A PHYTASE FROM *RHIZOPUS ORYZAE*: PRODUCTION, CHARACTERIZATION AND APPLICATION STUDIES

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

of

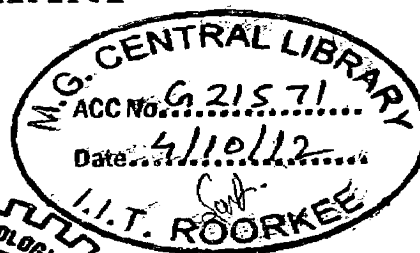
DOCTOR OF PHILOSOPHY

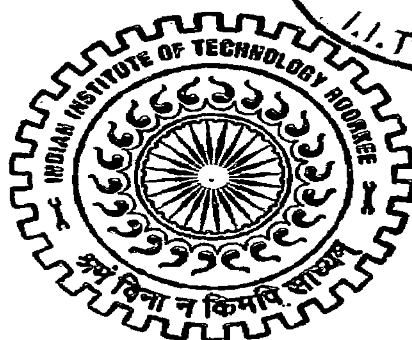
in

BIOTECHNOLOGY

by

RICHA RANI





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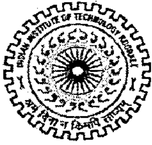

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**A Phytase from *Rhizopus oryzae*: Production, Characterization and Application studies**" in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee is an authentic record of my own work carried out during a period from July 2006 to October 2011 under the supervision of Dr. Sanjoy Ghosh, Asstt. Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(RICHA RANI)

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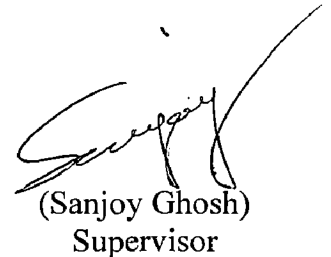

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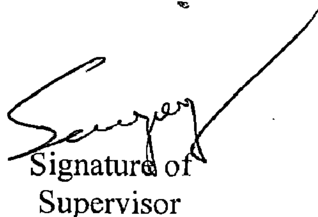

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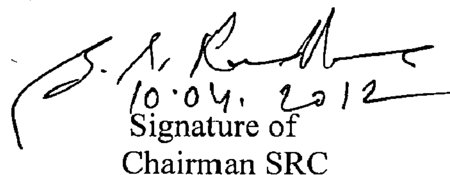

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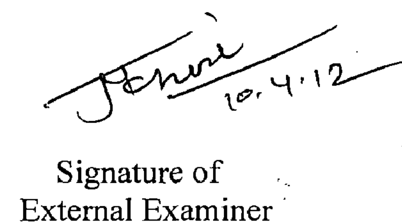

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ABSTRACT

ABSTRACT

Phytase is an important enzyme in the food/feed industry. It catalyzes the hydrolysis of phytate, an antinutrient compound present in cereals and grains, thereby, releasing orthophosphate and myo-inositol-6-phosphate with lower degrees of phosphorylation. Phytic acid is a strong chelator capable of complexing with a variety of metal ions under neutral and alkaline conditions, as well as with proteins and starch under acidic condition. Treatment with phytase increases not only the bioavailability of inorganic phosphorus but also the digestibility of proteins and the absorption of minerals from food/feed. The action of phytase also contributes towards reducing the pollution in surface and ground water caused by the phytate and phosphorus run-off from manure in intensive livestock regions.

This thesis presents the studies of a phytase from *Rhizopus* sp. The focus has been directed towards identification of a potential phytase producing microorganism, biochemical characterization of phytase, and optimization of parameters for the production of phytase, large volume production studies and evaluation of phytase potential in terms of phytate degradation.

A novel phytase from mesophilic fungal strain *Rhizopus oryzae* MTCC 1987 was identified. The phytase showed acid stable and pepsin resistant properties, with its optimum activity at 45°C and a dual pH profile at 1.5 and 5.5. Besides superior activity at physiological temperature (37-39°C), it demonstrated high thermostability with $t_{1/2}^{70°C}$ of 8.25 h, and a broad substrate specificity. All the tested metal ions (at 5 mM) exerted significant stimulatory effect except Fe^{2+}, Ni^{2+} and Cu^{2+}, however, the interesting feature of this phytase was insensitivity to heavy metal ions like Ba^{2+} and Ag^{+}.

Phytase was purified by using fractional ammonium sulphate precipitation and subsequent ion-exchange and gel filtration chromatography. Molecular size determination was carried out by SDS-PAGE and Gel filtration, and it was found to be ~34 kDa and ~34-36 kDa, respectively. Zymogram analysis displayed a single enzyme responsible for the phytase activity corresponding to ~34 kDa. Kinetic studies demonstrated a significant

low K_m value (2.42 x 10^{-4}mM), high catalytic efficiency (k_{cat}/K_m) (2.38 x 10^6 $M^{-1}s^{-1}$) and low activation energy value (28.99 kJ/mol). An uncompetitive inhibition was observed in presence of tested inhibitors (Fluoride > Phosphate > Vanadate).

Present study investigated the feasibility of various agro-industrial residues as solid substrate for phytase production, followed by optimization of culture parameters (substrate particle size, inoculum age, inoculum size, effect of supplementation of various surfactants and metal ions) under solid state fermentation (SSF). Linseed oilcake (LOC) and wheat bran (WB) (1:1) was found to be the most effective substrate with maximum phytase activity (37.43 U/gds) under optimal culture conditions. Present study also includes the statistical optimization of culture variables for the production of phytase under SSF.

The study also aimed at enhancing the phytase production using an improved strain of *Rhizopus oryzae* followed by statistical optimization of the medium components for submerged fermentation. Higher titer and higher productivity of phytase by improved strain can contribute to a significant reduction of the cost of phytase production from *R. oryzae* thereby, increasing its industrial utilization.

The ability of this phytase to completely degrade the phytate to its lower derivative, inositol tris-phosphate, was assessed by an improved method of HPLC analysis. It demonstrated the phytate degrading potency of this phytase using simple, precise and reproducible method using reversed-phase high performance liquid chromatography (RP-HPLC), equipped with variable wavelength detector (VWD). The feasibility of determining phytic acid and IP3 using this method was successfully demonstrated with minimal sample preparation and lower retention time at 246 nm. The results show that VWD system with UV spectrum specifically detects the phytate and its lower derivatives with a good resolution.

Furthermore, an *in vitro* study in milieu of physiological conditions revealed an unusual stability of this phytase at low pH with a degradation of 50% of phytate content. In other way, it can be stated that even under low pH condition (and at physiological temperature), this phytase has the ability to break three phosphomonoester bonds of phytate. These findings, taken together, demonstrate the new enzyme from *R. oryzae* as a novel phytase and render it of potential industrial interest.

A novel strain improvement procedure has also been developed to increase the yields of phytase production through adaptation of *R. oryzae* cells to subsequent heat and cold shock. The outcome suggests that an enhancement in phytase production by *R. oryzae* could be obtained by the combination of heat and cold treatment of spore suspension. The study was further extended to a comparative study on efficacy of wild and mutant strains pertaining to ash content and mineral extractability. The results confirm the effectiveness of this procedure as well as the enhanced efficacy of mutant strain in term of mineral extractability.

PUBLICATIONS

LIST OF PUBLICATIONS

PATENT:

1. "A novel phytase and process for enhancing phytase titer" Patent Application no-**1024/DEL/2010,** dated 29-04-2010.

PUBLICATIONS IN REFEREED JOURNALS:

1. **Rani, R.** and Ghosh, S. Production of phytase under solid–state fermentation using Rhizopus oryzae: Novel strain improvement approach and studies on purification and characterization. Bioresource Technology, **102**, 10641–10649, 2011.
2. **Rani, R.** and Ghosh, S. Statistical optimization of medium components for phytase production by mutant *R. oryzae* under Submerged Fermentation (Submitted).

PUBLICATIONS IN NATIONAL AND INTERNATIONAL CONFERENCES:

1. **Richa Rani,** Sanjoy Ghosh: Purification and characterization of a potential phytase from *Rhizopus oryzae*. Abstract published in 2nd World Congress on Biotechnology–2011, Philadelphia, USA, (Nov 29 - Dec 1, 2011).
2. **Richa Rani,** Sanjoy Ghosh: Value-added utilization of agro-industrial residues by *Rhizopus oryzae* for the production of a phytase of potential commercial interest. Full paper presentation made in World Congress on Biotechnology–2011, conducted by OMICS Publishing Group, Hyderabad, INDIA, (March 21-23, 2011).
3. **Richa Rani,** Sanjoy Ghosh: Isolation and characterization of a novel phytase from fungal strains. Full paper published in proceedings of 23rd National Convention of Chemical Engineers on Recent trends in Chemical Engineering, Indian Institute of Technology Roorkee, Roorkee, INDIA, pp 266-275, (October 5-7, 2007).

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

Completing a Ph. D. is truly a great marathon event, and I would not have been able to complete this long journey without the cooperation and guidance of a number of people to whom individually I owe a great sense of gratitude for their valuable contributions.

First of all, I thank the almighty GOD for his endless blessings bestowed on me and for providing me the strength and direction to make it possible to reach here.

At the outset, I would like to express my sincere regards to my supervisor Dr. Sanjoy Ghosh, Asstt. Professor, Department of Biotechnology, IIT Roorkee, who was always with me from the formative stages of this thesis to the final draft, for his ingenious advice, deep concerns, constitutive criticism and able guidance throughout the tenure of this study. Dr. Ghosh was instrumental in encouraging and inspiring me to cultivate critical approach to planning experiments, analyzing and presenting the data. I owe him high gratitude for the help extended to me and for showing me the path of leadership, shaping my carrier to the place where I stand confidently. I shall forever treasure the enriching experience of working with Dr. Ghosh as my guide.

I extend my profound gratefulness towards Prof. and Head, Mrs. Ritu Berthwal, Department of Biotechnology, IIT Roorkee, for providing all the facilities to accomplish my task smoothly and efficiently.

I would cherish the memorable moments, with my lab mates Rashmi, Gaurav, Pawan and Lalit. My special thanks to Vikas, Rohit, Shilpi and Meenu for their prolific help, whenever required. Without accounting Manish, Mayank and Ravi, this section will be incomplete. I express my deep appreciation to all of them.

My acknowledgement is due to Mr. V. P. Saini, technical assistant, and Mrs. Shashi Prabha, Department of Biotechnology, IIT Roorkee, for his unconditional help to carry out my research work.

I wish to extend my warmest thanks to all those who have joined me while travelling along this exciting road.

I would like to show my unforgettable thankfulness to Mr. Riyasat Ali, for his scientific support as well as his elderly support in tough time during this tenure.

I would like to express my deep and sincere thanks to Deepika, Research scholar, Department of Physics, for her untiring support in a number of ways in this study.

My heartfelt appreciation goes to my M. Tech. colleagues and other distinguished persons especially Vizi Narayanan for their kind support and immense help.

My gratitude is due to my mentors Prof. R. B. Narayanan, Late Prof. S. P. Gupta and Prof. M. R. Rahman, for their valuable suggestions and encouragement throughout my academic journey.

Words are not enough to express my indebted appreciation for my parents. My father and mother have always been very proud parents. It was their dream and ambition that made me strong enough to pursue higher studies. My papa's confidence in me and my mother's indefinable contributions in my life shaped my dreams and aspirations into reality. A special thanks to my mother, without her sacrifice, strict discipline and forbearance, my academic carrier would not have seen the light of the day. I also wish to make a special note of thanks to my elder sister Dr. Khushboo, brother-in-law Dr. Sanjeet, younger sister Rishu, younger brother Rohit and lovely Pihu, for their constant support and affection in all walks of my life.

I cannot express my feelings for the love and blessings showered on me by my Grandfathers Late Sri Ganauri Singh and Sri Lal keshwar Singh that always boosted my spirits throughout this academic pursuit. I would also like to affectionately acknowledge my mother-in-law Mrs. Usha Singh and father-in-law Mr. Dhanraj Singh, for their endless support, patience, love and encouragement that have put me on the right track and made this journey possible.

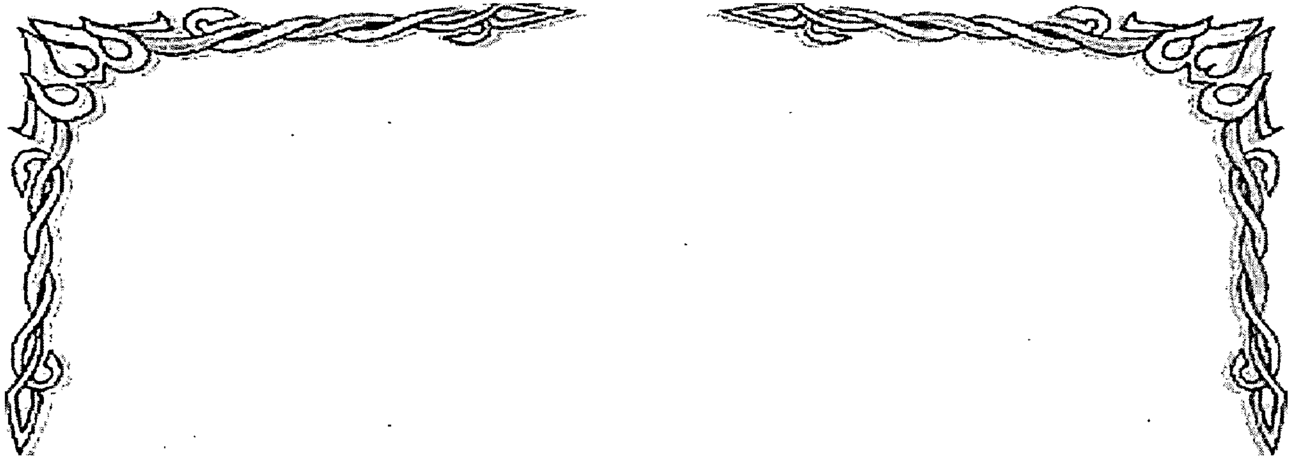
I want to hearty acknowledge and owe my loving thanks to my Husband Dr. Sudhir Kumar, for his steadfast support and insurmountable confidence in me that helped bring my work to conclusion in an exhaustive final stretch. I counted and relied on his ever-willing support and patience to face challenges with bold and positive attitude. He has been a never ending source of optimism, energy and strength for me all the way through my thesis work. It is his moral support, uplifting words of encouragement and refreshing sense of humour that kept me unbeaten throughout this work.

In the reminiscence, I might forget a few names. Many years from now those faces will flash back to my fading memories to me a feeling that I was lucky enough to spend some time with them.

Finally, yet importantly, I am immensely thankful to University Grants Commission (UGC) for providing financial assistance as JRF and SRF.

My thesis is a culmination of my parent's dream and their aspirations.

(RICHA RANI)

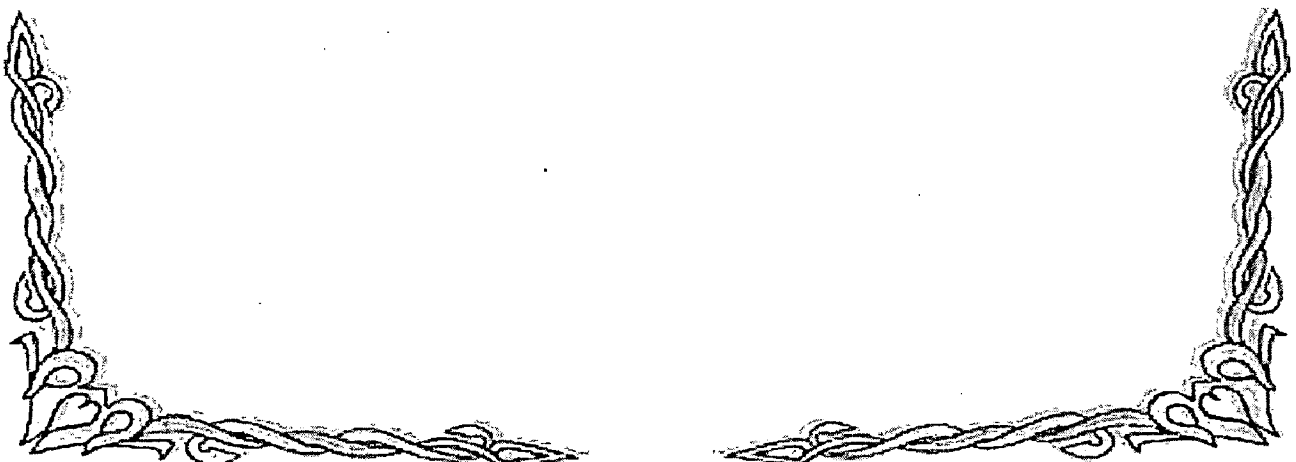


To,

My parents,

And

In loving memory of my Grandfather



ACRONYMS

ACRONYMS, ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
ε	Epsilon
µl	Micro liter
µg	Microgram
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic Acid
BPP	beta-propeller phytase
CaOC	Canola oil cake
CCD	Central Composite Design
cfu	Colony forming unit
COC	Coconut oil cake
CSC	Cottonseed oil cake
CV	Coefficient of Variation

D	dextro
DEAE	Diethylaminoethyl cellulose
DM	Dry mold
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FBP	Fructose–1, 6–biphosphate
g	gram
G-1-P	Glucose–1–phosphate
GOC	Groundnut oil cake
GRAS	Generally recognized as safe
h	Hour
HAP	Histidine acid phosphatase
HCl	Hydrochloric acid
Ins	Inositol
IPP	Inositol polyphosphate 1-phosphatase
IU	International unit
IUB	International Union of Biochemistry
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry

k_{cat}	Turnover number
kDa	Kilo Dalton
Kg	Kilo gram
K_m	Michaelis-Menten constant
L	levo
LOC	Linseed oil cake
M	mole
mg	Milligram
min	Minute
MIPP	Multiple inositol polyphosphate phosphatase
ml	milliliter
mM	Millimolar
mm	Millimeter
MOC	Mustard oil cake
MSGW	Monosodium glutamate wastewater
MTCC	Microbial Type Culture Collection
NADP	Nicotinamide adenine dinucleotide phosphate
NCIM	National Collection of Industrial Microorganisms
nm	nanometer
nmoles	nanomoles

NMR	Nuclear magnetic resonance
NRRL	Northern Regional Research Laboratory
OOC	Olive oil cake
P	Phosphorus
PAP	Purple acid phosphatase
PBD	Plackett–Burman design
PDA	potato dextrose agar
PEP	Phosphoenol pyruvate
Pi	Inorganic phosphate
PKC	palm kernel cake
*p*NPP	*para*-Nitrophenylphosphate
psi	pound per square inch
PTP	protein tyrosine phosphatases
R^2	coefficient of determination
R-5-P	Ribose-5-phosphate
RH	Rice husk
RP-HPLC	reversed–phase high performance liquid chromatography
rpm	Revolution per minute
RSC	Rapeseed cake
RSM	Response Surface Methodology
SBC	Soy bean cake

SCP	Single Cell Protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SGF	Simulated Gastric Fluid
SmF	Submerged Fermentation
SNP	Sodium–1–naphthyl phosphate
SOC	Sesame oil cake
SSF	Solid State Fermentation
SuOC	Sunflower oil cake
$t_{1/2}$	Half-life
TCA	Trichloroacetic acid
UV	Ultraviolet ray
V_{max}	Maximum enzyme velocity
VWD	Variable Wavelength Detector
WB	Wheat Bran

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CHAPTER 1
INTRODUCTION

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INTRODUCTION

1.1. Introduction

Conserving energy and resources has become vital since the increase of the modernization in our life style. The use of energy can act as an indicator for the environmental impact in the production of food/feed, as many environmental problems, like climate change, acidification potential and depletion of non-renewable resources, are related to energy consumption [242, 193]. Generally, we are encouraged to eat more products from plants and reduce consumption of meat, in an order to provide an adequate supply of micronutrients, but with fewer calories. A problem related to the consumption of more plant products is the presence of antinutritional compounds in vegetarian diets. Some specific nutrients (vitamins, amino acids, minerals etc) present in low concentrations or even absent in vegetable products, need to be supplemented to balance the nutrient value of foods and feeds. Some antinutritional factors (ANFs) such as phytate, protease inhibitors, lectins, anti-vitamins, saponins, estrogens, lysinolamine, carbohydrates from soluble fibers, and certain immunogenic proteins need to be inactivated or destroyed [207].

Phytate is the salt of Ins P_6 and the principal storage form of phosphorus in plant seeds. It accounts for 60-90% of total phosphorus contents in cereals, legumes, nuts and oilseeds. However, this phosphorus remains unutilized by monogastric animals (like human beings, dogs, birds, etc.) due to lack of or low level of phytase activity in the digestive tract [190, 239, 383]. Hence, addition of inorganic phosphorous, a non-renewable and expensive mineral, to feeds for monogastric animals is a common practice, which incurs costs and also contributes to water pollution, as it contributes up to 80% of total phosphorus in manures [16, 32, 102, 391, 392, 393]. The price of feed-grade phosphorus has been increased more than four-fold in the last few years [175, 177, 296, 298, 326].

Although phosphorus is not harmful in drinking water, its presence in water bodies can be of considerable concern and environmental significance. Addition of phosphorus, even in small quantities, to aquatic bodies is causing eutrophication of the aquatic system

which leads to accelerated growth of algae and other aquatic vegetation (Kerovuo et al., 1998; 2000). Requirements for better animal feed, environmental protection, lower food/feed cost and human health have prompted the fast development of research on phytases and their applications [383].

Furthermore, phytic acid also forms complexes with dietary minerals, proteins, amino acids and carbohydrates hindering their absorption in the body. To increase the availability of phosphorus, feed is either supplemented with non renewable and expensive phosphate. These problem can however be tackled by addition of phytase in animal feed, which is well proved to enhance the uptake of phosphorus and other minerals [329], protein, amino acids and carbohydrates [163] and energy [300].

Phytases (*myo*–inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8 and 3.1.3.26) catalyze the hydrolysis of phosphomonoester bonds of phytate (salts of myo–inositol 1, 2, 3, 4, 5, 6–hexakis dihydrogen phosphate), thereby releasing lower forms of *myo*–inositol phosphates and inorganic phosphate [294]. Phytases are produced by a large number of plants, animals and microorganisms; however, the phytases from microbial sources especially of fungal origin have supremacy in commercial industry [264].

An ideal phytase supplements must have following important properties: (a) high thermostability at pelleting temperatures (b) adequate activity at physiological temperature (c) able to withstand low as well as high pH in the digestive tract (d) resistant to proteolysis (e) *in vitro* broad substrate specificity and low sensitivity to metal ions (f) should be able to act at very low phytic acid concentration (low k_m value). However, no known phytase does have all the properties as mentioned above.

Although compendia of phytases are reported, however, a thermostable and acid stable phytase with broad substrate specificity is still highly desirable. In the present study, a novel phytase of commercial interest was identified from *Rhizopus oryzae* MTCC 1987.

Rhizopus oryzae, generally regarded as safe (GRAS) strain, is used in production of various enzymes, organic acids, aroma and mycotoxins because of its ability to utilize a variety of carbon sources, such as wheat bran, oil cakes [29, 30, 281], cassava, soybean and amaranth grain. Crude enzymes produced by GRAS strain on various feed supplements (bran, straw, oil cakes etc) could serve as a value–added supplement by providing other fungal proteins, sugars and some accessory enzymes along with the main enzyme source [30, 264].

In recent scenario, the interest in the recycling, upgrading and bioconversion of agro–industrial residues has increased drastically due to their disposal and looming environmental pollution problem. Effective utilization of these residues would not only help in curbing pollution but also pave the way for solid waste management and minimize the initial capital costs for the processes. Solid state fermentation (SSF) being a simple, low cost and self sustainable technologies with efficient utilization of these residues has served dual purpose of value addition and waste management [253]. The most inexpensive and high energy rich substrates for fermentation industry are represented by oil cakes and traditional agricultural by–products (wheat bran, rice bran, rice husk etc).

Several oil cakes such as coconut oil cake (COC), sesame oil cake (SOC), palm kernel cake (PKC), groundnut oil cake (GOC), cottonseed cake (CSC), soy bean cake (SBC), mustard oil cake (MOC), canola oil cake (CaOC), sunflower oil cake (SuOC) and olive oil cake (OOC) have been reported and have been used as a substrate for the production of industrial metabolites. Oil cakes being rich in proteins, carbohydrates and minerals offer a wide range of alternative substrates, thus find various applications in the bioprocess industry for the production of a wide spectrum of biometabolites (industrial enzymes, organic acids, antibiotics, biopesticides, vitamins and biofertilizer) [256, 257, 281].

Currently, the industrial potential of fungal enzymes has motivated research toward strain improvement. Herein, the effect of heat and cold shock (stress condition) on phytase production by *R. oryzae*, followed by a comparative study on efficacy of wild and mutant strains pertaining to mineral bioavailability was explored. Reports are available on strain improvement for enhancing the phytase production using physical and chemical mutagens [43, 319].

1.2. Scope of the thesis

In the present study, linseed oil cake (LOC) and wheat bran (WB) is used as raw materials for the production of phytase under SSF. Linseed oil cake is rich in phytic acid (~4.2%), protein content (32–36%), residual oil (2.81%), minerals and essential amino acids such as methionine, lysine, leucine etc [147] while wheat bran beside being a good source of carbon and nitrogen sources, has high phytate content and good support matrix properties [391]. Since, availability is also one of the important considerations for

development of a cost effective SSF process, data shows that the global output of linseed is estimated around 2.60 million tons per year with Canada (0.6 million tonnes), China (0.48 million tonnes), India (0.17 million tonnes) and United States (0.15 million tonnes) dominating the list of producers. (http://www.commodityonline.com/commodities/oil–oilseeds/linseedoil.php).

Optimization of culture conditions using statistical tools has been employed in enhancing the phytase production by various microorganisms during SSF [29, 30, 42, 330] and submerged fermentation [167, 330, 343, 385]. However, there is no literature report on optimization of the medium components and physical parameters for phytase production by *R. oryzae* under SSF as well as submerged fermentation. Additionally, a simple and effective strain improvement process was introduced to increase the phytase yield.

In the present study, a phytase of commercial interest was identified from *Rhizopus oryzae* MTCC 1987. It has been reported here for the first time the statistical optimization comprising Plackett–Burman design (PBD) and the path of steepest ascent (descent) method followed by a central composite design (CCD) and response surface methodology (RSM) of culture conditions for production of phytase by *Rhizopus oryzae* MTCC 1987 using inexpensive LOC in combination with WB as substrate under solid state fermentation (SSF). The study was further extended to strain improvement, and optimization of medium components for the production of phytase under submerged fermentation followed by bioreactor studies for its large volume production. The study was also carried out to characterize this phytase to explore its suitability in food and feed industry.

CHAPTER 2
REVIEW OF LITERATURE

CHAPTER 2
REVIEW OF LITERATURE

2.1. Phytic acid

Phytate was first identified more than a century ago. It is primary storage compound of phosphorus in cereals, legumes, nuts and oilseeds [203, 204, 238] and accounts for more than 80% of total phosphorus content. The partial availability of the phosphorus component of phytate to single–stomached species assumes importance as the world's rock phosphate reserves are not renewable, which could lead to a phosphorus supply crisis in the future [3, 221]. The global harvest of crop seeds and fruits contains an estimated 14.4 million tonnes of phytate phosphorus, which is equivalent to 65% of annual sales of phosphorus as fertilisers [204].

2.1.1. Chemical structure of phytic acid

Systemic name of phytic acid is *myo*–inositol–1,2,3,4,5,6–hexa*kis* dihydrogen phosphate [54, 211, 233]. It has 6 groups of phosphates attached to the inositol ring and the prefix "hexa*kis*" signifies that the phosphates are connected externally, not internally [162]. The three terminology phytic acid, phytate and phytin refer respectively, to the free acid, the salt and the calcium/magnesium salt. The adopted nomenclature of phytic acid is according to the set of rules suggested by Posternak [273]. Numbering direction of carbons in the inositol ring is denoted by D/L prefixes, where D and L annotate counterclockwise and clockwise, respectively. Trivial names for D–*myo*–inositol (1,2,3,4,5,6) hexa*kis*phosphate are InsP6, IP6 or Phytic acid.

According to International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) [155-157], atoms in the *myo*–inositol ring should always be numbered according to the D–configuration. Johnson and Tate [162] derived the structure of phytic acid from 31P–NMR and suggested axial position for the phosphate at C2 position, whereas an equatorial position for the other phosphates. In contrast, Blank et al. [28] who studied its structure by X–ray analysis concluded that the phosphate group at the C2 position is equatorial while other phosphates at remaining

carbon atoms are axial. Later, the study carried out by Costello et al. [55] supported the conformation suggested by Johnson and Tate [162].

The energetically most favourable conformation of phytic acid is shown in Fig. 2.1. The pK_a values determined for dissociating groups of phytic acid using 31P–NMR and pH titration methods by Costello et al. [55] concluded that six groups were in the strong acid range (pK_a 1.1 to 2.1), one in the weak acid range (pK_a 5.70), two with pK_a 6.8 to 7.6, and three in the very weak acid range (pK_a 10.0 to 12.0), although ionic strength of the solution and temperature influence these values [36, 55, 354]. These outcomes suggested that phytic acid has a strong potential for complexing multivalent cations and positively charged proteins, as it exists as a strongly negatively charged molecule over a wide range of pH values.

2.1.2. Functions of phytic acid

Phytic acid is associated with several physiological functions [61] and significantly influences the functional and nutritional properties of cereals, legumes, nuts and oilseeds (and food and feed derived thereof), by its ability to chelate nutritionally important proteins and minerals. Phytic acid plays several physiological roles in plants as well as in eukaryotes. Primarily, phytic acid in seeds and grains acts as (i) phosphorus storage, (ii) as an energy store, (iii) as mineral reserve in seeds, (iv) as a source of *myo*–inositol (a cell wall precursor), and (v) key factor in initiation of dormancy. In addition phytic acid is associated with several vital functions such as it acts as an antioxidant for the germinating seeds [278]. The role of phytic acid as a natural antioxidant in seeds during dormancy was earlier suggested by Graf et al. [106]. The property of phytic acid is attributed to its ability to bind and inactivate Fe ions in solution which in turn blocks the iron–driven hydroxyl radical formation (Fenton reaction). Its chelating ability is used in preservation of historic documents, since, it prevent iron (present in the historic ink along with gallic acid) catalyzed oxidation of the cellulose in the paper [235, 307].

In eukaryotes, it is associated with cell signalling pathways and cell differentiation [20], protein folding mechanism [206] and trafficking [322] and acts as precursors in the form of lower inositol phosphates due to its phosphate donor/acceptor capabilities. Their role especially that of inositol triphosphate (IP3) in signal transduction and regulation of

cell functions in plant and animal cells is a very active area of research [391]. An antagonist–stimulated increase in inositol (1,4,5)–trisphosphate (and inositol (1,3,4,5)–tetra*kis*phosphate) is often associated with an increase in cytosolic free Ca^{2+}, which subsequently triggers a variety of physiological events.

In mammalians, it has been shown to be efficient in cancer therapy [388]. The presence of undigested phytic acid in the colon may protect against the development of colonic carcinoma [70].

2.1.3. Occurrence and distribution

Phytic acid is widespread in nature and is the major ingredient in cereals, legumes, nuts and oilseeds [203, 204] (Table 2.1) that accounts for 60–90% of total phosphates. It occurs chiefly as salts of mono– and divalent cations (e.g. potassium–magnesium salt in rice and calcium–magnesium–potassium salt in soybeans) in discrete regions of cereal grains and legumes. During the ripening period of seeds and grains, it accumulates, accompanied by other storage substances such as starch and lipids in varied locations. In cereals, phytate lies mainly in the bran (aleurone layer, testa and pericarp), whereas in legume seeds, phytic acid accumulates in the cotyledons [293]. The endosperm of wheat and rice kernels is almost devoid of phytate, as it concentrates in the germ and aleurone layers of the cells of the kernel. Ferguson and Bollard [80] found that 99% of the phytate in dry peas was in the cotyledons and 1% in the embryo taxis. The highest amount of phytate among cereals is found in maize (0.83–2.22%) and among legumes in dolique beans (5.92–9.15%) (Table 2.1). Sesame, pumpkin/squash and flax (linseed) have 3.7–4.7% phytic acid on a dry weight basis whereas rapeseed (canola), sunflower and mustard have 2% (Table 2.2). Phytic acid is also present in most eukaryotic tissues as phosphoinositides or in complexes with proteins or metal ions [354, 382].

2.1.4. Antinutritive effects of phytic acid

The negative nutritional attributes of phytic acid are associated with its chelating ability as well as its conformational structure. Consumption of diet containing high phytic acid since long period has already been shown to be related to mineral deficiency and malnutrition [45, 212]. The capability of binding various nutritionally important minerals

and proteins and forming insoluble complexes, make it an antinutritional factor, since the solubility of the phytic acid–mineral complexes are low at the pH encountered in the major portion of the intestines. However, the reduced bioavailability of minerals due to phytic acid depends on several factors such as nutritional status of the consumer, concentration of minerals and phytic acid in the foodstuffs, ability of endogenous carriers in the intestinal mucosa to absorb essential minerals bound to phytic acid and other dietary materials, digestion or hydrolysis of phytate by phytase and/or phosphatase in the intestine, digestibility of the foodstuff and processing operations.

In human studies, it has been shown to inhibit absorption of iron, zinc, calcium, magnesium and manganese but not copper [33, 62, 74, 133, 199, 271, 292]. The unusual molecular structure of phytic acid has also been depicted as one of the key factors for its strong antinutritive effect [246]. When completely dissociated, the six phosphate groups of phytic acid carry a total of twelve negative charges which enables it to bind different mono–, di–, and trivalent cations and their mixtures, forming insoluble complexes, very effectively [293]. The formation of insoluble phytate–mineral complexes in the intestinal tract prevents mineral absorption and reduces the bioavailability of essential minerals [63, 64, 79, 207, 287, 386]. Copper and Zinc appears to be the trace elements of which the bioavailability is most influenced by phytic acid. The binding strength of phytic acid was found to be in the order of $Cu > Zn > Ni > Co > Mn > Fe > Ca$ by Vohra et al. [386] and $Zn > Cu > Co > Mn > Ca$ by Maddaiah et al. [207], respectively. Phytate also has negative effects on other nutrients. Phytate may influence starch digestibility through interaction with amylase, proteins associated with starch, calcium (which catalyzes amylase activity), or with starch itself [67, 163, 321, 349, 350].

Phytate also has been shown to form complexes with both dietary and digestive proteins [143] and to inhibit trypsinogen [37]; thus affecting the protein nutritional quality of feed ingredients. It interacts with proteins over a wide pH range. At a low acidic pH, phytic acid has a strong negative charge due to total dissociation of phosphate groups. Under these conditions a negative influence of phytic acid on the solubility of proteins can be expected because of the ionic binding between the basic phosphate groups of phytic acid and protonized amino acid (lysyl, histidyl and arginyl) residues [65, 85]. Under acidic conditions phytic acid is likely to bind tightly to plant proteins, since the isoelectric point of plant proteins is generally around 4.0–5.0. In the intermediate pH

range (6.0 to 8.0), both phytic acid and plant proteins have a net negative charge. However, under these conditions complex formation occurs between phytic acid and proteins. Possible mechanisms include direct binding of phytic acid to protonated α–NH_2 terminal groups and ε–NH_2 groups of lysine residues, and a multivalent cation–mediated interaction [45]. By binding to plant proteins, phytic acid decreases their solubility and digestibility, therefore also reducing their nutritive value.

In addition to complexing with minerals and proteins, phytic acid interacts with enzymes such as trypsin, pepsin, α–amylase and β–galactosidase, resulting in a decrease in the activity of these important digestive enzymes [67, 153, 336].

2.2. Phytase

Phytase (*myo*–inositol (1,2,3,4,5,6) hexa*kis*phosphate phosphohydrolases) belongs to a special class of phosphomonoesterases, which catalyzes the hydrolysis of *myo*–inositol (1,2,3,4,5,6) hexa*kis*phosphate (phytic acid, Ins P6) in a stepwise manner to inorganic monophosphate (Pi) and lower *myo*-inositol phosphate esters (Ins P5 to Ins P1), and, in some cases to free *myo*–inositol [221]. Phytases have the ability to hydrolyze variety of other natural and synthetic phosphorylated substrates which make it a unique phosphatase.

2.2.1. Classification of phytases

The International Union of Pure and Applied chemistry and International Union of Biochemistry and Molecular Biology (IUPAC–IUBMB) currently recognize three classes of phytases (i) 3–phytase (EC 3.1.3.8) (ii) 5–phytase (EC 3.1.3.72) and (iii) 6–phytase (EC 3.1.3.26) [32]. This classification is based on the initiation of dephosphorylation at specific position on the inositol ring of phytic acid by the enzyme (Fig. 2.2). 3–phytase act on the D–3 phosphate position leading to formation of D–*myo*–inositol (1,2,4,5,6) penta*kis*phosphate as product and is characteristics for microorganisms and filamentous fungi. 5–phytase initiates dephosphorylation with an attack of a first phosphate group at C5 of the inositol ring giving rise to D–*myo*–inositol (1,2,3,4,6) penta*kis*phosphate. The first phytase belonging to this family was detected from lily pollen [17, 32].

More recently, the extracellular phytate–degrading enzyme from *Selenomonas ruminantium* (PhyAsr) has also been described as a 5–phytase. It was suggested from the PhyAsr–hexasulphate structure that PhyAsr preferentially dephosphorylates InsP6 at the 5–phosphate position first [49]. 6–phytase, also known as 4/6 phytase, firstly act on the carbon atom next to C5 of inositol ring of the phytic acid and is typical for plant. Instead of official name of L–4– phytase, it is traditionally called as D–6–phytase and produces the same product.

Depending on the optimum pH of activity, phytases can be grouped as acidic, neutral or alkaline phophatases. Recently, on the basis of their structural differences and varied catalytic properties, Mullaney and Ullah [223] described three classes of phytases i.e. Histidine acid phosphatases (HAPs), β–propellar phytase (BPP) and Purple acid phytases (PAP). The X–ray structure of a novel phytate–degrading enzyme from *S. ruminantium* has recently been determined and suggests a new class of phytase; *i.e.*, the protein tyrosine phosphatase (PTP)–like phytases [49]. Most of the 3–phytases belong to HAPs or BPP. Soyabean phytase is a PAP phytase [144].

2.2.2. Occurrences

The research on phytase spans more than 85 years from its earliest report from rice bran [345] and blood of calves [213] until its first commercialization in Europe in 1994 by Gist–Brocades (now DSM) and sold by BASF under the trade name 'Natuphos'.

2.2.2.1. Phytases from fungal sources

Phytate hydrolyzing enzymes have been most commonly found in fungi, particularly from the *Aspergillus* sp. [178]. The phytase from *A. ficuum* was the first to be introduced in the market as a commercial product with the trade name of 'Natuphos' [391]. Phytate degrading enzymes have also been reported from many bacteria. The phytases from *Bacillus* [168, 175, 177] and *Escherichia coli* [118] have been well characterized, structures determined [129, 197]. Table 2.3 illustrates several examples as the source of microbial, plant and animal phytases. In view of ever increasing demand of phytases and more bio–efficacy of microbial phytases (1.5-fold more than from plant

phytases; [407]) over other sources [191, 348, 384], there is ongoing interest in isolating new microbial strains producing economical as well as effectual phytases.

Production of phytase from fungal sources are primarily reported in three domains of fungi i.e. mesophilic, thermophilic and thermotolerant [78]. Most of the reported phytases are from mesophilic fungi e.g. *A. niger* [39-41, 44, 71, 76, 77, 137, 164, 182, 266, 309, 338, 347, 372, 374, 375, 379, 381, 387, 402]; *A. tubingensis* [373]; *A. oryzae* [88, 183]; *A. usamii* [145]; *A. fumigates* [352, 395]; *A. ficuum* [72, 73, 179, 358, 360, 361, 364, 365, 397]; *A. carbonarius* [39, 266]; *A. flavipes* [405]; *A. awamori* [209]; *A. niveus* [11]; *Macrophomina phaseolina* [97]; *R. oligosporus* [40]; *Rhizopus sp.* [281, 389]; *Penicillium caseicolum* [390]; *Peniophora lycii* [165] and *Agaricus bisporus* [51].

Recently, phytase production by thermophilous fungi e.g. *Taloromyces thermophilous* [268]; *Myceliophthora thermophila* [216]; *Thermomyces lanugiosus* [19]; *Themoascus aurantiacus* [232]; *Rhizomucor pusillus* [42] and *Sporotrichum thermophile* [330, 331, 333-335] has also gained great attention.

However, phytase production in thermophilous fungi has not been explored adequately like their mesophilic counterparts. Production of phytase from thermotolerant *Aspergilli*, also received a considerable attention; e.g. *A. fumigates* [369, 371] and *A. niger* [208].

2.2.2.2. Plant sources

Phytase activity has been found in many plants, such as maize [184], barley [115], rye [119], Sunflower [6], canola seed [148] and lily pollen [314]. Unlike the phytases produced by microorganisms, it has been more difficult to purify plant phytases from contaminating nonspecific phosphatases [184]; thus, only a few phytases from plant sources have been purified to homogeneity and extensively characterized.

2.2.2.3. Animal sources

Phytate–degrading enzymes have been isolated from the intestinal mucosa of some monogastric animals [27, 46, 52, 399, 400]. A multiple inositol polyphosphate phosphatase (MIPP) displaying phytate–degrading activity was also identified in rat hepatic tissue, localized in the ER lumen. Although the MIPP mRNA could be found

ubiquitously in rat tissues, it was most highly expressed in the kidney and liver [56]. More recently, MIPP homologues have been cloned from mice and humans [46]. A phytate–degrading enzyme has also been purified and characterized from the protozoan *Paramecium* [86].

2.2.3. Market trends

The global market for feed enzymes is definitely one of the most promising segments in the animal feed industry. Regions like United state, China and South–East Asia hold a lot of potential for growth (Table 2.4). Phytase is one of the two major segments of feed enzyme market i.e. Phytase and Non–Starch Polysaccharides. The global market for enzymes is governed by various factors such as, predominant feed stock type, geography and regulations governing it and cultural differences. The combined market for enzyme was estimated at around $ 344 million in 2007. (http://www./frost.com/prod/servlet/market–inight–top.pag?docid=115387658)

Over the past 10 years, there has been an expanded use of phytase for hydrolysing the phytate [22-25, 57-59, 69, 84, 98, 101, 110, 134, 142, 194, 218, 247-250, 288-291, 317-318, 380] as evident from the fact that phytase accounts for 60–70% of the total $251 million global feed enzyme market [240]. Several commercially available phytases from different microbial sources have been reported and are summarized in the Table 2.5.

Several phytases from different sources were already reported in the literature including the commercial phytases from *Aspergillus* sp., however substantial amount of phytate was still left undegraded due to inadequate activity of the available phytases in milieu of required conditions for various reasons: (a) too little activity at physiological temperature (b) instability over a wide range of pH encountered in the gastrointestinal tract (c) less resistant to proteases (d) intolerant to pelleting temperature and (e) low specific activity.

In a recent study carried out by Boyce and Walsh [34], four commercially available phytases (Allzyme phytase produced by Alltech Ireland Ltd., Co. Meath; Natuphos produced by Gist–Brocades, Delfth, the Netherlands; Ronozyme P/Biofeed phytase produced by F. Hoffmann–La Roche Ltd., Switzerland and Novozymes A/S, Bagsvaerd, Denmark) were compared for their bio–efficacy and it revealed that none of them

satisfied all of the criteria of an ideal phytase for feed applications, such as resistance to proteases, denaturation under extreme temperatures and pH, etc.

2.2.4. Current research interest

At present, there is no single phytase which can fulfil all the criteria required for an ideal phytase such as high thermostability at pelleting temperatures, adequate activity at physiological temperature and pH, resistant to proteolysis, in vitro broad substrate specificity, high activity and low sensitivity to metal ions, to be used for all commercial and environmental applications. Most commonly strains employed for industrial applications are from *Aspergillus sp.*, mainly *A. ficuum* and *A. niger,* however, due to various limitations scientists, researchers as well as industrial people are still in an effort to identify phytases with physicochemical properties more suitable for use in animal feed.

The present research is mainly focused on to develop a phytase that shows (i) superior thermo stability at pelleting temperatures (60–95°C) (ii) an excellent enzyme activity at physiological temperature (39°C) (iii) good activity over a wide range of pH encountered in the digestive tract and (iv) high titre through strain improvement process.

2.3. Production of phytases

2.3.1. Production techniques

Approximately 90% of all industrial enzymes are produced in SmF, frequently using specifically optimized, genetically manipulated microorganisms. On the other hand, almost all these enzymes could also be produced in solid state fermentation (SSF) using wild–type microorganisms [81, 264]. The SSF processes offer several advantages over submerged fermentation (SmF) [146]. Interestingly, fungi, yeasts and bacteria that were tested in SSF in recent decades exhibited different metabolic strategies under conditions of solid state and submerged fermentation. Direct comparison of various parameters such as growth rate, productivity or volume activity favoured SSF in the majority of cases. It has also become clear that the cost–factor for the production of enzymes in most cases favours SSF over SmF. The low estimated costs of SSF are due to the rather traditional preferential claim of SSF, viz. SSF utilises complex, heterogeneous agricultural by–products as substrates and uses low–cost technology regarding sterility and regulation

demands. However, attempts to reduce costs by using cheap substrates have hampered biotechnological progress in SSF because of the strongly increased diversity in SSF research. There is no consensus on either, the methods, the microorganisms or the substrates used, that would allow comparison with other cultivation technologies. The broad spectrum of substrates used represents an especially severe problem. As already mentioned, one great advantage of SSF has always been the possibility of using substrates that are abundant, cheap, and not applicable to SmF. Production efficiencies in SmF is greatly influenced by filamentous fungal morphology such as cellular aggregation, mycelia pellet shape and size. SSF provides more similar culture conditions to the natural habitat resulting into higher productivity and enzyme yield.

Phytase production has been attempted in several microbial species by using both submerged as well as solid state fermentation techniques (Table 2.6). During past years, submerged fermentation (SmF) was largely employed for the production of industrial enzymes including phytases. However, in recent years solid state fermentation (SSF) has received much concern for the production of phytases. Fungal species from the *Aspergillus* genera are widely employed for phytase production [392, 393], although other mesophilic fungi like *Mucor racemosus* and *Rhizopus oligosporus* [30, 305], or the thermophilic fungi *T. aurantiacus* have also been employed. Methylotrophic yeast such as *P. pastoris* or *Hansenula polymorpha* exhibit great potential for producing high levels of *A. niger, E. coli,* and *A. fumigatus* phytases [210, 296-299]. The phytase production can be greatly enhanced by optimizing culture conditions, restriction of oxygen supply during passage, stabilization and screening process, and changes in codon usage of the phytase gene and modification of signal peptide [210, 342]. The technical control and the possibility of scaling up to an industrial level have limited the application of the solid state fermentation despite it having shown some advantages regarding use of agricultural by-products, as well as saving water and energy.

2.3.2. Oilcakes as potential substrate in SSF

The sustainability of the booming bioprocess industry depends on the progressive reduction of expensive nutrient inputs into fermentation media. The reutilization of or use of cheap agricultural and food–processing by–products such as oil cakes, is highly

favoured, to hasten the commercial feasibility of bioprocess technology. Over the past few decades, there has been an increased exploitation of organic residues from various sectors of agriculture and industries. In a recent review by Ramachandran et al. [282], utilization and biotechnological applications of several oilcakes such as sunflower oil cake (SuOC), sesame oil cake (SOC), soy bean cake (SBC), coconut oil cake (COC), mustard oil cake (MOC), palm kernel cake (PKC), groundnut oil cake (GOC), cottonseed cake (CSC), canola oil cake (CaOC), olive oil cake (OOC), rapeseed cake (RSC), in bioprocesses for the production of industrial bio–products is emphasised in detail. Earlier other crop residues such as bran, husk, bagasse, and fruit seeds were also shown to be utilised as a potential raw material in bioprocesses as they grant an excellent substratum for the growth of microorganism supplying the essential nutrients to them [251, 252, 256, 258–263]. Their application in bioprocesses also offers advantages in bioremediation and biological detoxification of hazardous compounds. Their application in the field of fermentation technology has resulted in the production of bulk–chemicals and value–added products such as amino acid, enzymes, mushrooms, organic acids, single–cell protein (SCP), biologically active secondary metabolites, etc. [230, 253, 256, 257, 372].

2.3.2.1. Oilcakes/meals

Oil cakes/oil meals are by–products obtained after oil extraction from the seeds. Oil cakes are of two types, edible and non–edible. Edible oil cakes have a high nutritional value; especially have protein content ranging from 15% to 50% (Table 2.7). Their composition varies depending on their variety, growing condition, geographical areas and extraction methods. Due to their rich protein content, they are used as animal feed, especially for ruminants and fish. Nonedible oil cakes such as castor cake, linseed cake, karanja cake, and neem cake are used as organic nitrogenous fertilizers, due to their NPK (nitrogen, phosphorus, potassium) content. Some of these oil cakes are found to increase the nitrogen uptake of the plant, as they retard the nitrification of soil. They also protect the plants from soil nematodes, insects, and parasites; thereby over great resistance to infection (www.itdgpublishing.org.uk).

Global oilseed production for 2009–10 is projected at 422.7 million tons (15% of total world crop production). From the world's total oilseed production, about 40% is

used for making oil & fats, whereas, approximately 25% is occupied by oil meal and cakes (http://www.fao.org/docrep/011/ai482e/ai482e06.htm).

Global output of linseed is estimated around 2.60 million ton per years with Canada, China, U.S and India dominating the list of producers. Canada is the leading producer and accounts for nearly 80% of the global trade in linseed. Global production of linseed oil is estimated between 0.6–0.7 ton while linseed meal ranges between 1.1–1.4 million tons.

Annual growth in oil cake production is projected to average 2.3% annually over the decade to 2012. India is one of the world's leading oilseeds producers. Total production currently stands at over 25 million tonnes per annum while the exports account for over 4.3 million tonnes of oil cake, valued at US$ 800 million annually (www.seaofindia.com). Of the total oil meal production increase of 23 million tonnes, 17 million tonnes is from developing countries including India, Brazil and Argentina. India is considered as the third largest producer of linseed in the world. India linseed is mainly cultivated as rabi crop in with October–November being the main sowing season. February–April is the main harvesting season in the country. Madhya Pradesh is the leading producer of the crop, which is broadly divided into two categories– peninsular and alluvial types according to the root formations. Oil markets in Indore, Kanpur, Agra and Gwalior are the main trading centres of linseed oil. Paint and allied industries are the main consumers of linseed oil accounting for nearly 70% of the total consumption. West Bengal, Maharashtra, Delhi and Uttar Pradesh are the main centres of linseed oil consumption in the country. (http://www.commodityonline.com/commodities/oil–oilseeds/linseedoil.php).

2.3.3. Statistical approaches in optimization of phytase production

For selecting a particular production technique, culture conditions, type of strain, nature of substrates and availability of nutrients should be taken into consideration [4, 89, 94, 195, 200-202, 217, 231, 254, 255, 267, 272, 283, 285]. Stockmann et al. [342] reported a phytase from *H. polymorpha* under oxygen limited conditions in SmF. They found that pre–culturing the strains in glucose medium under oxygen limitation resulted in increased production (25%) and removed 20 h lag phase which was normally observed

when there is no such limitation. The production of phytase from *Aspergillus ficuum* NRRL 3135 has been achieved by three different cultivation methods, namely, solid state [72, 73], semi–solid [135] and submerged fermentations [149, 362].

It was reported that there was a complete reduction of phytic acid content in canola meal using SSF by *A. ficuum* [227]. Ebune et al. [72, 73] also studied the phytase production by *A. ficuum* using canola meal and found that the age of inoculum had profound effect on enzyme synthesis. Krishna and Nokes [181] studied the effect of culture conditions, particularly inoculum age, media composition (wheat bran and full–fat soybean flour) and duration of SSF on the phytase production by *A. niger*. Kim et al. [172] studied phytase production from *Aspergillus* sp. 5990 (higher optimum temperature for catalytic activity than the commercial Natuphos from *A. ficuum* NRRL 3135 using SmF at 37°C, pH 7.0 where five–fold higher activity in liquid culture was obtained. Papagianni et al. [266] investigated qualitative relationship between medium composition, morphology and phytase production by *A. niger*. Recently, in a study carried out by Lan et al. [188, 189], phytase production in batch fermentation by *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle, showed glucose repression in rice bran–soybean milk (2:1) medium and optimum production was attained at pH 7.0, 39°C without any surfactant [376].

The conventional methods of optimization are extremely time consuming, tedious and expensive for a large number of variables [29, 30, 42, 331]. Optimization of all the variables by statistical experimental designs, Plackett–Burman (PB) and central composite design (CCD) of response surface methodology (RSM), can eliminate the limitations of 'one variable at a time' approach [341]. The use of response surface methodology in biotechnological processes is gaining immense importance for the optimization of enzyme production [29, 30, 42, 331]. There are, however, very few reports on the statistical optimization of phytase production in SSF [29, 30, 42]. RSM is a widely used statistical method based on the multivariate non–linear model for optimization of fermentation media [125, 60]. Considering the interactions of various parameters during the fermentation process, RSM examines the responses of several factors by varying them simultaneously with a limited number of experiments [166, 225].

A few approaches other than PB and RSM, have also been employed for optimization of phytase production. A different optimization strategy i.e. artificial neural

network pattern recognition model and DO/pH measurements–based on–line control strategy (ANNPRCtrl), was successfully implemented for maximizing the phytase production with recombinant *P. pastoris* [159]. The modified ANNPR–Ctrl increased the final phytase activity about three–fold compared with other control strategies.

Bai et al. [15] proposed a novel method to produce both phytase and single–cell protein in recombinant Pichia pastoris (transformed with a phytase gene (AppA–m) from *Escherichia coli*) fermentation using monosodium glutamate wastewater (MSGW) as the basal medium. The fermentation medium was optimized in shake flasks by single–factor test and RSM. The maximum phytase activity in the MSGW medium reached 3,380 U/ml, 84.2% of that in chemically defined medium, and the dry cell weight was 136 g/l. The production of phytase by feed–grade filamentous fungi *Aspergillus ficuum* NRRL 3135, after nutrient optimization and selection of the most appropriate carbon and nitrogen sources, was increased by 50% [29]. In another optimization study carried out by Bogar et al. [30], *M. racemosus* NRRL 1994 phytase production in solid–state fermentation was nearly doubled (from 14.5 IU/g DM to 26 IU/g DM) on optimized coconut oil cake at 71% moisture level, pH 5.5, incubation temperature 25°C. By employing PB and CCD, an overall 5– and 1.6–fold enhancement in phytase titres (324–1689 U/g dry yeast biomass) and biomass production (6.4–10 g/l) were obtained for cell–bound phytase production by *Pichia anomala* in a cost–effective cane molasses medium [167]. In case of phytase production by a marine yeast *Kodamaea ohmeri* BG3 in a cost–effective oats medium, an overall 9–fold enhancement in phytase activity (62.0 –575.5 U/ml) was observed by Li et al. [196] due to optimization. The optimum concentrations of significant variables that supported maximum enzyme activity were oats 1.0%, ammonium sulphate 2.3%, glucose 2.0%, NaCl 2.0% and initial pH 6.3. Sunitha et al. [343] optimized the medium for recombinant phytase production by *E. coli* BL21 using response surface methodology. A 2^3 central composite experimental design was used to study the combined effects of the medium components, tryptone, yeast extract and NaCl. The optimization of the medium increased the phytase production by 1.2–fold. The supplementation of 2 g/l glucose significantly enhanced the phytase production by 1.58–fold, showing an overall 2.78–fold increase compared to the unoptimized medium. Recently, statistical experimental designs were applied for the optimization of medium composition for phytase production by *S. cerevisiae*. The variables analyzed were

sucrose, sodium phytate and urea in mineral medium, and in cane molasses, sodium phytate and corn steep liquor complex medium. The 2^2 full factorial central composite design of response surface methodology was applied for optimization the concentrations of the significant variables. The optimum variables that supported maximum phytase activity in sodium phytate as substrate were 2.5% sucrose and 0.5% sodium phytate in mineral medium. In complex medium, cane molasses and sodium phytate at 6–10.8% and 0.3–0.6% supported maximum phytase activity respectively. An overall 10– and 5.3–fold improvement in phytase activity was achieved due to optimization in mineral medium and cane molasses medium, respectively [295]. In a similar study carried out by Singh and Satyanarayana [331], a two fold increase in phytase production by a thermophilic mould *Sporotrichum thermophile* was achieved due to optimization using statistical designs in a cost– effective cane molasses medium. Phytase production by a thermophilic mould *Sporotrichum thermophile* Apinis was also investigated in solid state fermentation (SSF) using sesame oil cake as the substrate. The optimum values of the critical components determined by central composite design of RSM for the maximum phytase production were glucose 3%, ammonium sulphate 0.5% and incubation period 120 h. An overall 2.6–fold improvement in phytase production was achieved due to optimization. Highest enzyme production (348.76 U/g dry mouldy residues) was attained at a substrate bed depth of 1.5 cm in enamel coated metallic trays. In submerged fermentation, Starch, Tween–80, peptone and sodium phytate at 0.4%, 1.0%, 0.3% and 0.3% supported maximum enzyme titres, respectively, leading to an overall 3.73–fold improvement in phytase production due to optimization [330]. Enhanced phytase yield was attained with the yeast *Pichia anomala* using RSM by Vohra and Satyanarayana [385]. Interactions were studied for three variables, viz. glucose, and beef extract and inoculum density using CCD. Highest enzyme yields were obtained when the glucose and beef extract concentrations in the medium were 2 and 0.5%, respectively.

2.4. Strain improvement

2.4.1. Introduction

A very few work on strain improvement for enhancing the phytase production is available in the literature. The reported work is focussed on increasing the phytase titre

by using physical and chemical mutagens, chiefly, UV radiation. Chelius and Wodzinski [43] reported for *A. niger* NRRL 3135 phytase, in which UV radiation was used for strain improvement, resulting in a 3.3 fold higher phytase (phyA) than the wild type strain. The production of mutant phyA was highly repressed 60% by the inorganic phosphate (0.006%, w/v), however, their approach was limited by lack of specificity and sensitivity to discriminate between phytase and acid–phosphatase activity during primary screening process. Later Shah et al. [319] reported for *A. niger* NCIM 563 by using combinations of physical and chemical mutagens resulting into two mutant strains N–1 and N–79 with an increase of 17 and 47% activity in comparison to parent strain respectively. Rodriguez et al. [299] improved the catalytic efficiency and thermostability of *E. coli* pH 2.5 acid phosphatase/phytase expressed in *P. pastoris* by site–directed mutagenesis. Tomschy et al. [353] identified monomeric, glycosylated wild type phytase from *A. niger* T213 with a three–fold lower specific activity than *A. niger* NRRL 3135 phytase. The sequence comparison studies and identification of candidate amino acids allowed an increase in specific activity of *A. fumigatus* phytase up to seven–fold [353]. Mullaney et al. [225] increased the phytase activity of *A. niger* NRRL 3135 phytase A (phyA) at intermediate pH levels (3.0–5.0) by site directed mutagenesis of its gene at amino acid residue 300. A single mutation, K300E, improved phytic acid hydrolysis at 37°C by 56 and 19% at pH 4.0 and 5.0, respectively.

2.5. Physicochemical properties of phytases

The majority of the characterized phytases are monomeric proteins, but both homo– and hetero–oligomers have been found [178]. The molecular mass of these phytases is variable; most monomeric proteins are in the range of 40–70 kDa.

2.5.1. pH activity

Phytases, based on their optimal pH for catalysis, can be divided into two major groups, acid and alkaline. Since phytase research is focused on finding an enzyme that shows adequate activity in the digestive tract of monogastric animals, most studies have aimed at acid phytases [224]. The acid phytases belonging to HAPs, can display a broad or narrow range of pH vs. activity profiles and show a variety of pH optima, most

commonly found at pH 2.5 and/or pH 4.5–6.0 [178, 224, 243]. The characterised phytases belonging to PAPs have been shown to display slightly acidic or neutral pH optima. These are, phytases from kidney beans and *A. niger*, which show optimal activity between pH 4.5–5.0 [144] and pH 6.0 [223], respectively. The phytate–degrading enzyme produced by *S. ruminantium*, PhyAsr, was reported to have substantial activity at pH 5.0, but no optimal pH has been reported [49]. Few alkaline phytases are also reported such as phytase from *Lilium longiflorum* pollen [314], legume seeds [313] and *Bacillus* species, however, the BPPs from *Bacillus* are the only extensively characterized class of alkaline phytase [168, 177, 356]. Most recently isolated and purified alkaline phytases was from *Bifidobacterium sp.* Alkaline phytases optimally hydrolyze phytate at a pH between 7.0 and 8.0, likely due to protonation of calcium binding sites at a lower pH resulting in poor binding of Ca^{2+} [242].

From physiologically relevant standpoint as well as from an applied perspective, a significant activity over a wide range of pH (pH 1.5–7.5) encountered in digestive tract is desirable to aid phytate degradation in various parts of the alimentary canal such as the salivary gland (pH 5.0–7.0), stomach (initial pH 5.5 following food ingestion; pH reaches in between 1.5–2.0 after the HCl secretion), upper part of small intestine (pH 4.0–6.0) and rest part of the small intestine (pH 6.4–7.2). The phytases with low pH value optima are found to be more effective in gastrointestinal tract in comparison to phytases with high pH value optima [93]. A study was carried out to assess the effect of pH on enzyme activity of the four available commercial phytases such as Allzyme phytase produced by Alltech Ireland Ltd., Co. Meath; Natuphos produced by Gist–Brocades, Delfth, the Netherlands; Ronozyme P/Biofeed phytase produced by F. Hoffmann–La Roche Ltd., Switzerland and Novozymes A/S, Bagsvaerd, Denmark and it showed that all phytases were active in the stomach (pH 2.5–5.0) but no activity in small intestine (pH 6.4–7.2) was found except some activity in the uppermost part of small intestine, where pH remained below 6.4 [34]. The extracellular phytase displaying two pH optima was reported for *A. ficuum* NRRL 3135 (*Phy. A.*) phytase at pH 2.5 and 5.5 [369, 393] with 48% less activity at pH 2.5 than at pH 5.5 [359]. Phytase from *A. fumigatus* was also found to exhibit dual pH optima but in the less acidic range, at pH 4.0 and pH 6.0–6.5 [270]. Some work were also attempted where phytases from several strains (*A. fumigatus, A. niger* and *E. coli*) were engineered to modify the pH profile for functioning well under

gastric conditions but did not show the desired activity. For instance, mutant phytase from *A. niger* [225] showed a good activity over a limited pH range i.e. pH 3.0–3.5 rather than whole pH range of stomach (pH 2.0–6.5).

To improve the pH activity profile, several mutational approaches such as modification of ionizable groups directly involved in substrate specificity or catalysis, replacement of amino acid residues in direct contact with residues located in the active or substrate specificity site by means of hydrogen bonds or salt bridges, or alteration of distant charge interactions by modification of the surface charge of the enzyme were also taken.

Similar strategy, aimed at improving the catalytic properties of the thermostable consensus–1 phytase, was employed by Lehmann et al. [192], in which all the divergent amino acid residues present in the active site of the consensus phytase was replaced by those of *A. niger NRRL 3135* phytase. The new phytase (consensus–7 phytase) featured a major shift in the catalytic properties that were similar to those of *A. niger NRRL 3135* phytase, however, the active site residues transfer resulted in a decrease in the unfolding temperature of consensus–7 phytase as compared to consensus–1 phytase.

2.5.2. Stability under gastric conditions

Other important feature desirable for phytases is the acidophilic characteristics which enable them to survive the acidic environment of the digestive tract and thus can be used as a potential candidate in feed applications. An effective phytase needs to be resistant to proteolysis in the animal digestive tracts. It is reported that intrinsic wheat phytase is more susceptible to inactivation by pancreatin and pepsin than *A. niger* phytase. In contrast, the *A. niger* phytase is less resistant to degradation by pepsin than recombinant *E. coli* phytase (r–AppA) [298]. In another comparison of *Bacillus subtilis* and *E. coli* with four recombinant fungal phytases, the fungal phytases were most susceptible to inactivation by pancreatin and pepsin, *Bacillus subtilis* was stable to pancreatin whereas *E. coli* was stable to both pancreatin and pepsin. Commercial phytases also need to resist degradation during production and storage. When the phytases from *A. fumigatus* and *Emericella nidulans* were expressed in *A. niger*, they were cleaved by proteases present in the culture supernatant [392]. The cleavage had no

effect on the activity of *E. nidulans* phytase, but significantly reduced the activity of *A. fumigatus* phytase activity. Site–directed mutagenesis at the protease sensitive sites of the *A. fumigatus* and *E. nidulans* phytases yielded mutants with significant reduced susceptibility to proteolytic degradation. With no loss in activity in the range of pH 1.0–2.5 and more than 80% of activity from pH 2.5 to 8.0 when incubated for 60 min, was reported for *Yersinia rhodei* [150]. *S. cerevisiae* CY strain retained more than 80% of initial activity in the range of pH 2.5–5.5 when kept at 4°C for 1 h [152].

Several studies for different phytases were carried out to know effect on enzyme activity in milieu of the gastric condition. The study carried out by Boyce and Walsh [34] for four commercial phytases under the simulated gastric conditions (pH 2.5 with pepsin, incubation duration of 2 h) revealed a significant decrease in activity in case of all phytases. Allzyme and Natuphos retained 79.8 and 72% of initial activity whereas Ronozyme and Novozyme significantly lost their activity (1.0% and 0.5%). In absence of pepsin, Allzyme and Natuphos displayed 83.8 and 77.5% of original activity whereas Ronozyme and Novozyme phytases showed a significant low activity of 7.6 and 4.6% respectively. The outcomes illustrated decrease in activity due to low pH in case of former two enzymes whereas loss in activity by later phytases was apparently due to instability at low pH as well as degradation by pepsin. A comparative study on five recombinant phytases was carried out by Huang et al. [150] to assess their stability and activity in simulated gastric fluid (SGF; 0.25 M glycine/HCl containing 2.0 mg/ml NaCl and 3.2 mg/ml pepsin) after incubating the enzymes in SGF at 37°C for 20 min. *Y. rhodei* retained 20% of original activity at pH 1.5 in contrast to a complete loss of activity by *E. coli, Y. pestis, A. niger* and *Y. intermedia* phytases. Konietzny [178] concluded that most microbial phytases are more pH stable than plant phytases as stability of most plant phytases was reduced significantly at $4.0 < pH > 7.5$, whereas most microbial enzymes are relatively stable at $3.0 < pH > 8.0$.

2.5.3. Temperature optima

A feed enzyme should exhibit significant catalytic activity within the range of 25–39°C [34]. Most of the phytases from yeasts, fungi and bacteria have optimum temperature value in the range of 45–60°C [234, 327, 366] with some exceptions. The

optimum temperature exhibited by *Aspergillus ficuum* NRRL 3135 [363], *Aspergillus niger* [226, 393], *A. niger* 307 [92], *Peniophora lycii* [190, 369], *Schwannomyces castellii* [316] and *Aspergillus fumigatus* [370] were found to be 58°C; 50°C, 56–58°C, 50–55°C, 77°C and 58°C respectively. Most fungal phytases have optimum temperature in the range of 50–60°C; predominantly the temperature optima displayed by mesophilic fungi are generally found to be below 60°C [40]. The phytases having high temperature optima value are generally not able to retain full activity in the gastrointestinal tract of animals. The *A. niger* ATCC 9142 (Gargova et al., 2003), *Rhizopus oligosporus* ATCC 22959 (both crude and purified) [40] and *Mucor hiemalis* Wehmer [35] phytases which possess optimum temperature of 65, 65 and 55°C respectively, displayed only 39, 68 and 66% of its maximum activity at porcine physiological temperature (39°C), so, a phytase with low optimum temperature value might have advantage over other phytases.

2.5.4. Substrate specificity

Many of the characterized HAPs exhibited a broad specificity for substrates with phosphate esters. It was thought that this was due to a mechanism that conforms to nonspecific acid phosphatase properties, since both HAPs and acid–phosphatases have the ability to hydrolyze synthetic phosphorylated substrates [99]. In *A. fumigatus*, the phytase crystal structure had revealed a large active site cleft [198] allowing it to accommodate a wide variety of phosphate esters [270]. In contrast, the *E. coli* phytase was more specific for phytate [118], and accordingly, the crystal structure of this enzyme revealed a smaller active site cleft [197]. HAPs can only hydrolyze InsP6 when it existed as a metal–free phytate [392, 393]. The positively charged active site cleft of HAPs did not favour a metal–InsP6 complex [180, 197, 198]. Chelating agents such as EDTA had been shown to stimulate the phytate–degrading activity of HAPs by removing divalent metal cations [392, 393].

The substrate specificity of alkaline phytases is far narrower than that of HAPs, exhibiting strict specificity towards phytate and having relatively no enzymatic activity on other phosphate esters [177]. The strict specificity of alkaline phytases was explained by the preference for phosphate bridge formation between Ca^{2+} and the two oxianions from the adjacent phosphate groups of InsP6 [130, 327]. When dealing with phytate

specifically, alkaline phytases require divalent cations such as Ca^{2+} or Sr^{2+} for catalytic activity. The crystal structure of the phytase from *B. amyloliquefaciens* revealed a negatively charged active site that provides a favourable electrostatic environment for the positively charged calcium–InsP6 complex [130]. Consequently, EDTA strongly inhibited alkaline phytase activity by removing the required divalent metal cations [168, 175].

2.5.5. Thermostability profile

Thermostability is the major essential feature of any enzyme destined for animal feed applications. Therefore, intensive phytase research and development was primarily directed toward screening of thermophiplilic and hyperthermophilic organisms for thermotolerant enzymes, mutagenesis of mesophilic enzymes to increase their thermostability, and design of formulations of chemical coating for protecting the enzymes from heat–denaturation.

Animal feed is commonly pelleted at 60–95°C to increase its digestibility, ensure a balanced diet [175, 177], for controlling microbial growth, preventing transmission of pathogens and reducing transport costs by increasing bulk density. Most of the known phytases lose their activities by 40% after treatment at 68°C for 10 min [359], thereby completely losing their activity during feed pelleting process in the temperature range of 60–95°C. The initial enzyme originating from *A. fumigatus* was not a genuine heat stable enzyme, as it possessed a low T_m (62.5°C), but it was refolded into a fully active conformation after cooling [394]. Uses of exogenous agents such as non–ionic detergent, trehalose, sorbitol etc have also been reported in the literature, rendering stability of phytase during pelleting. A study was carried out by Boyce and Walsh [34] to assess the thermostability of different commercial phytases such as Allzyme, Natuphos, Ronozyme P/Biofeed and Novozymes at 80°C for 5 min. The study revealed a significant loss of 65 and 24% of initial activity during the first min by Allzyme and Natuphos phytases respectively whereas Ronozyme P/Biofeed and Novozymes phytases were able to retain more than 80% of activity after 5 min. The most thermostable phytase was reported from *Aspergillus fumigatus,* which lost only 10% of initial activity at 100°C over a period of 20 min [270]. Another thermostable phytases from *Bacillus amyloliquefaciens* DS11

[171] and *Sporotrichum thermophile* [332] showed $t_{1/2}^{80°C}$ of 42 and 90 min respectively. The phytases from *A. fumigatus* and *A. niger* were completely denatured between 50 and 70°C [393]. The crude phytase from *Mucor hiemalis* Wehmer retained 64 ± 3%, 63 ± 2% and 61 ± 1% of its original activity after heating for 1, 3 and 5 min, respectively, at 80°C. The same phytase after purification was found significantly less thermostable than the crude preparation, retaining 21 ± 3% of its original activity after heating at 80°C for one min [35]. However, from an application perspective this was found not relevant as it is crude preparations that are added to feed to endow with accessory proteins and feed enzymes secreted into the SSF extract.

2.6. Applications of phytases

Although phytic acid is the principal storage form of phosphorus, it is not bioavailable for non–ruminant animals due to their lack of ability to metabolize it [236, 237]. This has had significant impact on modern intensive livestock productions due to consumption of grain based diets, as approximately 80% of phosphorus in cereal grains and legumes is present as phytate phosphorus [293]. A consequence is the need to supplement monogastric livestock diets with expensive and non–renewable inorganic phosphate to fulfil their nutritional requirement [236, 237]. The unutilized phosphorus of phytate present in the diet is excreted in the manure and is subsequently hydrolyzed by soil and water borne microorganisms. The released phosphate moves into rivers and lakes and can result in eutrophication of water supplies [279, 355]. During the past years, antinutritional attributes of phytate has been a matter of great concern for human populations, who depend on staples like wheat, rice and maize, as their major or only source of nutrition, as it can lead to mineral deficiency and malnutrition [45, 212].

Phytase has a wide range of applications as feed and food additive, in animal and human nutrition. Supplementation of phytase into animal feed has multiple benefits, mainly in improving availability of minerals, trace elements, amino acids and energy [132, 391,] and thereby decreasing the necessity to fortify the fodder. By increasing the utilization coefficient of organic phosphate and consequently reducing the amount of phosphate in manure, it contributes significantly toward environmental protection. For many years, potential of phytase has been well recognized in breadmaking procedures for

degrading the phytic acid content and improving the crumb texture. It helps in releasing the chelated cations such as calcium, which in turn promotes the activation of endogenous α–amylase [141], thus negating the need to supplement external α–amylase.

Recently, the phytases have become a subject of additional interest for their ability to produce lower inositol derivatives after phytic acid degradation. Even though mammalian cells are able to synthesize the inositol phosphates themselves [404], endogenous synthesis of phytic acid is minor [107, 108]. The inositol polyphosphates (IPPs) generated is useful from both kinetic and physiological aspects. IPPs have been recognized as having novel metabolic effects such as prevention of diabetes complications [302, 303], treatment of chronic inflammation [50], reduction in the risk of colon cancer [18, 105, 320] and kidney stone prevention [219, 243]. Additionally, the IPPs D–*myo*–inositol (1,3,4,5) tetra*kis*phosphate and D–*myo*–inositol (1,4,5) triphosphate have been found to stimulate intracellular Ca^{2+} release which affect cellular metabolism and secretion [140, 214, 274]. The growing list of research and pharmaceutical applications for specific IPPs has increased interest in the preparation of these compounds. The chemical synthesis of individual IPPs includes difficult steps and is performed at extreme conditions [26]. The separation of IPP isomers has also been reported to be difficult with most analytical approaches [113]. Since phytases hydrolyze InsP6 in an ordered and sequential manner, the production of IPPs and free *myo*-inositol using phytase is a potential and promising alternative to chemical synthesis [112, 116, 132, 391].

2.7. Tables of Chapter 2

Table 2.1 Phytate content in plants and plant products [294].

Cereals	Phytate Phosphorus (%)	Phytate (%)
Wheat	0.11–0.38	0.39–1.35
Corn	0.23–0.63	0.83–2.22
Popcorn	0.17	0.62
Triticale	0.14–0.53	0.50–1.89
Oat	0.20–0.33	0.70–1.16
Barley	0.21–0.33	0.75–1.16
Rye	0.15–0.41	0.54–1.46
Cereal milled		
Wheat flour	0.07–0.39	0.25–1.37
Wheat flour (India)	0.14–0.16	0.50–0.55
Wheat bran	0.80–1.44	2.85–5.11
Corn meal	0.23–0.30	0.81–1.07
Beans		
Soybeans	0.28–0.63	1.00–2.22
Faba beans	0.15–0.50	0.54–1.77
Dolique beans	0.17–0.26	5.92–9.15
Peas	0.06–0.33	0.22–1.22
Pigeon pea	0.20–1.97	0.71–7.00
Linseed	0.61–0.79	2.15–2.78
Peanuts	0.50	1.76
Chickpeas	0.08–0.35	0.28–1.26
Kidney beans	0.25–0.44	0.89–1.57
Bean products		
Peanut meal, defatted	0.48	1.70
Linseed meal, defatted	1.18	4.20
Bean based foods		
Tempe	0.27–0.30	0.67–1.08
Tofu	0.55–0.82	1.96–2.90
Idli	0.15	0.54
Dhokla	0.51	1.80

Table 2.2 Phytate content in Seeds/Grains/Fruits with seeds commonly eaten by human [204].

Plants	Structure	Phytate (%)
Sesame	Dry seed	4.71
Pumpkin/Squash	Embryo	4.08
Flax (linseed)	Dry seed	3.69
Rapeseed (canola)	Dry seed	2.50
Sunflower	Embryo	2.10
Mustard	Dry seed	2.00
Cashew	Embryo	1.97
Peanut	Seed in shell	1.70
Tomato	Seed only	1.66
Soybean	Dry seed	1.55
Almond	Dry embryo	1.42
Eggplant	Seed only	1.42
Beans	Dry seed	1.41
Watermelon	Seed only	1.36
Cucumber	Immature seed	1.07
Sorghum	Dry grain	1.06
Coco beans	Dry seed	1.04
Barley	Dry grain	1.02
Oats	Dry grain	1.02
Wheat	Dry grain	1.02
Peas	Dry seed	1.00

Table 2.3 Phytases from different sources.

Phytase sources	References
Fungi	
A. niger NRRL 3135	[224], [324]
A. flavus	[323]
A. terrus	[398]
A. ficuum	[29]
A. ficuum (phy A)	[174], [362]
A. ficuum (phy B)	[75], [367]
A. carneus	[96]
A. niger ATCC 9142	[41]
A. oryzae	[325]
A. fumigatus	[269], [406]
A. niger SK–57	[226]
Mucor sp.	[30], [323]
Penicillum sp.	[323]
P. caseoicolum	[12]
Cladosporium sp.	[277]
Peniophora lycii	[190], [368], [396]
Rhizopus oligosporus	[305], [344]
Rhizomucor pusillus	[42]
Sporotrichum thermophile	[330-335]
Yeasts	
Saccharomyces cerevisiae	[111],[136], [139], [234]
Schwanniomyces castelii	[186], [316]
S. occidentalis	[228]
Kluyveromyces fragilis	[187]
Candida tropicalis	[187]
Torulopsis candida	[187]
Debaryomyces castelii	[187]
D. castelii CBS 2923	[280]
Pichia pastoris	[136]
Bacteria	
Bacillus subtilis	[168], [169], [275]
B. subtilis (natto)	[326]

Table 2.3 Cont.....

B. amyloliquefaciens	[175], [177]
B. licheniformis	[356]
E. coli	[118], [245]
Klebsiella aerogenes	[346]
K. terrigena	[114], [117]
K. oxytoca	[158]
Pseudomonas sp.	[47], [154]
Enterobacter sp.	[403]
Citrobacter freundii	[66]
Citrobacter braakii	[173]
Mitsuokella multiacidus	[401]
S. ruminantium	[49]
Yersinia	[87], [150], [151]
Plant	
Maize, germinated	[184]
Rye	[119]
Lily pollen	[160], [314]
Hazel seed	[13]
Faba beans	[120]
Canola seeds	[148]
Sunflower	[6]
Crude extract wheat	[31]
Legume seeds	[313]
Wheat bran	[229]
Typha latifolia pollen	[138]
Barley	[115]
Animal	
Rat, intestinal mucose	[399], [400]
Rat, liver	[56]
Paramecium	[86]
Birds, reptiles, and fishes	[286]

Table 2.4 Global scenario in the feed enzyme market.

Regions	Market split by enzyme types		Global market share	Dominant raw material	Growth		Market potential
	NSP %	Phytase %	%		NSPs	Phytase	
Europe	65	35	44	Wheat, Barley	Modest	Seen to grow faster	Mature
U.S.	35	65	21	Corn, Soybeans	Seen to grow faster in coming years	Steady/ slow	Growing
China	75	25	16	Wheat, Barley	Show slow–steady growth in coming years	Seen to increase through penetration in swine	Good
South–East Asia	65	35	14	Corn, Soybeans	Show slow–steady growth in coming years	Thailand, Philippines and Indonesia seen to boost market	Good
Australia and NewZealand	70	30	4	Wheat, Barley	Slow growth in coming years owing to low farm animal	Improved growth in coming years	Less

Table 2.5 List of commercially available phytases [38].

Company	Country	Phytase source	Production strain	Trademark
AB Enzymes	Germany	*A. awamori*	*T. reesei*	Finase
Alko Biotechnology	Finland	*A. oryzae*	*A. oryzae*	SP, TP, SF
Alltech	USA	*A. niger*	*A. niger*	Allzyme phytase
BASF	Germany	*A. niger*	*A. niger*	Natuphos
BioZyme	USA	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	USA	*P. lycii*	*A. oryzae*	Bio–Feed Phytase
Fermic	Mexico	*A. oryzae*	*A. oryzae*	Phyzyme
Finn feeds International	Finland	*A. awamori*	*T. reesei*	Avizyme
Genencor International	USA	*P. simplicissimum*	*Penicillium funiculosum*	ROVABIO
Roal	Finland	*A. awamori*	*T. reesei*	Finase
Novozymes	Denmark	*A. oryzae*	*A. oryzae*	Ronozyme®, Roxazyme®

Table 2.6 Various production techniques and substrates used for the production of phytase from different microbial sources.

Sources	Production Technique	Substrate used	References
Fungi			
Aspergillus ficuum	SSF	canola meal	[72], [73]
A. niger	SSF	Dry olive wastes	[374]
A. niger ATCC 9142	SmF	corn starch media	[41]
A. niger	SSF	wheat bran, soybean meal	[39]
A. niveus	SSF	Wheat bran, corn bran, soy meal,	[78]
A. niger N 25	SmF	Starch	[44]
A. sp. 5990	SmF	Sucrose based medium	[172]
A. oryzae RIB–128	SmF	Dextrin	[88]
A. niger	SSF	Wheat bran, soy meal	[181]
A. oryzae AK9	SSF	Soybean meal	[183]
A. niger NCIM 563	SSF	Wheat bran, mustard cake, cowpea meal, groundnut cake, coconut cake, cotton cake, black bean flour	[208]
A. terreus	SmF	Glucose based complex medium	[216]
A. niger	SmF/SSF	Corn starch medium/wheat Bran and full fat soybean meal	[265]
A. niger van Teighem	SmF	Starch and glucose based medium	[377-379]
A. ficuum	SSF	Soybean cake	[29]
A. niger 307	SmF	Starch media	[311]
A. carbonarius	SSF	Canola meal	[8-10]
A. ficuum	SmF/ SSF	Glucose/canola meal	[227]
A. niger	SmF	Maize starch	[7]
A. sp. FS3	SSF	Citric pulp, apple pulp, soy bran, wheat bran, rice bran	[339]
A. niger NCIM 563	SmF	Rice bran–glucose–salt media	[21], [319]
A. niger NCIM 563	SmF	Dextrin and glucose based medium	[337]
A. niger CFR 335	SmF/SSF	Potato dextrose broth/wheat bran	[128]
Cladosporium sp.	SmF	Potato dextrose broth	[277]
M. racemosus NRRL 1994	SSF	Wheat bran, Groundnut cake, sesame cake	[301]
M. racemosus	SSF	Wheat bran, coconut cake, sesame cake	[30]
M. hiemalis Wehmer	SmF	Corn starch media	[35]
M. thermophila	SmF	Glucose based complex media	[216]
Rhizopus oligosporus	SmF	Corn starch media	[40]

Table 2.6 Cont.....

Rhizopus sp.	SSF	Coconut cake, sesame cake	[281]
Rhizopus oligosporus	SSF	Coconut cake	[305]
Rhizomucor pusilus	SSF	Wheat bran	[42]
S. thermophile	SmF	Cane molasses medium, starch	[330-31], [335]
S. thermophile	SSF	Sesame cake	[333], [334]
T. lanuginosus	SSF	Wheat bran	[19], [127]
T. aurantiacus	SmF	Glucose/Starch based media	[232]
Yeasts			
Arxula adeninivorans	SmF	Glucose and Galactose based medium	[310]
Candida kruesi	SmF	Glucose based medium	[276]
Cryptococcus laurentii	SmF	Glucose based medium	[270]
Debaryomyces castelli	SmF	Synthetic medium	[280]
Hansenula polymorpha	SmF	Glucose	[210]
Kodamaea ohmeri	SmF	Oats based medium	[196]
Pichia anomala	SmF	Cane molasses medium	[85], [86], [167]
S.accharomyces cerevisiae	SmF	Yeast extract and Succinic acid	[152]
S. cerevisiae	SmF	Mineral media and Cane molasses media	[295]
Schwanniomyces castelli	SmF	Wheat bran, cotton flour	[315]
Bacteria			
Bacillus sp. KHU–10	SmF	Maltose	[48]
B. amyloliquefaciens	SmF	Wheat bran and casein hydrolysate	[176]
Enterobacter sp.	SmF	Glucose based complex media	[403]
Escherichia coli	SmF	Glucose based complex media	[343]
E. coli	SmF	Monosodium glutamate water	[15]
Lactobacillus amylovorus	SmF	Glucose	[340]
Mitsuokella jalaludinii	SmF	Rice bran–soybean milk media	[188], [189]
Pantoea agglomerans	SmF	Luria–Bertani media	[109]
Peniophora lycii	SmF	Complex media	[190]
Yersinia intermedia	SmF	Luria–Bertani broth	[150], [151]
Yersinia kristeenenii	–	–	[87]

Table 2.7 Compositions of oil cakes.

	As % of dry matter				
Oilseed cake/meal	**Protein content**	**Phosphorus content**	**Methionine & Cysteine**	**Lysine**	**References**
Soybean meal	54	0.69	1.70	3.46	[68, 100]
Soybean cake	45–46	0.71	1.44	2.93	[68, 100]
Sunflower seed cake	34.1	1.30	1.36	1.19	[100]
Sunflower seed meal	42.7	–	1.70	1.49	[100]
Rapeseed meal	34	–	–	–	–
Groundnut cake	48–50	0.83	0.55	1.52	[68, 244]
Groundnut meal	37.3	0.75	0.48	1.34	[68, 244]
Cottonseed cake	26–48	1.34	1.04	1.52	[100]
Cottonseed meal	46.1	1.36	1.29	1.89	[100]
Copra cake	23–24	–	–	–	–
Palm kernel cake	18	0.60	0.94	0.75	[68, 100]
Linseed cake	30.5	0.96	1.34	1.07	[100]
Linseed meal	36	–	1.58	1.26	[100]
Sesame cake	37	1.41	1.89	1.08	[100]
Sesame meal	44	–	2.24	1.28	[100]
Coconut	18–22%	0.58	0.38	0.50	[68, 100]
Para rubber seed	20	0.47	–	–	[68, 100]

2.8. Figures of Chapter 2

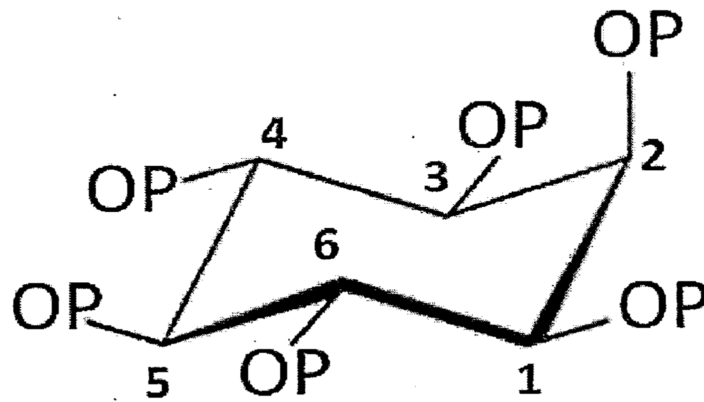


Fig. 2.1 *myo*–inositol (1,2,3,4,5,6) hexa*kis*phosphate (phytic acid)

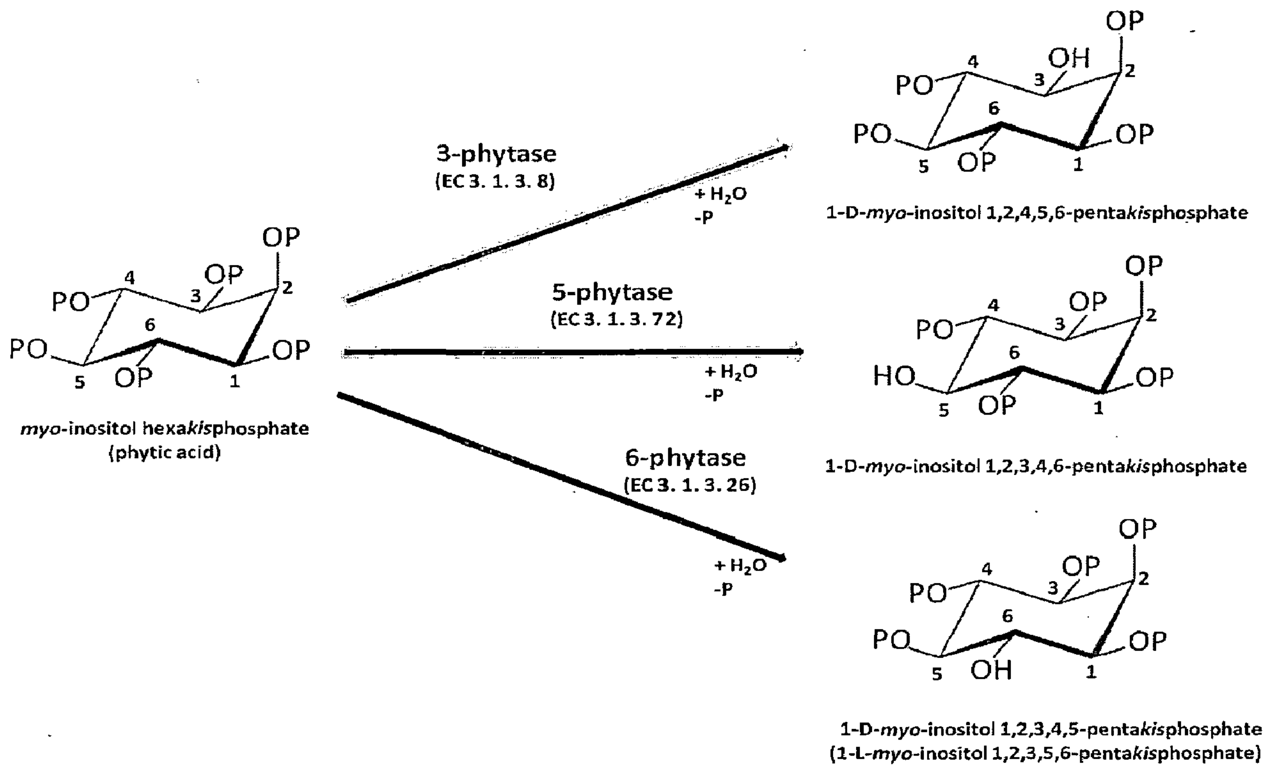


Fig. 2.2 Classification of Phytases

CHAPTER 3
LACUNAE AND RATIONALE

CHAPTER 3
LACUNAE AND RATIONALE

A compendium of phytases are reported from different sources, however, a thermostable and acid stable phytase with broad substrate specificity is still highly desirable. To work out this problem, scientific world is still trying to find out an ideal phytase especially produced from microbial source. Many of the researchers shifted towards recombinant technology and protein engineering; however, it affected the cost effectiveness of the resultant phytases. Despite multitude of advancements in this field, an economical and effective phytase is still an enigma yet to be solved by the researchers. In general, lack of thermal stability and resultant inactivation during pelleting process, and overall an economical production process has been major hall marks in this field. Moreover, phytases are not yet employed in food processing applications, however, there have been many studies focused on improving food quality for people in developing countries. Therefore, the present study accentuated with the following rationales:

1. Identification of a potential phytase producing microorganism.
2. Biochemical characterization of phytase.
3. Optimization of parameters for the production of phytase under Solid state fermentation (SSF) and Submerged fermentation (SmF).
4. Large volume production studies.
5. Evaluation of phytase ability in term of phytate degradation and mineral extractability.

CHAPTER 4
AIMS AND OBJECTIVES

CHAPTER 4
AIMS AND OBJECTIVES

The aim of the present study is to identify a microorganism producing an ideal phytase. To achieve major rationale of this study, following objectives were worked out:

1. Identification of a potential phytase producing microorganism.
2. Purification and characterization of phytase.
3. Optimization of parameters for the production of phytase in solid state fermentation (SSF) and large volume production studies.
4. Strain improvement procedure.
5. Optimization of parameters for the production of phytase in submerged fermentation (SmF) and large volume production studies.
6. Evaluation of potential application of phytase in terms of phytate degradation and mineral extractability.

CHAPTER 5
MATERIALS AND METHOD

CHAPTER 5
MATERIALS AND METHOD

5.1. Microorganism and inoculum preparation

Rhizopus oryzae (MTCC 1987) was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The fungal strain was routinely grown on potato dextrose agar (PDA) slants for 6 days at 30°C. Viable spores from slants were harvested by washing with 0.1% (v/v) Tween 80 and the spore suspension adjusted to ~$1x10^6$cfu/ml (colony forming units per millilitre) was used as inoculum for subsequent fermentations.

5.2. Screening of fungal strain for phytase production

Rhizopus oryzae was screened for phytase production on phytase screening medium (PSM) containing (w/v): 0.4% Sodium phytate, 1% D–glucose, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.5% NH_4NO_3, 0.01% $MnSO_4.H_2O$, 0.5% $CaCl_2.2H_2O$, 0.01% $FeSO_4.7H_2O$, 1.5% Agar–Agar and the medium was adjusted to pH 5.6 [149]. To eliminate the false positive halozones (zones of clearing) resulting from the microbial acid production, the counterstaining technique was performed according to Bae et al. [14]. In brief, the strain was grown on aforementioned phytase screening medium followed by flooding with 2% (w/v) aqueous cobalt chloride solution and were kept for 5 min incubation at room temperature. The cobalt chloride solution was thereafter replaced with a freshly prepared solution containing equal volume of 6.25% (w/v) ammonium molybdate and 0.42% (w/v) ammonium vanadate solution. After incubating for 5 min at room temperature, the plates were examined for zone of clearance against a contrast translucent background.

5.3. Solid state fermentation (SSF)

5.3.1. Phytase production

Initially, 5 g of dried substrates were taken into 250 ml Erlenmeyer flasks and were supplemented with one millilitre of distilled water. Substrates were sterilized at 121°C for 20 min, cooled and inoculated with one millilitre of spore suspension and fermentation

for stationary culture was carried out at 35°C for 96 h for phytase production. The fermented medium was extracted with Tween 80 (0.1% (v/v)) at 30°C on an orbital shaker at 200 rpm for 1 h and subsequently centrifuged at 10,000 x g for 10 min. The cell free crude extract was then used for phytase activity assay.

5.4. Evaluation of agro–industrial residues for phytase production

Various agro–industrial residues (individually and in combinations) namely, linseed oil cake (LOC), mustard oil cake (MOC), sunflower oil cake (SOC), rapeseed oil cake (ROC), wheat bran (WB) and rice husk (RH) were investigated for the production of phytase in SSF mode. These were purchased from local retail feedstuff outlets in Roorkee (Uttarakhand, India).

5.5. Effect of culture conditions on phytase production

5.5.1. Effect of particle size on phytase production

The oilcakes were firstly subjected to a sieving procedure employing mesh–size sieves of 4, 8, 12, 16 and 20 and then used as substrate for SSF. The smallest particles were of ~1.0 mm size, collected from fractions between meshes 16 and 20 (−16, +20), intermediate particles (~1.5 mm) were collected from fractions (−8, +12) and finally, heterogeneous oilcake (0.5–5.0 mm) was also used as substrate.

5.5.2. Effect of inoculum age and size on phytase production

The study was carried out in basal SSF medium containing WB and LOC (~1.0 mm) in equal ratio (1:1) and inoculated with inoculum of different age varying from one to seven days and inoculum size varying from 10–100% (v/w) at 35°C for 96 h.

5.5.3. Effect of metal ions on phytase production

Effect of various metal ions (Na^{+}, Mg^{2+} and Fe^{2+}), separately and in combinations, was studied at an overall concentration of 0.6% (w/v). The SSF medium optimized in the previous step was inoculated with 20% (v/w) of four days old inoculum and incubated at 35°C for 96 h for phytase production.

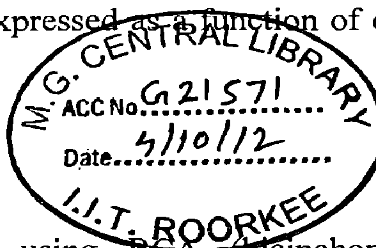
5.5.4. Effect of surfactants on phytase production

Effect of various surfactants like, Tween 20, Tween 40, Tween 80, Triton–X–100 and SDS on phytase production by *R. oryzae* was investigated at the level of 0.5% (v/w) in the SSF medium containing WB and LOC (~1.0 mm) in equal ratio (1:1) supplemented with salt solution containing 0.3% NaCl and 0.3% $MgSO_4.7H_2O$ and inoculated with 20% (v/w) of four days old inoculum. Fermentation was carried out at 35°C for 96 h for production of phytase.

5.6. Analytical methods

5.6.1. Phytase activity assay

Phytase activity was determined by estimating the inorganic phosphate released from sodium phytate [14]. One unit (U) of phytase is defined as the amount of enzyme required to release one nmol of inorganic phosphate (P_i) per second under the standard assay conditions. The enzyme yield was expressed as a function of dry substrate weight (U/gds).



5.6.2. Protein estimation

Soluble proteins were quantified using BCA (bicinchoninic acid) protein quantification kit (Sigma, USA). Soluble protein content was expressed as milligram of glucosamine per gram dry substrate (mg/gds).

5.6.3. Fungal biomass estimation

In SSF, fungal biomass was estimated by determining the *N*–acetyl glucosamine released by acid hydrolysis of the chitin [306]. Initially, 0.5 g of dried fermented material was mixed with two millilitre of concentrated sulphuric acid and was kept at 30°C for 24 h. The concentration of mixture was adjusted to 1 N, autoclaved for 1 h, neutralized with 1 N NaOH and then made up to 100 ml with sterile water. One millilitre of this mixture was incubated with one millilitre acetyl acetone reagent at 100°C for 20 min. After cooling, 6 ml of ethanol was added followed by the addition of one millilitre of Ehrlich reagent and were incubated at 65°C for 10 min. The glucosamine content was quantified

using glucosamine as standard at 530 nm. Biomass was expressed as milligram of glucosamine per gram dry substrate (mg/gds).

In SmF, fungal biomass was estimated by filtering the culture through a pre–weighed dry Whatman No. 1 filter paper circles. The mycelium was thoroughly washed with distilled water and dried at 80°C to a constant weight. The reducing sugars were estimated by dinitrosalicylic acid (DNS) method [215].

5.6.4. Determination of phytic acid content

The method is based on the estimation of indirect Fe (II) complex formation according to Garcia–Villanova et al. [91]. Briefly, the wheat bran samples (2 g) were extracted under magnetic agitation with 40 ml of extraction solution (5% Na_2SO_4 in 400 mM HCl) for 3 hours at room temperature. The suspension was centrifuged at 6000 rpm for 20 min and the supernatant was filtered and transferred to 250 ml Erlenmeyer flask. 20 ml of 5% Na_2SO_4, 20 ml of 20 mM $FeCl_3$ prepared in 160 mM HCl and 20 ml of 20% sulphosalicylic acid solution were mixed with the supernatant obtained above, shaken gently and the flasks were kept in boiling water bath for 15 min covered with aluminium foil, to minimize evaporation loss. The flasks were then allowed to cool under tap water and left standing. The ferric phytate precipitate can be observed here. One aliquot of 20 ml of clean floating liquid was transferred into a 250 ml beaker, adjusted to pH 2.5 by addition of glycine and diluted to 200 ml. The solution was then heated at 70–80°C and, whilst still warm, titrated with 10 mM EDTA sodium salt solution. The 4:6 Fe/P atomic ratios was used to calculate the phytic acid content.

5.7. Purification of phytase

5.7.1. Purification steps

SSF crude extract was subjected to fractional ammonium sulphate precipitation (40–80% saturation) with constant stirring. The precipitate was collected by centrifugation (15,000 x g, 20 min) and dissolved in 200 mM Tris–HCl, pH 8.0. The proteins after dialysis were loaded onto a DEAE Sepharose CL–6B column and eluted with various salt gradients in elution buffer (200 mM Tris–HCl, pH 8.0). Sephadex G–100 gel filtration column pre–equilibrated with 200 mM acetate buffer (pH 5.0), was used for desalting and separation of proteins based on size. Fractions showing high absorbance

at 280 nm were assayed for phytase activity. The proteins in the crude extract and the eluted fractions after size–exclusion chromatography were resolved by SDS–PAGE on a 12% separating gel topped with a 4% stacking gel in a vertical electrophoretic system. Gel staining was carried out using Coomassie Blue R–250 [185] and zymogram staining [14].

The zymogram staining was performed according to Bae et al. [14]. Firstly, the phytase was renatured by soaking the gel in 1% (v/v) Triton–X–100 for one hour at room temperature followed by soaking in 0.1 M sodium acetate buffer (pH 5.5) for one hour at 4°C. The gel was then incubated in substrate solution (0.4% (w/v) sodium phytate prepared in 0.1 M sodium acetate buffer; pH 5.5) at 39°C for 16 h. Phytase activity was visualized after treating the gel with cobalt chloride and ammonium molybdate/ammonium vanadate solution.

To check the homogeneity of the purified protein, Native–PAGE was carried out according to Casey and Walsh [40]. The enzyme purified by fractional ammonium sulphate precipitation and subsequent ion–exchange and size–exclusion chromatography was used for characterization studies.

5.7.2. Molecular weight determination and SDS–PAGE

The molecular properties of the purified fraction of *Rhizopus* phytase was determined by size–exclusion chromatography. 2 ml of phytase solution was loaded onto a Sephadex G–100 column preequilibrated with 0.1 M sodium acetate buffer (pH 5.5). Void volume was determined using Blue Dextran (2 mg/ml) and the column was calibrated with following protein standards (Sigma; 10mg/ml): glucose–6–phosphate dehydrogenase (120 kDa), creatine kinase (81 kDa), bovine serum albumin (68 kDa), carbonic anhydrase (36 kDa) and myoglobin (17 kDa). The eluted fractions were collected and assayed for phytase activity.

5.8. Characterization studies of phytase

5.8.1. Effect of pH

The pH optimum was determined over the range of pH 1.0–9.5 using 100 mM buffers: glycine–HCl (pH 1.0–3.5), acetate (pH 3.5–6.5), Tris–HCl (pH 6.5–8.5) and

Glycine–NaOH (pH 8.5–9.5) at 39°C. The activity measured in 0.1 M acetate buffer (pH 5.5) was taken as 100%.

5.8.2. pH stability studies

pH stability study for phytase were determined at 4°C after incubation of phytase for 6 h in the aforementioned buffers.

5.8.3. Effect of temperature

The temperature optimum was determined at different temperature varying from 25°–80°C. To check the thermal stability, the phytase was preincubated at 50°, 60°, 70° and 80°C up to 30 min, respectively, cooled to room temperature and assayed using the standard phytase assay method. Relative activity at each temperature was calculated relative to that at zero min (not incubated) taken as 100%.

5.8.4. Effect of metal ions, inhibitors, organic solvents and detergents on phytase

The effect of metal ions (Fe^{2+}, Na^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, k^{+}, Ag^{+}, Cu^{2+}, Na^{+}, $(NH_4)_2^{2+}$, Co^{2+}, Zn^{2+} and Ba^{2+}; at 1 mM and 5 mM), inhibitors (EDTA, citrate, oxalate, sodium azide, tartarate, iodoacetamide (at 1 mM and 5 mM) and β–Mercaptoethanol (at 5 and 10% (v/v)), organic solvents (Glycerol and ethyl alcohol; at 5 and 10% (v/v)) and detergents (Tween 20, Tween 40, Tween 80, Triton–X–100 and SDS; at 1 and 5% (v/v)) on phytase activity were determined by inclusion of these effectors in the standard phytase activity assay. The relative activity was then determined and expressed as percentage of that without any modulators.

5.8.5. Substrate specificity studies

Substrate specificity was carried out by using the phytase activity assay in 0.1 M acetate (pH 5.5) containing 2 mM tested substrate. Besides phytic acid, *p*NPP (*p*–nitro phenyl phosphate), NADP (Nicotinamide adenine dinucleotide phosphate), SNP (Sodium–1–naphthyl phosphate), FBP (Fructose–1, 6–biphosphate), R–5–P (Ribose–5–phosphate), G–1–P (Glucose–1–phosphate), PEP (Phosphoenol pyruvic acid), AMP (Adenosine monophosphate), ADP (Adenosine diphosphate) and ATP (Adenosine triphosphate) were tested as substrate.

5.8.6. Simulated Gastric Fluid (SGF) activity assay

To check the stability of phytase under gastric conditions, simulated gastric fluid assay was performed at pH 1.5, 5.5 and 7.5 according to Garrett et al. [93]. Enzyme stability in simulated gastric fluid (SGF: 84 mM HCl containing (mg/ml), NaCl 2.0 and Pepsin 3.2; [93] was determined by incubating the phytase and SGF (1:4) at 37°C at pH 1.5, 5.5 and 7.5. Aliquots of the digestion reaction mixture were removed at intervals and the residual phytase activity was determined according to the standard procedure. As a negative control, phytase was incubated in the SGF minus pepsin.

The kinetic constants K_m and V_{max} were determined with sodium phytate as substrate using Lineweaver–Burk plot (LB plot). To study the inhibition kinetics, the phytase was incubated in presence of fluoride, phosphate and vanadate at the concentration of 5×10^{-5} mM.

5.9. Statistical media optimization for the production of phytase in SSF

5.9.1. Plackett–Burman design (PBD)

PBD was employed for screening the most significant culture variables influencing the phytase production. Based on initial studies, eight assigned factors and three unassigned factors (dummy) were screened in a total of 12 runs. The detail of the design with the actual and predicted responses (phytase activity) is given in Table 5.1.

5.9.2. The path of steepest ascent (descent) method

To approach rapidly in close proximity to optimum response, the method of path of steepest ascent (descent) was performed (Table 5.2). The direction of the maximum increase in phytase activity was determined according to the estimated coefficient ratio from the fitted first–order model in PBD.

5.9.3. Central composite designs (CCD)

To determine the mutual interactions among the selected variables (mannitol, ammonium sulphate and K_2HPO_4/Na_2HPO_4) and their corresponding optimum concentrations, central–composite design (CCD) of response surface methodology (RSM) was used. A 2^3 factorial design having eight factorial points, six axial points and six

replicates at the centre point with a total number of 20 runs was formulated. The details of experimental design with coded and actual levels of each factor are summarized in Table 5.3 and Table 5.4.

A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response to the independent factors. The complete second–order polynomial model equation (1) to be fitted to the yield values was:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1, i \neq j}^{n-1} \sum_{j=2}^{n} \beta_{ij} x_i x_j \quad (1)$$

where, Y is the observed value of the response (phytase production); x_i (i = 1, 2 and 3) is the controlling factors; β_o is the offset term, and β_i (i = 1, 2 and 3), β_{ii} and β_{ij} (i = 1, 2; j = 2 and 3; and $i \neq j$) are the model linear, quadratic and interaction coefficient parameters, respectively.

5.9.4. Statistical analysis

The statistical software package 'Design–Expert®8.0.5, Stat–Ease Inc., Minneapolis, MN, USA was used for experimental design and subsequent regression analysis of the experimental data. All experiments were done in triplicate, and the average phytase activity was taken as the response.

For mineral analysis, one way analysis of variance (ANOVA) was applied to determine the significant differences at $P<0.05$ using SPSS software.

5.9.5. Validation

The optimum levels of medium components predicted by model was used for production of phytase in flasks (250ml) containing 5 g of LOC + WB (1:1, w/w)) The substrate (LOC + WB (1:1)) was supplemented with (w/w) 2.05% mannitol, 2.84% ammonium sulphate and 0.38% of K_2HPO_4/Na_2HPO_4 (1:1), 20 % v/w mineral solution (pH 7.6) with a final moisture content of 40% adjusted with addition of distilled water. The medium was autoclaved and inoculated with 20 % v/w inoculum. Fermentation was carried out at 30°C for 3 days. The fermented medium was extracted with Tween 80 (0.1% (v/v)) at 30°C on an orbital shaker at 200 rpm for 1 h. Cell free extract was used for phytase activity assay. All experiments were performed in triplicate and the data were presented as the mean ± SD.

5.10. Large volume production studies

5.10.1. Fermentation in flasks and trays

Flask level experiments were carried out under optimum conditions, predicted by the model, using 5–50 g of substrate (LOC + WB; (1:1)). The substrate was supplemented with (w/w): 2.05% mannitol, 2.84% ammonium sulphate and 0.38% of K_2HPO_4/Na_2HPO_4 (1:1), 20 % v/w mineral solution (pH 7.6) with a final moisture content of 40% (v/w). The medium was autoclaved and inoculated with 20% v/w of inoculum. Fermentation was carried out at 30°C for 72 h. The fermented medium was extracted with Tween 80 (0.1% (v/v)) at 30°C on an orbital shaker at 200 rpm for 1 h. Cell free extract was used for phytase activity assay. All experiments were performed in triplicate.

For tray fermentation, 50–1000 g of substrate was taken in enamel coated metallic trays (28 x 24 x 4 cm and 45 x 30 x 4 cm) with different bed thickness (0.2–2.0 cm). The fermentation was carried out under optimum condition as described above.

5.11. Strain improvement

5.11.1. Selection of best phytase producing strain

Fungal spore suspension was treated with a number of individual combinations involving maximum temperature range (70, 80 or 90°C), durations of heating and chilling (at 4°C) and number of cycles (Fig. 5.1a, 5.1b and 5.1c) and is summarized in Table 5.5. After optimizing the stress conditions, the treatment was applied up to six generations of cultures.

Stressed strains after each treatment were screened for phytase production on phytase screening medium (PSM) containing (w/v): 0.4% Sodium phytate, 1% D–glucose, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.5% NH_4NO_3, 0.01% $MnSO_4.H_2O$, 0.5% $CaCl_2.2H_2O$, 0.01% $FeSO_4.7H_2O$ and 1.5% Agar (pH 5.6) [149]. The spore suspension was plated and phytase activity was recorded after 72 h of incubation at 30°C by measuring the diameter of clear zone. The best strain was selected on the basis of larger diameter of the clear zone. After fermentation, crude extract of mutant strain was subjected to phytase activity assay as well as a semiquantitative method using SDS–PAGE band density analysis. For SDS–PAGE analysis, the crude proteins from both

unstressed (wild) and stressed (mutant) strains were resolved on a 12% separating gel topped with 4% stacking gel followed by Coomassie Brilliant Blue staining [185].

5.11.2. Evaluation of efficacy of wild and mutant strains

To justify the effectiveness of strain improvement process, a comparative study was performed to analyze the effect of fermentation of various feed ingredients with wild and mutant strain in terms of ash content and minerals (Fe, Zn and Ca) extractability. Percent ash content was determined using the standard official method [1,2].

For this, 2 g of samples were incinerated in Muffle furnace FO100 (Yamato Scientific Co., Ltd.) in a tarred crucible. With each set of samples, a blank was prepared in the same manner. The minerals (iron, zinc and calcium) were measured at the appropriate instrumental conditions (Table 5.6) using an atomic absorption spectrophotometer (AAS) (Avanta Grade M, GBC Scientific Equipment) and was quantified by standard curves made from standard mineral solutions (Fisher Scientific, Pittsburgh, PA). Concentration of iron, zinc and calcium was expressed as μg/g (ppm) on dry weight basis. Data are represented as mean ± standard deviation.

5.12. Submerged fermentation (SmF)

5.12.1. Phytase production

The basal medium in 250–ml Erlenmeyer flasks (50 ml) contained (% w/v): Glucose, 1.0; Peptone, 0.5; micronutrient solution (NaCl, 0.15; $MgSO_4.7H_2O$, 0.15; $MnSO_4.H_2O$, 0.005; $FeSO_4.7H_2O$, 0.005 and $CaCl_2.2H_2O$, 0.15) with initial pH adjusted to 5.6. The flask was inoculated with 5% inoculum concentration and kept for shaking at 200 rpm at 30°C in a shaker for 14 days. Crude extract was obtained after centrifugation at 10000 x g for 15 min in a refrigerated centrifuge. The cell free supernatant was subjected to phytase activity assay as well as a semiquantitative method using SDS–PAGE band density analysis. For SDS–PAGE analysis, the crude proteins from both unstressed and stressed cells were resolved on a 12% separating gel topped with a 4% stacking gel followed by Coomassie Brilliant Blue staining [185].

5.13. Strain improvement

In submerged fermentation mode, the strain improvement was performed in the same way as carried out in SSF mode (section 5.12.) and is summarized in Table 5.7.

5.14. Statistical media optimization for phytase production by stressed strain under SmF

5.14.1. Plackett–Burman design

The prerequisite for optimization for phytase production involving multiple inputs is to screen out the most influential inputs to determine the model output. PBD was employed for screening the most significant medium components and culture conditions influencing the phytase production. The PB design also incorporates insignificant dummy variables whose number is kept at one–third of all variables. These dummy variables introduce some redundancy required by the statistical procedure and its inclusion into an experiment allows an estimation of the experimental error of an effect. Based on initial studies, thirteen assigned factors and six unassigned factors (dummy) were screened in a total of 20 runs. The detail of the design with the response (phytase activity) is given in Table 5.8.

5.14.2. The path of steepest ascent (descent) method

To approach rapidly in the close proximity to optimum response, the method of path of steepest ascent (descent) was performed (Table 5.9). The direction of the maximum increase in phytase activity was determined according to the estimated coefficient ratio from the fitted first–order model in PBD.

5.14.3. Central composite design

To determine mutual interactions among the selected factors (mannitol, K_2HPO_4, Na_2HPO_4 and phytate) and their optimum concentrations, central composite design (CCD) of response surface methodology (RSM) was used. A 2^4 factorial design having sixteen factorial points, eight axial points and six replicates at the centre point with a total number of 30 runs was formulated. The details of experimental design with coded and actual levels of each factor are summarized in Table 5.10 and Table 5.11. A multiple

regression analysis of the data was carried out for obtaining an empirical model that relates the response to the independent factors. The following second–order polynomial model equation (1) was used to be fitted to the yield values:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1, i \neq j}^{n-1} \sum_{j=2}^{n} \beta_{ij} x_i x_j \quad (1)$$

where, Y is the observed value of the response (phytase production, Uml^{-1}); x_i (i = 1, 2, 3 and 4) is the controlling factors; β_o is the offset term, and β_i (i = 1, 2, 3 and 4), β_{ii} and β_{ij} (i = 1, 2, 3; j = 2, 3 and 4; and $i \neq j$) are the model linear, quadratic and interaction coefficient parameters, respectively.

5.14.4. Statistical analysis

The statistical software package 'Design–Expert®8.0.5, Stat–Ease Inc., Minneapolis, MN, USA was used for experimental design and subsequent regression analysis of the experimental data. All experiments were done in triplicate, and the average phytase activity was taken as the response.

5.14.5. Validation

The feasibility of the experimental model as well as regression equation with respect to phytase activity was performed under the optimal level conditions predicted by the model in varied volumes (0.25–1 L) of Erlenmeyer flask. Samples were drawn at desired intervals and were analyzed for phytase activity, biomass, protein content, pH and reducing sugars.

5.15. Bioreactor studies

The phytase production by *Rhizopus oryzae* was then studied in a 7–L bioreactor (Bioflo, New Brunswick Scientific, NJ, USA) with 5–L working volume. The medium was sterilized at 121°C for 15 min and the pH of the medium was not controlled but monitored. The medium was inoculated with *R. oryzae* spore suspension. The fermentation was carried out for 144 h at 30°C, and the impeller speed was adjusted at 250 rpm. Compressed sterile air was sparged into the medium at 0.4 vvm. Fermentation parameters were continuously monitored with microprocessor–controlled probes.

Samples were drawn at desired intervals and were analyzed for phytase activity, biomass and pH.

5.16. Evaluation of potential application of phytase in terms of phytate degradation and mineral extractability.

5.16.1. *In vitro* phytic acid degradation study

The stability and efficacy of *Rhizopus* phytase was investigated in the milieu of low pH (pH 2.0) and at physiological temperature (39°C) with wheat bran (2 g), as a source of phytic acid. The pH of the phytase preparation (5 Uml^{-1}) was adjusted before addition to the sample (wheat bran) and was incubated at 39°C. The samples were withdrawn at different time intervals and the residual phytic acid content was estimated according to Garcia–Villanova et al. [91].

5.16.2. HPLC analysis

Dynamics of phytate degradation was studied to determine the number of phosphomonoester bond breakage using standard phytase activity assay. It was further validated by reversed–phase high performance liquid chromatography (RP–HPLC) using Agilent 1200 series (Hewlett Packard, Palo Alto, CA, USA) liquid chromatography equipped with a variable wavelength detector (VWD 1200) and Agilent XDB eclipse C_{18} (250 x 4.6 mm) column. Phytate (IP6) and inositol tris–phosphate (IP3) (Sigma chemicals, St. Louis, MO) dissolved in the 100 mM sodium acetate buffer (pH 5.1) were used to calibrate the standard curve. Phytate hydrolysis experiment was carried out according to Graf and Dintzis [103] with some modifications. Purified phytase (0.47 U) was incubated with 0.29 mM phytate prepared in 100 mM sodium acetate buffer (pH 5.1), at 39°C. Reaction was stopped by denaturing the enzyme at 100°C for 10 min. Before injection, sample was filtered through 0.2μm syringe filter followed by addition of an equal amount 0.05 M HCl. A 100 mM sodium acetate solution (pH 5.1) was used as mobile phase with a flow rate of 1.0 ml/min.

Table 5.1 Design matrix for PBD with coded levels of independent factors.

Run	A	B	C	D	E	F	G	H	J	K	L	Phytase activity (U/gds)	
												Actual	**Predicted**
1	1	1	1	-1	-1	-1	1	-1	1	1	-1	**108.95**	**108.97**
2	-1	1	-1	1	1	-1	1	1	1	-1	-1	**132.05**	**130.80**
3	1	1	-1	-1	-1	1	-1	1	1	-1	1	**74.90**	**73.53**
4	-1	-1	1	-1	1	1	-1	1	1	1	-1	**138.65**	**138.55**
5	1	-1	-1	-1	1	-1	1	1	-1	1	1	**130.25**	**130.35**
6	-1	1	1	-1	1	1	1	-1	-1	-1	1	**104.90**	**106.15**
7	1	-1	1	1	1	-1	-1	-1	1	-1	1	**104.25**	**104.27**
8	1	1	-1	1	1	1	-1	-1	-1	1	-1	**97.45**	**97.43**
9	-1	-1	-1	1	-1	1	1	-1	1	1	1	**138.70**	**137.33**
10	-1	1	1	1	-1	-1	-1	1	-1	1	1	**93.67**	**95.04**
11	1	-1	1	1	-1	1	1	1	-1	-1	-1	**139.20**	**140.45**
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	**123.90**	**124.00**

C, F and J–dummy variables; **A**–Mineral salt solution, **B**–Mannitol, **D**–Initial pH, **E**–Fermentation time, **G**–Ammonium sulphate, **H**–Incubation temperature, **K**–Initial moisture content and **L**–K_2HPO_4/Na_2HPO_4.

Table 5.2 Design and results of path of steepest ascent (descent) experiment.

Experiment	**Factors**			**Phytase activity[a] (U/gds)**
	Mannitol (w/w %)	**Ammonium sulphate (w/w %)**	**K_2HPO_4/Na_2HPO_4 (w/w %)**	
1(zero level)	3	1.25	0.6	103.25 ± 0.21
2	2.5	1.50	0.5	117.19 ± 0.15
3	2.0	1.75	0.4	134.38 ± 0.45
4	1.5	2.00	0.3	139.22 ± 0.34
5	1.0	2.25	0.2	127.39 ± 0.37
6	0.5	2.50	0.1	114.78 ± 0.39

[a]Data are represented as means ± SD. n=3

Table 5.3 Range of variables used for response surface methodology.

Factor code	**Factor**	**Levels**				
		-α	**-1**	**0**	**+1**	**+α**
A	Mannitol (%)	0.24	0.75	1.50	2.25	2.76
B	Ammonium sulphate (%)	0.32	1.00	2.00	3.00	3.68
C	K_2HPO_4/ Na_2HPO_4 (%)	0.13	0.20	0.30	0.40	0.47

Table 5.4 Experimental design for CCD.

Run	Mannitol (A, w/w %)	Ammonium sulphate (B, w/w %)	K_2HPO_4/Na_2HPO_4 (C, w/w %)	Phytase activity (U/gds)	
				Experimental[a]	Predicted
1	2.25 (1)	3.0 (1)	0.4 (1)	148.73 ± 0.512	149.25
2	0.75 (-1)	1.0 (-1)	0.4 (1)	124.20 ± 0.721	123.07
3	1.50 (0)	2.0 (0)	0.3 (0)	144.51 ± 0.568	144.38
4	2.25 (1)	1.0 (-1)	0.4 (1)	119.81 ± 0.698	121.22
5	1.50 (0)	2.0 (0)	0.3 (0)	144.53 ± 0.546	144.38
6	1.50 (0)	2.0 (0)	0.3 (0)	144.51 ± 0.625	144.38
7	2.7615 (+α)	2.0 (0)	0.3 (0)	137.48 ± 0.642	136.81
8	1.50 (0)	3.682 (+α)	0.3 (0)	137.95 ± 0.465	137.73
9	1.50 (0)	2.0 (0)	0.1318 (-α)	124.20 ± 0.625	125.81
10	0.75 (-1)	3.0 (1)	0.2 (-1)	133.21 ± 0.547	131.98
11	0.2385 (-α)	2.0 (0)	0.3 (0)	134.20 ± 0.598	135.05
12	1.50 (0)	2.0 (0)	0.3 (0)	141.68 ± 0.477	144.38
13	2.25 (1)	1.0 (-1)	0.2 (-1)	116.69 ± 0.511	115.68
14	1.50 (0)	2.0 (0)	0.3 (0)	141.98 ± 0.479	144.38
15	1.50 (0)	0.318 (-α)	0.3 (0)	106.79 ± 0.501	107.59
16	0.75 (-1)	3.0 (1)	0.4 (1)	138.12 ± 0.445	138.73
17	0.75 (-1)	1.0 (-1)	0.2 (-1)	125.06 ± 0.502	124.13
18	2.25 (1)	3.0 (1)	0.2 (-1)	131.56 ± 0.564	131.69
19	1.50 (0)	2.0 (0)	0.4682 (+α)	137.16 ± 0.498	136.13
20	1.50 (0)	2.0 (0)	0.3 (0)	148.77 ± 0.522	144.38

[a]Data are represented as means ± SD. n=3

Table 5.5 Various treatment approaches and its effect on phytase activity under SSF.

S. No.	Temperature cycle (°C)	Time cycle (Heating/Chilling)	No. of cycles	Relative activity[a] (%)
1	80–4	90 sec–90 sec	10	97.4
2	80–4	5 min–5 min	3	92.1
3	80–4	3min–1min	5	114.2
4	80–4–80–4	2min–2min–3min–3min	3	95.0
5	80–4–80–4–80–4	1.5min–1.5min–3min–3min–4min 40sec–4min 40 sec	2	94.9
6	80–4	15min–15min	1	93.0
7	80–4	5min–1min	3	122.1
8	80–90–70	1min–1min–1min	5	–
9	Sinusoidal	16 min cycle	1	–
10	80–90–4	1.5min–1.5min–1min	5	117.9
11	70–80–4	1.5min–1.5min–1min	5	123.7
12	80–4	6min–1min	3	130.8
13	70–80–4	2.5min–2.5min–1min	3	129.4
14	70–80–4	3min–3min–1min	3	138.1
15	70–4	6min–1min	3	137.6
16	75–4	5min–1min	3	137.6
17	70–4	3min–1min	5	128.4
18	80–4	7.5min–1min	2	128.0
19	75–4	2min–1min	10	115.3
20	70–80–4	2min–2min–1min	5	112.5
21	75–4	5min–1min	3	117.2
22	75–4	6min–1min	3	117.6

– = No growth

[a]Phytase activity demonstrated by unstressed strain was regarded as 100% (148.77 ± 0.85 U/gds).

Table 5.6 Instrumental parameters for determination of elements by AAS.

Parameters	Fe	Zn	Ca
Primary Wavelength (nm)	248.3	213.9	422.7
Mode of Analysis (Flame type)	Air–Acetylene	Air–Acetylene	N_2O–Acetylene
Working range (mg/l)	2–9	0.4–1.5	1.0–4.0
Sensitivity (mg/l)	0.050	0.008	0.020

Table 5.7 Various treatment approaches and their effects on phytase activity under SmF.

S. No.	Temperature cycle (°C)	Time cycle (Heating/Chilling)	No. of cycles	Relative activity[a] (%)
1	80–4	1.5 min–1.5 min	10	98.5
2	80–4	5 min–5 min	3	85.9
3	80–4	3min–1min	5	99.4
4	80–4–80–4	2min–2min–3min–3min	3	96.1
5	80–4–80–4–80–4	1.5min–1.5min–3min–3min–4min 40sec–4min 40 sec	2	95.3
6	80–4	15min–15min	1	91.2
7	80–4	5min–1min	3	108.6
8	80–90–70	1min–1min–1min	5	No growth
9	Sinusoidal	16 min cycle	1	No growth
10	80–90–4	1.5min–1.5min–1min	5	105.1
11	70–80–4	1.5min–1.5min–1min	5	118.6
12	80–4	6min–1min	3	109.9
13	70–80–4	2.5min–2.5min–1min	3	114.5
14	70–80–4	3min–3min–1min	3	126.8
15	70–4	6min–1min	3	117.1
16	75–4	5min–1min	3	121.4
17	70–4	3min–1min	5	123.6
18	80–4	7.5min–1min	2	112.4
19	75–4	2min–1min	10	117.3
20	70–80–4	2min–2min–1min	5	122.9
21	75–4	6min–1min	3	120.6

[a]Phytase activity demonstrated by unstressed strain was regarded as 100%.

Table 5.8 Design matrix for PBD with coded levels of independent factors.

Run	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	R	S	T	Phytase activity (Uml^{-1})
1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	**8.9**
2	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	**9.7**
3	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	**8.9**
4	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	**10.4**
5	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	**11.2**
6	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	**9.8**
7	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	**8.8**
8	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	**6.8**
9	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	**8.2**
10	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	**7.7**
11	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	**9.6**
12	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	**9.0**
13	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	**9.84**
14	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	**9.2**
15	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	**10.6**
16	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	**8.6**
17	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	**8.7**
18	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	**10.5**
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	**9.9**
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	**8.23**

B, D, E, H, J and **M**–dummy variables; **A**–Glucose, **C**–Mannitol, **F**–Sucrose, **G**–Ammonium nitrate, **K**–Yeast extract, **L**– Peptone, **N**–KH_2PO_4, **O**–K_2HPO_4, **P**–Na_2HPO_4, **Q**–$ZnSO_4$, **R**–Sodium phytate, **S**–Tween 80 and **T**–M/N

Table 5.9 Design and results of path of steepest ascent (descent) experiment.

Experiment	Factors				Phytase activity[a] (Uml^{-1})
	Mannitol (%)	K_2HPO_4 (%)	Na_2HPO_4 (%)	Sodium phytate (%)	
1(zero level)	3.75	0.20	0.25	0.50	9.02 ± 0.34
2	3.50	0.30	0.30	0.60	10.22 ± 0.28
3	3.25	0.40	0.35	0.70	11.59 ± 0.33
4	3.00	0.50	0.40	0.80	12.32 ± 0.57
5	2.75	0.60	0.45	0.90	10.30 ± 0.24
6	2.50	0.70	0.50	1.00	8.69 ± 0.51

[a]Data are represented as means ± SD. n=3

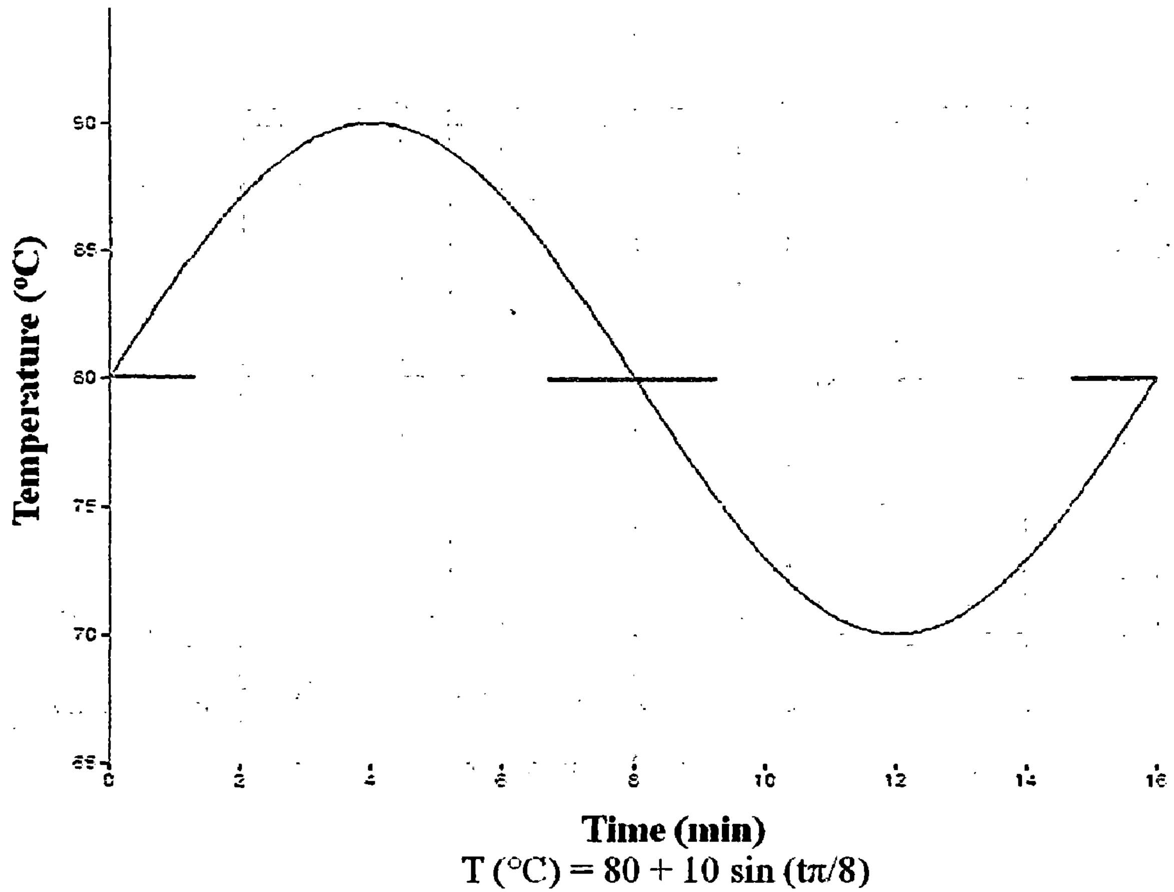
Table 5.10 Range of variables used for response surface methodology.

Factor code	Factor	Levels				
		-α	-1	0	+1	+α
A	Mannitol (%)	1.0	2.0	3.0	4.0	5.0
B	K_2HPO_4 (%)	0.1	0.3	0.5	0.7	0.9
C	Na_2HPO_4 (%)	0.2	0.3	0.4	0.5	0.6
D	Sodium phytate (%)	0.4	0.6	0.8	1.0	1.2

Table 5.11 Experimental design for CCD matrix of four variables in uncoded (coded) values with observed and predicted responses.

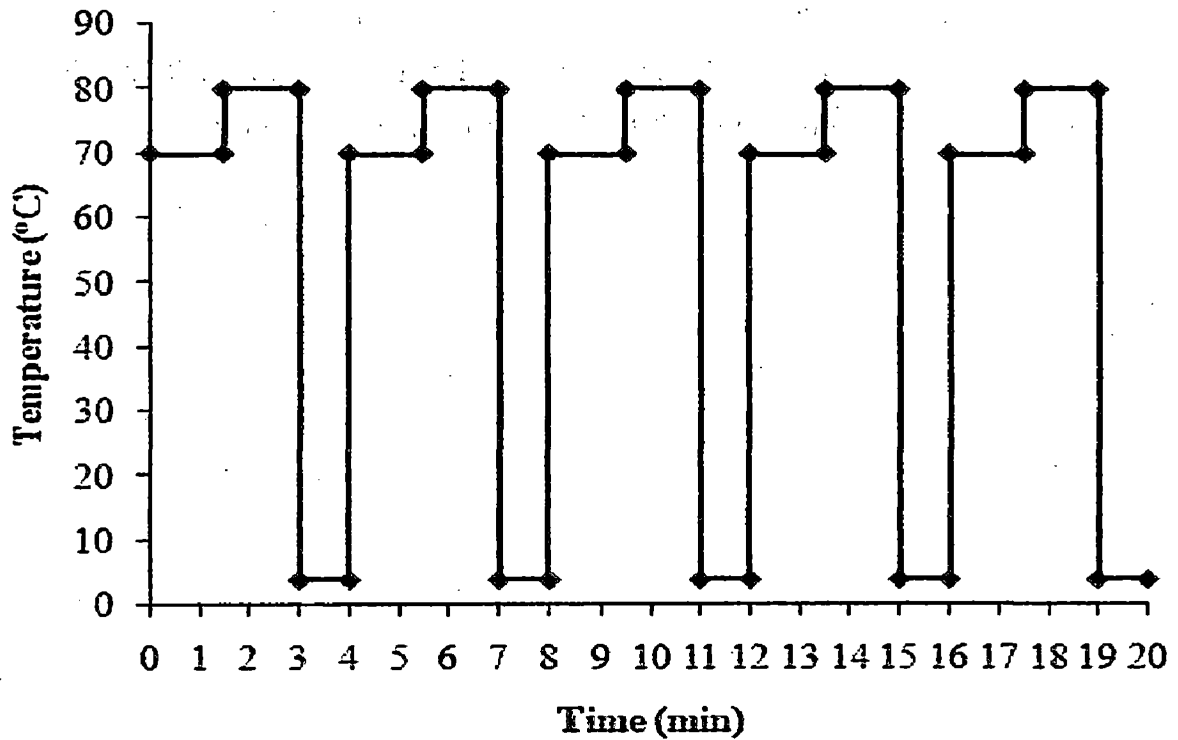
Run	Mannitol (%, A)	K_2HPO_4 (%, B)	Na_2HPO_4 (%, C)	Sodium phytate (%, D)	Phytase activity (Uml^{-1})	
					Observed[a]	Predicted
1	3.0 (0)	0.5 (0)	0.4 (0)	0.8 (0)	**14.25 ± 0.25**	**13.53**
2	4.0 (1)	0.3 (-1)	0.5 (1)	0.6 (-1)	**5.40 ± 0.31**	**5.40**
3	4.0 (1)	0.3 (-1)	0.3 (-1)	1.0 (1)	**12.45 ± 0.22**	**13.30**
4	2.0 (-1)	0.3 (-1)	0.3 (-1)	0.6 (-1)	**11.30 ± 0.41**	**11.29**
5	3.0 (0)	0.5 (0)	0.4 (0)	0.4 (-α)	**6.90 ± 0.77**	**6.88**
6	2.0 (-1)	0.3 (-1)	0.5 (1)	1.0 (1)	**12.70 ± 0.17**	**12.63**
7	1.0 (-α)	0.5 (0)	0.4 (0)	0.8 (0)	**10.12 ± 0.48**	**10.31**
8	4.0 (1)	0.3 (-1)	0.3 (-1)	0.6 (-1)	**11.90 ± 0.33**	**12.01**
9	3.0 (0)	0.1 (-α)	0.4 (0)	0.8 (0)	**12.80 ± 0.56**	**11.96**
10	2.0 (-1)	0.7 (1)	0.3 (-1)	1.0 (1)	**9.21 ± 0.31**	**9.70**
11	3.0 (0)	0.5 (0)	0.4 (0)	0.8 (0)	**13.03 ± 0.37**	**13.53**
12	2.0 (-1)	0.3 (-1)	0.5 (1)	0.6 (-1)	**10.10 ± 0.42**	**10.97**
13	2.0 (-1)	0.7 (1)	0.3 (-1)	0.6 (-1)	**12.00 ± 0.51**	**11.44**
14	3.0 (0)	0.5 (0)	0.4 (0)	0.8 (0)	**12.98 ± 0.24**	**13.53**
15	3.0 (0)	0.5 (0)	0.4 (0)	0.8 (0)	**13.97 ± 0.29**	**13.53**
16	3.0 (0)	0.5 (0)	0.4 (0)	0.8 (0)	**14.03 ± 0.65**	**13.53**
17	5.0 (α)	0.5 (0)	0.4 (0)	0.8 (0)	**9.27 ± 0.45**	**8.28**
18	3.0 (0)	0.5 (0)	0.4 (0)	1.2 (α)	**11.90 ± 0.25**	**12.01**
19	2.0 (-1)	0.7 (1)	0.5 (1)	0.6 (-1)	**10.98 ± 0.34**	**10.62**
20	4.0 (1)	0.3 (-1)	0.5 (1)	1.0 (1)	**10.10 ± 0.29**	**10.97**
21	4.0 (1)	0.7 (1)	0.5 (1)	1.0 (1)	**9.90 ± 0.42**	**10.41**
22	4.0 (1)	0.7 (1)	0.3 (-1)	1.0 (1)	**10.40 ± 0.43**	**9.94**
23	4.0 (1)	0.7 (1)	0.5 (1)	0.6 (-1)	**6.90 ± 0.13**	**7.24**
24	3.0 (0)	0.5 (0)	0.6 (α)	0.8 (0)	**11.50 ± 0.14**	**10.78**
25	2.0(-1)	0.7 (1)	0.5 (1)	1.0 (1)	**12.97 ± 0.22**	**13.17**
26	2.0 (-1)	0.3 (-1)	0.3 (-1)	1.0 (1)	**12.00 ± 0.24**	**11.96**
27	3.0 (0)	0.5 (0)	0.4 (0)	0.8 (0)	**12.89 ± 0.65**	**13.53**
28	3.0 (0)	0.5 (0)	0.2 (-α)	0.8 (0)	**14.00 ± 0.28**	**13.93**
29	4.0 (1)	0.7 (1)	0.3 (-1)	0.6 (-1)	**10.50 ± 0.35**	**11.06**
30	3.0 (0)	0.9 (α)	0.4 (0)	0.8 (0)	**11.50 ± 0.12**	**11.54**

[a]Data are represented as means ± SD. n=3

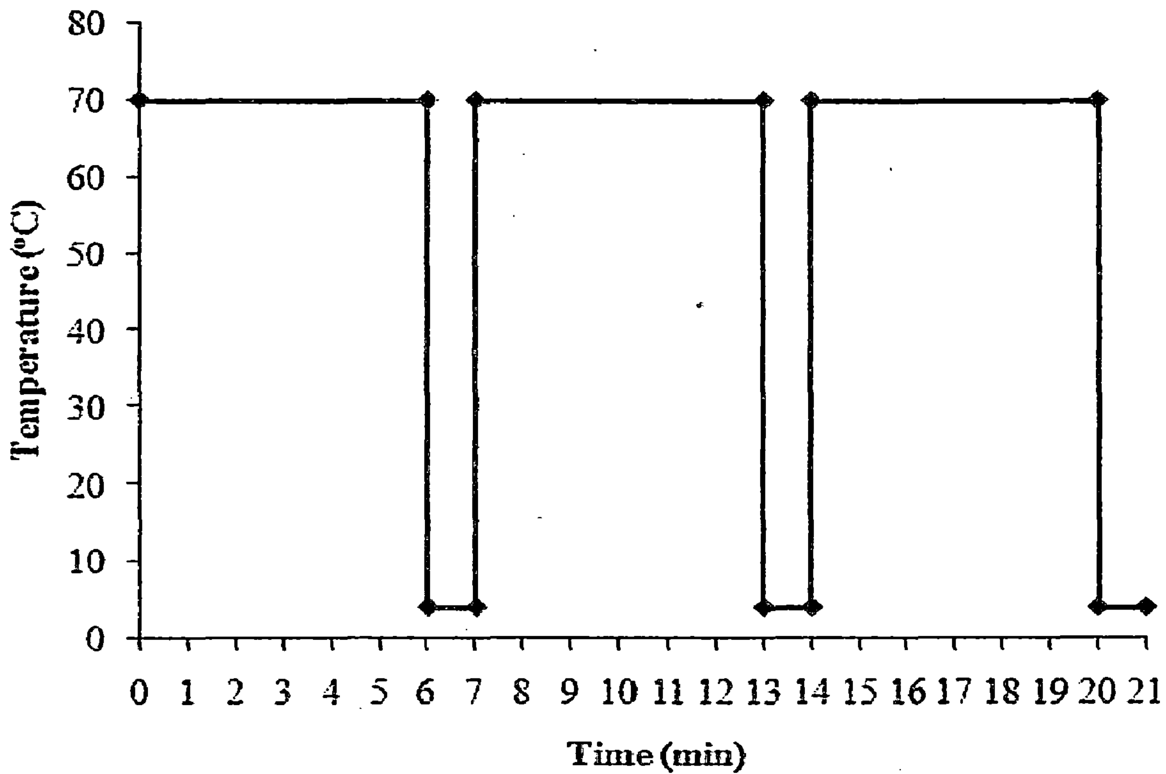


T (°C) = 80 + 10 sin (tπ/8)

(a)



(b)



(c)

Fig. 5.1 Various stress methods employed during strain improvement (a) Temperature range 90°C to 70°C; Time cycle – 16 min; Sinusoidal cycle, (b) Temperature range 70°C–80°C–4°C; Time cycle – 1.5 min–1.5 min–1.0 min; No. of cycle=5 and (c) Temperature range 70°C–4°C; Time cycle – 6.0 min–1.0 min; No. of cycle=3.

CHAPTER 6
RESULTS AND DISCUSSION

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6.1. Phytase activity of *Rhizopus oryzae*

Extracellular phytase activity of *Rhizopus oryzae* MTCC 1987 was first indicated by the formation of clear zone on petridish containing PSM (Fig. 6.1a) and was confirmed later by counterstaining technique (Fig. 6.1b) [284].

6.2. Solid state fermentation (SSF)

6.2.1. Agricultural residues as substrate for phytase production

Linseed oil cake (~1.0 mm) and wheat bran in an equal ratio (1:1), showed the highest phytase yield (17.68 ± 0.23 U/gds) at 96 h post fermentation (Table 6.1). Phytase production on WB was relatively low, whereas other substrates such as RH, MOC, SOC and ROC were found to be less effective for phytase production. The utilization of LOC and WB as solid mixed substrate for phytase production by *R. oryzae* under SSF has not been explored earlier. Maximum production in this combination may be attributed to high phytic acid content in LOC (3.95%) and WB (2.71%).

The phytic acid content in substrates was determined by the method according to Garcia–Villanova et al. [91]. As LOC and WB is rich in phytic acid content and are an economical substrate and supported maximum phytase production, it was selected as solid substrate for further optimization studies.

6.2.2. Effect of particle size on phytase production

Substrate particle size was found to be one of the most critical factors affecting phytase production. Maximum phytase activity was observed when *R. oryzae* was grown in SSF medium containing linseed oilcake of smallest particle size (~1.0 mm) mixed with wheat bran in an equal ratio. Linseed oilcake of intermediate particle size (~1.5 mm) resulted in a slight increase in phytase activity as compared to the mixed particle size containing particles smaller than 5.0 mm and bigger than 0.5 mm as shown in Table 6.2.

Molony et al. [220] reported that substrates with finer particles showed improved degradation of substrates due to an increase in surface area. Moreover, in solid state fermentation, the particle size of the substrate determines the void space occupied by air. Since culture growth is also affected by the rate of oxygen transfer, too small particle size is not desirable.

6.2.3. Effect of inoculum age and size on phytase production

The study revealed a steady increase in phytase activity with increase in the age of the spore inoculum and found to be maximum (19.54 U/gds) with four days old culture, after which the activity did not increase with an increase in the age of the inoculum (Fig. 6.2a). This inference was similar to the previous finding [72] where rate of phytase production by *A. ficuum* NRRL 3135 was shown to be increasing with increasing inoculum age upto five days.

Similarly, the effect of inoculum size was investigated by inoculating the fermentation medium with 4–days old inoculum at different inoculum size from 10 to 100% (v/w) and it was observed that an inoculum size of 20% (v/w) was optimum for maximum phytase production (Fig. 6.2b).

6.2.4. Effect of metal ions on phytase production

In the present study, none of the metal ions was found to have significant effect on phytase production as evident from the results summarized in Table 6.3. However, a slight increase in phytase activity was observed in case of combined Na^{+} and Mg^{2+} which showed that these metal ions might be acting as a cofactor for the phytase. The outcome was almost similar to that obtained by Gunashree and Venkateswaran [128] where metal salts were not found to stimulate the phytase production by *A. niger* CFR 335 in SSF. In yet another similar study, phytase activity from *R. oligosporus* was found to be unaffected or moderately stimulated by a range of metal ions [40].

6.2.5. Effect of surfactants on phytase production

Effect of various surfactants on phytase production is summarized in Table 6.4. It clearly showed that none of the tested detergents was able to make any enhancing effect

to the phytase activity in comparison to the control. It may be due to the fact that the linseed oilcake present in the SSF medium already contains residual natural oils which might be having increasing effect on phytase production. The result was different from that obtained for phytase production by *A. ficuum* [73] and *A. carbonarius* [9], where Tween 80 and sodium oleate were found to enhance the phytase production. However, the outcome was similar to the finding by Gunashree and Venkateswaran [128], where none of the tested surfactants favoured the phytase yield in SSF by *A. niger* CFR 335.

6.3. Purification and characterization of *Rhizopus* phytase

6.3.1. Purification of phytase

The phytase obtained from *R. oryzae* was purified using fractional ammonium sulphate precipitation and subsequent ion–exchange and gel filtration chromatography resulting into 26% enzyme recovery with purification of 20.7–fold and specific activity of 141.83 U/mg of protein (Table 6.5). The native molecular weight of the purified phytase was found to be ~34 kDa by gel filtration chromatography (Fig. 6.3) and SDS–PAGE analysis (Fig. 6.4a), respectively. Zymogram staining also confirmed the molecular mass of phytase from *Rhizopus* corresponding to be ~34 kDa (Fig. 6.4b). Purification to homogeneity and monomeric nature of this phytase was further confirmed by nondenaturing PAGE analysis (Fig. 6.4c). The molecular size of the phytase was found to be somewhat smaller than the molecular weight range of previously reported phytases (38–200 kDa) [391].

6.3.2. Kinetic characterization of phytase

The phytase manifested a K_m and V_{max} of 2.42 x 10^{-4} mM and 6.46 x 10^{-3} mMs^{-1}, respectively. The value for catalytic efficiency (k_{cat}/K_m) of the phytase was estimated to be 2.38 x 10^{6} $M^{-1}s^{-1}$ (Table 6.6 and Table 6.7). K_m value of the present phytase was significantly lower than the reported K_m values (10 µM–813 mM) of fungal phytases. The value was 20.66 and 111.57 times lower than *A. ficuum* NRRL 3135 (phy A) [362] and *A. niger* (Natuphos) [41] phytases, respectively. The phytase demonstrated an uncompetitive inhibition in presence of inhibitors in the order of fluoride > phosphate > vanadate, with their respective K_i values of 12.56 x 10^{-6} mM, 3.86 x 10^{-6} mM and 64.52 x 10^{-6} mM.

6.3.3. Physico–chemical characterization of phytase

6.3.3.1. Effect of pH on phytase activity

From physiologically relevant standpoint as well as from an applied perspective, a significant activity in the range of pH 3.5–7.0 is desirable to aid phytate degradation in various parts of the alimentary canal. The phytase in the SSF extract displayed a bihump profile i.e. two pH optima at 1.5 and 5.5, with higher activity at pH 5.5. The activity at pH 1.5 was 49% less in comparison to the activity at pH 5.5 (Fig. 6.5).

The extracellular phytase displaying two pH optima has previously been reported for *A. ficuum* NRRL 3135 phytase at pH 2.5 and 5.5, with 48% less activity at pH 2.5 than at pH 5.5 [357] and *A. fumigatus* phytase exhibiting dual pH optima but in the less acidic range (pH 4.0 and pH 6.0–6.5) [269]. Evidently the phytase from *Rhizopus* exhibited a superior activity between pH 3.5–7.5. Although the phytases from several strains (*A. fumigatus, A. niger* and *E. coli*) have been engineered to modify the pH profile for functioning well under gastric conditions however does not show the desired activity. Mutant phytase from *A. niger* showed a good activity over a limited pH range i.e. pH 3.0–3.5 rather than whole pH range of stomach (pH 2.0–6.5) [224]. Therefore the phytase obtained from *Rhizopus* have advantage over other available phytases.

6.3.3.2. pH stability studies

This *Rhizopus* phytase exhibited acidophilic characteristics which is desirable for a phytase to survive the acidic environment of the digestive tract and thus can be used in feed applications. It retained full activity at low pH value of 1.0 and 2.0 and exhibited more than 75% of initial activity over a wide range of pH 2.5 to 9.5, when stored at 4°C for 6 h (Table 6.8). With no loss in activity in the range of pH 1.0–2.5 and more than 80% of activity from pH 2.5 to 8.0 when incubated for 60 min, was reported for phytase obtained from *Yersinia rhodei* [150]. In another report, phytase from *S. cerevisiae* CY strain was found to retain more than 80% of initial activity in the range of pH 2.5–5.5 when kept at 4°C for one hour [152]. Generally, fungal phytases act efficiently in the range of pH 2–5 [93]. However, from an applied perspective, a phytase should exhibit a significant activity over a wide range of pH 1.5–7.5 to facilitate phytate degradation in

the digestive tract. The efficacy of *R. oryzae* phytase is evident from the fact that it retains activity over broader range of pH 1.0–9.5. Additionally, its acidophilic characteristic to survive the acidic environment of the digestive tract determines its utility in feed applications.

6.3.3.3. Effect of temperature on phytase activity

The phytase was found to be optimally active at 45°C (Fig. 6.6), which falls well within the range of most of the phytases from yeasts, fungi and bacteria (45°C–60°C) [391]. The optimum temperature value (45°C) being in lower side is an added advantage. Phytases having high temperature optima value are generally not able to retain full activity in the gastrointestinal tract of animals. The *Mucor hiemalis* Wehmer enzyme which possesses an optimum temperature of 55°C displays only 66% of its maximum activity at porcine physiological temperature (39°C) [35]. In comparison to that the *Rhizopus* phytase retains more than 80% of initial activity at this temperature (39°C).

6.3.3.4. Thermal stability studies

Thermal stability study suggest that the phytase was found to retain 100% activity when incubated at 50°C for 30 min and maintained >90% of its initial activity at 60, 70 and 80°C, respectively (Fig. 6.7). The $t_{1/2}^{70°C}$ for the phytase was estimated to be 8.25 h which shows strong thermal stability of the phytase. The most thermostable phytase so far reported was from *Aspergillus fumigatus,* which lost only 10% of initial activity at 100°C over a period of 20 min [269]. Another thermostable phytase from *Sporotrichum thermophile* showed $t_{1/2}^{80°C}$ of 90 min [332].

To survive the feed pelleting temperature and to avoid the additional expense of applying the phytase in pelletized form, the most economical approach would be to add a thermostable phytase directly into the feed. The phytase was found to be more stable than the other reported phytases [269, 332]. The study carried out for thermodynamic characterization also supported the high thermal stability of this phytase. The phytase revealed an activation energy (E_a) and entropy values of 23.92 kJ/mol and -264.89 J/(mol.K), respectively (Fig. 6.8). The E_a value was found to be significantly lower than the other phytases [280, 332].

6.3.3.5. Effect of metal ions, inhibitors, organic solvents and detergents on phytase activity

The study involving the effect of cations and inhibitors showed stimulatory effect on phytase activity, however, complete inhibition was observed in presence of Fe^{2+}, Ni^{2+} and Cu^{2+} (Table 6.9). Inhibitory effect by Cu^{2+} cation was similar to the result obtained for phytase from *T. lanuginosus* [126]. In contrary to *A. ficuum* phytase [362], the *Rhizopus* phytase showed stimulatory effect in presence of Zn^{2+}. The study also suggested the insensitivity of phytase to heavy metal ions (Ba^{2+} and Ag^{+}; 5mM) as indicated by a 5.5– and 2.5–fold increase in phytase activity, respectively. This unique feature of *Rhizopus* phytase can be utilized as an advantageous and an efficient mean to combat phosphate pollution in area where soil is polluted with substantial amount of unassimilated phytate and other contaminations.

Among inhibitors, the activity was enhanced by EDTA, sodium azide, tartarate but inhibited by oxalate at 5 mM concentration (Table 6.9). The result was different from earlier reported by Kerovuo et al. [168] where, EDTA was found to inhibit the phytase activity at 1 mM. The enzyme was inhibited by iodoacetamide even at low concentration with a loss of 20% and 90% activity at 1 and 5 mM concentration, respectively, whereas, an enhancing effect was observed for β–Mercaptoethanol at both concentration levels of 5 and 10% (v/v). Phytases from *Cladosporium sp.* FP–1 [277], and *Candida krusei* WZ–001 [276] showed similar effects in presence of iodoacetate and β–Mercaptoethanol, respectively.

Organic solvents were found to show stabilizing effect on phytase activity even at 5 mM concentration (Table 6.10).

An increase in activity by 8 to 12 fold was a characteristic feature observed in case of non ionic detergents at 5 mM concentration which may be attributed to change in conformation of enzyme in such a way that it favored the reaction (Table 6.10).

6.3.3.6. Substrate specificity of *Rhizopus* phytase

R. oryzae phytase was found to liberate phosphate from a range of phosphorylated compounds. More than 2.4–fold higher activity on ATP was observed as compared to that on sodium phytate (Table 6.11). The result was in contradiction to previously studied

phytase from *A. niger* ATCC 9142, where it showed high activity for sodium phytate but displayed significantly lesser activity for ATP, ADP, AMP and G–1–P [41].

6.3.3.7. Simulated Gastric Fluid activity assay

A phytase destined for feed applications should not lose its activity in milieu of gastric conditions. Phytase from *Rhizopus* was found to exhibit high stability and resistance to pepsin over broad range of pH, retaining > 93% and > 97% of original activity at pH 1.5 and 5.5 (Table 6.12). Several studies for different phytases were carried out to know the effect on enzyme activity in milieu of the gastric condition. The study carried out by Boyce and Walsh [34] for four commercial phytases revealed a significant decrease in activity in case of all phytases. In another comparative study on five recombinant phytases, carried out by Huang et al. [150], phytase from *Y. rhodei* retained 20% of original activity at pH 1.5 in contrast to complete loss of activity for phytases from *E. coli, Y. pestis, A. niger* and *Y. intermedia*. This study revealed that the *Rhizopus* phytase was more efficient than other phytases [150].

6.4. Statistical optimization of medium components for phytase production under SSF

6.4.1. Selection of influential culture parameters for phytase production

The application of Plackett–Burman design for screening critical culture parameters is a widely accepted technique. PBD was used for investigating the relative importance of eight independent factors for phytase production. The corresponding effects of these factors on the response (phytase activity) are given in Table 5.1. From the regression analysis, it was evident that A (mineral salt solution), B (mannitol) and L (K_2HPO_4/Na_2HPO_4) enhanced the phytase production at their low level whereas, high level of D (initial pH), E (fermentation time), G (ammonium sulphate), H (incubation temperature) and K (initial moisture content) supported high phytase yield. Based on analysis of total sum of squares and percent contribution, the most significant factors influencing phytase production were found to be B (mannitol), G (ammonium sulphate) and L (K_2HPO_4/Na_2HPO_4), respectively (Table 6.13). The regression model gave a model F–value of 177.34 with a corresponding model p–value (>F) of 0.0006, which shows the

model to be highly significant. Also, the coefficient of determination (R^2) indicates that the model could explain 99.79% of the total variations in the response. A very low value of coefficient of variance (C.V., 1.61%) further confirms the reliability of the model. The three medium components selected by PBD, were further optimized by the path of steepest ascent (descent) method. Plackett–Burman design has been widely used by many researchers for identifying the most influential factors for improving enzyme production [29, 30, 334].

6.4.2. Optimization by the path of steepest ascent (descent) method

The path of steepest ascent (descent) method was performed to approach rapidly in the close proximity to the optimum response by tracing direction of changing factors on the basis of PBD results. The highest response (139.22 U/gds) was observed at the concentration of mannitol (B), ammonium sulphate (G) and K_2HPO_4 / Na_2HPO_4 (L) of 1.5 % (w/w), 2.0 % (w/w) and 0.3% (w/w), respectively (Table 5.2). The corresponding levels were further considered as the '0' level values for factors in the design matrix of CCD.

6.4.3. Optimization using CCD of RSM

To fully explore the sub–regions of the response surface in the neighbourhood of the optimum, CCD with five coded levels was used for the three significant variables screened by PBD. The design matrix showing different combinations of mannitol, ammonium sulphate and K_2HPO_4/Na_2HPO_4 along with their corresponding experimental and predicted responses is presented in Table 5.4. The experimental results were analyzed using analysis of variance (ANOVA) which shows that the regression was statistically significant ($P < 0.0001$) at 95% of confidence level. The results for ANOVA analysis are summarised in Table 6.14. Application of multiple regression analysis on the experimental data resulted in the following quadratic model equation (2) explicitly explaining the phytase production:

$$Y = 144.38 + 0.52A + 8.97B + 3.07C + 2.04AB + 2.70AC + 3.00BC - 2.99A^2 - 7.69B^2 - 4.75C^2 \quad (2)$$

Where, *Y* represents phytase activity (U/gds), and A, B and C are the coded factors of mannitol (w/w %), ammonium sulphate (w/w %) and K_2HPO_4/Na_2HPO_4 (w/w %), respectively. In this case, linear terms (B and C), all the interaction terms (AB, AC and BC) and quadratic terms (A^2, B^2 and C^2) were found to be the most significant for phytase production. The statistical significance of the model equation was supported by the model high F–value of 59.46. Again, the quality of fit of the regression model was justified by high value of coefficient of determination (R^2 = 0.9817) which indicates an excellent correlation between the independent factors. At the same time, the predicted R^2 (correlation coefficient) value of 0.9404 was found in concordance with the adjusted R^2 value of 0.9651, suggesting a strong agreement between the experimental and predicted values of phytase production. The coefficient of variation (CV) indicates the degree of precision with which the treatments are evaluated, therefore, a very low value of CV (0.0159) demonstrates that the performed experiments were highly reliable and was performed with a better precision. Only 1.59% of the variations were not explained by the model.

In order to gain the better understanding of the effects of the significant factors on phytase production, the predicted model was represented as three dimensional response surface graphs and is shown in Fig. 6.9a – c. strongest interaction was observed between ammonium sulphate and K_2HPO_4/Na_2HPO_4. The optimum concentrations for the variables were calculated from the model equation (2) and were found to be: (w/w), mannitol, 2.05%; ammonium sulphate, 2.84% and K_2HPO_4/Na_2HPO_4 (1:1), 0.38%.

In present study, enhancing effect of mannitol for maximum phytase production seems counterintuitive as most literature showed glucose as the preferred carbon source for phytase production [29, 73]. Earlier, mannitol has been reported to have a role in stress tolerance and spore dispersal [304]. Because linseed oil cake contains some endotoxins, therefore, presence of mannitol might be having some protective role that resulted in enhanced phytase production. On the other hand, ammonium sulphate, an inorganic nitrogen source, supported maximum phytase production. This corroborates the results of Bogar et al. [29]. Other interesting finding observed in the present study was the maximum phytase production, when combination of K_2HPO_4 and Na_2HPO_4 (1:1; (w/w)) was used as a source of phosphorus. It might be due to the fact that K_2HPO_4 when combined with Na_2HPO_4 acts as a buffer for pH of the media. Optimized concentrations

of various carbon, nitrogen and phosphorus sources for phytase production have been previously reported by some researchers [30, 128].

6.4.4. Validation

The results from validation experiments showed a strong agreement between the maximum predicted response and the experimental response of 149.25 U/gds and 148.77 U/gds, respectively, thus supporting the high adequacy of the model. A marked enhancement in phytase activity was indicated by the corresponding increase in biomass as a function of glucosamine content (Fig. 6.10). Moreover, the statistical optimization for phytase production resulted in an overall 8.41–fold increase in phytase yield with a reduction in fermentation time from 96 h to 72 h.

From commercialization perspective, the phytase titer from *R. oryzae* seems to be low (Productivity, Q_p = 4750 IU/kg/day) (Table 6.15), however, the widely studied strains, mostly from *Aspergillus* sp. due to high phytase producing capacity, were found to be associated with respiratory allergy [5]. Moreover, for commercialization of feed enzyme, major concerns in general are the constraints of thermal stability, a good enzyme activity at physiological temperature, ability to hydrolyze the phytate phosphorus in digestive tract over wide range of pH and temperature and most importantly, free of health risks. Phytases are not yet employed in food applications, however, there have been many studies focused on improving food quality for people in developing countries. In this context, *Rhizopus* phytase could find potential in food processing applications since it is thermostable enough to withstand pasteurization and active during long–term storage of foods at room temperature. Beside this, the fermented food materials enriched with protein could be effectively used as such in animal feed, since it is a safe feed grade microorganism. *S. thermophile* was also found to produce low phytase titer (Q_p = 4184 IU/kg/day), however, the secreted phytase had its value due to its production from a thermophilic strain [334].

6.5. Large volume production of phytase

6.5.1. Cultivation in flasks and trays

Phytase production was sustainable in Erlenmeyer flasks of varied volumes and in trays, suggesting the feasibility of large volume production of phytase under SSF. Increase in amount of substrate and hence the bed height did not result in significant decrease in the phytase yield (Table 6.16). However, Singh and Satyanarayana [334] have reported a significant reduction in phytase production at below or above 1.5 cm of substrate bed height.

6.6. Strain improvement under SSF

6.6.1. Procedure and strain selection

Further enhancement in phytase yield was attained due to a novel strain improvement procedure, in which *R. oryzae* was exposed to subsequent heat and cold stress to study the response of the adaptative cells in terms of phytase production. The increase in phytase titer (38 and 59%, respectively) was observed up to second generation with standard deviations (1.01 and 0.89, respectively) (Table 6.17). This process was continued up to six generations with no further increase in phytase titer, rather maintained at a constant level. However, the third generation onwards, it showed higher standard deviations (4.54, 4.01, 4.39 and 4.37, respectively) as compared to the first two generations and these deviations remained almost constant.

Morphological changes that had been observed include short mycelium, cotton–like appearance and more and more whitish appearance compared to previous generation cultures. Another important morphological variation was that of lesser sporulation tendency in mutant culture. However, the reduction in sporulation tendency does not affect the phytase yield. The yield was found to be proportional to the rate of sporulation for unstressed strain and not in stressed strain.

Semiquantitative analysis further validates a remarkable enhancement in phytase production by 1.46– and 2.04–fold after first and second generation of strain improvement process, respectively (Fig. 6.11). Increase in phytase titer owing to strain

improvement has previously been reported by Chelius and Wodzinski [43] and Shah et al. [319].

Along the history of the improvement of strains, many different approaches have been used to enhance the production of various biotechnological products, since the production level of enzymes in naturally occurring strains is sometimes low for commercial exploitation. As mentioned earlier, a very few work on strain improvement for enhancing the phytase production is available in the literature, and are mainly focused on increasing the phytase titer by using physical and chemical mutagens, chiefly, UV radiation [43, 319]. Microorganisms respond differently to stress conditions such as low and high temperatures, osmotic or oxidative stress and high concentration of chemicals. Here, a simple, effective and rapid procedure for achieving the enhanced phytase titer from *R. oryzae* was investigated. As there are circumstantial data indicating that spores are much more resistant than their vegetative cell counterparts to a variety of treatments such as heat, radiations and oxidizing agents [95, 161], however, germicidal ultraviolet (UV) radiation, ion emission, and thermal treatment have also been studied with respect to their biocidal effects on microorganisms [121-124]. In the present study, mutation was attempted by direct but subsequent heat and cold treatments to the spore suspension of culture. Although, the actual mechanism of enhancing phytase production was not investigated in the present study, however, previous report suggest that heat stress acts as an oxidant and a mutagen causing increase in thermal viability and mutational frequency of the spore DNA [161]. On the other hand shifting to cold was done to prevent the spores from being damaged. The procedure was found to be positively affecting the phytase production ability of the stressed strain, which was later verified by increase in total protein content by stressed cells. The ability of stressed strain to produce more phytase is of great importance to the food and feed industry, since, the strain can survive the pelleting temperature and thus can directly be used as a source of phytase as well as other accessory enzymes secreted simultaneously during fermentation process. The results from the present investigation indicated that the increase in phytase production by the stressed strain was influenced by the temperature and time combination of the heat and cold stress.

6.6.2. Evaluation of efficiency of wild and mutant strains

To justify the effectiveness of this process, a comparative study was performed to analyze the effect of fermentation of various feed ingredients with wild and mutant strain in terms of ash content and minerals (Fe, Zn and Ca) extractability. Upto 1.8-fold increase in ash content was observed, when different phytate containing substrates were treated with crude phytase preparation obtained from wild strain. However, mutant strain showed upto 2.40–fold increase in ash content under similar treatment (Table 6.18). The mutant strain was found to be more efficient in extracting the iron from whole wheat flour, since, the mean difference in iron content between mutant and wild was significant ($p<0.0005$). On the other hand, except in semolina, no significant difference in mean content of zinc was observed for wild strain among the ingredients, but in whole wheat flour and beaten rice a significant difference with 1.14– and 1.40–fold increase in zinc content, respectively, was noted in case of mutant strain.

Based on calcium availability analysis, the mean difference of Ca content was found to be less significant ($p<0.038$) in wild strain fermented semolina, moderate significant in mutant strain fermented broken wheat ($p<0.003$), beaten rice ($p<0.004$) and maida flour ($p<0.003$) and highly significant in wild and mutant strain fermented cornflakes ($p<0.0005$) and mutant strain fermented gram flour ($p<0.0003$) (Table 6.18). Hence, the study confirms the effectiveness of this procedure as well as the enhanced efficacy of mutant strain in term of mineral extractability.

6.7. Submerged fermentation (SmF)

6.7.1. Strain improvement under SmF

Spore suspension of *R. oryzae* was exposed to subsequent heat and cold stress to study the response of the adaptative cells in terms of phytase production. Based on the results obtained from increase in diameter of clear zone and relative activity (Table 5.7), treatment found to be the optimum for maximum phytase activity was selected for successive treatments (Table 6.19). An increase of 27% and 43% in phytase activity was observed after first and second generation, respectively, with no further increase in the third generation. Semiquantitative analysis results also suggested a remarkable

enhancement in phytase production with an increase in protein content by 1.34– and 1.89–fold after first and second generation, respectively, of strain improvement process (Fig. 6.12).

6.7.2. Statistical optimization of medium components for phytase production by stressed strain under SmF

6.7.2.1. Selection of influential media components for phytase production

The prerequisite for optimization for production process involving multiple inputs is to screen out first the most influential inputs to determine the model output. In this context, PBD was employed for screening the most significant medium components and culture conditions influencing the phytase production. The PB design also incorporates insignificant dummy variables whose number is kept at one–third of all variables. These dummy variables introduce some redundancy required by the statistical procedure and its inclusion into an experiment allows an estimation of the experimental error of an effect. Based on initial studies, thirteen assigned factors and six unassigned factors (dummy) were screened in a total of 20 runs. The corresponding effects of these factors on the response (phytase activity) are shown in Table 6.20.

From the regression analysis, it was evident that A (glucose), C (mannitol), K (yeast extract), L (peptone) and Q ($ZnSO_4$) enhanced the phytase production at their low level whereas, high level of F (sucrose), G (ammonium nitrate), N (KH_2PO_4), O (K_2HPO_4), P (Na_2HPO_4), R (phytate), S (Tween 80) and T (M/N) supported high phytase yield. The regression model gave a model F–value of 30.14 with a corresponding model p–value (>F) of 0.0002, that shows the model to be highly significant. Also, the coefficient of determination (R^2) indicates that the model could explain 98.49% of the total variations in the response. A very low value of coefficient of variance (CV, 2.53%) further confirms the reliability of the model. Based on individual probability factor of failure (>F), the variables (mannitol (C), K_2HPO_4 (O), Na_2HPO_4 (P) and phytate (R)) with their corresponding probability less than 0.001 were considered significant (Table 6.20).

The four medium components identified above for significantly affecting the phytase production, were further optimized by the path of steepest ascent (descent) method.

6.7.2.2. Optimization by the path of steepest ascent (descent) method

The path of steepest ascent (descent) was performed to approach rapidly in the close proximity to the optimum response by tracing direction of changing factors on the basis of PBD results. The highest response (12.32 ± 0.57 Uml^{-1}) was observed at the following concentrations: mannitol (C), 3.0%; K_2HPO_4 (O), 0.5%; Na_2HPO_4 (P), 0.4% and phytate (R), 0.8%, respectively (Table 5.9). The corresponding levels were further considered as the '0' level values for all the four factors in the design matrix of CCD.

6.7.2.3. Optimization using CCD of RSM

To completely explore the sub–regions of the response surface in the neighborhood of the optimum, CCD with five coded levels was used for the four significant variables. The CCD design matrix showing different combinations of mannitol, K_2HPO_4, Na_2HPO_4 and phytate along with their corresponding experimental and predicted responses is presented in Table 5.11. The experimental results were analyzed using analysis of variance (ANOVA) which shows that the regression was statistically significant ($P < 0.0001$) at 95% of confidence level. The results for ANOVA analysis are summarised in Table 6.21. Application of multiple regression analysis on the experimental data resulted in the following quadratic model equation (3) explicitly explaining the phytase production:

$$Y = 13.53 - 0.51A - 0.10B - 0.79C + 0.96D - 0.28AB - 0.75AC + 0.15AD + 0.70BC - 0.60BD + 1.07CD - 1.06A^2 - 0.44B^2 - 0.29C^2 - 1.18D^2 \quad (3)$$

Where, Y represents phytase activity (U/ml), and A, B, C and D are the coded factors of mannitol, K_2HPO_4, Na_2HPO_4 and phytate, respectively. The statistical significance of the model equation was supported by the model high F–value of 18.52. Again, the quality of fit of the regression model was justified by the high value of coefficient of determination (R^2 = 0.9453) which indicates an excellent correlation between the independent factors. At the same time, the predicted R^2 (correlation coefficient) value of 0.8943 was found in concordance with the adjusted R^2 value of 0.7384, suggesting a strong agreement between the experimental and predicted values of phytase production. The coefficient of variation (CV) indicates the degree of precision

with which the treatments were evaluated. A lower value of CV (0.0676) demonstrates that the performed experiments were highly reliable and was performed with a better precision. Only 6.76% of the variations could not be explained by this model. The 'Lack of Fit F–value' of 1.71 implies that there is a 28.73% chance that a this large could occur due to noise. Furthermore, high value (16.012) of adequate precision that represents signal (response) to noise (deviation) ratio, indicates an adequate signal and suggested that the model can be used to navigate the design space. As per the regression analysis performed on the present model, linear effects of mannitol, Na_2HPO_4 and phytate, the interaction effect of Na_2HPO_4 and phytate and quadratic effects of mannitol and phytate were found to be most important for the production of phytase (Table 6.21).

In order to gain the better understanding of the interaction effects of the significant factors and their optimum concentrations, the predicted model was plotted as two dimensional response surface contour plots. The strong interaction effects of mannitol and K_2HPO_4 is evident from the contour plot shown in Fig. 6.13a, whereas, low interaction effect was observed between mannitol and phytate (data not shown). Moreover, the significant interaction effects among different phosphate sources (Fig. 6.13b–d) signify the important role of phosphorus sources in phytase production. Among the interactions, most significant is that between Na_2HPO_4 and phytate and the less significant is that between mannitol and phytate. The optimum concentrations for the variables were calculated from the model equation (2) and were found to be: mannitol, 2.50%; K_2HPO_4, 0.54%; Na_2HPO_4, 0.48% and phytate, 0.95%, respectively.

The statistical method employed for optimizing the medium components for maximum phytase production proved to be a valuable tool. Among the tested nutritional components, mannitol, K_2HPO_4, Na_2HPO_4 and phytate were identified as the most significant affecting the phytase production.

In the present study, enhancing effect of mannitol for maximum phytase production seems counterintuitive as most literature showed glucose, starch or sucrose as the preferred carbon source for phytase production [377]. In our SSF studies also, it was observed that mannitol was the most significant carbon source instead of glucose.

Earlier, mannitol has been reported to have a role in stress tolerance and spore dispersal [304], hence, presence of mannitol might be having some protective role that

resulted in enhanced phytase production. From the study, it can be concluded that different microbial strains have their own nutritional requirements for phytase production.

Another interesting finding observed in the present study was the phytase production at an overall high concentration of phosphorus (~1%) in contrast to that of the reported literatures. It might be due to the fact that presence of K_2HPO_4 and Na_2HPO_4 together served as a buffer for pH of the media that favored the phytase production. Most of the studied phytases were reported to be repressed at high phosphorus concentration [43, 377]. However, no significant inhibition or stimulation of phytase production was reported by Lan et al. [188] and Fredrikson et al. [84]. By using statistical media optimization approach, enhanced phytase yield has been successfully achieved earlier [29, 30].

6.7.2.4. Validation

Experiments were then carried out to determine the robustness of the optimal concentration of each factors estimated by statistical analysis. The results showed a strong agreement between the predicted and the actual response of 13.67 Uml^{-1} and 12.64 ± 1.45 Uml^{-1}, respectively, thus demonstrated the high adequacy of the model.

Phytase production was sustainable in Erlenmeyer flasks of varied volumes as well as in the bioreactor (Table 6.22) and therefore suggests the feasibility of phytase production at higher volume. The statistical optimization of media components resulted in an overall 2.22–fold higher phytase yield than that of the unoptimized medium (5.69 ± 1.35 Uml^{-1}). The maximum phytase production coincided with the decrease in pH of the fermentation broth and thus, pH can be used as an indicator of the initiation of the phytase production (Fig. 6.14).

In bioreactor studies, a significant reduction in the dO_2 level after 24 h was observed, which coincided with the increase in biomass production. Additionally, excessive foaming as well as high consumption of antifoam was observed, which is undesirable in any industrial fermentation process. Based on initial experiments in bioreactor, it might be due to the high inoculum size or presence of insufficient quantities of phosphate in the medium. An alternative approach to deal with this problem was to alter the C/N ratio of the medium. In this prospect, the sodium phytate in the medium was replaced with wheat bran which acts as a complex source of carbon, nitrogen and an

additional source of phytate phosphorus, however, overproduction of biomass, higher foam formation followed by sharp decline in phytase production was observed. It might be due to increase in the total content of phosphorus after addition of wheat bran to the media. The foam formation was finally controlled when the agitation rate was lowered from 250 to 200 rpm, with no dO_2 control. In bioreactor studies, the most significant effect was the reduction in fermentation time for maximum phytase production that resulted in increase in overall productivity by 3.11–fold (Table 6.22).

The results of validation experiments also supported the high adequacy of the model predicted by statistical tools. From the current studies in the bioreactor, it was concluded that the process for the production of phytase from *R. oryzae* could be easily optimized by controlling critical parameters. The bioreactor needed to be run at an increased level of phosphate in the medium and at low agitation rate to prevent excess foaming. Altering the C/N ratio of media negatively affected the phytase production from *R. oryzae*. Other studies have also shown that increased phosphate concentrations led to a shift from metabolite overproduction to overgrowth of the biomass [265, 312]. Further, the control of the dO_2 level was not necessary for the efficient production of the phytase. Apart from this, the pH of the system in the present case acts as the indicator of the initiation or end of the phytase synthesis. From this study, it can be concluded that the enzyme production can be easily increased to higher volume with no loss in enzyme activity. Again, a marked enhancement in productivity can be attributed to the controlled process parameters inside the bioreactor and supply of air (oxygen) during the process.

6.8. Phytate degrading potential of phytase

6.8.1. *In vitro* hydrolysis of phytic acid

The possibility of the relevance of the phytase from mutant strain for an effective use in food and feed applications was investigated by evaluating its phytate degrading ability under physiological conditions. For this, *Rhizopus* phytase was assessed in the milieu of digestive tract as it is essential for a phytase to be active under these conditions. The phytic acid content in untreated wheat bran was estimated to be in the range from 27 to 34 mg/g, which was in accordance with the phytic acid content estimated by the García–Estepa et al. [90]. Here, it is assumed that for each phosphate bond hydrolyzed by

the phytase, there is a reduction of 16.67% of phytic acid content in the substrate (16.67 x 6 = 100).

The result showing effect of treatment of wheat bran with phytase is summarized in Table 6.23 and it suggests that about 50% of phytic acid was reduced after incubation time of 210 min. Even under low pH condition and at physiological temperature the phytase showed the ability to break three phosphomonoester bonds of phytate. This *in vitro* phytate degrading ability of this phytase renders it as a potential candidate for food and feed applications.

6.8.2. Time course hydrolysis of sodium phytate

The study was carried out to estimate the release of inorganic phosphate per mole of phytic acid. The ratio (inorganic phosphate to phytic acid) of three indicates that the maximum number of phosphate released by this phytase was three out of six phosphomonoester bonds, with no further increase in bond cleavage when increasing the reaction time (Fig. 6.15). The study showed a rapid hydrolyzing activity of this phytase from IP6 to inositol pentakis–phosphate (IP5), however, a slow conversion of IP6 to inositol tetra–phosphate (IP4) and finally to IP3 was observed which suggested that the lower derivatives of inositol were less suitable substrates for the enzyme than the phytate or IP6.

6.8.3. HPLC characterization studies

The measurement of phytate hydrolysis is a crucial step that considerably influences the phytase characteristics. Above result suggesting IP3 as the end product of phytate degradation was further validated by an improved HPLC analysis method. In previously reported methods, refractive index of inositol phosphates (including various degradation products of phytic acid) solution was measured using RI detector. In the present study, the feasibility of determining phytic acid and IP3 by RP–HPLC equipped with variable wavelength detector (VWD) was successfully achieved at 246 nm.

The chromatogram of standard sodium phytate was found to be linearly proportional to the concentrations throughout, with R^2 value and retention time (R_t) of 0.988 and 1.23 ± 0.01 min, respectively. In a previous study carried out by Graf and

Dintzis (1982a), the retention time of phytate was found to be 1.40 ± 0.03 min. More than one peak with different R_t (1.26 ± 0.02 (major), 1.39 ± 0.04, 1.57 ± 0.02 and 1.88 ± 0.03 min) was observed in each chromatogram profile of standard IP3 (0.5–2.0 mg/ml). It might be due to the presence of different structural isomers of IP3 in the standard, also verified by the pattern of these small peaks observed in continuity with R_t of 1.26 ± 0.02 and 1.39 ± 0.04 min and R^2 value of 0.990 and 0.999, respectively.

Chromatogram profile for untreated and phytase treated phytic acid is illustrated in Fig. 6.16a and 6.16b. After 150 min of incubation period, no peak corresponding to IP6 was found in the chromatogram which clearly showed the ability of *Rhizopus* phytase to completely hydrolyze the intact phytate. In an earlier study by Casey and Walsh [40], phytase from *R. oligosporus* was shown to completely degrade the phytate; however, presence of IP5 as one of the hydrolyzed products of phytic acid was also found along with IP3. From an applied perspective, presence of IP5 is not desirable as it possesses inhibitory effect on iron absorption in humans; whereas, IP3 and IP4 in isolated form do not have negative effect [308].

Method previously reported for the determination of inositol phosphates and its lower derivatives have some limitations in terms of their inability to separate structural isomers, time consuming sample preparation, need for pre– or post– column derivatization, and by long analysis time. In this method, the sample preparation was much simpler and retention time was reduced. The results show that VWD system with UV spectrum specifically detects the phytate and its lower derivatives with a good resolution.

Table 6.1 Evaluation of different agro-industrial residues as substrate for phytase production under SSF.

Agro–industrial residues	**Phytase activity[a] (U/gds)**
WB	15.32 ± 0.41
RH	5.12 ± 0.33
MOC	7.69 ± 0.78
LOC	10.54 ± 0.51
SOC	0.12 ± 0.24
ROC	6.63 ± 0.37
WB + MOC (1:1)	8.79 ± 0.65
WB + ROC (1:1)	10.20 ± 0.14
WB + SOC (1:1)	9.05 ± 0.78
WB + LOC (1:1)	17.68 ± 0.23
WB + RH (1:1)	11.22 ± 0.39
WB + MOC + ROC (1.67:1.67:1.67)	9.12 ± 0.54
WB + SOC + LOC (1.67:1.67:1.67)	13.11 ± 0.41

[a]Phytase activity was estimated in the SSF crude extract after 96 h of fermentation of unoptimized medium at 35°C. Data are represented as means ± SD. n=3

Table 6.2 Effect of different particle size of linseed oil cake on phytase production.

Linseed oil cake		
Particle type	**Particle size**	**Phytase activity[a] (U/gds)**
Small	~1.0 mm	18.85 ± 0.67
Intermediate	~1.5 mm	17.02 ± 0.99
Heterogeneous (control)[b]	0.5-5.0 mm	16.54 ± 1.24

Data are represented as means of triplicate determinations±SD.

[a]Phytase activity in crude extract after 96 h growth in basal medium containing WB and LOC (1:1) at 35°C.

[b]Control refers to the basal SSF medium with LOC of heterogeneous type.

Table 6.3 Effect of different metal ions on phytase production.

Metal ions (0.6%, w/v)	**Phytase activity (U/gds)**
Control[a]	19.24 ± 0.95
Na^+	18.11 ± 0.22
Mg^{2+}	18.84 ± 0.21
Fe^{2+}	11.87 ± 0.31
$Na^+ + Mg^{2+}$	20.25 ± 0.21
$Na^+ + Fe^{2+}$	13.11 ± 0.16
$Mg^{2+} + Fe^{2+}$	12.32 ± 0.23
$Na^+ + Fe^{2+} + Mg^{2+}$	17.89 ± 0.19

[a]Control refers to the SSF medium without any metal ions. The experiments were carried out in basal medium containing WB and LOC (~1.0 mm) (1:1). The SSF medium was inoculated with 20% (v/w) of 4–days old inoculums and incubated at 35°C for 96 h.

Table 6.4 Effect of various surfactants on phytase production.

Surfactants (0.5%, v/w)	Phytase activity (U/gds)
Control[a]	20.04 ± 0.32
Tween 20	18.04 ± 1.20
Tween 40	18.09 ± 0.58
Tween 80	18.19 ± 0.67
Triton-X-100	15.20 ± 0.87
SDS	11.02 ± 0.97

[a]Control refers to the SSF medium without any surfactants.

The experiments were carried out in SSF medium containing WB and LOC (~1.0 mm) (1:1) supplemented with mineral salt solution containing 0.3% NaCl and 0.3% $MgSO_4.7H_2O$. The SSF medium was inoculated with 20% (v/w) of 4–days old inoculums and incubated at 35°C for 96 h.

Table 6.5 Showing the total activity, total protein content, specific activity, and purification fold of phytase at each purification steps.

Purification steps	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	5.83	6.95	0.84	1	100
Ammonium sulphate fractionation	8.09	5.82	1.39	1.65	84
DEAE Sepharose CL 6B	21.58	3.15	6.85	4.93	54
Sephadex G-100	116.3	0.82	141.83	20.7	26

Table 6.6 Calculated K_i values for different inhibitors.

Inhibitors (5 x 10^5 mM)	$K_m \times 10^4$ **(mM)**	$V_{max} \times 10^3$ **(mM/s)**	$K_i \times 10^6$ **(mM)**
No Inhibitor	2.42	6.46	-
Phosphate	0.27	3.9	12.56
Vanadate	0.096	2.5	3.86
Fluoride	0.95	3.8	64.52

Table 6.7 Calculated turnover number for *Rhizopus* phytase.

Temperature (K)	$k_{cat} (s^{-1})$	$(k_{cat}/K_m) \times 10^{-6} (M^{-1} s^{-1})$
293	0.268	12.76
303	0.482	4.68
313	0.575	2.38

Table 6.8 pH–stability profile of *R. oryzae* phytase.

pH	Relative activity[a] (%)
1.0	115
1.5	112
2.0	106
2.5	86
3.0	90
3.5	78
4.0	76
4.5	79
5.0	82
5.5	100
6.0	90
6.5	82
7.0	75
7.5	84
8.0	97
8.5	97
9.0	97
9.5	83

[a]Phytase activity at pH 5.5 (100 mM acetate buffer) was regarded as 100%. Enzyme stability was assessed at 4°C after incubating the phytase for 6 h in following buffers (100 mM): Glycine–HCl (pH 1.0 to 3.5), Acetate (pH 3.5 to 6.5), Tris–HCl (pH 6.5 to 8.5) and Glycine–NaOH (pH 8.5 to 9.5).

Table 6.9 Effect of various metal ions and inhibitors on phytase activity.

Cations and inhibitors	Relative activity[a] (%)	
	1mM	5 mM
Fe^{3+}	0	–
Na^{+}	27	15
Ca^{2+}	31	100
Mg^{2+}	77	222
Mn^{2+}	48	144
Ni^{2+}	0	–
K^{+}	84	201
Ag^{+}	75	221
Cu^{2+}	0	–
Na^{+}	88	170
$(NH_4)_2^{2+}$	98	247
Co^{2+}	24	137
Zn^{2+}	71	149
Ba^{2+}	84	443
EDTA	164	167
Tartarate	116	143
Iodoacetamide	82	18
Oxalate	112	0

[a]Phytase activity in absence of metal ions and inhibitors was regarded as 100%.

Table 6.10 Effect of organic solvents and detergents on phytase activity.

Organic solvents	**% (v/v)**	
	5	**10**
Control	100	100
Ethyl alcohol	123	158
Butan-2-ol	121	123
Detergents	**1**	**5**
Control	100	100
Tween 20	166.1	862.7
Tween 40	187.3	1249.1
Tween 80	197.6	1268.8
Triton X 100	175.8	927.1
SDS	38.3	12.5

Table 6.11 Substrate specificity profile for *Rhizopus* phytase.

Substrate	**Relative activity[a] (%)**
Sodium phytate	100
AMP	141
ADP	188
ATP	244
NADP	96
SNP	124
FBP	156
R–5–P	107
*p*NPP	100
G–1–P	100
PEP	167

[a]Phytase activity in presence of sodium phytate (2 mM) was regarded as 100%. Substrate specificity was determined after incubating the phytase with various phosphorylated compounds using the standard phytase assay.

Table 6.12 Effect of SGF on phytase activity[a].

	Incubation time (min)				
	(0)	**(30)**	**(60)**	**(120)**	(180)
	Relative activity (%)				
pH 1.5 only	100	97.04	96.38	95.61	93.46
pH 1.5 and Pepsin	100	97.15	96.45	95.44	93.39
pH 5.5 only	100	99.11	99.17	99.09	98.97
pH 5.5 and Pepsin	100	99.14	99.10	98.73	98.53
pH 7.5 only	100	99.00	99.01	98.69	98.61
pH 7.5 and Pepsin	100	98.84	98.87	98.76	98.37

[a]Phytase activities in the SGF (pH 1.5, 5.5 and 7.5) minus pepsin at zero minute was regarded as 100%. Enzyme proteolysis stability was assessed after incubating the phytase in simulated gastric fluid (pH 1.5, 5.5 and 7.5) (1:4) at 37°C using the standard phytase assay.

Table 6.13 Results of PBD analysis.

Factors (Code, Unit)	Low level (-1)	High level (+1)	SS[a]	Effect	Coef.[b]	Cont.[c] (%)	F-value	p-value
Mineral salt solution (A, v/w %)	20	60	492.4	-12.81	-6.41	9.98	141.9	0.0013
Mannitol (B, w/w %)	1.0	5.0	2214.9	-27.17	-13.59	44.92	638.6	0.0001
Initial pH (D)	5.6	7.6	47.08	3.96	1.98	0.95	13.58	0.0346
Fermentation time (E, h)	24	72	66.41	4.70	2.35	1.35	19.15	0.0221
Ammonium sulphate (G, w/w %)	0.5	2.0	1224.7	20.20	10.10	24.84	353.1	0.0003
Incubation temperature (H,°C)	25	30	77.88	5.09	2.55	1.58	22.46	0.0178
Initial moisture content (K, %)	30	40	67.55	4.75	2.37	1.37	19.48	0.0216
K_2HPO_4/Na_2HPO_4 (L, w/w %)	0.2	1.0	728.99	-15.59	-7.79	14.79	210.2	0.0007

R^2 = 0.9979,

R^2 (adjusted) = 0.9923

R^2 (predicted) = 0.9662

Coefficient of Variation (CV) = 0.0161

[a] Sum of Squares

[b] Coefficient estimate

[c] Contribution

Table 6.14 ANOVA analysis of regression model.

Source of variation	SS[a]	DF[b]	MS[c]	Coef.[d]	F-value	p-value
Model	2418.53	9	268.73	144.38	59.46	< 0.0001
A	3.09	1	3.09	0.52	0.68	0.4276
B	1039.92	1	1039.92	8.97	230.11	< 0.0001
C	121.90	1	121.90	3.07	26.97	0.0004
AB	25.26	1	25.26	2.04	5.59	0.0397
AC	44.38	1	44.38	2.70	9.82	0.0106
BC	65.93	1	65.93	3.00	14.59	0.0034
A2	125.23	1	125.23	-2.99	27.71	0.0004
B2	839.23	1	839.23	-7.69	185.70	< 0.0001
C2	319.91	1	319.91	-4.75	70.79	< 0.0001
Residual error	45.19	10	4.52			
Lack of fit	12.85	5	2.57		0.40	0.8332
Pure error	32.35	5	6.47			
Correlation (Total)	2463.73	19				

$R^2 = 0.9817$,

R^2 (adjusted) = 0.9651

R^2 (predicted) = 0.9404

Coefficient of Variation (CV) = 0.0159

[a] Sum of Squares

[b] Degree of freedom

[c] Mean square

[d] Coefficient estimate

Table 6.15 Comparison of phytase productivity of *Rhizopus oryzae* MTCC 1987 with other fungal strains during SSF.

Fungal strains	**Phytase activity[a] (IU/gds)**	**Phytase productivity (IU/kg/day)**	**References**
Rhizopus microsporous	1	432	[30]
R. oligosporous	5	1800	[30]
R. pusillus	9.18	1824	[42]
R. thailandensis	3	912	[30]
Aspergillus ficuum	4	1344	[72], [73]
A. carbonarius	2.5	840	[8], [9]
A. niger	5	1008	[76]
A. niger	6.9	1728	[76]
S. thermophile	20.92	4184	[334]
Rhizopus oryzae	**14.25**	**4750**	**Present work**

[a]One unit (IU) of phytase activity is defined as the amount of enzyme releasing one μmol of inorganic phosphorus per ml per minute under the assay conditions.

Table 6.16 Large volume production of phytase.

Apparatus	Capacity	Substrate amount (g)	Phytase activity[a] (U/gds)
Flask	250 ml	5	148.77 ± 0.33
Flask	500 ml	10	148.12 ± 0.69
Flask	500 ml	20	147.43 ± 0.54
Flask	1000 ml	40	147.55 ± 0.29
Flask	1000 ml	50	148.99 ± 0.23
Tray	28 x 24 x 4 cm	50	149.24 ± 0.68
Tray	28 x 24 x 4 cm	100	148.98 ± 0.35
Tray	28 x 24 x 4 cm	200	146.44 ± 0.58
Tray	45 x 30 x 4 cm	300	148.56 ± 0.56
Tray	45 x 30 x 4 cm	500	146.74 ± 0.87
Tray	45 x 30 x 4 cm	1000	145.98 ± 0.15

[a]Data are represented as means ± SD. n=3

Table 6.17 Results for successive treatments after optimization of stress conditions.

No. of generations	Temperature cycle (°C)	Time cycle (Heating/Chilling)	No. of cycles	Phytase activity[a] (U/gds)
Unstressed strain	–	–	–	148.77 ± 0.85
I	70–80–4	3min–3min–1min	3	205.30 ± 1.01
II	70–80–4	3min-3min–1min	3	236.54 ± 0.89
III	70–80–4	3min–3min–1min	3	220.02 ± 4.54

– = Not Applicable

[a]Data are represented as means ± SD. n=5

Table 6.18 Effect of fermentation with wild and mutant strain of different feed ingredients on ash content and mineral availability.

Ingredients		Ash (%)[a]	(µg/g DW)[b]		
			Fe	Zn	Ca
Semolina (UF)		0.82(±0.24)	47.23(±0.79)	20.63(±2.00)	33.25(±2.23)
(F)	Wild	1.49(±0.08)	65.75(±1.01)	32.06(±2.01)	26.03(±1.47)
	Mutant	1.98(±0.12)	68.19(±0.83)	36.77(±3.18)	27.99(±1.30)
Cornflakes (UF)		1.36(±0.13)	17.02(±3.40)	8.94(±2.25)	6.94(±0.25)
(F)	Wild	1.78(±0.95)	12.36(±2.45)	11.51(±1.50)	21.13(±2.50)
	Mutant	2.10(±0.55)	11.30(±0.70)	14.07 (±1.20)	26.54(±1.97)
Whole wheat flour (UF)		1.64(±0.57)	46.73(±2.29)	31.96(±1.01)	31.28(±2.75)
(F)	Wild	2.21(±0.06)	51.75(±2.29)	37.36(±2.13)	36.91(±3.03)
	Mutant	2.95(±0.11)	55.71(±4.67)	42.47(±1.49)	37.04(±2.01)
Broken wheat (UF)		1.95(±0.12)	42.30(±1.74)	26.79(±3.83)	32.43(±2.92)
(F)	Wild	2.59(±0.20)	45.27(±0.84)	23.95(±1.56)	38.87(±0.63)
	Mutant	3.01(±0.98)	47.90(±1.94)	22.15(±2.50)	41.05(±0.99)
Gram flour (UF)		2.38(±0.26)	21.06(±2.99)	38.72(±0.76)	67.75(±3.33)
(F)	Wild	3.04(±0.96)	47.75(±1.79)	40.51(±3.50)	73.90(±2.06)
	Mutant	3.46(±0.98)	48.78(±1.77)	39.62(±0.59)	80.69(±3.47)
Beaten rice (UF)		1.78(±0.47)	8.51(±1.50)	6.45(±0.72)	22.50(±0.51)
(F)	Wild	2.45(±0.27)	20.41(±0.86)	11.81(±3.23)	27.81(±0.86)
	Mutant	2.98(±0.32)	25.96(±0.45)	16.71(±3.63)	30.96(±1.00)
Maida flour (UF)		1.46(±0.07)	18.57(±1.64)	9.74(±1.21)	37.19(±1.77)
(F)	Wild	1.99(±0.38)	17.39(±0.59)	11.24(±2.68)	43.48(±3.50)
	Mutant	2.58(±0.34)	18.35(±1.42)	12.15(±2.13)	45.76(±1.74)

UF = unferment

ed; F = UF = unfermented; F = fermented.

[a]Ash content (%) are shown as means of triplicate determinations±SD.

[b]Data are represented as means of triplicate determinations±SD and expressed in µgram per gram dry weight of ingredients (µg/g DW).

Table 6.19 Results for successive heat and cold stress.

No. of generations	Temperature cycle (°C)	Time cycle (Heating/Chilling)	No. of cycles	Relative activity[a] (%)
Unstressed strain	–	–	–	100
I	70–80–4	3min–3min–1min	3	126.8
II	70–80–4	3min–3min–1min	3	142.7
III	70–80–4	3min–3min–1min	3	122.2

[a]Phytase activity demonstrated by unstressed strain was regarded as 100%.

Table 6.20 Results of PBD analysis.

Factors (Code, Unit)	**Low level (-1)**	**High level (+1)**	**SS[a]**	**Effect**	**Coef.[b]**	**Cont.[c] (%)**	**F–value**	**p–value Prob > F**
Glucose (A, %)	1.5	6.0	0.281	-0.237	-0.12	1.295	5.15	0.0637
Mannitol (C, %)	1.5	6.0	4.023	-0.897	-0.45	18.55	73.80	0.0001**
Sucrose (F, %)	1.5	6.0	0.222	0.211	0.11	1.026	4.08	0.0898
Ammonium nitrate (G, %)	0.25	0.5	0.548	0.331	0.17	2.526	10.05	0.0193
Yeast extract (K, %)	0.25	0.5	1.442	-0.537	-0.27	6.649	26.45	0.0021
Peptone (L, %)	0.25	0.5	1.235	-0.497	-0.25	5.696	22.66	0.0031
KH_2PO_4 (N, %)	0.1	0.3	0.929	0.431	0.22	4.283	17.04	0.0062
K_2HPO_4 (O, %)	0.1	0.3	5.649	1.063	0.53	26.05	103.6	<0.0001**
Na_2HPO_4 (P, %)	0.15	0.35	1.991	0.631	0.32	9.181	36.52	0.0009**
$ZnSO_4$ (Q, %)	0.00	0.002	0.418	-0.289	-0.14	1.926	7.66	0.0325
Sodium phytate (R, %)	0.2	0.8	3.065	0.783	0.39	14.14	56.23	0.0003**
Tween 80 (S, %)	0.2	0.4	0.895	0.423	0.21	4.126	16.41	0.0067
M/N (T, %)	0.2	1.0	0.659	0.363	0.18	3.038	12.09	0.0132

$R^2 = 0.9849$

R^2 (adjusted) = 0.9522

R^2 (predicted) = 0.8324

Coefficient of Variation (CV) = 0.0253

[a]Sum of Squares

[b]Coefficient estimate

[c]Contribution

**Factors selected with p–value (Prob > F) < 0.001

Table 6.21 ANOVA analysis of regression model.

Sources of variation	F– value	p–value Prob > F
Intercept	–	–
A	10.94	0.0048
B	0.46	0.5102
C	26.25	0.0001
D	39.00	< 0.0001
AB	2.14	0.1640
AC	15.83	0.0012
AD	0.66	0.4290
BC	13.79	0.0021
BD	10.25	0.0059
CD	32.31	< 0.0001
A^2	54.00	< 0.0001
B^2	9.50	0.0076
C^2	4.16	0.0594
D^2	67.38	< 0.0001

$R^2 = 0.9453$

R^2 (adjusted) = 0.8943

R^2 (predicted) = 0.7384

Coefficient of Variation (CV) = 0.0676

Table 6.22 Large volume production studies for phytase.

Apparatus	Capacity (ml)	Medium volume (ml)	Productivity ($Ul^{-1}h^{-1}$)
Flask	250	50	43.89
Flask	500	100	43.91
Flask	1000	200	43.87
Bioreactor	7000	5000	136.56

[a]The results are mean of four independent experiments.

Table 6.23 Phytate degradation result.

S. No	Incubation time (min)[a]	Phytate content in sample (%)	Reduction in phytate content (%)
1	0	2.706	–
2	30	1.914	29.26
3	60	1.914	29.26
4	90	1.848	31.71
5	120	1.782	34.15
6	150	1.650	39.02
7	180	1.518	45.90
8	210	1.452	49.80
9	240	1.452	49.80

6.10. Figures of Chapter 6

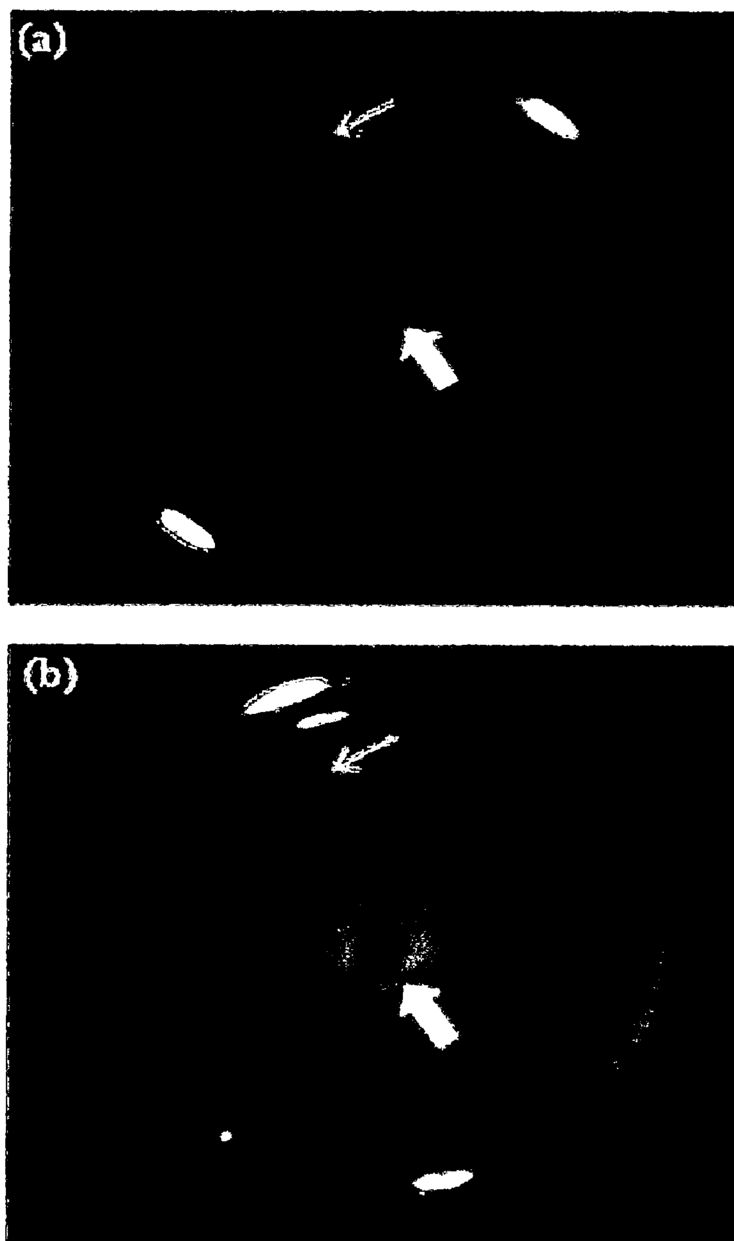
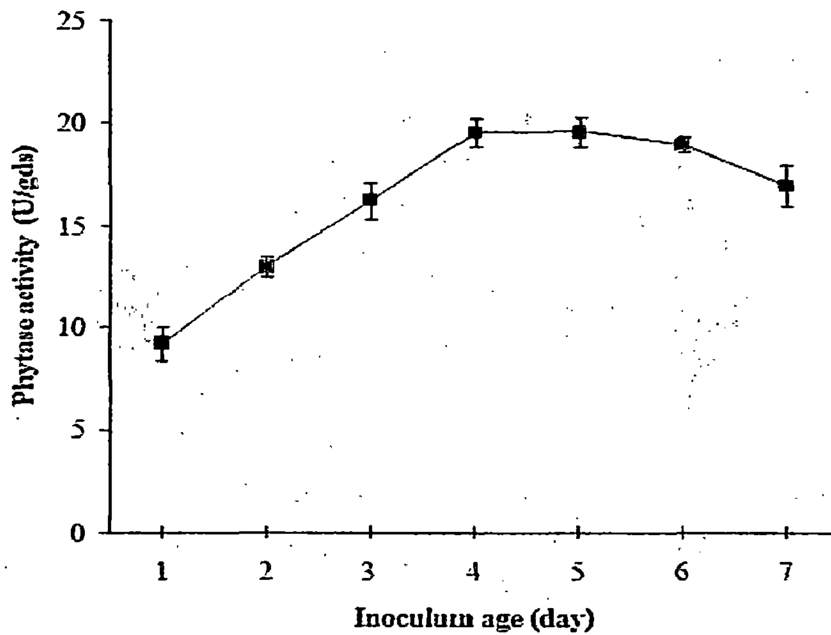
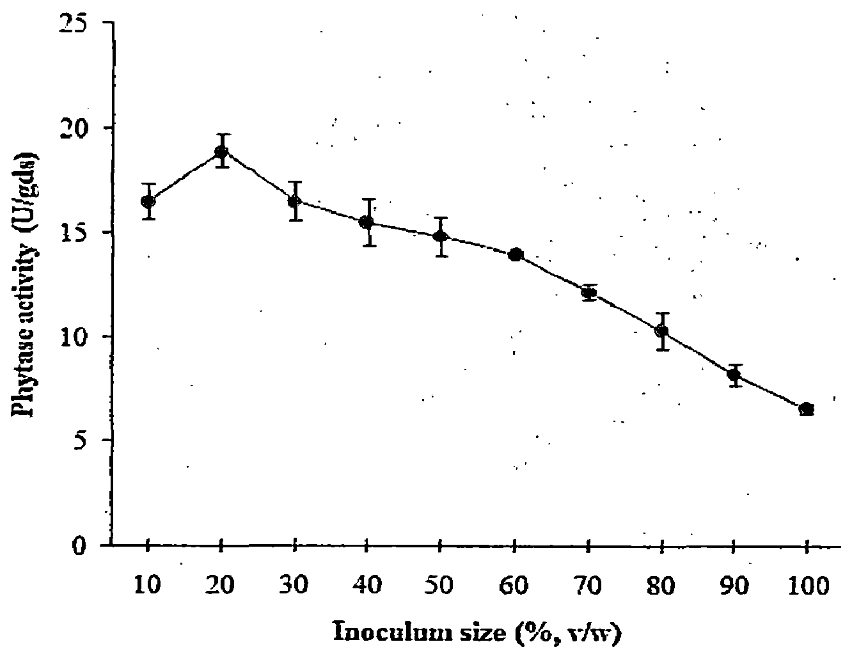


Fig. 6.1 Showing (a) phytate hydrolyzing ability of *Rhizopus* phytase on phytase screening medium (PSM) and (b) Zone of clearing after counterstaining method. Arrows in the figure correspond to fungal mycelia (Flat arrow) and zone of clearance (thin arrow).



(a)



(b)

Fig. 6.2 Effect of (a) inoculum age on phytase production inoculated with inoculum of different age varying from one to seven days and (b) effect of inoculum size on phytase production with inoculum size varying from 10 to 100% (v/w) in basal SSF medium containing equal ratio of wheat bran and linseed oil cake (~1.0 mm) after 96 h of fermentation at 35°C.

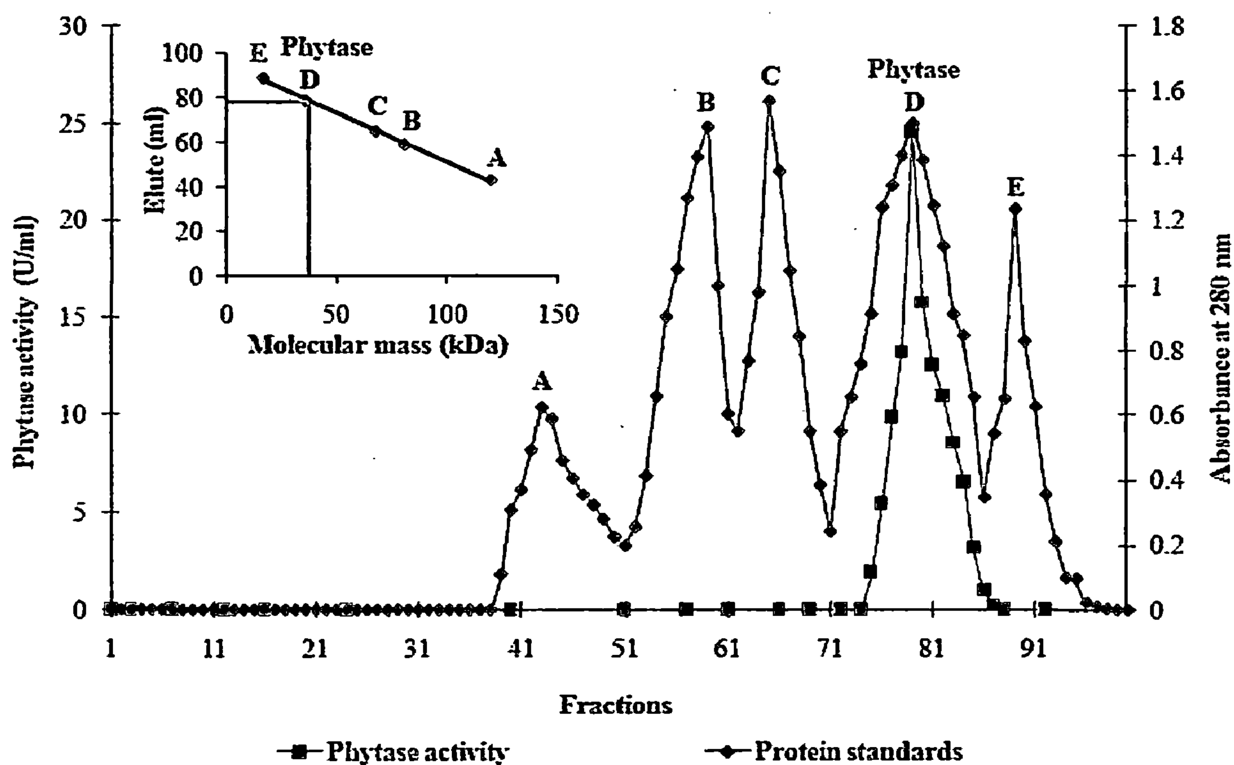


Fig. 6.3 Estimation of molecular size of *Rhizopus* phytase on Gel filtration column. The column was calibrated using glucose–6–phosphate dehydrogenase (A; 120 kDa), creatine kinase (B; 81 kDa), bovine serum albumin (C; 68 kDa), carbonic anhydrase (D; 36 kDa) and myoglobin (E; 17 kDa) as standard proteins.

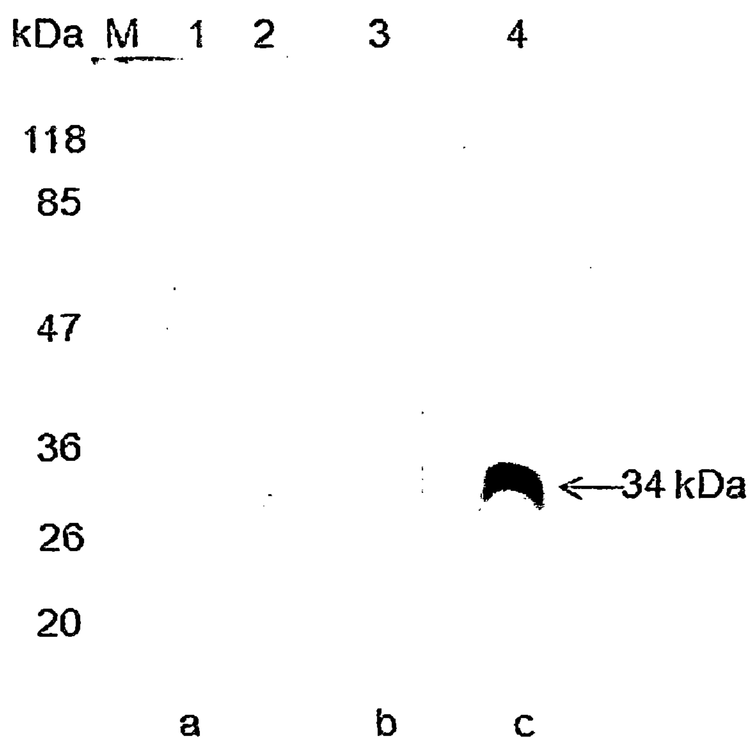


Fig. 6.4 Purification of phytase from *R. oryzae* (a) SDS–PAGE gel after Coomassie Brilliant Blue staining (b) Zymogram staining and (c) Nondenaturing (native) PAGE. The lanes on the gel correspond to molecular marker (Fermentas) (M); SSF crude extract (1); purified protein after size exclusion chromatography (2); zymogram analysis (3) and Native–PAGE (4).

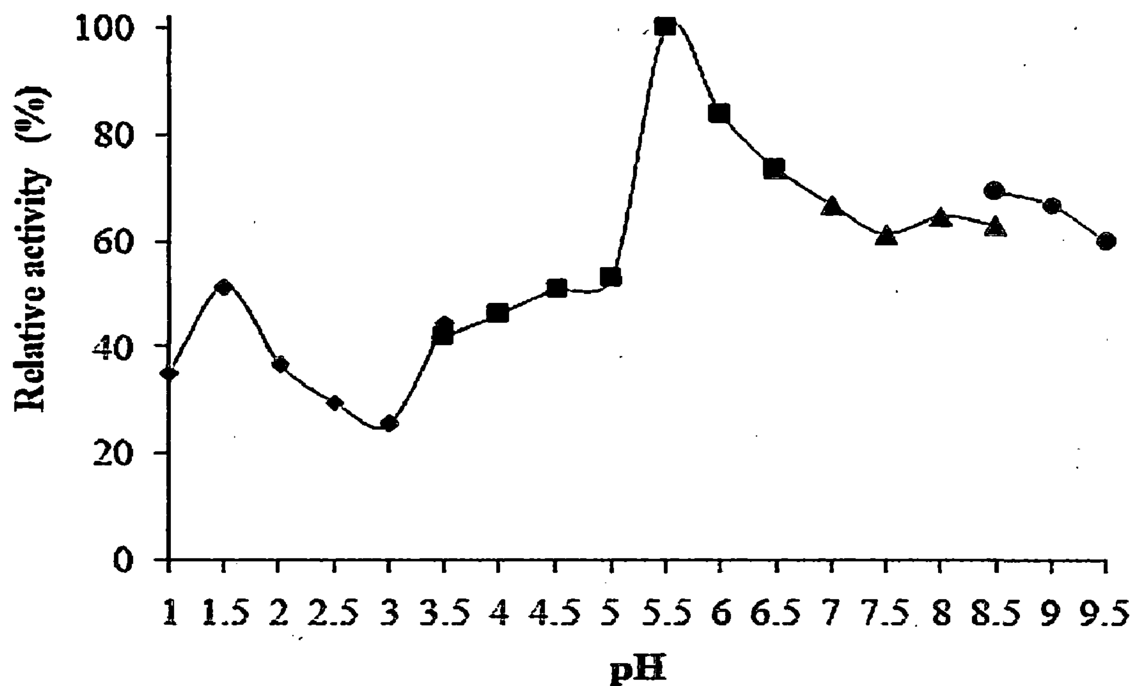


Fig. 6.5 Effect of pH on phytase activity. Phytase activity at pH 5.5 (100 mM acetate buffer) was regarded as 100%.

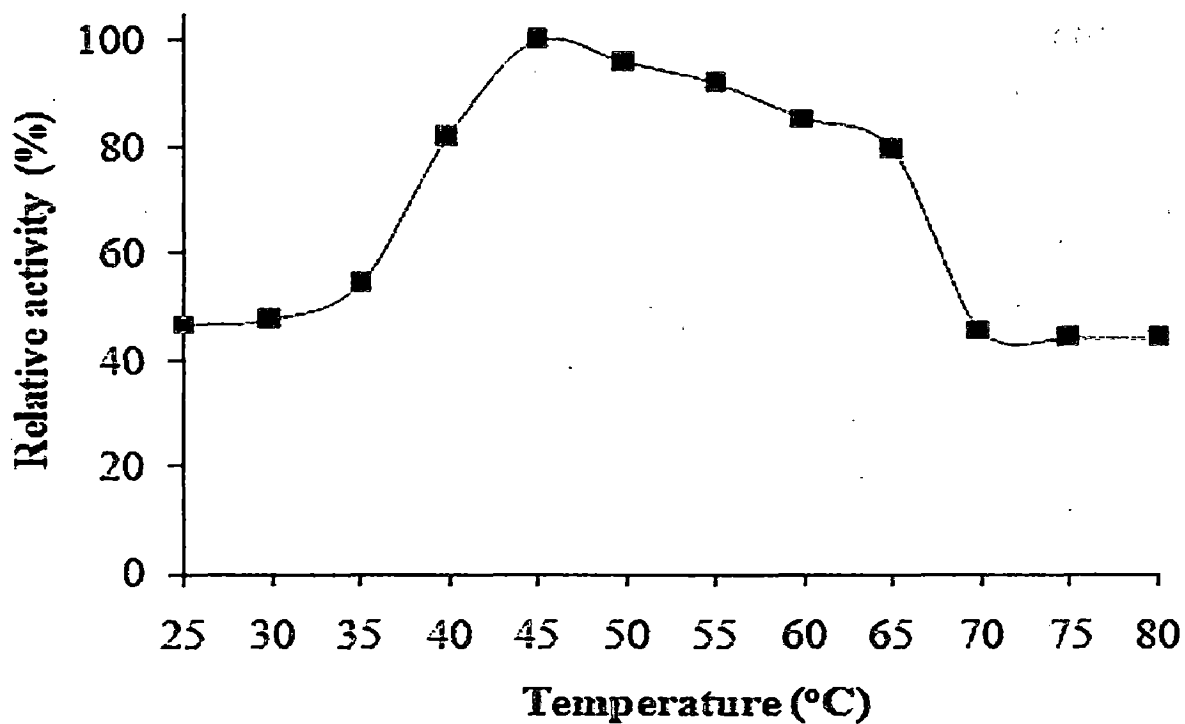


Fig. 6.6 Effect of temperature on phytase activity.

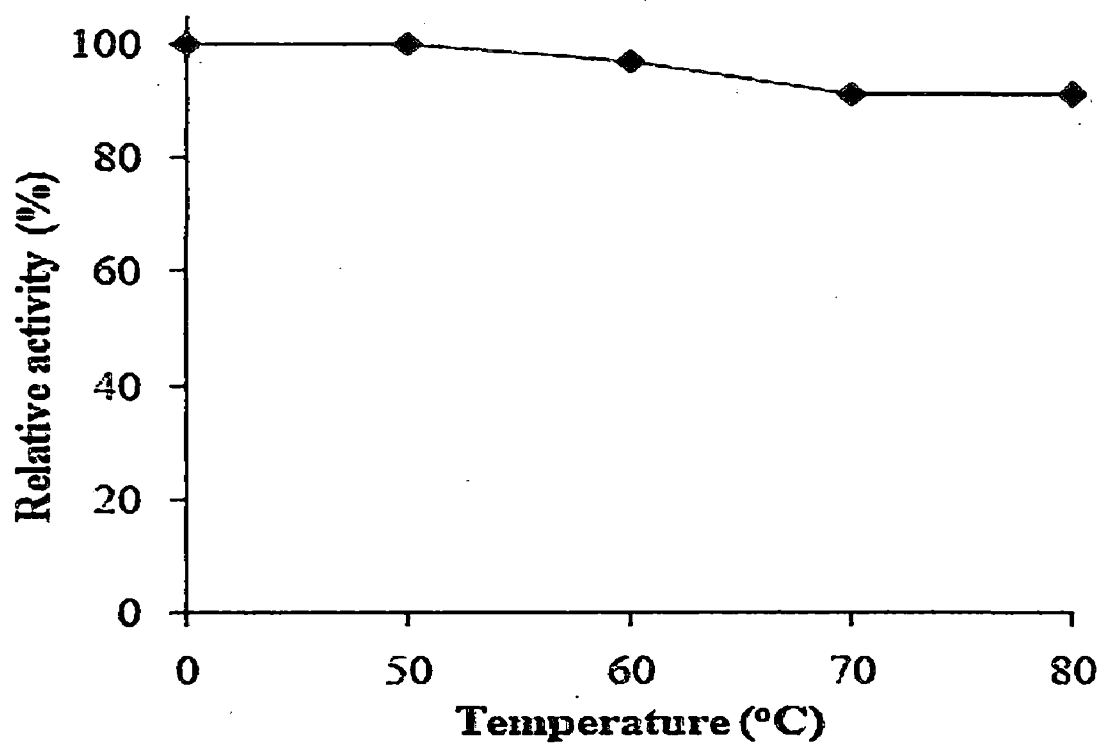


Fig. 6.7 Thermal stability of *Rhizopus* phytase. The activity of an unheated phytase was regarded as 100%.

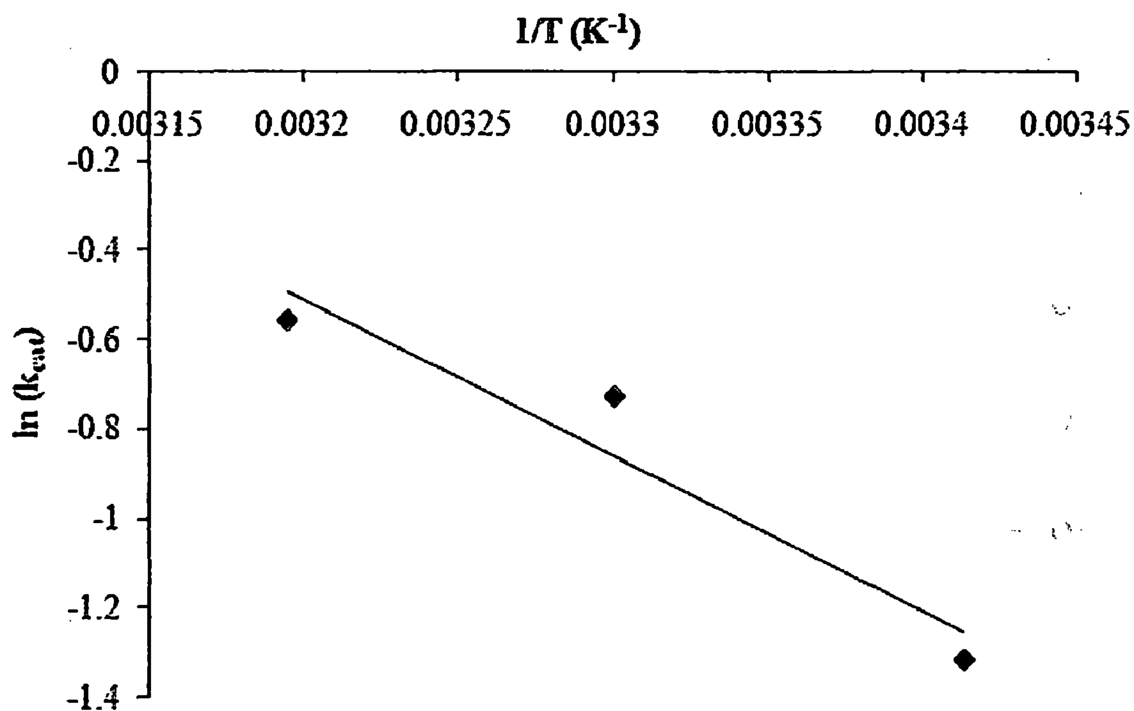
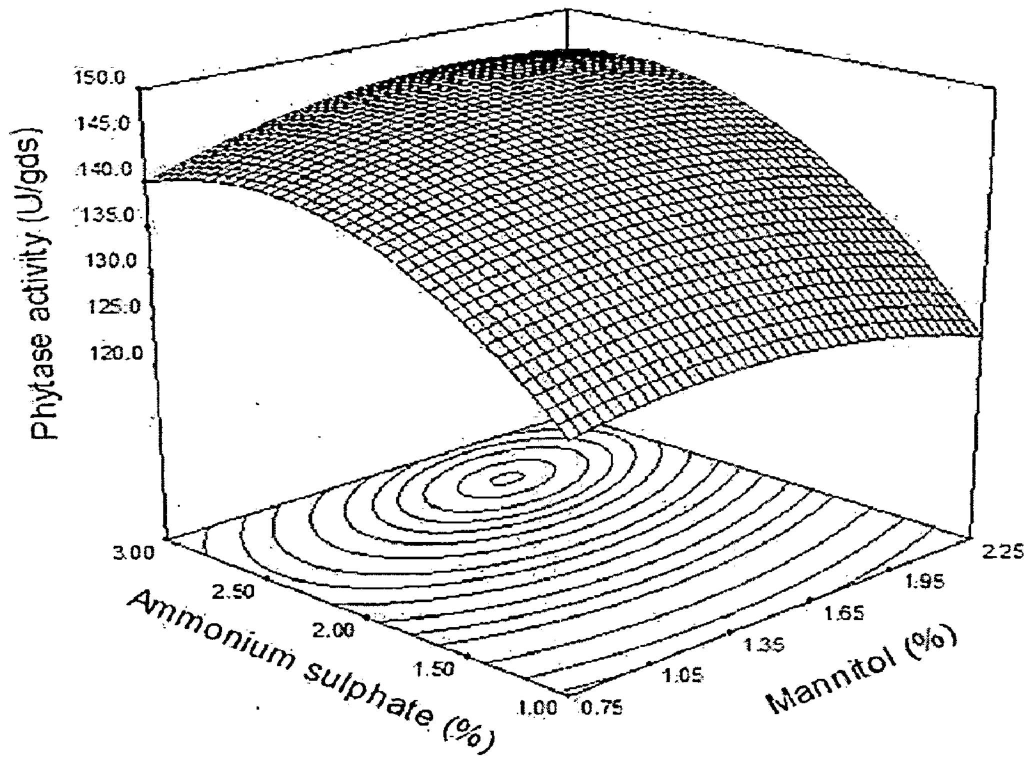
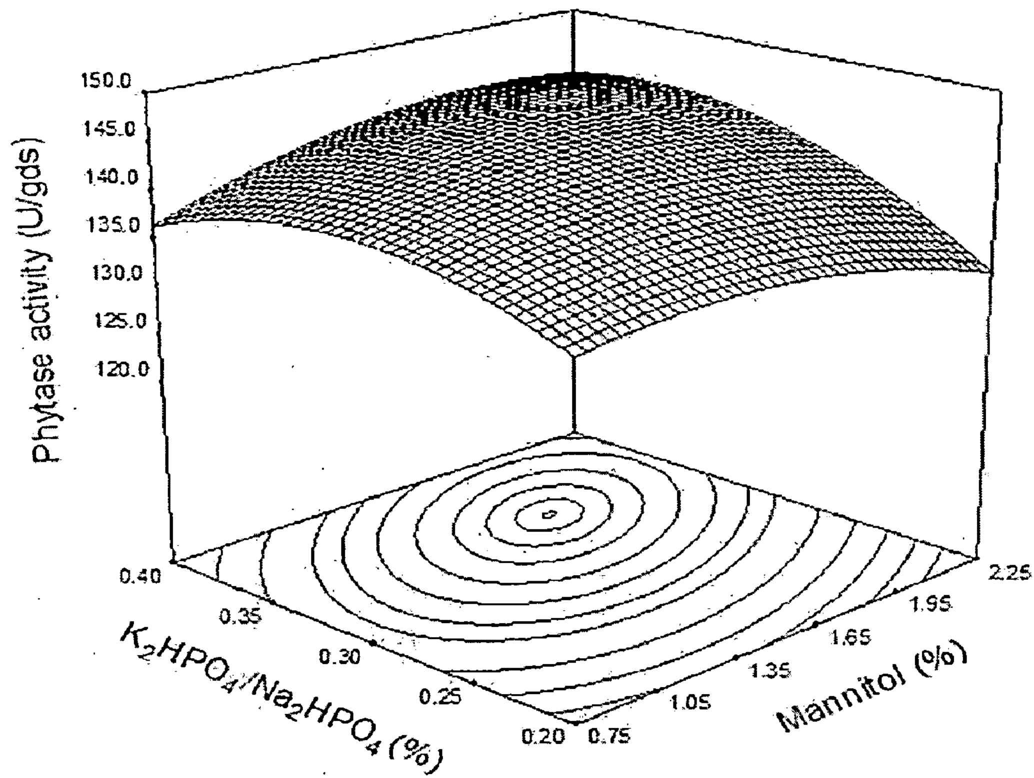


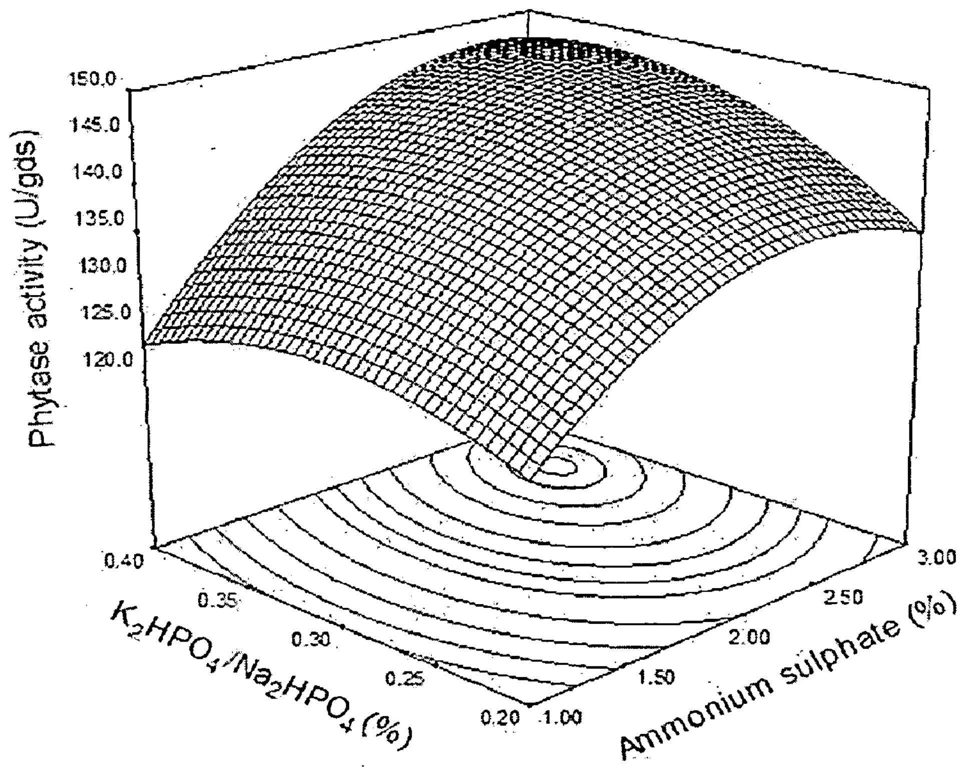
Fig. 6.8 Activation energy calculation.



(a)



(b)



(c)

Fig. 6.9 3D response surface plot showing interaction effects between

(a) mannitol and ammonium sulphate

(b) mannitol and K_2HPO_4/Na_2HPO_4 and

(c) ammonium sulphate and K_2HPO_4/Na_2HPO_4.

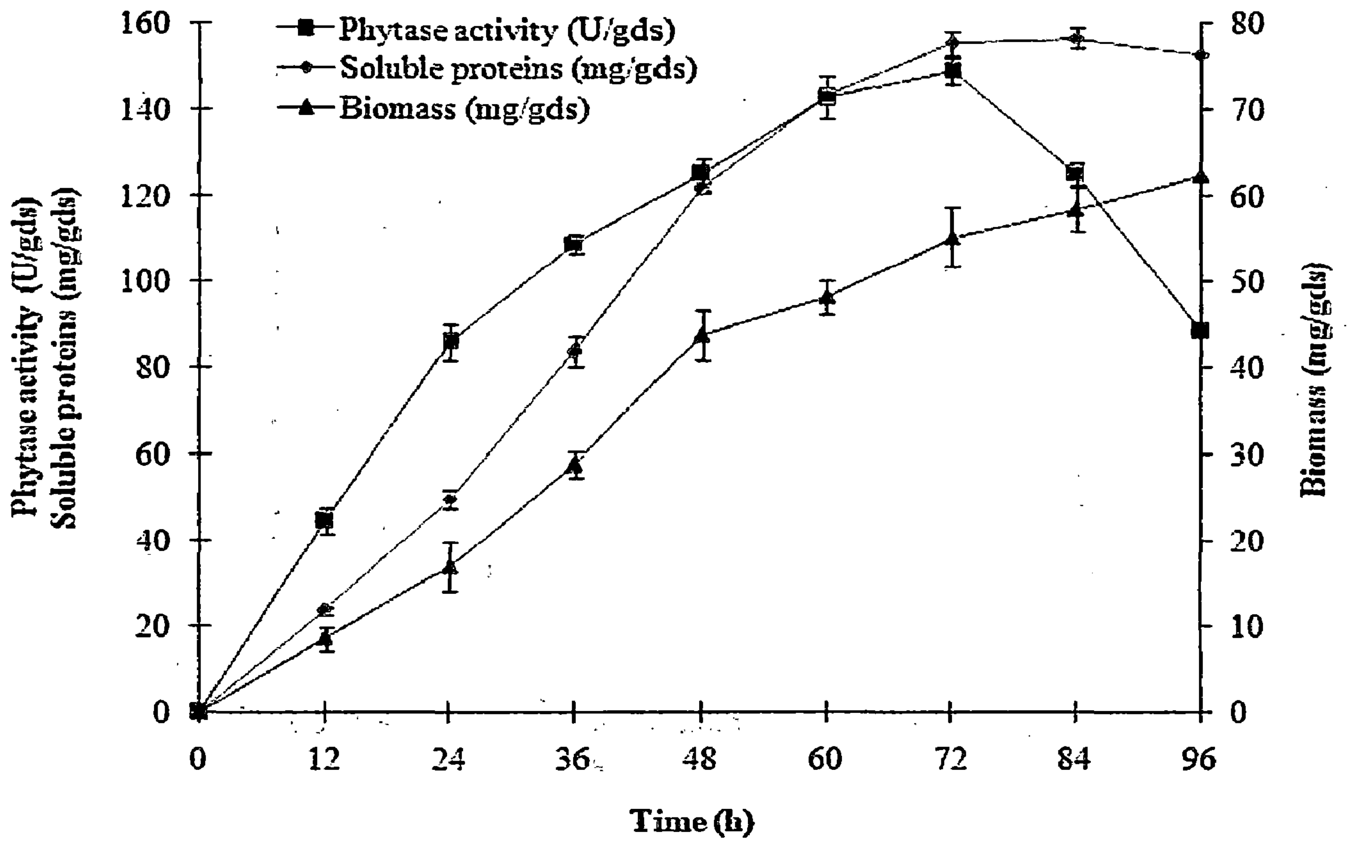


Fig. 6.10 Production profile of phytase by wild *R. oryzae* in optimized medium. Data are represented as means ± SD. n=3

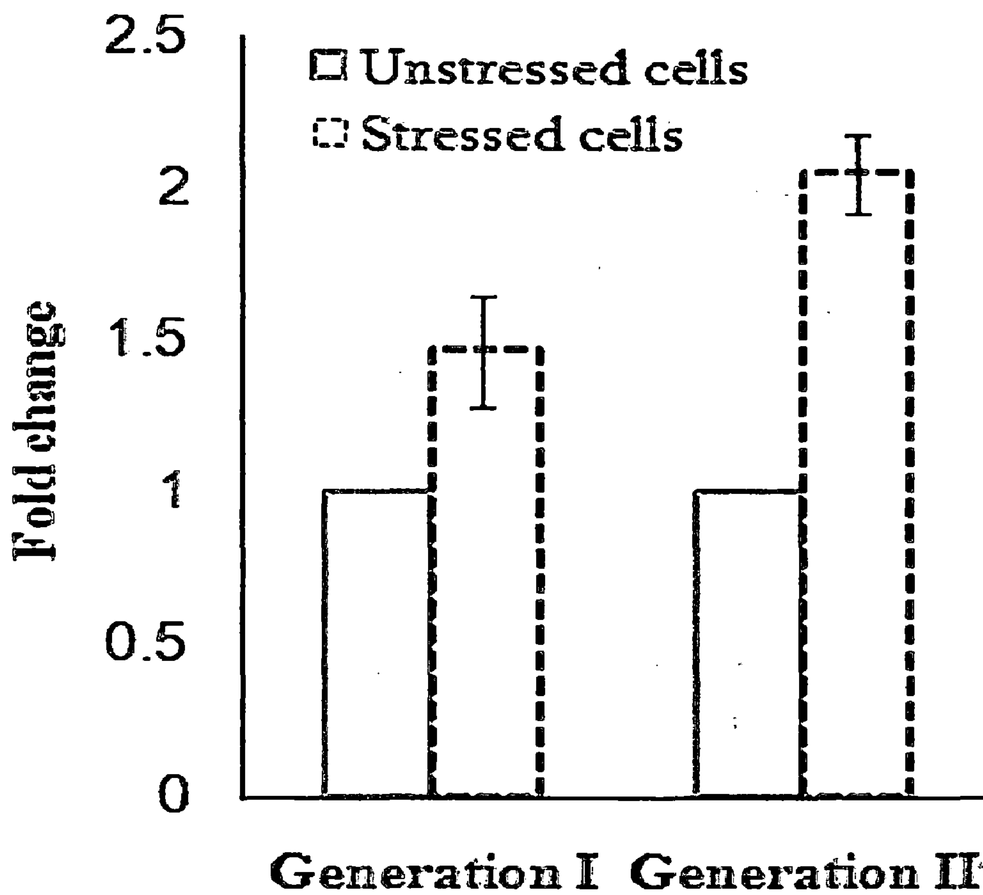


Fig. 6.11 Band density analysis showing fold change in phytase production ability of unstressed and stressed cells under SSF. Data are represented as means ± SD.

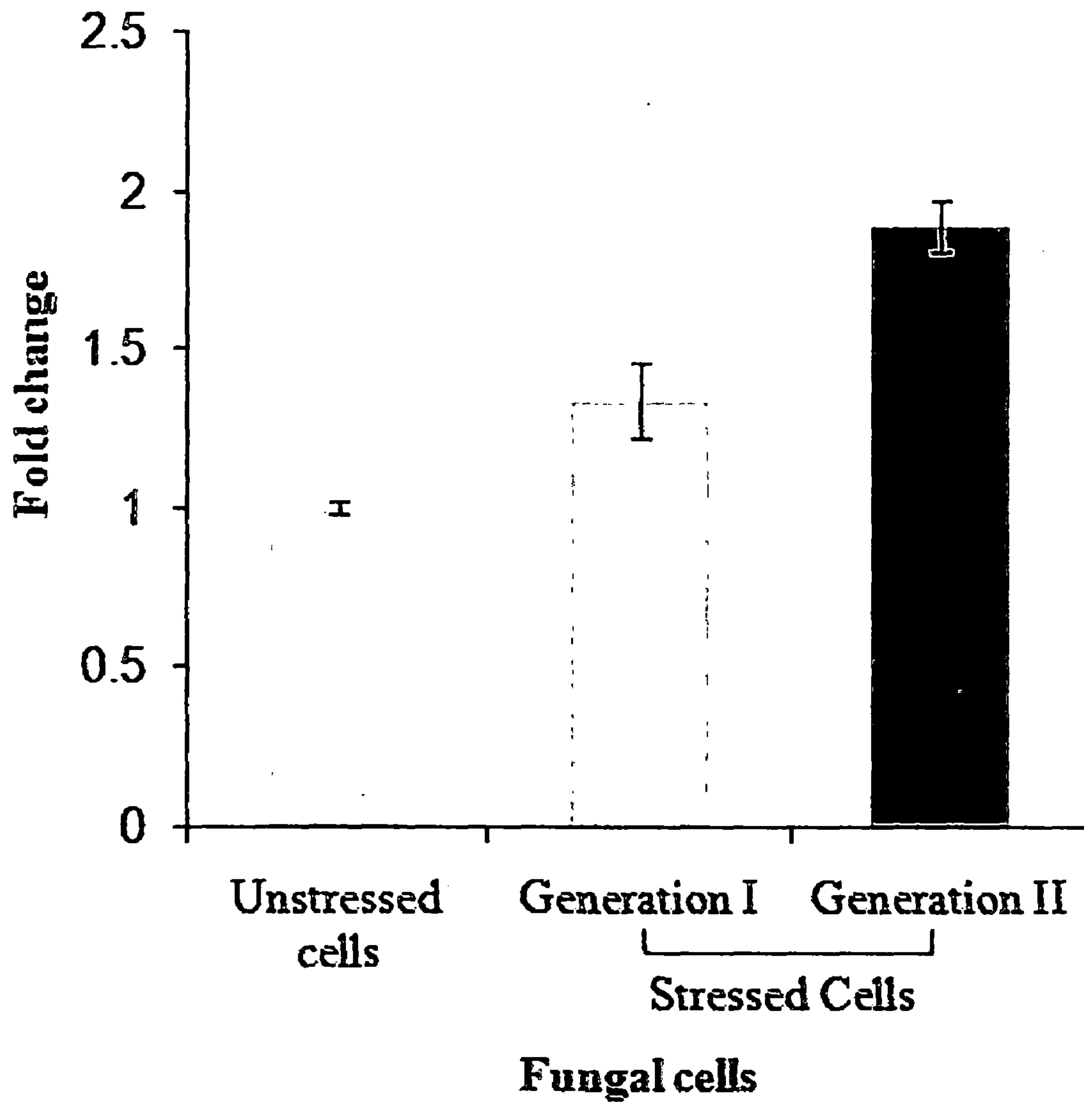


Fig. 6.12 Band density analysis showing fold change in phytase production ability of unstressed and stressed cells under SmF. Data are represented as means ± SD; n=3.

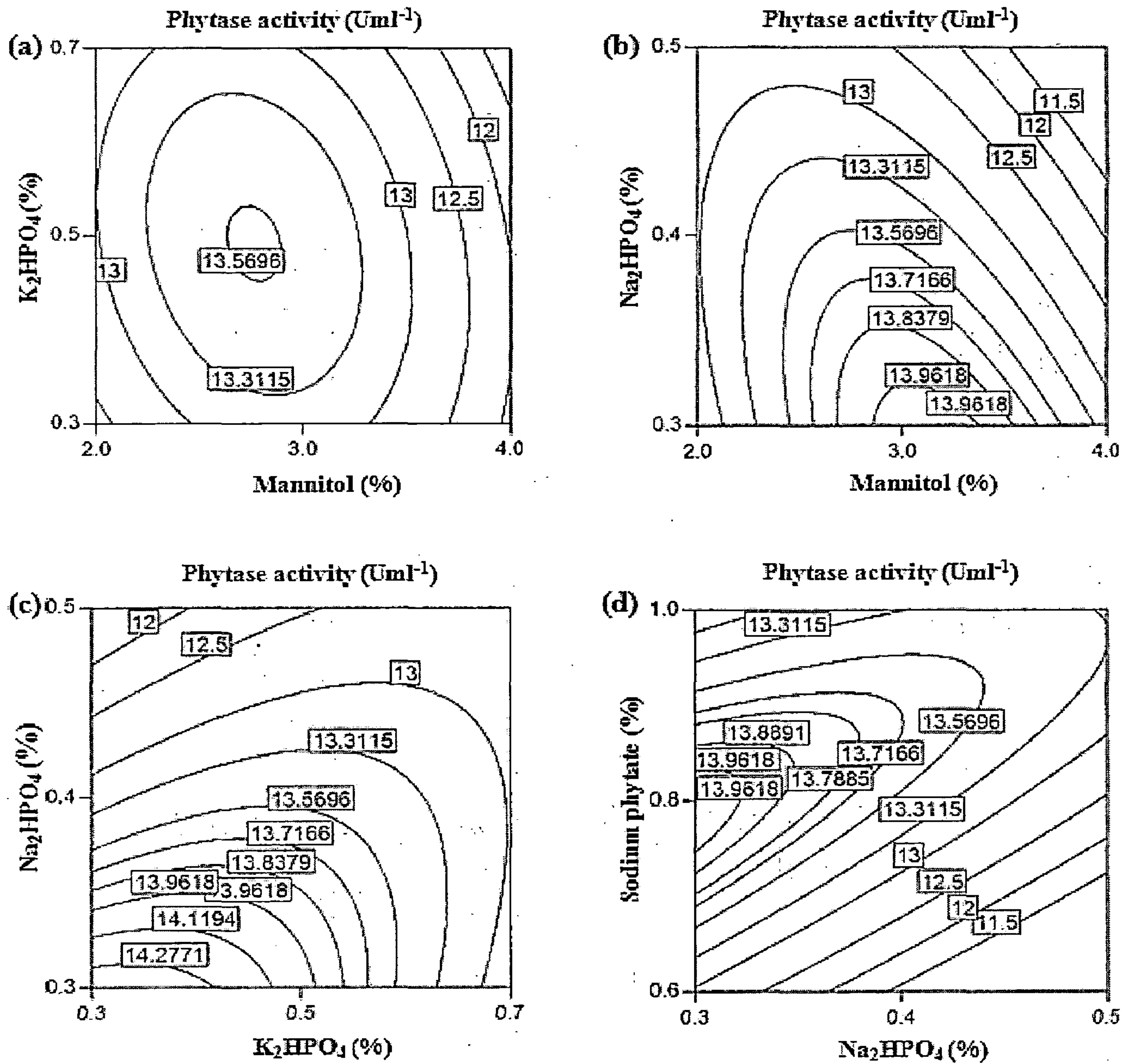


Fig. 6.13 Effect of interactions of various medium components on phytase production. Contour plots for (a) mannitol and K_2HPO_4 (b) K_2HPO_4 and phytate (c) K_2HPO_4 and Na_2HPO_4 and (d) Na_2HPO_4 and phytate.

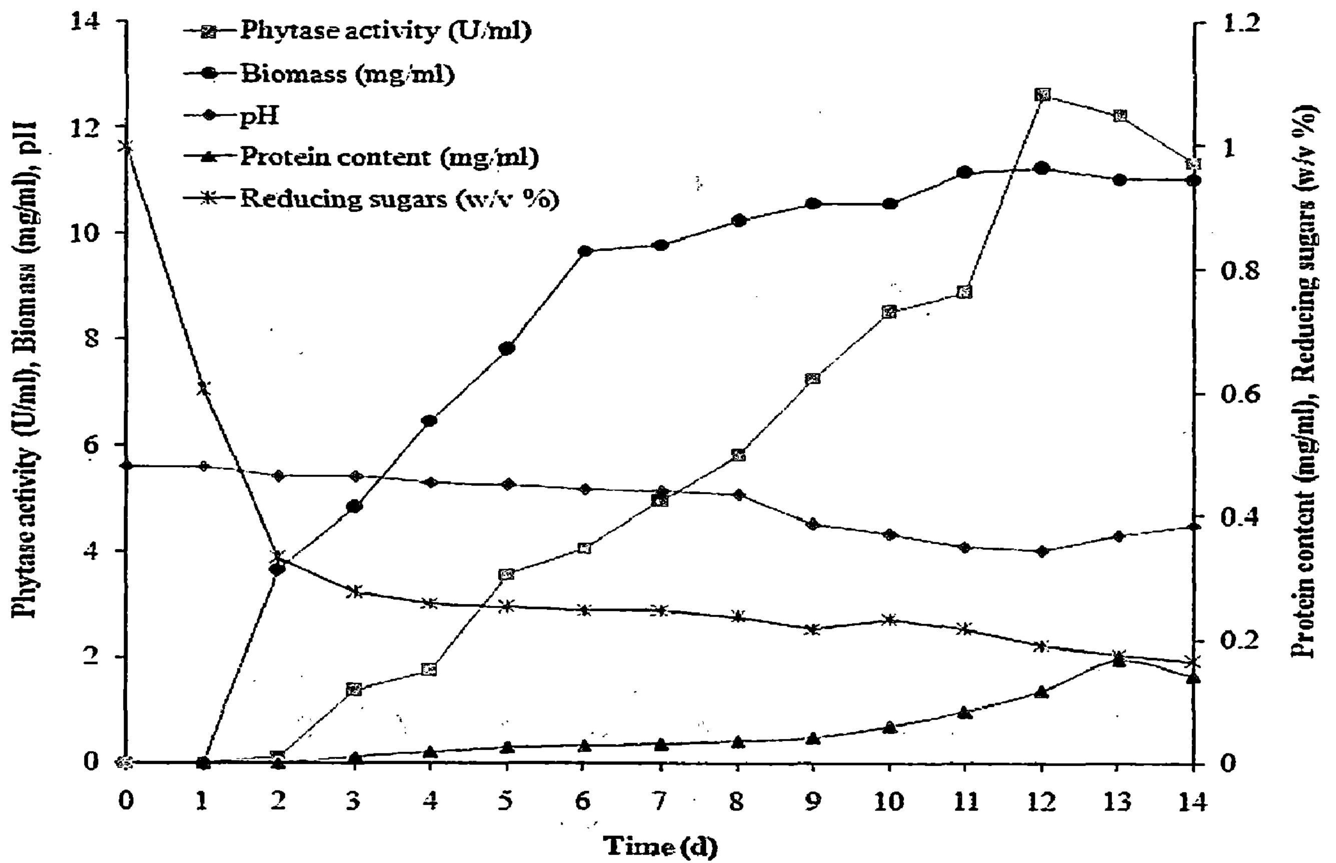


Fig. 6.14 Production profile of phytase by improved strain *R. oryzae* in optimized medium.

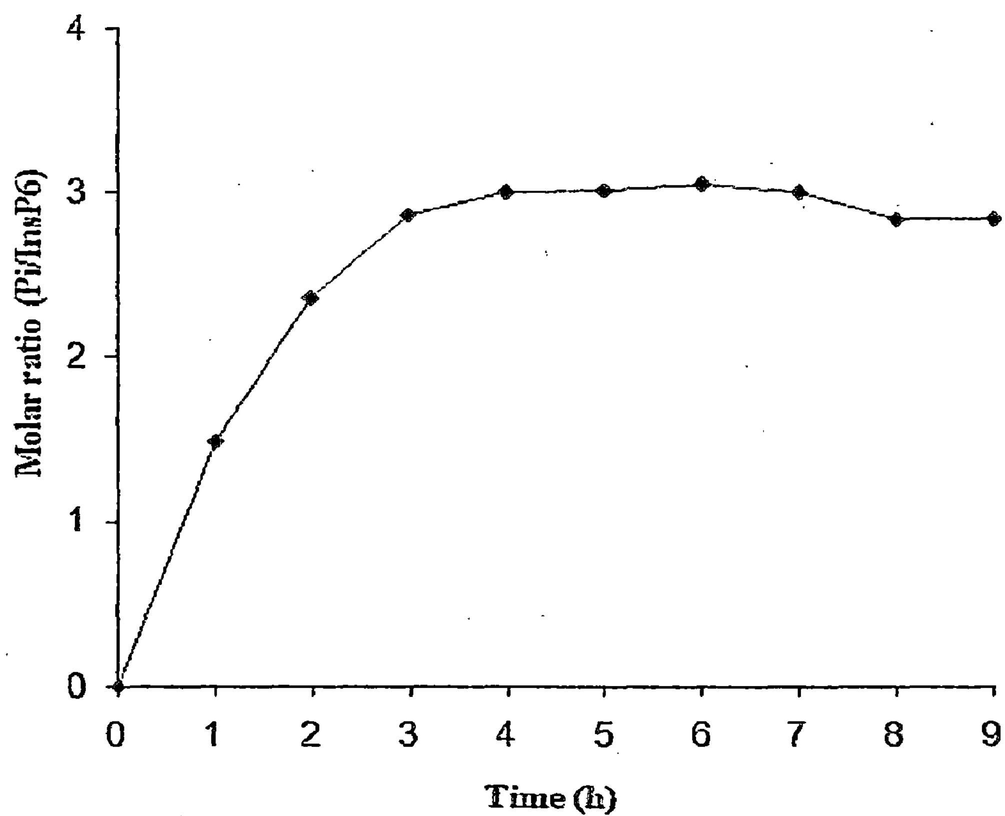


Fig. 6.15 Time course hydrolysis of sodium phytate by *Rhizopus* phytase.

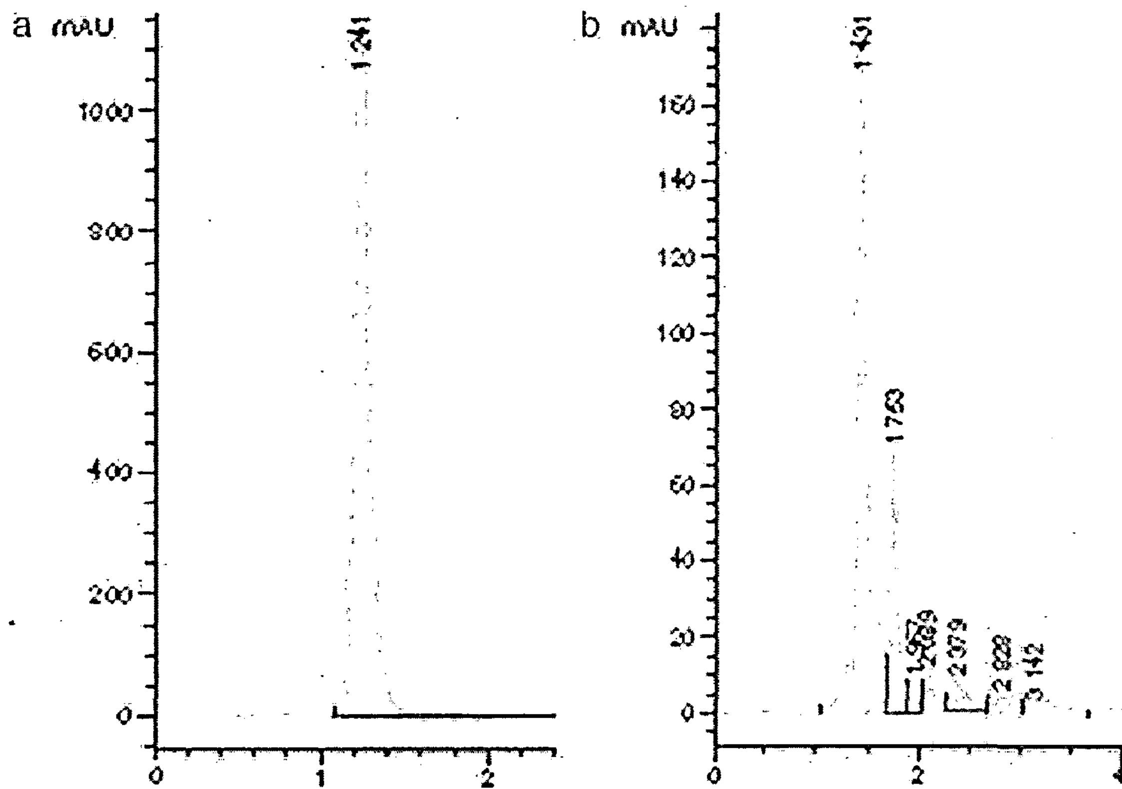


Fig. 6.16 Chromatograms profile after HPLC analysis (a) Untreated phytic acid (sodium salt) and (b) Sodium phytate treated with purified phytase.

CHAPTER 7 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

CHAPTER 7
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

7.1. Concluding remarks

Phytases have been of great interest for over a long period, owing to their high value from a nutritional as well as environmental perspective. Research has been devoted to find out organisms with suitable characteristics of phytase in nature, increasing the production yield by using statistical optimization of medium components, a suitable strain improvement approach, cloning and expression, and modifying the enzymatic properties to suit application requirements. So far, the main application of phytases has been as a feed additive. In this area, only a few acid phytases have been commercialized. They are active under acidic condition such as in the stomach of animals; however, they are not active under neutral and slightly alkaline conditions such as in the intestines of animals. Moreover, they are not efficient enough to withstand the high temperatures of feed pelletizing processes. Great interest has been addressed on mutation studies in order to increase the thermostability and modify the characteristics of phytases.

In present work, a highly thermoacidstable phytase, with a molecular weight of ~34 kDa, was identified from a mesophilic fungal strain *Rhizopus oryzae* MTCC 1987. Physico-chemical characterization studies showed several interesting properties of this phytase. The phytase from *Rhizopus* was found to exhibit bihump profile at pH 1.5 and 5.5, with a good activity observed in between pH 3.5-5.0. It displayed no loss in activity at lower pH and also depicted >80% of initial activity at pH 2.5. Another important characteristics of this phytase was to exhibit 45-80% of initial activity at 25-39°C, with >80% of initial activity at physiological temperature (39°C), which is a desired feature of an ideal phytase from application viewpoint. Thermostability is the major essential feature of any enzyme destined for animal feed applications, as the feed has to be commonly pelleted at 60-95°C so as to increase its digestibility, to ensure a balanced diet, for controlling microbial growth, for preventing transmission of pathogens and for

reducing the transportation costs by increasing bulk density. The present study revealed superior thermostability of this phytase showing no loss in activity at 50°C, and retaining >90% of its initial activity at 60, 70 and 80°C, when incubated for 30 min. The study carried out for thermodynamic characterization of phytase further supported the pronounced thermostability of this phytase. The phytase from *Rhizopus* was found to display pepsin resistant characteristics. Remarkable feature of the phytase was the insensitivity to heavy metal ions such as Ba^{2+} and Ag^{+} at high concentration of 5mM. Non-ionic detergents showed a significant enhancing effect on phytase activity, whereas anionic detergent (SDS) was found to inhibit the phytase activity even at 1% (v/v) concentration level. The above attributes of present investigation, such as high pH stability, high thermostability, insensitivity to proteolytic enzyme as well as heavy metal ions (Ba^{2+} and Ag^{+}), high residual activity at physiological temperature, good activity in wide range of pH encountered in digestive tract, broad range of substrate specificity, suggests this phytase as a prospective candidate intended for phytic acid degradation and mineral bioavailability in animal food and feed industry. Moreover, kinetic and thermodynamic characterization studies of the enzyme may help to fully understand the phytase from *R. oryzae*. Based on these studies, *Rhizopus* phytase was found to exhibit a low K_m value and high catalytic efficiency. The low K_m value further signified that the phytase produced required very low substrate concentration for gaining maximum catalytic activity. This implied that the enzyme can break even small amounts of phytate, if present.

Another important objective of the present study i.e. phytase production using agroindustrial residues under solid state fermentation, suggested the value-added utilization of these residues as substrates, resulting in an overall economical production process. Additionally, these studies pave the way for considering a wide variety of other cheap and renewable agro-industrial residues as raw materials for phytase production. Low cost linseed oilcake (LOC) when used in combination with wheat bran (WB) (1:1) was found to support the maximum mycelia growth of this strain, thereby phytase production, under solid state fermentation. This was the first report to study the phytase production by *R. oryzae* using LOC and WB, as mixed substrate. A marked enhancement in phytase production (17.69 U/gds-148.77 U/gds) was achieved due to optimization of culture conditions by 'one variable at a time' approach followed by the statistical

optimization of the medium components. Substrate particle size and initial culture conditions such as inoculum age and size were found to play a key role in affecting the phytase production most.

The work was further extended with the aim to increase the phytase titer through adaptation of *R. oryzae* cells to subsequent heat and cold stress via a novel strain improvement process. Based on the outcomes, the procedure was proved to be a simple and effective method for increasing the phytase yield by 1.50-fold in solid state production media, whereas, an increase in phytase titer by 43% was observed under submerged fermentation mode. This approach can contribute to a significant reduction of the cost of *R. oryzae* phytase production and consequently increase in its industrial utilization coefficients. The most noteworthy observation of this process was the less sporulation tendency in stressed culture. The reduction in sporulation did not affect the phytase yield in stressed strain whereas it was observed that the yield was found to be proportional to the rate of sporulation for unstressed strain.

Statistical optimization of medium components for phytase production by improved strain was also studied under submerged fermentation mode and resulted in an increase in phytase titer from 5.69 to 12.64 U/ml. The bioreactor studies showed a 3.11-fold increase in phytase productivity, suggesting the applicability of this process for the production of phytase for higher volume.

Based on thermal stability studies, *Rhizopus* phytase could find potential in food applications since it is stable enough to withstand pasteurization and active during long-term storage of foods at room temperature. Furthermore, the production of phytase by a GRAS strain would render them more advantageous for food applications, since the crude extract can directly be used as additional sources of enzyme along with the phytase. The benefit of the lower phosphorylation of inositol phosphate (i.e., IP3) for health would make this phytase more advantageous than other acid phytases since the end product is inositol trisphosphate, and not inositol monophosphate. Specific inositol phosphate derivatives are of pharmaceutical interest and require synthesis. For this, phytases represent a good choice compared to the chemical synthesis due to their specific activity during synthesis of enantiomer-pure products as well as their environmental friendliness.

Promising results were obtained when using thermostable phytase for hydrolyzing such IP6-metal ions complexes in various food ingredients. It is desirable for a phytase to

retain significant activity in milieu of digestive tract. Here, the *in vitro* study confirms the stability of this phytase at low pH and physiological temperature, further validating the potential of this phytase. Phytase from stressed *Rhizopus* sp. showed significant improvements with regard to the ash content or mineral extractability. Finally, an improved method for determining phytic acid and its lower derivatives based on absorption principle has shown the applicability of this method in a simple, faster and high-throughput manner. Method previously reported for the determination of inositol phosphates and its lower derivatives have some limitations in terms of their inability to separate structural isomers, time consuming sample preparation, need for pre- or post-column derivatization, and by long analysis time. In the present work, the feasibility of determining phytic acid and IP3 by reversed-phase high performance liquid chromatography (RP-HPLC) equipped with variable wavelength detector at 246 nm was successfully achieved with minimal sample preparation and lower retention time.

7.2. Future perspectives

- Scale up studies in bioreactor and solid state fermentation mode could help in proving the applicability of these processes on pilot and industrial scale.
- Further investigations to fully characterize this phytase by sequencing, crystallization, and X-ray studies of the enzyme structure could contribute to a better understanding of the structure-function relationships of the enzyme.
- The supplementation of *Rhizopus* phytase in food ingredients was found to increase the bioavailability of minerals as demonstrated in the study *in vitro*, and needs to be confirmed for chicken, poultry and other livestocks.

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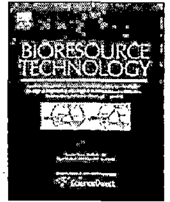
PAPER I

Bioresource Technology 102 (2011) 10641–10649

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech



Production of phytase under solid-state fermentation using *Rhizopus oryzae*: Novel strain improvement approach and studies on purification and characterization

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ARTICLE INFO

Article history:
Received 1 June 2011
Received in revised form 14 August 2011
Accepted 17 August 2011
Available online 8 September 2011

Keywords:
Phytase
Rhizopus oryzae
Linseed oil cake
Optimization
Strain improvement

ABSTRACT

Present study introduces linseed oil cake as a novel substrate for phytase production by *Rhizopus oryzae*. Statistical approach was employed to optimize various medium components under solid state fermentation (SSF). An overall 8.41-fold increase in phytase production was achieved at the optimum concentrations (w/w, mannitol, 2.05%; ammonium sulfate, 2.84% and phosphate, 0.38%). Further enhancement by 59% was observed due to a novel strain improvement approach. Purified phytase (~34 kDa) showed optimal temperature of 45 °C, dual pH optima at 1.5 and 5.5 and possesses high catalytic efficiency ($2.38 \times 10^6\ M^{-1}s^{-1}$). Characterization study demonstrates the phytase as highly thermostable and resistant to proteolysis, heavy metal ions, etc. Furthermore, an improved HPLC method was introduced to confirm the ability of phytase to degrade phytic acid completely and was found to be an efficient method.

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1. Introduction

In current scenario, the interest in the recycling, upgrading and bioconversion of agro-industrial residues has increased drastically due to their disposal and looming environmental pollution problem. Effective utilization of these residues would not only help in curbing pollution but also pave the way for solid waste management and minimize the initial capital costs for the processes. Solid state fermentation (SSF) being a simple, low cost and self sustainable technologies with efficient utilization of these residues has served dual purpose of value addition and waste management (Pandey, 2003). The most inexpensive and high energy rich substrates for fermentation industry are represented by oil cakes and traditional agricultural by-products (wheat bran, rice bran, rice husk, etc.).

Several oil cakes such as coconut oil cake (COC), sesame oil cake (SOC), palm kernel cake (PKC), groundnut oil cake (GOC), cottonseed cake (CSC), soy bean cake (SBC), mustard oil cake (MOC), canola oil cake (CaOC), sunflower oil cake (SuOC), and olive oil cake (OOC) have been reported and have been used as a substrate for the production of industrial metabolites. Oil cakes being rich in proteins, carbohydrates and minerals offer a wide range of alternative substrates, thus find various applications in the bioprocess industry for the production of a wide spectrum of biometabolites such as industrial enzymes, organic acids, antibiotics, biopesticides, vitamins, and biofertilizer (Pandey et al., 1999; Ramachandran et al., 2005).

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8 and 3.1.3.26) catalyze the hydrolysis of phosphomonoester bonds of phytate (salts of myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), thereby releasing lower forms of *myo*-inositol phosphates and inorganic phosphate. To increase the availability of phosphorus, feed is either supplemented with nonrenewable and expensive phosphate or with exogenous phytase. Phytases are produced by a large number of plants, animals and microorganisms. However, phytases from microbial sources especially of fungal origin have supremacy in commercial industry (Pandey et al., 2001). Inclusion of phytase in animal feed can alleviate these problems and has been shown to enhance the uptake of phosphorus and other minerals, protein, amino acids and carbohydrates and energy.

Rhizopus oryzae, generally regarded as safe (GRAS) strain, is used in production of various enzymes, organic acids, aroma and mycotoxins because of its ability to utilize a variety of carbon sources, such as wheat bran, oil cakes (Bogar et al., 2003a,b; Ramachandran et al., 2005), cassava, soybean and amaranth grain. Crude enzymes produced by GRAS strain on various feed supplements (bran, straw, oil cakes, etc.) could serve as a value-added supplement by providing other fungal proteins, sugars and some accessory enzymes along with the main enzyme source (Bogar et al., 2003b; Pandey et al., 2001).

Recently, the industrial potential of fungal enzymes has motivated research toward strain improvement. Hence, an effective

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0960-8524/$ - see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.biortech.2011.08.075

heat and cold shock (stress condition) method was developed to enhance the phytase production ability of *R. oryzae*, followed by a comparative study on efficacy of wild and mutant strains pertaining to ash content and mineral extractability.

In the present study, linseed oil cake (LOC) and wheat bran (WB) was exploited as raw materials for the production of phytase under SSF. Linseed oil cake is rich in phytic acid (~4.2%), protein content (32–36%), residual oil (2.81%), minerals and essential amino acids such as methionine, lysine, and leucine (Hossain and Jauncey, 1990) while wheat bran beside being a good source of carbon and nitrogen sources, has high phytate content and good support matrix properties (Wodzinski and Ullah, 1996). Since, availability is also one of the important considerations for development of a cost effective process, data shows that the global output of linseed is estimated around 2.60 million tons (mt) per year with Canada (0.6 mt), China (0.48 mt), India (0.17 mt) and United States (0.15 mt) dominating the list of producers. (http://www.commodityonline.com/commodities/oil-oilseeds/linseedoil.php).

Optimization of culture conditions using statistical tools has been employed in enhancing the phytase production from various microorganisms during SSF (Chadha et al., 2004; Bogar et al., 2003a,b; Singh and Satyanarayana, 2008). However, there is no literature reported to optimize the culture parameters for phytase production using *R. oryzae* under SSF.

Present study aimed at the statistical optimization of culture variables for the production of phytase under SSF using *R. oryzae* MTCC 1987. For the first time, the inexpensive LOC and WB are being revealed as a mixed substrate for phytase production under SSF. Additionally, an efficient and reproducible HPLC method is also demonstrated for the analysis of phytic acid and its degradation products. The study is further extended to purification and characterization of the phytase to explore its suitability in food and feed industry.

2. Methods

2.1. Microorganism and inoculum preparation

R. oryzae (MTCC 1987) was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The fungal strain was routinely grown on potato dextrose agar (PDA) (HiMedia, India) slants for 6 days at 30 °C. Viable spores from slants were harvested by washing with 0.1% (v/v) Tween 80 and the spore suspension adjusted to ~1 × 10^6 cfu/ml (colony forming unit per milliliter) was used as inoculum for subsequent fermentations.

2.2. Evaluation of agro-industrial residues and solid state fermentation (SSF)

Various agro-industrial residues (individually and in combinations) namely, linseed oil cake (LOC), mustard oil cake (MOC), sunflower oil cake (SOC), rapeseed oil cake (ROC), wheat bran (WB) and rice husk (RH) were investigated in this study and were purchased from local retail feedstuff outlets in Roorkee (Uttarakhand, India).

Initially, 5 g of agro-industrial residues was supplemented with 20% (v/w) of mineral salt solution ((w/w); 0.3% NaCl; 0.3% $MgSO_4.7H_2O$; pH 5.6) in 250 ml Erlenmeyer flask. Medium sterilized at 121 °C for 20 min was inoculated with 20% (v/w) inoculum and fermentation was carried out at 35 °C for 96 h. The fermented medium was extracted with Tween 80 (0.1% (v/v)) at 30 °C on an orbital shaker at 200 rpm for 1 h. Cell free extract was used for phytase activity assay. All experiments were performed in triplicate.

2.3. Fermentation in flasks and trays

Flask level experiments were carried out under optimum conditions, predicted by the model, using 5–50 g of substrate (LOC + WB; (1:1)). The substrate was supplemented with (w/w): 2.05% mannitol, 2.84% ammonium sulfate and 0.38% of K_2HPO_4/Na_2HPO_4 (1:1), 20% v/w mineral solution (pH 7.6) with a final moisture content of 40% (v/w). The medium was autoclaved and inoculated with 20% v/w of inoculum. Fermentation was carried out at 30 °C for 72 h. The fermented medium was extracted with Tween 80 (0.1% (v/v)) at 30 °C on an orbital shaker at 200 rpm for 1 h. Cell free extract was used for phytase activity assay. All experiments were performed in triplicate.

For tray fermentation, 50–1000 g of substrate was taken in enamel coated metallic trays (28 × 24 × 4 and 45 × 30 × 4 cm) with different bed thickness (0.2–2.0 cm). The fermentation was carried out under optimum condition as described above.

2.4. Strain improvement procedure

Fungal spore suspension was treated with a number of individual combinations involving maximum temperature range (70, 80 or 90 °C), durations of heating and chilling (at 4 °C) and number of cycles (Supplementary Table S1). After optimizing the stress conditions, the treatment was applied up to six generations of cultures.

Stressed strains after each treatment were screened for phytase production on phytase screening medium (PSM) containing (w/v): 0.4% sodium phytate, 1% D-glucose, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.5% NH_4NO_3, 0.01% $MnSO_4{\cdot}H_2O$, 0.5% $CaCl_2{\cdot}2H_2O$, 0.01% $FeSO_4{\cdot}7H_2O$ and 1.5% Agar (pH 5.6) (Howson and Davis, 1983). The spore suspension was plated and phytase activity was recorded after 72 h of incubation at 30 °C by measuring the diameter of clear zone. The best strain was selected on the basis of larger diameter of the clear zone. After fermentation, crude extract of mutant strain was subjected to phytase activity assay as well as a semiquantitative method using SDS–PAGE band density analysis. For SDS–PAGE analysis, the crude proteins from both unstressed (wild) and stressed (mutant) strains were resolved on a 12% separating gel topped with 4% stacking gel followed by Coomassie Brilliant Blue staining (Laemmli, 1970).

2.5. Analytical methods

Phytase activity was determined by estimating the inorganic phosphate released from sodium phytate (Bae et al., 1999). One unit of phytase is defined as the amount of enzyme required to release 1 nmol of inorganic phosphate (P_i) per second under the standard assay conditions. The phytase yield was expressed as a function of dry substrate weight (U/gds). Protein estimation was carried out using BCA protein quantification kit (Sigma, USA). Fungal biomass was estimated by determining the *N*-acetyl glucosamine released by acid hydrolysis of the chitin (Sakurai et al., 1977). Biomass was expressed as milligram of glucosamine per gram dry substrate (mg/gds). Percent ash content was determined using the standard official method (AOAC, 1980). For this, 2 g of samples were incinerated in Muffle furnace FO100 (Yamato Scientific Co., Ltd.) in a tarred crucible. With each set of samples, a blank was prepared in the same manner. The minerals (iron, zinc and calcium) were measured at the appropriate instrumental conditions (Supplementary Table S2) using an atomic absorption spectrophotometer (AAS) (Avanta Grade M, GBC Scientific Equipment) and was quantified by standard curves made from standard mineral solutions (Fisher Scientific, Pittsburgh, PA). Concentration of iron, zinc and calcium was expressed as μg/g (ppm) on dry weight basis.

2.6. Statistical optimization of phytase production

2.6.1. Plackett–Burman design (PBD)

PBD was employed for screening the most significant culture variables influencing the phytase production. Based on initial studies, eight assigned factors and three unassigned factors (dummy) were screened in a total of 12 runs. The detail of the design with the actual and predicted responses (phytase activity) is given in Supplementary Table S3.

2.6.2. The path of steepest ascent (descent) method

To approach rapidly in close proximity to optimum response, the method of path of steepest ascent (descent) was performed (Supplementary Table S4). The direction of the maximum increase in phytase activity was determined according to the estimated coefficient ratio from the fitted first-order model in PBD.

2.6.3. Central composite designs (CCD)

To determine the mutual interactions among the selected variables (mannitol, ammonium sulfate and K_2HPO_4/Na_2HPO_4) and their corresponding optimum concentrations, central-composite design (CCD) of response surface methodology (RSM) was used. A 2^3 factorial design having eight factorial points, six axial points and six replicates at the centre point with a total number of 20 runs was formulated. The details of experimental design with coded and actual levels of each factor are summarized in Table 1. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response to the independent factors. The complete second-order polynomial model equation (1) to be fitted to the yield values was:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1,i<j}^{n-1} \sum_{j=2}^{n} \beta_{ij} x_i x_j \quad (1)$$

where, Y is the observed value of the response (phytase production); x_i ($i = 1$, 2 and 3) is the controlling factors; β_0 is the offset term, and β_i ($i = 1$, 2 and 3), β_{ii} and β_{ij} ($i = 1$, 2 and 3; $j = 2$ and 3) are the model linear, quadratic and interaction coefficient parameters, respectively.

2.6.4. Statistical analysis

The statistical software package 'Design-Expert® 8.0.5, Stat-Ease Inc., Minneapolis, MN, USA was used for experimental design and subsequent regression analysis of the experimental data. All experiments were done in triplicate, and the average phytase activity was taken as the response.

2.7. Purification and characterization of phytase

SSF crude extract was subjected to fractional ammonium sulfate precipitation (40–80% saturation) with constant stirring. The precipitate was collected by centrifugation (15,000g, 20 min) and dissolved in 200 mM Tris–HCl, pH 8.0. The proteins after dialysis were loaded onto a DEAE Sepharose CL-6B column and eluted with various salt gradients in elution buffer (200 mM Tris–HCl, pH 8.0). Sephadex G-100 gel filtration column preequilibrated with 200 mM acetate buffer (pH 5.0), was used for desalting and separation of proteins based on size. Fractions showing high absorbance at 280 nm were assayed for phytase activity. The proteins were resolved by SDS-PAGE on a 12% separating gel topped with a 4% stacking gel and gels were subjected to Coomassie Brilliant Blue staining (Laemmli, 1970) and zymogram staining (Bae et al., 1999). Nondenaturing PAGE analysis was carried out according to Casey and Walsh (2004). The molecular size of native phytase was estimated on Sephadex G-100 gel filtration column equilibrated with 100 mM sodium acetate buffer (pH 5.5).

The phytase purified by fractional ammonium sulfate precipitation and subsequent ion-exchange and size-exclusion chromatography was used for characterization studies. The pH optimum was determined over the range of pH 1.0–9.5 using 100 mM buffers: glycine–HCl (pH 1.0–3.5), acetate (pH 3.5–6.5), Tris–HCl (pH 6.5–8.5) and Glycine–NaOH (pH 8.5–9.5) at 39 °C. Same buffers were used for assessing the pH stability profile of phytase at 4 °C for 6 h. The temperature optimum was determined between 25 and 80 °C. To check the thermal stability, the phytase was preincubated at 50, 60, 70 and 80 °C for 30 min, respectively, cooled to room temperature and assayed using the standard phytase assay. In order to determine the effect of metal ions and inhibitors, the phytase was incubated in presence of 1 and 5 mM of each at room temperature for 30 min along with the control. Substrate specificity was determined in 100 mM acetate buffer (pH 5.5) containing various

Table 1
Experimental design for CCD with actual and coded values of each factor.

Run	Mannitol (A, w/w%)	Ammonium sulfate (B, w/w%)	K_2HPO_4/Na_2HPO_4 (C, w/w%)	Phytase activity (U/gds)	
				Experimental[a]	Predicted
1	2.25 (1)	3.0 (1)	0.4 (1)	148.73 ± 0.512	149.25
2	0.75 (−1)	1.0 (−1)	0.4 (1)	124.20 ± 0.721	123.07
3	1.50 (0)	2.0 (0)	0.3 (0)	144.51 ± 0.568	144.38
4	2.25 (1)	1.0 (−1)	0.4 (1)	119.81 ± 0.698	121.22
5	1.50 (0)	2.0 (0)	0.3 (0)	144.53 ± 0.546	144.38
6	1.50 (0)	2.0 (0)	0.3 (0)	144.51 ± 0.625	144.38
7	2.7615 (+α)	2.0 (0)	0.3 (0)	137.48 ± 0.642	136.81
8	1.50 (0)	3.682 (+α)	0.3 (0)	137.95 ± 0.465	137.73
9	1.50 (0)	2.0 (0)	0.1318 (−α)	124.20 ± 0.625	125.81
10	0.75 (−1)	3.0 (1)	0.2 (−1)	133.21 ± 0.547	131.98
11	0.2385 (−α)	2.0 (0)	0.3 (0)	134.20 ± 0.598	135.05
12	1.50 (0)	2.0 (0)	0.3 (0)	141.68 ± 0.477	144.38
13	2.25 (1)	1.0 (−1)	0.2 (−1)	116.69 ± 0.511	115.68
14	1.50 (0)	2.0 (0)	0.3 (0)	141.98 ± 0.479	144.38
15	1.50 (0)	0.318 (−α)	0.3 (0)	106.79 ± 0.501	107.59
16	0.75 (−1)	3.0 (1)	0.4 (1)	138.12 ± 0.445	138.73
17	0.75 (−1)	1.0 (−1)	0.2 (−1)	125.06 ± 0.502	124.13
18	2.25 (1)	3.0 (1)	0.2 (−1)	131.56 ± 0.564	131.69
19	1.50 (0)	2.0 (0)	0.4682 (+α)	137.16 ± 0.498	136.13
20	1.50 (0)	2.0 (0)	0.3 (0)	148.77 ± 0.522	144.38

[a] Data are represented as mean ± SD $n = 3$.

phosphorylated substrates (2 mM): phytic acid, pNPP, NADP, sodium-1-naphthyl phosphate (SNP), fructose-1, 6-biphosphate (FBP), ribose-5-phosphate (R-5-P), Glucose-1-phosphate (G-1-P), phosphoenol pyruvic acid (PEP), AMP, ADP and ATP. To check the stability of phytase under gastric conditions, simulated gastric fluid assay was performed at pH 1.5, 5.5 and 7.5 according to Garrett et al. (2004). The kinetic constants K_m and V_{max} were determined with sodium phytate as substrate using Lineweaver–Burk plot. To study the inhibition kinetics the phytase was incubated in presence of Fluoride, phosphate and vanadate at the concentration of 5×10^{-5} mM.

2.8. Phytate degradation and HPLC analysis method

Dynamics of phytate degradation was studied to determine the number of phosphomonoester bond breakage using standard phytase activity assay. It was further validated by reversed-phase high performance liquid chromatography (RP-HPLC) using Agilent 1200 series (Hewlett Packard, Palo Alto, CA, USA) liquid chromatograph equipped with a variable wavelength detector (VWD 1200) and Agilent XDB eclipse C_{18} (250×4.6 mm) column. Phytate (IP6) and inositol tris-phosphate (IP3) (Sigma chemicals, St. Louis, MO) dissolved in the 100 mM sodium acetate buffer (pH 5.1) were used to calibrate the standard curve. Phytate hydrolysis experiment was carried out according to Graf and Dintzis (1982b) with some modifications. Purified phytase (0.47 U) was incubated with 0.29 mM phytate prepared in 100 mM sodium acetate buffer (pH 5.1), at 39 °C. Reaction was stopped by denaturing the phytase at 100 °C for 10 min. Before injection, sample was filtered through 0.2 μm syringe filter followed by addition of an equal amount 0.05 M HCl. A 100 mM sodium acetate solution (pH 5.1) was used as mobile phase with a flow rate of 1.0 ml/min.

3. Results and discussion

3.1. Agricultural residues as substrate for phytase production

Linseed oil cake (~1.0 mm) and wheat bran (1:1), showed the highest phytase yield (17.68 ± 0.23 U/gds) at 96 h post fermentation (Table 2). Phytase production on WB was relatively low, whereas other substrates were found to be less effective for phytase production. Maximum production in this combination may be attributed to high phytic acid content in LOC (3.95%) and WB (2.71%). The phytic acid content in substrates was determined by the method according to Garcia-Villanova et al. (1982).

Table 2
Evaluation of different agro-industrial residues as substrate for phytase production under SSF.

Agro-industrial residues	Phytase activity[a] (U/gds)
WB	15.32 ± 0.41
RH	5.12 ± 0.33
MOC	7.69 ± 0.78
LOC	10.54 ± 0.51
SOC	0.12 ± 0.24
ROC	6.63 ± 0.37
WB + MOC (1:1)	8.79 ± 0.65
WB + ROC (1:1)	10.20 ± 0.14
WB + SOC (1:1)	9.05 ± 0.78
WB + LOC (1:1)	17.68 ± 0.23
WB + RH (1:1)	11.22 ± 0.39
WB + MOC + ROC (1.67:1.67:1.67)	9.12 ± 0.54
WB + SOC + LOC (1.67:1.67:1.67)	13.11 ± 0.41

[a] Phytase activity was estimated in the SSF crude extract after 96 h of fermentation of unoptimized medium at 35 °C. Data are represented as mean ± SD n = 3.

3.2. Selection of influential culture parameters for phytase production

The application of Plackett–Burman design for screening critical culture parameters is a widely accepted technique. PBD was used for investigating the relative importance of eight independent factors for phytase production. The corresponding effects of these factors on the response (phytase activity) are given in Table 3. From the regression analysis, it was evident that A (mineral salt solution), B (mannitol) and L (K_2HPO_4/Na_2HPO_4) enhanced the phytase production at their low level whereas, high level of D (initial pH), E (fermentation time), G (ammonium sulfate), H (incubation temperature) and K (initial moisture content) supported high phytase yield. Based on analysis of total sum of squares and percent contribution, the most significant factors influencing phytase production were found to be B (mannitol), G (ammonium sulfate) and L (K_2HPO_4/Na_2HPO_4), respectively (Table 3). The regression model gave a model F-value of 177.34 with a corresponding model p-value (>F) of 0.0006, which shows the model to be highly significant. Also, the coefficient of determination (R^2) indicates that the model could explain 99.79% of the total variations in the response. A very low value of coefficient of variance (CV, 1.61%) further confirms the reliability of the model. The three medium components selected by PBD, were further optimized by the path of steepest ascent (descent) method. Plackett–Burman design has been widely used by many researchers for identifying the influential factors for improving phytase production (Bogar et al., 2003a,b; Singh and Satyanarayana, 2008).

3.3. Optimization by the path of steepest ascent (descent) method

The path of steepest ascent (descent) method was performed to approach rapidly in the close proximity to the optimum response by tracing direction of changing factors on the basis of PBD results. The highest response (139.22 U/gds) was observed at the concentration of mannitol (B), ammonium sulfate (G) and K_2HPO_4/Na_2HPO_4 (L) of 1.5% (w/w), 2.0% (w/w) and 0.3% (w/w), respectively (Supplementary Table S4). The corresponding levels were further considered as the '0' level values for factors in the design matrix of CCD.

3.4. Interaction analysis using CCD of RSM

To fully explore the sub-regions of the response surface in the neighborhood of the optimum, CCD with five coded levels was used for the three significant variables screened by PBD. The design matrix showing different combinations of mannitol, ammonium sulfate and K_2HPO_4/Na_2HPO_4 along with their corresponding experimental and predicted responses is presented in Table 1. The experimental results were analyzed using analysis of variance (ANOVA) which shows that the regression was statistically significant ($P < 0.0001$) at 95% of confidence level. The results for ANOVA analysis are summarized in Table 4. Application of multiple regression analysis on the experimental data resulted in the following quadratic model equation (2) explicitly explaining the phytase production:

$$Y = 144.38 + 0.52A + 8.97B + 3.07C + 2.04AB + 2.70AC + 3.00BC - 2.99A^2 - 7.69B^2 - 4.75C^2 \quad (2)$$

Where, Y represents phytase activity (U/gds), and A, B and C are the coded factors of mannitol (w/w%), ammonium sulfate (w/w%) and K_2HPO_4/Na_2HPO_4 (w/w%), respectively. In this case, linear terms (B and C), all the interaction terms (AB, AC and BC) and quadratic terms (A^2, B^2 and C^2) were found to be the most significant for phytase production. The statistical significance of the model equation was supported by the model high F-value of 59.46. Again, the quality

Table 3
Results of PBD analysis.

Factors (code, unit)	Low level (−1)	High level (+1)	SS[a]	Effect	Coef.[b]	Cont.[c] (%)	F-value	p-Value
Mineral salt solution (A, v/w%)	20	60	492.4	−12.81	−6.41	9.98	141.9	0.0013
Mannitol (B, w/w%)	1.0	5.0	2214.9	−27.17	−13.59	44.92	638.6	0.0001
Initial pH (D)	5.6	7.6	47.08	3.96	1.98	0.95	13.58	0.0346
Fermentation time (E, h)	24	72	66.41	4.70	2.35	1.35	19.15	0.0221
Ammonium sulfate (G, w/w%)	0.5	2.0	1224.7	20.20	10.10	24.84	353.1	0.0003
Incubation temperature (H, °C)	25	30	77.88	5.09	2.55	1.58	22.46	0.0178
Initial moisture content (K, v/w%)	30	40	67.55	4.75	2.37	1.37	19.48	0.0216
K_2HPO_4/Na_2HPO_4 (L, w/w%)	0.2	1.0	728.99	−15.59	−7.79	14.79	210.2	0.0007

R^2 = 99.79%, R^2 (adjusted) = 99.23%, R^2 (predicted) = 96.62%, Coefficient of variation (CV) = 1.61%.
[a] Sum of squares.
[b] Coefficient estimate.
[c] Contribution.

Table 4
ANOVA analysis of regression model.

Source of variation	SS[a]	DF[b]	MS[c]	Coef.[d]	F-value	p-Value
Model	2418.53	9	268.73	144.38	59.46	<0.0001
A	3.09	1	3.09	0.52	0.68	0.4276
B	1039.92	1	1039.92	8.97	230.11	<0.0001
C	121.90	1	121.90	3.07	26.97	0.0004
AB	25.26	1	25.26	2.04	5.59	0.0397
AC	44.38	1	44.38	2.70	9.82	0.0106
BC	65.93	1	65.93	3.00	14.59	0.0034
A^2	125.23	1	125.23	−2.99	27.71	0.0004
B^2	839.23	1	839.23	−7.69	185.70	<0.0001
C^2	319.91	1	319.91	−4.75	70.79	<0.0001
Residual error	45.19	10	4.52			
Lack of fit	12.85	5	2.57		0.40	0.8332
Pure error	32.35	5	6.47			
Correlation (Total)	2463.73	19				

R^2 = 98.17%, R^2 (adjusted) = 96.51%, R^2 (predicted) = 94.04%, Coefficient of variation (CV) = 1.59%.
[a] Sum of squares.
[b] Degree of freedom.
[c] Mean square.
[d] Coefficient estimate.

of fit of the regression model was justified by high value of coefficient of determination ($R^2 = 0.9817$) which indicates an excellent correlation between the independent factors. At the same time, the predicted R^2 (correlation coefficient) value of 0.9404 was found in concordance with the adjusted R^2 value of 0.9651, suggesting a strong agreement between the experimental and predicted values of phytase production. The coefficient of variation (CV) indicates the degree of precision with which the treatments are evaluated, therefore, a very low value of CV (1.59%) demonstrates that the performed experiments were highly reliable and was performed with a better precision. In order to gain the better understanding of the effects of the significant factors on phytase production, the predicted model was represented as three dimensional response surface graphs and is shown in Fig. 1a–c. The optimum concentrations for the variables were calculated from the model equation (2) and were found to be (w/w): 2.05% mannitol, 2.84% ammonium sulfate, and 0.38% K_2HPO_4/Na_2HPO_4 (1:1). In present study, enhancing effect of mannitol for maximum phytase production seems counterintuitive as most literature showed glucose as the preferred carbon source for phytase production (Bogar et al., 2003a; Ebune et al., 1995). Earlier, mannitol has been reported to have a role in stress tolerance and spore dispersal (Ruijter et al., 2003). Because linseed oil cake contains some endotoxins, therefore, presence of mannitol might be having some protective role that resulted in enhanced phytase production. On the other hand, ammonium sulfate, an inorganic nitrogen source, supported maximum phytase production. This corroborates the results of Bogar et al. (2003a). Other interesting finding observed in the present study was the maximum phytase production, when combination of K_2HPO_4 and Na_2HPO_4 (1:1; (w/w)) was used as a source of phosphorus. It might be due to the fact that K_2HPO_4 when combined with Na_2HPO_4 acts as a buffer for pH of the media. Optimized concentrations of various carbon, nitrogen and phosphorus sources for phytase production has been previously reported by some researchers (Bogar et al., 2003b; Gunashree and Venkateswaran, 2008).

3.5. Validation of experimental model

The results from validation experiments showed a strong agreement between the maximum predicted response and the experimental response of 149.25 and 148.77 U/gds, respectively, thus supporting the high adequacy of the model. A marked enhancement in phytase activity was indicated by the corresponding increase in biomass as a function of glucosamine content (Fig. 2). Moreover, the statistical optimization for phytase production resulted in an overall 8.41-fold increase in phytase yield with a reduction in fermentation time from 96 to 72 h.

From commercialization perspective, the phytase titer from *R. oryzae* seems to be low (productivity (Q_p) = 4750 IU/kg/day) (Supplementary Table S5), however, the widely studied strains, mostly from *Aspergillus* sp. due to high phytase producing capacity, were found to be associated with respiratory allergy (Adhikari et al., 2004). Moreover, for commercialization of feed enzyme, major concerns in general are the constraints of thermal stability, a good enzyme activity at physiological temperature, ability to hydrolyze the phytate phosphorus in digestive tract over wide range of pH and temperature and most importantly, free of health risks. Phytases are not yet employed in food applications, however, there have been many studies focused on improving food quality for people in developing countries. In this context, *Rhizopus* phytase could find potential in food processing applications since it is thermostable enough to withstand pasteurization and active during long-term storage of foods at room temperature. Beside this, the fermented food materials enriched with protein could be effectively used as such in animal feed, since it is a safe feed grade microorganism.

3.6. Production of phytase in flasks and trays

Phytase production was sustainable in Erlenmeyer flasks of varied volumes and in trays. Increase in amount of substrate and hence the bed height did not result in significant decrease in the phytase yield (Supplementary Table S6). However, Singh and Satyanarayana (2008) have reported a significant reduction in phytase production at below or above 1.5 cm of substrate bed height.

Further enhancement in phytase yield was attained due to a novel strain improvement procedure, in which *R. oryzae* was exposed to subsequent heat and cold stress to study the response of the adaptive cells in terms of phytase production. Heat stress acts

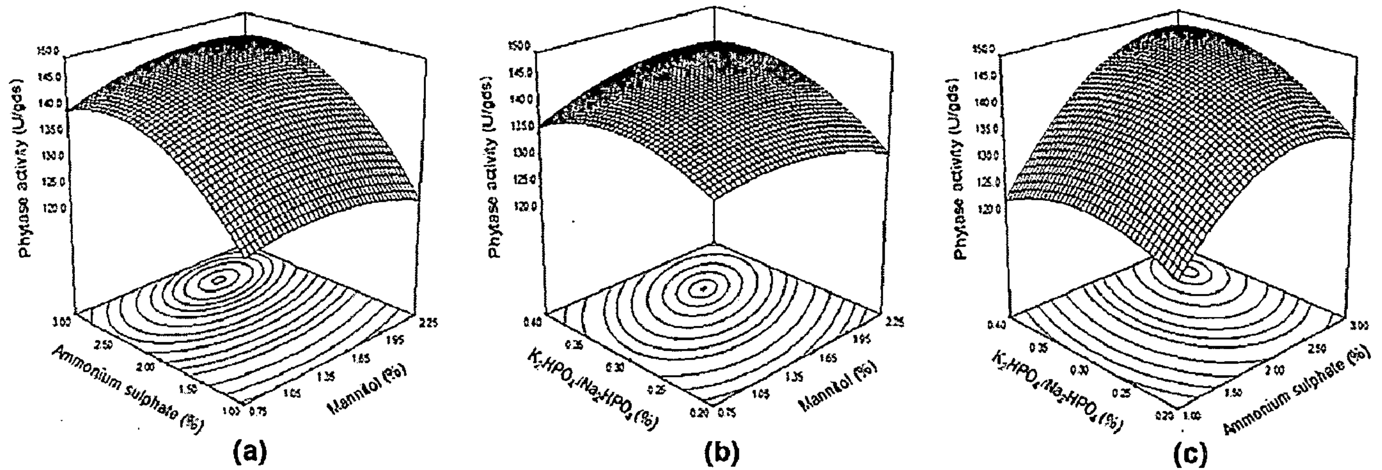

Fig. 1. 3D response surface plot showing interaction effects between (a) mannitol and ammonium sulfate, (b) mannitol and K_2HPO_4/Na_2HPO_4, (c) ammonium sulfate and K_2HPO_4/Na_2HPO_4.

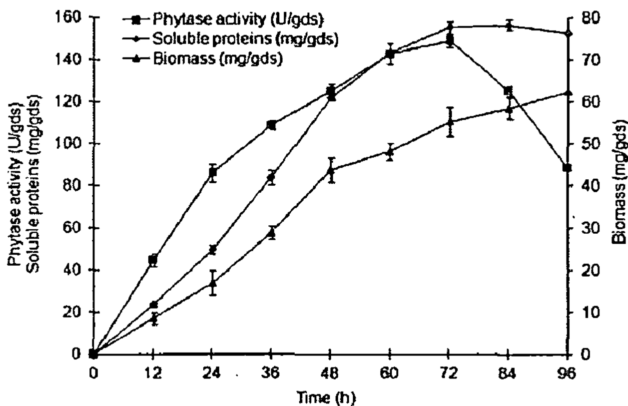

Fig. 2. Production profile of phytase by wild *R. oryzae* in optimized medium. Data are represented as means ± SD $n = 3$.

as an oxidant and a mutagen causing increase in thermal viability and mutational frequency of the spore DNA (Johansson et al., 2011). On the other hand shifting to cold was done to prevent the spores from being damaged. The increase in phytase titer (38% and 59%, respectively) was observed up to second generation with standard deviations (1.01 and 0.89, respectively) (Supplementary Table S7). This process was continued up to six generations with no further increase in phytase titer, rather maintained at a constant level. However, the third generation onwards, it showed higher standard deviations (4.54, 4.01, 4.39 and 4.37, respectively) as compared to the first two generations and these deviations remained almost constant.

Morphological changes that had been observed include short mycelium, cotton-like appearance and more and more whitish appearance compared to previous generation cultures. Another important morphological variation was that of lesser sporulation tendency in mutant culture. However, the reduction in sporulation tendency does not affect the phytase yield. The yield was found to be proportional to the rate of sporulation for unstressed strain and not in stressed strain.

Semiquantitative analysis further validates a remarkable enhancement in phytase production by 1.46- and 2.04-fold after first and second generation of strain improvement process, respectively (Supplementary Fig. S1). Increase in phytase titer owing to strain improvement has previously been reported by Chelius and Wodzinski (1994) and Shah et al. (2009).

The efficacy of phytase from stressed and unstressed strains was investigated by analyzing the effect of fermentation of various feed ingredients in terms of minerals extractability. The increase in ash content (1.14 to 1.33-fold) is validating the extent of extraction of different minerals (Table 5). In other words, more the ash content is, the more is the mineral availability.

3.7. Purification and characterization of phytase

The phytase obtained from *R. oryzae* was purified using fractional ammonium sulfate precipitation and subsequent ion-exchange and gel filtration chromatography resulting into 26% phytase recovery with purification of 20.7-fold and specific activity of 141.83 U/mg of protein. The native molecular weight of the purified phytase was found to be ~34 kDa by gel filtration chromatography (Supplementary Fig. S2) and SDS-PAGE analysis (Supplementary Fig. S3a). Zymogram staining also confirmed the molecular mass of phytase from *Rhizopus* to be ~34 kDa (Supplementary Fig. S3b). Purification to homogeneity and monomeric nature of this phytase was further confirmed by nondenaturing PAGE analysis (Supplementary Fig. S3c). The molecular size of the phytase was found to be somewhat smaller than the molecular weight of previously reported phytases (38–200 kDa) (Wodzinski and Ullah, 1996). The phytase manifested a K_m and V_{max} of 2.42×10^{-4} mM and 6.46×10^{-3} mM s^{-1}, respectively, whereas the catalytic efficiency (k_{cat}/K_m) of the phytase was estimated to be 2.38×10^{6} $M^{-1} s^{-1}$. K_m value of isolated phytase was significantly lower than the reported K_m values (10 μM–813 mM) of fungal phytases. The value was 20.66 and 111.57 times lower than *Aspergillus ficuum* NRRL 3135 (phy A) and *Aspergillus niger* (Natuphos) phytases, respectively. The phytase demonstrated an uncompetitive inhibition in presence of inhibitors in the order of fluoride > phosphate > vanadate, with their respective K_i values of 12.56×10^{-6}, 3.86×10^{-6} and 64.52×10^{-6} mM.

Phytase displayed dual pH optima at 1.5 and 5.5, with higher activity at pH 5.5 (Supplementary Fig. S4a). Additionally, phytase was found to retain 100% activity at low pH value of 1.0 and 2.0 and exhibited more than 75% of initial activity over a wide range of pH 2.5–9.5 (Supplementary Table S8). Generally, fungal phytases act efficiently in the range of pH 2–5 (Garrett et al., 2004). However, from a physiologically relevant standpoint and from an applied perspective, a phytase should exhibit a significant activity

Table 5
Effect of fermentation with wild and mutant strain of different feed ingredients on ash content and mineral availability.

Ingredients	Ash (%)[a]	(μg/g DW)[b]		
		Fe	Zn	Ca
Semolina (UF)	0.82(±0.24)	47.23(±0.79)	20.63(±2.00)	33.25(±2.23)
(F) Wild	1.49(±0.08)	65.75(±1.01)	32.06(±2.01)	26.03(±1.47)
Mutant	1.98(±0.12)	68.19(±0.83)	36.77(±3.18)	27.99(±1.30)
Cornflakes (UF)	1.36(±0.13)	17.02(±3.40)	8.94(±2.25)	6.94(±0.25)
(F) Wild	1.78(±0.95)	12.36(±2.45)	11.51(±1.50)	21.13(±2.50)
Mutant	2.10(±0.55)	11.30(±0.70)	14.07(±1.20)	26.54(±1.97)
Whole wheat flour (UF)	1.64(±0.57)	46.73(±2.29)	31.96(±1.01)	31.28(±2.75)
(F) Wild	2.21(±0.06)	51.75(±2.29)	37.36(±2.13)	36.91(±3.03)
Mutant	2.95(±0.11)	55.71(±4.67)	42.47(±1.49)	37.04(±2.01)
Broken wheat (UF)	1.95(±0.12)	42.30(±1.74)	26.79(±3.83)	32.43(±2.92)
(F) Wild	2.59(±0.20)	45.27(±0.84)	23.95(±1.56)	38.87(±0.63)
Mutant	3.01(±0.98)	47.90(±1.94)	22.15(±2.50)	41.05(±0.99)
Gram flour (UF)	2.38(±0.26)	21.06(±2.99)	38.72(±0.76)	67.75(±3.33)
(F) Wild	3.04(±0.96)	47.75(±1.79)	40.51(±3.50)	73.90(±2.06)
Mutant	3.46(±0.98)	48.78(±1.77)	39.62(±0.59)	80.69(±3.47)
Beaten rice (UF)	1.78(±0.47)	8.51(±1.50)	6.45(±0.72)	22.50(±0.51)
(F) Wild	2.45(±0.27)	20.41(±0.86)	11.81(±3.23)	27.81(±0.86)
Mutant	2.98(±0.32)	25.96(±0.45)	16.71(±3.63)	30.96(±1.00)
Maida flour (UF)	1.46(±0.07)	18.57(±1.64)	9.74(±1.21)	37.19(±1.77)
(F) Wild	1.99(±0.38)	17.39(±0.59)	11.24(±2.68)	43.48(±3.50)
Mutant	2.58(±0.34)	18.35(±1.42)	12.15(±2.13)	45.76(±1.74)

UF = unfermented.
F = fermented.
[a] Ash content (%) are shown as mean (±SD).
[b] Data are represented as mean (±SD) and expressed in microgram per gram dry weight of ingredients (μg/g DW).

over a wide range of pH 1.5–7.5 to facilitate phytate degradation in the digestive tract. The efficacy of *R. oryzae* phytase is evident from the fact that it retains activity over broader range of pH 1.0–9.5. Additionally, its acidophilic characteristic to survive the acidic environment of the digestive tract determines its utility in feed applications.

The phytase was found to be optimally active at 45 °C (Supplementary Fig. S4b), which falls well within the range of most of the phytases from yeasts, fungi and bacteria (45–60 °C) (Wodzinski and Ullah, 1996). The optimum temperature value (45 °C) being in lower side is an added advantage, since, phytases having high temperature optima value are generally not able to retain full activity in the gastrointestinal tract of animals.

Thermal stability study suggest that the phytase was found to retain 100% activity when incubated at 50 °C for 30 min and maintained >90% of its initial activity at 60, 70 and 80 °C, respectively (Supplementary Fig. S5). The $t_{1/2}^{70°C}$ for the phytase was estimated to be 8.25 h which shows strong thermal stability of the phytase. To survive the feed pelleting temperature and to avoid the additional expense of applying the phytase in pelletized form, the most economical approach would be to add a thermostable phytase directly into the feed. The phytase was found to be more stable than the other reported phytases (Pasamontes et al., 1997; Singh and Satyanarayana, 2009). The study carried out for thermodynamic characterization also supported the high thermal stability of this phytase. The phytase revealed an activation enthalpy (E_a) and entropy values of 23.92 kJ/mol and −264.89 J/(mol K), respectively. The E_a value was found to be significantly lower than that of the other phytases (Ragon et al., 2008; Singh and Satyanarayana, 2009).

The study involving the effect of cations and inhibitors showed stimulatory effect on phytase activity, however, complete inhibition was observed in presence of Fe^{2+}, Ni^{2+} and Cu^{2+} (Supplementary Table S9). In contrary to *A. ficuum* phytase (Ullah and Gibson, 1987), the *Rhizopus* phytase showed stimulatory effect in presence of Zn^{2+}. The study also suggested the insensitivity of phytase to heavy metal ions (Ba^{2+} and Ag^{+}; 5 mM) as indicated by a 5.5- and 2.5-fold increase in phytase activity, respectively. This unique feature of *Rhizopus* phytase can be practiced as an advantageous and an efficient mean to combat phosphate pollution in area where soil is polluted with substantial amount of unassimilated phytate and other contaminations. EDTA and tartarate resulted in an increased phytase activity except iodoacetamide, whereas a complete inhibition was observed for oxalate at 5 mM (Supplementary Table S9). The result was different from earlier reported by Kerovuo et al. (1998) where, EDTA was reported to inhibit the phytase activity at 1 mM.

R. oryzae phytase was found to liberate phosphate from a range of phosphorylated compounds. More than 2.4-fold higher activity on ATP was observed as compared to that on sodium phytate (Supplementary Table S10). The result was in contradiction to previously studied phytase from *A. niger* ATCC 9142, where it showed high activity for sodium phytate but displayed significantly lesser activity for ATP, ADP, AMP and G-1-P (Casey and Walsh, 2003).

A phytase destined for feed applications should not lose its activity in milieu of gastric conditions. Phytase from *Rhizopus* was found to exhibit high stability and resistance to pepsin over broad range of pH, retaining >93% and >97% of original activity at pH 1.5 and 5.5 (Supplementary Table S11). Based on this study, the *Rhizopus* phytase was found to be more efficient than other phytases (Huang et al., 2008).

3.8. Time course hydrolysis of sodium phytate

The study was carried out to estimate the release of inorganic phosphate per mole of phytic acid. The ratio (inorganic phosphate to phytic acid) of three indicates that the maximum number of phosphate released by this phytase was three out of six phosphomonoester bonds, with no further increase in bond cleavage when increasing the reaction time (Supplementary Fig. S6). The study showed a rapid hydrolyzing activity of this phytase from IP6 to inositol pentakis-phosphate (IP5), however, a slow conversion of IP6 to inositol tetra-phosphate (IP4) and finally to IP3 was observed which suggested that the lower derivatives of inositol were less suitable substrates for the phytase than the phytate or IP6.

3.9. HPLC characterization studies

The measurement of phytate hydrolysis is a crucial step that considerably influences the phytase characteristics. Above result suggesting IP3 as the end product of phytate degradation was further validated by an improved HPLC analysis method. The chromatogram of standard sodium phytate was found to be linearly proportional to the concentrations throughout, with R^2 value and retention time (R_t) of 0.988 and 1.23 ± 0.01 min, respectively. In a previous study carried out by Graf and Dintzis (1982a), the retention time of phytate was found to be 1.40 ± 0.03 min. More than one peak with different R_t (1.26 ± 0.02 (major), 1.39 ± 0.04, 1.57 ± 0.02 and 1.88 ± 0.03 min) was observed in each chromatogram profile of standard IP3 (0.5–2.0 mg/ml). It might be due to the presence of different structural isomers of IP3 in the standard, also verified by the pattern of these small peaks observed in continuity with R_t of 1.26 ± 0.02 and 1.39 ± 0.04 min and R^2 value of 0.990 and 0.999, respectively.

Chromatogram profile for untreated and phytase treated phytic acid is illustrated in Supplementary Fig. S7a and b. After 150 min of incubation period, no peak corresponding to IP6 was found in the chromatogram which clearly showed the ability of *Rhizopus* phytase to completely hydrolyze the intact phytate. In an earlier study by Casey and Walsh (2004), phytase from *R. oligosporus* was shown to completely degrade the phytate; however, presence of IP5 as one of the hydrolyzed products of phytic acid was also found along with IP3. From an applied perspective, presence of IP5 is not desirable as it possesses inhibitory effect on iron absorption in humans; whereas, IP3 and IP4 in isolated form do not have negative effect (Sandberg et al., 1999).

Method previously reported for the determination of inositol phosphates and its lower derivatives have some limitations in terms of their inability to separate structural isomers, time consuming sample preparation, need for pre- or post-column derivatization, and long analysis time. Additionally, in previously reported methods refractive index of inositol phosphates (including various degradation products of phytic acid) solution was measured using RI detector. In the present study, the feasibility of determining phytic acid and IP3 by RP-HPLC equipped with variable wavelength detector (VWD) was successfully achieved at 246 nm. In this method, the sample preparation was much simpler and retention time was reduced. The results show that VWD system with UV spectrum specifically detects the phytate and its lower derivatives with a good resolution.

4. Conclusions

Present study has demonstrated an inexpensive LOC and WB as potential substrate for phytase production. A marked enhancement in phytase production was achieved due to statistical optimization of culture parameters. An effective strain improvement procedure could aid in an economical phytase production and thus increase in its industrial utilization. Phytase characterization study revealed some exclusive features of *Rhizopus* phytase (stability over broad pH and temperature, insensitivity to heavy metal ions, etc.) that render it as potential candidate for commercial purpose. Furthermore, the results obtained from improved method of HPLC analysis establish it as an accurate and reproducible analytical method.

Acknowledgements

The authors greatly appreciate the financial assistance in the form of junior research fellowship by University Grants Commission, New Delhi, India.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.08.075.

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