the enzymatic activity, solubility and digestibility [Kies et al. 2006]. Because of such effects phytic acid is classified as antinutrient.

Table 1 Total phosphorus, Phytate P 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
Rye	3.05	1.95	63.9
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

The undigested phytate excreted by the animals is degraded by microorganisms in the soil and this released phosphorus at high concentrations gets into the rivers and causing serious

threat of phosphorous pollution. Higher phosphorus run off accelerates the process of eutrophication, which results into cyanobacterial blooms, hypoxia and death of aquatic animals [Vats et al. 2005].



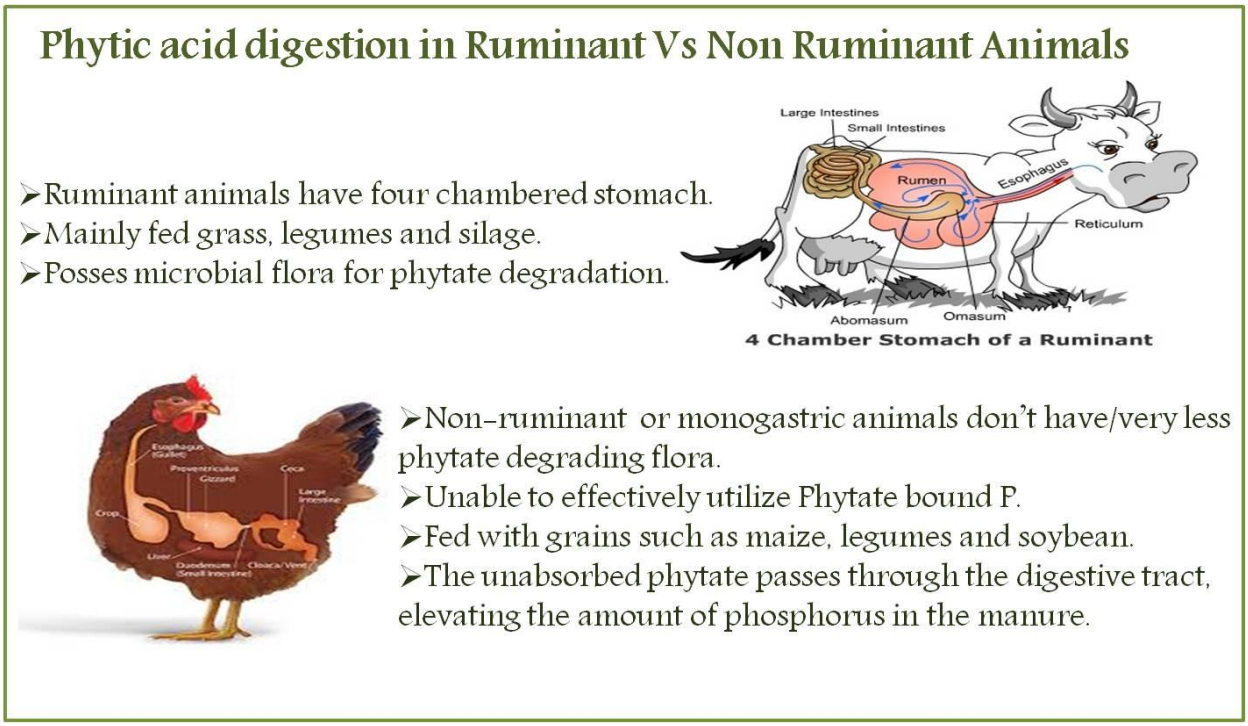
The importance of phytic acid as a source of phosphorus, its ability to cause undesirable ecological effects and antinutritive properties has stimulated research for ways for its dephosphorylation. Phytase is one of the practical approaches for it.

2. Phytase:

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase) catalyze the hydrolysis of phytate to *myo*-inositol pentakisphosphate (IP5) or to less phosphorylated *myo*-inositol phosphates IP3 or IP. Phytase were originally proposed as an animal feed additive to enhance the value of plant material in animal feed by liberating orthophosphate. Besides, addition of phytate-degrading enzymes can improve the nutritional value of plant-based foods by

enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in the stomach and it has also been seen as a way to reduce the level of phosphate pollution in the areas of intensive livestock management [Vats et al. 2005, Singh et al. 2011].

Phytase was identified for the first time in the rice bran as an enzyme, which could mediate the production of various phosphatidyl inositols as intermediates or as end products [Suzuki et al. 1907]. Phytases are widespread in nature, occurring in plants, microorganisms, and animal tissues. However, Industrial interest is mainly focused on microbial phytase for various applications. Most of the naturally occurring extracellular phytases having high thermal stability and a broad pH range were identified from fungi and was first to commercialised [Casey and Walsh 2003]. Although research on this enzyme persisted for over a hundred years, it has grown exponentially during the past two to three decades. The scientific and practical significances of phytase are best attested by its recent distinction as one of the ten most important discoveries in swine production in the past century [Cromwell 2009]. At the first international phytase summit in 2010, the current global phytase market was estimated to account for more than 60% of the total feed enzyme market and to be worth $350 million annually. The current inclusion rate of phytase in all diets for swine and poultry is approximately 70% [Lei et al. 2013].



2.1 Types of phytase:

Although all phytases enable phosphate monoester hydrolysis of phytic acid, the enzyme is diverse. They can be classified based on site of action, pH optimum and structure.

2.1.1 On the basis of site of dephosphorylation:

They are broadly classified into two types depending on their preferential attack on carbon atom present in phytic acid: 3 phytase and 6 phytase as classified by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) in consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN). But now, other phytases such as 2-, 4- and 5-phytases have also been described. Phytases, especially those of fungal origin (E.C. 3.1.3.8), often split the phosphate group at the C3 (carbon) of the inositol ring and are called 3-phytases. Plant phytases (E.C. 3.1.3.26) act preferentially at the C6 carbon, and are called 6-phytase. This had also been reported in some basidiomycetes fungi [Lassen et al. 2001]. An alkaline 5-phytase (E.C. 3.1.3.72) from lily pollen was found to start phytate hydrolysis at the D-5 position [Barrientos et al. 1994].

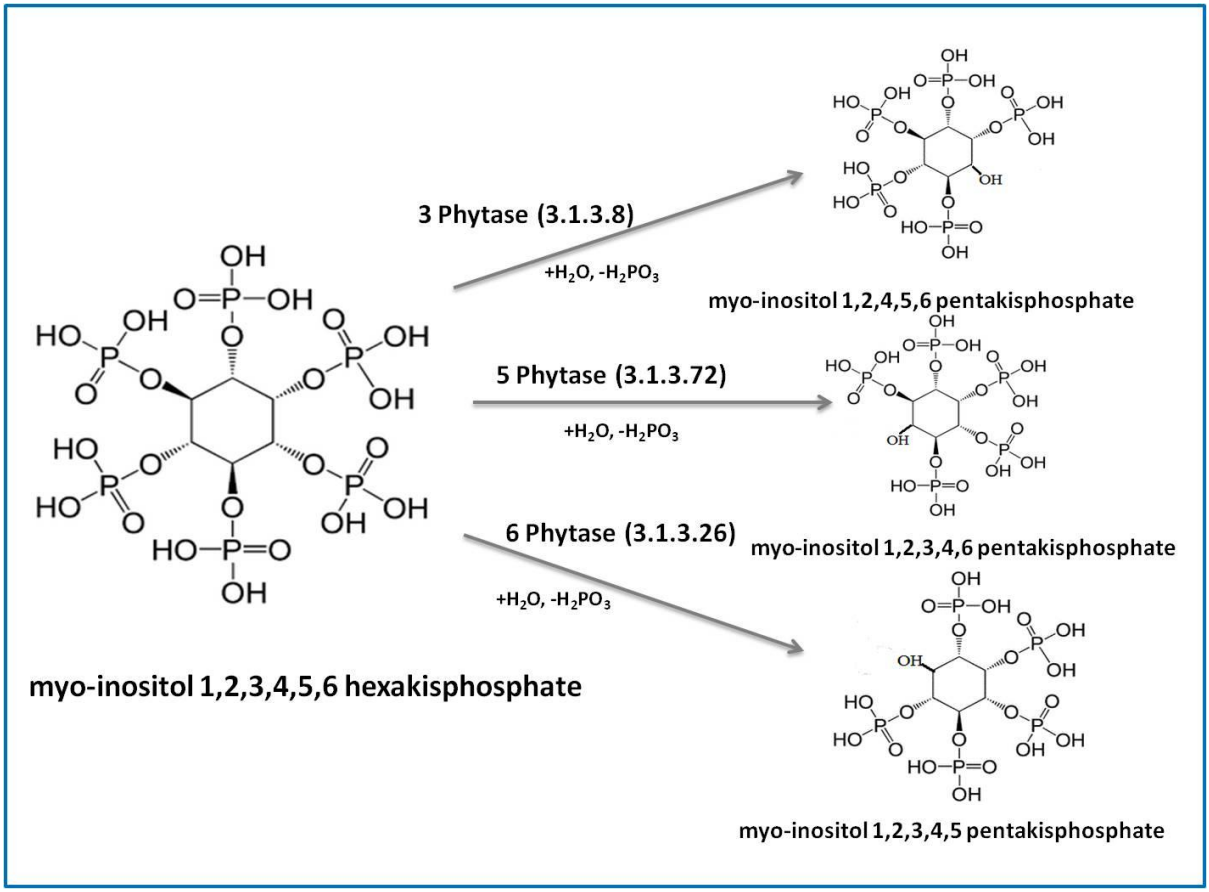
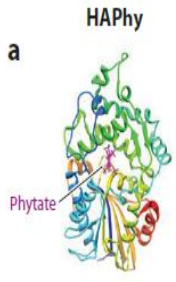
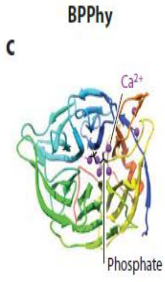
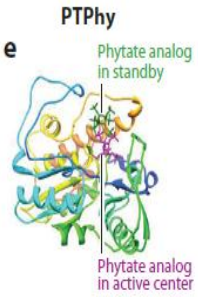
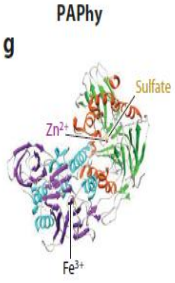


Figure 1 Phytate hydrolysis by different class of phytases and their end products

2.1.2 On the basis of catalytic properties:

On the basis of their catalytic properties phytases have been classified in four class .The classification and main characteristics of each class are given in Table 2. [Mullaney and Ullah 2003, Lei et al. 2013]

Table 2 Different class of phytase and their properties

	Histidine Acid Phosphatases (HAP)	**β-Propeller Phosphatases (βPP)**	**Purple Acid Phosphatases (PAP)**	**Protein Tyrosine Phosphatases (PTP)**
Activity pH	Active at acidic pH	Active at alkaline pH	Active at acidic pH	Active at acidic pH
Structural characteristics	Conserved active site hepta-peptide motif RHGXRXP at N terminal and the catalytically active dipeptide HD at C terminal	Six bladed propeller folding architecture with six Ca^{2+} binding site and the enzyme activity is dependent on calcium.	Seven conserved residues in five conserved motifs DXG, GDXXY, GNH (D/E), VXXH and GHXH involved in co-ordination of dinuclear Fe-Fe or Fe-Zn centre in the active site.	Cysteine containing (Cys 241) P loop HCXXGXXR(T/S)
Occurrence	Phytases from fungi and *E. coli* belong to the HAPhy.	The enzymes in this group are mainly *Bacillus* phytases and from legume seeds	These are found as a homodimeric glycoprotein in mainly plant species.	It is comparatively a newly described class in anaerobic ruminal bacterium *Selenomonas ruminantium.*
Application	All commercialized feed enzymes belong to HAPhy family	No commercial application till now, possibility to use in fish feed and agriculture	No commercial application is envisioned till now.	No commercial application is envisioned till now.
Secondary Structure				

2.2 Histidine Acid Phosphatases (HAP):

HAP shows broad substrate specificity and hydrolyzes metal-free phytate at acidic pH, and produces myo-inositol monophosphate as the final product. Phytases belonging to this class are the most widely studied and utilized today. Evidence from site-directed mutagenesis studies established the importance of certain amino acid residues that make up the substrate specificity site in fungal HAP. Mutating these key amino acids leads to changes in substrate affinity and the pH profile [Mullaney et al., 2002]. It is noted that two indispensable motifs, including substrate-binding site and catalyzation domain, are conserved in the phytases. The active site motif consist of RHGXRXP is generally located at N terminal while catalyzation motif is found at the C-terminal and consists of distinct HD element. The catalytic histidine in this sequence initiates a two-step reaction that results in the hydrolysis of phosphate monoesters.

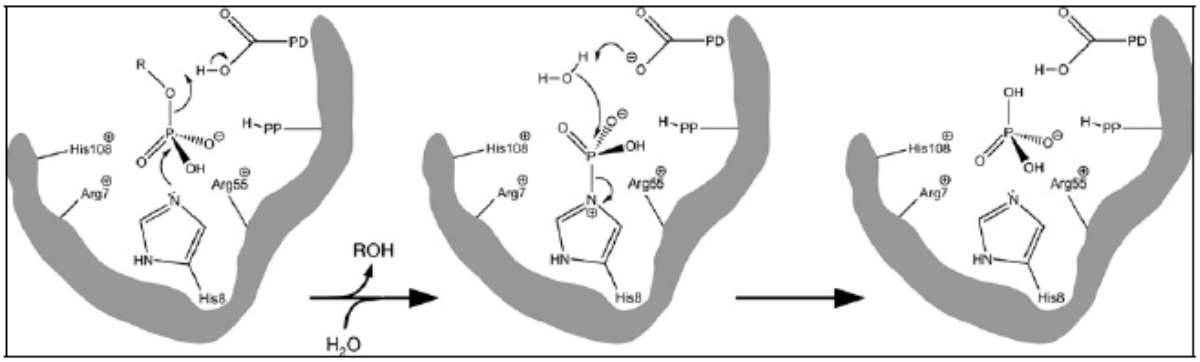


Figure 2 Phytase catalytic mechanisms

At the tertiary level, a typical 'pocket' structure is shown based on the interaction of key residues in the motifs. When the 'pocket' space is touched by the substrate, the conserved sequence in the substrate-binding site interacts with the phosphate groups in the substrate to form a complex of enzyme-substrate. The HD elements in the catalyzation domain further function to release the phosphate group from the substrate [Mullaney et al., 2000, Loewus and Murthy, 2000]. While not directly involved in the catalytic mechanism of HAP, the

conservation of an eight-cysteine motif appears to be essential to maintain the proper molecular structure necessary for the enzyme activity in fungal phytases [Mullaney and Ullah, 2005].

The term Histidine Acid Phytase (HAPhy) has been advanced to designate the HAPs that can accommodate phytate as a substrate. Both prokaryotic and eukaryotic HAPhys are known and they share little sequence homology other than the conserved active site motif. Among prokaryotic phytases, the one produced by *Escherichia coli* is the best-characterized HAPhy [Greiner et al., 1993]. In eukaryotes, HAPhys have been cloned in maize and in a number of fungal isolates. The most widely studied fungal phytases are from *A. niger* and *A. fumigatus*. Wyss et al. [1999] divided HAP phytases into two groups on the basis of the catalytic properties. There are two HAP phytases from *A. niger* NRRL 3135, that is phyA with high specific activity for phytic acid and phyB with low specific activity for phytic acid. Another difference between phyA and phyB phytases is that the active form of phyA is a monomer, whereas it is tetramer in phyB . Today, the major application for HAPhys is in the hydrolysis of phytate in cereal and grains in animal feed.

2.3 Phytase sources:

Although phytase has been isolated and studied in large number of microbes, plants and animal tissues, for commercial application microbial source are advantageous. Among microorganism also mainly *Aspergillus* and *E. coli* phytase are available as feed enzyme. [Vats et al. 2005, Singh et al. 2011]. The first phytases produced on a commercial scale were either derived from fungal strains mutated via standard procedures or by recombinant DNA technology. [Cao et al. 2007]. However, effectiveness of these phytase supplements is low because of the lack of essential characteristic, and thus the quest for an ideal phytase continues. Since fungal phytases are more promising and has broader applicability, we are focusing here on fungal phytases.

2.4 Fungal phytases:

Fungi have been used to produce various commercially important products like enzymes (cellulases, xylanases, ligninases and pectinases), proteins and organic acids. They have the benefit of extracellularity and higher productivity. They also secret accessory enzymes which may improve nutritional quality and digestibility of animal feed [Bogar et al. 2003, Bhavsar et al. 2011].

2.4.1 Screening methods for phytase producing micro-organism:

Several screening programmes have been carried out aiming at the isolation of phytase producing micro-organisms. A simple and rapid method was described for determining microbial phytase by evaluating the inorganic orthophosphate released on the hydrolysis of sodium phytate at pH 5.5[Engelen et al. 1994]. Bae et al [1999] developed a method for detecting phytase activity on differential agar media, in which the disappearance of precipitated calcium or sodium phytate was an indication of enzyme activity. However, this technique was unable to differentiate between phytase activity and acid production. The above mentioned assay is performed with phytate as a substrate and degradation of phytic acid to the amount of P released. Chen [1998] developed a bioassay method using the washed cells of *Corynebacterium glutamicum* as an indicator strain for the screening of extracellular phytase.

However, the phytase screening media and assay has limitations. The traditional endpoint assay is time-consuming and is uses toxic regents. This method does not give a very detailed picture of the actual mechanism of phytase action and other methods including chromatographic separation followed by quantification of the lower inositol phosphates are therefore sometimes employed making it time consuming. Thus, a simple, fast and nontoxic kinetic method was developed by Tran et al. [2011] for high throughput assaying of phytase, overcoming the limitations of traditional phytase assay methods. This assay is based on the

principle that IP6 forms stable turbid complexes with positively charged lysozyme in a wide pH range, and the hydrolysis of IP6 in the complex is accompanied by a decrease in turbidity monitored at 600 nm.

2.4.2 Screening of fungi for phytase activity:

Shieh and Ware [1968] tested over 2000 microorganisms isolated from soil. Extracellular phytase activity was observed in only 30 isolates. They all were filamentous fungi, 28 of them belonged to the genus *Aspergillus,* one species belonging to *Penicillium* and other one to *Mucor*. *A. niger* strains were identified as the best producers. While Howson and Davis [1983] surveyed 84 fungal species for phytase production. Of all the organisms surveyed, *A. niger* NRRL 3135 was identified as the most potent phytase producer in corn starch [Shieh and Ware, 1968] and semi synthetic [Howson and Davis, 1983] media. Lissitskaya et al. [1999] screened microorganisms producing phytase using museum and soil samples, wherein it was found that moulds metabolized P more effectively than bacteria.

Phytase have been detected in different fungal classes with differing biochemical properties. Phytases from ascomycetes fungi and yeast belonged to 3 phytase classes whereas phytases from basidiomycetes fungi falls in a different cluster and belonged to 6 phytase class [Lassen et al. 2001].

Phytase from thermophilic fungi:

Thermophilic fungi have also been screened for phytase activity. They have been seen as a source of novel phytases with desirable properties of thermal stability, significant shelf life and organic solvent tolerance [Maheshwari et al. 2000]. Several thermophilic molds (*R. pusillus*, *M .thermophila*, *and T. lanuginosus*) have been isolated from composts and soils and screened for phytase production [Mitchell et al. 1997, Berka et al. 1998, and Chaddha et al. 2004].

Phytases from Yeasts:

Phytase production by yeasts has also been studied and reviewed [Singh and Satyanarayana 2015]. Both conventional and non-conventional yeasts are known to secrete phytases. *Candida tropicalis, Torulopsis candida, Debaryomyces castelii*, *Kluyveromyces fragilis* and *Schwanniomyces castellii* were able to hydrolyse phytate when cultivated in the medium containing phytate as a sole source of phosphorus. *S. castellii* secreted the highest phytase titres as compared with other yeasts [Lambrechts et al. 1992, Segueilha et al. 1992]. Vohra and Satyanarayana [2001] and Quan et al. [2002] reported the production of a cell-bound phytase from *P. anomala* and *Candida krusei* WZ-001 respectively. *Aruxla adenininvorns* a non-conventional yeast like *P. anomala* was shown to assimilate phytate as a sole source of carbon and phosphorus. This ability was associated with a high phytase activity secreted into the medium [Sano et al.1999].

An account of different fungal classes producing phytase with their biochemical properties is given in the Table 3.

Table 3 Phytase producing fungi among different classes of fungi and their properties.

Enzyme Class	Fungi	Type of fermentation	pH optima	Temperature optima (°C)	Reference
Ascomycetes					
3 Phytase	*Aspergillus sp.*	SmF	5	30	Shieh and Ware 1968,1969
	A. niger	SmF	2.5 & 5	58	Shieh and Ware 1969
	A. ficcum	SmF	2.2 & 5.5	55-58	Ullah 1988
	A. niger	SmF	2.5	60	Ullah and Phillipy 1994
	A. oryzae	SmF	6.4	37	Shimizu 1993
	A. terries	SmF	5- 5.5	70	Mitchell et al. 1997, Wyss et al. 1999
	A. niger NCIM 563	SSF	5	50	Mandvivala and Khire 2000
	A. niger van teighem	SmF	5.5	30	Vats et al. 2005
	N. crassa	SmF	5.5/3.5	60	Zhou et al. 2006
	A.niger NCIM 563	SmF	2.5/4	60	Soni et al. 2007
	P. oxalicum		4.5	55	Lee et al. 2007
	A. japonicas		5.5	50	Promdonkoy et al. 2009
	E. parvum		5.5	50	Fugthong et al. 2010
	Penicillium sp 07		5.5-6	50	Zhao et al. 2010
	N. spinosa		5.5	50	Pandee et al. 2011
	A.tamari	SmF	8.5	28	Shah et al. 2012
	A.oryzae SBS 50	SmF	5	35	Singh 2013
	S. castellii	SmF	4.4	37	Segueilha et al. 1992
	A. adeninivorans	SmF	5.5	28	Sano et al. 1999
	C. krusei	SmF	4.6	40	Quan et al. 2002
	P. anamola	SmF	6	25	Vohra and Satyanarayana 2011
	M. thermophila *	SSF	5.5	45-50	Mitchell et al. 1997
	*A.fumigates SRRC 322**	SmF	5	37	Mullaney et al. 2000
Not classified	*T. lanuginosus**	SmF	6	65	Berka et al.1998
	*T. aurantiacus**	SmF	5.5	55	Nampoothiri et al.2004
	*T. auranticus**	SSF	6	45	Hassouni et al. 2006
	S.thermophila *	SSF	5	60	Singh and Satyanarayana

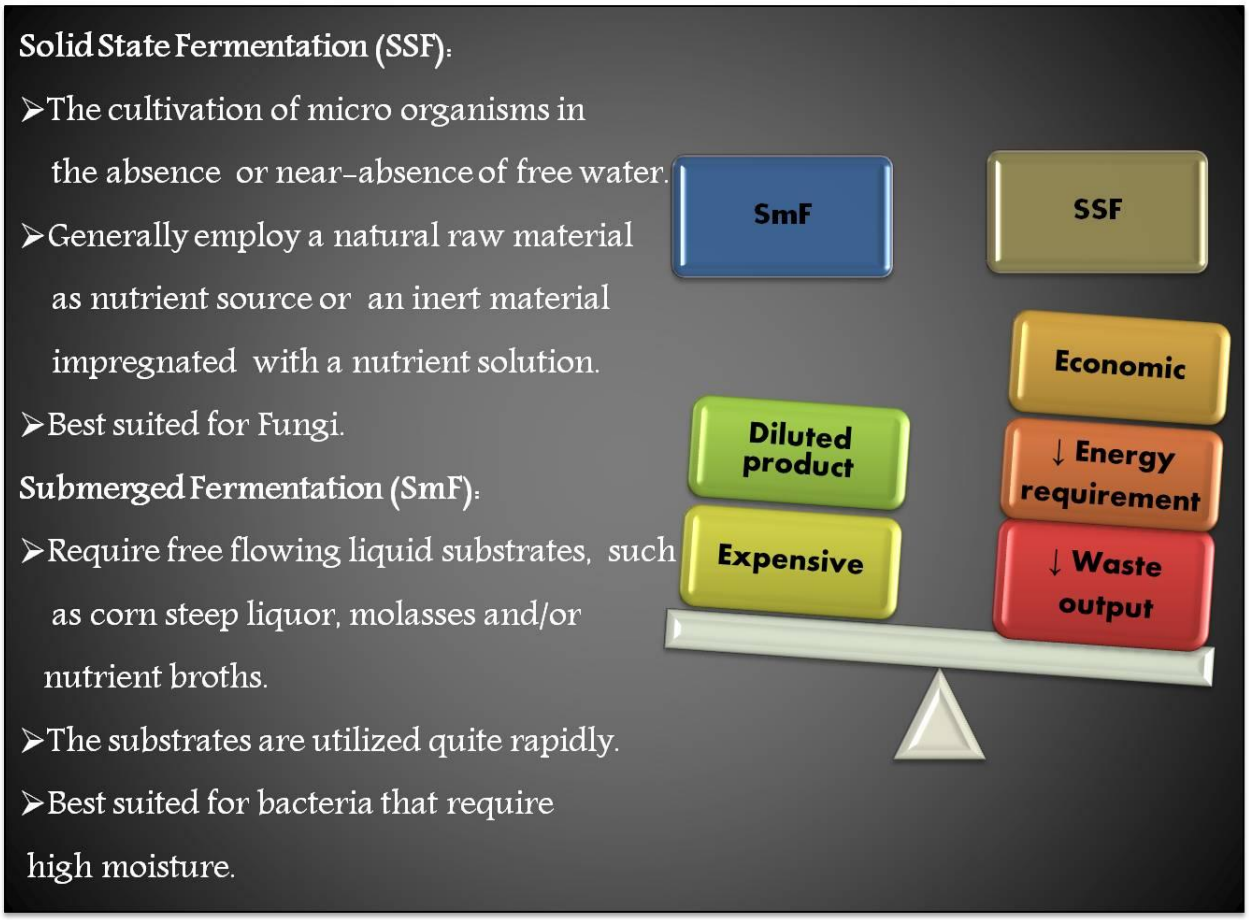
					2006b
	T. lanuginosus TL-7*	SSF	5.5	45	Gulati et al. 2007
	*S.thermophila **	SmF	5	45	Singh and Satyanarayana 2008c
Basidiomycetes					
6 phytase	*P. lycii*	SmF	4-5	40-60	Lassen et al. 2001
	A. pediadis		5-6		
	Ceriporia sp.		5-6		
	T. pubesons		5-6		
Not classified	*A. bispora*	SSF	5.5	37	Callopy et al. 2004
	G. frondosa				
	L. edodes				
	P. cornucopia				
	Rhizoctonia sp.	SmF	5	50	Marlida et al. 2010
	F. verticellioides		4		
	S. commune	SSF	5	50	Salmon et al. 2012
Zygomycetes					
Not classified	*R. oligosporus*	SmF	5.5	27	Casey and Walsh 2004
	*R. pusillus**	SSF	8	70	Chadha et al. 2004
	R. oryzae	SSF	5.5	30	Ramchandran et al. 2005
	M. recemosus	SSF	5.5	30	Roopesh et al. 2006

*thermophiles.

2.4.3 Production Method for Fungal Phytase:

Phytase can be produced on industrial level by submerged (SmF) and solid state fermentation (SSF). SmF has largely been employed as a production technology for commercial phytases. However, in recent years solid state fermentation (SSF) has gained significant interest for the production of phytase. The type of strain, culture conditions, nature of the substrate and availability of the nutrients are critical factors affecting the yield and should be taken into consideration when selecting a particular production technique. For example, a filamentous fungus in SmF is exposed to hydrodynamic forces but in SSF the surface of the solid particles

acts as the matrix for the culture. Phytase production has been studied under SmF and SSF; and various studies have reported that enzymatic production under SSF has several advantages in comparison to that of SmF. This includes lower waste water output, reduced energy requirements, simpler fermentation media, easier aeration, reduced bacterial contamination etc. [Pandey et al. 1999]. SSF is more economic as various agricultural and industrial residues in different ratio can be used as substrate and crude product can act as value added supplement in animal feed. The product spectrum from SSF is also very different from that obtained in SmF. However, the mechanisms underlying these differences are not understood. Despite of advantages of SSF, SmF is preferred for commercial production due to ease of operation, up scaling and less variability [Bhavsar et al. 2008]. Because of the inadequate understanding of the morphogenesis of filamentous microorganisms, fungal morphology is often a bottleneck of productivity in industrial production [Wucherpfennig et al. 2011]. Recently, significant advances were made in understanding the physical (process engineering) aspects of SSF but the information on physiology and molecular genetics is limited. To obtain an optimized production process, it is of significant importance to gain a better understanding of the molecular and cell biology of these microorganisms as well as the relevant approaches in biochemical engineering. Due to low productivities and lack of ideal characteristics, the quest for the discovery of new wild type phytases and improving the existing phytases production methods continues.



2.4.4 Critical factors of phytase production and optimization:

Optimization is important for maximizing production and yield, and at the same time for minimizing the cost. No single media composition can be assigned for optimum phytase production by different fungal strain; however certain common factors need to be considered while optimizing the production. The critical factors which affect phytase yield under SmF or SSF includes culture conditions, type of strain, nature of substrate and availability of nutrients. Krishna and Nokes [2001] studied the effect of culture conditions, particularly inoculum age, media composition (wheat bran and full-fat soybean flour) and duration of SSF on the phytase production by *A. niger*. They reported that phytase production was strongly growth associated in SSF where younger fungal inoculum, being in active growth phase, contributes to the active utilization of the substrate and further phytase synthesis. Bogar et al. [2003] reported phytase production by *A. ficuum* NRRL3135, *Mucor racemosus* NRRL1994

and *Rhizopus oligosporus* NRRL 5905 on canola meal, cracked corn, soybean meal and wheat bran in SSF. Both *A. niger* CFR 335 and *A. ficuum* produced high phytase titres in wheat bran solid substrate medium [Shivanna and Venkateswaran, 2014]. Enzyme production was further enhanced using mixed substrate medium comprising wheat bran, rice bran and groundnut cake. A maximum enzyme activity (9.6 and 8.2 U/ml) was observed in SmF by *A. niger* CFR 335 and *A. ficuum* in potato dextrose broth. The fermentation conditions (pH 5.3, 30 °C, 54.5% moisture content) of phytase production in SSF using canola oil cake with no additional nutrients by *R. oligosporus* were reported by Sabu et al. [2002]. *A. niger* NCIM563 produced high phytase on cowpea meal followed by wheat bran [Mandviwala and Khire, 2000]. Optimisation studies were carried out using Plackett-Burman (PBD) and Central Composite Designs(CCD) showing the improved production when wheat bran was supplemented with additional carbohydrate and nitrogen source [Bhavsar et al [2011]. *Thermoascus aurantiacus* TUB F43 synthesized phytase in a medium containing glucose and starch as carbon sources and peptone as a nitrogen source at 45 °C, 150 rpm and pH 5.5 after 72 h of fermentation [Nampoothiri et al., 2004]

Phosphate concentration is also one of the important factors for phytase production. High phosphate conditions are known to repress the synthesis of acid phosphatases and phytases, while limiting phosphate conditions result in their expression. In a survey of phytase producing microorganisms, *A. ficuum* produced highest amount of phytase (113 nkat/ml in shake flask in 5 days) when the inorganic phosphorus content was in the range of 0.0001–0.005%, optimum being 0.4 mg/100 ml with 8% cornstarch [Wodzinski and Ullah 1996]. Han and Gallagher [1987] also confirmed that high phosphorus concentration inhibited phytase synthesis by *A. niger* NRRL 3135 while Fredrikson et al. [2002] showed that repression of phytase synthesis by inorganic phosphorus was less significant in a medium of higher composition complexity.

Al-Asheh and Duvnjak [1994] studied the effect of surfactants such as Tween-80, Triton-X-100, Na-oleate on the phytase production and reduction of phytic acid content in canola meal by *A. carbonarius* in SSF. The phytase production increased in the presence of sodium oleate (1%) and Tween-80 suggesting alteration of the cell permeability, resulting in the higher release of enzyme. Similarly Mandviwala and Khire [2000] reported 30% increase in phytase activity of *A. niger* NCIM 563 when 0.5% Triton-X-100 was added to the production medium. Enhanced phytase production by *Thermoascus aurantiacus* was observed in a semi synthetic medium using Tween-20 and Tween-80, while Triton X 100 exerted a negative effect on enzyme production (Namapoothiri et al., 2004).

Response surface methodology (RSM):

RSM can be defined as a statistical method that uses quantitative data from appropriate experiments to determine & simultaneously solve multivariant equations. It has been successfully used in many optimization studies. The conventional one variable at a time (OVAT) approach is time consuming and laborious because it involves the variation of a single variable while maintaining others at a constant level. The true optimum value is missed due to the lack of the interaction of components. While response surface methodology is more systematic, efficient and explores the relationships between several variables . It uses a sequence of designed experiments to obtain an optimal response.

2.4.5 Downstream processing:

Downstream processing is an integral part of any product development process. Downstream processing, involving recovery and formulation, incurs 70% of the overall production cost. Phytase technology for separation and purification, employing a chromatographic process, has evolved slowly as compared to production. Most of these approaches were employed for analytical purposes especially for biochemical, molecular and structural characterization of

enzyme. Fungal extracellular phytases are obtained from the culture filtrate in rather high yields. Conventional method includes common biochemical techniques such as ammonium sulphate fractionation, acetone precipitation, gel filtration, ion-exchange chromatography, affinity chromatography and RP HPLC [Lim and Tate 1973, Skowronski 1977, Ullah 1988, Ullah and Dischinger 1990]. To purify the phytate degrading enzyme from *A. niger* NRRL 3135 a three-step protocol has been proposed, including ion-exchange chromatography and chromatofocusing. The enzyme was purified 22-fold with a recovery of 58% [Ullah and Gibson 1987]. Casey and Walsh [2004] purified and characterized thermostable extracellular phytase from *A. niger* ATCC 9142 by ion-exchange, gel filtration and chromatofocusing steps. Soni et al [2010] purified two extracellular phytase produced by *A.niger* NCIM 563 under SmF using ion-exchange chromatography, followed by gel filtration. Bhavsar et al [2012] showed phytase extraction in a single step from fermentation broth by a liquid–liquid extraction process using ATPE. High phytase recovery (98.5%) within a short time (3 h) and improved thermo-stability was attained by ATPE in comparison to 20% recovery in 96 h by chromatography process.

Phytases are susceptible towards inactivation, thus for enhanced stability, they are often formulated as solid-state proteins produced by spray drying, lyophilization or granulation. A dry formulation greatly reduces the likelihood of chemically and biologically mediated inactivation. Thus, there is a growing interest for fast and economic processes, which will simplify phytase down streaming technology. Alternatively the phytase produced by GRAS organism on suitable substrate can be directly applied in feed application purpose. However suitability of such feed supplement in terms of stability, efficiency and safety has not been evaluated.

2.4.6 Biochemical Properties:

The following properties of phytase are of practical significance:

2.4.6.1 pH and Temperature Optima:

Most of the so far described fungal phytate degrading enzymes belong to the acidic ones and their pH optima range from 4.5 to 6.0.(Table 3). For animal feed application acid phytase is preferred over alkaline phytase because of its ability to work at stomach pH. In contrast to most fungal phytases, *A. fumigatus* phytase has a broad pH optimum; at least 80% of the maximal activity is observed at pH values between 4.0 and 7.3 [Ullah et al. 2000]. *A. niger* NRRL 3135 secreted two different phytases, one with pH optima at 5.5 and 2.5, and the other at 2.0; as such, these enzymes were designated as phyA and phyB, respectively [Wyss et al. 1999]. The phytases of thermophilic molds are also acidic in nature. Phytases of *T. lanuginosus* [Berka et al. 1998] and *S. thermophila* [2006] were optimally active at pH 6.0 to 6.5.Soni at al.[2007] described two different phytases produced by *A. niger* NCIM 563 active at pH 2.5 and 5 respectively.

Phytases employed in feed, work inside digestive tract of animal. The body temperature of poultry and pig are in range of 37 to 39° C while fish has lower body temperature. However the temperature optima of most fungal phytases range from 45 to 60ºC. An enzyme should be sufficiently active at the body temperature of targeted animal to serve the purpose. Phytase of *A. oryzae* [Shimizu 1993] and *A. niger* NRRL 3135 [Shieh et al 1969] exhibited optimum activity at 37°C and at 58°C, respectively. Among the thermophilic fungi, *Thermomyces aurantiacus* phytase exhibited optimum activity at 55°C [Nampoothiri et al. 2004], and that of *Rhizomucor pusillus* at 70°C [Chadha et al. 2004]. Phytase from *S. thermophile* [Singh and Satyanarayan 2008] were optimally active at 60°C.(Table 3).

2.4.6.2 pH, Thermal and protease stability:

Broad pH stability is the one of the most desirable characteristic for phytase which determines its ability to remain stable and act efficiently in the digestive tract of poultry and pig. Most isolated phytases are active within the pH range 4.5– 6.0 and the stability of enzyme activity is decreased dramatically by pH values lower than 3 or higher than 7.5 [Wyss et al. 1999]. Soni et al [2007] showed that two phytases produced by *A. niger* NCIM 563 was stable in the pH range 1.5–3.5 and other one stable in the wider pH range, 2.0–7.0.

Thermo-stability is the main constraint for ideal phytase designing because commercial feed processing involves pelleting at high temperatures (60–80ºC) .Feed enzymes need to be heat stable to avoid substantial activity loss during this process. Thermo-stability of any given phytase is decided by its ability to resist heat denaturation and its ability to refold appropriately into the native-like, fully active conformation after heat denaturation [Ullah 1988]. The phytases of thermophilic molds are highly thermo-stable compared to their mesophilic counterparts. An extremely thermostable phytase from *A. fumigatus* was reported by Pasamontes et al. [1997]. The phytase of *S. thermophile* retained 100% of its activity at 60ºC for 5 h with a $t_{1/2}$ of 16 h at 60ºC and 90 min at 80ºC [Singh &Satyanarayana 2006b]. The thermostability of *A. fumigatus* phytase, *A. niger* phytase, and *A. niger* acid phosphatase was investigated based on circular dichroism, fluorescence studies, and enzyme activity. *A. fumigatus* phytase alone could refold into a fully active native conformation even after heat denaturation at 90°C. In feed pelleting experiments performed at 85°C, the recovery of enzyme activity was significantly higher for *A. fumigates* phytase (51%) than that of *A. niger* phytase (31%) or *A. niger* acid phosphatase (14%) [Wyss et al. 1998]. Fungal phytases from *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia* sp., and *Trametes pubescens* showed high degree of refolding after thermal unfolding, as evidenced by differential scanning calorimetric studies [Lassen *et al*., 2001]. *A. niger* phytase expressed in *P. pastoris*, upon

glycosylation, revealed enhanced (60%) thermostability in comparison to the unglycosylated form [Han et al. 1999]. The higher thermal tolerance disclosed by *A. fumigatus* phytase was attributed to the differences in the post-translational modifications rather than to the primary structure of the enzyme [Rodriguez *et al*., 2000]. Recombinant DNA technology and enzyme engineering are the ways for the production of thermally viable phytases.

The effectiveness and limitations of food and feed supplementation with phytate-degrading enzymes may also depend on their sensitivity to protease digestion. It was shown that the phytases from *A. niger* was more stable in the presence of pepsin or pancreatin than the corresponding enzyme from wheat [Phillippy, 1999].Furthermore, the *Aspergillus* enzyme is more resistant to trypsin and less resistant to pepsin and pancreatin than the phytase enzyme from *E. coli* [Rodriguez et al., 1999b]. High stability of the *E. coli* phytase against inactivation caused by pepsin was also confirmed by Golovan *et al*. [1999] .*Bacillus* phytase was extremely resistant to papain, pancreatin, and trypsin, but was found susceptible to pepsin [Kerovuo et al. 2000]

2.4.6.3 Substrate specificity of phytases:

Phytases with acidic pH optima, in general, are known to show broad substrate specificity. Enzymes from *A. niger* [Casey and Walsh 2003], *A. fumigatus*, *E. nidulans*, *Myceliophthora thermophila* [Wyss et al. 1999] were found to show broad substrate specificity for InsP6, glucose- 1-phosphate, glucose-6-phosphate, fructose-1-phosphate, fructose-6-phosphate, *p*-nitrophenyl phosphate, AMP, ADP, ATP, 1-naphthylphosphate, 2-naphthylphosphate, and phenyl phosphate.

2.4.6.4 Molecular weight:

Phytases are high molecular weight proteins ranging between 40 and 700 kDa. The majority of phytases characterized so far acted like monomeric proteins with molecular masses between 40 and 70 kDa. However, some phytate degrading enzymes appear to be made up of

multiple subunits. Phytase of *S. castellii* has a molecular weight of 490 kDa with a glycosylation of around 31% [Seguilha et al. 1992]. The glycosylated protein was tetrameric, with one large subunit (MW 125 kDa) and three identical small subunits (MW 70 kDa). Purified phytase from *A. fumigatus* revealed a protein with a molecular mass of 60 kDa by SDS-PAGE [Pasamontes et al. 1997]. The molecular masses of the monomeric form of phyA, phyB and acid phosphatase were estimated by SDS-PAGE as 85, 65 and 85 kDa, respectively. An extracellular phytase and an extracellular acid phosphatase were purified from *A. oryzae* K1 and their molecular masses were 60 and 70 kDa, respectively [Shimizu, 1993]. The phytase of *A. niger van Teighem* was a 353 kDa homopentameric protein with a monomeric molecular mass of 66 kDa [Vats and Banerjee 2005], while the phytase of *S. thermophile* is a homopentameric 456 kDa glycosylated protein with a monomeric mass of 90 kDa [Singh and Satyanarayana 2008], and that of *P. anomala* is a homohexamer with a molecular mass of 390 kDa [Kaur et al., 2010]. The *A. oryzae* [Shimizu 1993] were homodimeric proteins, while a homohexameric structure was proposed for the *A. terreus* enzyme [Yamamoto et al., 1972]. Soni et al. [2010] reported two different phytase from *A. niger* NCIM 563, one is a tetrameric 264 kDa and other one is monomeric 150kDa protein

Fungal and several plant phytases have been found to be glycosylated with a carbohydrate content of 27.3% [Ullah 1988]. Glycosylation may have an effect on the catalytic properties, thermal stability or the isoelectric point of an enzyme.

.2.4.6.5 Effectors of Enzyme Activity:

Metal ions have been shown to modulate phytase activity. Most phytases characterized so far are inhibited by Cu^{2+}, Zn^{2+} and Fe^{2+} or Fe^{3+} .but it is difficult to determine whether the inhibitory effect of various metal ions is the result of binding to the enzyme or the formation of poorly soluble metal ion-phytate complexes. The appearance of a precipitate while adding Fe^{2+} or Fe^{3+} to the assay mixtures suggests a decrease of the active substrate concentration by

the formation of poorly soluble iron-phytate [Konietzny et al. 1995]. Wyss et al [1999] reported that Cu^{2+} considerably depressed the enzyme activities of *E. nidulans* and *A. terrus* phytases, and that several metal ions inhibited *A. fumigates* phytase. On the other hand phytase activity from *A. ficuum* NRRL 3135 is unaffected by calcium ions but inhibited by iron [Ullah and Gibson 1987]. Bhavsar et al [2011] showed retention of phytase activity in the presence of Pb^{2+} (125% for 5 mM) and Ag^{2+} (50% for 1 mM) which provides an opportunity for phytate hydrolysis in soils contaminated with heavy metals. Phytase from *Aspergillus niger van Teighem*, however, was not affected by most metal ions, inhibitors and organic solvents [Vats et al. 2005]. Molybdate and vanadate are also known to inhibit phytase. It has been suggested that these transition metal ions exert their inhibitory effects by forming complexes that resemble the trigonal bipyramidal geometry of the transition state [Ullah et al. 2000].

Reducing reagents, such as 2-mercaptoethanol, dithiotreitol and reduced glutathione have no major effect on the activity of fungal enzymes. This suggests that these enzymes either do not have any free and accessible sulphydryl group or that free sulphydryl groups play a negligible role in enzyme activity and structure [Wyss 1999].

The hydrolysis product orthophosphate was recognized as a competitive inhibitor of enzymatic phytate degradation. Besides the hydrolysis product, the substrate phytate was also reported to act as an inhibitor of many phytate-degrading enzymes [Ullah and Gibson 1987] .With high substrate concentrations; the charge because of the phosphate groups may affect the local environment of the catalytic domain of the enzyme. This might inhibit conversion of the enzyme-substrate complex to enzyme and product.

2.5 Commercially available phytases and current status:

The research on phytase spans 87 years from its discovery by Suzuki et al. in 1907 until its commercialization in 1991 which emphasized on practical use and cost effective production

of enzyme [Vats et al. 2005]. Even after commercial availability, its market developed slowly in next 15 years. This was because in the first 15 years after introduction, use of phytase provided no cost benefit to the use of inorganic phosphorous. But now phytase market has reached to $350 million and it is increasing by 10% every year [Lei et al. 2013]. Two main factors contribute to current intensified phytase research and increased annual sale:

[A] Society's awareness towards pollution

[B] Increasing cost of inorganic phosphate.

The first phytases produced on a commercial scale were either derived from fungal strains mutated or by recombinant DNA technology. Practical application of phytase in animal feed for the removal of phytic acid was demonstrated in several studies [Simons et al. 1990, Jongbloed et al. 1992].

Since 2007 cost of inorganic phosphate has been increased six fold because traditional sources of non-renewable inorganic phosphorus are becoming either limited or prohibited. These factors necessities the application of phytase. Practically, typical dietary phytase supplementation at 500– 1,000 units/kg of feed virtually replaces the routine addition of 1% dicalcium phosphate or 0.18% phosphorus to a diet. The average cost of supplemental phytase at 1,000 units/kg of feed is approximately $0.5–$2/ton of feed [Lei et al. 2013].

The Natuphos produced by Gist-Brocades, when supplemented in feed, resulted in enhanced utilization of phytin phosphorus by monogastric animals. The quantitative determination of released phosphate upon phytase supplementation indicated that if the phytase were used in the diets of all monogastric animals in the United States, it would preclude 8.23×10^7 kg phosphorus worth $\$1.68 \times 10^8$ from entering the environment [Bhavsar and Khire 2014].

From Laboratory to Market : Story of Commercialization

In 1962, International Minerals & Chemicals (IMC) made initial efforts to develop a commercial phytase. Although IMC supplied the animal-feed industry with inorganic (rock) phosphates, it foresaw the potential to market a phytase that would hydrolyze phytic acid in soybean and other plant meals. In those efforts, over 2,000 microorganisms were screened for phytase production. The project was terminated in 1968 when the company failed to identify an organism that produced phytase activity sufficiently high to be commercially viable. However, IMC's pioneering attempt provided a very valuable isolate of *Aspergillus (ficuum) niger* NRRL 3135 (ATCC 66876).

Application of recombinant DNA technology in the 1980s led to another milestone in the development of phytase as an animal-feed additive. The *A. niger NRRL* 3135 strain served as an excellent candidate to test this new technology's ability to yield a phytase acceptable in the marketplace. Dr. Rudy Wodzinski, a former member of the IMC phytase research team, assisted the Agricultural Research Service of the US Department of Agriculture in the 1980s after learning of its interest in exploiting recombinant DNA technology for agriculture-related projects. With his initiative, a phytase research project was started by the USDA in 1984. This federally funded research rendered the very first cloning of a partial sequence of a phytase gene (phyA) by which in turn guided the later cloning of the full sequence of the gene and its over expression to produce the first commercialized phytase '**Natuphos**', launched in 1991

Now the industry focus has been shifted to Phytase engineering to improve enzymatic properties, such as thermostability, pH activity profile, and protease resistance that determine its efficacy in field application. Because of its commercial importance, HAPhy has been the most-studied target of phytase engineering, and these engineering efforts have produced *A. niger* PhyA, *E. coli* AppA and AppA2. Expression of phytase by native *A. niger* in solid-state fermentation systems has been applied for the commercial production of Allzyme_ SSF. [Ramesh and Devegowda 2005], a natural mixture of enzymes that includes phytase. However, most phytases are produced by over expressing the selected genes in either native or heterologous hosts. Native over expression has been used for the production of PhyA by *A.niger* (Natuphos, Table 4). Heterologous expression has been performed in fungi, yeast, bacteria, and plants. [Lei and Stahl 2001]. Commercialized phytases are produced mainly in

fungi and yeast hosts. A list of commercially available phytases is given in Table 4. [Lie et al. 2013, Bhavsar et al. 2014]

Table 4: Commercially available phytases, their source and properties

Company	Product	Phytase origin	Type of phytase	Expression	pH	T ºC
Alltech	AllZyme	*A.niger*	HAP 3	*A.niger*	6	55
ABVista	Finase EC	*E. coli AppA*	HAP 6	*T. reesei*	4.5	55
ABVista	Finase PL	*A.niger*	HAP 3	*T. reesei*	2.5	-
BASF	Natuphos	*A.niger*	HAP 3	*A.niger*	2.5&5-5.5	65
Enzyvia	Optiphos	*E.coli AppA2*	HAP 6	*P. pastoris*	3.4,5	58
Dupont Industrial Biosciences	Phyzyme XP	*E.Coli AppA*	HAP 6	*S.pombe ATCC 5223*	4.5	55
ABVista	Quantum	*E.coli phy9X*	HAP 6	*P.pastoris DSM 15927*	4.5	-
Novozyme &DSM	Ronozyme	*P. lycii*	HAP 6	*A.oryzae DSM 14223*	4,4.5	50-55

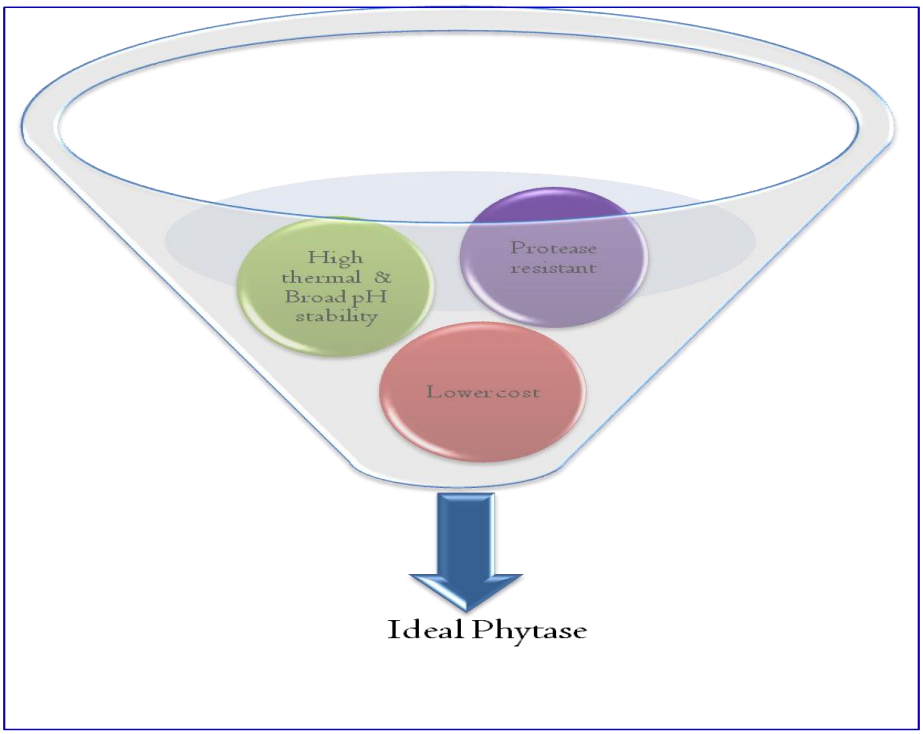
2.5.1 Problems:

In spite of the growing list of novel and engineered phytases, transition for commercialization has been limited. For example, the remarkable laboratory success in creating consensus phytase has not resulted in development of new commercial products in spite of its development by industry over a decade ago. An ideal phytase must possess at least three characteristics:

[A] The ability to effectively hydrolyze phytate in the upper digestive tract of the animal,

[B] Resilience to the 65–80°Ctemperatures of feed pelleting, and

[C] Cheap production costs.



The quest for ideal phytases has taken two routes: identifying novel wild-type (WT) phytases in nature and engineering desired characteristics in known phytases. However it is now realized that any single phytase may never be 'ideal' for all feeds and foods, so there is need for an array of application-specific phytases to match the phytate hydrolysis conditions in the functional sites of phytase in swine, poultry, and fish. For example, the stomach pH in finishing pigs is much more acidic than that of the weanling pigs [Jongbloed et al. 1992]. Thus, phytase with optimum pH close to 3.0 will perform better in the former than in the latter. For poultry, an enzyme would be beneficial if it is active over a broad pH range,that is, acidic (stomach) to neutral (crop) [Quan et al. 2004] . Phytases used for aquaculture applications require a lower temperature than those used for swine or poultry [Cao et al. 2007] .Therefore, the choice of an organism for phytase production and development is dependent upon the target application using directed evolution and protein engineering.

3. Applications of phytase:

3.1 Major Applications of phytase:

Phytase has direct application in animal and human nutrition and in agriculture. Importance and effects of phytase in these fields are discussed below:

3.1.1 Animal nutrition:

Monogastric animals such as poultry, swine and fish shows negligible or no phytase activity in their digestive tract. Consequently, phytates cannot be metabolized by these animals, thus creating a need to enhance phosphate and mineral bioavailability via phytase supplementation. Most importantly, the improved utilization of the phosphate in feed results in a substantial reduction in the phosphate content in animal manure, and hence decreases phosphate load on the environment in areas of intensive animal agriculture. High dietary P bioavailability reduces the need for supplemental inorganic P such as mono and dicalcium-phosphate (MCP, DCP). Thus phytase turn plant phytate into a very valuable P for animal nutrition.

3.1.1.2 Poultry feed:

The inclusion of feed enzymes in poultry diets to enhance nutrient utilisation and growth performance has become routine these days. Possibly, Warden and Schaible [1962] were the first to show that exogenous phytase enhances phytate-P utilisation and bone mineralisation in broiler chicks. After its commercially introduction in 1991, It was considered that the use of microbial phytases would be confined to areas where financial penalties are imposed on excessive P levels in effluent from intensive pig and poultry units [Chesson, 1993]. Contrary to this forecast, the inclusion of phytase feed enzymes in monogastric diets has been far more widely accepted and now exceeds that of Non starch polysaccharide (NSP) degrading enzymes [Bedford, 2003]. Several hundred investigations into the effects of various microbial phytases on growth performance of poultry have been completed. Phytate degradation is correlated with increases in P retention, tibia ash, weight gain, feed intake, nitrogen (N) retention, feed efficiency, apparent metabolisable energy (AME) and Ca retention. These

parameters are basis for checking the efficiency of supplemental phytases. Predictably, the addition of phytase to P inadequate diets has been consistently shown to enhance growth performance. In the study of Simons et al. [1990], phytase addition (1500 FTU kg−1) to diets containing 4.5 g kg−1 total P increased weight gain (733 g *versus* 338 g) and feed efficiency (1.50 *versus* 1.85) of broilers from 0 to 24 days of age. Subsequently, Cabahug et al. [1999] reported that phytase addition (400 and 800 FTU kg−1) to 2.3gkg−1 non-phytate-P diets increased weight gain (18.8%), feed intake (9.0%) and feed efficiency (7.9%) of broiler chicks from 7 to 25 days of age. Generally, responses to phytase in feed intake and weight gain are more robust and consistent than feed efficiency responses. Rosen [2002], from multi-factorial analyses of phytase feeding trials, argues that feed efficiency responses to phytase have been declining with time, which he attributes to concurrent improvements in broiler strains, feeds and management techniques.

Phytase supplementation of P-adequate broiler diets has been shown to generate equivocal growth performance responses, which may be mediated by dietary nutrient specifications. Nutrient specification levels, phytate concentrations and phytase inclusion rates in broiler diets are critical, interactive variables. It is likely that high nutrient specification levels may accommodate the anti-nutritive properties of dietary phytate concentrations and negate responses to phytase supplementation. Consequently, one approach is to decrease nutrient specifications appropriately and counter potential reductions in growth performance with phytase supplementation, which has been shown to be economically viable [Selle et al., 2003b]. Phytase inclusion level varied from 500U to 12000U and the results showed that increasing phytase inclusions are associated with substantial increases in total tract phytate degradation ranging from 0.403 to 0.948 and these increases are numerically most pronounced at the highest phytase inclusion rate of 12,000 FTU kg−1. [Shirley and Edwards 2003]. At such extreme inclusion rate, however, the possibility arises that any minor

enzymatic side-activities that may be present in the phytase preparation may become significant, impacting independently on nutrient utilisation.

In poultry birds without microbial phytase supplementation the phytase activity is mainly observed in caecum but degradation of phytate in the caecum did not contribute to the total P retention. Studies showed that it is crucial to add microbial phytase in the diet to increase the hydrolysis of phytate in the upper part of the digestive tract, to reduce the negative effect of phytate on nutrient digestion and to improve P utilization in poultry. With the pH ranging from 5.2 to 5.8 in the crop and a pH of 2.8 in the proventriculus, these GI segments were expected to be the major sites of exogenous phytase in poultry. While low activity in the lower part of the small intestine may be due to the activity of endogenous digestive protease. However, different phytases differ in their resistance to endogenous protease. [Dersjant et al. 2015]

When phytate is incompletely hydrolyzed in the stomach, it may result in reprecipitated phytate in the small intestine. Liebert *et al.*[1993] measured phytate P concentration in the digestive tract in chickens and observed that supplementation of 1000 FTU kg−1 phytase in the diet reduced phytate P content in the crop, stomach and small intestine compared to the control diet. At the end of the small intestine, phytate P disappearance was up to 65%, while it was only 15–23% in non-phytase-supplemented diets. Among different digestive sections, the phytate P content was lower in the crop and stomach and increased in the small intestine in all treatments, but to a lesser degree in the phytase-treated group, indicating reprecipitated phytate in the small intestine due to increased pH. Similar results were also reported by Onyango *et al.*[2005].

Phytase supplementation of layer diets:

Compared to broiler chicks, phytase inclusion in diets for laying hens has been the subject of less research. The underlying reason may be that the P requirement for layers has not been

established and it may be complicated by the failure to recognise the contribution of digestible phytate-P in layers [Boorman and Gunaratne, 2001]. A number of evaluations of Phytase supplementation of layer diets have been done by various researchers. The thrust of these studies, in which diets were based mainly on maize and soyabean meal, is that phytase supplementation of layer diets permits reductions in non-phytate-P for layers, and consequently P excretion, without compromising performance and egg quality [Selle and ravindran 2007]. It is noteworthy that phytase inclusion levels (250–300 FTU kg−1) in layer diets are generally lower than for broilers and this may be related to the longer retention of feed in the crop, which facilitates phytate degradation. It is possible that phytase enhances Ca availability [Sohail and Roland 2000] and Ca influences phytase efficacy. It may be instructive to focus attention on the effects of dietary Ca levels when evaluating phytase supplementation of layer diets. Tri calcium phosphate (TCP) is an inorganic P source generally used in layers diet, thought to be contaminated with fluoride, which may have adverse effects on egg production. It will be of significance to reduce or eliminate TCP by supplementing phytase. For example, Um and Paik [1999] reduced TCP in maize–soy layer diets from 14.0 to 7.0 and 0 g kg−1,which were supplemented with 500 FTU kg−1 phytase. Phytase significantly increased egg production (86.3% *versus* 84.5%) in the high P diet and egg production in the lower P, supplemented diets was not compromised. In another study involving TCP, Lim et al. [2003] concluded that phytase supplementation improved egg production and reduced percentages of broken and soft eggs and P excretion.

Recommended inclusion rates of contemporary phytase feed enzymes may be conservative but, as discussed, appropriate dietary formulations may be needed to realise benefits from higher additions. It remains possible that inherently more effective phytase feed enzymes, with the capacity to degrade the majority of phytate in broiler diets, will be developed. Alternatively, the simultaneous inclusion of phytase with other exogenous

enzymes may be beneficial, particularly if substrate access is enhanced. The simultaneous inclusion of phytase with β-galactosidase, β-glucanase and xylanase has been investigated in maize, barley and wheat based diets, respectively, by Juanpere et al. [2005] and Cowieson and Adeola [2005]. The data suggest that phytase in combination with carbohydrase and protease has additive effects in nutritionally marginal broiler diets. Thus, further research into enzyme 'cocktails' is justified.

3.1.1.3 Swine:

In the pig's stomach, the pH is normally at 2–2.5 when it is relatively empty and increases after feeding. Newly weaned piglets have a low capacity to secrete hydrochloric acid (HCl); therefore the pH level in the stomach can be higher than in growing pigs. In the first part of the small intestine the pH ranges from 3.5 to 5.5 [Kemme et al. 1998] . Jongbloed *et al.*[1992] observed that the pH of digesta in the duodenum was about 6 at feeding, decreased to 5 at 1 h after feeding and remained at about 4 from 2 to 5 h after feeding. Therefore a microbial phytase that has broad pH stability and high activity at low pH will be more effective in swine industry. In pigs, without microbial phytase supplementation in the feed the main phytase activity was observed in the colon. With microbial phytase supplementation, the main active site was the stomach and upper part of the small intestine. Yi and Kornegay [1996] investigated the site of phytase activity in the GI tract of young pigs fed a diet containing *A. niger* fungal phytase and found that it was higher in the digesta of the stomach than the digesta from the upper small intestine. It has been reported that 30- 50% of dietary phytate P can be released by exogenous phytase supplementation allowing reductions in dietary non-phytate phosphorous of 0.1-0.2%, and decreasing fecal P concentrations from 35 to 50% [Mroz et al 1994]. Mroz *et al.* (1994) also reported improved ileal digestibility of protein and amino acids in 45-kg pigs receiving corn-tapioca-soybean meal diet supplemented with *A. niger* phytase but Traylor *et al.* [2001] reported in growing pigs that

A. niger phytase supplementation to soybean meal did not significantly improved apparent and true ileal protein and amino acid digestibility demonstrating that the response of animals to phytase varies depending on feed type and age of animals among other factors. Similarly, Liao *et al.* [2005] reported no improvement in ileal digestibility of protein and amino acids in weanling pigs fed *A. niger* supplemented diets. Diets fortified with phytase could increase bone phosphorous, bone calcium and bone ash compared with pigs which were fed with unsupplemented phytase diets [Jongbloed et al. 1992, Mroz et al. 1994].

3.1.1.4 Fish

Plants by products are considered as a promising source of protein and energy which can be used for the formulation of economical and environment friendly aqua feeds. However, anti-nutritional factors like phytate disturb the physiology of digestive tract; limiting the overall fish growth [Cao et al. 2007] Moreover the impact of phosphorous excreted from fishes is even more important than other animals because it is released to the water bodies directly causing algal bloom. Many studies have demonstrated that phytase supplementation can improve P availability of chelated phytate P in plant based feed while reducing P excretion [Cain and Garling 1995, Rodehutscord and Pfeffer 1995, Baruah et al. 2004]. Fish can be classified into two big types with totally different pH value in the digestive systems: gastric and agastric fish. Ji [1999] reported that the pH value was 6.8–7.3 in the digestive systems of agastric fish which showed poor efficacy of phytase, while the gastric fish with lower pH value in their digestive systems gained much better results of phytase addition. Schafer and Koppe [1995] reported that 20% and 40% of phytate-P can be released by the phytase addition of 500 and 1000 U/kg respectively in carps fed with soybean meal based diet. Yu and Wang [2000] found that in soybean meal based diet for crucian carp, 60% and 80% of phytate-P can be released by the phytase addition of 500 and 1000 U/kg, respectively. Phytate-P is converted to available-P by phytase which can be utilized directly by aquatic

animals. Thus, the utilization rate of P can be considerably enhanced by phytase. It was reported that the effect of 1000 U/kg phytase addition is equivalent to that of 0.85–1.28% monocalcium phosphate [Cheng and Hardy 2002]; also phytase replacement can avoid the toxin compromise of fluorin brought by inorganic-P input, thus enhancing the security of feed.

Most phytases have an optimal pH in the range of 4.5–6.0 and a temperature range of 45–60°C. Outside the optimal range of pH and temperatures the action of phytase is reduced. More data for neutral and alkaline phytases are required to evaluate the potential of these enzymes for commercial application in aqua feed. In addition, the optimum supplement doses of phytase in diets of different fish species also vary greatly and needs further research. The including methods of phytase into fish diets also needs further study to find out an ideal way to maximize the phytase efficacy and keep lost at the minimum level. The use of phytase in fish feed will expand along with the need of cost-effective feed and environmental protection concern increasing.

3.1.2 Phytase in Human nutrition:

The small intestine of humans has limited ability to digest phytates, resulting in adverse nutritional consequences with respect to metabolic mineral absorption. Phytic acid (PA) containing 12 dissociable protons with pKa values ranging from 1.5 to 10 is a highly reactive and potent chelator of several mineral ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}. It forms insoluble salts, at normal acidity (pH 3.0–6.8), in the human digestive tract, thereby reducing the bioavailability of these critical mineral nutrients for absorption.[Costello et al. 1976] Mucosal phytase and alkaline phosphatases, even if present in the human small intestine, do not play a significant role in the phytate digestion [Sandberg and Anderson 1988]. The processing and manufacturing of human food by including phytase can provide a way out for these problems. Up to now, no phytase product for a relevant food application is available in

the market. Research in this field focuses on better mineral absorption or technical improvement of food processing. Using phytase, Simell and co-workers [1989] reported the preparation of a phytate-free soy protein isolate with increased solubility at low pH (pH 3) compared to the control soy protein isolate .Additions of *A. niger* phytase to flour containing wheat bran increased iron absorption in humans [Sandberg et al. 1996]. There are many successful attempts to use phytase in brewing to improve alcohol production [Fujita et al. 2001], in bread making to improve proofing time, width/height ratio of bread slices, specific volume, and crumb firmness [Haros et al. 2001], in dephytination of soy milk [Ushashree et al. 2012] and in separation of soybean b-conglycinin and glycinin [Saito et al. 2001]. Haros et al. [2001] investigated the possible use of phytase in bread making. Different amounts of fungal phytase were added in whole wheat breads and it was shown that phytase is an excellent bread-making improver. Besides reduction in phytate content in dough and fresh breads, fermentation time was shortened by phytase addition without affecting the dough pH. An increase in bread volume and an improvement in crumb texture were also observed. The main achievement of this activity was the shortened fermentation period without affecting the pH of the bread dough. An increase in bread volume and an improvement in crumb texture were also observed. Application of immobilized *E. coli* phytase and fusion protein in the dephytinization of soy milk led to a 10% increase in the release of inorganic phosphate at 50°C relative to free fusion protein.[Ushashree et al. 2012] Applications of phytase in human foods can equal, if not exceed, those in animal feeds. However, several issues have limited wide implementation of phytase in human nutrition. The main issue is the consumer perception of recombinant phytase and the lack of readily available native phytase. Thus search for a higher phytase producing native strain is continued.

3.1.3 Phytase in agriculture:

Phosphorus (P) is one of the major plant nutrition. However in soil it is mainly present in organic form (phytic acid and its derivatives). The ability of plant to utilize this P can be improved by phytase addition. Findenegg and Nelemans [1993] studied the effect of phytase (phyA) on the availability of phosphorus from phytic acid in the soil for maize plants and they show that phytin hydrolysis increased when phytase was added to the soil which also stimulated plant growth. Thermophilic mould, *Sporotrichum thermophile*, and phytase from it both have been promoted the growth of wheat seedlings. The growth and inorganic phosphate content of the plants were better than the control [Singh and Satyanarayana 2010]. Phytase and phosphatases producing fungi were used as seed inoculants, to help attain higher P nutrition of plants in the soils containing high phytate phosphorus [Yadav and Tarafdar, 2003]. The efficiency of different organic P compounds' hydrolysis by different fungi indicated that the fungi have enough potential to exploit native organic phosphorus to benefit plant nutrition. These approaches can be applied as a strategy for boosting the productivity in agriculture and horticulture. The ability of plants to use phosphorus from low phosphate or phytate containing media and/or from soil is improved when soil/media are inoculated with microorganisms, possessing the ability to exude phytase or when a purified phytase is added.

The effect of fungal phytase on plant growth at pot and tray level, compared with commercial fertilizers pertaining to chemical and physiological parameter and as soil amendment, was studied. Phytase was efficient in reducing the phytic acid content of soil by about 30% while simultaneously increasing the phytate phosphate availability by 1.18-fold. [Gujar et al. 2013]

One more approach to increase plant phosphorus availability is expression of phytase in the roots of transgenic plants. Expression of phytases in plants has enormous potential in improving plant phosphorus acquisition and phyto remediation. Stable transgenic rice and wheat plants, expressing fungal phytases, exhibited enhanced bioavailability of phosphate and iron [Lucca et al. 2002].

3.2. Potential applications:

Until now phytase has been mainly seen as animal feed additive but besides increasing nutritional value of animal feed and combating phosphorus pollution, phytase has many more applications. (**Figure 2**)

3.2.1 Pulp and Paper Industry:-

A thermostable phytase along with appropriate xylanase activity have been suggested as powerful additive in pulp and paper industry. The enzymatic degradation of phytic acid during pulp processing would not result in the production of toxic and mutagenic by-products, and at the same time aging of paper can be prevented [Liu et al. 1999]. Therefore, the exploitation of phytases in the pulp and paper process could be environmentally friendly and would assist in the development of cleaner technologies.

3.2.2 Chemical Synthesis:

3.2.2.1 Semi synthetic peroxidise:-

When vandate ion is substituted in histidine acid phosphatase (HAPs) it could act as peroxidase. Incorporation of vandate ion into the active site of *A. niger* NRRL 3135 phytase, thus transformed native phosphohydrolase into semi synthetic peroxidase. The “new” enzyme was able to catalyse enantio selective oxidation of pro-chiral sulphides and was found to be stable for 3 days with only slight loss in turnover number [Correia et al. 2008].

3.2.2.2 Lower myo-inositol phosphate synthesis:

There is a continuous demand of inositol phosphates and phospholipids, which play an important role in cell signalling pathways [Billington 1992]. Potential health values of certain inositol phosphates are well known [Siren et al. 1991]. The most extensively studied positive aspect of *myo*-inositol phosphate (InsP1, 4, 5 and InsP1, 3, 4, 5) is the potential for reducing the risk of colon cancer. InsP5 plays an important role in modulation of the oxygen-binding affinity of hemoglobin in the erythrocytes of avian, amphibian, and reptilian species while

InsP4 and InsP3 was found to act as secondary messenger in controlling intracellular calcium level [Craxton et al. 1997]. Surprisingly, the esters of inositol triphosphate have been shown to exert significant inhibitory effects against retroviral infections including HIV [Siren 1998].Phytase and phytase-producing micro-organisms can be immobilized on a variety of matrices as cost effective bioreactors for large-scale production of these compounds. Phytases of different origin may lead to the production of different isomers. Enzymatic hydrolysis has the advantages of stereo specificity and mild reaction conditions. In addition to usage as drugs, *myo*-inositol phosphate derivatives can be used as enzyme substrates for biochemical and metabolic investigations and as chiral building blocks [Laumen and Ghisalba 1994].

3.2.3 Phytase in medicine:

The application of phytase in human health and medicine may represent an exciting new avenue. Pagano et al. [2007] demonstrated the non phosphorus-related benefit of high–dietary phytase supplementation for bone development in young pigs. Because pigs are an excellent model for humans, it would be interesting to test whether pig results can be translated into humans. For instance, phytase could be used alone or in combination with other reagents, such as strontium, to treat or prevent osteoporosis. A recent human study suggested a potential role for zinc and/or phytase in enhancing the efficacy of botulinum toxin in the treatment of cosmetic facial rhytids, benign essential blepharospasm, and hemifacial spasm [Koshy 2012].

3.2.4 Phytase in pesticide removal:

Organophosphorus compounds are organic compounds containing phosphorus and they are used primarily in pest control. Pesticide residue in harvested crops and vegetables are poisonous. They contain phosphor ester bonds which can be hydrolysed by phytase. It is reported that P removal from organophosphorus reduces their toxic property. Thus phytase

can be formulated to act as scavenger for pesticides. [Patent publication no. CN100406548 C].

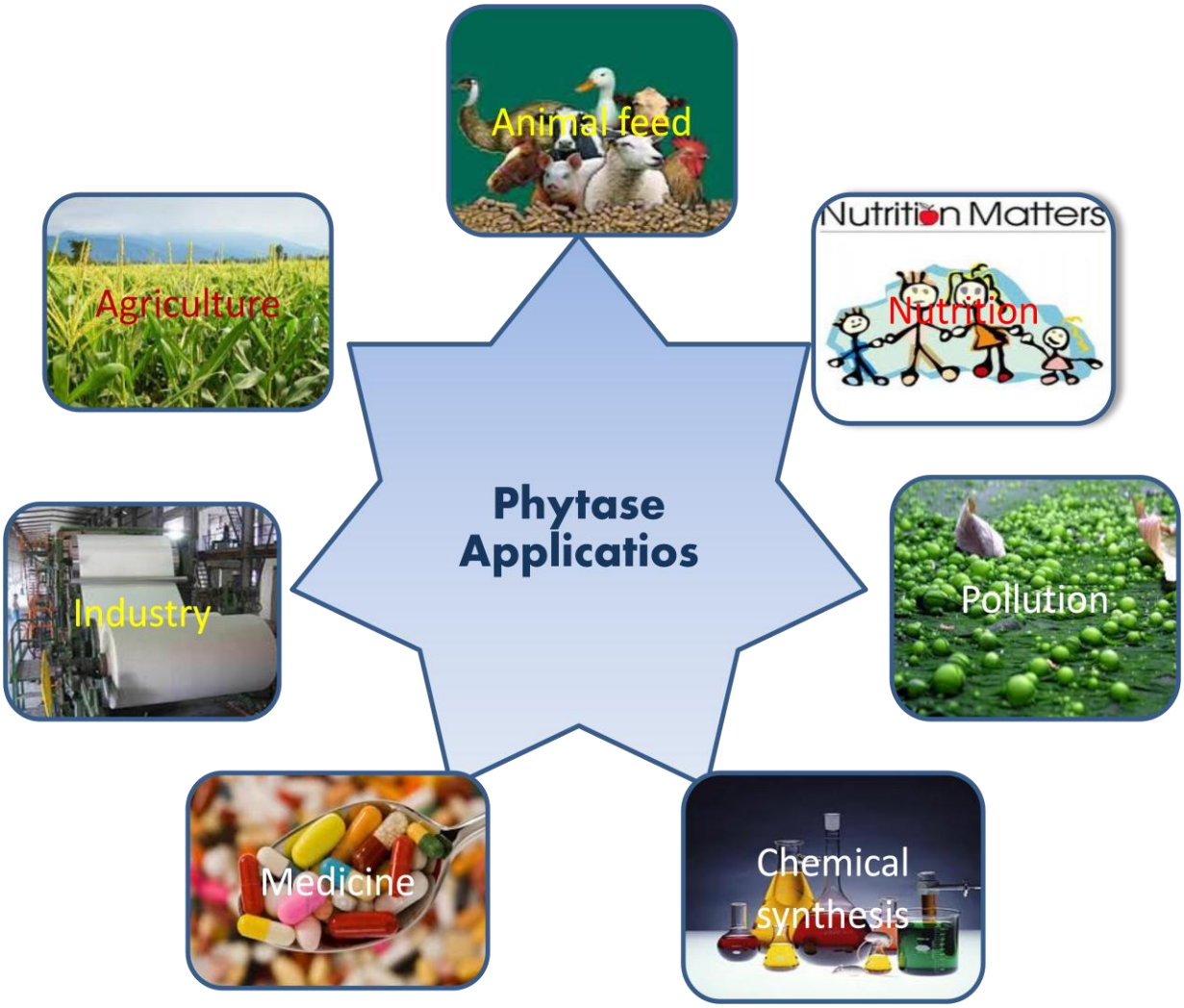


Figure 3 Applications of phytase

Conclusion:

The increasing public awareness of environmental protection, along with a concurrent price rise and supply shortage of conventional feed phosphorus sources, will lead to a greater demand for phytase in the future. Since 1907 so many phytases from different sources are recognized, produced, isolated and modified but still the quest for so called Ideal phytase is ongoing. Currently available commercial phytases are used indistinctively in diets for all purposes, but no single available phytase is ideal for or compatible to digestive-system conditions in all species while substantial loss of phytase activity during feed pelleting arguably remains the most limiting factor for its feed application. Both traditional and biotechnological approaches have been applied successfully to overcome such limitations .There are various constraints related to these innovations: consumer's acceptance of genetically modified products, potential increase of soluble phosphorus in animal excreta, and allergic reactions to phytase powder exposure. Apart from its role in animal feed varied application of phytase need to be explored. Further insights in the development of application oriented phytases will open a new era in its bioprocessing and widen the horizons of its applicability and efficiency. New market segments such as aquaculture and agriculture will provide new opportunities for phytase. The novel roles of metabolic intermediates from phytate degradation should be studied. Thus in future phytase will be able to exert a larger socioeconomic impact than for animal feed industry alone.

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Chapter 2

Phytase Production Optimization under Solid State Fermentation

Summary: SSF is a low cost, higher production method and provide preferable conditions for fungal growth. Our studies deals with use of ground nut oil cake and response surface methodology for phytase production employing SSF. Placket–Burman design (PBD) was used to evaluate the effect of 11 variables and it was found that production was primarily influenced by four variables namely glucose, dextrin, distilled water, and $MgSO_4.7H_2O$. Further optimization of selected variables using Box-Behnken design (BBD) leads to about 37 fold increase in enzyme production from 15 IU/gds to 550 IU/gds. A comparison with available reports of phytase production proves that this the highest phytase production reported with SSF. The existing SSF processes for phytase production are facing engineering challenges due to low productivity. Hence high yield of phytase overcome the above limitations show the potential of oilcakes using SSF.

1. Introduction:

Phosphorus (P) is an essential constituent of life, but, unlike carbon and nitrogen, most of the P is stored in soil and rocks on earth in the form of phosphates. While plants can absorb this phosphate directly from the soil, animals obtain it through their diet [Cordell et al. 2011]. In plants roughly 60–80% of P is tied up in phytic acid, an organic acid that is not digestible by monogastric animals. Phytic acid thus ends up as manure, which after going into water bodies is degraded by aquatic microorganisms to release P which causes widespread eutrophication [Singh and Satyanarayana 2011]. It would be advantageous to find ways to make this bound P available and digestible to animals in some suitable way. Supplementation of feed with exogenous phytase, a phosphohydrolase, is a feasible solution because it releases the bound P from phytic acid. Besides providing nutritional benefits, the addition of enzyme will also alleviate environmental problems by reducing the P excretion by the animals and the observed eutrophication [Cromwell 2009].

Phytases are produced by a large number of plants, animals and micro-organisms. Phytases from microbial sources, especially those from fungal origin, have been widely employed in industries [Pandey et al. 2001]. Commercially they are produced by Submerged Fermentation Techniques (SmF) but this process has certain disadvantages because it uses synthetic growth media that yields diluted product that makes downstream processing especially difficult. On the other hand, enzyme production by Solid State Fermentation (SSF) technique is well established as a simple, cost-effective, self sustainable process [Godoy et al. 2009, Oruza et al. 2009, Ang and Suraini 2013, Sadaf and Khare 2014,Salgado et al. 2014]. The SSF permits growth of microorganisms on moist insoluble solid supports and in the absence of free water, is a comparatively inexpensive technique for enzyme production in concentrated and large amounts. Also by utilizing agro-industrial waste, it

serves the dual purpose of value addition as well as waste management [Graminha et al. 2008].

Traditionally, agricultural by-products and oil cakes form low-cost energy rich substrates for the fermentation industry. An oil cake available globally on very large scale is obtained from the groundnut crop. This crop is grown on nearly 24 million hectares and in as many as 90 countries with a total annual production of over 36 million tons [www.icrisat.org]. Two thirds of the total crop produce is used for oil production and the remaining as food. Groundnut cake (GOC), an agro-waste and by-product after the oil extraction process is an excellent livestock feed with advantages in terms of low cost, stability and availability all throughout the year. The oil cake composition in terms of protein, crude fibre and amino acids is well reported.[Kuo 1967, Owusu 1970] The cake contains 45-60% protein, 22- 30% carbohydrate, 3.8-7.5% crude fibre and 4-6% minerals [Desai et al. 1999]. Thus use of oil cake for solid state fermentation for production of animal feed enzymes such as phytase is ideal cheap substrate. Then, it may also become possible to dry the SSF product (koji) and mix it directly in animal feed as a value-added food supplement without the need for any downstream enzyme concentration steps.

To make the process feasible, we also need to improve the productivities of SSF by identifying suitable conditions for up-scaling. Productivity can be increased by optimizing the growth media and in this connection Response Surface Methodology (RSM) has been demonstrated as a powerful statistical tool for the optimization of associated fermentation parameters. The advantage of RSM is that it identifies the most sensitive process factors and their optimal values for maximizing the enzyme production by carrying out a few designed set of experiments [Elibol 2004].

For all the above reasons, the present work was undertaken with the objective of evaluating the suitability of GOC as a raw material in an SSF process and further to

optimize experimental conditions by RSM to obtain high yields of phytase using a generally recognized as safe (GRAS) fungi, namely, *Aspergillus niger* NCIM 563. The use of filamentous fungi in SSF is advantageous because it mimics their growth similar to a natural environment [Moo- youn et al. 1983]. We have earlier studied the *A. niger* strain for phytase production using wheat bran and obtained high yields [Bhavsar et al. 2011]. To make the GOC process more attractive, studies of phytase activity in dried koji form has also been determined.

2. Materials and methods:

2.1 Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo. Agriculture residues were purchased from a local market. The GOC was purchased from the local market and dried at 70°C overnight and ground. It is sieved through filter of 0.4 mm and 0.8 mm to get different particle size.

2.2 Fungi and inoculum preparation

A. 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 using sterile saline solution containing 0.01 % Tween 80 to obtain $5x10^{7}$ spores /mL and used as inoculum for SSF.

2.3 Phytase production and extraction

The un-optimized medium contained 10 g of agricultural residue moistened with 10 mL distilled water in a 250-mL Erlenmeyer flask, sterilized at 121ºC and 15 psi for 30 min. On cooling the fermentation medium was inoculated with spore suspension at a concentration of $5x10^{6}$ spores /g of GOC and incubated for 4 days at 30ºC. The fermentation medium included

additional components, namely glucose, dextrin, $FeSO_4.7H_2O$, $MnSO_4$, $MgSO_4.7H_2O$, triton X 100 and KCl at concentration values as required by the statistical design of experiments (DOE). The concentration of variables is expressed in gram (g) of variable per 100g of GOC (w/w %) and volume (v) per 100g of GOC (v/w %). For up-scaling of phytase production GOC (50, 100 and 200gm) was moistened with the optimized medium in enamel-coated metallic trays having dimensions 28 x 24 x4 cm, 45 x30 x 4 cm, and 80 x 40 x 4 cm. The trays were covered with aluminium foil and sterilized at 121°C for 20 min

For phytase extraction from koji 50 mL of 2% of an aqueous solution of $CaCl_2 \cdot 2H_2O$ was added to each flask containing 10 g fermented GOC. Flasks were kept on a rotary shaker at 200 rpm for 2 h at room temperature. The suspension was squeezed through a double layer of muslin cloth and centrifuged at 5000 G for 20 min at 4ºC to obtain the liquid enzyme extract. To obtain dried fermented koji for potential application in feed, the fermented substrate was dried in an oven at 50°C for 36 h and ground to powder till constant dry weight obtained.

2.4 Analytical methods

The solution for analysis of phytase consisted of 3 mM sodium phytate with 100 mM glycine-HCl buffer (pH 2.5) and 100 µl of liquid enzyme extract solution. After incubating for 30 min at 50 °C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [Heinohen and Lathi 1981]. A freshly prepared solution of acetone: 5N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl 1M citric acid was added and absorbance was measured at 370 nm against blank. Blank consists of buffer and substrate solution and treated in the same way as sample. One unit of phytase 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 enzyme activity IU/gds (i.e., gram of dry substrate). Each experiment was carried out in triplicate and the values

reported are the mean of three such experiments that had a variability of 2-4%. Enzyme activity has also been determined at pH 5.5 as the *A. niger* NCIM 563 has been reported to produce two different phytase with different pH optima [Soni and Khire 2007]. Concentration of the protein was determined using Lowry method with bovine serum albumin as standard [Lowry et al. 1951].

2.5 Media optimization using response surface methodology

Media optimization was done in two stages. Initially, a Plackett-Burman Design (PBD) was employed to identify the most sensitive factors affecting the yield of enzyme [Plackett and Burman 1946]. The choice of variables was made on the basis of earlier studies employing SmF and SSF for phytase production [Soni and Khire 2007, Bhavsar et al. 2011]. In the present study, PBD was used to screen 11 variables, viz., glucose (A), dextrin (B), $NaNO_3$ (C), distilled water (D), $MgSO_4.7H_2O$ (E), $FeSO_4.7H_2O$ (F), KCl (G), incubation period (H), particle size (J), triton X 100 (K), and $MnSO_4$ (L). The total number of trials to be carried out was *n+1* where *n* is the number of variables (equal to 11). Each independent variable was tested at two levels, a high (+1) level and a low (-1) level as shown in Table 1. The PBD experimental DOE was generated using Design-Expert Software (DES) version 7.1.2 (Stat-Ease, Minneapolis, MN, USA).

Table 1: Selected variables for PBD and their assigned levels for studying phytase production

S. No.	Code	Variables	Level 1	Level 2
1	A	Glucose (w/w %)	10	50
2	B	Dextrin (w/w %)	5	20
3	C	$NaNO_3$ (w/w %)	2	8
4	D	Distilled water (mL)	100	200
5	E	$MgSO_4.7H_2O$ (w/w %)	1	5
6	F	$FeSO_4.7H_2O$ (w/w %)	1	5
7	G	KCl (w/w %)	1	5
8	H	Incubation Time (days)	4	8
9	J	Particle Size (mm)	0.4	0.8
10	K	Triton X 100 (v/w %)	0.5	1
11	L	$MnSO_4$ (w/w %)	1	5

On the basis of analysis of PBD results, four variables, viz., glucose (A), dextrin (B), distilled water (C), and $MgSO_4.7H_2O$ (D), were chosen for further optimization by RSM using Box-Behnken Design (BBD) [Box and Behnken 1960]. In BBD each variable was studied at the three levels described in Table 2.

Table 2: Selected variables for BBD and their assigned levels for studying phytase production

Variables	Variable code	Unit	Levels		
			1	2	3
Glucose	A	w/w %	5	15	25
Dextrin	B	w/w %	2.5	6.3	10
Distilled water	C	mL	150	200	250
$MgSO_4.7H_2O$	D	w/w %	0.5	1.5	2.5

The BBD was constructed using DES and analysed using ANOVA to obtain a best fit mathematical model for predicting the experimentally observed response values. Optimized values of the four variables studied in BBD were obtained for the fixed values of the other less sensitive variables studied in the PBD. All experiments were carried out in triplicates and their mean values are presented as responses.

2.6 Scanning electron microscopy

Samples for SEM were mounted on brass stubs. Specimens were then coated with a thin layer of gold (100 Å) in a gold coating unit, model E 5000, Polaron Equipment Ltd (Cambridge, UK) and were viewed with an SEM Leica Stereoscan 440 (Leica, Cambridge, UK) at an accelerating voltage of 10 kV, and beam current 25 Pa.

2.7 Thermo-stabilization of phytase in koji

Fermented koji was dried at 50°C for 36 h in presence of different thermo-stabilizers at 5% and 10% concentration. Thermo-stabilizers used were skimmed milk, sorbitol and mannitol.

Thermostabilizers are blended with koji to make homogenized mix. The phytase activity of dried koji with stabilizer and without stabilizer is compared and expressed as % residual activity with respect to phytase activity before the drying process. Phytase activity was determined by modified ammonium molybdate method as described in Analytical methods.

To determine shelf life of above dried koji, the koji was packed in a poly bag and kept at room temperature. Phytase activity was determined at regular intervals of 15 days.

Apart from these the toxicity testing of dry koji was done at Central Avian Research Institute (CARI) for possible aflatoxin contamination.

2.8 Efficacy of phytase 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) with the pH adjusted by HCl or NaOH, viz., 1.5, 2.0, 2.5, 3.5, 4.5, 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. 50 U of enzyme is added either in liquid or dried koji form and incubated by agitation at 37°C for 60 min. The amount of released P was determined as described in Analytical methods while the total P (Control) was quantified by ICP-AES analysis as described in the above section. This was considered as 100% to determine P release at different pH conditions.

2.9 Dephytinization and increase in mineral bioavailability from soybean flour

The ability of phytase to degrade phytic acid (PA) and increase the bioavailability of PA bound minerals from plant based food was studied using soybean flour (SBF). This was quantified using an inductively coupled plasma atomic emission spectroscopy (ICP-AES). The SBF was dried in oven at 80ºC. One gram of dried SBF was weighed in crucible and heated at 500ºC for 4h 30min. After cooling to room temperature, the sample was mixed with

5mL HNO_3: $HClO_4$ in the ratio 4:1 and heated at 120–140ºC until a clear solution was obtained. It was then filtered through Whatman filter paper (0.2 mm, Pall Corporation, Port Washington, NY). The filtrate was used for the determination of total mineral analysis of untreated SBF and considered as control (100%). For determination of mineral mobilization after phytase treatment, 1 g of dried SBF was suspended in 10 mL of 0.2M glycine-HCl buffer (pH 2.5) containing 50 unit of enzyme in liquid form and incubated at 37ºC on a rotary shaker at 100 rpm for 1h. Samples were centrifuged at 10,000 G for 10 min at 4ºC, supernatant filtered and the filtrate collected. The filtrate was then diluted with distilled water to adjust the signal to lie in the calibration range for the elements viz., Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+}. Mineral contents in the samples were measured by ICP-AES and expressed as % mineral release as compared to control. An identical experiment was carried out with dried koji (50 U of enzyme) by replacing the liquid enzyme by the former. Experiments were conducted in duplicate to calculate the mean values and standard errors.

Phy I& Phy II :

The high phytase producing *A. niger* NCIM 563 strain was screened in our lab in year 2000. Later on production in Submerged fermentation conditions and characterization studies by Soni (2007 and 2010) proves that it produces two types of phytase enzyme differing in bio chemical properties. They are named as Phy I and Phy II. Significant biochemical properties are given below:

Characteristics	Phy I	Phy II
pH		
Optimum	2.5	5.0
Stability	1.5-9	4-8.5
Temperature		
Optimum	55°C	55°C
Stability	40 % at 65°C (1.5hr)	55% at 65°C (1.5hr)
Isoelectric point	3.65	3.95
Molecular mass(kDa)		
Native	264 (Tetramer)	150 (Monomer)
Subunit	66(SDS)	150
MALDI	66	74

3. Results and Discussion:

The results obtained using GOC as biomass for enhanced phytase production is discussed here. It may be pointed out that our first study employing only GOC (10g) and distilled water (10mL) as basal medium at 28°C showed a low phytase yield of 15 IU/gds on the 4th day of SSF. To corroborate the finding, Figure 1 shows the SEM image of SSF media before and after the fungal hyphae growth.

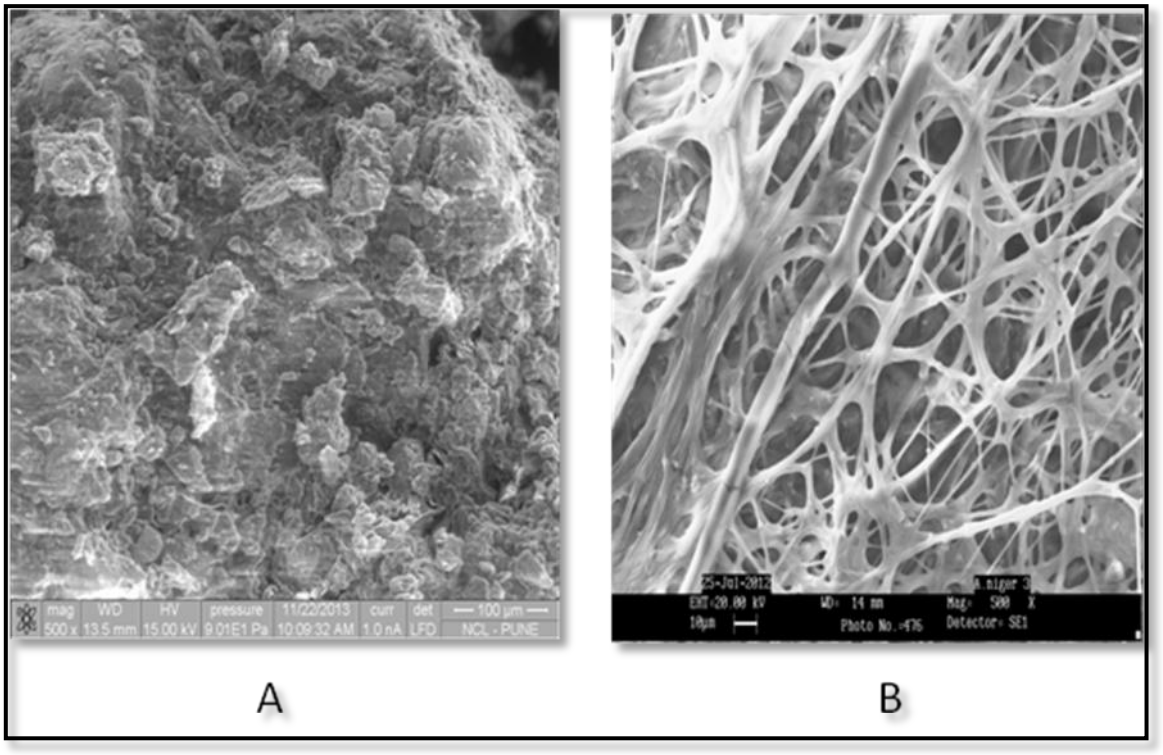

Figure 1 SEM image of *A. niger* NCIM 563 grown on GOC: A: Before growth, B; After growth

3.1 Screening the factors affecting phytase production

We then undertook studies to screen the media variables that could significantly affect the observed phytase production. For this purpose, a PBD design of experiments (Table 3) was carried out involving eleven selected variables. The experimentally obtained response values of phytase production (IU/gds) are also reported in Table 3 for the 12 runs planned in the

PBD study. The results showed a maximum phytase production of 275.54 IU/gds in run number 11.

Table 3: PBD for the selected variables along with the experimentally obtained phytase production response values

Run no.	A	B	C	D	E	F	G	H	I	J	K	Phytase production (IU/gds)
1	1	1	2	1	2	2	1	2	2	2	1	10.86
2	2	2	2	1	1	1	2	1	2	2	1	4.00
3	2	2	1	2	2	2	1	2	1	2	1	60.52
4	2	1	2	2	1	2	2	1	1	1	1	173.12
5	1	1	1	1	1	1	1	1	1	1	1	76.62
6	2	1	1	1	2	1	2	2	1	2	2	0.008
7	2	2	1	1	1	2	1	2	1	1	2	1.02
8	2	1	2	2	2	1	1	1	2	1	2	79.5
9	1	2	2	1	2	2	2	1	1	1	2	3.6
10	1	2	1	2	2	1	2	2	2	1	1	85.25
11	1	1	1	2	1	2	2	1	2	2	2	275.54
12	1	2	2	2	2	1	1	1	1	2	2	114.25

A linear model fit to the chosen 11 variables identified four variables as the most significant and in terms of actual factors was obtained as

Phytase production = 155.05 - 54.53A - 44.63B + 128.55D - 79.22E (1)

Here A, B, D, E corresponds to levels of glucose, dextrin, distilled water and $MgSO_4.7H_2O$, respectively. The above linear model satisfied all the necessary statistical tests for its acceptance. Thus, the obtained model F-value of 23.97 indicated that the model is very significant and that there is only a 0.04% chance that a model F-value this large could occur due to noise. The values of "Prob > F" was less than 0.05, for all the four terms and brought out their importance. The coefficient of determination R^2 provides a measure of how much of the variability in the observed response can be explained by the model. The R^2 was found to

have a high value of 0.93. Also, the 'Pred- $R^{2'}$ of 0.77 is in reasonable agreement with 'Adj-$R^{2'}$ of 0.89. The 'Adeq Precision', a measure of the signal-to-noise ratio, was found to have a high value of 14.89 and this indicated the signal to be strong. All the above tests reiterate that the model equation (1) can be used to navigate the design space. In fact, using the above model it was found that phytase production was significantly affected by the above four factors and that they accounted for 93.81% of the total contribution to the response. The remaining variables accounted for only 6.19% and thus PBD identified those to be much less significant for the ranges of levels employed in the chosen variables. The Pareto chart given in Figure 2 additionally shows and compares the significance and the contributions of the different factors. The factors D (distilled water) and E ($MgSO_4.7H_2O$), lie above the Bonferroni limit and are almost certainly significant while those above the t-value limit, viz., A (glucose) and B (dextrin) are possibly significant and recommended to be considered in further optimization studies.

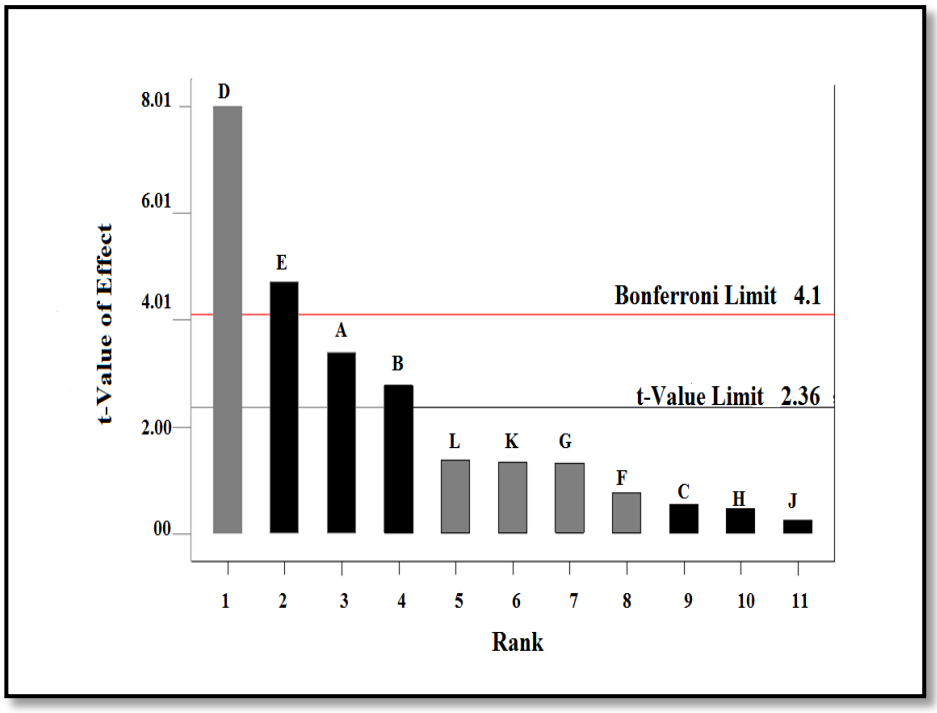

Figure 2 Pareto chart showing the contributions of different factors A-L by PBD analysis

Therefore, the four media components identified by PBD, as significant factors, for phytase production were further optimized by a suitably chosen BBD of experiments generated by DES at three levels. Table 4 gives the BBD involving all the four significant variables along with the experimentally obtained phytase activity for all the 29 experiments. It is seen that run number 24 showed remarkably high phytase production of 524 IU/gds while run number 12 showed the lowest phytase production of 57 IU/gds. The wide variation in activity shows the sensitivity of the process to the experimental conditions and provides the justification for having carried out systematic process optimization studies by RSM. Production of enzyme has been determined by checking its activity at pH 2.5 however as proved earlier by Soni et al. (2007), *A. niger* NCIM 563 produces two different type of phytases (Phy I & Phy II). Hence we determined the production of other phytase also at pH 5. The activity/production of Phy II was found to be 3 times less as compared to Phy I. Therefore we have decided to include the data of Phy I optimization only.

Table 4: BBD for the selected variables along with the experimentally obtained phytase production response values

Run no.	A Glucose (w/w %)	B Dextrin (w/w %)	C Distilled Water (mL)	D $MgSO_4.7H_2O$ (w/w %)	Phytase production (IU/gds)
1	3	2	1	2	83
2	2	3	3	2	335
3	2	1	2	3	399
4	1	2	3	2	254
5	2	2	1	1	140
6	1	2	2	3	131
7	1	2	1	2	120
8	3	2	2	1	177
9	2	1	3	2	376.2
10	3	2	3	2	169
11	2	3	2	3	281
12	3	3	2	2	57.96
13	2	2	3	3	233

14	2	2	1	3	180.9
15	2	1	2	1	406.7
16	3	1	2	2	423
17	1	2	2	1	436
18	2	2	2	2	494
19	2	2	3	1	400.2
20	2	2	2	2	493
21	2	1	1	2	176
22	2	2	2	2	501
23	3	2	2	3	271
24	1	3	2	2	524
25	2	2	2	2	494
26	1	1	2	2	168
27	2	3	2	1	470
28	2	2	2	2	516
29	2	3	1	2	59

The response data was regressed successfully using actual factors in a backward elimination regression to obtain a hierarchical quadratic model with interactive terms and given by

Phytase production = $-3747.01+57.05A+136.86B+32.47C+54.32D-4.80AB+9.96AD-16.73BD- 1.52A^2-3.66B^2-0.08C^2-46.54D^2$ (2)

ANOVA analysis of the above model satisfied the statistical tests. The model F-value of 24.82 implied the model to be significant suggesting that there exists only a 0.01% chance that the model F-value this large could arise due to noise. The value of correlation coefficient (Pred R^2) for phytase production was 0·80 and suggests a strong agreement between the observed and model predicted values for phytase production. The coefficient of determination $R^2 = 0.94$, suggests that 94 % of the variability is explained by Equation (2). The obtained signal-to-noise ratio value of 14.85 shows an adequate signal for Equation (2)

to navigate the design space. For the model terms C, D, AB, AD, BD, A^2, B^2, C^2 and D^2 the values of "Prob > F" <0.05 was satisfied and indicated their significance.

Figure 3(a-c) bring out the nature of the complex nonlinear interactions between Glucose and Dextrin; Dextrin and $MgSO_4.7H_2O$; and Glucose and $MgSO_4.7H_2O$, respectively, in the form of 3D plots where the pairs of variables are varied systematically to form a grid while keeping the other variables fixed for phytase production using Equation 2.

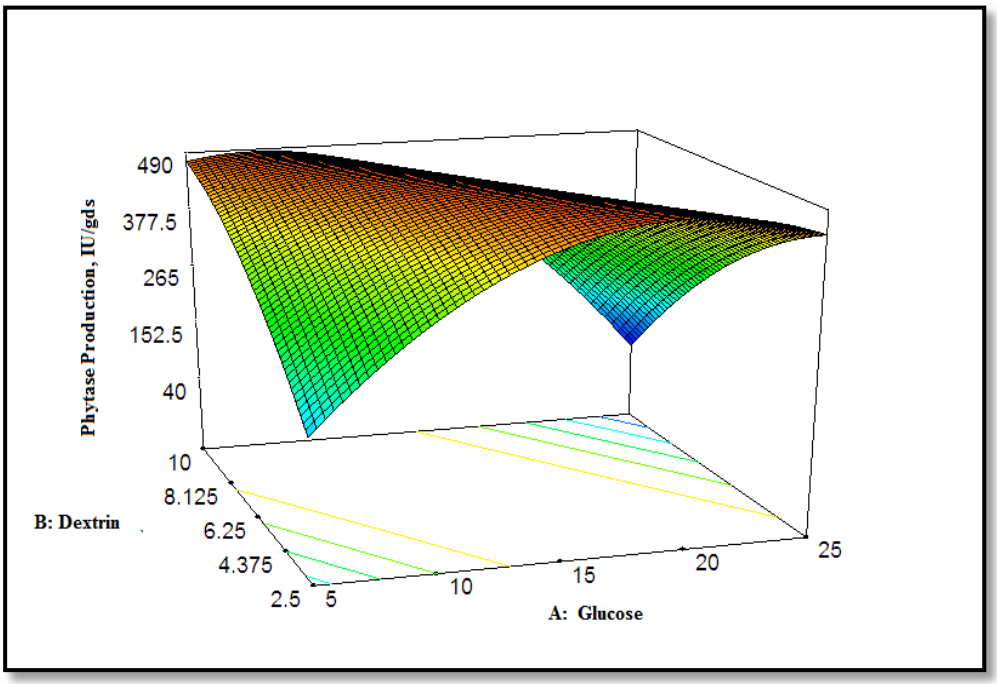

Figure 3a

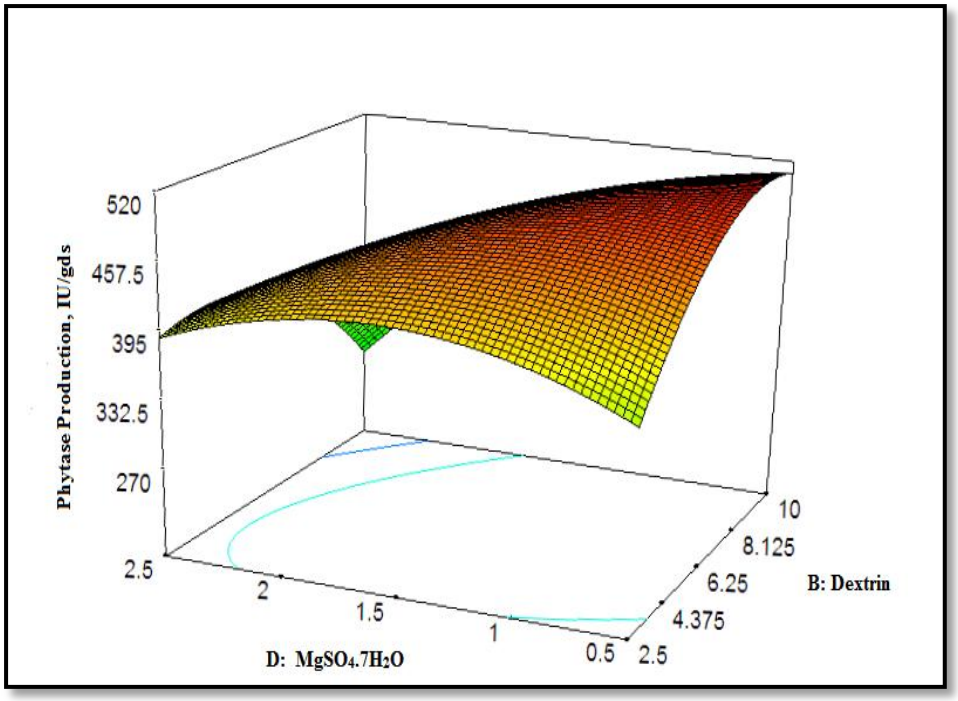

Figure 3b

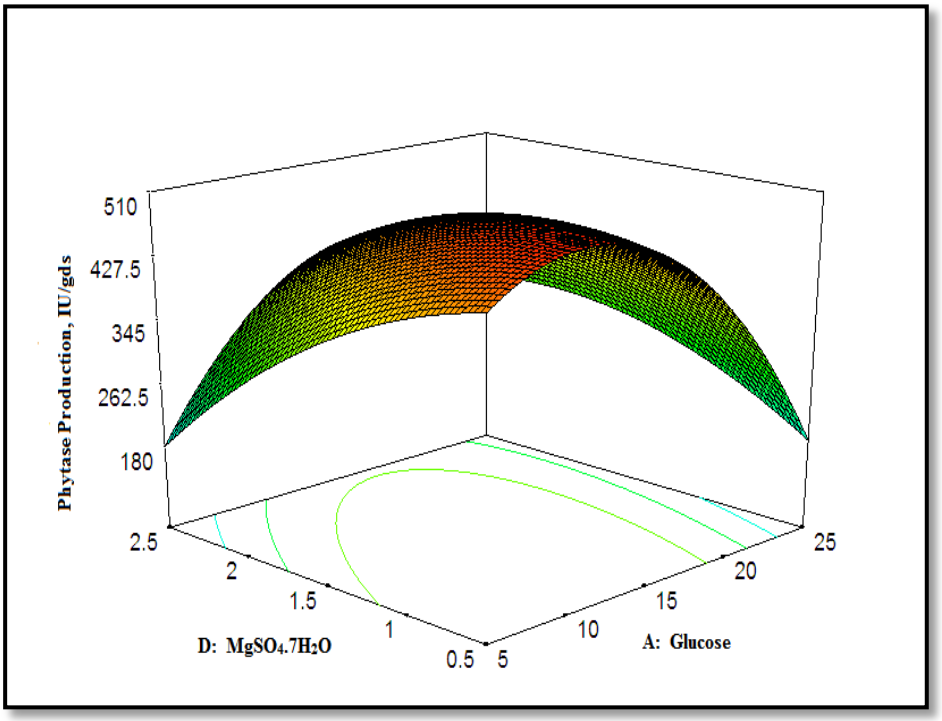

Figure 3c

Figure 3 3D surface plot between media variables using BBD model: (3a) Glucose and Dextrin; (3b) $MgSO_4.7H_2O$ and Dextrin and (3c) Glucose and $MgSO_4.7H_2O$.

The composition of BBD optimized media for maximum production of phytase (524 IU/gds) was obtained as 5 g of glucose, 10 g of dextrin, 200 mL of distilled water and 1.5g of $MgSO_4.7H_2O$ per 100g of GOC, with the rest of the variables kept at the mean value of the corresponding levels employed in PBD. The plot between predicted *vs.* actual values of phytase production (Figure 4) showed a good model fit.

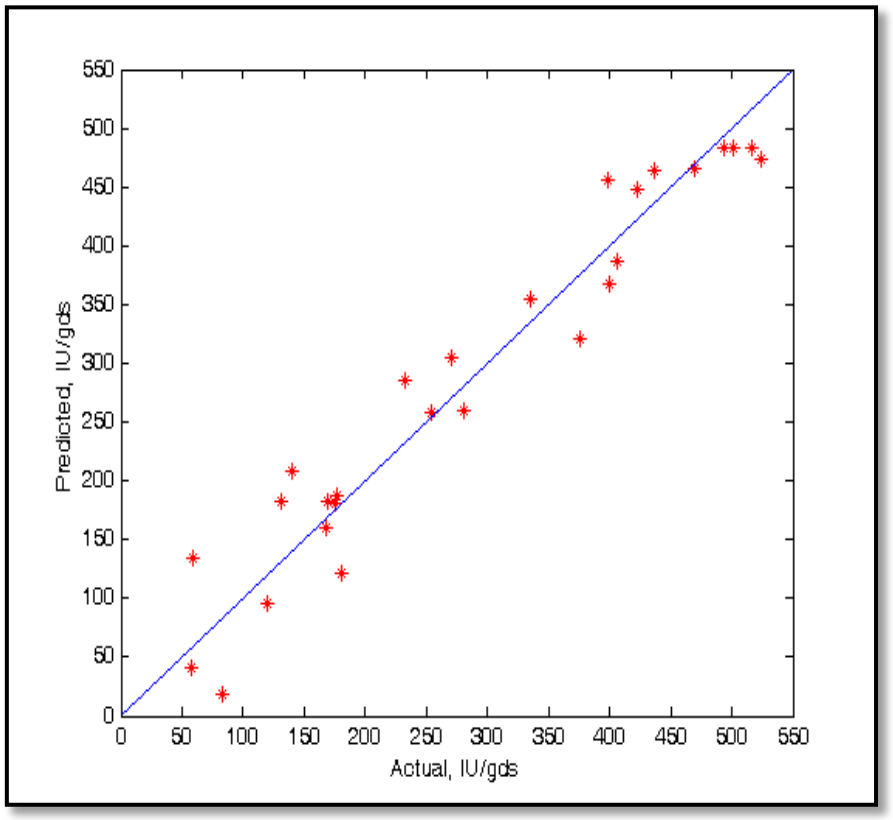

Figure 4 BBD model predictions Vs actual response values of phytase production

To validate the model, time course experiments for phytase production was carried out under the optimized conditions predicted by RSM. The best experimental response for phytase production was 550 IU/gds on fourth day and this is slightly better than the results of BBD (Figure 5). Thus, a remarkable 36.6 fold enhancement in phytase yield from 15 IU/gds to 550 IU/gds was attained by following the RSM optimization procedure.

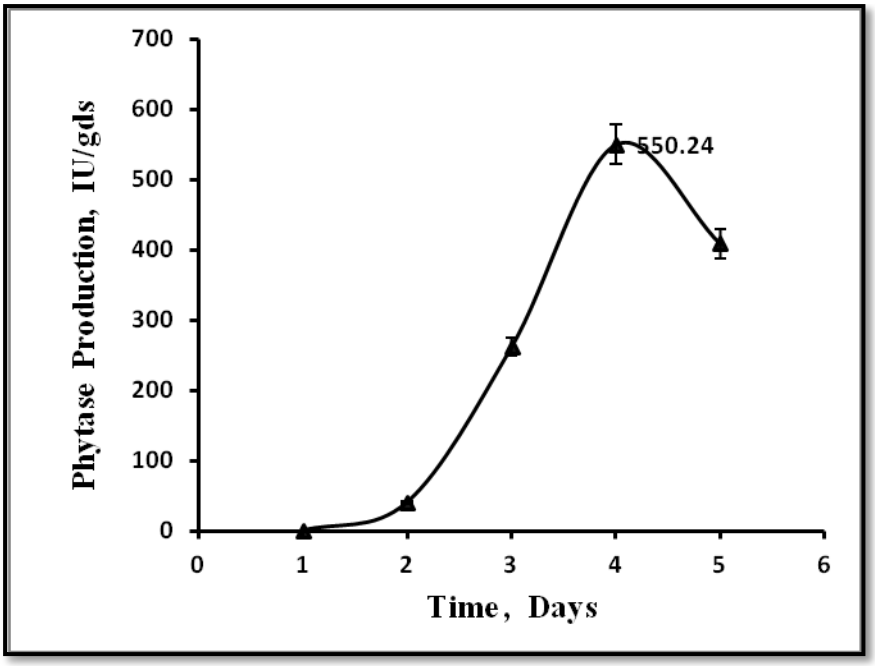

Figure 5 Time course of phytase production using BBD optimized media conditions

We also compared the observed phytase production activities using GOC with other studies reported in the literature using different microorganisms and growth media. The comparison is reported in Table 5 and it is clearly seen here that the present study outperforms the others in phytase production with the observed high activity of 550 IU/gds. The present system, in fact, near doubles the activity reported using wheat bran and sesame oil cake which by themselves show high activity of 250 and 282 IU/gds [Bhavsar et al. 2013, Singh et al. 2011].The comparison clearly recommends the use of GOC for phytase production with *A. niger* [Table 5]. Up-scaling in trays were also giving proportionate phytase production.

Table 5: Comparison of responses reported using other SSF bioprocesses with fungi

Microorganism	Substrate	Optimization by RSM	pH	Production (IU/gds)	Reference
***A. niger* NCIM 563**	**Groundnut oil cake**	**Yes**	**2.5**	**550**	**Present work**
S. thermophile	Sesame oil cake	No	5	282	[Singh and Satyanarayana 2006]
A. niger NCIM 563	Wheat bran	Yes	2.5	250	[Bhavsar et al. 2013]
A. niger NCIM 563	Wheat bran	Yes	5.6	154	[Bhavsar et al. 2011]
R. oryzae	Linseed cake	Yes	5	149	[Rani and Ghosh 2011]
S. commune	Wheat bran	Yes	5	113.7	[Salmon et al. 2012]
A. niger FS3	Citric by products	No	5	96	[Spier 2011]
A. oryzae	Soy meal	Yes	-	58.7	[Chen 2014]
A.niger NB2	Olive waste	No	-	58	[Vassiley 2007]
A.niger FS3	Citric pulp bran	Yes		40.25	[Spier 2011]
A. ficuum	Lentils	Yes	-	32	[Bennet and Yang 2012]
A. ficuum NTG-23	Waste vinegar	Yes	1.3	8.72	[Wan 2011]

3.2 Thermo-stabilization

As pointed out in the Introduction we need to study the robustness of the produced phytase by testing for its thermo-stability and activity directly in dried koji. The major constraint in application of phytase in animal feed is the reduced thermal stability during the pelleting process. Pelleting stability is generally improved by protected formulation and thermo-stability coatings [Yanez et al. 2013]. The protein or enzyme stabilizers used are non reducing sugars, organic and inorganic salts and polyols [Obon et al. 1996]. We studied the effect of stabilizers skimmed milk, sorbitol, and mannitol at 10% concentration on the phytase obtained from *A. niger* koji by SSF (Figure 6) as described in Materials and methods. The results obtained show that the enzyme is protected at high temperature (50ºC for 36h) especially with skimmed milk and sorbitol. Thus, it is evident that use of additives like skim

milk or sorbitol (10 %) to fermented koji before drying is useful for loss of phytase activity during drying process and this is the normal practice used in SSF process so koji can be directly used as supplement in animal feed. Phytase is found to be active in this dried koji form stored at room temperature. Stability is checked for up to 6 months.

Feed Analysis report showed the koji to be toxin free and safe to use as feed additive, so koji can be directly used as supplement in animal feed.

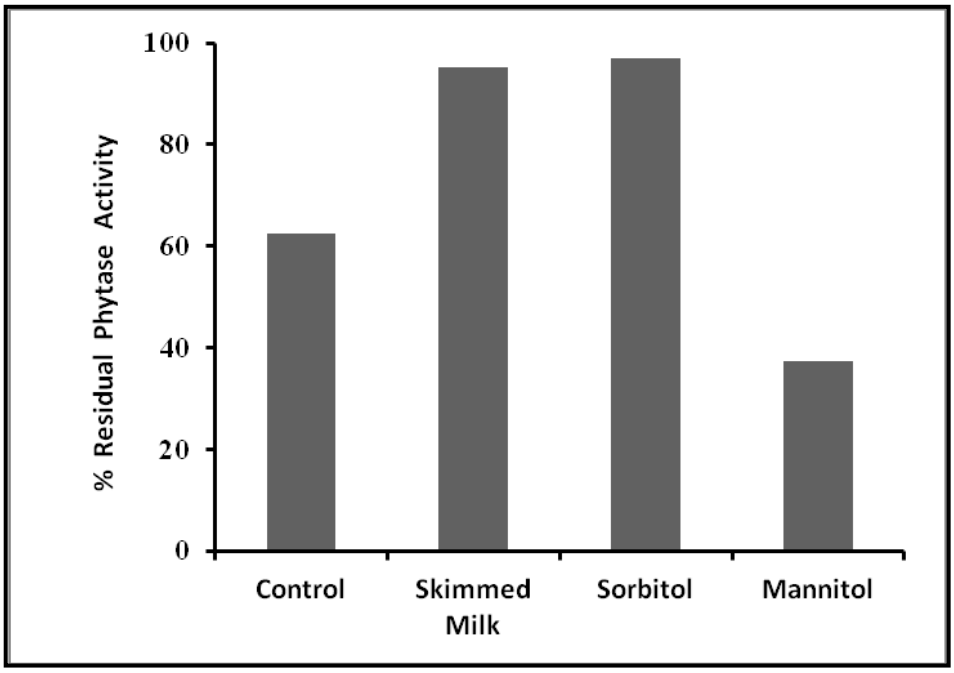

Figure 6 Thermo-stabilization of solid state phytase using different stabilizers

3.3 Enzyme stability in simulated gastric fluid

The pH optima and pH stability profile of phytase determines its ability to act efficiently in the digestive tract of poultry. Digestive tract pH of poultry varies from 5.5 in crop to 2.5 in stomach. To act efficiently, the enzyme must be stable and active at broad pH range [Lei and Stahl 2001]. Our liquid enzyme extract and dried koji both exhibited high efficacy in phytate hydrolysis by releasing more than 60% of P at highly acidic pH (1.5-3.5). The significant P release was maintained at higher pH values (4.5-6.5) and therefore under simulated gastric conditions (Figure 7). By this we can deduce that the catalytic efficiency of *A. niger* NCIM

563 SSF phytase will be maintained in both crop and stomach of poultry because it retains activity over a wide pH range without any need for reactivation.

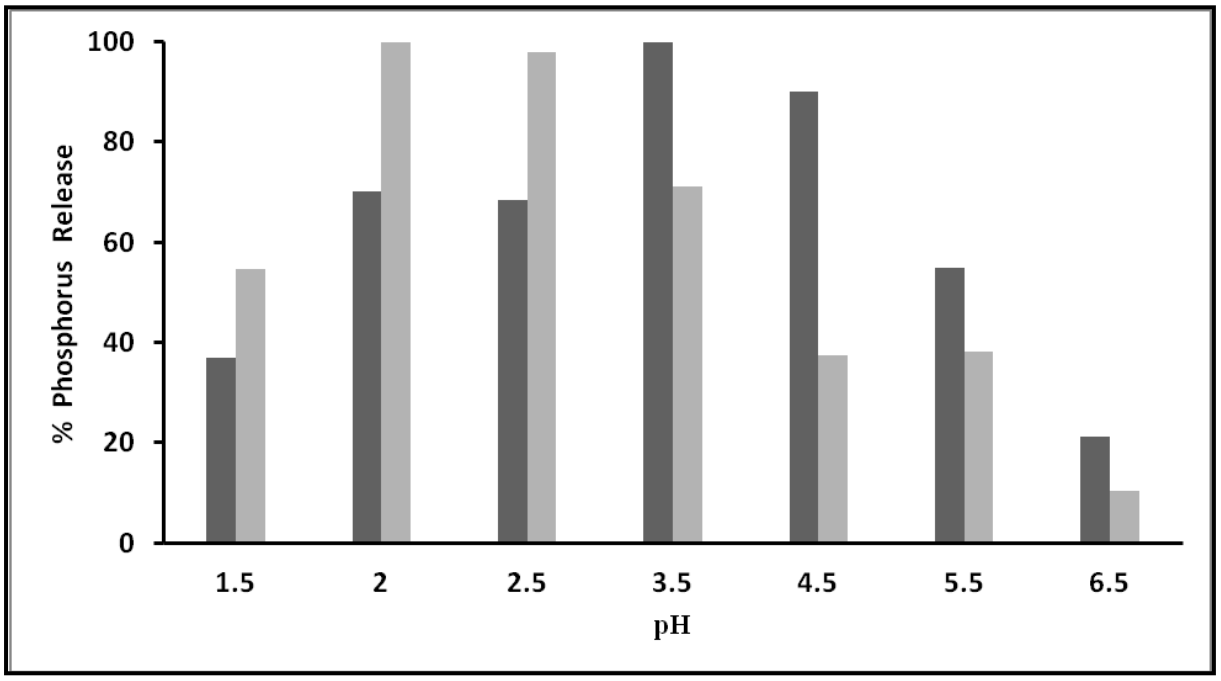

Figure 7 P release efficiency of solid state phytase in simulated gastric fluid conditions at different pH. . ■ Liquid enzymes extract □ Dried koji

3.4 Action of SSF phytase on minerals and their availability

Phytate is a negatively charged ion of phytic acid and therefore has an affinity for positively charged minerals, trace elements and proteins. This phytate-mineral interaction affects mineral bioavailability in animal feed and is therefore a well-known anti-nutrient [Urbano et al. 2000]. However, the bound minerals get released by phytase addition, and it neutralizes the anti-nutritional effect of phytic acid. We studied the effect of dried koji and liquid enzyme extract on the bioavailability of minerals like Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+}. It was seen that SSF phytase released more than 50% of bound mineral ions (Figure 8) and the results obtained further support the applied perspective potential of phytase obtained from GOC and *A. niger* in animal feed.

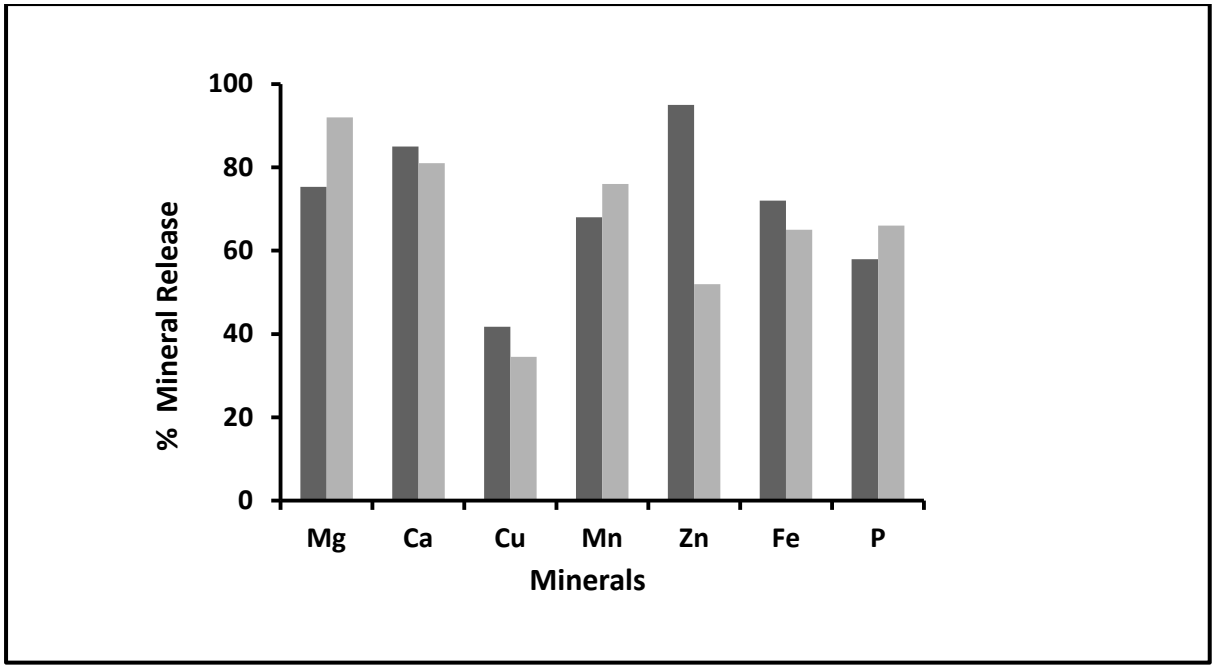

Figure 8 % Minerals release by phytase in liquid and solid form

Liquid enzyme extracts Dried koji

Conclusion:

Employing a low-cost SSF methodology using the fungal micro-organism *A. niger* for phytase production with groundnut oil cake (an agro-industrial waste), the present study, has brought out its potential for application in food processing and feed industries. Using RSM, the maximum phytase yield obtained was 550 IU/gds, which is 36.6 fold greater than the basal medium activity before optimization of the fermentation parameters. In fact, this high production of phytase is even two fold-higher than the best reported. Importantly, the phytase produced offers the possibility of using it as a direct food supplement in the dried koji form and thereby offers considerable scope for application. It would be worthwhile to study the growth kinetics to further understand the mechanisms responsible for the phytase production from a fermentation view point so that suitable scale-up procedures for technology development becomes possible.

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Chapter 3

In vitro and in field Poultry study of Phytase Efficiency

Summary: The application of phytase in poultry feed and its efficiency in releasing P and minerals in vitro and in vivo was studied. Phytase enzyme produced by *A. niger NCIM 563* by solid state fermentation was applied in poultry feed study. For application the koji obtained after SSF was dried and made in a powder form. First, the ability of enzyme in the dried powder to dephytinize the poultry feed under simulated gastric condition was studied. Positive results guide us towards in field poultry study. Poultry feed was designed including our enzyme and a 42 days feed trial was conducted on broilers. Results indicated that reduction of dietary phosphorus in the diet with the supplementation of phytase could maintain growth performance and skeletal development and reduce phosphorus and phytic acid in the litter. Our enzyme was able to replace up to 0.1% phosphorus in poultry feed. Thus with a minimum downstream processing we were able to prepare a low cost feed supplement with high phytase activity and efficiency in poultry feed.

1. Introduction:

Exogenous phytase supplementation of poultry diets is a common practice to improve phosphorus (P) nutrient management. Poultry feed is largely composed of grains, cereals, vegetable oil cakes, meals and there by products. The storage form of P in plant seeds is phytate, the salts of *myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate or phytic acid which may hold approximately 70% of the total P in plants [Olukosi 2013**].**

Unfortunately, phytate is not hydrolyzed in the poultry gut and phytate-bound phosphate remains unabsorbed. P is an essential and limiting nutrient in poultry feed, Thus feed require the exogenous addition of phosphate to avoid phosphorus deficiency. This not only increases the basic food costs, but also the unabsorbed Phytate and excess P in excreta run off to water bodies that leads to serious environmental pollution [Garret et al. 2004].

In addition phytic acid is a strong chelating agent with six reactive groups which binds to cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}. Under gastrointestinal pH conditions, insoluble metal phytate complexes are formed which make the metals unavailable for absorption in the intestinal tract. It also reduces digestibility of proteins, starch and lipids by forming insoluble complexes, as a result affecting metabolism and growth performance of animal [Woyengo and Nyachoti 2013].

The best way to solve these problems is careful use of natural resources by phytase enzyme supplementation in the diet. Phytases are myo-inositol hexaphosphate phosphohydrolase that are able to hydrolyse phytate. Phytases catalyse the stepwise removal of inorganic orthophosphates from phytic acid. The capacity of this feed enzyme to release phytate-bound P and reduce P excretion is well studied [Simons et al. 1990, Shirley and Edward 2003, Selle et al. 2007, Cowieson et al. 2011, Chen et al. 2013]. In this direction, a great number of phytase producing microorganisms have been isolated. The first phytases produced on a commercial scale were either derived from fungal strains mutated via standard

procedures or by recombinant DNA technology. Microbial phytase supplementation at 250-500 units/kg in diet increased body weight gain and feed intake in broiler chicken [Selle and Ravindran 2007]. Since relatively large quantity of enzyme is required for feed trial experiment, very few studies are available on feeding of lab produced phytase to poultry birds [Ahmad et al. 2000, Paik et al. 2000, Vohra et al. 2011, Lan et al. 2012]. Our lab has reported the high phytase producing strain of *Aspergillus niger* NCIM 563. [Mandviwala and Khire 2000] We have optimized the phytase production under solid state fermentation [Buddhiwant et al. 2015]. For application in poultry feed the koji obtained after SSF was dried and made in a powder form. A phytase which has high activity at low pH and a wide pH range would be expected to hydrolyze phytate more quickly and thus more efficiently reduce the anti nutritional effect of phytate [Dersjant-Li et al. 2015]. Our enzyme has an optimum pH of 2.5 and broad pH stability. The ability of enzyme in the dried powder to dephytinize the poultry feed in vitro and in vivo conditions was studied. Poultry feed was designed including our enzyme and a 42 days feed trial was conducted on broilers. Despite of availability of commercial enzymes, phytase produced in high yield by a relatively inexpensive system is attracting feed industries worldwide and our phytase may provide an alternative.

2.1 Materials and Methods:

2.1 Chemicals:

All the chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo. The designed poultry feed was manufactured and supplied by a local feed manufacturer and day old chicks were supplied by Venketeshwara hatcheries.

2.2 Phytase production:

The fungal phytase was produced by *A. niger* NCIM 563 employing solid state fermentation (SSF) as reported earlier [Buddhiwant et al. 2015] .The SSF media consist of 100 g Ground nut oil cake (GOC),5 g of glucose, 10 g of dextrin, 200 mL of distilled water, 1.5 g of $MgSO_4.7H_2O$, 3 g of $FeSO_4.7H_2O$, 3 g of KCl and 3 g of $MnSO_4$. Fermentation medium was sterilized at 121ºC and 15 psi for 30 min. On cooling the medium was inoculated with spore suspension of *A. niger* NCIM 563 at a concentration of $5x10^6$ spores /g of GOC and incubated for 4 days at 30ºC. For application purpose the whole fermented substrate (koji) was dried in an oven at 50°C for 36 h with 10% skimmed milk powder till constant dry weight obtained. Then koji was ground to get in powdered form. This dried koji has been sent for analysis to analytical facility at Central Avian Research Institute, (CARI) U.P. India.

2.3 Analytical method

2.3.1 Phytase and phytic acid analysis

Phytase reaction mixture consisted of 3 mM sodium phytate with 100 mM Glycine-HCl buffer (pH 2.5) and 100 µl of crude enzyme solution. After 30 min at 50 °C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [Heinohen and Lathi 1981]. A freshly prepared solution of acetone: 5N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl 1M citric acid was added and absorbance was measured at 370 nm. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1 µmol phosphorus/min/ml under standard assay conditions. Production was reported as U of phytase per g of dry substrate (U/gds). Water soluble P in excreta was also determined by ammonium molybdate method.

Phytic acid (PA) analysis in feed and litter samples were carried out on the basis of the modified procedure of Latta and Eskin [1980]. Briefly, 1 g of samples extracted with 2.4% HCl (20 ml) by incubating on rotary shaker at 150 rpm for 1 hr at room temperature. After

centrifugation, the supernatant was diluted with double volume of distilled water, and 10 ml of the diluted supernatant was loaded on to a column containing an AG-1-X8 anion exchange resin (200–400 mesh chloride form). Interfering compounds and inorganic phosphorus were removed by washing with distilled water (10 ml) followed by 0.1 M NaCl (10 ml). Bound PA was eluted with 0.7 M NaCl (10 ml).The PA content was determined by modified wade reagent method.

2.3.2 Dephytinization of poultry feed under in vitro condition

The ability of enzyme to dephytinize the poultry feed in dried powder form was studied at different pH. For that one gram of feed 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) with the pH adjusted by HCl or NaOH, viz., 1.5, 2.0, 2.5, 3.5 and 4.5 as required. Different pH was maintained to check the pH stability profile .The solutions were incubated with agitation at 39 °C for 30 min, and the pH again adjusted to the corresponding values. 50 U of enzyme was added and the whole mixture was incubated by agitation at 39°C for 60 min. The amount of phytic acid before and after enzyme treatment was determined as described in Analytical methods.

2.4 Broiler experiment

2.4.1 Experimental design

The broiler experiment was done at Department of Poultry Science, KNP College of veterinary sciences, Shirwal, Pune., India. The 42 days trial was conducted in a completely randomized design. A total of one hundred mix sexed, day-old Cobb broiler chicks, vaccinated against Newcastle disease were obtained from commercial hatchery. The chicks were distributed randomly into five groups, 20 chicks in each group. The chicks were housed in standard poultry deep litter house fitted with heating arrangements, feeders, waterer, 24 h light and proper air ventilation, and reared under standard conditions (Figure 1). The feed and

fresh drinking water were provided ad libitum during the entire experimental period.

Figure 1 The experimental site for broiler feed trial

2.4.2 Experimental diets

Three phased diets were used. The pre-starter, starter and finisher diet was fed from 0 to 2 weeks, 3 to 4 weeks and 5 to 6 weeks of age respectively. The dietary treatments were consisted of normal phosphorus (NP) group without any phytase enzyme , two low-phosphorus (LP1 and LP2) groups and two LP groups supplemented with phytase (LPP1 and LPP2) at 500 U/kg diet, respectively, to full fill the phosphorus requirements. Ingredient and nutrient composition of all the corn soya based diets are given in Table 1. The diets were formulated based on Nutrient Requirement of Poultry published by ICAR (2013).

Table 1 Ingredient and nutrient composition of feeds during 3 growth phase

	Pre-starter (0-2 weeks)					Starter (3-4 week)					Finisher (5-6 week)				
Ingredient composition (g/kg):															
	NP	LP1	LPP1	LP2	LPP2	NP	LP1	LPP1	LP2	LPP2	NP	LP1	LPP1	LP2	LPP2
Maize	603	607	607	611	611	634	638	638	642	642	695	699	699	703	703
Soya Doc	300	300	300	300	300	250	250	250	250	250	179	179	179	179	179
Full fat soya	0	0	0	0	0	25	25	25	25	25	40	40	40	40	40
LSP	15	15	15	15	15	13	13	13	13	13	11	11	11	11	11
PBS premix	17	17	17	17	17	15	15	15	15	15	14	14	14	14	14
Maize gluten	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
MCP	15	11	11	7	7	13	9	9	5	5	11	7	7	3	3
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Nutrient composition (%):															
Crude fibre	2.15	2.2	2.21	2.1	2.1	2.52	2.87	2.9	2.5	2.92	3	3.15	3.01	2.81	2.96
Crude protein	24.5	24.5	25.88	24.5	25.88	23.45	23.45	24.72	23.45	24.72	21.52	21.52	22.90	21.52	22.90
C/P	21.93	21.96	21.96	22	22	20.96	20.99	20.99	21.03	21.03	18.91	18.95	18.95	18.98	18.98
Lysine	1.27	1.27	1.27	1.27	1.27	1.14	1.14	1.14	1.14	1.14	1	1	1	1.01	1.01
Methionine+Cystine	0.91	0.92	0.92	0.92	0.92	0.83	0.83	0.83	0.83	0.83	0.78	0.78	0.78	0.78	0.78
Threonine	0.78	0.78	0.78	0.78	0.78	0.72	0.72	0.72	0.73	0.73	0.66	0.66	0.66	0.66	0.66
Energy	2899.9	2913.1	2913.1	2962.3	2962.3	2982.2	2995.4	2995.4	3008.6	3008.6	3073.1	3086.3	3086.3	3099.5	3099.5
Tryptophan	0.21	0.21	0.21	0.21	0.21	0.2	0.2	0.2	0.2	0.2	0.17	0.17	0.17	0.18	0.18
Arginine	1.24	1.24	1.24	1.24	1.24	1.16	1.16	1.16	1.16	1.16	1	1.01	1.01	1.01	1.01
Ca	1	0.93	0.93	0.86	0.86	0.96	0.83	0.83	0.76	0.76	0.85	0.77	0.77	0.72	0.72
Total P	0.76	0.68	0.68	0.60	0.60	0.70	0.62	0.62	0.54	0.54	0.66	0.58	0.58	0.50	0.50
Available P	0.5	0.42	0.42	0.34	0.34	0.45	0.37	0.37	0.29	0.29	0.43	0.35	0.35	0.27	0.27
Phytate P	0.26	0.26	0.26	0.26	0.26	0.25	0.25	0.25	0.25	0.25	0.23	0.23	0.23	0.23	0.23
Phytic acid*	0.92	0.92	0.92	0.92	0.92	0.88	0.88	0.88	0.88	0.88	0.84	0.84	0.84	0.84	0.84
Phytase (U)			500U		500U			500U		500U			500U		500U

2.4.3 Measurements, sample processing and lab analysis

Physical parameters of growth such as average weekly gain (g gain/bird/week), feed intake (g feed/bird/week), and feed conversion (feed: gain) were calculated using the body weight and feed intake measurements taken during 7 days interval. At the end of 42 days, four birds were selected randomly per group and sacrificed by cervical dislocation. Prior to sacrificing blood samples of birds were collected into a sterile centrifuge tube .The blood was centrifuged at 3000 rpm for 10 min, and serum was separated and stored at -20°C for estimation of Ca and phosphorous. Samples were sent to a pathology laboratory for estimation.

The left tibia bones were collected after removing the muscles and adhering structures, cleaned by dipping into boiling water for 5 min. After drying in hot air oven (80°C) the bone morphology including weight, length and width of the bone were recorded. Analysis of bone ash, Ca and P content were done at AFAQL, Veterinary College and research institute, Namakkal, Tamilnadu.

Excreta samples were collected at the end of 2nd, 4th and 6th week from each group from four different places with in one pen and pooled. The samples were stored at -20°C until they were dried in a forced air oven (at 55°C for 48 hours) and then ground for analysis. P and phytic acid was analysed as described in analytical section.

3. Results and discussion:

With minimum downstream processing enzyme was prepared in powder form. Enzyme was stable and active in this form and feed analysis study for possible aflatoxin and ochratoxin contamination, proves it to be safe to use in poultry feed. (Data not shown)

3.1 In vitro dephytinization and P release from poultry feed

The enzyme in dried koji form was able to hydrolyze phytate effectively and dephytinizes more than 90% of PA at acidic pH (2-2.5) (Figure2).

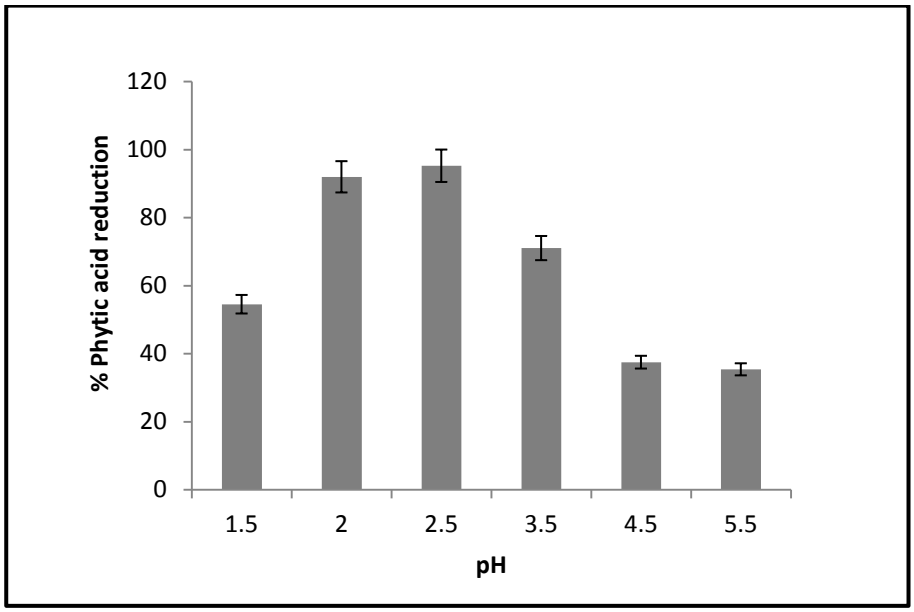

Figure 2 Dephytinization of poultry feed in vitro

Digestive tract pH of poultry varies from 2.5 to 5.5. To act efficiently, the enzyme must be stable and active at broad pH range. By in vitro dephytinization experiment we can deduce that the catalytic efficiency of *A. niger* NCIM 563 SSF phytase will be maintained inside poultry gut. Besides the dried koji has a mixture of enzyme because *A. niger* NCIM 563

produces two phytases having activity at pH 2.5 and 5. However it is difficult to ascertain that which enzyme is working in which part of digestive system.

3.2 Growth performance

Although improved growth performance of broiler by phytase addition is well studied, these trials were mainly done with commercial phytases. Studies indicated that low P without phytase supplementation resulted in poor performance with lower feed intake and poor feed efficiency. The phytase supplementation improved P availability, thus overall growth. Reports of efficiency of lab made phytase in broiler feed are very few. We have optimized the phytase production under solid state fermentation and up scaled it to tray levels, which enables us to produce enzyme in sufficient quantity to study its effects on poultry. The body weight gain and feed intake of LP1 and LP2 are lower as compared to NP. Inclusion of phytase in LPP1 was able to restore growth performance and it is comparable with NP. There is slight improvement in LPP2 group as compared to LP2 but it is not up to the positive control (NP). (Table 2) This trend is observable in all the phases of growth. The feed conversion ratio was non significant during the experimental period. Thus our enzyme at 500 U/kg of feed was able to replace up to 0.1% of non-phytin P without compromising growth performance. Paik et al 2003, showed that NPP (Non phytate P) level in broiler diets could be reduced by approximately 0.2% by the microbial phytase supplementation at 600U/kg [Paik 2003].

Table 2: Growth performance of broilers during 6 weeks of feed study

Day of analysis	Average body weight gain per bird (g)					Average feed intake per bird (g)				
	NP	LP1	LPP1	LP2	LPP2	NP	LP1	LPP1	LP2	LPP2
0	52.6	54.0	53.0	52.9	53.5	-	-	-	-	-
7	115.3	107.0	116.1	107.4	114.0	123.6	125.6	113.8	106.5	115.4
14	195.1	187.5	200.3	150.8	165.3	236.4	216.95	230.2	186.9	194.8
21	273.9	261.2	268.6	224.8	240.2	369.4	323.3	310.9	302.3	309.6
28	345.6	330.4	351.7	308.5	320.7	706.8	659.3	690.0	551.7	591.8
35	405.2	380.7	414.9	340.5	356.5	710.3	711.6	716.3	588.6	600.3
42	428.6	390.2	425.8	305.3	328.5	839.5	744.3	811.1	606.1	664.7

In our studies we need to try higher concentration of enzyme to maximize the P utilization. Manobhavan et al. [2016] concluded by experimenting with higher doses of phytase that super doses of phytase in low-phosphorus diet were beneficial than the normal standard dose (at 500 FTU/kg) of phytase in broiler chicken diet.

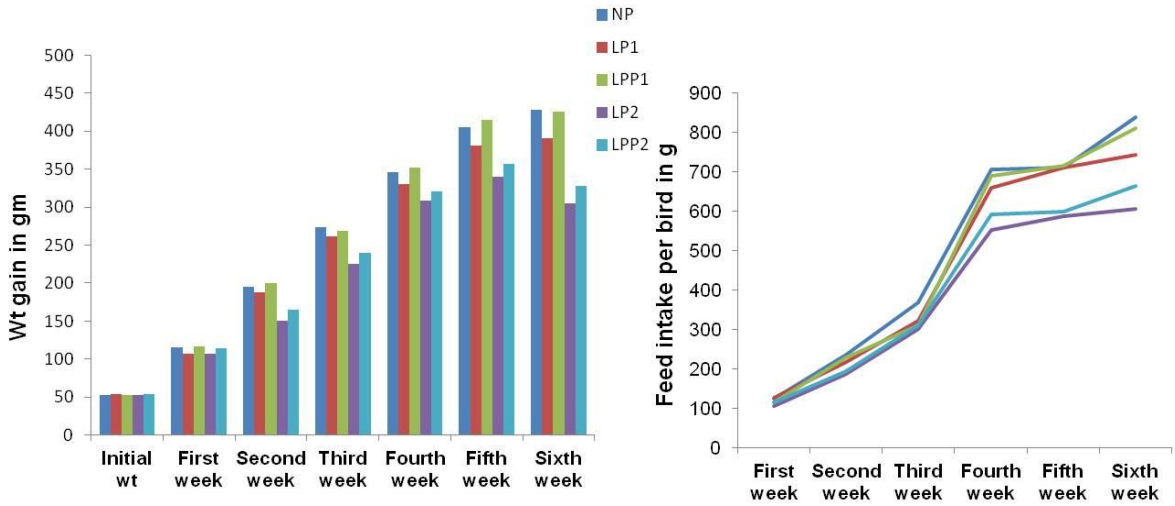

Figure 3 Wt gain and feed intake per bird during six weeks of feed trial

3.2 Bone morphometry and mineralization

Bone morphology and mineralization are considered as important parameters to check P utilization. Bone weight (g/kg body weight) and Ca & P content of phytase supplemented group (LPP1& LPP2) was higher as compared to low P group (LP1&LP2) (Table3). Tibia diameter was also better by phytase addition in low P diet (Figure 4). Rutherfurd et al. [2012] and Guo et al. [2009] reported that tibia weight was significantly higher in phytase-supplemented group. Rousseau et al. [2012] reported that tibial diameter/mid-shaft width was improved by phytase supplementation of 500 FTU/kg in low-P diets but no improvement in tibia bone length.

Table 3: Bone weight and ash, Ca & P content among different groups

	Gender	**Bone weight (g/kg of body wt)**	**Bone Ash (%)**	**Ca (%)**	**P (%)**
NP	**Male**	6.13	39.61	13.98	6.01
	Female	5.14	40.78	13.97	6.53
LP1	**Male**	5.32	39.77	13.98	6.3
	Female	4.35	36.87	13.06	4.62
LPP1	**Male**	5.64	41.84	14.36	6.22
	Female	6.00	38.81	13.99	6.4
LP2	**Male**	4.85	36.11	11.95	4.93
	Female	4.03	37.53	12.99	5.32
LPP2	**Male**	6.06	40.35	14.36	5.94
	Female	5.46	35.43	11.95	5.27

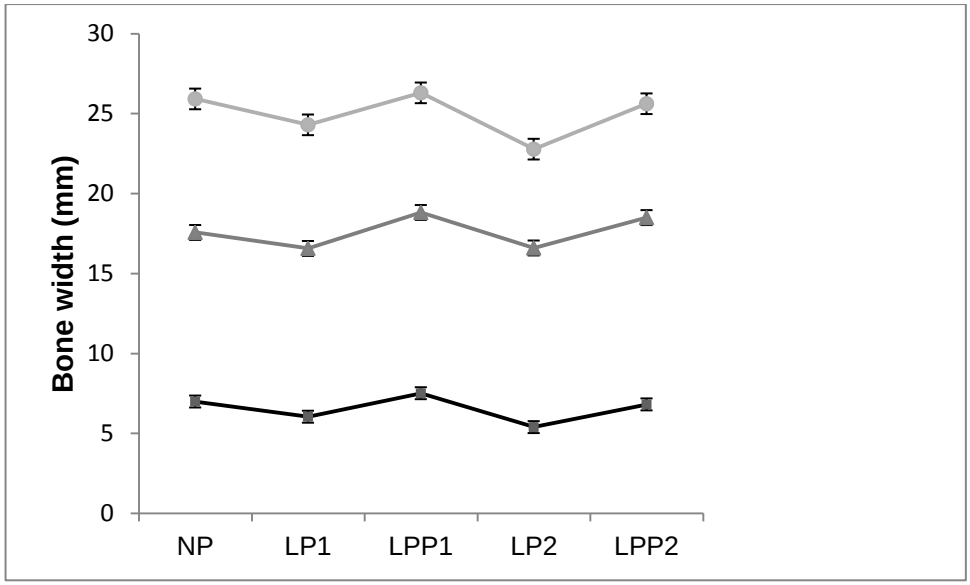

Figure 4 Variation in bone width among groups

Blood serum Ca and P concentration was not much affected by supplemental phytase. Angel et al. also find that blood Ca level was not influenced by the applied treatments, possibly because Ca homeostasis in the extracellular fluid is maintained by the joint effects of the various other factors [Angel et al. 2006].

3.3 Phosphorus and phytic acid in excreta

Phosphorus and phytate excretion in poultry manure is of great ecological concern as they pollute water bodies. Phytase supplemented low P diet showed reduction in P excretion.

Several researchers [Musapuor et al. 2006, Sukumar et al. 2013] reported reduction in P excretion in low-P diet and the reduction was higher on phytase supplementation in the low-P diet. Lalpanmawia et al. reported that phytase supplementation (at 500 FTU) in low-P diet reduced P excretion by 30% when compared to normal P-fed group [Lalpanmawia et al. 2014]. If dietary concentrations of inorganic P are not reduced appropriately when phytase is included, excretion of available P will increase, and thus the potential exists for the increases in excreted soluble P [Angel et al. 2005]. Thus we determined total P, phytic acid and water

soluble P content of all the experimental groups. The reduction in P and phytic acid excretion in phytase supplemented group was 30-40% as compared to normal P fed diet. Figure 5 shows the total P (including phytate P and WSP) intake and excretion among different treatment groups. Water-soluble P concentrations in litter were not affected by the concentration of P fed relative to bird requirements when phytase was added to the diet [Applegate et al. 2003]**.**

Reduction of dietary P in the diet with the supplementation of our lab made fungal phytase could sustain live performance and skeletal development while reducing total and soluble P in the litter.

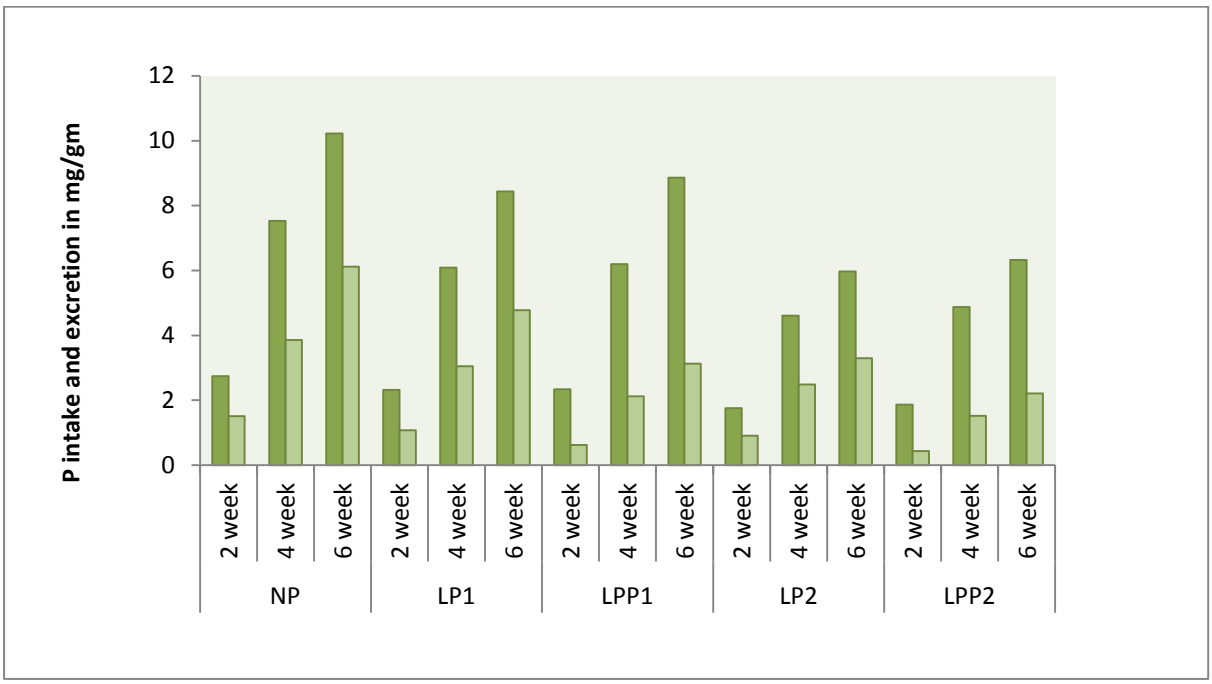

Figure 5 P intake and excretion among different groups at the end of three phases

Conclusion:

It is commonly accepted that microbial phytases are alternative economical P source and their dietary inclusion generates bio available P and reduces the P load on the environment. Despite of availability of commercial phytases, research is open for efficient low cost phytase product which could be applied widely in rapidly growing livestock production. Our phytase produced on cheap agriculture residue via solid state fermentation and with minimum downstream processing provide a good option. Its efficiency in poultry diet is comparable to available commercial phytases. Further studies on larger scale and with optimized dose may prove its potential for large scale applications.

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Chapter 4

Phytase Production on Poly-Urethane foam and its application in bread making

Summary: Solid state fermentation (SSF) is a preferable method for fungal fermentation as it provides a solid support for good mycelial growth. Agricultural residues can be utilized for this purpose. These residues besides providing support also act as nutrient media however; there is a problem of uniformity with this support. These residues can be replaced with inert solid supports like polymeric urethane and styrene foam (PUF, PSF). PUF is particularly reported for SSF as it is porous, cheap, reusable and less complicated product recovery is an added advantage. In our study we have selected PUF as a reliable solid support for phytase producing fungus *A.niger* NCIM 563. Total phytase production found to be 3600 U on 4^{th} day of fermentation with 1g PUF and 25ml liquid media. After three successive cycle of fermentation, production reached to 5000U. In the present study we have applied this Phytase during bread making process. Phytase breaks down the phytic acid of flour which leads to dephytinization and augment soluble mineral content of bread up to 90%.

1. Introduction:

Solid state fermentation (SSF) is an age old technique for microbial production of various products and in the last 10-15 years it has gained renewed interest because of its advantages over submerged fermentation (SmF) like concentrated products, lower cost, simpler fermentation requirement and lower waste output [Yoon et al. 2014]. SSF is defined as the growth of microorganisms on moist solid supports. Two types of SSF systems can be defined based on the nature of the solid support used. The first and most commonly used system involves cultivation on natural substrates like agro-industrial residues. The second system, which is not as frequently used, involves cultivation on an inert support impregnated with a liquid medium [Bhargav et al. 2008]. Natural substrates in SSF system serves both as a support and a nutrient source. However they have certain disadvantages. The carbon source constitutes an essential part of their structure and during growth of the microorganism the solid medium is degraded, and as a result, the geometric and physical characteristics of the medium changes [Ooijkaas et al.2000]. In most of these systems it is not possible to separate residual solid substrate from biomass, therefore, direct estimation of growth is difficult, and Moreover product recovery also becomes difficult and challenging. These complications can be overcome by using biologically inert materials. There are reports of use of polystyrene [Gautam et al. 2002] and polyurethane foam [Majumdar et al. 2015; Mata-Gómez et al. 2015] as inert carriers for SSF with fungi.

Polyurethane foam presents high porosity, low density and comparatively high water absorption. It has been widely used in several fields like enzyme immobilization [Nakamura et al. 1999], production of extra-cellular microbial enzymes [Majumdar et al. 2015; Mata-Gómez et al. 2015], and production of organic acids [Banik & Mukhopadhyay 2014], alcohol production [Baptista 2006] etc. using different types of

microbes. The extracellular products of interest can be easily extracted from the inert support, and therefore products can be obtained with fewer impurities and the support can be reused. In this work, PUF was used as solid inert support for phytase producing fungus *A. niger* and an optimised media based on previous studies [Buddhiwant et al. 2015] has been used. The results showed that the fungus grows very well on PUF based medium and gave good quality enzyme production. The enzyme can be easily extracted and the inert support of PUF which immobilizes fungi, could be reused in semi-continuous fermentation. The extracted enzyme has been concentrated and partially purified by ultra-filtration in single step and then application studies were done.

Phytase, a phosphohydrolase enzyme degrades phytic acid (myo-inositol hexakisphosphate or InsP6) and its salts (phytate) into free phosphorus and lower myo-inositol. This phytate is a common constituent of plant based foods such as cereals and legumes [Singh and Satyanarayana 2011]. The increasing demand for whole grain based food products has resulted in interest in phytic acid also, because it is widely known that phytate have adverse effects on the bioavailability of multivalent cations, especially Zn^{2+}, Ca^{2+}, Mg^{2+}, and Fe^{3+}. To reduce the phytate content and increase the nutritional value of whole grain bread ,application of commercial fungal phytase have been reported [Haros et al. 2001; Park et al. 2011].

We have reported the application of our phytase enzyme produced by SSF on inert carrier PUF during bread making procedure to dephytinize and fortify the bread with available minerals.

2. Materials and methods:

2.1 Chemicals

All the chemicals used were of analytical grade and obtained from leading manufacturers. Agriculture residues, PUF and commercial blend of whole wheat flour and multigrain flour were purchased from a local market.

2.2. Fungi and inoculum preparation

A. niger NCIM 563 was used in the present study from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India. The stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4^{o}C. Spores from 7 days old PDA slant were harvested using sterile saline solution containing 0.01 % Tween 80 to obtain $5x10^{7}$ spores/ml and used as inoculum for SSF.

2.3 Media preparation

PUF was cut into 0.5 cm^3 cubes and washed with warm water several times. It is dried in oven at 70°C overnight and stored for use. 1 g of PUF was taken into 250ml flask and autoclaved. Groundnut Oil Cake (GOC) extract was prepared by boiling 10 g of GOC in 27 ml of distilled water for 1 hr. It is then filtered and cooled for media preparation. Liquid media of following composition was prepared separately: 3g Glucose, 1.25 g Dextrin, 0.4 g $FeSO_4$, 0.4 g KCl, 0.3 g $MnSO_4$ and 0.1 g KNO_3 dissolved in 25ml of GOC extract in 100ml conical flask. It was autoclaved, cooled and inoculated by 1 ml of spore suspension. It was mixed well and poured into 250 ml flask containing 1 g of PUF. Inoculated PUF is incubated at 30°C.

2.4 Variation in growth conditions

PUF size, Concentration and depth of media layer were varied in different experiment to see the effect on enzyme production. PUF was cut into pieces of different size, washed and dried.

Fermentation media was prepared as mentioned, inoculated and incubated. PUF concentration was varied in range of 0.5g, 1 g, 1.5g and 1.75g. For depth analysis thickness of media is increased in flask by increasing media concentration. (*Figure 1*)

Figure 1: PUF media variation

a: PUF pieces, b: PUF concentration, c: Size and d: Media depth

2.5 Enzyme extraction and Purification

Enzyme extraction was carried out using 40ml of sterilized distilled water. The fermented substrates were mixed with distilled water and the flasks were kept on a rotary shaker at 200 rpm for 30 min. After this, the PUF was squeezed under sterile conditions. Liquid supernatant was centrifuged at 5000 × g for 20 min at 4°C. The supernatant was used for enzyme assay. After extraction enzyme was first centrifuged to get a clear supernatant and then concentrated and purified by ultra-filtration with 100 kD membrane filter. Squeezed PUF pieces were either reused for production or dried and used for biomass determination.

2.6 Analytical method

2.6.1 Phytase and protein assay

Phytase reaction mixture consisted of 3 mM sodium phytate with 100 mM Glycine-HCl buffer (pH 2.5) and 100 µl of crude enzyme solution. After 30 min at 50 °C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [Heinohen & Lathi 1981]. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1 µmol phosphorus/min/ml under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments. Production was reported as total production in U per flask on 4^{th} day of fermentation and U/ml. Concentration of protein was determined using Lowry method using BSA as standard [Lowry et al. 1951].

2.6.2 Phytic acid (PA) and mineral analysis

The control and test samples during bread making were collected and freeze dried .These samples were then made in a powder form and used to estimate the residual PA content. Briefly, 1 g of phytase treated and untreated control samples extracted with 2.4% HCl (20 ml) by incubating on rotary shaker at 150 rpm for 1 hr at room temperature. After centrifugation, the supernatant was diluted with double volume of distilled water, and 10 ml of the diluted supernatant was loaded on to a column containing an AG-1-X8 anion exchange resin (200–400 mesh chloride form). Interfering compounds and inorganic phosphorus were removed by washing with distilled water (10 ml) followed by 0.1 M NaCl (10 ml). Bound PA was eluted with 0.7 M NaCl (10 ml).The PA content in control and test samples were determined by modified wade reagent method. [Latta and Eskin 1980]

The ability of phytase to increase bioavailability of PA bound minerals in bread was quantified using an inductively coupled plasma atomic emission spectroscopy (ICP-AES). For determination of mineral mobilization in phytase treated test bread sample, 1 g of dried

sample was suspended in 9 ml simulated gastric fluid (SGF; 250 mM Glycine–HCl containing 2.0 mg/ml NaCl and 3.2 mg/ml pepsin pH 2.5) and incubated at 37ºC on a rotary shaker at 150 rpm for 1 h. Samples were centrifuged at 10,000× g for 10 min at 4ºC, supernatant was filtered (0.2 mm, Pall Corporation) and filtrate was collected. Filtrate was diluted with distilled water for adjusting the signals in the calibration ranges of each element. Mineral contents in the samples were measured by ICP-AES. Total mineral analysis of whole wheat flour and multigrain flour was also done to compare the % release of minerals.

2.7 Biomass determination

Fungal growth was measured by dry weight determinations. The PUF pieces were washed with distilled water and subsequently dried overnight at 80 °C. Because of the inert character and the exactly known weight of PUF in every sample, the dry weight of biomass was estimated by subtracting the weight of PUF from the total weight after drying.

2.8 Bread making

Three types of bread were made: whole wheat flour, multigrain bread and flat Indian bread (Chapati). The bread dough formula for 100g consisted of wheat flour /multigrain flour (70g), fine wheat flour (maida, 30g), dry yeast (3g), salt (2.0 g), sugar (2.0 g) and water up to optimum absorption. Multigrain flour includes whole wheat, soya, bengal gram (chana), oat, maize and psyllium husk. The ingredients were mixed properly and phytase was added (500U/100g) for comparative purposes. After mixing, resting (10 min), dividing, kneading and resting (10 min), dough was mechanical shaped, proofed (at 35-40°C for 2 h or up to optimum volume increment) and baked (150 ºC, 45min). After mixing and resting a sample of dough was collected, called as unfermented dough. After baking, loaves were cooled for 2 hr at room temperature. Samples were also removed from fermented dough and finished bread *(Figure 2)*. Certain physicochemical characteristics of breads including weight, specific

volume, and width /height ratio of the central slice, moisture content and texture were also checked.

Chapati is the unfermented flat Indian bread consists of whole wheat flour, pinch of salt and water. After mixing and kneading the flour is kept for 30 min and flat chapatis were made. These were baked on flat pan.

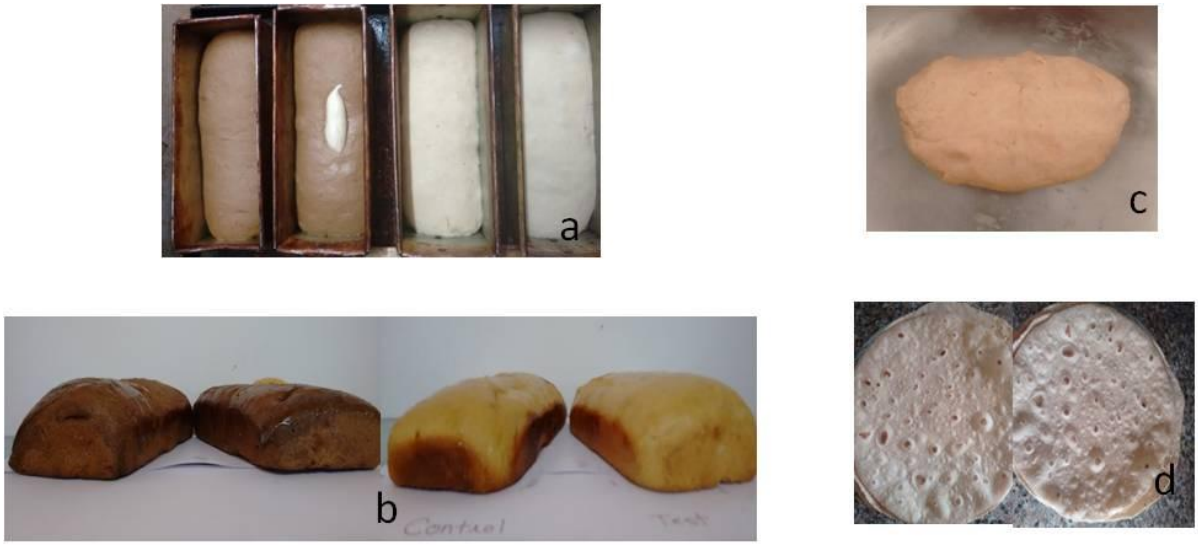

Figure 2 Bread making

a: Fermented dough, b: Finished bread, c: Dough for chapati , d: chapati

Results and Discussion:

Polyurethane foam impregnated with optimized liquid media gave a suitable environment for fungal growth. Extracellular protein impurities are less and further processing of enzyme becomes easy.

3.1 Phytase production on PUF media

Based on previous studies in our lab for optimum phytase production by *A. niger NCIM* 563 we tried optimized synthetic media [Soni & Khire 2007], Wheat bran extract containing media [Bhavsar et al. 2011] and Ground nut oilcake extract containing media [Buddhiwant et al. 2015]. For proper enzyme extraction and washing of PUF pieces 40 ml of sterilized distilled water was used on 4th day of fermentation. PUF was squeezed in sterile conditions to

get around 60 ml of extract. Maximum production was obtained with GOC based media. Total phytase production found to be 3600 U (60 U/ml) on 4th day of fermentation with 1 g PUF and 25 ml of liquid media with GOC extract. While Protein concentration was 160 mg (2.6 mg/ml). In our previous report of phytase production on oilcake [Buddhiwant et al. 2015] total production per flask was 5240 U, on 4th day, however in this study extracellular protein production is much less and specific activity is almost doubled. Natural substrate may induce various extracellular protein secretions. Thus by using above fermentation method we could reduce the protein impurities obtained with solid natural substrate. Recently Sato et al. [2016] reported phytase production by *R. microsporus var. microsporus* biofilm on different inert support using sugarcane bagasse as carbon source in modified NBRIP media. The production on PUF was found to be 70 U/mg of protein. While In our production study phytase production was 22.5 U/mg of protein. This less specific activity is due to the higher amount of protein in GOC extract. Both the studies shows that biofilm on inert support can be used effectively to produce high levels of extracellular phytase.

3.2 Reuse of PUF

After squeezing enzyme from PUF, it was reused by adding fresh sterilized 25 ml of media. On PUF fungus has been immobilized. The reused PUF was harvested on 3rd day of growth. It was observed that enzyme production decreased by 30% but protein concentration has been increased. Similarly PUF was reused second time. During third cycle production was decreased by 50%. The total production exceeds 4800 U with same 1g PUF in three cycles.

It becomes difficult to press the PUF after that because of heavy growth of biomass. (Table 1) Immobilised PUF has been reported for production of bioactive compounds [Ferrari et al. 2013] and has been recycled up to 12 times for bacterial growth. This semi continuous system can be up-scaled and designed to give continuous production. This technique may assist to

overcome some problems of both types of fermentation, which are e.g. the large amounts of water used in SmF and the difficulties in scaling-up of SSF [Couto & Toca-Herrera 2007].

Table 1 Semi continues fermentation using PUF

	Total Enzyme production (U)	**Total biomass (g)**	**Total protein (mg)**
1st use of PUF	2200	1.21	100
2nd use of PUF	1550	1.56	160
3rd use of PUF	1126	1.93	184
Total production /flask	4876		

3.3 Variation in growth conditions

3.3.1 Effect of PUF concentration

PUF concentration was varied in range of 0.5g, 1 g, 1.5g and 1.75g. It was observed that in 250 ml flask 1g PUF+ 25 ml media is most favourable. In flask liquid media settles down and growth was more on PUF pieces embedded in media. So increase in PUF concentration doesn't lead to increase in production while less concentration of PUF leads to bio film formation on surface only.

3.3.2 Effect of Particle Size of PUF

PUF was cut into pieces of different size, washed and dried. Fermentation media was prepared as mentioned, inoculated and incubated. With increase in size, production increases. PUF size of $1cm^3$ was found to be most ideal for enzyme production. Small size may not allow mycelia bonding and growth and larger size may limit nutrient and mass transfer.

3.3.3 Depth Analysis

In 250 ml flask media concentration was varied so as to change the thickness of media layer. With increasing thickness enzyme production increases but after a limit it decreases. Limiting air and improper media distribution may hamper the enzyme production.

Figure 3 Summarises the optimum conditions for maximum phytase production.

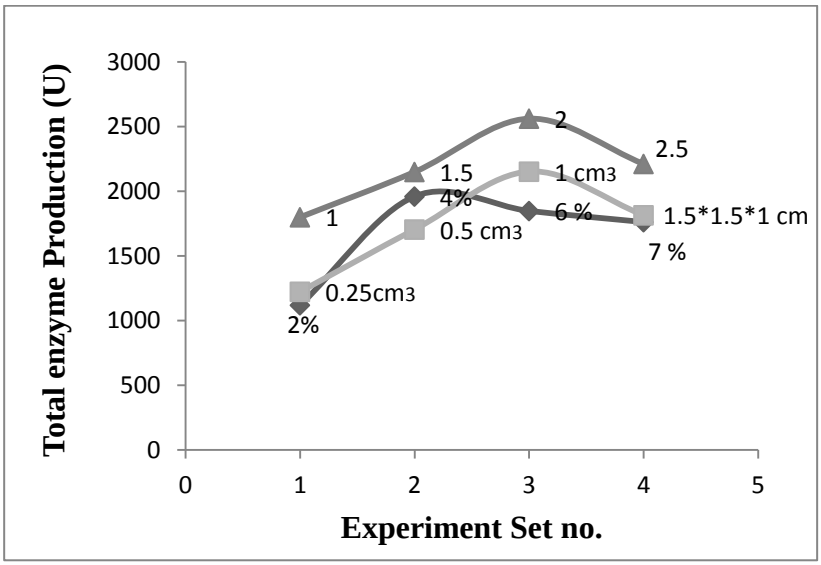

Figure 3 Optimum conditions for phytase production

PUF concentration, PUF Size , Depth of media

3.4 Microscopic observation of growth

Microscopic and SEM examination of the PUF cubes, showed mycelia tangled in the polyurethane network having large spaces filled with air (*Figure 4*). Thus, it seems reasonable to assume that SSF culture on PUF was not as limited for oxygen transfer.

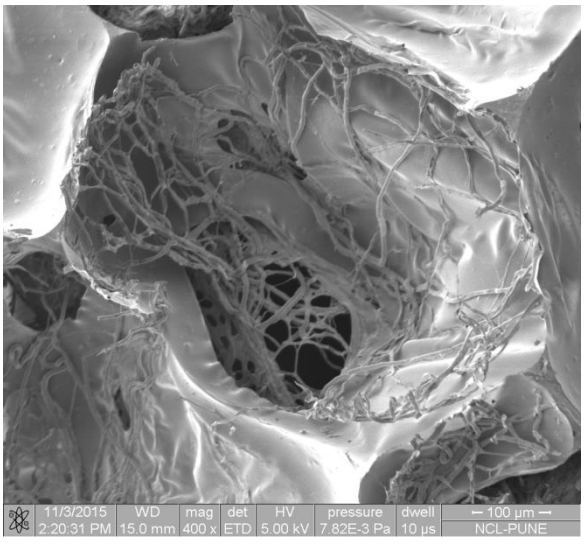

Figure 4 SEM image of fungal mycelia entangled in PUF

3.5 Effect of phytase addition on the phytate content of bread

Phytic acid or phytate analysis of various samples during bread making was done as described in section 2.6.2. Phytic acid content of test dough has been decreased considerably after proofing/fermentation. For whole wheat bread there was a reduction of 91.3% PA while for multigrain bread a reduction of 91.5 % is observed. The added enzyme mainly acts during proofing stage. There is not much difference between PA content of fermented dough and finished bread.(Table 2) Indigenous phytase activity of flour and yeast has also been checked (Data not shown) and it is found to be insignificant. Thus the added phytase is mainly responsible for dephytinization of product.

Table 2 PA analysis during bread making

Bread Types	Phytic Acid Content (mg/g)		% Decrease in PA content in Test
	Control	**Test**	
Whole wheat Bread			
Unfermented dough	7	7	91.2
Fermented dough	5.4	0.65	
Finished bread	5.2	0.61	
Multigrain flour bread			
Unfermented dough	9	9	91.5
Fermented dough	6.2	0.82	
Finished bread	6.2	0.76	
Chapati (Indian flat bread)			
Dough	7.2	7.2	43.05
Dough (30 min)	5.9	4.6	
Finished Chapati	5.6	4.1	

Phytase addition does not much affect the quality of bread. It decreases the width/height ratio of the central bread slices by about 16 %. Therefore, a slight improvement of the loaf shape was obtained in the presence of phytase. In addition, the specific volume index was increased by up to 6 %. Other parameters are constant for control and test finished bread.

In case of chapati, PA content has been decreased by 43 %.This less reduction may be because of less time for enzyme to act as the process of making chapati does not include fermentation and proofing steps.

3.6 Mineral bioavailability

Availability of soluble minerals in finished bread (Zn^{2+}, Ca^{2+}, Mg^{2+}, and Fe^{3+}) was determined by ICP-AES method as described under analytical methods. As compared to control, bread with added phytase shows significantly more soluble mineral content. Soluble mineral content of test sample is 80-90% as compared to 30-40% of bread without added phytase (*Figure 5*). This increase in soluble minerals is interrelated with reduction of phytic acid by phytase addition.

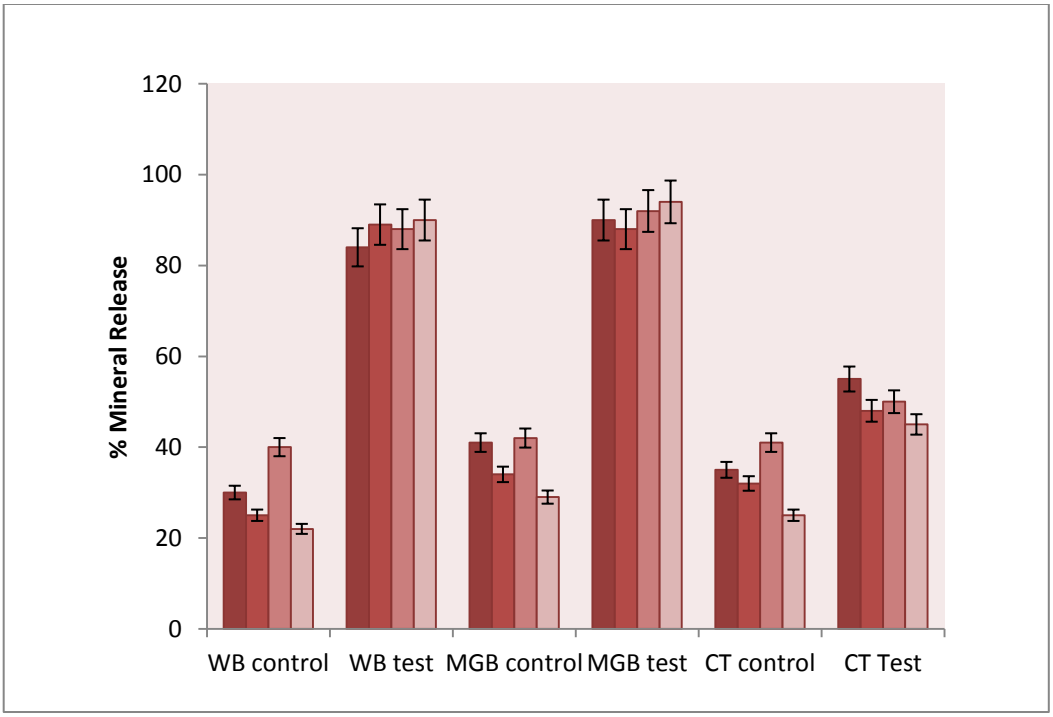

Figure 5 % Mineral release in finished bread samples

Zn^{2+} Ca^{2+} Mg^{2+} Fe^{2+}

WB: Wheat Bread, MGB: Multigrain Bread, CT: Chapati

Conclusion:

This work signifies a low-cost SSF methodology using PUF for higher enzyme production and its application in food industry. PUF present several benefits in the fermentation. It has good productivity; it provides surface support for adsorption of the fungi; the fungi adsorbed on PUF gives a prospect to be reused in successive cycles of fermentation. The semi-continuous SSF process has promising applications for the production of other bioactive compounds also. The phytase enzyme produced using above media was able to reduce phytic acid content and increase the soluble mineral content of bread without affecting the quality of product. This provides a way for nutritional quality improvement for cereal based product.

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Conclusion

The whole study can be summarised as follows:

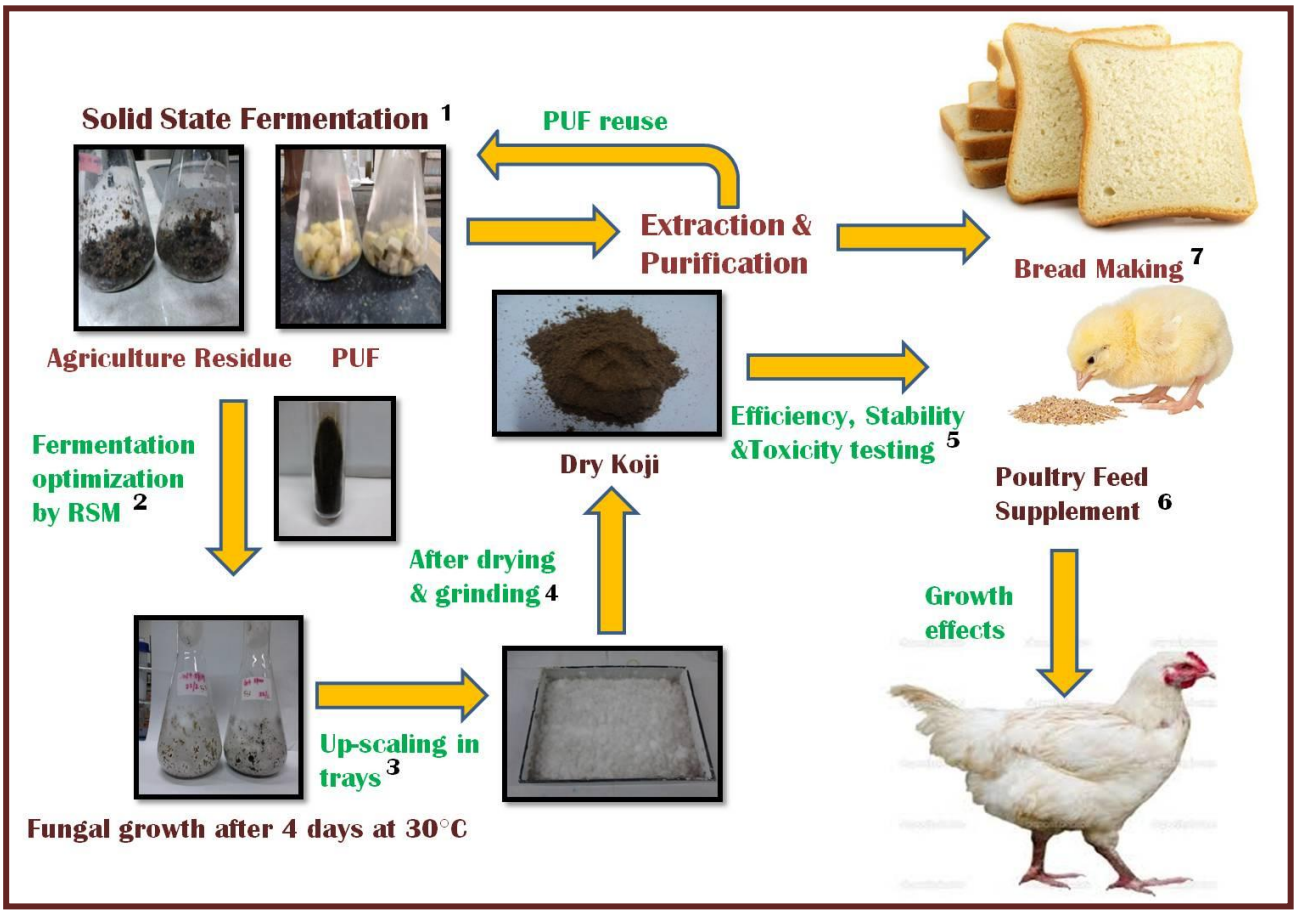

Figure 1 Pictorial summary of whole study

1. Phytase production under solid state fermentation utilizing agriculture residue as well as inert support.

2. Production optimization by statistical methods.

3. Up-scaling to tray level.

4. Preparation of dry koji.

5. Analysis of koji for enzyme stability, efficiency and toxicity.

6. Application of koji as feed mix in mash broiler feed and growth study.

7. Application of enzyme produced on PUF during bread making to increase nutritional value.

Major conclusion from the study:

1. Solid state fermentation was proved to be an economic and sustainable process for phytase production by *A. niger* NCIM 563.

2. Ground nut oilcake as substrate with added optimized nutrients by statistical method gave enhanced yield of enzyme. Higher yield is the basic requirement for any successful commercial application of enzyme.

3. For application purpose there is no need of any extensive downstream processing. Phytase in dried koji was sufficiently active and stable. Moreover it was aflatoxin free. Thus it provides the convenience of direct addition of koji as supplement in poultry feed.

4. Phytase addition in poultry feed has positive effects in P deficient experimental group. Enzyme at 500U/kg was able to replace up to 0.1% P in feed without any adverse growth effects on broiler birds.

5. Phytase production on inert carrier like poly urethane foam gave an alternate technique for cleaner and efficient enzyme production. PUF has advantage of reusability and easier processing to get pure enzyme.

6. When applied during bread making process enzyme was able to reduce phytic acid content of bread hence it proved that our enzyme has potential to improve the nutritional value of grain based food.

Future perspectives:

1. SSF up-scaling: Tray fermentation studies for up scaling gave promising results. However the process can be improved using advanced bioreactors that provide accurate moisture and temperature control, as well as optimized O_2 mass transfer.

Similarly for production on PUF, a bioreactor can be designed for semi-continuous production of enzyme. Fungal mycelia immobilized on PUF can be used repetitively, if provided with fresh media and the enzyme can be squeezed after suitable production time.

2. Poultry feed application: We have done application studies with a small group of birds. In this study our phytase prove to be effective in restoring P deficiency in experimental groups. Moreover the enzyme does not require any intense downstream processing before applying in mash broiler feed. Thus we have provided a simple and cost effective alternative for phytase in feed application.

However before exploiting the enzyme potential in feed, a large level feed trial will be required to optimize the dose and response.

3. Food based application: Phytase has been successfully applied for nutritional enrichment of bread without affecting product quality. Various grain based product including soya products, baby food product can be treated with phytase to make them more digestible and mineral rich.

4. Commercialization: For feed application various enzyme preparations are available in market. Our phytase enzyme production process with minimum down streaming and direct applicability provides a cheap alternative. Enzyme is in solid dry koji powder form which can be directly and easily mixed in mash feed. It has an enormous potential for commercialization.

Although potential of phytase for phytic acid reduction is well reported, it has not been exploited in food industry. There is no commercial phytase available for food application purpose. Several issues have limited wide implementation of phytase in human nutrition. The main issue is the consumer perception of recombinant phytase and the lack of readily available native phytase. Our enzyme has been produced by native strain *Aspergillus niger* which is generally recognized as a safe (GRAS) for food applications.

Research papers published / accepted / communicated for publication:

1. P. Buddhiwant, K. Bhavsar, J. M. Khire [2013] Phytase isozymes from *Aspergillus niger* NCIM 563 under solid state fermentation: Biochemical characterization and their correlation with submerged phytases. **(Published in 'Process Biochemistry' 48 (2013) 1618–1625)**

2. P. Buddhiwant, K. Bhavsar, R. Kumar, J. M. Khire [2015] Phytase production by solid state fermentation of ground nut oilcake by *A niger*: A Bioprocess optimization study for animal feedstock application.**(Published in 'Preparative Biochemistry and Biotechnology' 46 (2016) 531-538)**

3. P. Buddhiwant, M.S. Dharne, J. M. Khire, Phytase production under solid state fermentation utilizing polyurethane foam as inert support and its application in bread fortification. **(Published in 'Advances in Applied Research' 8 (2016) 86-91)**

4. P. Buddhiwant, S. Agawane, A. Kadam, M. S. Dharne, J. M. Khire, Study of efficiency of lab made fungal phytase on broiler growth performance and phosphorus excretion. **(Communicated)**

5. K. Bhavsar, P. Buddhiwant, P. shah, P. Gujar [2013] Phytase isozymes from *A. niger* NCIM 563. **(Published Book chapter Biotechnology Beyond Borders.)**

Posters presented:

- Title: "Phytase isozymes from A. niger NCIM 563 under solid state fermentation."

9th BRSI convention & international conference on industrial biotechnology & Indo-Italian workshop on food biotechnology: industrial processing, safety and health .November 21-23, 2012 At: **Punjabi University, Patiala.**

- Title: "Media engineering for enhanced production of phytase under Solid state fermentation by *Aspergillus niger* NCIM 563."

Indo- Mexican workshop, Biotechnology: Beyond borders. October 7-9, 2013, At: **CSIR-National chemical laboratory, Pune.**

- Title: "Solid state phytase from *Aspergillus niger*: Bioprocess optimization using agro-industrial waste and its application."

55th annual conference of Association of Microbiologist of India. November 12-14, 2014 At: **Tamil Nadu Agricultural University, Coimbatore.**

- Posters presented at CSIR-NCL on science day in the year 2012 & 2014.

CSIR 800 summary:

CSIR 800 program has been launched by CSIR to sensitize the research students with the societal issues. The major objective of the CSIR-800 project is to create and nurture a sense of social consciousness and responsibility by participation in Science & Technology activities relevant to the nation.

By keeping the above objective in mind we have selected our project work related to waste processing in surrounding area. It was entitled as "**Study of SWaCH model for efficient residential solid waste management in NCL colony and improved process for Bio-waste degradation".** Solid waste management (SWM) in NCL colony is managed by SWaCH, an autonomous enterprise that provides front-end waste management services. They collect wastes door to door, segregate in dry and waste and then process it. The wet and biodegradable waste composted by using microbial inoculants. Our objective was:

- ✓ Study of SWaCH model in NCL colony area and its effectiveness.
- ✓ An efficient formulation for faster processing of bio waste.
- ✓ A Survey of awareness among public in different parts of pune regarding waste management.
- ✓ Suggestions and ideas of people for better SWM.

We have prepared a microbial consortia consist of fungal and bacterial cultures from NCIM and analysed its efficiency in composting bio-wastes. It shows good results. Our inoculum was able to degrade waste in short period as compared to commercial product. A quantitative and up-scaled study may further provide a better product for commercialization.

Survey and awareness regarding waste management gave an insight into the problems faced by citizens as well as the waste pickers. We have tried to communicate the problems and also suggested possible solutions. Overall, the project was a great learning experience and awakens our social responsibility.