Studies on phytase from *Williopsis saturnus* NCIM 3298 and its applications

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By

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CERTIFICATE

This is to certify that the work in the Ph.D. thesis entitled **"Studies on phytase from *Williopsis saturnus* NCIM 3298 and its applications"** submitted by **Ms. Anupama Pable** was carried out by the candidate under my supervision at the NCIM Resource Center, CSIR-National Chemical Laboratory, Pune, India. The material obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION

I hereby declare that the thesis entitled **"Studies on phytase from *Williopsis saturnus* NCIM 3298 and its applications"** submitted for Ph.D. degree to the Savitribai Phule Pune University has been carried out at CSIR-National Chemical Laboratory, Pune 411008, India. This work is original and has not been submitted in part or full by me for any degree or diploma to any other university. Materials obtained from other sources have been duly acknowledged in the thesis. The results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

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Dedicated
To the memory of my father
to my mother
and
lovely husband and daughter Veena

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Anupama

Abbreviations

A°	Angstrom
ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
AL	Alkaline phytase
ATP	Adenosine triphosphate
BBD	Box Behnken Design
BPP	β propeller phytase
CAP	Cysteine Acid Phosphatase
°C	Degree celsius
CM	Carboxymethyl
CP	Chickpea
DEAE	Diethylaminoethyl cellulose
DTT	Diethriotol
DWB	Dry weight biomass
EDTA	Ethylene diaminetetraacetic acid
FDA	Food and Drug Administration
g, mg, µg, ng	gram, milligram, microgram, nanogram
GRAS	Generally regarded as safe
HAP	Histidine acid phosphatase
HPLC	High-performance liquid chromatography
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	Kilo dalton
K_m	Michaelis constant
L, mL, µL	Liter, milliliter, microliter
LMM	Liquid minimal medium
M, mM, µM	molar, millimolar, micromolar
MGYP	Malt extract-glucose-yeast extract-peptone medium
MW	Molecular Weight
NADP	Nicotinamide adenine dinucleotide phosphate

NCBI	National Center for Biotechnology Information
NCIM	National Collection of Industrial Microorganisms
OVAT	One Variable Approach
P	Phosphorus
Pi	Inorganic phosphorus
PA	Phytic acid
PAP	Purple acid phosphatases
PBD	Placket Burman Design
pI	Isoelectric point
PMSF	Phenylmethyl-sulphonilfluoride
PSM	Phytase screening medium
R^2	Coefficient of Determination
rpm	Revolutions per minute
RSM	Response surface methodology
RT	Room temperature
SmF	Submerged fermentation
SSF	Solid state fermentation
UV	Ultra violet

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Thesis Abstract

Phytic acid (PA) (*myo*-inositol 1,2,3,4,5,6 hexakisphosphate) is the principle storage form of phosphorus comprising 1-5% by weight in cereals, legumes, oil seeds and nuts. It is primarily present as a salt of monovalent and divalent cations (Fe^{2+}, Mn^{2+}, K^{+}, Mg^{2+} and Ca^{2+}). Phytic acid exists as a highly negatively charged ion over a broad pH range and therefore has a tremendous affinity for food components with positive charge(s), such as minerals, trace elements and proteins. It is considered an anti-nutrient because it acts as a strong chelator of minerals such as Mn^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}/Fe^{3+}. The formation of insoluble mineral-phytic acid complexes at physiological pH is regarded as the main reason for the poor mineral bioavailability, essentially because these complexes are difficult to assimilate in the animal and human gastrointestinal tract. Also, phytic acid binds to proteins and makes them more resistant to proteolytic digestion.

Monogastric animals are unable to hydrolyze the phytic acid because they lack phytate degrading enzymes. Therefore, inorganic phosphorus is supplemented in the diets for poultry and piggery to meet their nutritional requirement for phosphorus. After digestion, unhydrolyzed phytate of the feed is released in the nearer water bodies through animal excreta. Ultimately it leads to the eutrophication of the water bodies in the areas of intensive animal agriculture. Therefore, the degradation of the phytate in feeds is of nutritional and environmental importance in order to increase the bioavailability of essential dietary minerals and to decrease environmental pollution, respectively.

Phytase (EC 3.1.3.8) (myo-inositol hexakisphosphatephosphohydrolase) catalyzes the hydrolysis of PA to inorganic monophosphate and lower phosphoric

esters of myo-inositol, in some cases to free myo-inositols. Many organisms, such as fungi, yeasts and bacteria are able to produce the phytase. Several reports have shown that supplementation of phytase in animal feed increases the availability of phosphorus for animal digestion through degradation of the PA. Many fungal phytases are commercially available and used in the poultry and swine feeds to increase bioavailability of phytate bound phosphorus and essential minerals. The great potential for use of phytase in processing and manufacturing of food for human consumption has been proposed, but it has not been applied to date. Research in this field has focused on obtaining the appropriate phytase producing microbial strain for improvement of nutritional value of plant based foods.

There are very few reports on yeast phytases due to lack of cost effective production and low expression levels. Most of the yeasts are non-pathogenic and non-toxic for animals and human consumption. Moreover, yeasts are a rich source of proteins and vitamins and hence whole cell application of yeast as a phytase source in poultry and swine feeds will sustainably improve productivity of the livestock by increasing the availability of the phosphorus and minerals through dephytinization. Hence, there is a need to identify yeasts with higher production and explore other applications of phytases.

This study entitled "**Studies on phytase from *Williopsis saturnus* NCIM 3298 and its applications**" was designed to study the production and characterization of phytase from yeast under submerged fermentation condition and its applications to improve the nutritional value of the feed ingredients.

Screening of yeasts from NCIM culture collection for phytase activity

This chapter discusses about the screening of yeasts from NCIM culture collection for phytase production and selection of high phytase producing yeast for further study.

Screening method was used to reveal the ability of different yeast strains to utilize PA as a sole phosphorus source. Two minimal media, phytase screening medium (PSM) and liquid minimal medium (LMM) were used to investigate the ability of the yeasts to grow on media with PA as the sole phosphorus source. Among 600 screened yeast strains 40 showed a zone of clearance on PSM. Of the 40 strains only 11 grew significantly in LMM. Five yeast strains (*Zygosaccharomyces bisporus* NCIM 3265 and 3296, *Williopsis saturnus* NCIM 3298, *Zygosporium priorionus* NCIM 3299 and *Schizosaccharomyces octosporus* NCIM 3297) showed comparable growth with *S. cereviciase. W. saturnus* NCIM 3298 was selected for further study due to its strong growth in screening medium and high phytase production in Na-phytate containing minimal medium.

Production of phytase by *W. saturnus* NCIM 3298 and its characterization

This chapter discusses high level phytase production by *W. saturnus* NCIM 3298 using one variable approach and an integrated statistical optimization approach involving the combination of Plackett Burman design (PBD) and Box-Behnken design (BBD). Key media components which influenced the phytase production were identified by applying PBD two factorial design. Four statistically significant factors, namely, citric acid, $FeSO_4$, pH and glucose, identified by PBD were further selected for optimization study using BBD. After media optimization by PBD and BBD, phytase production improved from 46 IU/g DWB to 297 IU/g DWB. This indicating that there is about 6.45 fold increase in phytase production after media optimization. It was found that citric acid is an important factor in the medium and maintains the phytase production at higher side even after 24 hrs of incubation by controlling the pH of the medium.

Cell bound phytase possessed an optimal pH of 5.5 and an optimal temperature of 60 ^{o}C. *Km* value for cell bound phytase is 0.2 mM. Phytase activity was moderately stimulated in presence of 1mM Co^{2+}, Ba^{2+}, Ca^{2+} and 10 mM K^{+}, Na^{+}, $Mg^{2+.}$ It is strongly inhibited in presence of 10 mM Hg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Ba^{2+}. Localization studies were carried out by earlier reported methods. Yeast cells were subjected to physical and chemical permeabilization by using freeze-thaw and detergents, respectively. Extracellular phytase activity was not detected after, freeze-thaw and detergents, permeabilization treatment. Enzymatic hydrolysis of the yeast cell wall released phytase in the lysis buffer. Therefore, the results of localization of enzyme experiment conclude that the studied yeast phytase is cell wall bound.

Applications of *W. saturnus* NCIM 3298 phytase to improve the nutritional value of feed

Cereal grains and their by-products are excellent source of starch, proteins and minerals such as P, Fe, Mg, Ca, and Zn and thus constitute a bulk of the livestock feed. Generally used cereals grains in poultry feed are soybean, sorghum, maize, wheat, rice, bajara and chickpea. Although the starch in cereals is highly digestible, most of the other nutrients such as minerals, proteins are not easily digested and absorbed because of anti-nutritional factors, mainly PA, present in the cereals. Thus supplementation of phytase in feed is required to increase the productivity of livestock. Effect of phytase was checked on chickpea, feed source of poultry, to increase the bioavailability of phosphorus and other minerals (Fe^{2+}, Ca^{2+}, and Zn^{2+}). Two fold increases in phosphorus and 80% decrease in phytate content were seen after treatment with phytase. Minerals availability of chickpea determined by Inductively Coupled Atomic Emission Spectroscopy (ICP-AES) was increased in

phytase treated chickpea as compared to control by 28%, 32% and 39% for Zn^{2+}, Fe^{2+} and Ca^{2+} respectively.

Effect of phytase on phosphorus liberation from soybean, maize, sorghum, rice and wheat was checked. Phytase treatment showed about 2.6, 2.5, 2.0, 2.3 and 1.3 fold increase in phosphorus content for soybean, maize, sorghum, rice and wheat respectively, as compare to control. This indicates that studied yeast phytase has a potential application in feed industry.

Summary and future directions

Phytase is one of the important enzymes in the animal feed and it is the most widely used feed enzyme in the world, included in ~90% of poultry - and ~70% of pig diets. Addition of phytase in the feed improves nutritional value of the feed ingredients. Yeast is a rich source of proteins and vitamins; also it is safe for animal consumption. Therefore, application of whole cell yeast as phytase source in the feed will significantly increase the nutritional quality of the feed. In present study, various yeasts from NCIM culture collection were screened for phytase production. After screening, *W. saturnus* NCIM 3298 was selected for studies based on its phytase production ability. Phytase production of *W. saturnus* NCIM 3298 was optimized using PBD and BBD methods of media optimization. Phytase from studied yeast was found very efficient in releasing the phytate bound phosphorus from soybean, maize, chickpea, sorghum, rice and wheat.

In future, phytase from *W. saturnus* NCIM 3298 can be evaluated for its effect on the growth and productivity of livestock. Further it can be used to decrease anti-nutrient effects of the human food.

CHAPTER 1

Introduction

Phosphorus (P) is an essential component and an energy conduit of life despite that it does not have a cycle to constantly replenish its supply. It plays an important role in energy metabolism, metabolic regulation, and signal transduction pathways. Furthermore, P is a vital mineral that has most important function in bone and tissue formation in poultry birds. Poultry feeds are generally based on plants, especially cereals and pulses, which are a rich source of carbohydrates, proteins, fats and essential minerals. A small proportion of the total P in cereals and pulses is inorganic phosphates (Pi) while phytate P is the most predominant organic form. About 70-80% of the total P in the seed is present in organic form, phytic acid (PA). Remaining 20-30% of Pi is only available for consumption, which represent one third of the total P value. Monogastric animals, especially poultry birds and swine are unable to utilize phytate bound P because they lack the enzyme that can hydrolyze the PA and release the bound P. Thus, these animals able to utilize only one third of the total P present as free inorganic form.

PA exists as a highly negatively charged ion over a broad pH range because p*Ka* values (total 12) of PA are in the range of 1.8 to 9.7 and six of them are at 1.87 [1]. Hence, PA has a tremendous affinity for food components with positive charge(s) and under normal physiological conditions it chelates essential minerals such as calcium, magnesium, iron and zinc. Furthermore, it also has an affinity towards positively charged amino acids, proteins and other biomolecules. PA present in a vegetarian diet is responsible for iron and zinc deficiency and ultimately leads to

anaemia [2, 3]. PA is therefore called as anti-nutrient factor that present in plant derived feeds and foods.

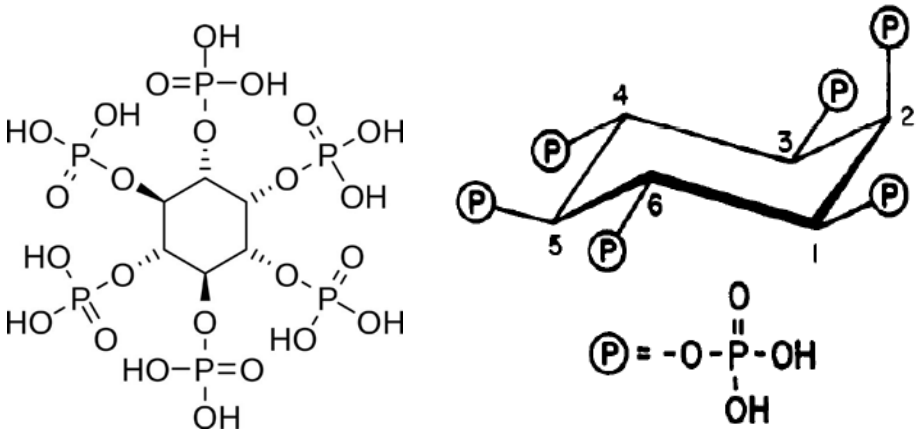
Poultry and piggery feed is supplemented with dicalcium phosphate, inorganic P source, so as to fulfil the P requirement of these animals. This supplemental inorganic P however, does not reduce the antinutritive effect of PA. Excretion of PA and excess inorganic P can lead to accumulation of P in soil and water bodies and subsequently to eutrophication of fresh water streams. P is non-renewable and at the current extraction and usage rate, the existing phosphate reserves will be exhausted in next 80 years.

1 Phytic acid

PA, a cyclic compound, was discovered by Posternak in 1903. The chemical description of the PA is *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (IUPAC-IUB, 1977) as it is derivative of *myo*-inositol and has six phosphate groups that are not internally connected [4]. PA is called as phytate when phosphate groups of it interact with positively charge metals and form a salt. It is primarily present as a salt of the potassium, magnesium and calcium. Phytate is ubiquitous and also a principle storage form of P in plant seeds and grains, comprising 0.5-5% (w/w) [5]. Phytate is normally found in the outer (aleuron) layers of cereal grains and in the endosperm of the legumes and oil seeds. Molecular weight and molecular formula of PA are 660 and $C_6H_{18}O_{24}P_6$, respectively.

1.1 Structure of phytic acid

Structural conformation of PA was studied by Johnson and Tate using 31PNMR [6]. They suggested the orientation of six P on *myo*-inositol ring; the phosphate at 2-position is in axial position and the other phosphates are in an equatorial position [6]. Studies of Costello *et al.* [7] supports the structural conformation suggested by Johnson and Tate [4]. The most favourable conformation of PA is shown in Fig. 1. Furthermore, Costello and co-workers [8] determined p*Ka* values for dissociating groups of PA using 31P-NMR and pH titration methods. They concluded that six groups have p*Ka* values in the strong acidic range (p*Ka* 1.1 to 2.1), one in the weak acidic range (p*Ka* 5.70), two with p*Ka* 6.80 to 7.60, and three in the very weak acidic range (p*Ka* 10.0 to 12.0). This suggests that PA has a high potential to form strong complexes with the multivalent cations and positively charged proteins because it exists as a strong negatively charged molecule over a wide pH range.



(a) **(b)**

Figure 1: Structure of phytic acid.

(a) Primary structure of *myo*-inositolhexakisphosphate

(b) Energetically most favourable confirmation of PA [Depicted from Graf [9]]

1.2 Occurrence

The total P and Phytate-P content of the common poultry feed are presented in Table 1. Phytate has been found in cereal grains and legumes up to a level of approximately 1% to 5% by weight. PA occurs mainly as salts of mono- and divalent cations, potassium magnesium salt in rice and calcium-magnesium-potassium salt in soybeans, in separate regions of cereals and legumes. It accumulates in seeds and grains during ripening along with other storage substances such as starch and lipids. In cereals and legumes PA accumulates in the aleurone particles and globoid crystals, respectively [1]. PA is deposited in seeds during the seed development and its concentration changes as the kernels mature, reaching a maximum when kernels are ripe [10]. Apart from cereals and legumes, phytate has also been found in potatoes [11], crested wheatgrass [12], *Wolffiella floridana* [13], Bengal gram seed and Black gram [14].

1.3 Role of phytic acid

Many physiological roles have been suggested for PA in seeds and grains, which include PA as an energy store, as a source of cations and P, as a source of *myo*-inositol (a cell wall precursor), and initiation of dormancy. Graf and Eaton [15] reported the antioxidant property of PA, which was based on the assumption that PA effectively blocks iron-driven hydroxyl radical formation. PA could prevent the coronary heart diseases. This assumption was based on the studies explaining the role of phytate, which may influence the aetiology of heart [16] and significantly lowered serum cholesterol and triglyceride levels in animals [17]. Numerous studies have reported that PA as an anti-cancer agent [18-21] and anti-oxidant agent [15, 22, 23].

Table 1. Total P and phytate P of common poultry feedstuffs [Depicted from Tyagi *et al.* [24]]

Ingredient	Total P (%)	Phytate P (%)	% of total P
Cereals/Millets			
Maize	0.39	0.25	64
Rice	0.15	0.09	60
Wheat	0.44	0.27	61
Sorghum	0.30	0.22	73
Barley	0.33	0.20	61
Bajara	0.31	0.23	74
Oilseed meals			
Groundnut meals	0.60	0.46	77
Soybean meal	0.88	0.56	64
Cotton seed meal	0.93	0.78	82
Sunflower meal	0.90	0.45	51

1.4 Negative aspects of phytate

PA has been known as an anti-nutrient because of its molecular and chemical structure. At physiological pH, PA carries an overall negative charge as it dissociates 9 out of 12 protons. Therefore, it has a strong affinity for positively charged cations and it effectively binds different mono-, di-, and trivalent cations and their mixtures, forming insoluble complexes. These insoluble complexes are unavailable for

absorption and thus cause the mineral deficiency. The order of the ability of the mineral cations to form complexes with phytate *in vitro* has been reported as Cu^{2+} > Zn^{2+} > Cd^{2+} at pH 3–7 [25]. Among all minerals, bioavailability of Zn^{2+} is most influenced by PA in human and animals [26, 27].

Due to the negative charge on the PA at physiological pH, it forms very strong complexes with proteins and resists their hydrolysis by proteolytic enzymes [3]. Phytate-protein interactions are pH dependent. At a pH value lower than the isoelectric point of the protein, a cationic group of the basic amino acids, arginine, histidine and lysine bind to the phosphate groups of the phytate and form binary protein–phytate insoluble complexes [28]. These insoluble complexes dissolve only below pH 3.5. Such complex formation may affect the protein structures that can hamper enzymatic activity, protein solubility and protein digestibility. It has also been reported that protein-phytate complexes are less likely to be digested by proteolytic enzymes [29]. In addition to diet proteins, PA also interacts with important digestive enzymes such as trypsin, pepsin, α-amylase and β-galactosidase, resulting in a decrease in the metabolic activity [30-32].

Rickard and Thompson reported that phytate may bind with starch either directly, via hydrogen bonds, or indirectly via proteins associated with starch [33]. Further, they have also mentioned that the phytate-carbohydrate complex reduces the solubility and hence digestion of the carbohydrates. The effect of PA on starch digestibility was studied *in vitro* and related it to the blood glucose response (glycemic index) by Yoon *et al.* [34]. In their study, they observed that at physiological pH and temperature, rate of starch hydrolysis was reduced by 50% in the presence of Na-phytate.

Phytate forms 'lipophytins' (complexes with lipid and its derivatives), along with other nutrients. It is reported that dietary phytate or inositol phosphate ester fractions inhibit the *in vitro* activity of lipase [35]. Furthermore, lipid digestibility, blood lipid concentration and hepatic fatty acid synthesis studied in mice or rats were also affected by the dietary phytate and its derivatives [36, 37].

Interaction of PA with minerals, proteins and starch is shown in Fig. 2.

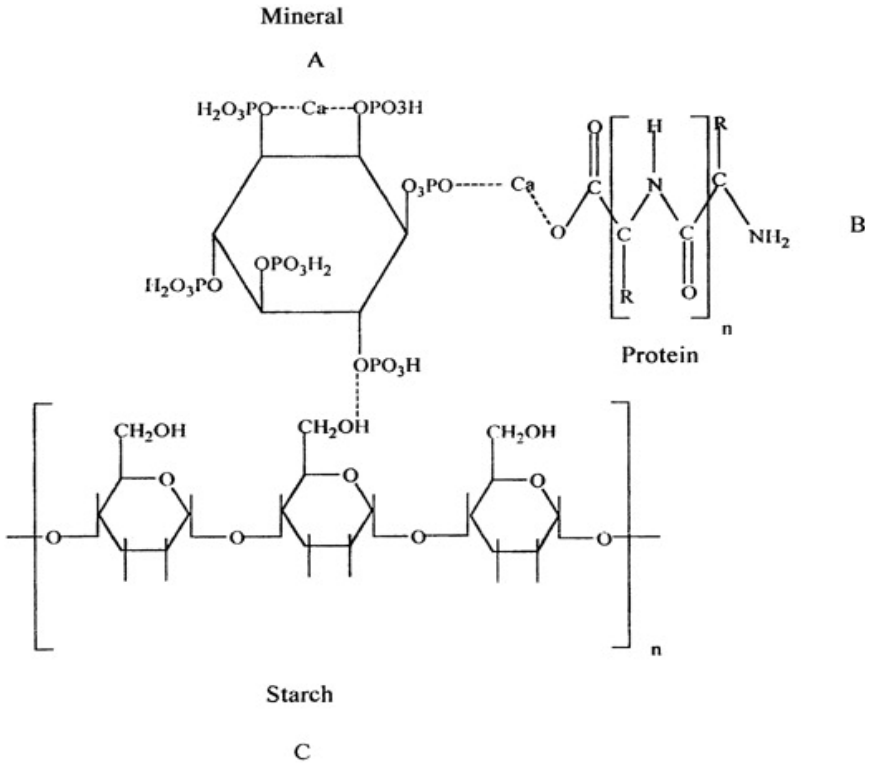


Figure 2. Interaction of PA with minerals (A), proteins (B) and starch(C) [Depicted from Oatway *et al.* [38]]

2 Degradation of PA

The hydrolysis of PA is prerequisite for improving the nutritional value because the removal of P groups from the inositol ring decreases the mineral binding strength of phytate. Moreover, this results in increase bioavailability of essential dietary minerals

and preservation of environmental recourses. PA has been hydrolysed by dehulling, soaking, cooking, germination, autoclaving methods [39, 40]. Hydrolysis of PA by autoclaving decreases the nutritional value of the feed. Though germination is good way to get rid of the PA, but % decrease in PA was not enough to inhibit its anti-nutrient effect [40].

2.1 Phytase

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) catalyzes the sequential hydrolysis of *myo*-inositol hexakisphosphate (PA) to inorganic monophosphate and lower *myo*-inositol phosphates, and in some cases to free *myo*-inositol (Fig. 3). It is categorized as a phosphoric monoester hydrolase. Ever since discovery of first phytase in rice bran by Suzuki *et al.* [41] in 1907, many different types of phytases have been identified and described from microorganisms and plants.

Hydrolysis of phytate into *myo*-inositol and inorganic P and release of metal ions is shown in Fig. 3.

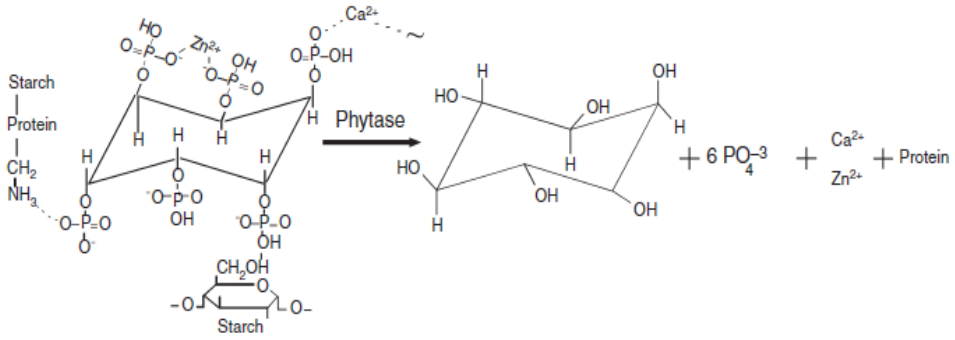


Figure 3. Hydrolysis of phytate by phytase into inositol, phosphate and other divalent elements. The removal of phosphate groups by phytase results in the release of metals, metal-binding enzymes and proteins. [Adapted from Yao *et al.* [42]]

2.2 Classification of phytase

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) in consultation with the Joint Commission on Biochemical Nomenclature (JCBN) classified the phytases in three classes based on the position (3, 6 or 5) on the inositol ring where the dephosphorylation is initiated (Fig. 4.)

1) **3-phytases** (EC 3.1.3.8) catalyze the hydrolysis of PA to yield 1, 2, 4, 5, 6-pentakisphosphate and does not always completely dephosphorylate PA. This type of phytase is produced by fungi and bacteria except *E. coli*, which produces 6-phytase [43].
2) **6-phytases** (EC 3.1.3.26) give 1,2,3,4,5- pentakisphosphate as the first product along with Pi. This class of enzymes hydrolyze PA completely. They are present in grains, oil seeds and higher plants except soybean, which has 3-phytase [44].

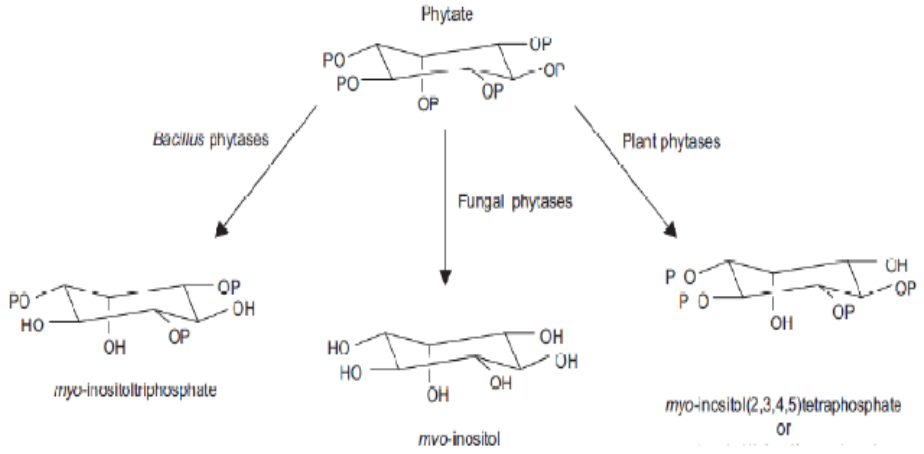


Figure 4. Schematic diagram showing end products resulting from phytate hydrolysis mediated by different phytases

3) **5-phytases-** (EC 3.1.3.72) generally occur in *Medicago sativa*, *Phaseolus vulgaris*, and *Pisum sativum*. Hydrolysis of phytate initiate at the fifth phosphate group.

Based on pH optima of the enzyme activity, phytases are classified into two major classes, Acidic and Alkaline phosphatases (Fig. 5). Most of the studies have focused on acidic phytases with high specific activity for the preferred substrate, PA, because this type phytase would sustain and function effectively in the digestive tract of monogastric animals. Acidic phosphatases are further categorised into three classes viz., histidine acid phosphatases (HAP), Cysteine acid phosphatases (CAP), and purple acid phosphatases (PAP).

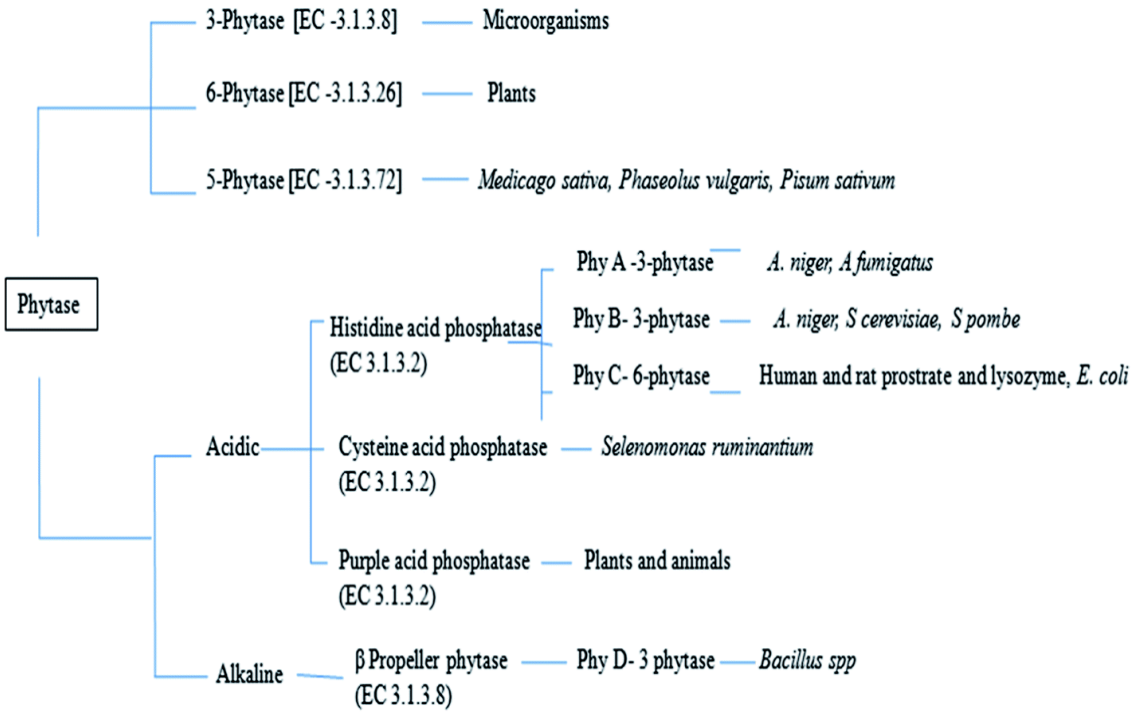


Figure 5. Classification of phytases [Adapted from Bhavsar and Khire [45].

1) Acid phosphatases:

A. Histidine acid phosphatases (HAP): Phytases belonging to this class are the most widely studied and utilized today. Representatives of this large class of enzymes are known to occur in animals, plants and microorganisms [46]. This class shows broad substrate specificity and hydrolyzes metal-free phytate at the acidic pH range and produces *myo*-inositol monophosphate as the final product. All members of HAPs share a conserved active site motif, RHGXRXP, unique to this enzyme class [47]. Most reported HAPs are identified in *Enterobacteriaceae* and *Aspergillus* group. HAPs from bacteria are superior in substrate specificity, resistance to proteolysis, and catalytic efficiency. The term Histidine Acid Phytase (HAPhy) has been advanced to designate the HAPs that can accommodate phytate as a substrate [48].

B. Cysteine acid phosphatases (CAP): This type of phytase reported in an anaerobic ruminal bacterium, *Selenomonas ruminantium* [49].

C. Purple acid phosphatases (PAP): These enzymes have been identified and characterized from numerous plants, animal and some bacteria. All members of this group contain a characteristic set of seven amino-acid residues involved in metal ligation [50].

2) Alkaline phytase (AP):

Alkaline phosphatases are a group of enzymes with low substrate specificity that catalyze the hydrolysis of phosphate esters at an alkaline pH. APs occur widely in nature, and are found in microorganisms, animals. Most of the APs are homodimeric enzymes and each catalytic site contains three metal ions, i.e., two Zn and one Mg, necessary for enzymatic activity [51].

2.3 Occurrence

Phytases are produced in a wide range of plant, microorganisms and animal tissues. Microorganisms show either intracellular or extracellular phytase production.

2.3.1 Microbial sources

Phytases have been reported in fungi, yeast and bacterial strains. Shieh and Ware [52] screened over 2000 soil microbial isolates for phytase production, most of them were intracellular phytase producer while only 30 showed extracellular phytase activity and those were all fungal strains. Other studies [53-55] confirmed *A. niger* strains to be the best producers of extracellular phytase. Other fungal strains, *Penicillum, Mucor, Sporotrichum thermophile, Aspergillus fumigatus* have been reported for phytase production.

Phytase has also been detected in various bacteria, e.g. *Aerobacter aerogenes* [56], *Pseudomonas* sp. [57], *Bacillus subtilis* [58], *Klebsiella* sp. [59], *B. subtilis* (natto) [60], *Escherichia coli [61]*, *Enterobacter* sp. 4 [62] and *Bacillus* sp. DS 11 (later designated as *B. amyloliquefaciens*) [58]. The only bacteria producing extracellular phytase are those of the genera *Bacillus* and *Enterobacter*. *E. coli* phytase is a periplasmic enzyme.

Yeasts were reported for intracellular and extracellular phytase production. Extracellular phytase production was observed in *Arxula adeninivorans* [63], *Schwanniomyces castellii* [64], *Pichia spartinae* [65], and *P. rhodanensis* [65]. Cell bound phytase activity was reported in *Pichia anomala* [66], *Candida krusei* WZ-001 [67], *Saccharomyces cerevisiae* [68], *Rhodotorula gracilis* [69], etc.

2.3.2 Plant sources

Phytases have been reported in rice, wheat, soybeans, maize, corn seeds, mung beans, fababean, rye, and other legumes and oil seeds. PA is a principal storage form of P in seed and pollen. Germination of seeds and pollens requires immobilization of phytate bound P, which is carried out by indigenous plant phytase. Saxena reported a significant amount of phytase activity associated with the roots of both monocot and dicot plants [70]. This suggests that phytase is secreted from plant roots as a mechanism of the mineralization of P from organic P source from the soil.

2.3.3 Animal sources

As compared to phytases of the bacteria and fungi, very little research of animal phytases has been undertaken. First evidence on animal phytase was given by Patwardhan that showed the phytase activity in the intestinal preparation of rat [71]. Thereafter, many authors reported the presence of phytase activity in the intestine of cow and pig [72, 73]. Bitar and Reinhold reported the phytase activity in the rat, human and chicken intestine [74], however, intestinal phytase did not appear to be of a great significance in rats [75].The normal human small intestine has a limited ability to digest undegraded phytate [76] but dietary phytase may be an important factor in phytate hydrolysis.

2.4 Production of phytase

2.4.1 Screening

Among the all phytase producing sources, microbes are the choice of the phytase producer because of ease of handling and high yield. Several bacteria, yeasts and fungi have been screened for phytase activity using different screening methods.

Howson and Davis reported that microorganisms expressing phytases show zones of clearing on agar media containing sodium or calcium phytate [77]. However, it has been revealed that the halo size of the zone is not quantitatively correlated with the amount of phytase activity [65, 78]. Therefore, confirmatory assays using both solid and liquid media are mandatory. Over 200 fungal strains were screened for phytase production using a two-step procedure [79]. Sano *et al.* screened about 1200 yeast strains for their efficiency to grow on phytate as a sole source of carbon and phosphate [63]. The vast majority of the strains were unable to grow, however, *Arxula adeninivorans* showed a particularly vigorous growth. This capacity was correlated with the presence of a high activity of secreted phytase. About 122 yeast strains from 62 species were screened for phytase activity using a microtiter plate with the PA as a sole P source [80]. Large differences in both extra- and intracellular phytase activities were observed among species. Strain-specific extracellular phytase activities were detected in *P. anomala*. Yanke *et al.* investigated the presence of phytase activity in 344 strains of obligately anaerobic ruminal bacteria [81]. Measurable activities were demonstrated in strains of *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella ruminicola*, *Mitsuokella multiacidus* and *Treponema* spp.

2.4.2 Production studies

Phytase production studies were carried out using submerged fermentation (SmF) and solid state fermentation (SSF) techniques. SmF has mostly been employed as the production technology [55, 82, 83] on a commercial level, however, there are very few reports on the use of SSF for phytase production [84, 85]. Recently SFF has gained much interest for the production of phytase. Several bacterial strains (wild or genetically modified) such as *Lactobacillus* [86], *E. coli* [43], *B. subtilis* [87], *B.*

amyloliquefaciens [58], *Klebsiella sp*. [59], etc., have been used for phytase production under SmF. Most of the reported bacteria produce intracellular or cell bound phytases with the exception of *Bacillus* and *Enterobacter* genera that produce extracellular phytases and *E.coli* who produces periplasmic phytase. The available phytase preparations used as feed additives are of fungal origin, produced by recombinant strains under submerged fermentation condition. Several investigators have optimized the nutritional and physical parameters to obtain maximum production of yeast phytases. Sano *et al.* reported the extracellular phytase production from *Arxula adeninivora* [63]. Cell bound phytase production and characterization has been studied in *Candida krusei* WZ-001 [67]. Many studies reported that phytase synthesis repressed by the presence of phosphate in the medium. Cell bound phytase production from *P. anomala* has also been extensively studied using statistical media optimization methods [82, 88, 89]. Phytase production have been tried using different substrates which includes wheat bran, rice bran, cane molasses, soybean whey and meal and canola oil cake.

The culture conditions, type of strain, nature of the substrate and availability of nutrients should be taken into consideration in selecting a particular production technique, as they are the critical factors affecting the yield. Phytase production has been improved by using different techniques such as mutagenesis and strain improvement, statistical media optimization approach using RSM, etc. Very few reports are present on amelioration of phytase activity using mutagenesis. Chelius and Wodzinski [90] isolated a phytate hydrolysing mutant with 3.3-fold higher phytase (phyA) than the wild type strain during the strain improvement studies of *A. niger* NRRL 3135 by UV radiation. Phytase production in mutant strain was highly repressed by 0.006% (w/v) Pi. Rodriguez *et al.* [91] improved the catalytic efficiency

and thermostability of *E. coli* pH 2.5 acid phosphatase/phytase expressed in *P. pastoris* by site-directed mutagenesis. Bhavsar *et al.* used combinatorial approach of statistical optimization and mutagenesis for improved production of acidic phytase by *A. niger* NCIM 563. About 6 fold increase in phytase production was observed after this study [92].

To get economically feasible level phytases production, which is not observed in wild-type organisms, cloning and expression of phytase genes in suitable host organisms is necessary in order to reach higher productivities. Different heterologous expression systems and hosts, plants, bacteria, and fungi including yeast, have been evaluated. As expected, each system bears some unique advantages, along with certain limitations. Since most of the phosphate in plants is present in the form of PA, heterologous phytase production in crop plants will reduce phosphate load on agricultural ecosystems besides improving phosphate bioavailability [91]. The *A. niger* phyA gene has been successfully expressed in tobacco seeds [93] or leaves [94] and soybean cells [95]. However, thermostability and phytase performance under adverse conditions are still a major concern. Phytase from *B. subtilis* has been introduced into the cytoplasm of tobacco cells that resulted in an equilibrium shift of inositol biosynthesis pathway, thereby making more phosphate available for primary metabolism [96]. Han *et al.* expressed *A. niger* phytase gene (*phyA*) in *S. cerevisiae* to determine the effects of glycosylation on the phytase's activity and thermostability. They observed that expressed phytase in *S. cerevisiae* had more thermostability and molecular weight as compared to the commercial enzyme [97]. Han *et al.* also expressed *A. niger* NRRL 3135 *phy*A in two strains of *P. pastoris* and found high level phytase activity in both hosts [98].

2.4.3 Effect of phosphorus concentration

In general, phytase production depends on medium P contents. A high level of Pi inhibits phytase production while low level result in their expression. Han *et al.* reported that high P content (more than 10 mg Pi/100 g substrate) in the medium inhibits phytase production. However, the degree of P inhibition was less apparent in semisolid medium than in liquid medium for *Aspergillus ficuum* phytase [99]. Quan *et al.* obtained maximum phytase production in a medium containing 0.5 mg Pi/100 mL while 5 mg Pi/100 mL phosphate concentration in the medium inhibited the phytase activity [100]. However, Lan *et al.* [101] found that phytase production by *M. jalaludinii* neither inhibited nor stimulated after phosphate supplementation (0.05-0.5%) to the production medium, consisting of rice bran-soybean milk, whereas Fredrikson *et al.* [78] observed that the repression of phytase synthesis by Pi was less significant in a complex medium. Vohra and Satyanarayana reported that phytase activity of *P. anomala* was not inhibited in the presence of Pi up to 10 mM [66].

2.4.4 Effect of medium ingredients and inoculum

Sano *et al.* [63] found that when glucose was replaced by galactose, *A. adeninivorans* secreted high levels of phytase into the culture medium. *S. castellii* CBS 2863 found to be secreted high level of phytase at high pH (above 6) and dilution rate in continuous culture conditions [102]. Phytase production by *S. thermophile* has been increase in the presence of Tweens (Tween-20, 40 and 80) and sodium oleate in the medium [103]. Similarly, Mandviwala and Khire reported a 30% increase in phytase activity of *A. niger* NCIM 563 when 0.5% Triton-X-100 was added to the production medium [104]. A source of carbon and nitrogen in the medium affects the phytase production. Vohra and Satyanarayana found that the inoculum density to be an

insignificant variable in phytase production during media optimization using RSM [88]. Roopesh *et al.* observed that phytase synthesis increased as inoculum size increases to 1 mL. Higher concentrations of inoculum, however, showed inhibitory effects on phytase synthesis [84, 105].

2.5 Purification of phytases

Various methods have been used for purifying enzymes, including salt/solvent precipitation, ultrafiltration, ion exchange and gel filtration chromatography. Two periplasmatic phytases from *E. coli* were purified using ammonium sulphate precipitation (25% to 80% saturation) followed by CM-Sepharose CL 6B chromatography, DEAE-Sepharose CL 6B chromatography, Phenyl-Sepharose CL 4B chromatography, and Mono S HR 5/5 chromatography [43]. An extracellular phytase from *Bacillus* sp. DS11 was purified to homogeneity by acetone precipitation and phenyl-sepharose, Resource S, and Superose 12 column chromatographies [58]. A novel neutral and heat-tolerant phytase from a newly isolated strain *Bacillus nealsonii* ZJ0702 was purified to homogeneity by ammonium sulphate precipitation followed by DEAE-sepharose chromatography, Sephadex G-100 chromatography [106]. Bhavsar *et al.* applied a single step aqueous two-phase extraction (ATPE) method for the purification of phytase from *Aspergillus niger* NCIM 563 and further compared with the traditional multi-step procedure involving salt precipitation and column chromatography. In their study, they attained high phytase recovery (98.5%) within a short time (3 h) and improved thermostability by ATPE in comparison to 20% recovery in 96 h by chromatography process. The ATPE method, therefore, seems to be an interesting alternative for simultaneous partitioning and purification of phytase [107].

Phytase from *S. castellii* was purified by anion exchange and gel filtration chromatography [64]. Cell bound phytase of *P. anomala* was purified to near homogeneity by a two-step process of acetone precipitation followed by anion exchange chromatography using DEAE-Sephadex [66]. Partial purification of phytase from *A. adeninivorans* was achieved by Sephadex G50 filtration and/or DEAE chromatography [63].

2.6 Biochemical and molecular characterization of phytases

The physiochemical properties of some reported phytases are given in Table 1.

Table 2. Physicochemical and kinetic properties of phytases from different sources

Sources	Mole. Wt. (kDa)	Optimum		K_m (mM)	Substrate specificity	Ref.
		pH	Temp(^{o}C)			
Fungi						
Aspergillus ficuum (*PhyA*)	85	2.5, 5.0	58	0.027	Specific	[83]
A. ficuum (*PhyB*)	68	2.5	63	0.103	Broad	[53]
A. niger SK-57	60	2.5, 5.5	50	0.019	Specific	[54]
A. niger ATCC 9142	84	5.0	65	0.10	Broad	[55]
A. oryzae	120	5.5	50	0.33	Broad	[108]
Bacteria						
Bacillus sp. DS11	44	7.0	70	0.55	Specific	[58]
Citrobacter braakii	47	4.0	50	0.46	-	[109]
Klebsiella terrigena	40	5.0	58	-	-	[59]
Lactobacillus sanfrancesis	40	4.0	45	-	Broad	[86]

B. subtilis	36-38	6.0-6.5	60	0.55	Specific	[87], [110]
B. licheniformis (*phyA*, *phyL*)	44,47	4.5-6.0	55, 65	-	Specific	[111]
Escherichia coli	42	4.5-5.0	-	-	-	[43]
Yeast						
Arxula adeninivorans	-	4.5	75	0.25	Specific	[63]
Schwanniomyces castellii	490	4.4	77	0.038	-	[64]
Saccharomyces. cerevisiae	120	2.0- 2.5 5.0-5.5	55-60	-	-	[97]
P. anomala	64	4.0	64	0.20	-	[66]
P. rhodanensis	-	4.0-4.5	70-75	0.25	-	[65]
P. spartinae	-	4.5-5.5	75-80	0.33	-	[65]
Candida krusei WZ-001	330	4.6	40	0.03	-	[67]

2.6.1 pH and temperature optima and stability

The pH and temperature optima of phytases from various sources are presented in Table 2. The pH optima for microbial phytases have varying from 2.2 to 8. Most of the fungal phytases have an optimum pH between 4.5 and 5.6 except *A. fumigatus* phytase, which has a broad pH optimum and at least 80% of the maximal activity is observed at pH values between 4.0 and 7.3 [112]. The pH optima for most of the bacterial phytases have been reported at 6.5-7.5 while *Citrobacter braakii* and *Lactobacillus sanfrancesis* have maximum activity at pH 4. *A. niger* NRRL 3135 and *Citrobacter freundii* phytases differ from other phytases in having two pH optima (pH 2.5 and 5.5). Nearly all yeast phytases have a pH optimum towards the acidic side

between 4.0 and 5.5. The temperature optima of phytases vary from 45 to 77 ^{o}C. *Bacillus* sp. Strain DS11 phytase [98] had a temperature optimum at 70 ^{o}C, which is higher than the temperature optimum for other bacterial phytases in general. It was also very thermostable: 100% residual activity after 10 min incubation at 70 ^{o}C (in the presence of $CaCl_2$). Phytases from most of the fungal species are optimally active between 50 to 65 ^{o}C. Phytases of *A. adeninivorans, S. castellii* and various *Pichia* spp. were found to be active in the range of 75-80 ^{o}C, while the phytase of *C. krusei* WZ-001 was optimally active at 40^{o}C. In the animal feed industry, phytases with high temperature optima are desirable as feed pelleting involves a brief exposure to 80-85 ^{o}C for a few seconds.

2.6.2 Molecular characteristics

Molecular weights of phytases from various sources are shown in Table 2. Phytases are high molecular weight proteins ranging from 40-500 kDa. Bacterial phytases found to be smaller than fungal and yeast phytases. The predicted size of the fungal and yeast phytases was less than that of the experimental size, indicating heavy glycosylation. *A. niger* NRRL 3135 native phytase is 27% glycosylated. Molecular weight of *P. anomala* phytase was 64 kDa, while phytase from *S. castellii* and *C. krusei* WZ-001 had a molecular weight of 490 kDa and 330 kDa, respectively. Phytase from *C. krusei* WZ-001 was composed of two different subunits while *S. castellii* phytase showed about 31% glycosylation, which are responsible for high molecular weight of phytase in these organisms. Glycosylation may have several effects on the enzyme properties, it may influence the catalytic properties or have an impact on the stability of the enzyme; also it may influence the pI of the protein. Han and Lei [98] studied the role of glycosylation in the functional expression of *A. niger*

phytase (*phyA*) in *Pichia pastoris*. Their results showed that glycosylated phytase from *P. pastoris* had an identical capacity of PA hydrolysis and slightly improved thermostability as compared to the same enzyme over expressed in *A. niger*.

2.6.3 Substrate specificity and kinetic parameters

Phytases usually exhibit broad substrate specificity, with a high affinity for phytates. Substrates, structurally not similar to PA, such as ADP, ATP, *p*-nitrophenyl phosphate, phenyl phosphate, α- and β-glycerophosphate, fructose 1, 6-bisphosphate, glucose 6-phosphate and 3-phosphoglycerate are frequently hydrolyzed by phytases. Phytase from *Bacillus* sp. has been described as very specific for PA [60]. The same result was reported for alkaline phytase isolated from lily pollen [113]. Along with this, many other alkaline phytases also exhibit high substrate specificity for phytate and have no enzymatic activity on other phosphate esters [58, 87]. Among the yeasts, *S. castellii, P. anomala* and *C. krusei* WZ-001 exhibited broad substrate specificity. Six phosphate groups of PA may be released by phytases at different rates and in different order under different conditions. Kerovuo *et al.* studied the PA hydrolysis by *Bacillus* phytase [114]. They observed that this enzyme hydrolysed only three phosphates from PA and preferred the hydrolysis of every second phosphate over that of adjacent ones. Wyss *et al.* [112] investigated the kinetics of phosphate release and the kinetics of accumulation of reaction intermediates, as well as the end products of PA degradation by various phytases. They found that all studied fungal phytases released five of the six phosphate groups and the end product being *myo*-inositol 2-monophosphate when excess enzyme is used. Among yeasts, the pathways of dephosphorylation of phytate have been studied for *C. krusei*, *P. rhodanensis and S.*

cerevisiae and the intermediates and end products of the reaction are shown in Fig. 4.

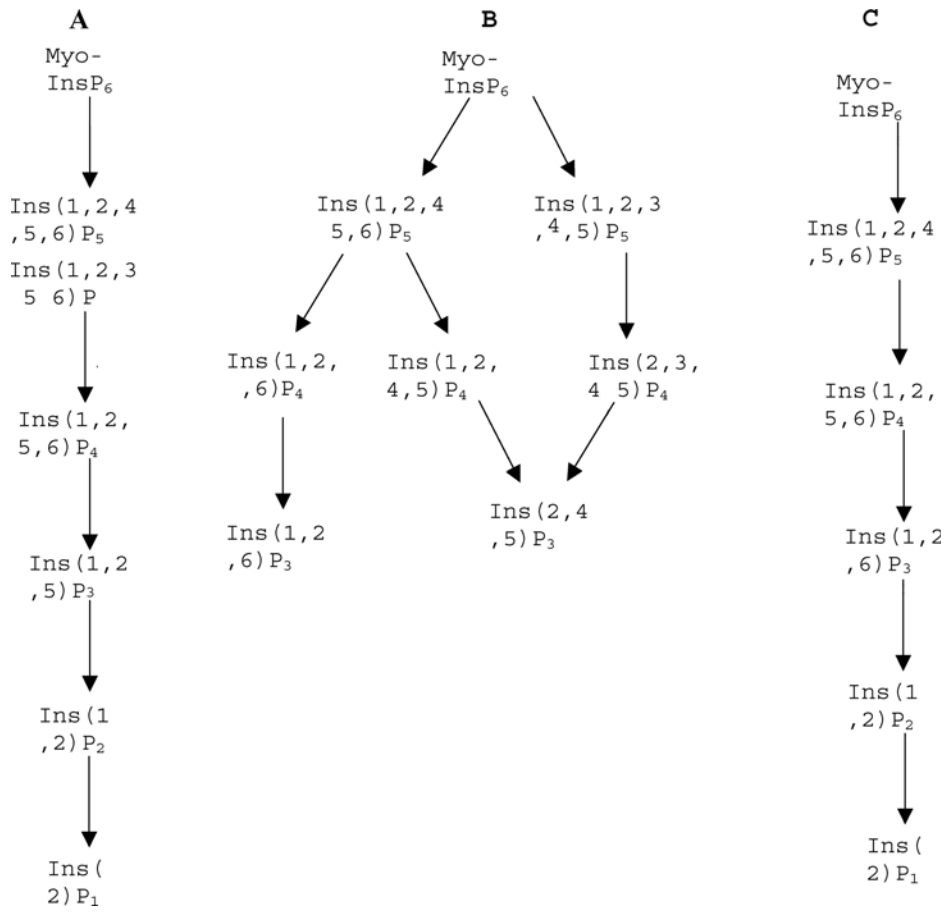


Figure 6. Pathways of dephosphorylation of IP6 catalyzed by the phytases of (A) *C. krusei,* (B) *P. rhodanensis* and (C) *S. cerevisiae*. [Depicted from P. Kaur *et al.* [115]

The K_m values for phytases from various sources are presented in Table 2. Wyss *et al.* [112] studied the kinetic parameter of phytases from different fungal strains. In their study, they found that the *A. terreus* 9A1 and CBS phytases followed Michaelis-Menten kinetics, with K_m values of 10.6 6 0.9 and 23.2 6 2.2 mM, respectively. However, the K_m values of the *A. fumigatus* and *A. niger* phytases could not be determined reliably due to the limited sensitivity of the assay, while the *M.*

thermophila and *E. nidulans* phytases displayed non-Michaelis- Menten behaviour [112]. The apparent K_m value for *Shigella* sp. CD2 phytase for phytate as determined by Lineweaver-Burk plot was 0.25 mM [116]. The K_m values of *P. spartinae* and *P. rhodanensis* phytases were 0.30 m*M* and 0.25 m*M*, respectively; these values are similar to that of *A. adeninivorans* but higher than that of *S. castellii* (0.038 m*M*). The K_m value for *P. anomala* phytase for PA was 0.20 mM and Vmax was 6.34 μmol/mg protein/min. Among the all reported yeast phytases, *C. krusei* phytase showed a lowest K_m value for PA.

2.6.4 Effect of modulators on phytase activity

Effect of different metal ions on phytase activity has been studied. Metal ions have been shown to modulate phytase activity. In general, Cu and Zn inhibit the phytase activity irrespective of the microbial source. Zn^{2+}, Ba^{2+}, Cu^{2+} and Al^{3+} had a negative influenced on phytase from *Enterobacter* sp. [62]. Wyss *et al.* [112] reported that Cu^{2+} considerably decreases the enzyme activities of *E. nidulans* and *A. terrus* phytases. The cations Zn^{2+} and Cu^{2+} (0.5mM) caused around 50% inhibition of enzyme activity, while 5 mM Zn^{2+} and Cu^{2+} were highly inhibitory for phytase from *S. castellii* [64]. Phytase of *C. krusei* WZ-001 and *P. anomala* was strongly inhibited by Zn^{2+} and Mg^{2+} and Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+}, respectively. Augspurger *et al*. [117] reported that pharmacological levels of Zn^{2+} supplementation in the diet (1500 mg kg^{-1} in pigs and 800 mg kg^{-1} in chickens) reduced the P-releasing efficacy of phytase in young pigs and chickens. Supplementation of 200 mg kg^{-1} Cu^{2+}, however, did not affect the response of chickens to phytase. It is difficult to conclude whether the inhibitory effect of various metals is due to direct binding of the metal ion to the

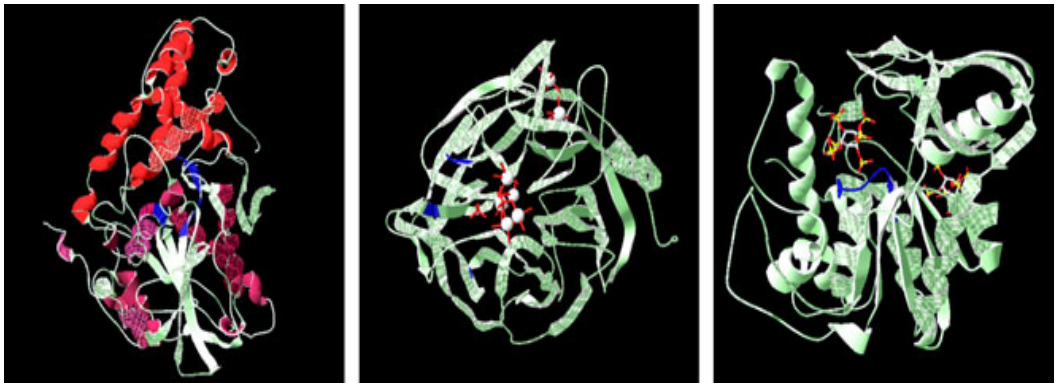
enzyme or metal ions complexes with PA and thereby decrease the active substrate concentration.

The activity of *A. fumigatus* phytase was stimulated up to 50% by EDTA, whereas EDTA had no major effects on the enzymatic activities of other fungal phytases tested (*E. nidulans*, *A. niger* and *A. terrus*). Phytase activity from *Enterobacter* sp. 4 [62], *B. subtilis* (natto) N- 77 [58] and other *Bacillus* sp. [87, 110] was greatly inhibited by EDTA suggesting that a metal ion is needed for the activity. Alkaline phytate-degrading enzymes were greatly inhibited by EDTA. Sodium azide, DTT, β-mercaptoethanol, EDTA, toluene, glycerol, PMSF, iodo-acetate and N-bromo-succinimide did not show an inhibitory effect on phytase from *P. anomala* while activity was highly inhibited by SDS. Furthermore, the enzyme was inhibited by 2,3-butanedione, indicating the involvement of arginine residues in catalysis [66]. Fungal phytase activity has been shown to be inhibited by substrate concentrations exceeding 1 mM [112]**.** The phytase activity of *Candida krusei* WZ-001 was stimulated by β -mercaptoethanol and dithiothreitol (DTT) while it was inhibited by iodoacetate, *p*-chloromercuribenzoate and PMSF [67].

2.6.5 Crystal structure of phytases

Crystal structure analyses of a number of phytases have been determined and which revealed a range of distinct folds for these enzymes and have allowed their biophysical properties to be rationalized in terms of their structure. The structural features of several phytases have been established and X-ray crystallographic studies have confirmed that they belong to a class with a novel catalytic mechanism [118]. The elucidation of the 3-D molecular structure of different phytate degrading enzymes has enhanced our understanding of the linkage between the molecular structure of the

molecule and its catalytic function. Study with crystal structures of a novel, thermostable phytase determined in both the partially and fully Ca^{2+} loaded states revealed that a new folding architecture of a six-bladed propeller for phosphatase activity [118]. Crystal structure analysis of *Escherichia coli* phytase with a resolution of 2.5 Å showed two domains, one contains five alpha-helixes and two beta-sheets, and the other includes six alpha-helixes and nine beta-sheets [119]. The crystal structure of phytase of *D. castellii* was determined at a resolution of 2·3 Å. It observed that this structure was very similar to that of *A. ficuum* phytases and can be divided into two parts: a large alpha-helical/beta-sheet domain with a six-stranded beta-sheet, and a small alpha-helical domain [120]. The crystal structure of the phytase from *Klebsiella* sp. ASR1 determined at 1.7 Å resolution. The overall structure of *Klebsiella* phytase showed similarity to other histidine-acid phosphatases, such as *E. coli* phytase, glucose-1-phosphatase and human prostatic-acid phosphatase though having low sequence similarity with a later one [121]. The polypeptide chain is organized into an alpha and an alpha/beta domain, and the active site is located in a positively charged cleft between the domains.



(a) (b) (c)

Figure 7. Swiss-Pdb viewer-prepared molecular models from the National Center for Biotechnology Information (NCBI)'s website (http://

www.ncbi.nlm.nih.gov), representing three types of phytases: (a) 1IHP, PhyA, a histidine acid phosphatase; (b) 1H6L Ts-Phy, a *β* propeller phytase; (c) 1U26, SrPhy, a cysteine phytase [Depicted from Yao *et al.* [42]]

Kostrewa *et al.* studied the crystal structure of phytase from *A. ficuum* at 2.5 Å resolution [122]. It is a high molecular weight acid phosphatase and the structure has an alpha/beta-domain similar to that of rat acid phosphatase and an alpha-domain with a new fold. A three dimensional model of *A. ficuum* phytase (1IHP) from the National Center for Biotechnology Information's (NCBI) website is shown in Fig. 7(a) The crystal structure of *Bacillus amyloliquefaciens* phytase (TsPhy) at 2·1 Å resolution revealed a six-bladed *β*-propeller in which each blade consists of a four- or five-stranded antiparallel beta-sheet (Fig. 7(b)) [123]. *Selenomonas ruminantium* phosphatase (SrPhy) shows a third, dual-specificity phosphatase type with a conserved cysteine (C241) in its so-called P loop [49]. Fig. 7(c) shows 1U26 to underscore the structural differences in three classes of enzyme.

2.7 Protein engineering of phytase

Thermostability is prerequisite for the successful application of phytase in animal feed which are exposed to 60–90 °C during the pelleting process. Furthermore, a phytase must be optimally active in the pH range prevalent in the digestive tract so that it can use in animal feed preparation. Although many phytases with varying properties are known, there is no single wild-type enzyme that is perfect or ideal for field applications. Theoretically, an 'ideal' phytase should be catalytically efficient, proteolysis-resistant, thermostable and cheap [124]. Thus engineering phytases to improve their catalytic properties under various conditions is of great interest. Zhao *et*

al. [125] introduced random mutation in protease resistance phytase gene of *Penicillium* sp. using Mn^{2+}-dIT. In their study, they identified two mutants, 2-28 and 2-249, with improved thermal stability and optimal temperature and pH. The mutants also retained their high resistance to pepsin. They further observed the thermostability of phytase from mutants after heat treatment at 100°C for 5 min. The two mutant proteins retained about 72.81% and 92.43% of the initial activity, respectively. Tomschy *et al.* [126] engineered the pH activity profiles of *A. fumigatus* and consensus phytases by rational mutagenesis for the possible industrial applications of phytase in animal feed. They reported that decreasing the negative surface charge of the *A. fumigatus* Q27L phytase mutant by glycinamidylation of the surface carboxy groups of Asp and Glu residues lowered the pH optimum by ca. 0.5 unit, but also resulted in 70 to 75% inactivation of the enzyme. Brugger *et al.* [127] tested the effect of different low molecular weight additives on thermostability of phytases from various fungal strains and consensus phytases. This study revealed that polyethylene glycols increase the thermostability of all phytases in a molecular weight-dependent fashion. Furthermore, the enhancement in thermostability was observed after crosslinking of the carbohydrate chains of *A. fumigatus* and consensus phytase using sodium periodate and adipic acid dihydrazide resulted in the formation of oligomeric forms. Zhu et al [128] improved the thermostability of *E. coli* (AppA) by 23.3% as compared to wild type using error-prone PCR and high-throughput screening.

2.8 Immobilization

Immobilization of cells or enzymes is one of the excellent ways to increase the stability and life of the enzyme. Immobilized enzyme can be reused and therefore reduce the cost of the process. Phytases act sequentially on *myo*-

inositolhexakisphosphate to liberate various lower isomers. Thus, an efficient immobilized bioreactor could be used to produce various isomers of PA besides rendering the molecule non-chelator of metal-ions, proteins, etc. [129]. A few studies have been made on the application and properties of immobilized phytases. *A. ficuum* phytase was covalently immobilized on Fractogel TSK HW-75 containing 2-oxy-l-alkylpyridinium salts. Immobilized phytase then used for hydrolysis of PA in packed-bed reactor [130]. Quan *et al.* reported the immobilization of cells of *C. krusei* capable of producing phytase in Ca-alginate gel beads and used that for the preparation of *myo*-inositol phosphates. The optimum temperature for the enzyme activity of the immobilized cells was 15 ^{o}C higher than that of free cells. At a flow rate of 1.30 mL/min, a mixture of *myo*-inositol-2-monophosphate, *myo*-inositol-1,2,5-triphosphate and *myo*-inositol-1,2,5,6-tetrakisphosphate was produced and which were separated using ion-exchange chromatography [129]. Heat tolerance of *E. coli* phytase was improved by covalently immobilizing it on NHS-activated Sepharose [131]. Permeabilized cells of *P. anomala* capable of producing phytase have been immobilized using Ca-alginate [132].

2.9 Market trends

Feed enzymes (protease, xylanase, phytase, amylase, cellulase, lipase, β-glucanase) are gaining importance as they play an important role in improving the feed digestibility, cost reduction, nutrient absorption, and better performance. Phytases have well-recognized and documented enzyme that has application in the animal feed industry, particularly for poultry and pig feeds. Recent studies showed additional benefits; improved mineral availability, human nutrition, soil amendment, beyond the reduction of the environmental impact of P pollution. Due to concerns about

environmental pollution, 22 countries have adopted the use of phyA, produced from *A. niger* NRRL 3135, as a feed additive. The FDA has approved "generally recognized as safe (GRAS)" petition for use of phytase in food, and it has been marketed as an animal feed enzyme in US since 1996. Replacing dicalcium phosphate in wheat bran diets with phytase as 25 g/100 kg reduces the feed cost by about INR 1/kg [133].

2.10 Application of phytase

Plant-based sources are the main feed ingredients for animals in the world. They are rich of carbohydrates, protein, dietary fibre, vitamins and non-nutrients [134]. Moreover, they also constitute the antinutrients, which adversely affect the absorption of nutrients. AP, among the all antrinutrients, is of prime concern in monogastric animals since they lack phytate degrading enzyme. Phytase preparations therefore have a wide range of applications in animal and human nutrition to improve the nutritional value. The first commercial phytase product Natuphos® was launched in 1991 [135]. Now market volume of the phytase is in the range of 150 million Euro and new generations of phytase have been developed and commercially available.

The major application of phytase is as a feed supplement to improve P bioavailability in plant feed-stuffs via the enzyme-mediated hydrolysis of phytate. Moreover, the improve utilization of the phytate phosphate results in a substantial decline in the phosphate content in animal manure and consequently less phosphate load on the environment in areas of intensive animal agriculture. Application of phytase in animal feed also reduces the need for supplemental inorganic P such as mono- and dicalcium-phosphate. As inorganic phosphate is non-renewable resource,

the use of phytase is an effective way for natural resource management of P on a global scale.

2.10.1 Application in animal nutrition

Monogastric animals are unable to metabolize PA and thus nutrients bound to it are also unavailable because of lack of phytate-degrading enzyme in their gastrointestinal tract. Generally, PA degradation can occur in the digestive tract and/or in the feed before consumption [136]. It is seen that the use of phytase in feed degrades the PA and thus improves the P bioavailability and reduces the chelating ability of PA. Furthermore, use of phytase in feed reduces the level of phosphate pollution in intensive livestock areas by avoiding the addition of exogenous phosphate [137]. The FDA (The Food and Drug Administration) has approved the phytase preparation as GRAS and recent trend in the market also have clearly shown phytase as an important enzyme and feed supplement. The use of phytase as a feed additive has been approved in 22 countries due to serious concerns regarding environmental pollution.

Phytase is incorporated into commercial poultry, swine, and fish diets to improve the availability of P, minerals and amino acids since last two decades. There are many reports that demonstrating the efficacy of microbial phytases to release phytate-bound P using animal system [138, 139]. The evolved *E. coli* phytase was found effective in improving broiler growth performance, bone characteristics, and retention of P, Ca, N, and a number of amino acids [61]. Addition of phytase in corn-soybean meal diets for pig increases the absorption of the corn meal P and therefore reduces the percentage of dietary P that excretes in the faeces [140]. Van der Klis *et al.* [141] reported that in maize–soy diets, containing 2.4 g phytate-P kg^{-1}, 500 FTU phytase activity kg^{-1} substantially increased ileal degradation of phytate. Phytases

were also found to improve the minerals bioavailability and hence growth and productivity of animals. Fungal and bacterial phytases have been evaluated individually and in combination for their efficacy as feed additives in poultry. Several yeast strains have also been studied for their dephytinization ability but none of them is yet commercialized. Yeasts themselves are good source of proteins and vitamins and hence will be excellent feed additive as a phytase source.

2.10.2 Application in human nutrition

Diet of the vegetarian peoples and in most of the developing countries constitutes the cereals and legumes, which are rich in PA content. PA, as it has strong affinity for positively charge metal ions cause mineral deficiency in people on vegetarian diet. Vegetarians, elderly people consuming high amounts of cereals, people in undeveloped countries and babies eating soy-based infant formulas take in large amounts of phytate and hence more susceptible for mineral deficiency [142]. Physiological condition of human gastrointestinal track is unable to absorb the insoluble phytate-mineral complex. Though soaking, germinations and cooking methods have shown decrease in phytate content; phytase treatment may be more efficient in reducing the phytate content. Therefore, processing and manufacturing of human food is also a possible application field for phytase.

Degradation of PA during bread making has been known to effect mineral bioavailability for many years. Phytase shows potential as a bread making improver because first, it improves the nutritional value of bread by decreasing phytate content, and second, it releases bound calcium and thereby promotes the activation of endogenous α-amylases, which increases the bread volume and improves texture [143]. The non-heme iron absorption in humans almost doubles from wheat bread

rolls treated with fungal phytase compared to bread untreated with phytase [144]. Addition of phytase to the porridge along with amylase improves the absorption of Fe by another 3-fold [145]. Addition of *Bifidobacterium* strain contributed to *myo*-inositol hexakisphosphate (phytate) hydrolysis during breads making results into higher mineral availability [145].

2.10.3 Application in aquaculture

Fishmeal production from wild-catch sources is not sufficient to fulfil the global aquaculture need and hence suitable alternatives have to be found for sustainable and environmental friendly aquaculture. A plant-based source seems to be the ideal alternative to for aquafeed [146]. However, plant-based feed is rich in PA and like poultry; aquatic animals are inefficient in hydrolyzing the PA. High PA content feed reduces the bioavailability of nutrients like minerals and proteins to the fish. Use of phytase treatment in aquafeed will reduce the aquaculture pollution and will also improve the bioavailability of nutrients. Various reports on use of phytase as feed additive in aquaculture suggest that phytase supplementation could enhance the bioavailability of P and other minerals, thereby decreasing P-load in the aquatic environment [7, 147].

2.10.4 Preparation of *myo*-inositol derivatives

Derivatives of the PA, mono, bis, tris, and tetrakisphosphate, play a crucial role in trans-membrane signalling processes as well as in calcium mobilization from intracellular store in animal as well as in plant tissues [6, 148]. Certain derivatives of PA have been suggested to have positive effects on heart disease by controlling hypercholesterolemia and atherosclerosis [16]. Many studies have explained the

potential of *myo*-inositol phosphates (InsP1,4,5 and InsP1,3,4,5) for reducing the risk of colon cancer [19, 149]. The esters of inositol triphosphate were found to alleviate conditions associated with abnormal levels of neuropeptide Y (NPY), such as arthritis and asthma, besides acting as a pain killer [150]. *myo*-inositol phosphates have to be available in pure form and sufficient quantity to study its physiological effects. Non-enzymatic methods are attempted to synthesize the defined isomers of the different *myo*-inositolphosphates but resulted in mixtures of *myo*-inositol pentakis-, tetrakis-, tris-, and bisphosphate isomers. Moreover, purification of these isomers from the mixture is difficult and too costly. The stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation by the *E. coli* phytase was established using a combination of high-performance ion chromatography analysis and kinetic studies. High-performance ion chromatography revealed that the enzyme degrades *myo*-inositol hexakisphosphate by stepwise dephosphorylation via D/L-Ins(1,2,3,4,5)P_5, D/L-Ins(2,3,4,5)P_4, D/L-Ins(2,4,5)P_3 or D/L-Ins(1,2,4)P_3, D/L-Ins(1,2)P_2 or Ins(2,5)P_2 or D/L-Ins(4,5)P_2 to finally Ins(2)P or Ins(5)P. Kinetic studies showed that the *myo*-inositol pentakisphosphate intermediate produced either by the wheat or *E. coli* phytases are not identical [151]. Thus this suggests that the intermediates of PA hydrolysis by phytases are not identical and it is the characteristic of that enzyme. Hydrolysis of PA using *S. cerevisiae* phytase resulted in the production of D-Ins(1,2,6)P_3, D-Ins(1,2,5)P_3, L-Ins(1,3,4)P_3 and Ins(1,2,3)P_3 [152].

2.10.5 Phytases in soil amendment

P is an essential plant nutrient that limits agricultural production on a global scale. Approximately 30–80% of the total P in soils is present in organic form [153]**.** Inorganic P fertilizers are applied to fulfil the P requirement; however, only 10% -

20% of fertilizer P is available for utilization by crops in the first year after application. Phytate constitutes ~50% of the total organic P pool in the soil and is poorly utilized by plants [154], therefore, application of phytases are of particular interest in agricultural field. Transgenic *Nicotiana tabacum* plants expressing a chimeric phytase gene accumulated 3.7-fold more P than vector control plants when supplied with phytate [155]. Extracellular phytase activities have been reported under phosphate stress conditions in tobacco [96], barley [156], tomato, alfalfa [157], and so on. Extracellular phytase from transgenic strain of *Bacillus mucilaginosus*, a rhizosphere soil organism, has able to degrade the soil PA and able to promote tobacco growth and also increases phosphorous content in the plant, thereby potentially limiting eutrophication [158].

3 Genesis of thesis and objectives

There are many reports on phytase production by fungal, yeast and bacterial strains. Several fungal phytases are commercialized up to industrial scale for animal feeds due to their acid tolerance and higher yield. All phytase preparations that available in the market are of fungal origin and produced by recombinant strains. Fungal phytases have been extensively used in animal feed, but they have not been used in human food as yet.

Yeasts could be served as a good source of phytase for feed and food application because most of them are non-pathogenic and food grade. Moreover, they are rich in proteins and vitamins and hence routinely used in animal feed to increase the productivity. Thus application of yeast phytases in feed or food will hydrolyze the PA so as to increase bioavailability of mineral and P also it will act as a protein and vitamin source. Though there are many reports on yeast phytases none of them has

made entry into the market, because of low productivity and cost effective production. Hence there is a need to identify yeasts with high phytase yield and to develop an economical process for large scale phytase production. In this study, we selected the strain of *Williopsis saturnus* for phytase production. Use of *W. saturnus* has been reported to be useful in food processing industry as it has ability to produces the killer toxin, a protein which displays a cytocidal activity against a wide range of microorganisms including yeasts and fungi. Hence, in present study, we focused on high phytase production by *W. saturnus* NCIM 3298 as it has good prospects in food processing industry.

3.1 Objectives of the study

The study entitled as "Studies on phytase from *Williopsis saturnus* NCIM 3298 and its applications" was taken up with the following objectives.

1) Screening of yeasts from NCIM resource center for phytase activity and selection of high phytase producing yeast strain
2) Production of phytase by *W. saturnus* NCIM 3298 using Response Surface Methodology (RSM)
3) Characterization of cell bound phytase from *W. saturnus* NCIM 3298
4) Application of *W. saturnus* NCIM 3298 phytase to improve the nutritional value of livestock feed

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CHAPTER 2

Screening of yeast strains from NCIM resource center for phytase activity

This chapter discusses about the screening of 600 yeast strains from NCIM resource center for phytase production. Screening methods were used to reveal an ability of various yeast strains to utilize PA as a sole phosphorus source. Two minimal media, Ca-phytate containing phytase screening medium followed by liquid minimal medium were used for screening. Among 600 screened yeast strains, *W. saturnus* NCIM 3298 was selected for further studies due to its strong growth and high phytase production in the liquid medium. Maximum phytase production in minimal liquid medium for *W. saturnus* NCIM 3298 was 7 IU g/ DWB. Biochemical and physiological characterization studies revealed that the strain under study was *W. saturnus* var. *saturnus*.

Part of the work presented in this chapter is published

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1. Introduction

Increasing human population needs to shift from agricultural practices to animal productions to reduce the burden on agricultural products. Animal productivity can be increased by giving the nutritionally rich feeds to animals. Cereals, legumes and oilseeds are a good source of carbohydrates, proteins and all essentials minerals [1, 2] and hence supplemented in animal feed to increase the productivity [3]. Along with nutrients some anti-nutritional factors are present in cereals and legumes, which reduced the nutritional quality of these feed ingredients [4]. PA, the sole source of P, is one of the anti-nutrient which interfere with the assimilation and absorption of phosphorous, minerals, proteins as it has high affinity for these nutrients [5, 6].

Monogastric animals are unable to utilize the phytate bound P as they lack the PA degrading enzyme, therefore, excess Pi is supplemented in the feeds to meet their P requirement. Unutilized PA and excess Pi are out in the excreta and thereafter released in the nearer water bodies. Moreover, increased concentration of P and nitrogen of water bodies leads to eutrophication, formation of algal blooms.

Use of phytases in the monogastric animal feeds have been known for to increase the availability of P, starch, protein, amino acids and positively charge metals, to seize the anti-nutritional effects of phytate, to decrease environmental pollution and to eliminate the addition of inorganic phosphate in animal feed. In addition, phytase hydrolysis of PA sometimes gives different derivatives of *myo*-inositol, lower phosphate esters, some of which are considered to be pharmacoactive and important intracellular secondary messengers [7]. The FDA has approved "generally recognized as safe

(GRAS)" petition for use of phytase in food, and it has been marketed as an animal feed enzyme in US since 1996 [8].

Fungal and bacterial phytases have been studied extensively for their applications in animal feed [8-10]. Many fungal phytases are commercially available in the market. Yeasts are good source of proteins and vitamins therefore used in animal feed to increase the productivity [11]. However, phytases from yeast has limitations for commercialization because of costly large scale production and low phytase activity. Extracellular phytase activity has been reported for *Arxula adeninivorans*, *Schwanniomyces occidentalis, Kluyveromyces lactis, Pichia anomala, Torulaspora Delbrueckii* and *Saccharomyces cerevisiae* [12-14]. There are reports on cell associated phytase production by yeast strains such as *Pichia anomala, Rhodotorula gracilis, Candida krusei, Saccharomyces cerevisiae* and *Cryptococcus laurentii* ABO510 [15-18]. Maximum cell associated and extracellular phytase production reported for *Pichia anomala* and *Arxula adeninivorans*, respectively [12, 15]. Nakamura *et al.* [13] and Olstorpe *et al.* [19] have screened 738 and 122 yeast strains, respectively, for phytase activity. Though there are many reports on yeast phytases, none of them has yet commercialized. The high price of commercial phytase currently restricts widespread application of phytase as feed supplement. Hence, there is a need to identify organism with higher production and explore other applications of phytases. Since yeasts are rich source of proteins and vitamins, nutritious value of the feed can also be further increased by application of the whole cells as phytase.

The present work was, therefore, directed toward screening of yeast strains from NCIM resource center for phytase production followed by selection of the best strain on the basis of its phytate hydrolysing capacity and its applicability in the feeds and foods.

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, Bacto, Sigma, Qualigen and Merck.

2.2 Yeast strains

The yeast strains used in this study were procured from in house culture collection, NCIM Resource Centre, CSIR-National Chemical Laboratory, Pune, India. 600 yeast strains belonging to 15 different species from culture collection were taken for screening. The cultures under study were maintained by periodic transfer on malt extract-glucose-yeast extract-peptone (MGYP) (per 100 mL): malt extract, 0.3 g; yeast extract, 0.3 g; glucose, 1 g; peptone, 0.5 g; agar, 2 g and pH 5.5; slants at 28 ^{o}C for 3 days and preserved at 4±1 ^{o}C.

2.3 Growth media and culture conditions

Screening of yeast strains for phytase production was carried out with slight modifications according to Howson and Davis and Lambrechts *et al*. [18, 20]. Two minimal media, opaque Ca-phytate containing Phytase Screening Medium (PSM) and

Na-phytate containing Liquid Minimal Medium (LMM) were used to investigate the ability of the strains to grow on media with PA as the sole P source. PSM contained (per 100 mL) glucose, 2 g; NH_4NO_3, 0.5 g; KCl, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.03 g; $FeSO_4 \cdot 4H_2O$, 0.001 g; $MnSO_4 \cdot H_2O$, 0.001 g; Ca-phytate, 0.5 g; agar, 2 g and pH 5.5. LMM contained (per 100 mL) glucose, 2 g; NH_4NO_3, 0.5 g; KCl, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.03 g; $FeSO_4 \cdot 4H_2O$, 0.001 g; $MnSO_4 \cdot H_2O$, 0.001 g; Na-phytate, 0.2 g and pH 5.5. Media and Na-phytate were autoclaved separately at 121 ^oC for 20 min. pH of the media were adjusted using NaOH.

2.3.1 Growth test on PSM

For screening on solid media, overnight grown cultures of yeast strains in MGYP broth were spot inoculated on PSM and incubated at 28 ^oC. Thereafter, growth on solid media examine for the presence of zone of hydrolysis around the colony. Cultures showing zone of clearance around the colony were selected for next screening step.

2.3.2 Growth test on LMM

Colonies which showed clear zone around on PSM were further evaluated for phytase production by the growth in LMM to eliminate false-positive test on PSM due to acid production. Yeast strains were grown in MGYP broth; biomass was harvested by centrifugation at 6,000 x *g* for 10 min at 4 ^oC and then washed three times with sterile saline to remove medium phosphate. Saline washed cells were inoculated in 10 mL of LMM and incubated at 30 ^oC for 48 h on rotary shaker (200 rpm) to test presence of the growth (Turbidity). Culture turbidity was monitor by taking the absorbance at 600 nm. More the turbidity more the ability of the organism to hydrolyzed the phytate.

For estimation of phytase activity in LMM, both cell free supernatants and biomass were collected and assayed for phytase in the extracellular and cell-bound fractions. To estimate the cell bound phytase activity, biomass was harvested by centrifugation at 6000 x *g* for 10 min at 4 ^{o}C, washed trice with distilled water to remove medium phosphate. Washed biomass then used for phytase assay. To estimate the dry yeast biomass, the cell pellet was washed twice with distilled water and dried in pre-weighed tubes at 80 ^{o}C up to a constant weight. For extracellular phytase activities measurement, supernatant were collected and kept in refrigerator until activity measurements were performed.

2.4 Analytical method

Both cell associated and extracellular phytase activities were determined using whole cells and culture supernatant, respectively. Phytase activity was determined with slight modifications according to Ullah and Gibson [21], including phosphate determination as described by Heinonen and Lahti [22]. For determination of cell-associated phytase activity, the cells were washed thrice with distilled water and then suspended in the 200 mM acetate buffer pH 5.0. The standard reaction mixture contained 200 mM acetate buffer pH 5.0, 1 mM Na-phytate and suitably diluted enzyme, in a final volume of 0.5 mL. The reactions were carried out at 50 ^{o}C, unless indicated otherwise. Negative controls were prepared from samples mixed with acetate buffer without Na-phytate. Samples were incubated at 50 ^{o}C for 10 min, and the reaction was stopped by addition of freshly prepared 4 mL of acid molybdate reagent (2 volumes of acetone: 1 volume of 5 N H_2SO_4: 1 volume of 10 mM ammonium molybdate) and 400 µl of 1 M citric acid. The

liberated P was determined at 370 nm in a spectrophotometer using acetate buffer blank. One unit (IU) of phytase activity was defined as the amount of enzyme that released 1 µmol P per minute under standard assay conditions. Cell associated and extracellular phytase activities were expressed as IU/g dry weight cell biomass (DWB) and IU/mL, respectively.

2.5 Biochemical and physiological characterization of *W. saturnus* NCIM 3298

2.5.1 Carbon assimilation

The *W. saturnus* NCIM 3298 was tested on different carbon sources (D-glucose, D-galactose, sucrose, trehalose, melibiose, D-lactose, xylose, rhamnose, maltose, cellobiose, D-melezitose, starch, glycerol, L-arabinose, inositol, D-sorbitol, methyl-α D-glucopyranoside, N-acetyl glucosamine, D-saccharose, D-threhalose, D- raffinose, adonitol, xylitol, calcium-2-ceto-gluconate and citrate) for carbon assimilation ability. Each tube contained 10 mL minimal medium with 1% carbon source. The inoculated tubes were incubated at 28 ºC for 48 h.

2.5.2 Nitrogen assimilation

Nitrogen assimilation test was performed by growing the yeast strain in minimal medium containing 0.5% nitrogen source [NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NaNO_2$, $NaNO_3$ and KNO_3]. The inoculated tubes were incubated at 28 ºC for 48 h.

2.5.3 Maximum growth temperature

The yeast strain was inoculated in 10 mL minimal medium containing 1% glucose and incubated at different temperatures *viz*., 30 ºC, 35 ºC, 37 ºC and 40 ºC for 48 h.

2.5.4 Glucose concentration tolerance

The yeast strain was inoculated in 10 mL minimal medium containing 50% and 60% glucose and incubated at 28 ^{o}C for 48 h.

3. Results and discussion

3.1 Screening of yeast strains for phytase activity

About 600 yeast strains were spot inoculated on PSM plates to assess their growth on PA as a sole P source. All yeasts were able to grow on PSM, but only 40 yeasts showed zone of clearance around the colony after 4 to 7 days of incubation at 28 ^{o}C. A zone of clearance for *W. saturnus* NCIM 3298 is given in Fig. 1. It was observed that the five yeasts, *Zygosaccharomyces bisporus* NCIM 3265 and NCIM 3296, *W. saturnus* NCIM 3298, *Zygosporium priorionus* NCIM 3299 and *Schizosaccharomyces octosporus* NCIM 3297, which showed zone of clearance on PSM, have not been previously reported for phytase production. Along with them, 26 strains of *Saccharomyces cerevisiae*, 4 strains of *Pachysolen tannophilus*, 2 strains of *Candida* and a strain of *Zygosaccharomyces rouxii, Torulaspora delbrueckii* and *Metschnikowia pulcherrima* also showed zones of clearance on PSM, which have already been previously reported for phytase production [13, 18, 20, 23].

Lambrechts *et al.* [20] did not observed hydrolysis of phytate by *P. anomala* on solid medium. In this study, we also observed the similar results for two strains of *P. anomala* on solid medium. In contrast to that Olstorpe *et al.* [19] observed the growth of three *P. anomala* on solid media with phytate as a sole phosphorus source. Phytate degradation on solid medium cannot be considered as only criteria for screening of yeast strains, as organism not producing zone of clearance has also been reported for phytase production [19]; but to narrow down the screening experiments this method is very useful. It has also been suggested that the size of clear zone is not quantitatively related with the amount of secreted phytase [13]. Therefore, growth test in both, solid and liquid media is mandatory to confirm the phytase production as microorganism producing phytase on solid phytate media may not produce the enzyme in liquid broth or vice versa [24, 25].

Hydrolysis of Ca-phytate from PSM did not provide a clear evaluation of phytase activity, and an alternative testing method in LMM was therefore used according to Olstorpe *et al*. [19]. Growth in LMM at 28 ^{o}C was monitored by measuring optical density (OD) at 600 nm after 48 h. The relative capabilities of strains to utilize PA as a sole P source were determined by comparing OD_{600nm} in LMM with the negative control (PA-depleted minimal medium).

Forty strains showing zones of clearance on PSM grew in LMM but to the varying extents as shown in Fig. 2. In minimal liquid culture, we observed both species and strain specific differences in growth that were not clear on solid medium. Of the tested yeast strains, five strains of *S. cerevisiae*, and NCIM 3296, *W. saturnus* NCIM 3298, and *S. octosporus* NCIM3297 grew very well with OD value more than 2.0. A

strain of *Z. biosporus* NCIM 3265, *Z. priorionus* NCIM 3299 and *S. cerevisiae* NCIM 3053 showed the OD value in the range of 1.4 to 1.7. Four strains of *P. tannophilus*, two strains of *Candida*, a strain of *T. delbrueckii*, *Z. rouxii* and 19 strains of *S. cerevisiae* were unable to enter the log phase and two strains of S. *cerevisiae* were unable to grow at all, although they exhibited zones of clearance on PSM. Thus, a total of 11 yeast strains were found to grow in LMM. A large difference in growth on LMM was observed for 25 strains of *S. cerevisiae* indicating that phytase activity may be strain specific, but not a species specific. The strains of *S. cerevisiae* have been reported for phytase production [19, 23] but the enzyme activities associated with them are too low for commercialization. Therefore, strains of *S. cerevisiae* with high growth were not selected for phytase production though having more growth as compared to other tested strains.

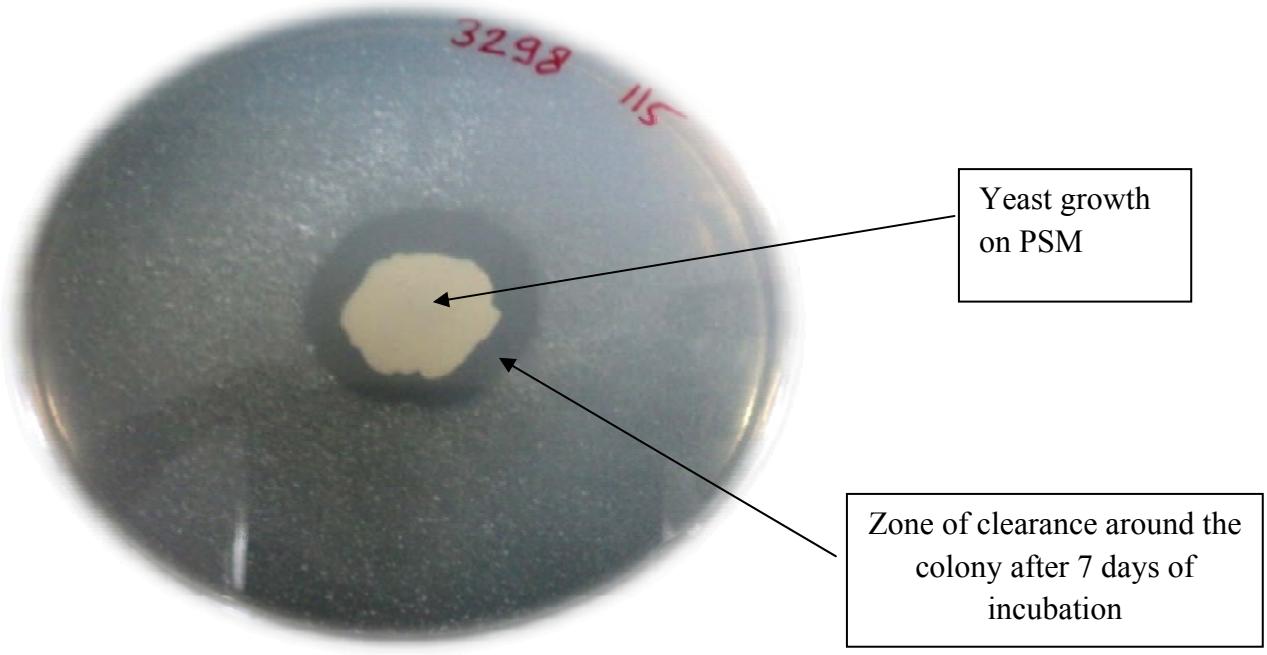

Figure 1: Screening of yeast strains on Ca-phytate containing medium for phytase activity. Cultures were grown on PSM medium (0.5% Ca-phytate) at 28 ^{o}C (pH 5.5).

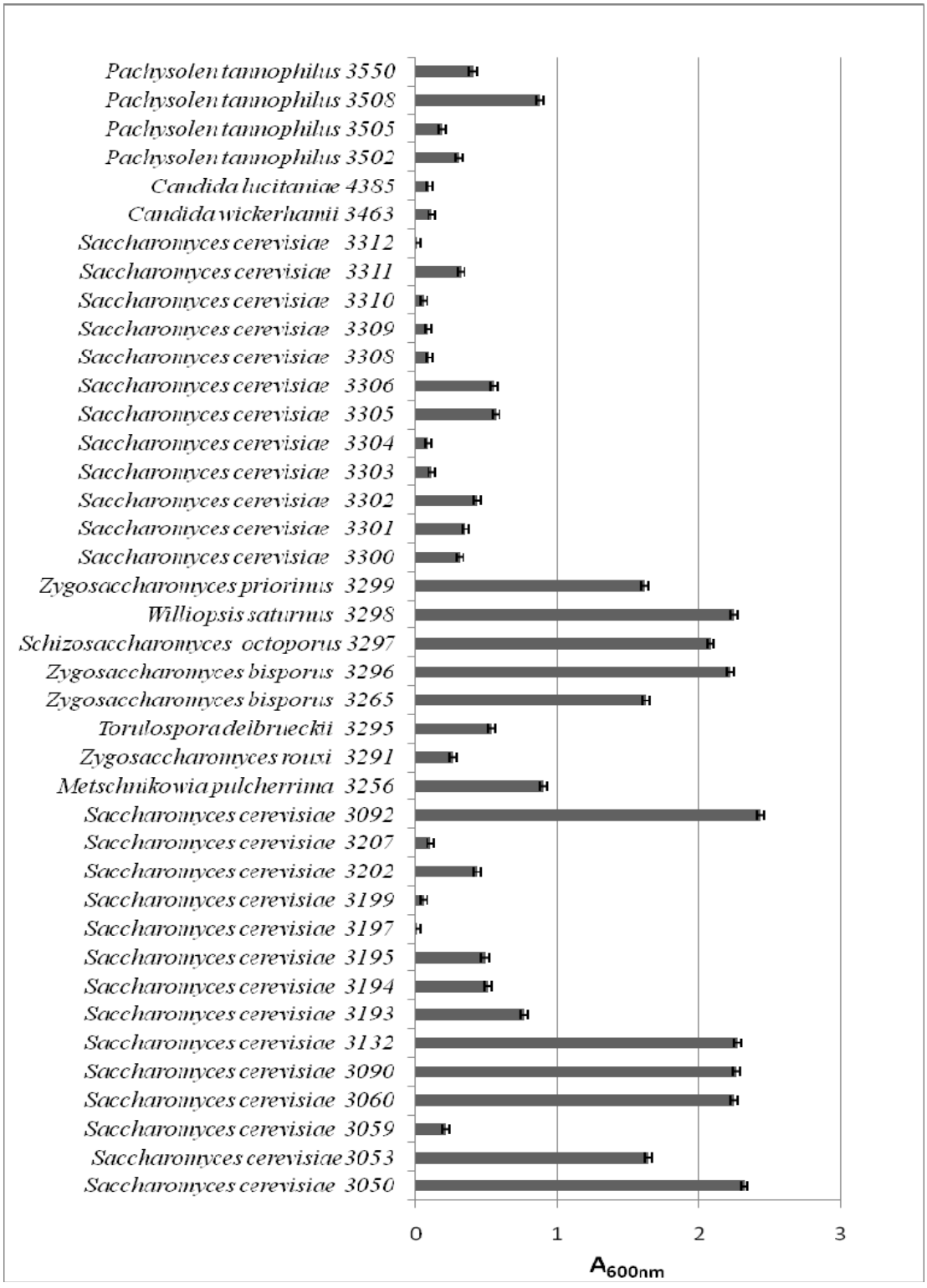

Figure 2: A_{600nm} values of yeast strains grown in MMP medium at 28 ^{o}C for 48 h. Results are average of two independent experiments.

Five yeasts *viz., Z. bisporus* NCIM 3265, *Z. bisporus* NCIM 3296, *S. octosporus* NCIM 3297, *W. saturnus* NCIM 3298 and *Z. priorionus* NCIM 3299 showed extensive growth in LMM and they were selected for phytase production studies because of lack of significant study for phytase production in these strains.

3.2 Phytase activity in the selected strains

Cell-associated and extracellular activities obtained for *Z. bisporus* NCIM 3265, *Z. bisporus* NCIM 3296, *W. saturnus* NCIM 3298, *S. octosporus* NCIM 3297 and *Z. priorionus* NCIM 3299 are shown in Table 1. Highest extracellular phytase activities were detected after 48 h, while cell-associated phytase activities were detected at 24 h of fermentation. Our results show that, for the all tested strains, cell-associated phytase activities were higher than extracellular activities. However, in contrast to that Nuobariene *et al*. [23] observed higher extracellular phytase activities as compare to intracellular. Cell bound phytase activities have been reported for *C. krusei* [17], *Pichia anomala* [15] and *Schwanniomyces castellii* [26]. Therefore, further studies were conducted with cell-associated phytase. Observed extracellular phytase activities of the all tested strains were comparable with the extracellular activities reported for *A. adeninivorans, S. cerevisiae* and *P. anomala* [19, 23]. Among the all tested yeasts, *W. saturnus* NCIM 3298 showed higher extracellular phytase production (0.180 IU/mL). However, extracellular activities of studied yeast were very less as compared to the extracellular phytases activities of reported fungal strains and hence they are not considered in further study.

Cell associated activities showed by *Z. bisporus* NCIM 3265, *Z. bisporus* NCIM 3296, *S. octosporus* NCIM 3297, *W. saturnus* NCIM 3298 and *Z. priorionus* NCIM 3299 were 13, 6, 5, 7 and 11 IU/g DWB, respectively. These values are higher or comparable with *Candida krusei* (201 U/mg cells), *Pichia anomala* (6 U/g cells) and *Cryptococcus laurentii* ABO 510 (4.55 U/g cells) as reported by Quan [17], Vohra and Satyanarayana [15] and Van Staden *et al*. [27], respectively; although it should be noted that these assays were not all performed under similar conditions.

Table 1. Extracellular and cell-associated phytase activity

Yeast strain	**Extracellular Phytase activity (U/ mL)**	**Intracellular phytase activity (IU/g of DWB)**
Z. bisporus NCIM 3265	0.153	13
Z. bisporus NCIM 3296	0.121	6
S. octoporus NCIM 3297	0.120	5
W. saturnus NCIM 3298	0.180	7
Z. priorionus NCIM 3299	0.099	11

Cells were grown in LMM medium (0.2% Na–phytate) at 28 ^{o}C (pH 5.5). Results are expressed as the mean of three replicated measurements.

Z. bisporus NCIM 3265, *W. saturnus* NCIM 3298 and *Z. priorionus* NCIM 3299 showed higher phytase activities than *Z. bisporus* NCIM 3296 and *S. octosporus* NCIM 3297 though the growth of earlier strains were less as compared to later strains in LMM. This suggests that the high OD value of the liquid medium does not reveal the high phytase production. Further, for *Z. bisporus* NCIM 3265 and *Z. bisporus* NCIM 3296,

though they are belong to the same species, a large difference in phytase production was observed among them, indicating that phytase activity may be strain specific.

Most of the *Zygosaccharomyces* species are reported as food spoiling yeasts [28, 29] therefore, they cannot be used for food or feed applications. Thus, *Z. bisporus* NCIM 3265 and *Z. priorionus* NCIM 3299 were not considered for phytase production studies though having higher phytase activity as compared to other tested strains. Therefore, *W. saturnus* NCIM 3298 selected for further studies based on its high phytase production ability in LMM, which was higher as compared to earlier reported yeasts. *W. saturnus* has also been reported for killer toxin production that displays a cytocidal activity against a wide range of microorganisms [30, 31]. Goretti *et al*. and Liu *et al*. studied the effect of killer toxin of *W. saturnus* on the growth of food spoilage yeasts and the biocontrol of spoilage yeasts and moulds in yogurt, respectively [32, 33]. Thus, *W. saturnus* NCIM 3298, a multifunctional organism, that has potential application in feed and food for more than one purpose including source of phytase, vitamins and proteins and inhibitor of food spoiling organisms.

3.3 Biochemical and physiological characterization of *W. saturnus* NCIM 3298

Biochemical and physiological tests such as the ability to assimilate different carbon and nitrogen sources, growth temperatures and glucose concentration tolerance were performed for characterization of *W. saturnus* NCIM 3298. Results for these tests are given in Table 2. Studied yeast was able to utilize glucose, sucrose, trehalose, mannitol, maltose, cellobiose, xylose, D-melezitose, L-arabinose, D-sorbitol, methyl-α D-

glucopyranoside, D-saccharose, D-threhalose and glycerol but unable to utilize galactose, melibiose, D-lactose, rhamnose, starch, inositol, L-arabinose, adonitol, xylitol, calcium-2-ceto-gluconate and citrate. Carbon utilization results for *W. saturnus* NCIM 3298 are similar with other *W. saturnus* strains reported by Kurtzman *et. al.* [34] and Liu [35]. According to Kurtzman *et. al.* [34] *Williopsis* species are xylose assimilating but unable to utilize inositol. The same results were observed for *W. saturnus* NCIM 3298, xylose assimilating and inositol non assimilating strain.

Table 2. Biochemical and physiological characteristics of *Williopsis saturnus* NCIM 3298

Characteristic	**Result**	**Characteristic**	**Result**
Carbon assimilation		**Nitrogen assimilation**	
D- Glucose	+	NH_4NO_3	+
D-Galactose	-	$(NH4)_2SO4$	+
Sucrose	+	$(NH_4)_2HPO_4$	+
Trehalose	+	$NaNO_2$	+
Melibiose	-	$NaNO_3$	+
Mannitol	+	KNO_3	+
D-Lactose	-	**Growth temperature (^oC)**	
Xylose	+	25	+
Rhamnose	-	30	+
Maltose	+	35	W
Cellobiose	+	40	-

D- Melezitose	+	**Biochemical reaction**	
Starch	-	50% Glucose	+
Glycerol	+	60% Glucose	-
L-Arabinose	-	Extracellular polysaccharide production	-
Inositol	-	**Colony colour**	Tannish white
Methyl-α D-Glucopyranoside	+	**Colony morphology**	Spheroidal, Smooth, Butyrous
D-Sorbitol	+		
N-Acetyl Glucosamine	-		
D-Saccharose	+		
D-Threhalose	+		
D- Raffinose	-		
Adonitol	-		
Xylitol	-		
Calcium-2-Ceto-gluconate	-		
Citrate	-		

Scored for response to tests: -, negative; ＋, positive; w, weak

Cells were grown in the medium as mention in materials and methods at pH 5.5.

We observed that, *W. saturnus* NCIM 3298 was able to assimilate all nitrogen sources, ammonium and nitrate, which were used in this study. Based on physiological tests results, carbon and nitrogen assimilation studies, and key characters of species in the

genus *Williopsis* given by Kurtzman *et. al.* [34] studied strain is may be *W. saturnus* var. *saturnus*. The yeast strain under study showed a good growth at 28 and 30 ^{o}C, but unable grew at 40 ^{o}C and grew weakly at 35 ^{o}C. The colony was smooth, butyrous and tannish white in colour. The cells were spheroidal, ellipsoidal. Extracellular polysaccharide production was not observed in this yeast. *W. saturnus* NCIM 3298 tolerate glucose concentration up to 50%, but was unable to grow at 60% glucose.

In summary, the present study screened almost 600 yeast strains for their ability to hydrolyze the PA. Though all of them were able to grow on solid screening medium, only 40 yeast strains showed a zone of clearance around the colony. Of the 40, 11 yeasts showed a good growth in liquid medium. *W. saturnus* NCIM 3298 was found to be a better phytase producing yeast.

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CHAPTER 3

Production of phytase by *Williopsis saturnus* NCIM 3298 and its characterization

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High level cell bound phytase production by *W. saturnus* NCIM 3298 was carried out using an integrated statistical optimization approach of Plackett Burman Design (PBD) followed by a Box-Behnken Design (BBD) of experiments. Four statistically significant factors, *viz*., citric acid, $FeSO_4$, pH and glucose were identified by PBD and further selected for optimization study by response surface methodology (RSM) using BBD. After the media optimization studies by PBD and BBD, the phytase production improved from 46 IU/g DWB to 297 IU/g DWB. Therefore an overall 6.45 fold enhancement was attained in phytase production after media optimization. Cell bound phytase had an optimal pH of 5.0 and an optimal temperature of 50^{o}C. K_m value for cell bound phytase was 0.2 mM. Localization studies revealed the cell bound nature of the phytase. None of the detergents and solvents was able to increase the phytase activity. Phytase activity was moderately stimulated in presence of 1mM Co^{2+}, Ba^{2+}, Ca^{2+} and 10 mM K^{+}, Na^{+}, Mg^{2+}. It was strongly inhibited in presence of 10 mM Hg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Ba^{2+}.

1. Introduction

Phosphorus (P) is an essential component of life that is required for the survival of all living organisms. In livestock cultivation, animals get their P requirement through their diets which mostly consist of cereals, pulses and oil seeds. PA normally occurs in nature and constitutes the principal storage form of P (60-90%) and inositol in plants, legumes and oil seeds [1]. PA acts as an anti-nutrient because it forms strong complexes with amino acids, proteins, starch and nutritionally important metal ions such as calcium, zinc, magnesium and iron, and therefore decreases their bioavailability [2, 3]. Monogastric animals, poultry and piggery, diet is mainly derived from the plant based sources and hence rich in PA. However, monogastric animals are unable to utilize the PA, a sole source of P in cereals and pulses, because they lack the intrinsic enzyme that hydrolyses PA [4, 5]. Excess of inorganic P (Pi), therefore, is supplemented in animal diet to fulfil the P need. Since these animals are unable to utilize PA bound P, the unhydrolyzed phytate is released through animal excreta. The excreta when discharged in near water bodies can cause their undesirable eutrophication [6]. Therefore, phytate-rich ingredients have become a major interest among researchers and nutritionists with a view to formulate the feed for animals. Addition of Pi in the feed is one of the ways to meet the P requirement of the monogastric animals but the sources of Pi are non-renewable. Thus, supplementation of feed with phytase will increase the bioavailability of phosphate along with other minerals and decrease P pollution in areas of intensive poultry and piggery farms [7, 8]. Also, phytases are known for dephosphorylation of PA into optically active myoinositol derivatives which are considered to be important intracellular secondary messengers [9, 10].

To obtain a good source of phytase, a variety of microorganisms have been screened for their ability to produce phytase. Several phytases of fungal origin are available in the market and are widely employed in animal feed due to their acid tolerance and higher yield [11]. Though most of the yeasts are non-pathogenic and nontoxic to animals and human, there are very few reports on phytase producing yeasts and its large scale production. Yeasts are rich source of proteins, vitamins and P [12, 13] and hence whole cells of the yeast can be used as phytase sources to improve the nutritional value of the feed. Yeasts have been reported for both extracellular and cell associated phytase production but extracellular activities are too low as compared to fungal phytase secretion. Also, there is no defined medium for optimum production of yeast phytases on commercial scale as each yeast has its own special growth conditions and specific substrates for phytase production.

As cell associated activities in yeasts are high as compare to extracellular, there is need to improve cell bound phytase production by using submerged or solid state fermentation for developing a commercial process. There are various ways to achieve the highest phytase production such as optimization of one variable at a time (OVAT), media optimization using RSM followed by growth kinetics studies. Phytase production is ameliorated by employing statistical optimization techniques rather than the conventional OVAT approach because statistical optimization gives the optimum media formulation with minimum number of experiments in short time [14]. Vohra and Satyanarayana studied the effect of glucose, beef extract and inoculum density on the cell bound phytase production by the yeast *Pichia anomala* using response surface methodology [15]. Very few reports are available on yeast cell bound phytase production using response surface methodology because of the low productivities and handling of yeast cells.

The present work is therefore directed toward the screening and selection of the media components and their concentration by using statistical media optimization methods, PBD and BBD, to improve phytase production by the yeast *W. saturnus* NCIM 3298. *W. saturnus* is known as killer yeast, which inhibits the growth of food spoilage organisms including fungi and yeasts. Therefore, it has been used as bio-control agents in the preservation of foods [16, 17].

2. Materials and methods

2.1 Chemicals

Phytic acid sodium salt and yeast lytic enzyme (β-1, 3-glucanase from *Rhizoctonia solani*) were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO), USA. All other chemicals and solvents used were of analytical grade. Cane juice, cane molasses and various agriculture residues were purchased from a local market.

2.2 Yeast and culture conditions

W. saturnus NCIM 3298 was used in the present study from NCIM Resource Center, CSIR-NCL, Pune, India [18]. The stock culture was maintained on MGYP agar slants and stored at 4 ^{o}C. Yeast culture from MGYP slant was inoculated in glucose-yeast extract-peptone broth (YGP) and incubated at 28^{o}C for 16 h. Culture OD was adjusted to 1.5 by taking absorbance at 660nm and it used as inoculums for production studies.

2.3 Phytase production in Erlenmeyer flasks

250 mL Erlenmeyer flasks containing 50 mL unoptimized medium (per 100 mL): glucose, 2 g; yeast extract, 0.3 g; NH_4NO_3, 0.5 g; citric acid, 0.5 g; $FeSO_4$, 1mM and pH 5.5; were inoculated with the 16 h old yeast inoculum (3%) and incubated on a

rotary shaker at 28 ^{o}C for 24 h (200 rpm). Various carbon and nitrogen (organic and inorganic) sources were supplemented in the unoptimized medium to study their effect on phytase production. Effect of pH and temperature on phytase production was also studied using unoptimized medium. All optimization tests were performed in 250 mL flasks containing 50 mL medium at 30 ^{o}C at 160 rpm. The cells were harvested and washed thrice with distilled water to remove media ingredients and then used to determine cell bound phytase activities. Various agricultural and industrial waste substrates were used for phytase production (per 100 mL): glucose, 2 g; NH_4NO_3, 0.5 g and yeast extract, 0.3% with cane molasses, 10% (w/v), cane juice, 50% (v/v); soy whey, 10% (v/v); 5% rice bran extract or 1% chickpea extract. All these media were adjusted to pH 5.5 using NaOH. All experiments were carried out in triplicates.

2.4 Analytical method

Phytase activity measurements were carried out at 50°C as described earlier in chapter two. The reaction mixture consisted of 3mM sodium phytate buffered with 200 mM acetate buffer (pH 5.0). Enzymatic reactions were started by the addition of 5 mg yeast cells in Na-phytate substrate. After 10 min at 50°C, the liberated inorganic phosphate was measured with slight modification of the ammonium molybdate method [19]. A freshly prepared solution of acetone: 5N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) followed by 400µl 1M citric acid was added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1µm P/min under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments. Negative controls were prepared from cells

mixed with acetate buffer without Na-phytate. Cell associated phytase activities were expressed as IU/g of dry weight biomass (DWB).

2.5 Optimization using response surface methodology

2.5.1 Screening of key media components using PBD

Two level full factorial design of PBD was employed for identification of key fermentation parameters, physical and chemical, which significantly increase the cell bound phytase production by *W. saturnus* NCIM 3298. According to PBD the screening was carried out in N+1 trials, where N is number of variables [20]. Seven independent variables viz., glucose, NH_4NO_3, yeast extract, citric acid, incubation period, $FeSO_4$ and pH were tested at two levels, high and low in a set of 12 trials. The PBD experimental design matrix with the name and actual level of the variables used is shown in Table 1. The matrix was constructed using DES software (version 7.1.2, Stat-Ease, Minneapolis, MN, USA). The experiments were carried out in triplicate and the average phytase production was taken as the response. The variables which showed higher percent contributory effect on cell bound phytase production were selected for further optimization studies using response surface methodology by a Box-Behnken Design (BBD) of experiments. Percentage values were calculated by adding the total sum of squares and then taking each term's sum of squares and dividing by the total.

Table 1. PBD matrix for screening of key medium components and their assigned level for cell bound phytase production by *W. saturnus* NCIM 3298

Variable	Level 1	Level 2
Glucose (%)	2.0	10
NH_4NO_3 (%)	0.2	2.0
Yeast extract (%)	0.1	0.5
Citric acid (%)	0.2	2.0
Incubation period (h)	16	36
$FeSO_4$ (mM)	0.5	3.0
pH	5.0	7.0

2.5.2 Optimization of components by BBD

After selection of key factors by PBD, the BBD was employed to determine optimum levels of the selected factors for enhancing the cell bound phytase activity. The BBD [21] method is an independent quadratic design with no embedded factorial or fractional factorial points. Here the variables combinations lie at the midpoints of the edges of the variable space and at the centre. It requires 3 levels for each factor. Furthermore, the number of experiments (N) required for the development of BBD is defined as $N = 2k\,(k - 1) + C_o$ (where k is number of variables and C_o is the number of central points).

In the present study, the BBD employed four factors, viz., citric acid, $FeSO_4$, pH and glucose at three levels again using DES, version 7.1.2 (Stat-Ease, Minneapolis, MN, USA). The selected significant factors and their levels used in the

BBD experimental runs are given in Table 2. Total 30 experiments were conducted with five replicates at the center point for estimating the purely experimental uncertainty variance. Three-dimensional response surface plots were drawn to illustrate the relationship between the responses and the experimental levels of each independent variable. The quadratic equation model in DES was used to study the optimum values of the variables and the behavior of the system. All experimental runs of the BBD were carried out in triplicates and their mean values are presented.

Table 2. Selected factors and their assigned levels for BBD to study phytase production

Variable	Level 1	Level 2	Level 3
Citric acid (%)	0.05	0.28	0.50
$FeSO_4$ (mM)	0.05	0.78	1.5
pH	4.5	5.5	6.5
Glucose (%)	5.0	8.5	12.0

2.6 Localization of Enzyme

To find the location of phytase, cells were subjected to physical, chemical and enzymatic treatment according to Bindu *et al*. and Van Staden *et al*. [22, 23]. *W. saturnus* NCIM 3298 was grown in glucose-yeast extract medium for 24 h. Cells were harvested by centrifugation at 6000 x *g* for 15 min, and washed thrice with distilled water. Washed cell pellet was suspended in 100 mM sodium acetate buffer (pH 5.0). Physical permeabilization was done by freezing the cells in liquid nitrogen followed by thawing at RT and this procedure was repeated up to 15 cycles. Chemical permeabilization was performed on cells suspended in 100 mM sodium acetate buffer

(pH 5.0) for 2 h at RT in the presence of permeabilizing agents *viz*., 1% (v/v) Triton X-100, 1% (v/v) Tween-80, 0.1% (w/v) sodium dodecyl sulfate (SDS) and 1% (w/v) EDTA. For enzymatic treatment, cells were treated with yeast cell wall lytic enzyme (1mg/ mL) obtained from *Rhizoctonia solani* for 2 h and then centrifuged at 6000 x *g* for 15 min at 4 ^{o}C. Lytic enzyme treated cells were also subjected to lysis using sonicator for 15 min.

2.7 Characterization of cell bound phytase

The optimum pH was determined by measuring the enzyme activity between pH 2.0-8.0 using 200 mM buffers; glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-6.0) and Tris-HCl (pH 7.0-8.0) at 50 °C and optimum temperature was determined over temperature range 20-70 ^{o}C at pH 5.0. The thermal stability was studied by incubating the cells at different temperatures up to 80 ^{o}C for different time intervals and the residual enzyme activity was determined using standard assay conditions and compared it with the control, without incubation as 100%.

The effect of various metal ions such as Cd^{2+}, Hg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, K^{+}, Na^{+} and Ba^{2+} on cell bound phytase activity was measured at 50 ^{o}C for 10 min as described earlier by adding metal ions with final concentration of 1 mM or 10 mM to the cell suspension in 200 mM acetate buffer (pH 5.0) and 3 mM sodium phytate along with control, i.e. phytase reaction without metal ions at 50^{o}C. Effect of different detergents *viz*., ionic detergent, sodium dodecyl sulphate; non-ionic detergents, Triton X-100, Tween 80, Brij C-10, n-Dodecyl *β*-D-maltoside and Octyl *β*-D-glucopyranoside; zwitterionic detergent, CHAPS and solvents namely toluene, acetone, diethyl ether, benzene, DMSO and butanol (0.1% or 0.5%) on phytase activity was studied by incubating cells with solvents and detergents for 15 min. The

residual activity was then assayed under standard assay conditions considering enzyme activity without solvent and detergent as 100%.

The rate of hydrolysis of substrate (sodium phytate) was determined by varying the substrate concentrations in the range of 0.01-1 mM. Assays were carried out at 50 °C under standard assay conditions mention earlier. The kinetic rate constant, K_m, was determined graphically from Lineweaver-Burk plotting.

3. Results and discussion

3.1 Effect of carbon sources on phytase production

To study the effect of various sugars on cell bound phytase production by *W. saturnus* NCIM 3298, fermentation medium was supplemented either with glucose, sucrose, glycerol, maltose, sorbitol or cellobiose at 2 g/100 mL medium. Effect of carbon sources on phytase production is given in Table 3. Among all tested sugars, glucose and sucrose were found good carbon source for phytase production as compare to others and gave 33 and 30 IU/g DWB phytase production, respectively. Glycerol containing medium was found to be a good carbon source for biomass production (data not given) but unable to give high phytase production. By varying the concentration of glucose from 2 to 10 g/100 mL medium did not show significant variation in phytase synthesis. Mayer *et al.* [24] reported that glucose is the most productive substrate for high level phytase production by yeast *Hansenula polymorpha*. However, galactose was found to be a better carbon source than glucose for phytase production by *Arxula adeninivorans* [25]. Sorbitol, cellobiose and glycerol did not show significant effects on phytase production and gave 11, 5 and 8 IU/g DWB phytase production, respectively. For *P. anomala*, a high phytase titre was attained in a synthetic medium that contained 4% glucose [26].

Table 3: Effect of various sugars on cell bound phytase production by *W. saturnus* NCIM 3298

Carbon source (g/100 mL)	Phytase production (IU/g DWB)
Glucose (2g)	30
Glucose (4g)	33
Glucose (6g)	32
Glucose (8g)	30
Glucose (10g)	30
Sucrose (2g)	30
Glycerol (2g)	8
Maltose (2g)	14
Sorbitol (2g)	5
Cellobiose (2g)	11

The culture was grown at 28 ^{o}C with shaking (200 rpm) as described in material and methods. The values given in the Table are the average of two independent experiments.

3.2 Effect of organic and inorganic nitrogen sources on phytase production

Effect of various inorganic and organic nitrogen sources (0.5 g/100 mL) on phytase production was studied and the results are given in Table 4. Maximum phytase production (40 IU/g DWB) was obtained in presence of ammonium nitrate and yeast extract. Medium containing potassium nitrate, sodium nitrate, ammonium sulphate or tryptone also favoured comparable phytase production. Phytase production was severely inhibited in presence of beef extract and di-ammonium hydrogenphosphate. Kwong-Bun Li reported that beef extract in the medium inhibits the α-Glucosidase

production by *Lactobacillus acidophilus* [27]. In contrast to that beef extract found a better nitrogen source for phytase production by *P. anomala* [15]. For *A. adeninivorans,* yeast extract (1%) and peptone (1%) were served as good nitrogen sources for phytase synthesis [25]. Ammonium sulfate (0.1%), inorganic nitrogen source, was used for phytase production by *S. castellii* [28].

Table 4. Effect of nitrogen sources on phytase production by *W. saturnus* NCIM 3298

Nitrogen source	Phytase activity (IU/g DWB)
0.5 g/100 mL	
NH_4NO_3	40
$(NH_4)_2SO_4$	32
$(NH4)_2HPO_4$	10
$NaNO_3$	28
KNO_3	38
0.3 g/100 mL	
Yeast extract	40
Beef extract	01
Peptone	14
Tryptone	24

Phytase production using different inorganic nitrogen sources was carried out in yeast extract containing medium similarly for organic nitrogen sources it was carried out in presence of NH_4NO_3. The culture was grown at 28 ^oC with shaking (200 rpm) as described in Material and Methods. The values given in the Table are the average of two independent experiments.

3.3 Effect of pH and temperature on phytase production

The effect of pH and temperature on phytase production by *W. saturnus* NCIM 3298 was studied after 24 h of incubation in shake flask condition. Production of phytase was determined at initial pH values in the range from 2.0 to 7.0 (Fig. 1). Phytase synthesis increased gradually with increase in initial pH values and maximum phytase production was attained at pH 6. Further increase in pH decreased the phytase production drastically. Considerable activity observed when initial pH was adjusted towards acidic conditions. This in contrast with phytase production by *P. anomala* which was highest at pH 7 and very low activity was detected at low pH [26].

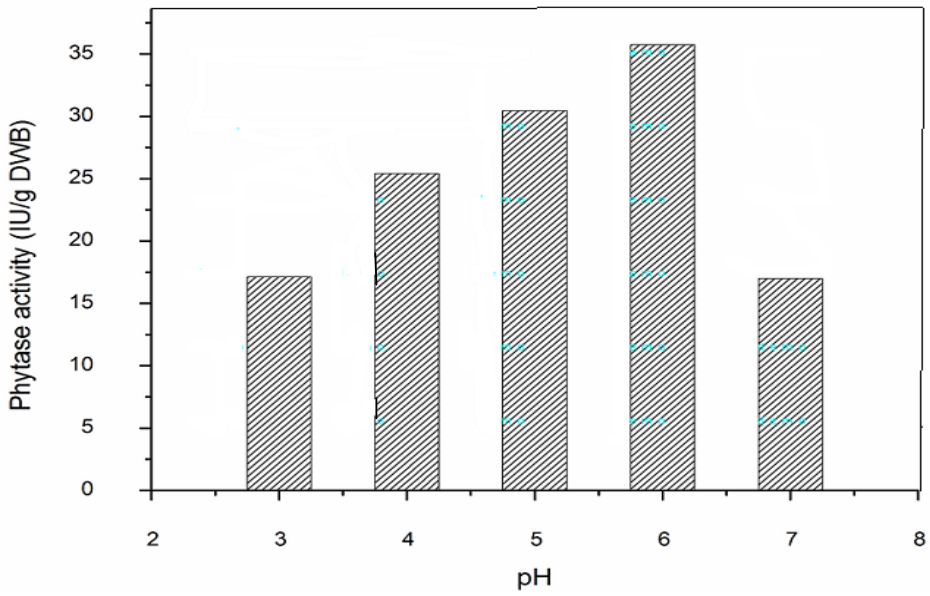


Figure 1. Effect of pH on cell bound phytase production by *W. saturnus* NCIM 3298.

The culture was grown at 28 ^{o}C with shaking (200 rpm) as described in material and methods. The values given in the table are the average of two independent experiments.

Effect of incubation temperatures in the range from 20 ^{o}C to 35 ^{o}C on phytase production is shown in Fig. 2. The *W. saturnus* NCIM 3298 grew best at 28 ^{o}C and 30 ^{o}C and less growth was observed at 35 ^{o}C. Maximum phytase production was observed at 28 ^{o}C and 30 ^{o}C. Similar observation was recorded by Quan *et. al*. for

Candida krusei, which showed maximum phytase production at 30 ^{o}C [29]. Phytase production at 20^{o}C was less and this in contrast to the phytase production by *P. anomala* and *Cryptococcus laurentii* AL_{27} which were optimum at 20 ^{o}C and 24 ^{o}C, respectively [26, 30]. For *A. adeninivoran,* high levels of phytase secretion were obtained at 44 ^{o}C during its active growth phase [25].

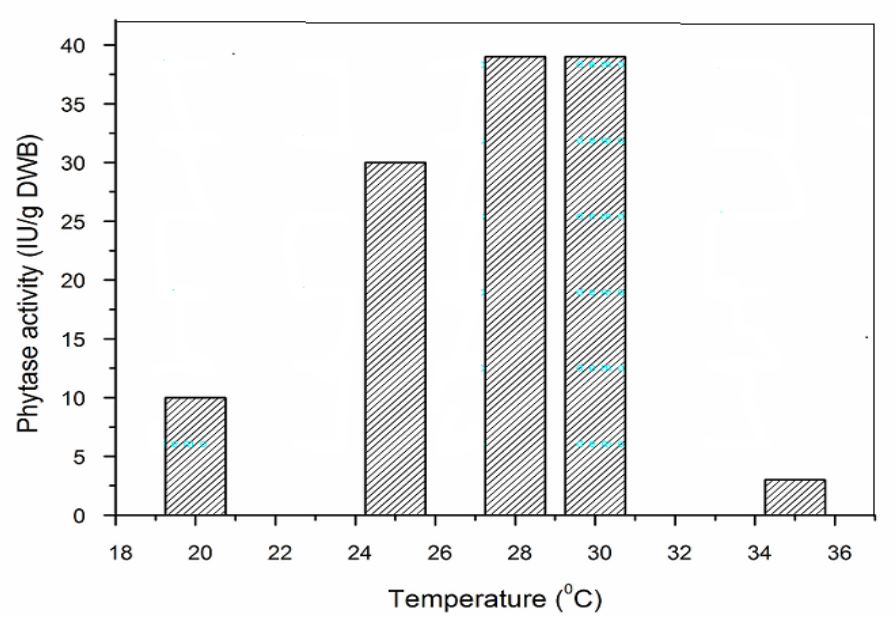


Figure 2. Effect of temperature on cell bound phytase production by *W. saturnus* NCIM 3298.

The culture was grown at different temperatures with shaking (200 rpm) as described in material and methods. The values given in the table are the average of two independent experiments.

3.4 Optimization of phytase production by RSM

Effect of seven selected factors on phytase production was investigated using PBD to identify key ingredients and the fermentation conditions to get maximum yield of cell bound phytase. The PBD design matrix for seven selected variables along with the experimental values of responses is shown in Table 5. The PBD design matrix for seven different variables showed a wide range of phytase production (10 to 269 U/g DWB). This large difference between low and high value of phytase production

suggests that the response is strongly influenced by the component(s) present in the designed medium and it also reflects the importance of carrying medium optimization studies. The maximum phytase production (269 IU/g DWB) was obtained in trial number 4. The best fit linear model to the orthogonal DOE fitting the predicted response for cell wall bound phytase was obtained as (IU g/ DWB):

Phytase activity = 134.03 + 19.27 x [glucose] - 53.07 x [citric acid] - 33.43 x [$FeSO_4$] - 23.20 x [pH] (1)

Table 5. Experimental designs used in PBD studies for seven selected independent factors along with experimental values of phytase production

Run No.	Factor							Phytase Production (IU/g DWB)
	Glucose (%)	NH_4NO_3 (%)	Yeast extract (%)	Citric acid (%)	Incubation period (h)	$FeSO_4$ (mM)	pH	
1	10	0.2	0.5	2.0	16	3.0	7.0	45
2	2.0	0.2	0.1	2.0	16	3.0	7.0	10
3	10	0.2	0.1	0.2	36	0.5	7.0	211
4	2.0	0.2	0.1	0.2	16	0.5	5.0	269
5	10	2.0	0.1	0.2	16	3.0	5.0	219
6	10	2.0	0.1	2.0	36	3.0	5.0	91
7	2.0	2.0	0.5	2.0	16	0.5	5.0	72
8	10	0.2	0.5	2.0	36	0.5	5.0	170
9	2.0	2.0	0.5	0.2	36	3.0	7.0	116
10	10	2.0	0.5	0.2	16	0.5	7.0	184
11	2.0	0.2	0.5	0.2	36	3.0	5.0	123
12	2.0	2.0	0.1	2.0	36	0.5	7.0	98

The culture was grown at 30 ^oC as described in material and methods. The values given are the average of three independent experiments

Carrying out ANOVA studies gave the sum of squares for each individual factor and the percentage of contribution for each individual term (Table 6). The analysis of half-normal plot, the total sum of squares and percent contribution showed that phytase production was influenced by glucose, citric acid, $FeSO_4$ and pH. Other variables showed very small percent contribution and they were therefore considered insignificant. Citric acid was found to be important factor in phytase production and its contributory effect was more that 50%. Earlier Spier *et al.* [31] reported the use of citrate pulp in phytase production medium by fungal isolates using solid state fermentation. This study however presents the first report on use of citric acid in the production medium for phytase production by a yeast *W. saturnus* NCIM 3298.

Table 6. Analysis of PBD for % contribution of each variable in phytase production by *W. saturnus* NCIM 3298

Variable	Contribution (%)
Glucose	6.76
NH_4NO_3	0.30
Yeast extract	4.45
Citric acid	51.21
Incubation period	0.02
$FeSO_4$	20.33
pH	9.79

The ANOVA was studied further to test the acceptability of PBD model equation 1. It gave a model F-value of 12.93 implying that the selected model is significant and there is only a 0.24% chance that a model F-value could occur due to noise (Table 7). The values of "Prob >F" less than 0.0500 indicates that the model terms are

significant. The coefficient of determination R^2=0.8808 was obtained and it provides a measure of how much of the variability in the observed response values can be explained by the analysis.

Table 7. ANOVA for the selected factors by PBD

Source	Sum of squares	Mean square	F-value	*p*-value (Prob>F)
Model	58119.64	14529.91	12.93	0.0024
Glucose	4454.45	4454.45	3.96	0.0868
Citric acid	33792.85	33792.85	30.06	0.0009
$FeSO_4$	13413.45	13413.45	11.93	0.0106
pH	6458.88	6458.88	5.75	0.0477
Residual	7868.61	1124.09		
Cor Total	65988.25			

Media components, *viz*., glucose, citric acid, $FeSO_4$ and pH were identified as key variables for phytase production using PBD. The concentrations of the 3 chemical factors and the pH were further optimized by carrying out the BBD runs. Design matrix of BBD for four selected factors with their actual level and effect on phytase production is shown in Table 8. Maximum phytase production of 297 IU/g DWB was obtained in run number 3.

Table 8. Experimental design for optimization of phytase production using BBD

Run No.	Factor				Phytase Production (IU/g DWB)
	Citric acid (%)	$FeSO_4$ (mM)	pH	Glucose (%)	
1	0.05	1.5	5.5	8.5	267
2	0.28	0.78	6.5	12.0	211
3	**0.28**	**1.5**	**4.5**	**8.5**	**297**
4	0.28	1.5	6.5	8.5	279
5	0.05	0.78	5.5	5.0	219
6	0.05	0.78	6.5	8.5	181
7	0.05	0.78	5.5	12.0	181
8	0.28	0.05	6.5	8.5	111
9	0.28	0.78	6.5	5.0	148
10	0.28	0.05	5.5	5.0	125
11	0.05	0.05	5.5	8.5	133
12	0.28	0.78	5.5	8.5	294
13	0.50	0.78	4.5	8.5	168
14	0.50	1.5	5.5	8.5	253
15	0.50	0.78	6.5	8.5	235
16	0.28	0.78	5.5	8.5	289
17	0.28	0.05	4.5	8.5	57
18	0.28	0.78	5.5	8.5	291
19	0.50	0.78	5.5	5.0	199

20	0.28	0.78	4.5	5.0	208
21	0.05	0.78	4.5	8.5	176
22	0.28	1.5	5.5	12.0	147
23	0.28	0.78	5.5	8.5	292
24	0.28	1.5	5.5	5.0	234
25	0.28	0.78	5.5	8.5	293
26	0.50	0.05	5.5	8.5	139
27	0.28	0.05	5.5	12.0	70
28	0.28	0.78	5.5	8.5	292
29	0.50	0.78	5.5	12.0	271
30	0.28	0.78	4.5	12.0	266

The culture was grown at 30 ^{o}C as described in material and methods. The values given are the average of three independent experiments

By applying multiple regression analysis on the experimental data, the following best fit quadratic equation was found to explain the phytase production (IU/g DWB),

$$\text{Phytase production} = 272.63 + 70.23 \times [FeSO_4] - 67.81 \times [FeSO_4]^2 - 39.71 \times [pH]^2 - 46.72 \times [\text{Glucose}]^2 \quad (2)$$

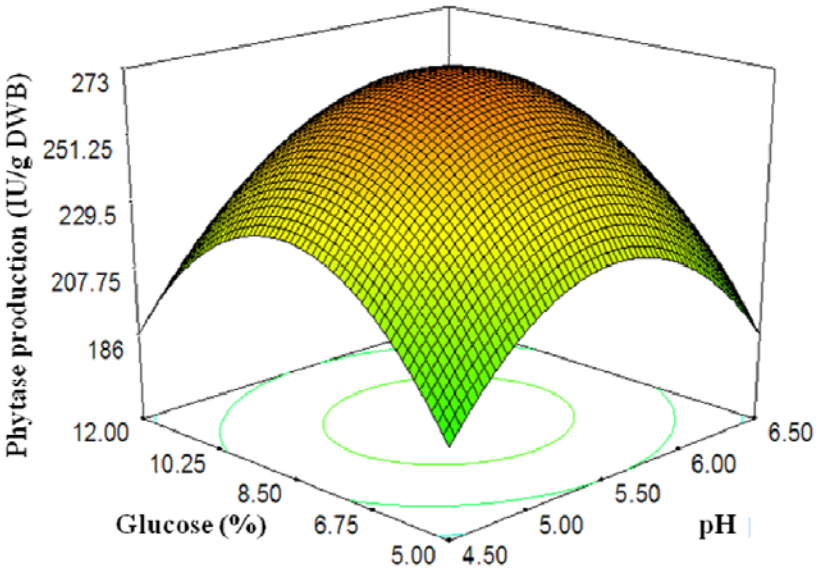
The analysis of variance (ANOVA) for the obtained quadratic regression model demonstrated that the values of the determination coefficient (R^2) and the adjusted determination coefficient (Adj. R^2) were 0.7168 and 0.6714, respectively. These indicate that a high degree of correlation exists between the observed and predicted values. The coefficient of determination $R^2 = 0.717$ implies that 71.7% of the variability is explained by the model. The Predicted $R^2 = 0.5818$ is in reasonable agreement with the Adjusted $R^2 = 0.6714$. The obtained Model F-value of 15.82 implies that the model is significant and there is only 0.01% chance that a model F-

value this high could occur due to noise. Also, values of Prob >F less than 0.0500 (<0.0001) indicated that the model terms are significant. The lack-of-fit F-value = 581.17 implies it is significant and that there is only a 0.01% chance that the lack-of-fit value this large could occur due to noise. Adequate precision measures the SNR and the obtained value of 11.148 for the given model is greater than 4 and it indicates that the model equation 2 is significant for phytase production. For phytase production, $[FeSO_4]$, $[FeSO_4]^2$, $[pH]^2$ and $[Glucose]^2$ are significant model terms. Table 9 summarizes the results of the ANOVA study.

Table 9. ANOVA for response surface reduced quadratic model of BBD

Source	**Sum of squares**	**Mean square**	**F value**	***P*-value (Prob>F)**
Model	1.070E+005	26754.03	15.82	< 0.0001
$FeSO_4$	59187.46	59187.46	34.99	< 0.0001
$[FeSO_4]^2$	32183.91	32183.91	19.03	0.0002
$[pH]^2$	11040.61	11040.61	6.53	0.0171
$[Glucose]^2$	15278.40	15278.40	9.03	0.0060
Residual	42287.9	1691.52		
Lack of Fit	42269.77	2113.49	581.17	< 0.0001
Pure Error	18.18	3.64		
Cor Total	1.493E+005			

(a)



(b)

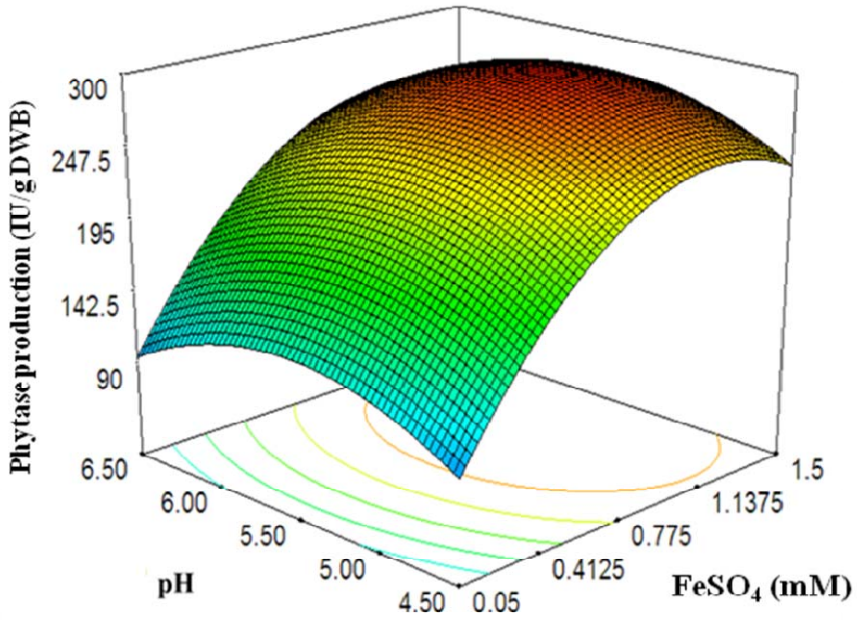


Figure 3. 3D Surface plots showing the effect of (a) glucose and pH and (b) $FeSO_4$ and pH on phytase production of *W. saturnus* NCIM 3298.

The 3D response surface graphs display the characteristic effects of key process variables on cell bound phytase production. Fig. 3 (a) demonstrates the effect of glucose and pH on phytase production while other two factors namely $FeSO_4$ and citric acid concentration are maintained at 0.78 mM and 0.28 g/100 mL, respectively. Fig. 3 (b) demonstrates the effect of pH and $FeSO_4$ on phytase production while the other two factors namely glucose and citric acid concentration are maintained at 8.5 and 0.28 g/100 mL, respectively.

The cell bound phytase production behavior was studied under optimized fermentation conditions by monitoring the response obtained as a time-course for 48 h. The basal observation with an unoptimized medium (per 100 mL): glucose, 4 g; citric acid, 0.5g ; NH_4NO_3, 0.5g; yeast extract, 0.3 g and 1 mM $FeSO_4$, pH 5.5) showed a phytase production of 46 IU/g DWB whereas studies with the optimized levels found by BBD gave phytase production of 299 IU/g DWB. The result thus shows a remarkable 6.4 times increase in phytase production. The experiment also validated the model.

It was found that citric acid played an important role in phytase production. Phytase production in absence of citric acid declined drastically after 24 h of incubation as shown in Fig. 5. However in presence of citric acid, phytase production continued even after 24 h of fermentation with slight decrease in production rate (Fig. 4).

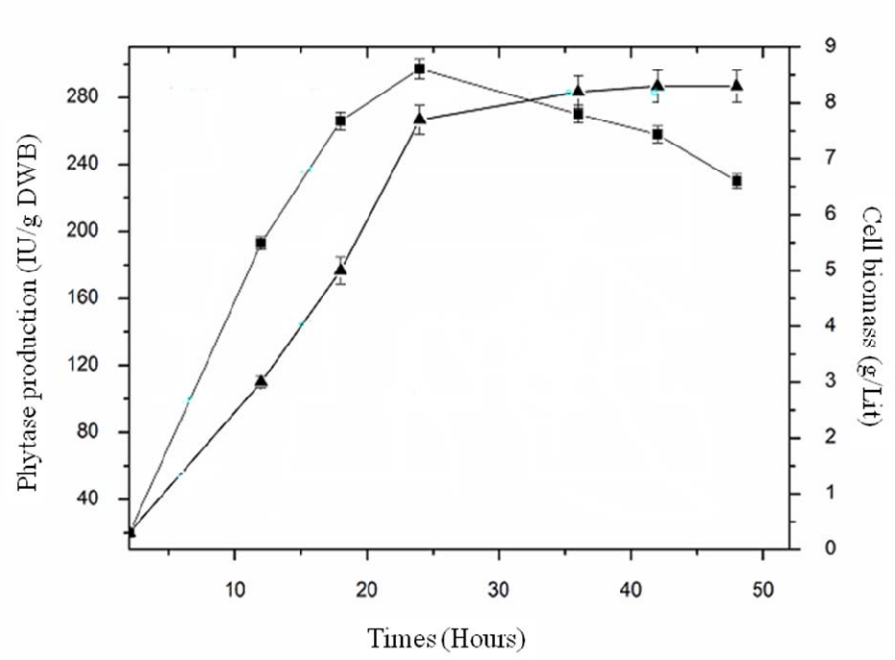


Figure 4. Time course of cell bound phytase production by *W. saturnus* NCIM 3298 using optimized medium.

Phytase production (IU/g DWB) (--- ■--); Biomass (g/Lit) (--- ▲-)

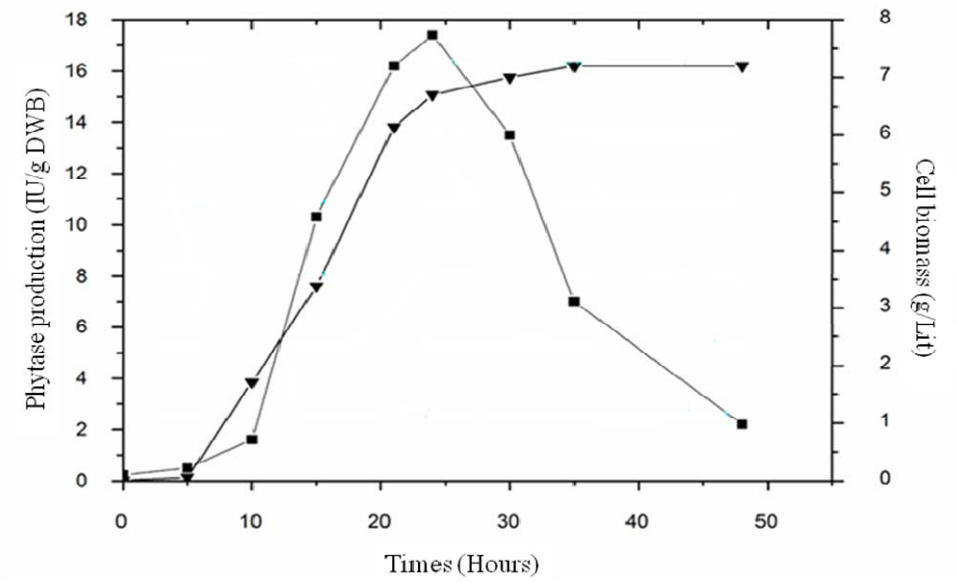


Figure 5. Time course of cell bound phytase production by *W. saturnus* NCIM 3298 using unoptimized medium.

Phytase production (IU/g DWB) (--- ■--); Biomass (g/Lit) (--- ▲-)

Phytase production in presence of citric acid was declined to 265 IU/g DWB at 48 h of incubation with small increase in cell biomass. Moreover, the pH of optimized medium remained same as initially adjusted value (pH 4.5) whereas in medium without citric acid it declined from 5.5 to 3.0 after 24 h of incubation. This may be due to the buffering capacity of the citric acid that prevented change in pH during fermentation and this could be responsible for observed phytase production even after 24 h of fermentation in optimized medium. Other reason for higher contribution of the citric acid for enhanced phytase production could be chelation of the P from the medium. Citric acid is known for its metal ions chelating property. Removal of P from the medium or low P medium enhances the phytase production [29, 32].

Table 10. Comparison of cell bound phytase production by *W. saturnus* NCIM 3298 with other yeast strains grown under submerge conditions

Phytase source	**Cell bound phytase activity (IU/g DWB)**
W. saturnus NCIM 3298	297
Pichia anomala	120 [15]
Pichia anomala	1781* [33]
Cryptococcus laurentii ABO510	4.55 [23]
Cryptococcus laurentii AL_{27}	25 [30]
Candida krusei	201* [29]
Saccharomyces cerevisiae CY	135.09 [34]

*** Activity calculated as nmol/sec**

The optimized results showed a production of 297 IU/g DWB which is the highest as compared to other reported cell bound phytases (Table 10). Our experiments using

RSM gave a total of 6.45 fold increase in phytase production which was more compared to 5.0 fold increased reported for *P. anomala* using Central Composite Design method [15].

3.5 Up scaling

Cell bound phytase production was performed in 50 mL medium in 250 mL flask as mentioned in Section 2.3. The procedure was up scaled using optimized medium from 50 mL in 250 mL Erlenmeyer flask to 2 lit in 2.5 lit fermenter (Table 11). Up scaling the phytase production from 50 mL shaking flak condition to 2 lit fermenter showed reproducibly in phytase production i.e. 297 IU/g DWB. These results are therefore encouraging further for optimization under pilot scale conditions.

3.6 Phytase production using agricultural residue and industrial waste

Different agricultural and industrial wastes were tested for phytase production as mention in Table 11. Cane molasses and cane juice media were found to be good substrates for cell bound phytase production by *W. saturnus* NCIM 3298. Phytase production for cane molasses and cane juice medium were 153 and 198, respectively. Kaur and Satyanarayana reported the use of cane molasses for phytase production by *P. anomala* [33].

Table 11. Up scaling of phytase production in optimized medium and phytase production using agricultural and industrial waste

Properties	Cell bound phytase activity (IU/g DWB)
Up scaling using optimized media	
50 mL in 250 mL flask	297
300 mL in 1000 mL flask	296
2000 mL in 2500 mL fermenter	303
Production using agriculture waste	
5% Rice bran extract	64
1% Chickpea extract	72
Production using industrial waste	
50% Cane juice	198
10% Cane molasses	153
5% soy whey	109

The culture was grown at 28 ^{o}C (pH 5.5) as described in material and methods. The values given are the average of two independent experiments

Cane molasses and cane juice media (70–80 mM) contained more free inorganic phosphorus compared with glucose-yeast extract media (20 mM) and still showed higher phytase production. This implies that inorganic phosphorus in these media does not suppress the phytase production. Fredrikson *et al*. suggested that, either inorganic phosphorus in complex medium was not as effective in repressing phytase production, or promotion of phytase synthesis in complex medium was governed by a component absent in the minimal medium [35]. Rice bran extract, chickpea extract and soy whey were also found to be good substrates for phytase production. Soy whey has been used employed in the medium for extracellular phytase production by *S.*

cerevisiae strain using RSM [36]. Similarly, combination of Rice bran and soybean meal extract has been used for thermo-acido-tolerant phytase production by soil bacterium [37].

3.7 Localization of Enzyme

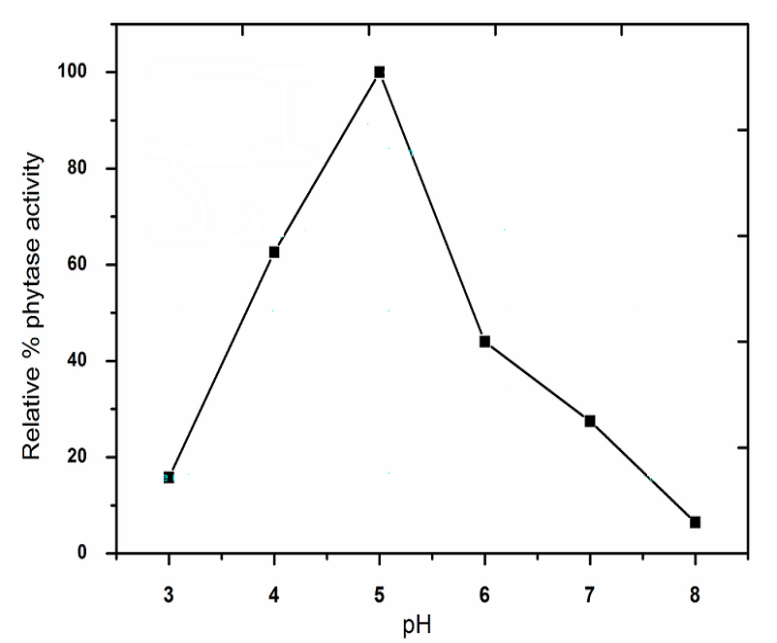
The phytase activity of *W. saturnus* NCIM 3298 was associated with the whole cells, whereas no phytase activity was found in the cell-free extract after permeabilization with physical and chemical methods. Correspondingly, treatment with the lysing enzyme or sonication increased the phytase activity in the cell-free extract, suggesting that the *W. saturnus* NCIM 3298 phytase is cell bound but not the intracellular or not bound on the outer surface of the cell. Cell associated phytase activity have also been found in other yeasts and bacteria, such as *P. anomala, C. krusei* and *E. coli* [26, 29, 38].

3.8 Characterization of cell bound phytase from *W. saturnus* NCIM 3298

As depicted in Fig. 6, the pH and temperature optima for cell bound phytase from *W. saturnus* NCIM 3298 were pH 5.0 and 50 ^{o}C, respectively. This pH value is comparable with those of other yeast phytases, with optima between 4.0 and 5.5 [26, 29, 39]. Temperature optima for *P. anomala* and *C. laurentii* ABO510 were reported at 60 ^{o}C, while for *C. krusei* this value was at 40 ^{o}C. The phytase was active over pH range 3.0 to 7.0 and temperature range of 30 to 80 ^{o}C. More than 60% of the activity was retained at acidic pH between 4.0 and 5.0. Thermal stability of the enzyme was determined by exposing the cells to temperatures of 70 ^{o}C and 80 ^{o}C for 5 min. Yeast retained about 75–80% phytase activity after exposure to a temperature of 70 ^{o}C for 5

min, but no residual activity was observed after exposure to a temperature of 80 ^{o}C for 5 min (data not shown).

(a)



(b)

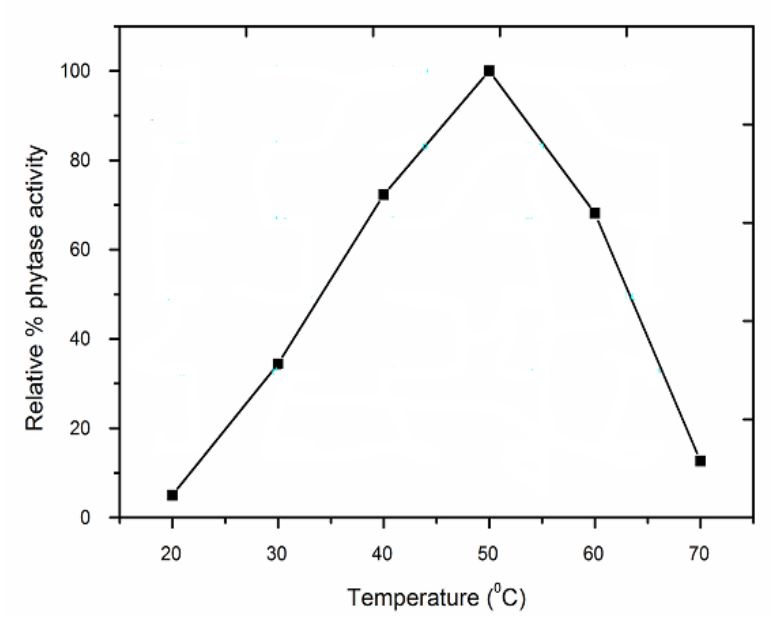


Figure 6. Effect of (a) pH and (b) temperature on cell bound phytase activity of *W. saturnus* NCIM 3298. The values are the mean of three independent experiments.

Effect of different ionic and non-ionic detergents was studied as shown in Table 12. None of the detergent was able to stimulate the enzyme activity. Enzyme activity in presence of non-ionic detergents; Brij C 10, Tween 80, Triton-X 100, n-Dodecyl *β*-D-maltoside, Octyl *β*-D-glucopyranoside was slightly inhibited. Anionic detergent (SDS), even at low concentration severely inhibited the phytase activity. Zwitterionic detergent CHAPS had no effect on enzyme activity. Acetone, butanol, DMSO and diethyl ether had inhibitory effect on enzyme activity and about 55% to 60% activity lost in presence of these solvents. However, toluene and benzene showed moderate inhibitory effect on enzyme activity. Various solvents such as alcohol, isopropanol, acetone, butanol and DMSO have also been reported to permeabilize yeast cells [40]. Triton X, SDS, Tween 80, Digitonin and CTAB have been shown to inhibitor for phytase activity from *R. gracilis* [41]. Quan *et al.* has also been reported the inactivation of *C. krusei* phytase in presence of 1% SDS [29].

Table 12. Effect of detergents and solvents on *W. saturnus* NCIM 3298 phytase activity

Effect	Relative enzyme activity (%)
Control	100
Brij C 10 (0.1% w/v)	78.6
CHAPS (0.1% w/v)	72.3
n-Dodecyl *β*-D-maltoside (0.1% w/v)	46.9
Octyl *β*-D-glucopyranoside (0.1%w/v)	40.0
SDS (0.5% w/v)	26.7
SDS (0.1% w/v)	37.7
Tween 80 (0.5%v/v)	69.2

Tween 80 (0.1%)	78.8
Triton X-100 (0.1% v/v)	70.0
Toluene (20% v/v)	62.6
Benzene (10% v/v)	60.5
Butanol (40% v/v)	41.9
Diethyl ether (50% v/v)	39.8
Acetone (50% v/v)	45.2
DMSO (15% v/v)	39.3

The activity in the absence of a detergents and solvents was defined as 100%. The values are the mean of three independent experiments.

The effect of metal ions was studied by adding metal ion at 1 mM and 10mM final concentration in a reaction mixture using 200 mM Na-acetate buffer, pH 5.0 at 50 ^{o}C (Table 13). The cations; Na^{+}, K^{+} and Mg^{+} did not significantly affect the phytase activity when added at a concentration of 1mM but showed a stimulatory effect at 10 mM this in contrast to phytase of *C. krusei* WZ-001 that was strongly inhibited by Mg^{2+} (1 and 5 mM). Ba^{+2} had stimulatory effect at concentration of 1mM while at 10mM concentration it exhibited strong inhibitory effect. However, phytase activity intensely inhibited by Cu^{2+} and Zn^{2+} (1and 10 mM) this was similar to inhibition of phytase from *P. anomala* and *S. castellii* [28, 42]. Effect of Fe^{2+} on phytase activity was same for both concentrations. Fe^{2+} was strong inhibitor of phytase activity [42]. 10 mM concentration of Cd^{2+}, Hg^{2+}, Mn^{2+} and Ca^{2+} was found inhibitory for enzyme activity.

The K_m for cell bound phytase was 0.2 mM when sodium phytate was used as substrate which is similar to the K_m value reported for purified phytase of *P. anomal*

[42]. K_m values reported for *S. castellii* [28], *C. krusei* WZ-001[43] and *A. adeninivorans* [25] were 0.038, 0.03 and 0.25 respectively.

Table 13. Effect of metals on phytase activity from *W. saturnus* NCIM 3298

Metal ions	Residual Relative activity (%)	
	1 mM	10 mM
Control	100	100
Cd^{2+}	70.4	11.1
Hg^{2+}	75.5	15.3
Mn^{2+}	87.7	25.6
Co^{2+}	102.6	42.0
Cu^{2+}	44.1	-1.1
Zn^{2+}	16.7	10.2
Ba^{2+}	127.1	17.6
Fe^{2+}	60.3	51.7
Mg^{2+}	86.3	129.1
Ca^{2+}	98.8	46.3
Na^{+}	95.8	137.2
K^{+}	94.7	160.4

The activity in the absence of a metal ion was defined as 100%. The values are the mean of three independent experiments.

In summary, the present study optimized the level of medium components using PBD and BBD for improved phytase production. The phytase production increased from 46 to 297 IU/ g DWB after media optimization and accounts for an overall 6.45 fold

increase in phytase production. Citric acid was found to be a key media variable whose contribution was about 50% among the all tested variables in PBD study. $FeSO_4$, pH and glucose showed significant effects while yeast extract, NH_4NO_3 and incubation period did not show considerable effect on phytase synthesis. The pH and temperature optima for phytase activity were 5.0 and 50 ^oC, respectively. Permeabilizing agents showed inhibitory effect on cell bound phytase activity. Of the different metal ions, Co^{2+}, Ba^{2+}, Ca^{2+} (1mM) and K^+, Na^+, Mg^{2+}(10 mM) were found enzyme activity stimulators whereas Hg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Ba^{2+} (10 mM) were strong inhibitors for phytase activity. Localization studies revealed that phytase activity was cell bound but not intracellular. Cane molasses, cane juice, soy whey, rice bran and chickpea extract, were found a good substrates for phytase production.

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CHAPTER 4

Applications of *Williopsis saturnus* NCIM 3298 phytase to improve the nutritional value of feed

This chapter discusses about the application of phytase from *W. saturnus* NCIM 3298 for improved availability of P of different cereals and pulses, which are commonly used in the poultry feeds. Addition of phytase to grinded chickpea significantly enhanced the mineral bioavailability by approximately 40%, 32.1% and 42% for Fe^{2+}, Zn^{2+}, and Ca^{2+}, respectively. HPLC analysis revealed that there was 80% decrease in the total PA content in phytase treated chickpea (CP). P availability from soybean, rice, maize, sorghum and wheat after phytase treatment was improved by 2.6, 2.3, 2.5, 2.0 and 1.3 times, respectively, as compared to the control. This suggests that the addition of phytase has significant role for improved P and minerals availability from feeds and foods.

1. Introduction

PA frequently occurs in nature and constitutes the principal storage form of P (60-90%) and inositol in plants, legumes and oilseeds [1, 2]. It is primarily present as a salt of monovalent and divalent cations (Fe^{2+}, Mn^{2+}, K^{+}, Mg^{2+} and Ca^{2+}) and accumulates in seeds during the maturation period. PA is, therefore, a common constituent of plant derived foods.

The chemical structure of PA is indicative of its strong chelating ability. PA has six phosphate groups, corresponding to 12 dissociable protons, six of these are strongly dissociated (p*Ka* 1.1 to 2.1) while other six are weakly dissociated (p*Ka* 4.6 to 10.0). PA, therefore, exists as a highly negatively charged ion over a broad pH range and thus has a tremendous affinity for food components with positive charge(s), such as minerals, trace elements and proteins [3]. It is considered an anti-nutrient because it acts as a strong chelator of divalent and trivalent minerals such as Mn^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}/Fe^{3+} [4]. The formation of insoluble mineral-PA complexes at physiological pH is regarded as the main reason for the poor mineral bioavailability, essentially because these complexes are difficult to assimilate in the animal and human gastrointestinal tract [3]. Minerals are involved in activation of intracellular and extracellular enzymes, in regulation of critical pH levels in body fluids necessary for the control of metabolic reactions and in osmotic balance between the cell and its environment. Lopez *et al.* reported that deficiency of any one of the essential minerals can result in severe metabolic disorders and compromise the health of the organism [5].

Legumes and cereals are good sources of proteins as well as dietary minerals such as Fe^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}. Jambunathan *et al*. and Attia *et al*. have mention that, the content of Fe^{2+}, Zn^{2+} and other minerals is generally high in legumes [6, 7].

However, their applications as food are limited by PA, which acts as an anti-nutrient, resulting in decline of nutritive value of these legumes. To increase the bioavailability of minerals from legume-based foods, enzymatic degradation of the PA and its dephosphorylated isomer inositol pentaphosphate is desirable [8].

Phytases are PA degrading enzymes present in the plants, animal tissues and a large number of bacteria and fungi. However, monogastric animals, fishes and humans are unable to utilize dietary PA, due to lack of adequate levels of phytases. Therefore, hydrolysis of PA content in the foods and feeds before or after consumption using external phytases is essential to improve their nutritional value. Several reports have shown that supplementation of phytase in animal feed increases the availability of P for animal digestion through degradation of the PA [9, 10]. The great potential for use of phytase in processing and manufacturing of food for human consumption has been proposed [11-13], but it has not been applied to date. Research in this field has focused on obtaining the appropriate phytase producing microbial strain, improvement of nutritional value of plant based foods as well as improving the techniques of food processing [3].

Increasing demand for the poultry products in India needs increase cultivation of broilers and layers. Poultry feed is mainly based on cereals and pulses. India is second most in the world for CP production. CP is a rich source of proteins, carbohydrates, minerals and hence can be a good feed ingredient for poultry. The objective of the present study, therefore, was to determine the effect of dephytinization using phytase on P, zinc, iron and calcium bioavailability of CP. We have also tried to find out the effect of phytase on the P availability from rice, maize, sorghum, wheat, and soybean.

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. AG-1-X8 anion exchange resin (200-400 mesh chloride form) was purchased from sigma chemicals. The cereals and pulses; rice, maize, sorghum, wheat, soybean and CP, used in this study were purchase from local market.

2.2 Preparation of Cell-Free Extract

Enzyme extraction was carried out in two steps. Cells from *W. saturnus* NCIM 3298 were washed thrice with distilled water and resuspended in lysing buffer (5 mM mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 M KCl, 2 M sorbitol, 0.2 M acetate buffer pH 5.0) containing 1 µg/mL of lytic enzyme. Cells were incubated at RT on rotary shaker (100 rpm) for 6 h and subsequently sonicated for 15 min. The homogenate was centrifuged at 6,000 x *g* for 20 min at 4 ^{o}C. Supernatant was dialyzed against 0.1 M Na-acetate buffer pH 5.0 to remove excess salt and lyophilized to concentrate the enzyme solution. This crude extract of enzyme was used for dephytinization of CP.

2.3 Phosphate Liberation from chickpea (CP)

To determine the amount of phosphate liberated from chickpea after phytase treatment, 1 g of grinded CP sample was suspended in 10 mL 200 mM acetate buffer (pH 5.0). Suspension was incubated with and without the supplementation of 1 IU/mL of crude phytase of *W. saturnus* NCIM 3298 at 37 ^{o}C on a rotary shaker (100 rpm).

Aliquots were obtained at fixed intervals and centrifuged at 6000 x *g* for 15 min. The liberated Pi was quantified by standard procedure as mentioned in analytical method.

2.4 Phosphate Liberation from Soybean, Rice, Maize, Sorghum and Wheat

To determine the amount of phosphate liberated from soybean, rice, maize, sorghum and wheat after phytase treatment, 1 g of grinded sample of each grain was suspended in 10 mL 200 mM acetate buffer (pH 5.0). Suspensions were incubated with and without the supplementation of *W. saturnus* NCIM 3298 cells corresponds to 1 IU/mL activity at 37 ^{o}C on a rotary shaker (160 rpm) for 2 h. Aliquots were obtained at fixed intervals and centrifuged at 6000 x *g* for 15 min. The liberated Pi was quantified by standard procedure as mentioned in analytical method.

2.5 Analytical Methods

2.5.1 Inorganic phosphorus estimation

After enzymatic treatments to cereals and pulses, aliquots were subjected to P estimation. The liberated P was measured by a modification of the ammonium molybdate method. A freshly prepared solution of acetone: 5N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400µl 1M citric acid was added to the sample mixture. Absorbance was measured at 370 nm. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments.

2.5.2 Mineral Analysis

The ability of phytase to increase bioavailability of PA bound minerals (Ca^{2+}, Fe^{2+},Zn^{2+}, Mn^{2+} and Mg^{2+}) from CP was quantified using an Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Digestion of CP for total mineral

analysis was carried out according to Friel and Ngyuen [14], with slight modification. CP was dried in oven at 80 ^{o}C until constant weight was obtained. One gram of dried CP was weighed in crucible and heated at 500 ^{o}C for 4.30 h. After cooling at RT, samples in crucibles were mixed with 5 mL of HNO_3 : $HClO_4$ (4:1) and heated at 120–140 ^{o}C until a clear solution was obtained, which was filtered through Whatman filter paper (0.2 μm, Pall Corporation, Port Washington, NY). This filtrate was used for total mineral analysis in untreated CP. For determination of mineral mobilization after phytase treatment, 1 g of dried CP was suspended in 10 mL of 200 mM Na-acetate buffer pH 5.0, containing 1 IU/mL of crude phytase and incubated at 37 ^{o}C on a rotary shaker at 100 rpm for 2 h. Samples were centrifuged at 10,000 x *g* for 10 min at 4 ^{o}C, supernatant was filtered through Whatman filter paper and filtrate was collected. Filtrate was diluted with distilled water for adjusting the signals in the calibration ranges of each element. Zn^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+} contents in the samples were measured by ICP-AES. The experiment was conducted in duplicate, and the mean values and standard errors reported.

Mineral availability (%) was calculated as follows:

Mineral availability (%) = 100 × *Y*/*Z*, where *Y* is the element content of the bioavailable fraction (mg mineral element / 100 g CP), and *Z* is the total Zn^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+} content (mg mineral element / 100 g CP).

2.5.3 Determination of PA using High-Performance Liquid Chromatography

PA extraction, clean-up and quantification: The leftover pellet of CP after phytase treatment was used to estimate the residual PA content. PA extraction and clean up were carried out on the basis of the modified procedure of Latta and Eskin [15]. Briefly, 1 g of phytase treated and untreated CP were extracted with 2.4% HCl (20

mL) by incubating on rotary shaker at 150 rpm for 1 h at RT. 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 P were removed by washing with distilled water (10 mL) followed by 100 mM NaCl (10 mL). Bound PA was eluted with 700 mM NaCl (10 mL). The elutant was evaporated to dryness with a Speedvac concentrator at 40 ^{o}C, and the residue was dissolved in mobile phase (0.008 N, H_2SO_4). The PA content in CP before and after phytase treatments were determined using a high-performance liquid chromatography (HPLC) system (Dionex India Ltd, Mumbai, Maharashtra, India) equipped with UV/RI-detectors. An ion exclusion column (Aminex, HPX-87H, Biorad, Hercules, CA) was used at a temperature of 30 ^{o}C with 0.008 N H_2SO_4 as a mobile phase at flow rate of 0.6 mL/min. The solution was centrifuged at 10,000 x *g* for 5 min to remove any suspended material prior to injection of 50 µL into HPLC. The concentration of PA was quantified based on standard curve created with Na-phytate (100–1000 µg). The experiment was conducted in duplicate; the mean values are presented.

3. Result and discussion

3.1 Effect of phytase on PA content of the CP

Dephytinization ability of the phytase from *W. saturnus* NCIM 3298 was studied by incubating it with the CP for 2 h. The PA content of the phytase treated and untreated CP are shown in Fig. 1. PA content in untreated CP that estimated using HPLC was 950 mg/100 g and was found to be comparable with previous values reported by Chitra *et al*., Nestares *et al*. and El-Adawy *et al*. [16-18]. In this study, we observed

that about 80% of the total PA content of CP was hydrolyzed by the phytase treatment after 2 h at 37 ^{o}C. Lopez *et al*., also, reported the hydrolysis of whole wheat flour phytate, which was observed after 2 h of incubation with lactic bacteria strains [19].

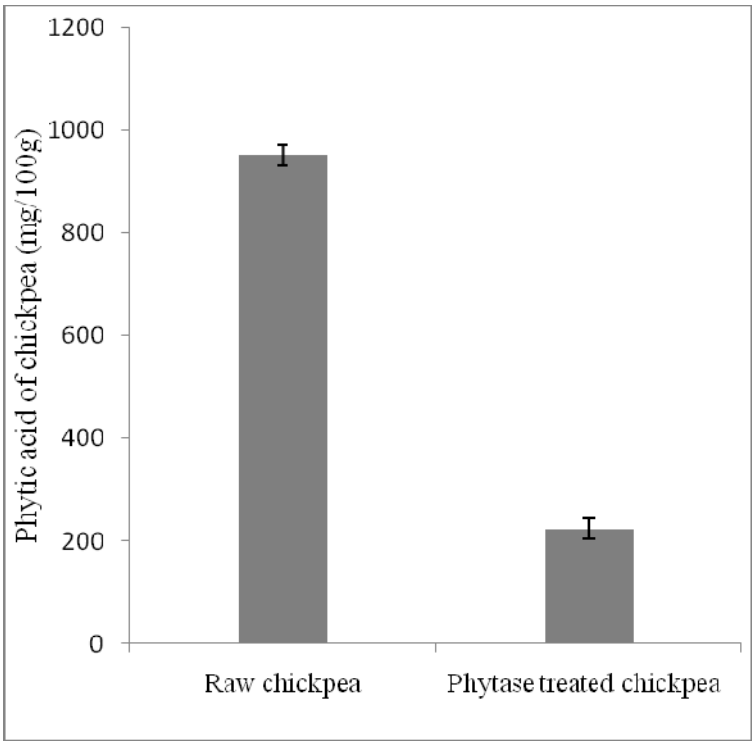


Figure 1. PA content (mg/100 g) in phytase treated and untreated CP at 37 ^{o}C in 10 mL acetate buffer (pH 5.0) after 2 h. Results are expressed as the mean of two independent experiments.

Duhan *et al*. reported that the cooking of soaked chickpea seeds lowered PA content by 20-26% [20], whereas fermentation reduced the PA content by 26-39% [16]. Our results showed that the phytase treatment in the processing of CP was very efficient in hydrolyzing PA as compared to the previously reported treatments [16, 20]. This result, therefore, suggest the potential use of studied yeast phytase in releasing PA-bound P from various commercial cereals and pulses based foods and feeds.

3.2 Effect of phytase on phosphorus liberation from CP

Treatment of phytase to CP was further studied to determine the time-course of the P release. Results for P liberation from CP with and without phytase treatment are shown in Fig. 2. Difference between phytase treated and untreated CP with respect to P liberation was very significant. Free P content of the raw chickpea was 236 mg/100 g estimated as described in material and methods. Free P content teported by El-Adawy was comparable with the present study [18].

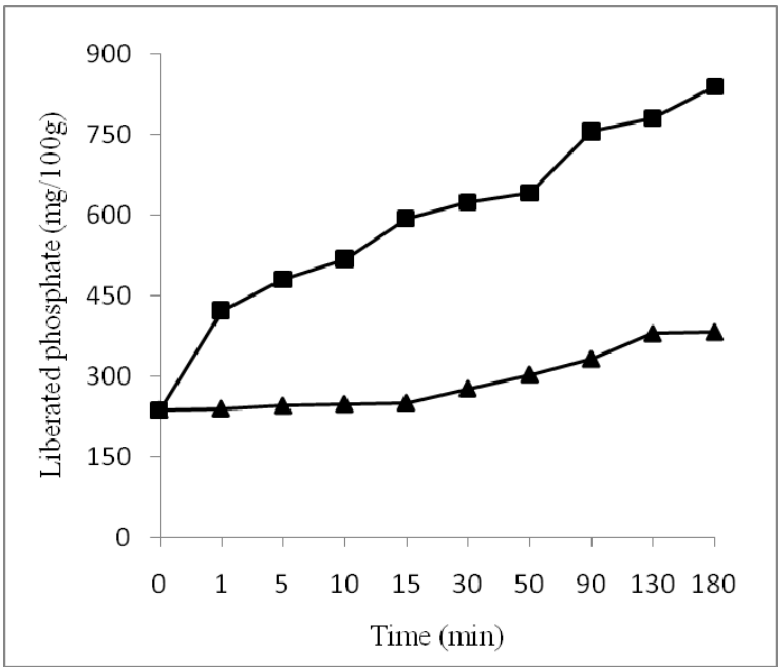


Figure 2. Release of phosphate from CP after the treatment of crude cell-free extracts at 37 ^{o}C in 10 mL acetate buffer (pH 5.0) for 2 h. Results are expressed as the mean of three independent experiments.

For untreated control, free P content was increased to 380 mg/100 g, whereas for phytase treated CP it was increased to 780 mg/100 g after 2 h of incubation. Thus about 2.0 times more P release was observed in phytase treated CP than control suggesting that studied phytase is more efficient in releasing phytate bound P. Furthermore, the value for liberated P was about six times more than that of

hydrolyzed phytic acid in terms of mM (data not given) and hence suggests that the liberated P was of PA.

3.3 Effect of phytase on mineral availability of CP

Effect of dephytinization on mineral bioavailability was studied by analyzing the minerals content of the CP before and after phytase treatment. Mineral content (Zn^{2+}, Fe^{2+} and Ca^{2+}) of raw CP are shown in Table 1. The average concentration of Zn^{2+}, $Fe^{2+}$and Ca^{2+} in untreated CP were 2.1, 2.4 and 136 mg per 100 g, respectively, on a dry weight basis determined using ICP-AES. The estimated concentrations of minerals in untreated CP were comparable with that of Alajaji and Srinivasa [21]. Many researchers have mentioned that the phytate/mineral molar ratios are indicators of mineral bioavailability and it can be used to predict its inhibitory effect on the bioavailability of the minerals [22, 23]. Molar ratio of phytate:iron less than one showed adequate availability of iron, while more than one regarded as indicative of poor iron bioavailability [24]. Zinc absorption is greatly reduced and resulted in a zinc deficiency when the phytate/zinc molar ratio is 15 [22]. In this study, we observed the molar ratio of Phytate:Zn and Phytate:Fe was above 15 for CP, which hence predicted a low rate of Zn and Fe absorption for this legume.

Table 1. Phytic acid, phosphorus and mineral content of raw chickpea

Treatment	PA mg/ 100 g	mg/ 100 g of dry weight			PA/Zn Molar ratio	PA/Fe Molar ratio
		Fe	Zn	Ca		
Raw	950	2.4	2.1	136	44	33

Data are expressed as mean of two replicated measurements on a dry weight basis

The molar ratio of phytate: Zn and phytate: Fe was found 44 and 33, respectively. These results suggested that hydrolysis of PA is required to improve mineral bioavailability of CP.

Effect of phytase on mineral availability of CP is given in Fig. 3. Mineral accessibility in phytase untreated CP were 40%, 32.1% and 42% for Fe^{2+}, Zn^{2+} and Ca^{2+}, respectively. In phytase-treated CP, the accessibility of Fe^{2+}, Zn^{2+} and Ca^{2+} increased to 71%, 57% and 76% respectively, which was significantly higher than untreated CP. This study suggests that dephytinization of CP has a significant influence on mineral availability. Sandberg and Svanberg [25] have shown that the digestion of cereal samples with phytase led to increase in accessibility of Fe^{2+} significantly. Similar observation was also recorded in the present study. The increase in mobilization of Zn^{2+}, Fe^{2+} and Ca^{2+} was 25%, 30% and 34%, respectively, suggesting that improved accessibility was achievable.

The correlation between release of minerals and liberation of phosphate from PA showed that about 25–35% increase in mineral release was observed per 2 times more phosphate released from phytase treated CP. *In vitro* solubility of Ca^{2+}, Fe^{2+} and Zn^{2+} in CP was improved after the application of phytase. The minerals were released in the order $Ca^{2+} > Fe^{2+} > Zn^{2+}$. Jin and Ma earlier reported that cation capacity to bind PA was in the order $Cu^{2+} > Zn^{2+} > Fe^{2+} > Ca^{2+}$, and the stability of the mineral–phytate complex was in the order $Zn^{2+} > Cu^{2+} > Ca^{2+}$ [26]. In the present study, it was observed that Zn^{2+} was less accessible as compared with Ca^{2+}, which may be due to weaker binding power of Ca-phytate complex than Zn-phytate complex. The mineral content of legumes is generally high, but the presence of PA inhibits its mobilization by chelating the minerals [27].

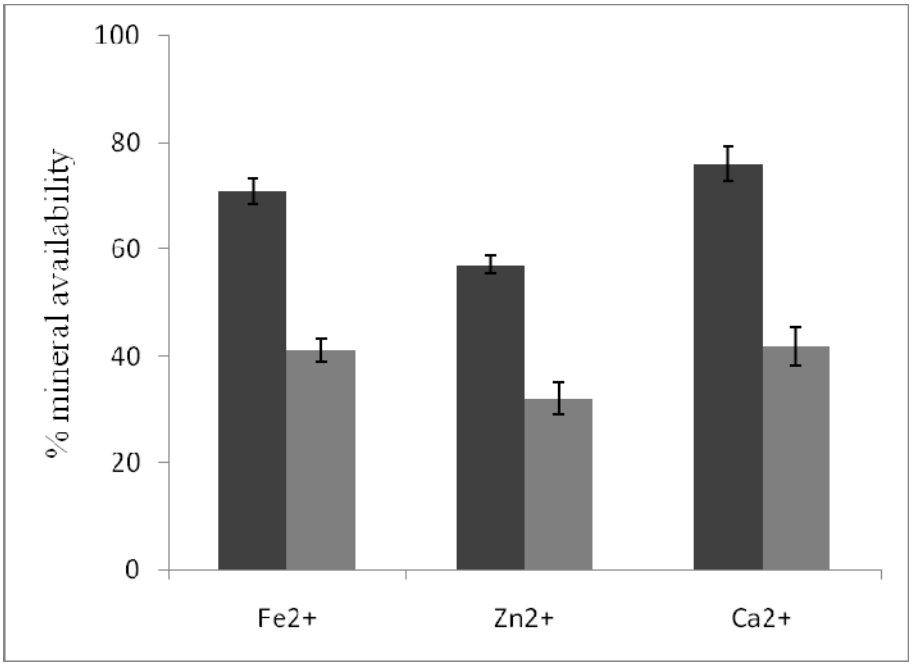


Figure 3. % Mineral mobilization for CP after treatment of crude phytase at 37 ^{o}C in 10 mL acetate buffer (pH 5.0) over 2 h. Results are expressed as the mean ± SD of duplicate samples.

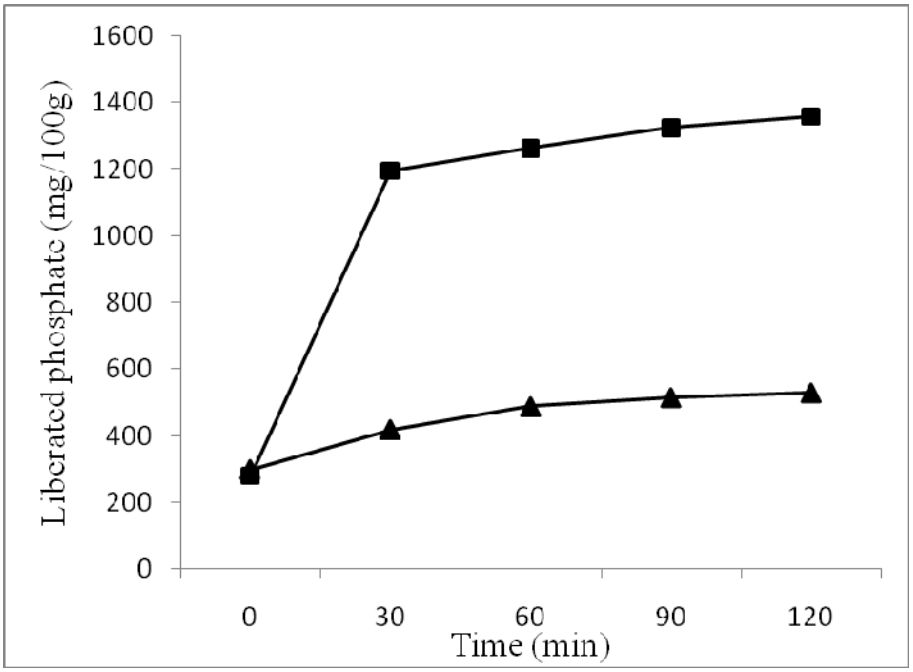
Phytase treated ( ), Untreated Control ( )

3.4 Phosphate liberation from Soybean, Rice, Maize, Sorghum and Wheat

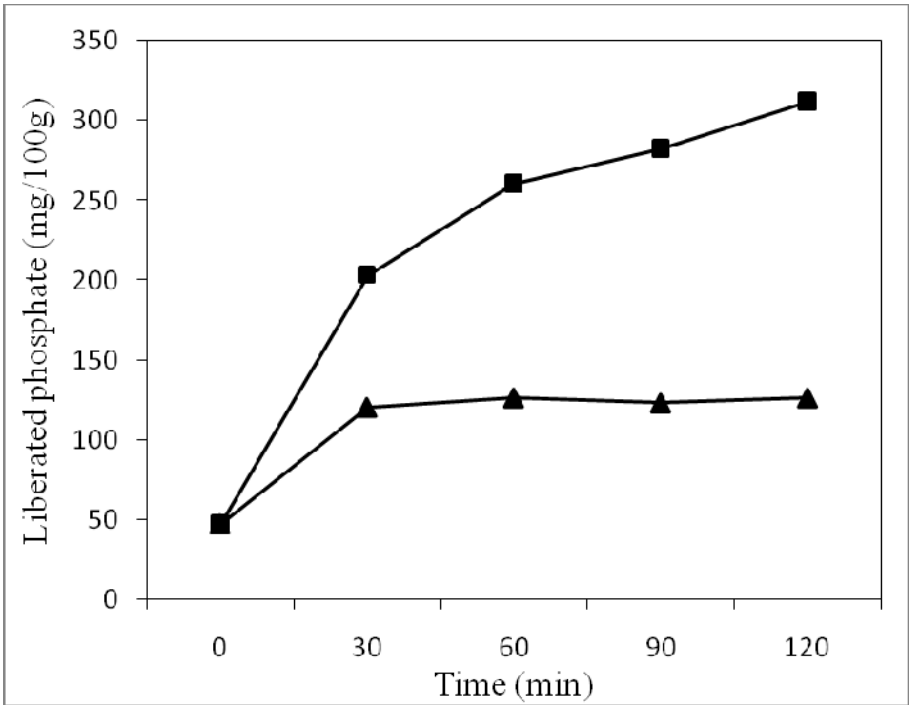
In vitro studies were carried to examine the effect of phytase on P liberation from soybean, rice, maize, sorghum and wheat, which are generally supplemented in poultry feed. Given the same enzyme activity, measured at pH 5.0 using Na-phytate as a substrate, the amount of inorganic P released by the cereals and pulses after incubation with and without phytase was determined for 2 h (Fig. 2). Observed concentrations of P in phytase treated soybean, rice, maize, sorghum and wheat were comparable with the values reported by Anderson [28], Wilcox [29], Afify Ael [30], Radhakrishnan [31], Liang [32], Erdal [33] and Sokrab [34] for free P present in these grains. Supplementation of the soybean, rice, maize, sorghum and wheat with yeast phytase resulted in significant increase in P liberation as compared to control. The

increase in P liberation was correlated with increased time of phytase treatment. Effect of phytase was more significant for soybean, maize, and sorghum. The values for released inorganic P were 1357, 312 and 393 mg/100 g for soybean, maize and sorghum, respectively. These values were significantly higher than the control values, 529, 129 and 199 mg/100 g for soybean, maize and sorghum, respectively. Thus, about 2.6, 2.5 and 2.0 times more inorganic P were liberated in the presence of phytase from soybean, maize and sorghum, respectively. In phytase treated soybean, about 90% inorganic P was liberated within first 30 min as compared to total P released after 2 h incubation. Bilyeu at el. reported that Pi was rapidly liberated from soybean meal, and reached a plateau within 4 h after bacterial phytase treatment [23]. In case of the maize and sorghum P was liberated gradually.

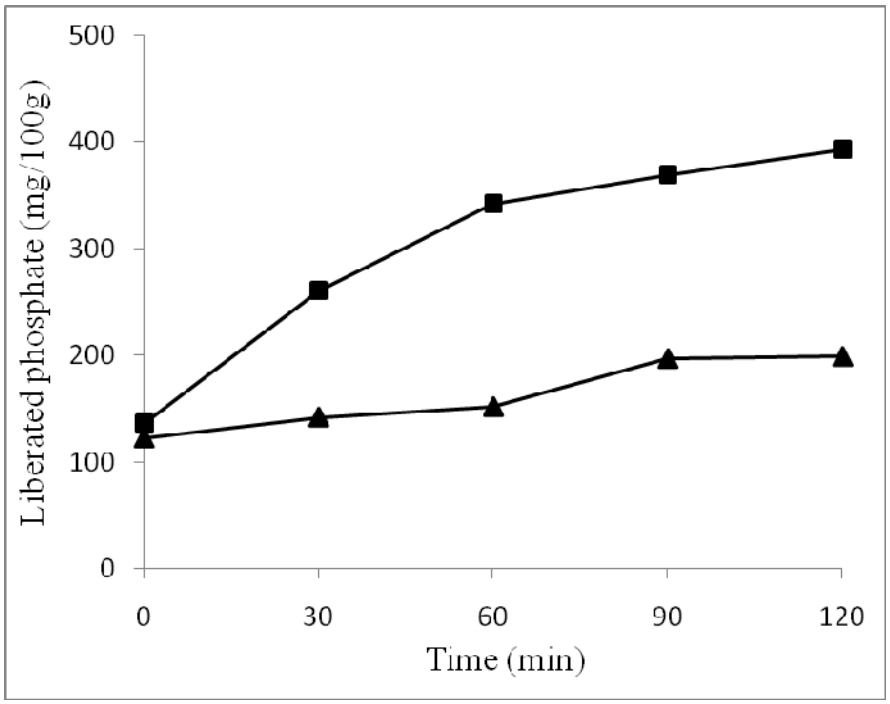
(a) Phosphate liberation from soybean



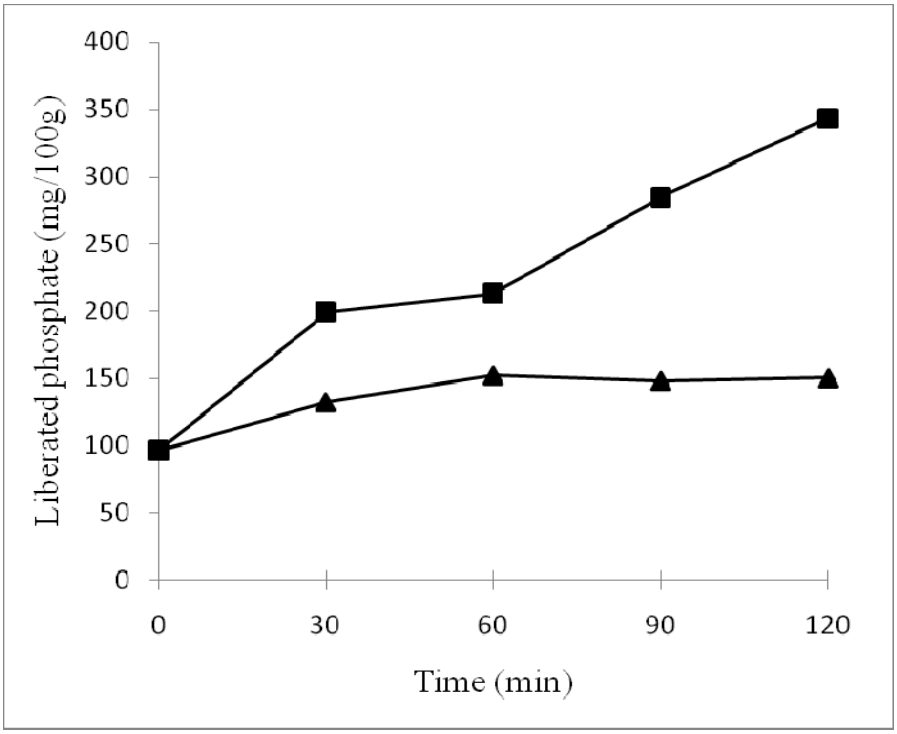
(b) Phosphate liberation from maize



(c) Phosphate liberation from sorghum



(d) Phosphate liberation from rice



(e) Phosphate liberation from wheat

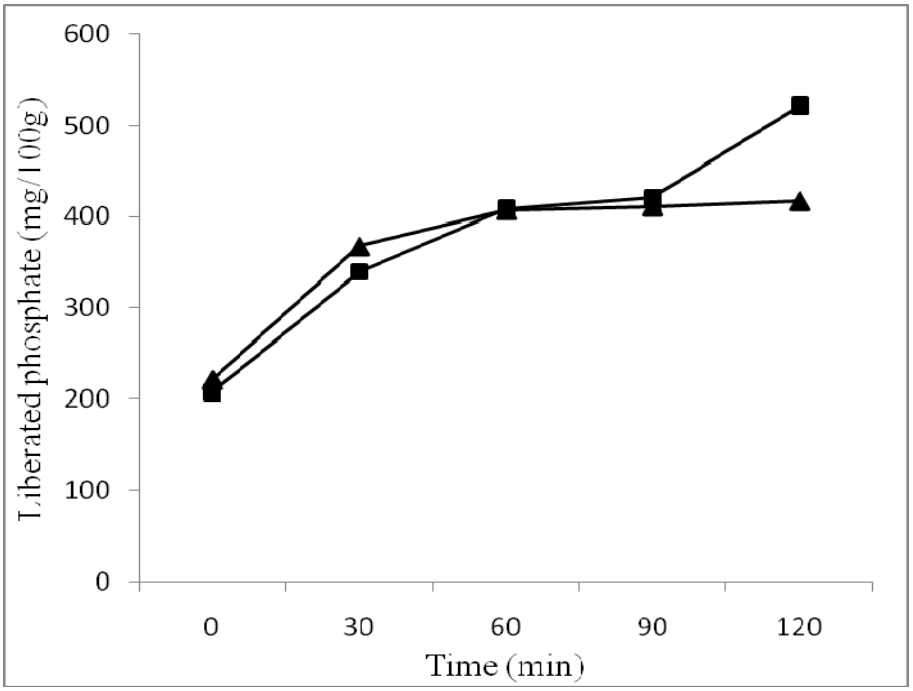


Figure 4. Release of phosphate (mg/100 g) from (a) Soybean, (b) Maize, (c) Sorghum, (d) Rice and (e) Wheat after the cell bound phytase treatment at 37 ^{o}C in acetate buffer (pH 5.0) for 2 h.

Phytase treated (--- ■--), Control (--- ▲--)

Supplementation of phytase in the grinded rice did not show any significant effect until 1 h, after that there was rapid increase in P liberation observed. Total of 2.3 times more P release was seen in phytase treated rice. In case of phytase treated wheat, P liberation was coincided with the control until 90 min of incubation followed by slight increase in P availability at 120 min. Overall a less significant effect was observed in phytase treated wheat as compared to the other tested cereals. Only 1.3 fold increased in P availability was observed in phytase treated wheat.

The released of Pi as a result of phytase treatment has also been observed in rice, wheat and corn containing poultry feed [10, 35]. Many researchers reported the use of yeast phytases for animal feed pre-treatment or supplementation. Segueilha *et al.* reported that the phytase from *S. castellii* was able to hydrolyze the PA from wheat bran and glandless cotton flour [36]. The application of cell-bound phytase of *P. anomala* to the feed of broiler chicks resulted in improved growth, better P retention in the body and reduced excretion of P in the faeces [10]. Matsui *et al.* [37] observed that dietary yeast phytase improved bioavailability of P in growing pigs, but its efficacy was less than that of *A. niger* phytase. Furthermore, they mentioned that the lower efficacy of yeast phytase was because of low stability of yeast phytase in the presence of pepsin. Veide and Andlid suggested that a high-phytase S. *cerevisiae* strain may be suitable for the production of food-grade phytase and can directly use during food production [38].

In summary, we evaluated the ability of phytase from *W. saturnus* NCIM 3298 to reduce the phytate content so as to improve the bioavailability of P and essential minerals of CP. Phytate content of the CP reduced by 80% leading to increase in the bioavailability of P, Zn^{2+}, Fe^{2+} and Ca^{2+}. Furthermore, phytase treated rice, soybean, wheat, maize and sorghum also showed an increased availability of P as

compared to control. Thus, the ability of *W. saturnus* 3298 phytase to degrade the PA and to enhance the mineral mobilization from CP and to increase the P availability of various cereals and pulses suggests that this phytase may be utilized for processing and manufacturing of the feeds and foods.

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CHAPTER 5

Summary and future directions

The present work includes production, characterization and application of phytase from *W. saturnus* NCIM 3298. The objectives of the study were the screening of the high phytase producing yeast strain from NCIM resource center, the characterization of the enzyme and its applications in the animal feed diet. Screening was carried out using two step procedure followed by a selection of high phytase producing strain. Production studies were performed using media optimization by RSM. Phytase applications were studied for improved availability of the phytate bound P and minerals of cereals and pulses.

1. Screening of yeast strains from NCIM Resource center for phytase activity

This study discusses about the screening of phytate hydrolyzing yeasts from NCIM resource center. 600 yeast strains were screened for phytase production in a two-step procedure. Only 40 showed a zone of clearance around the colony, the first step of screening. In the second step only 11 strains showed strong growth in the phytate containing medium. Of the 11 strains, five were selected for their phytase activity as they have not been previously reported for phytase production. Of the five, *W. saturnus* NCIM 3298 was selected for further study as other either did not show good phytase activity or reported as food spoiling yeasts. The maximum phytase production showed by *W. saturnus* NCIM 3298 was 7 IU g/ DWB in minimal liquid medium. Furthermore, this strain was also able to secrete the phytase after 24 h and maximum production was at 48 h. *W. saturnus* NCIM 3298 was able to assimilate xylose and

nitrate, a carbon and nitrogen source, respectively, which are the key characteristics of *Williopsis* sp.

2. Production of phytase by *Williopsis saturnus* NCIM 3298 and its characterization

This study discusses about production of phytase using an integrated statistical optimization approach involving the combination of PBD and BBD. Amongst the seven selected factors in PBD study, citric acid, $FeSO_4$, pH and glucose were found a phytase synthesis influencing factors. Citric acid found a key factor whose contribution was about 51 % in phytase production. The role of citric acid is may be phosphorus sequestering or buffering agent. Phytase production after PBD, increased to 267 IU/g DWB, which indicated about 5.8 fold increase as compared to unoptimized medium. Key media components obtained in PBD were further selected for optimization using BBD of RSM. After media optimization by BBD phytase activity was further improved to 297 IU/g DWB, which represented overall 6.45 fold increase in phytase synthesis as compare to unoptimized medium. Biochemical characterization studies of cell bound phytase revealed that the pH and temperature optima for enzyme activity were 5.0 and 50 ^{o}C, respectively. *Km* value for cell bound phytase was 0.2 mM. Enzyme localization study was performed using previously reported methods and it was observed that the enzyme activity was cell bound but not the intracellular. Permeabilization study using different detergents and solvents revealed that none of them is effective in ameliorating enzyme activity. Effect of different metal ions on enzyme activity was tested and it was observed that phytase activity was moderately stimulated in the presence of 1mM Co^{2+}, Ba^{2+}, Ca^{2+} and 10 mM K^{+}, Na^{+}, Mg^{2+}. It is strongly inhibited in the presence of 10 mM Hg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Ba^{2+}.

3. Application of phytase from *Williopsis saturnus* NCIM 3298 to increase bioavailability of phosphorus from grains and pulses

In this study, we discuss about the application of *W. saturnus* NCIM 3298 phytase to improve availability of P from cereals and pulses. Furthermore, we also tried to find out the correlation between dephytinization and mineral bioavailability using CP as a phytate source. Addition of phytase to CP significantly enhanced release of P and about two times more P was liberated in treated CP. Furthermore, 80% dephytinization of CP significantly increases the mineral bioavailability by 40%, 32.1% and 42% for Fe^{2+}, Zn^{2+}, and Ca^{2+}, respectively, as compared to control. These results indicate that the phytase from *W. saturnus* NCIM 3298 is effective in dephytinization of CP. The present study further investigated the effect of phytase supplementation on P availability from rice, maize, sorghum, soybean and wheat, which are high phytate containing cereals and pulses. The results of P availability experiment from cereals and legumes revealed that studied phytase was effective in increasing the P availability of all tested grains except wheat. The effect of phytase was more prominent on soybean. However, more in-depth studies need to be conducted to understand the role phytase on dephytinization and mineral availability of these cereals and pulses.

4. Scope for future work

a. Studies on up-scaling for phytase production using optimized medium at pilot and subsequently large scale fermentor could be undertaken for commercialization of phytase.

b. The whole cells as an enzymes source from *W. saturnus* NCIM 3298 could potentially be used at an animal trial level in order to assess the applicability in animal feed.

c. The above mentioned strain could be exploited in human food preparation to study its effect on mineral availability, especially for Zn and Fe whose deficiency is more prevalent in people from developing countries.

d. Genetic improvement of this natural yeast strain could be undertaken to exploit maximum phytase production capacity which may increase its chances for industrial applications as dietary yeast supplement and whole cell bio-catalyst.

Publications

1. **Anupama Pable**, Pradnya Gujar and J. M. Khire (2014). Selection of phytase producing yeast strains for improved mineral mobilization and dephytinization of chickpea flour. **Journal of Food Biochemistry** 38:18-27. (IF:1.02)

2. **Anupama Pable**, Ravi Kumar and J. M. Khire. Optimization of phytase production by *Williopsis saturnus* NCIM 3298 by response surface methodology. (Manuscript ready for communication)