PROCESS OPTIMIZATION FOR THE PRODUCTION OF MICROBIAL PHYTASE AND ITS APPLICATION IN FEED INDUSTRY

Thesis Submitted
in Partial Fulfillment of the Requirements for the
Degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

PRIYA
(2K19/PHDBT/05)

Under the Supervision of

Prof. Jai Gopal Sharma
Department of Biotechnology
Delhi Technological University

Prof. Bhoopander Giri
Department of Botany
Swami Shraddhanand College
University of Delhi

To the
Department of Biotechnology

DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road, Delhi-110042, India

OCTOBER, 2024

DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road, Delhi-42

CANDIDATE'S DECLARATION

I, Priya hereby certify that the work which is being presented in the thesis entitled **"Process Optimization for the Production of Microbial Phytase and its Application in Feed Industry"** in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy, submitted in the Department of Biotechnology, Delhi Technological University is an authentic record of my own work carried out during the period from 19th July 2019 to 1st July 2024 under the supervision of Prof. Jai Gopal Sharma, Department of Biotechnology, Delhi Technological University, Delhi and Co Supervision of Prof. Bhoopander Giri, Department of Botany, Swami Shraddhanand College, University of Delhi, Delhi, India.

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.

Priya

Date: 16th Oct 2024

Candidate's Signature

Priya

2K19/PHDBT/05

This is to certify that the student has incorporated all the corrections suggested by the examiners in the thesis and statement made by the candidate is correct to the best of our knowledge.

Jai Gopal

Signature of Supervisor(s)

Signature of External Examiner

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road, Delhi-42

CERTIFICATE BY THE SUPERVISOR(s)

Certified that **Priya** (2K19/PHDBT/05) has carried out their search work presented in this thesis entitled **"Process Optimization for the Production of Microbial Phytase and its Application in Feed Industry"** for the award of **Doctor of Philosophy** from Department of Biotechnology, Delhi Technological University, Delhi, under our supervision. The thesis embodies results of original work, and studies are carried out by the student herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution.

Date:

Prof. Jai Gopal Sharma
(Professor)
Department of Biotechnology
Delhi Technological University
Delhi-110042

Prof. Bhoopander Giri
(Professor)
Department of Botany
Swami Shraddhanand College
University of Delhi, Delhi-110036

21.10.24

Prof. Yasha Hasija
HoD & DRC Chairperson
Department of Biotechnology
Delhi Technological University
Delhi-110042

Process Optimization for the Production of Microbial Phytase and its Application in Feed Industry

Priya

ABSTRACT

Phytase breaks the phytic acid molecule and liberates inorganic phosphate and myo-inositol esters. It also releases other biomolecules attached to the phytate and helps to overcome the anti-nutritive effect. Due to the lack of certain features of an ideal phytase, its application on a commercial level is limited. As a feed additive, the demand for phytase is continuously increasing in the market of feed enzymes. Phytase increases the bioavailability of nutrients and enhances the nutritional value of feed. It decreases the cost of livestock feed and load on phosphate reserves. Further, phytase is also involved in reducing the level of phosphorus, and eutrophication in water bodies, thus mitigating environmental issues. In the current investigation, among the screening of various microbes, *Penicillium oxalicum* PBG30 was selected as the most potent source of phytase. This fungus was isolated from a rotten orange sample. For the cost-effective production of phytase, the solid-state fermentation method was applied. In the SSF method, wheat bran was used as a substrate due to its high nutritional value and cost effectiveness. For the enhancement of the phytase level, optimization was performed using traditional and statistical approaches. One variable at a time (OVAT), a traditional method, is used to study various parameters like the amount of wheat bran, moistening media, substrate and moisture ratio, incubation temperature, incubation days, and pH. The production of phytase applying OVAT method reached 200.407 ± 6.01 U/g DMR with 10 g wheat bran, mixed with moistening media (0.5% Urea, 0.1% $MgSO_4.7H_2O$, 0.1% KCl and 0.1% $FeSO_4.7H_2O$) of pH 7.0 in a ratio of 1:2, having incubation of 5 days at 30°C. The OVAT approach leads to a 2.4-fold increase in the phytase production. However, the main drawback of OVAT is that it examines factors one by one, and not the interaction among the factors, making the process costly and time-consuming, hence, statistical optimization was conducted. The statistical optimization includes Plackett-Burman design (PBD) and Response Surface Methodology (RSM). PBD screened the critical variables essential for the production, while RSM analyzed the interaction among the variables and provided the exact amount of that variable required for the production. The four significant variables, viz., magnesium sulphate, pH, incubation days and Tween-80 were identified by PBD during the study that

was further examined by RSM. Magnesium sulphate (0.75%), pH (7.0), incubation days (5), and Tween-80 (3.5%) were the parameters that influenced the phytase production majorly and enhanced the phytase production by 4.4-fold. The highest production (373.32 ± 3.28 U/g DMR) was obtained when 5 g wheat bran supplied with moistening media (0.5% ammonium sulphate, 0.01% $FeSO_4$, 3.5% Tween 80 and 0.75% $MgSO_4$) of pH 7.0 in a ratio of 1:2 having incubation of 5 days at 30°C. For sustainable production, phytase is produced in trays on a large scale. The amount of phytase was recorded as the highest in the trays, resulting in a 5.6-fold increase in its production level. After statistical optimization, the production level of phytase enhanced up to 5.6-fold. Besides phytase, *P. oxalicum* PBG30 also produces cellulase (51.06 U/g DMR), amylase (86.20 U/g DMR), xylanase (18.05 U/g DMR) and lipase (7.05 U/g DMR). The phytase was partially purified by ammonium sulphate precipitation and dialysis methods and exhibited a 4.9-fold purification with a 55.31% yield. The biochemical characterization was done to determine the properties of phytase isolated from *P. oxalicum* PBG30. The phytase was optimally active at 70°C and pH 3.0. *P. oxalicum* PBG30 phytase showed broad substrate specificity with various substrates which was found maximum with sodium phytate. Phytase is positively affected by organic solvents such as ethanol, methanol, isopropanol, butanol, DMSO, and acetone. Tweens and Triton-X-100 considerably increased the phytase activity, while SDS, EDTA, ß-ME, DTT, and sodium molybdate decreased the activity. The impact of different metal ions was also studied and varied with the microbial species. *P. oxalicum* PBG30 phytase is found thermostable with a $t_{1/2}$ value of 1 h, stable in pH 3.0 and pH 5.0 and protease resistant. The K_m and V_{max} values were measured as 4.42 mM and 909.1 U/ml, respectively. Phytase showed a long storage life at 4°C and -20°C. *P. oxalicum* PBG30 phytase was immobilized with the calcium alginate method and exhibited reusability up to 5 cycles. Phytase efficiently degrades phytic acid and secretes inorganic phosphate and other nutritional by-products. The phytase was supplied in fish feed that shows maximum liberation of inorganic phosphate (33.986 mg/g of feed), reducing sugars (134.4 mg/g of feed), and soluble protein (115.52 mg/g of feed) with the addition of 200 U of phytase in 0.5 g of feed mixed with 20 ml buffer and incubated at 37°C for 6 h. In another experiment, phytase was added into experimentally prepared fish feed with different doses, and the phytic acid hydrolysis was observed. The maximum reduction of phytic acid was 46.6% occurred with a 1500 FTU/kg phytase dose. Moreover, the effect of phytase was studied in the hydrolysis of insoluble phytate-metal and phytate-protein and found efficient in releasing inorganic phosphate with time. In addition, vanadium inhibits

the phytase activity and enhances the peroxidase activity, thus allowing the phytase to perform as haloperoxidase. The present study depicts the cost-effective production of phytase and its utilization as a feed additive in the feed industry.

ACKNOWLEDGEMENTS

In the journey of PhD research work, every researcher faced certain problems and challenges that would be overcome by hard work, sincerity, and an obstinate attitude. A lot of individuals provided guidance and support throughout the duration to accomplish this research work. First and foremost, I express my gratitude to God for granting me the opportunity to do this work and bestowing their blessings upon me. During my tenure, he provides me strength, peace of mind, patience, and calmness to deal with every challenge.

Behind every successful student, a mentor plays the most important role. I would like to thank my supervisor, Prof. Jai Gopal Sharma, and co-supervisor, Prof. Bhoopander Giri for their continuous support, guidance, and encouragement throughout the execution of this study. Your advice on both research as well as on my career was inevitable and invaluable.

I would like to give special thanks to Prof. Bijender Singh, Department of Biotechnology, Central University of Haryana, Haryana for his proficient guidance, appraisal, cooperation in providing facilities to carry out experimental work, and comments at all steps of my research work.

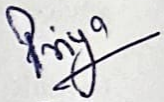
My sincere thanks to the University Grant Commission (UGC) New Delhi for providing me financial assistance and DTU for providing facilities to complete my research work. A special mention to Ex-VC (Prof. Yogesh Singh), former VC (Prof. Jai Prakash Saini), and current VC (Prof. Prateek Sharma) for allowing me to work in this elite University. I would also like to thank Prof. Yasha Hasija (Head & DRC Chairperson), Department of Biotechnology, DTU, Prof. Pravir Kumar (Former HoD & DRC Chairman), Prof. Jai Gopal Sharma (Former HoD), all faculty members and office staff for their support, progressive environment and guidance in all research work as well as the administrative work.

I pay my sincere thanks to Dr. Krishna Kant Sharma, all faculty members, research scholars, and staff members of Department of Microbiology, Maharshi Dayanand University, Haryana. I would also like to thank Prof. Rina Chakrabarti, research scholars, and laboratory staff of Aquaculture Research Laboratory, Department of Zoology, DU for providing essential amenities, and infrastructure for pursuing the research work. A vote of thanks to the Division of Plant Pathology, IARI, for the identification of microbial sample.

I owe my special thanks to my senior cum friend Dr. Pragya who guided and supported me a lot during my experimental work. I have no words to express my admiration for my seniors, colleagues, and juniors for their moral support throughout my academic endeavours.

I have no words to thank my family for their selfless love, infinite blessing, eternal sacrifice, and support in the most challenging time. Credit goes to my mother Ms. Durga Devi, who taught me the value of hard work without whose support the study would have remained a distant dream. Luckily, I have the luxury of being surrounded by loving friends who cheered me on with their positivity and encouragement.

I extend my heartfelt appreciation to every individual who has played a role, big or small, in assisting me along this path. Your contributions have been indispensable and deeply appreciated.



Priya

Dedicated

To

My Family…

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ADC	Apparent digestibility coefficient
ANPU	Apparent net protein utilization
ANOVA	Analysis of variance
HD	Aspartic acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
ß-ME	ß-mercaptoethanol
BPP	ß-propeller phytase
CCD	Central Composite design
CLEA	Cross-linked enzyme aggregates
CP	Crude protein
CPME	Crude protein metabolizable energy
CZE	Capillary zone electrophoresis
DEAE	Diethylaminoethyl
DoE	Design of experiments
DHA	Docosahexaenoic acid
DMR	Dry mouldy residue
DMSO	Dimethyl sulfoxide
DNS	Dinitrosalicylic acid
DTT	Dithiothreitol
EAAs	Essential amino acids
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture Organization
FCR	Feed conversion ratio
FDA	Food and Drug Administration
FPLC	Fast protein liquid chromatography
FI	Feed intake

FT-NIR	Fourier transform near-infrared spectroscopy
GC	Gas chromatography
GE	Gross energy
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
HAP	Histidine acid phosphatase
HPLC	High-performance liquid chromatography
IARI	Indian Agricultural Research Institute
ICP-MS	Inductively coupled plasma mass spectrometry
ITCC	Indian type culture collection
ITS	Internal transcribed spacer
LB	Luria Bertani
MEGA	Molecular Evolutionary Genetics Analysis
MHA	Mueller Hinton agar
MSA	Multiple Sequence Alignment
NA	Nutrient agar
NMR	Nuclear magnetic resonance
OVAT	One variable at a time approach
PA	Phytic acid
PAP	Purple acid phosphatase
PAGE	Poly acrylamide gel electrophoresis
PBD	Plackett-Burman design
PCR	Polymerase Chain reaction
PDA	Potato dextrose agar
PER	Protein efficiency ratio
PGPR	Plant growth promoting rhizobacteria
PGPF	Plant growth promoting fungi
PSM	Phytase screening medium
PUFAs	Polyunsaturated fatty acids
PVA	Polyvinyl alcohol
RSM	Response surface methodology
SmF	Submerged fermentation
SDS	Sodium dodecyl sulfate

S.E	Standard error
SGR	Specific growth rate
SSF	Solid state fermentation
SSSF	Semi-solid fermentation
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography
UV-VIS	Ultraviolet–Visible spectrophotometry
WG	Weight gain
NaCl	Sodium chloride
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$CoCl_2.2H_2O$	Cobalt chloride dihydrate
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
$MnCl_2.4H_2O$	Manganese chloride tetrahydrate
$ZnCl_2.7H_2O$	Zinc chloride heptahydrate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
Cu^{2+}	Copper ions
Zn^{2+}	Zinc ions
Ni^{2+}	Nickel ions
Co^{2+}	Cobalt ions
Mn^{2+}	Manganese ions
Fe^{3+}	Ferric ions
Ca^{2+}	Calcium ions
Fe^{2+}	Ferrous ions
Mg	Magnesium
Ca	Calcium
K	Potassium
Na	Sodium

Al	Aluminum
Sr	Strontium
Cr	Chromium
Pb	Lead
His	Histidine
Leu	Leucine
Ile	Isoleucine
Phe	Phenylalanine
Lys	Lysine
Val	Valine
Asp	Aspartic acid
Thr	Threonine
Glu	Glutamine
Ala	Alanine
Pro	Proline
Gly	Glycine
Ser	Serine
%	Percentage
$	Dollar
°C	Degree Celsius
$t_{1/2}$	Half-life
K_m	Michaelis constant
V_{max}	Maximal velocity of the reaction
IP6	Myo-inositol hexakisphosphoric acid
IP5	Myo-inositol penta phosphoric acid
IP4	Myo-inositol tetra phosphoric acid
IP3	Myo-inositol tri phosphoric acid
IP2	Myo-inositol bis phosphoric acid
IP	Myo-inositol phosphoric acid/ Inositol phosphates
M	Molar
g	Gram
h	Hour
ml	Millilitre

kDa	Kilo Dalton
mM	Millimolar
µM	Micromolar
U	Enzymatic unit
FTU	Phytase activity unit
cm	Centimeter
min	Minutes
mm	Millimeter
nm	Nanometer
rpm	Revolutions per minute
3D	3-Dimensional
P	Phosphorus
µg/ml	Micrograms per milliliter
mg/ml	Milligram per millilitre
µmol/ml/min	Micromole per milliliter per minute
µmol/min/mg	Micromole per minute per milligram
U/g	Unit per gram
U/ml	Unit per millilitre
mg/g	Milligram per gram
µM/min	Micromolar per minute
FTU/kg	Enzymatic unit per kilogram
OTU/kg	Enzymatic unit per kilogram
U/gds	Unit per gram of dry solid substrate
U/g DMR	Unit per gram of dry mouldy residue
U/mg	Unit per milligram
g/l	Gram per liter
g/ml	Gram per milliliter
w/v	Weight/Volume
v/v	Volume/volume

Chapter-1

INTRODUCTION
&
REVIEW OF
LITERATURE

CHAPTER 1

INTRODUCTION

1.1 General

Health and food safety are serious concerns as people are suffering from the problem of malnutrition all over the globe. Environmental pollution and waste management are the other current issues that must be resolved in the interest of upcoming generations. As, the global human population exceeds 8 billion, with India at the top (UN, 2022), the demand for food has increased significantly. To accomplish this, the availability of healthy food is the principal aspect that needs to be emphasized. Plants and animals are important sources of food, but their nutritional value is severely affected due to the deficiency of nutrients, mainly phosphorus (P). P is an essential element for the growth and development of living organisms and is involved in various metabolic processes for survival, therefore, should be mandatory in the diet (Priya et al. 2023a). Plant food contains fruits, vegetables, seeds, nuts, grains, legumes, beans, and oils while animal food includes meat (poultry, beef, pigs), eggs, seafood, milk, cheese, and yogurt. Most humans prefer animal food over plant food due to the high amount of protein content, nutrients, and vitamins (Miller et al. 2022).

The aquaculture industry is rapidly growing in the animal food sector. According to FAO (2023), India's total food production from the aquaculture sector was 8.64 million tonnes in 2020. Fish is an important source of dietary food consumed worldwide and carries 16% of animal protein. Besides, it is a good source of essential amino acids (EAAs), polyunsaturated fatty acids (PUFAs), especially omega-3 PUFAs, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Priya et al. 2023a). The nutritive value of fish solely depends upon the formulation of a balanced diet (Afzal et al. 2020). Fishmeal is the prime constituent in fish feed, highly proteinaceous, having excellent amino acid and fatty acids profile, vitamins, minerals, digestibility, palatability, and low carbohydrate content. However, due to its excessive use, rising fish production costs (40%–60%), and limited global supply, it is a prerequisite to be replaced with a sustainable alternative source of protein such as protein-rich plants (Priya et al. 2023a). In the last two decades, fishmeal has been replaced with plant protein ingredients. The different plant meals utilized in the aquaculture industry for fish feed are

canola, soybean, sunflower, safflower, rapeseed, and cottonseed meals (Hussain et al. 2021, Priya et al. 2023a).

However, plant-based foods are nutritionally deficient due to the presence of phytic acid (PA), a principal storage form of phosphorus. PA acts as an anti-nutritional component by chelating metal ions, proteins, and other biomolecules and restricts the bioavailability of these nutrients. The complex form of PA is known as phytate. Due to the lack of phytate-degrading enzymes in the gastrointestinal tract (GIT) of monogastric animals, P becomes unavailable and affects their health. The supplementation of inorganic P from finite P reserves is one way to attain the insufficiency of P, but they are limited in stock and non-renewable. This additional inorganic P and phytate-associated P when excreted into water bodies causes algal blooms, hypoxia, and death of marine animals, leading to severe environmental issues (Handa et al. 2020, Singh et al. 2024). Due to nutritional and environmental issues, researchers were focused on the phytate degrading enzyme i.e. phytase, which is involved in the phytate breakdown by releasing the bounded P and other nutrients and making them available for the organisms. Since P becomes available, it abolishes the need for additional inorganic P and thus protects the P reserves. It also reduces the P level in aquatic bodies and preserves the environment from P pollution (Kumari and Bansal 2021a).

Phytase is an important feed industry enzyme and has a high market value around the globe. Food and Drug Administration (FDA) approved phytase as generally recognized as safe (GRAS) in the feed industry and it is utilized in more than 22 countries (Priya et al. 2023a). Currently, the market value of phytase is enhancing yearly at a 6.3% rate and is predicted to be more than $1 billion by 2025 (Kumari and Bansal 2021a). In terms of revenue, phytase occupies 83.6% market share (Thakur et al. 2022). The leading phytase-producing companies are Novozymes, DuPont (Danisco), AB enzymes, DSM, and BASF (Vasudevan et al. 2019, Zhou et al. 2022). The phytase market is categorized based on the type of available phytase (powder, granulated, liquid, thermostable), application in animals (poultry, pigs, aquaculture, pharmaceuticals), and geographical area. Phytase are mostly found in powder and granular form, used in poultry feed and North America is the highest producer of phytase (Priya et al. 2023a). The first commercial phytase is Natuphos, discovered in 1991, and comes under first-generation phytase. Natuphos E is the advanced version that came up in the market in 2016 with improvements in its properties (Singh et al. 2024). The majority of the commercial phytases are sourced from fungi and come under the Histidine acid phosphatase (HAP)

category. Recombinant phytases are also available on a commercial scale nowadays. OptiPhos is a recombinant phytase that comes under the second generation of phytase (Priya et al. 2023a).

The research on phytase has been ongoing for the past century and rapidly emerged over a few decades. Thorough work has been conducted on the optimization and production of phytase from different sources (bacteria, fungi) and its application in different fields like the food industry, plant growth, and animal nutrition (poultry, pigs, fish). However, the criteria for ideal phytase (thermostable, protease-resistant, wide pH range, substrate specificity, catalytic efficiency, and performance in GIT of animals are still not fulfilled and their production cost is also high (Handa et al. 2020). The need to produce a cost-effective phytase with the properties of an ideal phytase is still under consideration.

Although various commercial phytases have been studied in improving fish health, the impact of supplementation of phytase in the fish feed contains many gaps, like the accomplishment of desired properties for an ideal phytase required during the feed processing, cost-effective production of phytase, the effect of phytase on feed material before feeding, the exact location and mechanism of phytate hydrolysis in the fish GIT, the response of phytase in varied pH of GIT, applied form of phytase in the feed as a supplement, therefore more investigation is also needed in these aspects. The focus of the present work is to produce a cost-effective phytase suitable for the feed industry, evaluate its features to meet the desired properties of an ideal phytase, and study its effects on feed for enhancing the nutritive value.

1.2 Objectives

Phytase is widely used in the food and feed industry. It ameliorates the nutritive value of food and feed by overcoming the negative effect of phytic acid. In the aquaculture industry, the involvement of phytase as a fish feed supplement is complicated due to a variety of fish species with different characteristics. The study aims to produce a cost-effective phytase from fungal species and optimize its production followed by purification and characterization of a phytase that can fulfil the properties of an ideal phytase and its utilization in the feed industry. The objectives of this study are as follows:

Objective 1. Isolation and identification of potent phytase-producing microbe.

Objective 2. Process optimization for the production of microbial phytase.

Objective 3. Purification and characterization of the microbial phytase.

Objective 4. Immobilization and application of the phytase in the feed industry.

This study showed the production of phytase from *Penicillium oxalicum* by solid-state fermentation method using wheat bran as substrate. Solid-state fermentation is adopted for the work because it uses agricultural waste or lignocellulosic biomass as a substrate and requires a low amount of water. By using these waste materials, SSF resolves the disposal issue that occurs through agro-residues and biomass, one of the environmental challenges. The conversion of these waste into value-added products is beneficial from both economic and environmental point of view. Wheat bran was selected for the study due to its easy availability and low cost. It also acts as an inducer for phytase production during the fermentation process. The production of phytase is optimized through the statistical approach of PBD and RSM techniques. During the PBD and RSM methods, many factors are optimized simultaneously in a single set of experiments. To check the sustainability of production, phytase is produced on a large scale in trays. This entire use of SSF, wheat bran, and optimization through statistical methods makes the phytase production cost-effective. Further, the enzyme is partially purified and characterized to know the biochemical properties of phytase. The study of optimal temperature and pH, thermostability, pH stability, storage life, and action of digestive enzymes helps in determining the ideal properties of phytase. Also, the effect of phytase in the presence of metal ions, detergents, inhibitors, and organic solvents is evaluated. Immobilization of phytase is done with calcium alginate to improve the catalytic activity and to check its reusability. Finally, the application of phytase in the feed industry is studied to determine the influence of phytase in the amelioration of nutrition and environmental pollution management. The involvement of phytase in hydrolyzing phytate-metals and phytate-protein complexes was assessed. The action of phytase in liberating inorganic phosphate, and reducing sugars and protein content from fish feed was checked. Also, reduction of phytic acid through phytase in fish feed was examined.

1.3 Review of Literature

1.3.1 Phytic acid

Myo-inositol hexakisphosphoric acid, IP6 (phytic acid) is the principal storage of organic P found in all plant seeds. It exists in the free form (without metal) and complex structure (with metal) at acidic and neutral pH (Priya et al. 2023a). It is present in cereals (aleurone layer), legumes (globoid crystals), nuts, and oilseeds and occupies 1-5% by weight (Singh et al. 2020). As its name indicates, there are six phosphate groups attached to myoinositol which can bind with different metal ions. Its chemical formula is $C_6H_{18}O_{24}P_6$. In plant seeds, phytate constitutes 65-80% of the total P (Gampa et al. 2023).

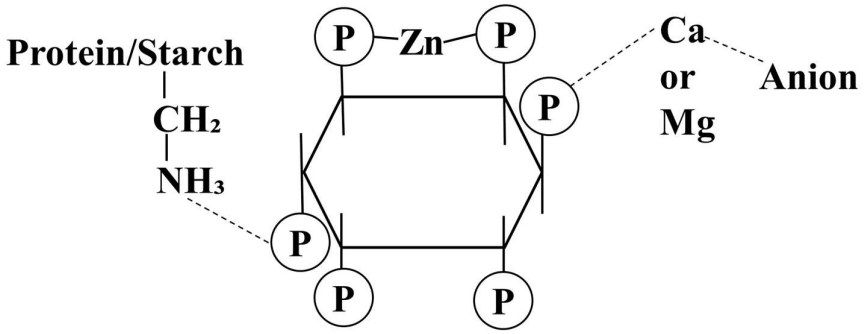


Fig. 1.1 Structure of phytate molecule

Within the pH of the GIT, phytic acid gets deprotonated becomes negatively charged, and exists as a phytate molecule by making a binary complex with cations. It also binds to proteins, amino acids, digestive enzymes, and other biomolecules. In the acidic stomach region, positively charged amino acids bind to phytate and form phytate-protein complex and reduce protein digestibility. While in the alkaline intestine, the breakdown of phytate-protein association occurs in the existence of cations, here the negative charge accommodates over protein molecules and binds to cations. Thus, a ternary structure is formed as a phytate-cation-protein, which resists the protease action (Kryukov et al. 2021, Gampa et al. 2023). The ruminant animals possess phytase activity in the microbes of the gut region, thus degrading the phytate complex and utilizing P and other nutrients, but non-ruminant animals are unable to do so and are thus affected by the problem of malnutrition. Asian countries severely face such a

situation as they rely on a cereals-based diet which is rich in phytate amount (Song et al. 2019). The phytate-minerals complex also leads to a deficiency of minerals creating various disorders that impact the health of children and human beings. In the order of reduction in stability, the binding affinity of phytate towards metal is as follows: Cu^{2+} >Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+} (Sun et al. 2021). Phytate binds with proteins in acidic and alkaline conditions and impairs the protein structure, decreasing the enzymatic activity, solubility, and proteolytic digestibility (Joudaki et al. 2023). Phytate also binds with digestive enzymes such as pepsin, trypsin, and amylase and lowers their activity. Phytate decreases the energy and amino acids availability by changing the release of endogenous substances such as digestive enzymes, HCl, and mucin in GIT (Sharma et al. 2020). Phytate makes a lipophytin complex with lipid molecules and restricts lipid availability (Coban and Demirci 2017).

Apart from anti-nutritional behaviour, phytate is beneficial for human health. Phytate has anti-carcinogenic, antineoplastic, antimicrobial, and antioxidant properties (Kumar et al. 2021). It prevents dental cavities and stone formation in the kidney due to the hypocholesterolemic effect (Nassar et al. 2021, Rizwanuddin et al. 2023a). It also protects from cardiac disease, neurodegenerative disorders, and breast cancer (Abdulwaliyu et al. 2019). It has potential against toxic metal ions and also regulates insulin secretion (Bloot et al. 2023). Phytate also limits putrefaction in fruits and vegetables by restricting the polyphenol oxidase (Rizwanuddin et al. 2023a). This contrasting behaviour of phytate whether being beneficial or detrimental needs further exploration. The reported intake of phytate in India is 670-2500 mg (Sharma et al. 2020), and the limit of phytate content in the food and feed should be balanced because a low concentration of phytate is friendly, but its excess amount raises nutritional and environmental issues (Handa et al. 2020).

The phytic acid can be measured through several methods like ferric precipitation, colorimetric, titration, spectroscopic (UV-VIS, ICP-MS, NMR, FT-NIR), sensor-based (electrochemical biosensors and nanoprobes), chromatographic (HPLC, GC, TLC), and electrophoresis (PAGE, CZE) {Sivakumaran and Kothalawala, 2018, Marolt and Kolar, 2020}. The most commonly used colorimetric method for PA analysis is Wade's test. During the protocol, a pink colour developed when PA reacted with the Wade reagent (a mixture of $FeCl_3$ and sulphosalicylic), and the intensity of the colour decreased as the level of phosphorus increased in the mixture (Wade and Morgan, 1955). This method is easy to perform, accurate, and less costly. The estimation of PA in wheat, cereals, soybean, maize, rye, sunflower, and

oats through Wade's method has been mentioned in the literature as well (Gao et al. 2007, Agostinho et al. 2016, Pragya et al. 2023b).

Phytic acid can be broken down either by traditional food processing methods or an enzymatic approach. In food processing treatment, activation of endogenous phytase is done through milling, soaking, germination, autoclaving, cooking, fermentation, malting, ion exchange, and acid hydrolysis methods. However, these methods are less efficient, time-consuming, and lower the nutritive value of food (Handa et al. 2020, Joudaki et al. 2023). Therefore, enzymatic hydrolysis is preferred in which exogenously supplemented phytase hydrolysis the phytate molecule, improves the nutritional value, and reduces the P pollution.

1.3.2 Phytase

Phytase, chemically known as myo-inositol hexakisphosphate phosphohydrolase (EC 3.1.3.8) comes under the class of acid phosphatases. It catalyzes the phytic acid and releases inorganic P and lower forms of myo-inositol sequentially (Filippovich et al. 2023). Initially, it dissociates IP6 into IP5 completely and then similarly degrades further inositol IP5 into IP4 and so on (Dersjant-Li et al. 2015). It also releases phytate-associated minerals, proteins, digestive enzymes, and other biomolecules. The phytase enzyme was first reported in rice bran by Suzuki et al. (1907).

According to the nomenclature committee of the International Union of Biochemistry and Molecular Biology, there are mainly two types of phytases: 6-phytase in which phytase acts on the ester bond at the 6th position and is found in plants while 3-phytase in which phytase act on the 3rd position and commonly present in microbes, but exceptions are remaining there. Also, some other phytases like 5-phytase in *Selenomonas ruminantium* and pollen of *Lilium longixorum*, and 4-phytase in rye have been reported (Outchkourov and Petkov 2019).

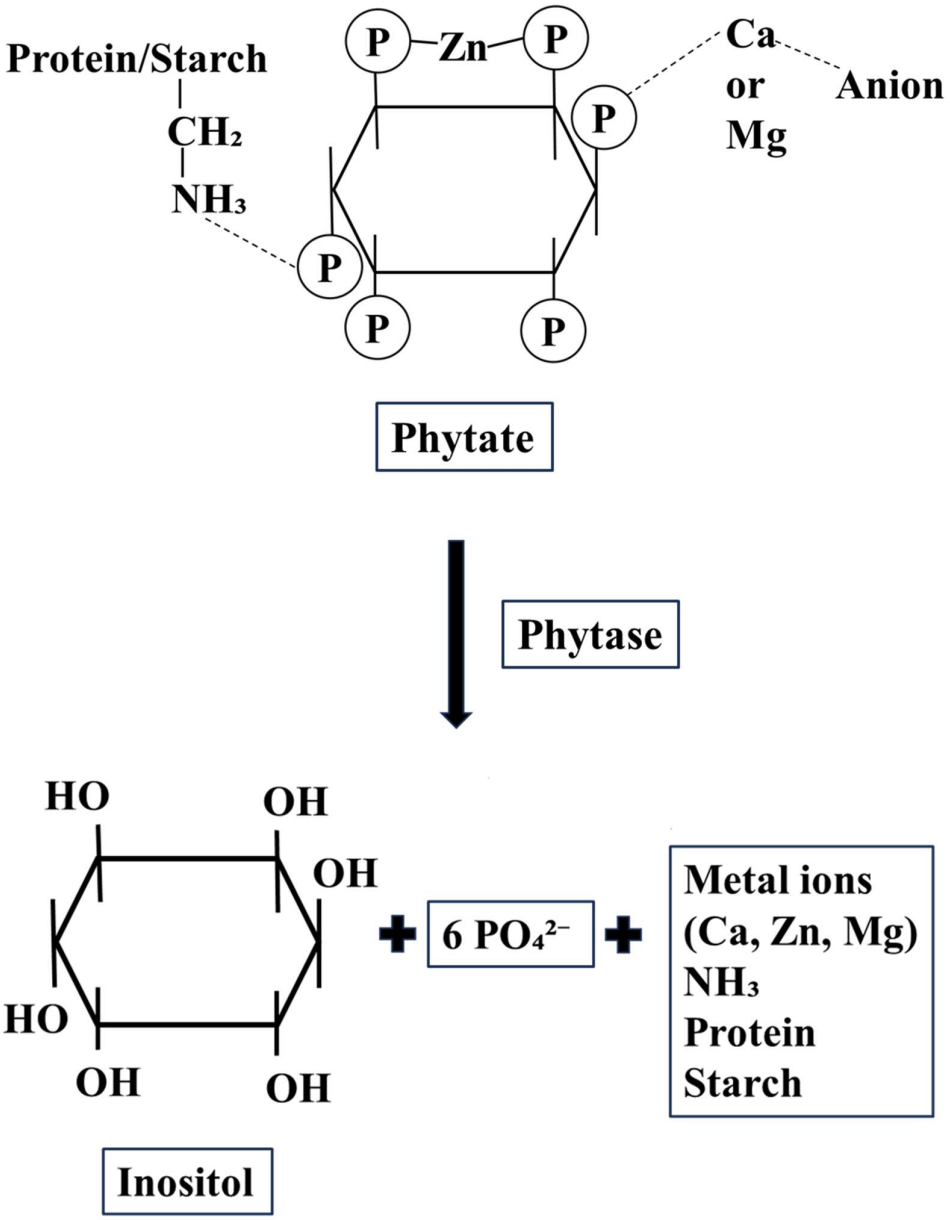


Fig. 1.2 Hydrolysis of phytate molecule by phytase enzyme

Another classification of phytases is based on the catalytic properties and they are of four types, i.e., Histidine acid phosphatase (HAP), ß-propeller phytase (BPP), Cysteine phosphatase, and purple acid phosphatase (PAP) (Singh et al. 2020). In HAP, the enzyme catalytic site is located at the interface of the conserved N-terminal active site motif RHGXRXP domain and the C-terminal of aspartic acid (HD) domain. The mechanism behind the reaction is the nucleophilic attack of histidine (RHGXRXP) on the P and the formation of

an intermediate–covalent phosphohistidine and then HD donates a proton to oxygen of scissile phosphomonoester bond make the pH acidic. It includes microbes and plants. The first commercial phytase “Natuphos” is HAP. ß-propeller phytase e.g., *Bacillus* spp. lack such type of motif and need Ca^{2+} for enzyme activity. It dissociates only three phosphates from phytate and has a narrow substrate range with pH varying from neutral to alkaline. Structurally, it has a hexameric assembly similar to a six-bladed propeller. At the top of a propeller, a substrate binding site is present. PAP consists of Fe^{2+}, Fe^{3+}, Mn^{2+}, and Zn^{2+} in catalytic centres and is therefore called purple or pink phytases. It is found in plants, fungi, and mammals. Cysteine phosphatase resembles the catalytic mechanism with protein tyrosine phosphatase (Singh et al. 2024). It was first reported in *S. ruminantium* (Mullaney and Ullah, 2006). The active site is found at the junction of larger and smaller domains, enclosing with a P loop (Chen et al. 2015). Further based on pH, phytases are acidic, alkaline, and neutral. Fungal phytases and some bacterial species belong to acidic phytases having a pH range of 2.0-6.0, while plants and Bacillus spp. comes under alkaline phytases (Singh et al. 2024). Only a few neutral phytases have been reported in some bacteria and fungi (Singh et al. 2020).

Phytases can be obtained from plants, animals, archaea, and microorganisms (bacteria, yeasts, fungi) (Singh et al. 2020). It can be found in intracellular, extracellular, or cell-bound forms (Joudaki et al. 2023). In plants, phytases have been reported in rice, wheat, maize, soybean, rye, barley, beans, spinach, lettuce, oilseeds, and grass. Among these wheat, barley, and rye possess a high amount of phytase. However, the extraction of phytase from plants is a tedious, time-consuming, and costly process (Rizwanuddin et al. 2023b). In animals, phytase has been reported in the calf liver and blood of lower vertebrates but has low efficiency (Joudaki et al. 2023). Bacteria, fungi, and yeasts are included under microbial sources of phytase. A major portion of the work has been done with the production and optimization of microbial phytase, characterization, and its application in various fields. Fig. 1.3 demonstrates the framework that enlightens the extraction of phytase enzyme from microbes, upstream and downstream techniques, applied form of phytase, and its application in different sectors.

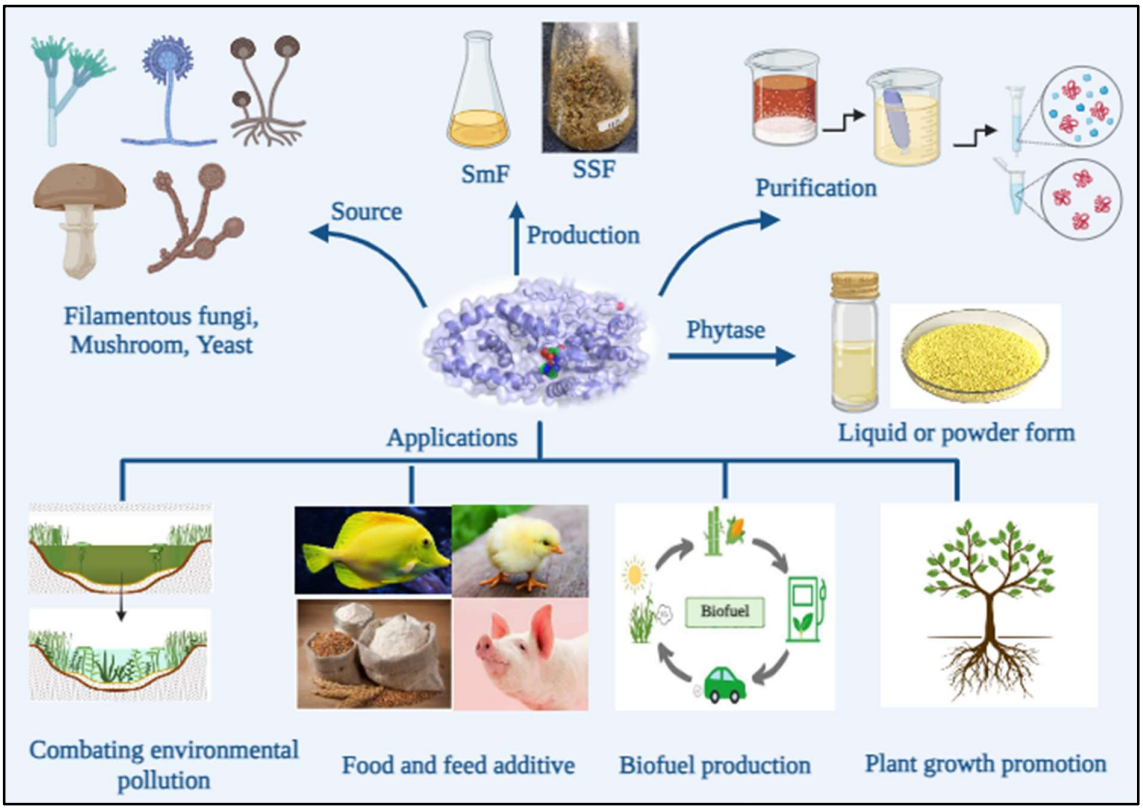


Fig. 1.3 Outline view of bioprocessing of phytase along with its application

1.3.3 Microbial source of phytase

Microbes are a preferred source of phytase because of easy culturing, fast growth, high yield, and the production of stable and cost-effective phytase (Priya et al. 2023a). Microbial phytase breaks down the phytate molecule completely as compared to endogenous plant phytase (Song et al. 2019). Phytase can be isolated from three different sources of microbes – bacteria, fungi, and yeasts. Among microbes, the presence of phytase can be intracellular and extracellular. The majority of fungal phytases are extracellular, while bacteria and yeasts are cell-bound with some exceptions (Jain et al. 2016). Microbial phytase can be obtained from the soil, plant rhizosphere, gut region of animals, and extreme environmental conditions. As shown in Table 1.1, an enormous number of microbes have been isolated for the production of phytase enzymes. Bacterial phytases have been isolated from *Pseudomonas* sp., *Bacillus* sp., *Prevotella* sp., *Raoutella* sp., *Pantoea* sp., *Mitsuokella multiacidus*, *M. jalaludinii*, *Aerobacter aerogenes,*

Citrobacter braakii, Klebsiella sp., *Enterobacter* spp., *Bifidobacterium* sp., *Megasphaera elsdenii, Lactobacillus* sp., *S. ruminantium* and *Escherichia coli* (Jain et al. 2016; Rizwanuddin et al. 2023a, Rizwanuddin et al. 2023b). Bacterial phytases perform mostly in alkaline regions, usually produced by SmF and are phytate-specific making them less preferable as compared to fungal phytases which are thermostable, broad substrate specificity, protease-resistant, and possess chitin wall (Rebello et al. 2017, Singh et al. 2024). As compared to fungal phytase, they are smaller in size due to the occurrence of glycosylation in fungal phytase (Hussain et al. 2021). Yeast phytases are present in both extracellular and cell-bound forms. *Arxula adeninivorans, Pichia anomala, P. kudriavzevii, Saccharomyces cerevisiae, Clavispora lusitaniae, Kluyveromyces marxianus, Millerozyma farinosa, Candida glabrata, Wickerhamomyces anomalus, Hanseniaspora guilliermondii,* and *Debaryomyces nepalensis* are some examples of yeasts species containing phytase enzyme (Gessler et al. 2018).

At the commercial level, fungi are the preferred source for phytase production due to the release of extracellular phytase. In the case of fungi, the genera *Aspergillus, Penicillium, Mucor, Rhizopus, Trichoderma,* and *Myceliophthora,* are frequently involved in phytase production (Joudaki et al. 2023). Fungal phytases are acidic (2.0-5.0) in nature and come under the HAP category. They can perform in a wide range of temperatures and are highly thermostable. Fungi have chitin walls that help them to survive in extreme environmental conditions, are cultured easily, and produce a high yield of enzymes (Rizwanuddin et al. 2023a and b). Due to these peculiar features, fungi are preferred over bacteria. Various mesophilic and thermophilic fungi have been mentioned in literature for phytase production. *A. niger* is the first fungi species that shows the phytase activity. After that, different species of *Aspergillus (A. ficuum, A. oryzae, A. flavus, A. niger, A. tubingensis, A. niger van teighem, A. fumigatus, A. carbonarius, Ganoderma applanatum, G. stipitatum, Grifola frondosa, Mucor racemosus, M. piriformis, Penicillium purpurogenum, Rhizopus oligosporus, R. oryzae, Schizophyllum commune, Trametes versicolor*, *Humicola nigrescens*, *N. sitophila, Cladosporium* species, *M. piriformis, Agaricus bisporus, Agrocybe pediades, Ceriporia* sp., *Lentinula edodes* have been reported for the production of phytase (Jatuwong et al. 2020a, Singh et al. 2024). Thermophilic molds like *Myceliopthora thermophila, Thermomyces lanuginosus, Sporotrichum thermophile, Chaetomium thermophilum* ATCC58420, *Rhizomucor miehi* ATCC22064, *Thermomucor indicae-seudaticae* ATCC28404 were also found as phytase producers. Filamentous fungi can be grown by both SSF and SmF methods.

Table 1.1 Location and fermentation method used by phytase-producing microbes.

Source	Location	Fermentation	References
Fungi			
A. niger	Extracellular	SSF, SmF	Sandhya et al. 2015
A. niger NCIM 563	Extracellular	SmF, SSF	Buddhiwant et al. 2016
A. niger UFV-1	Extracellular	SmF	Monteiro et al. 2015
A. niger CFR 335	Extracellular	SSF	Gunashree and Venkateswaran 2015
A. niger NT7	Extracellular	SSF	Kumari and Bansal 2021a
A. niger 7A-1	Extracellular	SSF	Neira-Vielma et al. 2018a
A. niger var. *phoenicis* URM4924	Extracellular	SmF	Nascimento et al. 2022
A. fumigatus NF191	Extracellular	SmF	Gangoliya et al. 2015
A. flavus ITCC 6720	Extracellular	SSF	Gaind and Singh 2015
A. tubingensis SKA	Extracellular	SmF	Qasim et al. 2017
Muscodor sp.	Extracellular	SmF	Alves et al. 2016
R. oligosporus MTCC	Extracellular	SSF	Suresh and Radha 2015
R. microsporus var. microsporus	Extracellular	SmF	Sato et al. 2016
R. oryzae	Extracellular	SSF, SmF	Kanti and Sudiana 2018
S. thermophile BJTLR50	Extracellular	SSF	Kumari et al. 2016
T. lanuginosus SSBP	Extracellular	SmF	Makolomakwa et al. 2017
T. lanuginosus IMI 096218	Extracellular	SSF	Bujna et al. 2016
N. sitophila	Extracellular	SSF, SmF	Kanti and Sudiana 2018

N. crassa	Extracellular	SmF	Kanti and Sudiana 2016
Hypocrea lixii SURT01	Extracellular	SmF	Thyagarajan et al. 2016
T. purpurogenum NSA20	-	SSF	Ahmed et al. 2022
Ganoderma sp. MR-56	-	SmF	Salmon et al. 2016
G. frondosa	Extracellular	SmF, SSF	Huang et al. 2018
T. aurantiacus SL16W	Extracellular	Semi SSF	Tanruean et al. 2021
Bacteria			
Streptomyces luteogriseus R10	Extracellular	SmF	Aly et al. 2015
Enterobacter sp. ACSS	Extracellular	SmF	Chanderman et al. 2016
E. aerogenes	Extracellular	SmF	Muslim et al. 2018
E. intermedius PHY03	Extracellular	SmF	Aziz et al. 2015
E. cloacae strain PSB-45	Extracellular	SmF	Kalsi et al. 2016
Rahnella aquatilis JZ-GX1	Extracellular	SmF	Li et al. 2021
B. subtilis subsp. *subtilis* JX292128	Extracellular	SSF	Das and Ghosh 2015
B. subtilis US417	Extracellular	SmF, SSF	Singh et al. 2013
B. subtilis DR6	Extracellular	SmF	Kammoun et al. 2012
B. subtilis KT004404	Extracellular	SmF	Javaid et al. 2022
Serratia sp. PSB-15	Extracellular	SmF	Kalsi et al. 2016
C. koseri PM-7	-	SmF	Tripathi et al. 2017
Pediococcus acidilactici SMVDUDB2	Extracellular	SmF	Bhagat et al. 2020
P. acidilactici BNS5B	Extracellular	SmF	Sharma and Shukla 2020
Lactic acid bacteria	Intracellular	SmF	Goswami et al. 2017
Pantoea vagans 3.2	Intracellular	SmF	Suleimanova et al. 2013
Lactobacillus brevis	Intracellular Extracellular	SmF	Sumengen et al. 2012

Bacillus coagulans	Intracellular	SmF	Chatraei and Emtiazi 2019
Yeast			
Cystobasidium minutum (Rhodotorula minutum)	Extracellular	SmF	Gowthami and Gunashree 2023
Cyberlindnera jadinii CJ2	Cell bound, Extracellular	SmF	Capusoni et al. 2021
K. lactis CBS 2359	Cell-bound	SmF	Capusoni et al. 2021
K. marxianus Y1058	Cell-bound	SmF	Capusoni et al. 2021
K. marxianus	Extracellular	SmF	Pires et al. 2019
Hanseniaspora uvarum UMY 514	Cell-bound	SmF	Capusoni et al. 2021
H. uvarum UMY 571	Cell-bound	SmF	Capusoni et al. 2021
Brettanomyces bruxellensis CBS 2499	Cell-bound	SmF	Capusoni et al. 2021
Lachancea thermotolerans CBS 6340	Cell-bound	SmF	Capusoni et al. 2021
Torulaspora delbruekii CBS1466	Cell-bound	SmF	Capusoni et al. 2021
Zygosaccharomyces kombutchaensis CBS 8849	Cell-bound	SmF	Capusoni et al. 2021
Meyerozyma guilliermondii UBOCC-A-214008	Cell-bound	SmF	Capusoni et al. 2021
M. guilliermondii UBOCC-A-214143	Cell-bound	SmF	Capusoni et al. 2021
Rhodotolura mucilaginosa UBOCC-A-214036	Cell-bound	SmF	Capusoni et al. 2021
R. mucilaginosa JMUY14	Extracellular	SmF	Yu et al. 2015
R. diobovata Mo38 UBOCC-A-208033	Cell-bound	SmF	Capusoni et al. 2021
C. atlantica Mo31 UBOCC-A-208026	Cell-bound	SmF	Capusoni et al. 2021
C. tropicalis BOM2	Extracellular	SmF	Ogunremi et al. 2020
D. hansenii BIO2 UBOCC-A-208002	Cell-bound	SmF	Capusoni et al. 2021
Williopsis saturnus NCIM 3298	Cell-bound	SmF	Pable et al. 2019

Schwanniomyces (f. Debaryomyces) occidentalis	Cell-bound	SSF	Molina et al. 2021
P. guilliermondii EX15 UBOCC-A-208004	Cell-bound	SmF	Capusoni et al. 2021
P. kudriavzevii TY13	Extracellular	SmF	Hellstrom et al. 2015
P. kudriavzevii OG32	Extracellular	SmF	Ogunremi et al. 2020
P. membranifaciens S3 (MG663581)	Extracellular	SSF	Soman et al. 2020
S. cerevisiae Finarome	Extracellular	SmF	Klosowski et al. 2018

1.3.4 Production and optimization of microbial phytase by SSF

Fermentation is the most frequent method used for enzyme production. Two types of fermentation processes are commonly utilized for phytase production, solid-state fermentation (SSF) and submerged fermentation (SmF). A new upcoming approach for the production of enzymes is the semi-solid fermentation method (SSSF), which is similar to SSF, but requires more water than SSF. SSF is the method in which microbes are grown on a solid matrix and the process is carried out in the absence or less amount of water. In this process, the substrate acts as a solid matrix and requires adequate moisture for the growth of microorganisms. It provides a similar environment to the microbes from where they are isolated. In SmF, microbes are grown in a flask having liquid nutrient media (Handa et al. 2020). Phytase yield is influenced by various factors such as type of strain, culture parameters, nature of substrate, and bioavailability of nutrients (Handa et al. 2020). The SmF method has been largely used for phytase production in the case of bacterial species. For eg., *B. amyloliquefaciens* DS11 and *L. amylovorus* B4552 (Hussain et al. 2021), *B. subtilis* KT004404. However, some fungal species are also found to produce phytase by SmF like *A. niger* var. *phoenicis* URM 4924 (Nascimento et al. 2022). Traditionally, SmF is used for the production of enzymes at a commercial scale but SSF is preferred nowadays due to its advantages over SmF.

SSF includes simple media, easy aeration, less water requirement, high product yield, low requirement of energy, decreased bacterial contamination, less space requirement, easy operation, economical cheaper, less time consuming, and high reproducibility (Rizwanuddin et al. 2023a, Singh et al. 2024). It uses agricultural wastes as a solid material that promotes the

growth of microorganisms. Agricultural residues are by-products left over after harvesting and processing of crops. They are accumulated every year in millions of tons and have been a cause of environmental pollution. To mitigate this issue, researchers are utilizing these by-products in the production of enzymes or bioethanol formation. They are highly nutritive due to the presence of cellulose, hemicellulose, lignin, and other polysaccharide components. They are lignocellulosic and promote the growth of microorganisms (Jatuwong et al. 2020b). Phytase production in SSF through the utilization of agricultural waste (wheat bran, wheat straw, rice bran, rice straw, oil cakes, corn cobs, corn bran, sugarcane bagasse, cassava bagasse, oat bran and soybean bran, coffee pulp, husks, fruit peels and pulps, oat husk, sawdust, sorghum straw, soybean meal, citrus peels, and coconut oil cakes has been reported in various literature (Jatuwong et al. 2020b, Hussain et al. 2021, Ahmed et al. 2022, Singh et al. 2024). Semi-solid fermentation is the least common method and reported in *Thermoascus aurantiacus* SL16W (Tanruean et al. 2021).

Filamentous fungi can be produced through both SSF and SmF, however, SSF is a more common method for the production of phytase from these fungi. For instance, *R. oligosporus, S. thermophile, G. frondosa, A. zeae, A. aculeatus, P. adiposa, A. niger, A. tubingensis, A. oryzae* are the fungi that produced phytase through the SSF method (Suresh and Radha 2015, Kumari et al. 2016, Huang et al. 2018, Pires et al. 2019, Saxena et al. 2020, Jatuwong et al. 2020b, Kumari and Bansal 2021a, Mahendran et al. 2022, Pragya et al. 2023a). The production of phytase from yeasts and a few bacterial species has also been done using the SSF method (Soccol et al. 2017, Jatuwong et al. 2020a). Table 1.2 demonstrates the production of phytase by using different substrates.

Though the production of phytase and cellulolytic enzymes from *T. aurantiacus* SL16W has been achieved using rice bran as a substrate (Tanruean et al. 2021), wheat bran is the most commonly used substrate for the production of phytase. It is rich in carbon, nitrogen, and amino acids and a high amount of phytic acid. Due to the presence of phytate, it acts as an inducer for the production of phytase (Chanderman et al. 2016), and provides support for the growth of fungi, easy availability, and low cost (Kumari and Bansal 2021a). The utilization of wheat bran by different fungi in phytase production has been mentioned such as *A. awamori* NRC-F18, *A. niger* F-258, *A. oryzae* F -923, *S. cerevisiae* F-307, *A. fumigates* NRCF-113, *A. oryzae* F -937, *T. viride* NRCF-107 (Elkhateeb and Fadel 2022), *A. tubingensis* (Mahendran et al. 2022), *A. niger* NT7 (Kumari and Bansal 2021a), *A. aculeatus* APF1 (Saxena et al. 2020),

A. oryzae SBS50 (Sapna and Singh 2015), *R. oligosporus* MTCC 556 (Suresh and Radha 2015).

Table 1.2 Optimization conditions of fungal phytase produced through the SSF method.

Source	**pH**	**Temperature**	**Substrate**	**References**
A. flavus ITCC 6720	6.0	37	Mustard cake	Gaind and Singh 2015
A. oryzae SBS50	5.0	30	Wheat bran	Sapna and Singh 2015
A. niger	5.0	30	Rice bran	Sandhya et al. 2015
R. oligosporus MTCC 556	5.5	30	Mixed substrate	Suresh and Radha 2015
A. niger NCIM 563	-	30	Groundnut oil cake	Buddhiwant et al. 2016
S. thermophile BJTLR50	-	45	Mixed substrate	Kumari et al. 2016
A. niger	-	30	Soybean meal	Saithi and Tongta 2016
A. ficuum	8.2	27	Potato waste	Tian and Yuan 2016
A. tubingensis SKA	5.0	30	Wheat bran	Qasim et al. 2017
FSS223	7.0	65	Wheat bran	Yaseer et al. 2018
FSS296	5.0	60	Soybean powder	Yaseer et al. 2018
G. frondosa	-	25	Brown rice	Huang et al. 2018
N. sitophila	-	30	Rice straw powder and soybean curd residue	Kanti and Sudiana 2018
A. niger 7A-1	-	28	Triticale	Niera-Vielma et al. 2018a
A. ficuum PTCC 5288	5.5	30	Wheat straw	Shahryari et al. 2018
A. zeae B	4.0	28	Corn meal	Pires et al. 2019
T. atroviride	-	30	Maize DDGS	Prado et al. 2019

A. niger Str 3	-	30	Coconut oil cake and rice brand	Kanti et al. 2020
A. aculeatus APF1	3.0	50	Wheat bran	Saxena et al. 2020
P. adiposa	6.0	30	Water hyacinth	Jatuwong et al. 2020b
A. niger NT7	5.0	35	Wheat bran	Kumari and Bansal 2021a
A. niger	6.0	35	Rice polish	Mahmood et al. 2021
A. awamori NRC-F18	6.0	30	Rice straw	Elkhateeb and Fadel 2022
T. purpureogenus NSA20	5.5	30	Potato peel	Ahmed et al. 2022
A. terreus	4.5	40	Rice bran	Chaudhari and Peshwe 2022
A. tubingensis	5.0	30	Wheat bran	Mahendran et al. 2022
A. oryzae SBS50	5.0	30	Mixed substrate	Pragya et al. 2023b
P. purpurogenum	8.0	27	Maize cob and corn bran	Rizwanuddin et al. 2023a

The optimization is a process to identify the best factors involved in enhancing the production of enzymes significantly. Optimization can be done by traditional and statistical methods. One variable at a time (OVAT) is an unconventional or traditional approach that analyses variables one by one during the experiment. Once it fixes the level of one variable, then it examines others. OVAT is a tedious, costly, and time-consuming process. Statistical optimization is the most adopted approach in enhancing the production of enzymes. The method includes Placket Burman Design (PBD) and Response Surface Methodology (RSM) which analyze multiple parameters in a single experiment. With the help of mathematics and statistics, an experimental model was designed to analyze the influence and importance of parameters and their interaction in increasing the production yield (Singh et al. 2024, Kumari and Bansal 2021b). It reduces the time, number of experiments, and cost taken during the OVAT experiment. PBD identifies critically important factors useful for the production of enzymes while RSM depicts the interaction level among the critical factors. Optimization of variables is necessary to increase the production level of enzymes. Various factors including

substrate, amount of substrate, moisture level, pH, temperature, incubation days, carbon and nitrogen source, detergents, amount, and age of inoculum need to be optimized for the highest production. Different carbon sources (glucose, sucrose, maltose, dextran, galactose, lactose, mannitol, fructose, starch), nitrogen sources (ammonium nitrate, ammonium sulphate, urea, yeast extract, potassium nitrate, sodium nitrate, beef extract, peptone, tryptone), detergents (Tween 20, Tween 40, Tween 60, Tween 80, Triton-X-100) may be employed to the production media for the enhancement in the yield of an enzyme.

The optimization of phytase has been performed via both OVAT and statistical ways {Jatuwong et al. 2020b, Kumari and Bansal 2021(a, b), Mahmood et al. (2021, 2022), Pragya et al. 2023 (a, b), Singh et al. 2024}. The optimized conditions for phytase isolated from *A. awamori* NRC- F18 was 5 g rice straw moistened with media (1:2) of pH 5.5 containing glucose and ammonium phosphate as a carbon and nitrogen source incubated at 30ºC for 6 days (Elkhateeb and Fadel 2022). The production of phytase by *A. tubingensis* is found maximum when the moistening media was supplemented with 75 mM glucose, 1% peptone, 1mM zinc sulphate, and 1% sodium chloride along with culture conditions of pH 5.0, 30ºC, 200 × 10^4 (v/v) inoculum level and 5 days (Mahendran et al. 2022). The best culture conditions for *A. flavus* ITCC 6720 were 0.5% malt extract and glucose with a moisture level of 58%, 10% of 3 days old inoculum, incubation period of 4 days, 37ºC incubation temperature and 6.0 pH along with mustard cake substrate (Gaind and Singh 2015). Similarly for *A. niger*, the growth conditions were rice bran substrate along with the incorporation of glucose and ammonium nitrate with pH 5.0 and 30ºC for 4 days (Sandhya et al. 2015). Also, other fungal studies represented phytase production in *R. oligosporus* MTCC 556 (Suresh and Radha 2015), *A. niger* Str3 (Kanti et al. 2020), *Neurospora sitophila* (Kanti et al. 2020), *T. aurantiacus* (Tanruean et al. 2021), and *A. niger* (Kumari and Bansal 2021a) were optimized through OVAT.

In most of the cases, OVAT is performed initially for considering the useful parameters and then statistical optimization is designed upon it. Kumari and Bansal (2021b) identified a few variables like the amount of substrate, temperature, incubation period, pH, mannitol level, and ammonium sulphate through OVAT and finalized 5 g wheat bran with 2% mannitol and 0.5% ammonium sulphate in media of pH 4.3 incubated at 35°C for 5 days via RSM. In the *P. adiposa* phytase production, the reported chief components were water hyacinth with 85% moisture content of liquid medium having pH 6.5 along 30ºC incubation temperature for 7 days through statistical method (Jatuwong et al. 2020b). Statistical optimization has also been

performed in various fungi such as *A. oryzae* SBS50 (Sapna and Singh 2015, Pragya et al. 2023a), *A. niger* NCIM 563 (Buddhiwant et al. 2016), *S. thermophile* BJTLR50 (Kumari et al. 2016), *A. ficuum* (Wang et al. 2017), *Talaromyces purpureogenus* NSA20 (Ahmed et al. 2022) via PB and RSM. Similarly, in other studies, the involvement of PBD, and RSM has been mentioned as well (Pragya et al. 2023a, Niera-Vielma et al. 2018a, Shahryari et al. 2018, Qasim et al. 2017, Kumari et al. 2016, Buddhiwant et al. 2016), as shown in Table 1.3.

The optimization and improvement in strain through mutations or transgenic and recombination production are the other important techniques performed to enhance the amount of microbial phytase. For the upgradation of phytase properties at the molecular level, the phytase variant is developed by protein engineering technology via two approaches directed evolution and rationale design (Chen et al. 2015).

Table 1.3 Statistical optimization of phytase production by PBD & RSM experiments

Source	**Optimized conditions via PB & RSM**	**References**
A. oryzae SBS50	Triton-X-100 3.0%, magnesium sulphate 0.4%, moisture ratio of 1:3.5 with an incubation time of 48 h.	Sapna and Singh 2015
A. niger NCIM 563	5 g of glucose, 10 g of dextrin, 200 ml of distilled water and 1.5 g of $MgSO_4.7H_2O$	Buddhiwant et al. 2016
S. thermophile BJTLR50	yeast extract 1.0 %, Tween 80 2.5 % and incubated period 48 h	Kumari et al. 2016
R. oligosporus MTCC 556	3.0 g dextrose, 2.5 g ammonium nitrate, substrate size of 80 mesh, 10 mg calcium chloride - 116 h	Suresh and Radha 2016

A. niger 7A-1	Dextrose (14%), lactose (0.6%), Tween 80 (2%), and potassium chloride (0.15)	Niera-Vielma et al. 2018a
A. ficuum PTCC 5288	0.17 g glucose, 0.068 g ammonium sulfate, 655 g moisture content	Shahryari et al. 2018
W. saturnus NCIM 3298	glucose, citric acid, yeast extract, $FeSO_4$, pH	Pable et al. 2019
P. adiposa	85% moisture content, pH 6.5, 30°C, 7 days	Jatuwong et al. 2020b
A. niger NT7	5 g wheat bran, 2% (w/w) mannitol, 0.5% ammonium sulfate, pH 5, 35 °C, 5 days	Kumari and Bansal 2021b
A. niger	incubation period (6 days), incubation temperature (35°C), initial pH (6), NH_4NO_3 (0.75%), and tween-40 (0.6%)	Mahmood et al. 2022
A. fumigatus	2.75 ml mineral solution, 1.05 ml Ca-phytate, 0.105 g $CaCl_2.2H_2O$, inoculum level 390 spores/ml	Thakur et al. 2022
A. oryzae SBS50	2% glucose, 0.4% $MgSO_4 \cdot 7H_2O$, 2.5% Tween 80, and 1:4 moisture ratio	Pragya et al. 2023a

1.3.5 Purification and characterization of microbial phytase

The purification of an enzyme is a necessary step to extract the desired molecule of interest. It removes the unwanted molecules and concentrates the desired enzyme by increasing its purification fold. It includes different stages of precipitation, and dialysis followed by column chromatography. Precipitation is the most commonly used method for purification and

can be done by ammonium sulphate, acetone, and ethanol followed by dialysis and column chromatography (ion exchange, gel filtration) for the complete purification of the enzyme. After chromatography, electrophoretic analysis is done by SDS-PAGE to show the band of purified enzyme. Biochemical characterization helps in determining the properties of the enzyme, its structure and function, and its perspective in different fields. Table 1.4 demonstrates the purification techniques used by the phytase with their biochemical characteristics.

The phytase is a monomeric protein and its molecular mass varies from 35-700 kDa (Rebello et al. 2017). It demonstrates different characteristics (optimum temperature, optimum pH, substrate specificity, resistance from proteolysis) which depend on the type of microbial species used for extraction (Pragya et al. 2021). Microbial phytases are thermostable and exhibit a wide range of temperatures. Most of the fungal phytases are HAP and their pH lies between 2.0-6.0 with some exceptions such as *A. bisporus* (pH 5-8) and *R. microsporus* var. *microspores* (pH 9.0) (Kaur et al. 2021). The optimal temperature value varies from 37- 67 ºC (Jatuwong et al. 2020a). Also, the effect of metal ions, inhibitors, organic solvents, detergents, and chaotropic agents was studied to depict the nature of phytase. Niera-Vielma et al. (2018b) isolated thermostable phytase from *A. niger* 7A-1 and purified it up to 7.4-fold with a 15.5% yield. The K_m and V_{max} values were 220 μM and 25 μM/min respectively. It showed maximum specificity with sodium phytate. Similar biochemical properties were recorded in *A. flavus* ITCC 6720 (Gaind and Singh 2015). The purification fold was reported at 34.72, demonstrating specificity towards sodium phytate and p-nitrophenyl phosphate. A thermostable and protease-resistant phytase was partially purified from *A. aculeatus* APF1 through ammonium sulphate precipitation and dialysis, showing an optimal pH of 3.0 and temperature of 50ºC, respectively (Saxena et al. 2020).

Table 1.4 Purification and characteristics of microbial phytase

Source	**MW (kDa)**	**Optimum temperature**	**Optimum pH**	**Purification process**	**Reference**
R. oligosporus MTCC 556	-	55	5.5	Ammonium sulphate precipitation,	Suresh and Radha 2015

				ultrafiltration, Sephadex G-25 column	
A. flavus ITCC 6720	30	45	7.0	Acetone precipitation, Macro-prep high Q column, Ultra-filtration	Gaind and Singh 2015
S. thermophile expressed *in E. coli*	55	60	5.0	Ni-NTA column chromatography	Ranjan et al. 2015
A. niger CFR 335	66	30	4.5	Ammonium sulphate fractionation, Dialysis, DEAE-Sephadex G-250, G-150, G-50 column chromatography	Gunashree and Venkateswaran 2015
S. thermophile expressed *in P. pastoris*	70	60	5.0	Ultrafiltration, fast protein liquid chromatography (FPLC), gel filtration chromatography column [Sephacryl S-200HR(16/60)]	Ranjan and Satyanarayana 2016
A. oryzae SBS50	80	50	5.0	Ammonium sulphate precipitation, Dialysis, ion	Sapna and Singh 2017

				exchange (SP-Sepharose), gel filtration (P-60)	
A. niger 7A-1	89	56	5.3	microfiltration, ultrafiltration, and DEAE-Sepharose column chromatography	Niera-Vielma et al. 2018b
A. zeae B	-	50	Neutral pH	-	Pires et al. 2019
Aspergillus fumigatus	118	40	6.0	Ammonium sulphate precipitation, Dialysis, ion exchange (DEAE-Sephacel column), gel filtration (sephacryl S200)	Sanni et al. 2019
P. adiposa	-	42	5.0	Ammonium sulphate precipitation	Jatuwong et al. 2020b
A. aculeatus APF1	25–35	50	3.0	Ammonium sulphate precipitation, Dialysis, ion exchange (SP-Sepharose), gel filtration (P-60)	Saxena et al. 2020
A. niger NT7	-	60	2.6, 4.8	-	Kumari and Bansal 2021b

Aspergillus tubingensis TEM 37	48	45	2.0, 5.5	ultrafiltration	Caliskan-Ozdemir et al. 2021
T. purpureogenus NSA20	-	37	5.5	Ammonium sulphate precipitation, Dialysis	Ahmed et al. 2022
A. fumigatus	62	56	5.5	Ammonium sulphate precipitation, Dialysis, Sephadex G-100 column chromatography	Thakur et al. 2022
A. niger BIONCL8	65	40	2.1	Ammonium sulphate precipitation, Dialysis, DEAE Sephadex A-50, Bio-Gel P-60 column chromatography	Bhandari et al. 2023

1.3.6 Immobilization of microbial phytase

The immobilization of enzymes is a process in which the enzyme is physically confined within a matrix so that it can avoid extremely harsh conditions like variations in temperature and pH. It restricts the structural and functional changes that occur due to environmental conditions (Filippovich et al. 2023). Besides, it increases the stability of the enzyme and can be reused for biocatalytic reactions. The immobilization process is greener, highly efficient, and economical. The immobilization process is dependent upon the interaction between the enzyme and support matrix and can be done by various approaches- adsorption, encapsulation, entrapment, cross-

linking, and covalent bonding (Filippovich et al. 2023, Pragya et al. 2021). The use of nanoparticles in the immobilization is latest in this field and more efficient for the food and feed industry (Pragya et al. 2021**,** Filippovich et al. 2023). The type of immobilization technique adopted for the process must be selected carefully as it affects the properties of the enzyme. Also, the matrix used in the immobilization should be cheaper, compatible, inert, physically strong, easily dissolved with enzyme (hydrophilic), and resistant to microbes (Gampa et al. 2023). Immobilized enzymes are advantageous as they protect the enzyme from denaturation, provide favourable surroundings for catalysis and have no loss of activity (Filippovich et al. 2023). Immobilization of phytase with several techniques has been mentioned in the literature (Coutinho et al. 2020, Lopes et al. 2021, Pragya et al. 2021, Gampa et al. 2023).

The different matrix have been used for the immobilization of microbial phytases. A phytase isolated from *A. oryzae* SBS50 was immobilized on aluminum oxide activated with glutaraldehyde, showing better characteristics useful for the poultry feed industry than free enzyme (Gampa et al. 2023). Also, this enzyme was immobilized with calcium alginate and found effective in food ingredients (Pragya et al. 2023a). Coutinho et al. (2020) investigated the immobilization of phytase onto hydroxyapatite nanoparticles and applied it in animal feed because of its high thermostability, acidic pH, protease resistance nature, and 100% recovery rate. Similarly, phytase was immobilized on monodisperse mesoporous silica nanoparticles via physical adsorption (Xin et al. 2020**)**. Another study showed the effect of KIT-6 silica mesoporous containing phytase on *Medicago truncatula* plant and reported that it is effective in releasing P for plant growth (Trouillefou et al. 2015). Sirin et al. (2017) immobilized the phytase of *Geobacilllus* sp. TF16 in chitosan and Ca-alginate matrix and found that chitosan-associated phytase represents 38% efficiency while phytase with Ca-alginate matrix depicts 42%. They further analyzed the effect of both immobilized phytases in dephytinizing soymilk. The comparison of free and immobilized phytase was examined by entrapping *Penicillium purpurogenu* GE1 on alginate/carrageenan beads (Awad et al. 2015) and encapsulated phytase with alginate (Hidayatullah et al. 2020). The cross-linking method is a carrier-free approach that mainly depends on the covalent association between the molecules. It requires a cross-linking agent that makes a complex of enzymes with surrounding molecules. Tirunagari et al. (2018) mixed the phytase enzyme in soymilk and formed CLEA (cross-linked enzyme aggregates) phytase with 100% efficacy. Similarly, phytase-bound magnesium nanoparticles were cross-linked with graphene oxide (Dutta et al. 2017). Phytase obtained from *Lactarius*

piperatus mushroom was immobilized on magnetic chitosan nanoparticles, and its dephytinizing effect was examined in cereal products (Onem et al. 2016). The phytase isolated from cowpea seed was immobilized into polyvinyl alcohol (PVA) nanofiber, and its biochemical properties were compared with free phytase (Kamaci and Peksel 2022**).** Belho and Ambasht (2021) immobilized phytase on chitosan microspheres activated via glutaraldehyde and studied its structure and kinetic properties. The phytase was immobilized with iron-modified zeolite and its effect was determined in food and feedstuffs (Lopes et al. 2021). The immobilization of phytase on monodisperse mesoporous silica nanoparticles through the adsorption method (Xin et al. 2020) and on carbon nanotubes (Naghshbandi and Moghimi 2020) along with its physiochemical characteristics has been studied as well.

1.3.7 Applications of microbial phytase

Phytases have diversified applications in different fields like human nutrition, animal nutrition, aquaculture, plant growth, therapeutic, bioethanol formation, and other biotechnological applications. Since the target applications are broad, a wide range of microbial phytases are used. For instance, poultry requires neutral phytase whereas acidic phytase performs well in pigs and fish. Also, the optimal temperature of phytase varies with poultry and aquaculture feed. (Rizwanuddin et al. 2023a, Singh et al. 2024).

1.3.7.1 Human nutrition

The minerals present inside human food are restricted from utilization due to the presence of phytate complex and cause health disorders. A deficiency of iron intake causes anaemia in human beings mainly young women, newborns, adults, and pregnant women (Sharma et al. 2020). Dephytinization is a process of eliminating phytate from the food grains by supplementing phytase in the food. During the action, inorganic P and other nutritional components are liberated that have been studied as well in wheat flour, rice flour, maize, oats, sorghum, wheat-soy flour blend, sesame oil cake, and soy milk (Saxena et al. 2020, Joudaki et al. 2023, Bhandari et al. 2023). Phytase is also involved in improving the bread quality and nutritive value after adding phytase to the dough. In the presence of phytase, fermentation time is reduced, the pH of the dough remains unchanged, and enhancement occurs in the volume of bread and crumb texture (Handa et al. 2020, Sharma et al. 2020). Chapathi, made from wheat

flour is the staple food in India and contains phytic acid which decreases up to 45% after the addition of *Candida versatilis* phytase (Kaur et al. 2021, Joudaki et al. 2023). The involvement of phytase has been reported in dephytinizing tandoori, naan, rabadi, and other fermented products (Ranjan et al. 2015, Joshi and Satyanarayana 2015).

1.3.7.2 Plant growth

P is an important nutrient component essential for the growth and development of plants (Jatuwong et al. 2020a). In soil, P found in organic and inorganic forms. Organic P contains phytic acid which holds 50-80% of total soil P while the rest available as inorganic P (Singh et al. 2020). Phytic acid is found either adsorbed on clays or as insoluble phytate in acidic and alkaline soils. By chelation, it restricts the minerals essential for soil fertility and plant growth (Sadaf et al. 2022). Chemical fertilizers are used as a substitute for accomplishing the requirement of P in plants. But these fertilizers are of high cost, having high transportation rate and low quality of P and leading to environmental pollution. The supplementation of phytase is an effective and sustainable approach for the mobilization of P from the breakdown of insoluble phytate complexes (Joudaki et al. 2023). In the rhizosphere, phytase-possessing microbes are present that hydrolyze phytic acid and release P, which gets utilized by the plant to improve its growth (Kaur et al. 2021). Also, phytase-containing microbes such as plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF) or direct phytase can be incorporated into the soil for the degradation of insoluble phytate (Singh et al. 2024). Transgenic plants can also be developed by inserting a phytase gene inside the plant so that P becomes accessible for utilization. Such plants contain low levels of phytic acid in their seeds (Singh et al. 2020, Kaur et al. 2021).

1.3.7.3 Formation of semi-synthetic peroxidase

Peroxidases are those enzymes that catalyze the oxidation reactions of H_2O_2 (Kaur et al. 2021). Due to the structural similarity between vanadium-dependent peroxidase and acid phosphatases, phytase is involved in the synthesis of haloperoxidase. Vanadium acts as a substitute against phosphate analog and inhibit the activity of phosphatase enzymes (Sharma et al. 2020). When the incorporation of vanadium ion takes place in the active site of HAP, it

forms a semi-synthetic peroxidase, which catalyzes the enantioselective oxidation of prochiral sulphides with H_2O_2. Among the metal oxoanions, only vanadate leads to the formation of semisynthetic peroxidase (Singh et al. 2024). The modification of phytase into haloperoxidase through insertion of vanadium has been reported in *P. anomala* (Joshi and Satyanarayana 2015), *S. thermophile* (Singh et al. 2018) and *A. oryzae* (Pragya et al. 2023b). These semi-synthetic peroxidases are involved in pharmaceutical uses such as anti-microbial properties, diagnostic purposes, immunoassays (Kaur et al. 2021).

1.3.7.4 Lower myo-inositol phosphates

Upon hydrolysis of phytate, various myo-inositol intermediates are released. Lower myo-inositol forms (IP, IP2, IP3, IP4, IP5) are involved in the signaling process (transmembrane and calcium signaling pathway) {Sharma et al. 2020}. They are pharmaceutically important as involved in anti-inflammatory, antiangiogenic, chronic renal failure, and other health disorders (Fig. 1.4) {Handa et al. 2020}. They also act as drug blockers by inhibiting the enzyme. Food containing unsaturated fatty acid undergoes oxidation reaction leading to spoilage. Phytase acts as a natural, potent, and non-toxic antioxidant. It possesses anti-neoplastic and anti-tumor properties, thus preventing the growth of cancer-causing cells in breasts, cervical, and prostate. It works against coronary heart disease, restricting the agglomeration of platelets hence limiting cardiovascular disorders. Phytase governs the release of insulin and controls diabetic conditions. Phytase is also involved in preventing tooth decay that occurs due to demineralization (Sharma et al. 2020).

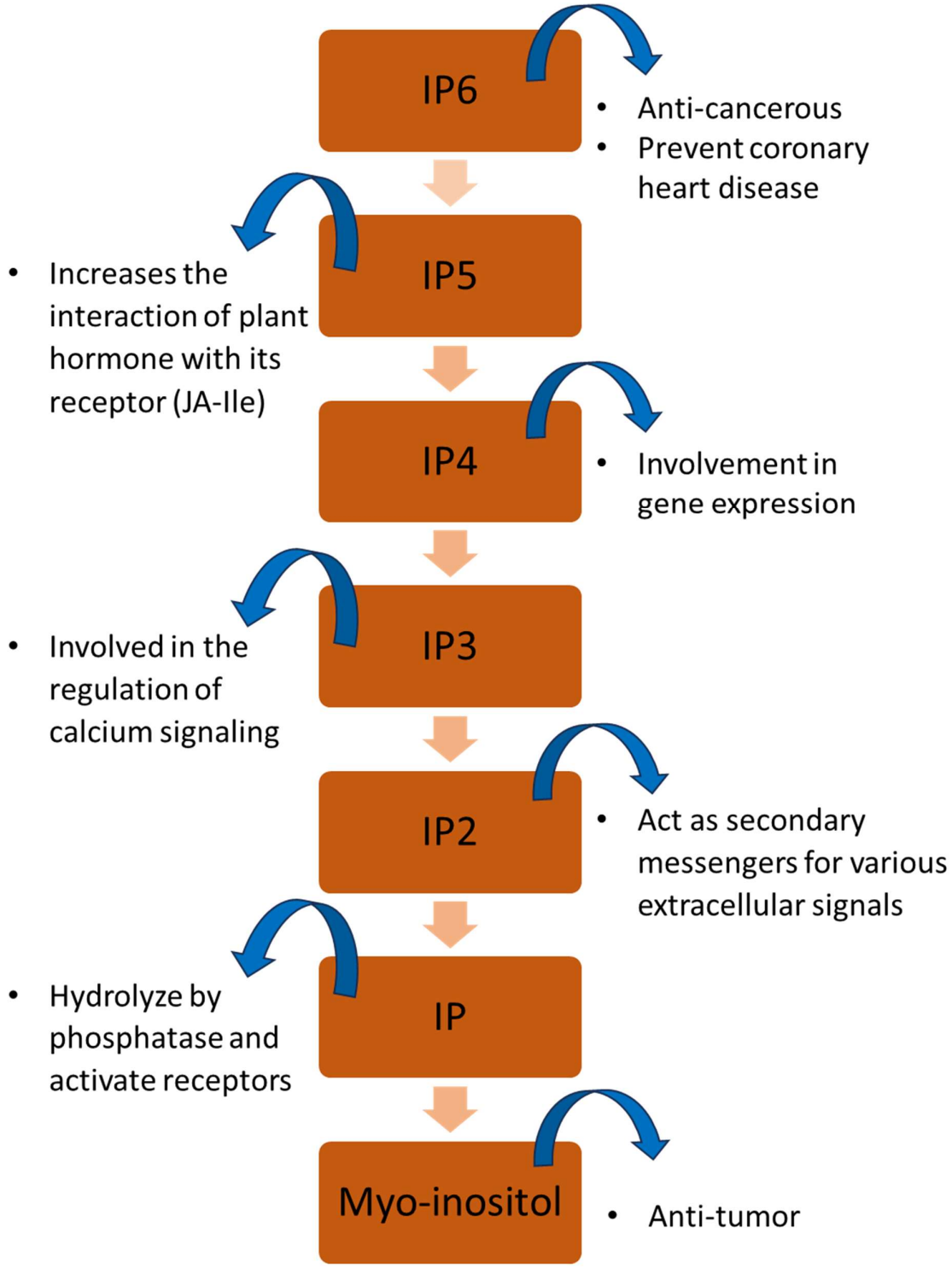


Fig. 1.4 Role of IP6 and its lower inositol esters

1.3.7.5 Bioethanol production

Though phytase is not directly involved in the bioethanol formation, it increases the production of starchy and lignocellulosic biomass (Kumari and Bansal 2021a), supports degrading waste, and makes economical bioethanol along with other enzymes (Rebello et al. 2017). In the paper and pulp industry, the aging of paper is the main issue that needs to be considered. Therefore, phytase is employed to detach phytate content from industrial raw materials. Hydrolysis of phytate is safer and releases non-toxic and non-carcinogenic by-products during the process (Rebello et al. 2017, Zhou et al. 2022). Phytase is used as a biosensor in the detection of estrogenic activity in wastewater (Kaur et al. 2021). In agriculture, organophosphorus pesticides are generally used for killing insects and pests that remain in the soil and become a part of the food chain. It is a harmful chemical for animals and human beings. Phytase deteriorates this toxic component that accumulates in the environment. (Shah et al. 2017, Singh et al. 2024).

1.3.8 Role of phytase in animal nutrition

The monogastric animals including pigs, hens, turkeys, birds, and fishes lack phytase enzymes, therefore, supplementation of phytase in feed is useful in overcoming the malnutrition and environmental pollution that occurs due to the accumulation of phytate. The purpose of using exogenous phytase in the feed is to ameliorate the P level in animal nutrition and reduce the P level in the environment. Phytase not only enhances the P in the diet but also increases other essential minerals for the monogastric animals. As an additive, phytase contributes 70% to animal feed (Rizwanuddin et al. 2023a). The phytase has been incorporated in more than 90% of poultry and 70% of swine feed (Sharma et al. 2020). There are various phytases used for animal feed at the commercial scale (Table 1.5). Phytase as a feed additive enhances the growth parameters, and digestibility of nutrients and minerals thus improving animal health and nutrition.

Table 1.5 List of commercially available phytases in the market

S. No.	Commercial Name	Source	Company	Type	Reference
1.	Natuphos®	*A. niger*	BASF, Germany	3-phytase	Bentes et al. 2020, Fries et al. 2020
2.	Natuphos®E	Genetically engineered in *A. niger*	BASF Corporation	Hybrid-6-phytase	Rizwanuddin et al. 2023a, Correa and Araujo 2020
3.	Ronozyme®P	*Peniophora lycii* expressed in *A. oryzae*	DSM, Switzerland	6-phytase	Xie et al. 2020, Handa et al. 2020
4.	Ronozyme®NP	Genetically modified in *A. oryzae*	Novazymes/ DSM	6-phytase	Rizwanuddin et al. 2023a, Correa and Araujo 2020, Handa et al. 2020
5.	Ronozyme® HiPhos	*C. braakii* expressed in *A. oryzae*	Novazymes/ DSM	6-phytase	Rizwanuddin et al. 2023a, Correa and Araujo 2020, Handa et al. 2020
6.	Phyzyme®XP	*E. coli* expressed in *Schizosaccharomyces pombe*	Danisco, UK	6-phytase	Handa et al. 2020, Xie et al. 2020, Rizwanuddin et al. 2023a
7.	OptiPhos®	*E. coli* expressed in *Pichia pastoris*	Huvepharma	6-phytase	Handa et al. 2020, Xie et al. 2020
8.	Finase® P/L	*A. niger* expressed in	AB Vista	3-phytase	Dersjant-Li et al. 2015, Jatuwong et al. 2020a

		Trichoderma reesei			
9.	Allzyme® SSF	*A. niger*	Alltech	3-phytase	Rizwanuddin et al. 2023a, Handa et al. 2020
10.	HiPhorius™	Genetically expressed in *A. oryzae*	DSM	-	Rizwanuddin et al. 2023a
11.	AxtraPHY®	*Buttiauxella* spp. expressed in *T. reesei*	Danisco Animal Nutrition, Dupont, UK	6-phytase	Correa and Araujo 2020, Handa et al. 2020
12.	SP, TP, SF	*A. oryzae*	Alko Biotechnology	-	Handa et al. 2020
13.	Amaferm	*A. oryzae*	BioZymes	-	Handa et al. 2020
14.	Quantum®	*E. coli* expressed in *P. pastoris*	AB vista	6-phytase	Handa et al. 2020, Rizwanuddin et al. 2023a
15.	Quantum Blue®	*E. coli* expressed in *T. reesei*	AB vista	6-phytase	Dersjant-Li et al. 2015
16.	Rovabio	*Penicillium funiculosum*	Adisseo	-	Jatuwong et al. 2020a

Most of the phytases used in animal feed belong to the HAP category (Kaur et al. 2021). Phytase degrades phytate and releases P and other nutrients which get utilized by animals (Handa et al. 2020). The addition of phytase (500-1000 U) in the animal diet can be used in place of 1 g of inorganic phosphate from phytate and lower the P level by 30-50% in the environment (Kaur et al. 2021). Phytase is far better than using monocalcium phosphate in the diet, because it decreases 17 times more global warming, 110 times acidification, and 700 times

eutrophication (Rebello et al. 2017). Fig. 1.5 demonstrates factors that significantly affect phytase activity and its response towards animal species.

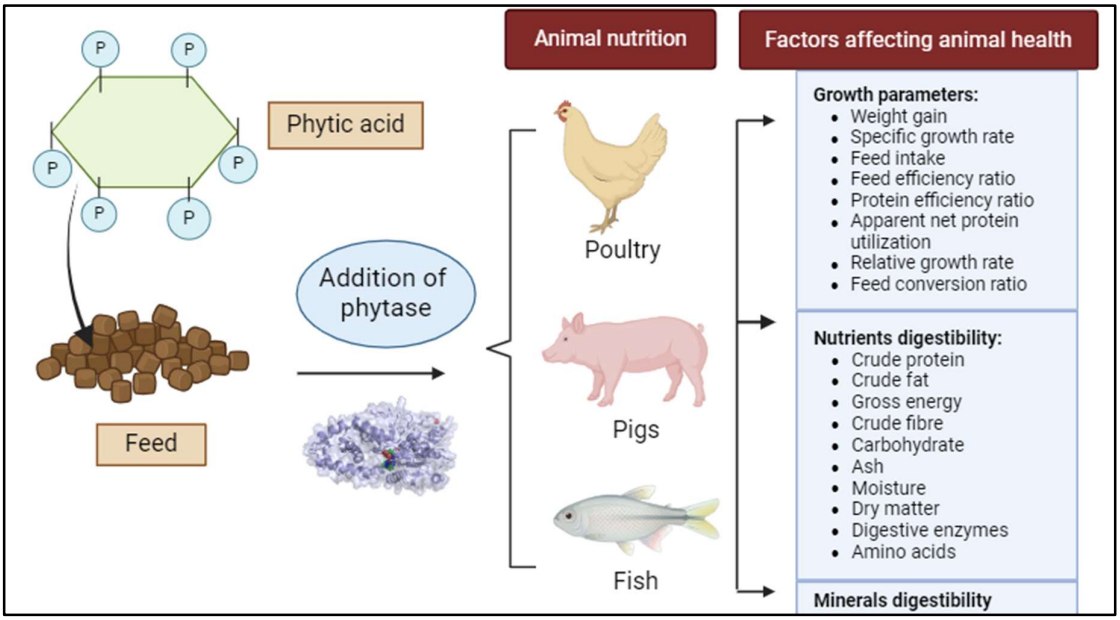


Fig. 1.5 Application of phytase in monogastric animal nutrition and health

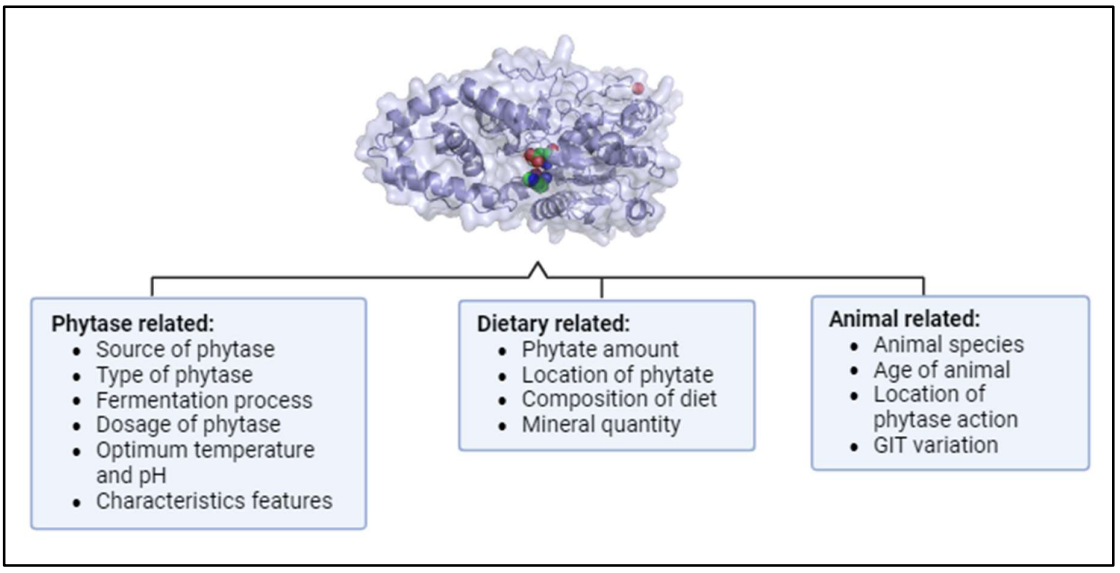


Fig. 1.6 Factors affecting phytase activity in animal nutrition

1.3.8.1 Poultry

The addition of phytase to the broiler diet affects the growth parameters by increasing weight gain, feed intake (FI), and reducing feed conversion ratio. Further, it enhances the utilization of minerals and nutrients necessary for eggshell and bone development (Abd-El-Hack et al. 2018**).** The effect of phytase in improving the growth performance of broilers has been studied by adding phytase developed from *A. niger* in P-deficient diets (Kaur et al. 2021) and *E. coli* and *A. niger* phytase in low-CPME diet (Attia et al. 2021). A balanced ratio of Ca and P affected the growth performance and nutrient digestibility of the broiler (Ajith et al. 2018). In laying hens, supplementation of phytase (5000 FTU/kg) from *E. coli, A. niger,* and *T. reesei* in the diet enhances the quality of the egg, reproductive morphology, and productivity (Saleh et al. 2021). The effect of microbial phytase was also studied in pullets having a diet containing high and low amounts of non-phytate P and observed improvement in pullets fed with low levels of non-phytate P (Jing et al. 2021). Different dose of *A. niger* phytase was provided to the diet and examined its impact on the quality of eggs and utilization of Ca and P in hens (Englmaierova et al. 2015). Providing a high dose of phytase (20,000 FTU/kg) influences the rate of egg production positively (Kim et al. 2017). Phytase with a super dose (≥ 2500 FTU/kg) showed improvement in energy, protein, and phosphorus digestibility in comparison with industrial commercial doses (Woyengo and Wilson 2019).

1.3.8.2 Pigs

The supplementation of phytase to the pigs enhances the nutrients and minerals digestibility by using P from phytate-P. It also reduces the P level in feces (Jatuwong et al. 2020a). The addition of phytase in the pig diet showed that growth parameters were increased in the starter period and mineral levels of Ca and P were enhanced in bones and meat (Buzek et al. 2023). The effect of phytase in improving the mineral digestibility of Ca, P, Mg, Zn, Cu, and Fe was studied at 1000 FTU/kg in grower-finisher pigs (Czech et al. 2022). The impact of engineered phytase on pigs' performance and bone mineralization was evaluated (Wisniewska et al. 2020). The investigation of P level in the diet and body parts of pigs was done to know the involvement of phytase in the availability of P (Tsai et al. 2020). The supplementation of phytase with a super dose amount (2500 FTU/kg) positively influences the health performance of the nursery phase of pigs more than the grow-out phase (Holloway et al. 2019). Similarly, phytase 20,000

FTU/kg dose of phytase showed a significant enhancement in the nutrient digestibility and minerals level in different organs of pigs (Zeng et al. 2015). Irrespective of diet either wheat or corn-based, 500-2000 FTU/kg dose of phytase demonstrated an improvement in nutrient digestibility and retention with the maximum at 2000 FTU/kg dose (Dersjant-Li et al. 2017).

1.3.8.3 Aquaculture

The effect of phytase on fish growth performance can be determined by studying various parameters including weight gain (WG), FI, feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), and apparent net protein utilization (ANPU). Except for FCR, other variables increase in the presence of phytase (Priya et al. 2023a). Relative growth, PER, FI, and FCR increased after the addition of phytase in the *Oreochromis niloticus* diet (Norag et al. 2018; Rachmawati et al. 2018). Besides, the supplementation of feed with phytase has shown improvement in the growth performance of several fishes such as *Clarias gariepinus* (Uzezi et al. 2019), *Labeo rohita* (Ali and Kaviraj 2018), *Cirrhinus mrigala* (Hussain et al. 2017), *Psetta maxima* (Danwitz et al. 2016), *Pelteobagrus fulvidraco* (Cheng et al. 2016), *Ictalurus punctatus* (Chen et al. 2018), *Chanos chanos* (Rachmawati et al. 2017), *Pangasius hypophthalmus* (Rachmawati and Samidjan 2018b), and *Lates calcarifer* (Yudhiyanto et al. 2017). The effect of commercial and non-commercial phytases was compared in *L. rohita* (Roy et al. 2016) and *Cyprinus carpio* (Salman and Alkhafaji 2020) and increased growth was obtained in both cases. The inclusion of phytase in a methionine-supplemented diet showed a significant improvement in the growth of *Myxocyprinus asiaticus* (Chu et al. 2016). A positive effect of neutral phytase was recorded on the growth performance, nutrients, and mineral digestibility of *Carassius auratus* (Nie et al. 2017). Rodrigues et al. (2020) stated that *Rhamdia quelen* acquires high FCR in a sorghum feed with 1500 FTU/kg of phytase.

The supplementation of phytase in the feed also enhances nutrients and minerals accumulation in the bone, plasma, and whole body of fish. The assimilation of crude protein (CP), crude fat, gross energy (GE), and minerals can be determined by calculating the apparent digestibility coefficient (ADC). The digestibility of CP, crude fat, GE, and minerals improved significantly due to the supplementation of fish diet with phytases. The involvement of phytase in increasing protein digestibility in fish is contradictory as in some cases it increases, decreases, or remains unaffected (Baruah et al. 2017). The involvement of phytase in increasing

protein digestibility is not direct, in fact it is an outcome of phytase action on the digestibility of amino acids (Kryukov et al. 2021).

Terrey et al. (2023) studied the effect of microbial phytase with 750 OTU/kg dose in increasing the growth parameters and P retention level in *Salmo salar*. The influence of phytase on *C. gariepinus* performance, antioxidant, and antibacterial properties, and immunological parameters was found significant when mixed with a soybean diet (Adeshina et al. 2023). Supplementation of phytase improves the proximate composition of *L. rohita* fed on a DDGS diet (Naseem et al. 2021). The maximum enhancement in the growth performance, nutrients, and mineral digestibility was observed in *C. carpio* at 950 FTU/kg (Shahzad et al. 2021a), *C. catla* at 900 FTU/kg (Shahzad et al. 2020a) dose of phytase with moringa plant diet. Nutrients (dry matter, CP, GE) and minerals and digestibility increase in *O. niloticus* after adding 1500 FTU/kg phytase to a P-deficient diet. Moreover, the bioavailability of amino acids such as His, Leu, Ile, Phe, Lys, Val, Asp, Thr, Glu, Ala, Pro, Gly, and Ser increased by spraying phytase on the top of pellets (Pontes et al. 2021). Similarly, the addition of 950 FTU/kg phytase in a moringa-based diet helps in retaining maximum nutrients and improves hemato-immunological factors (Shahzad et al. 2021b). A diet containing 20% safflower with 2000 IU/kg phytase positively influences the growth factors and digestibility of *Oncorhynchus mykiss* and lowers the P level in feces (Cantas and Yildirim 2020). In another study, the addition of 2500 FTU/kg dose of phytase in the *O. mykiss* diet enhances their growth and P utilization at 11 and 15 ºC water temperatures (Lee et al. 2020). The role of phytase in degrading phytate and releasing lower esters of myo-inositol in feces was found higher in *O. mykiss* than in *S. salar* (Greiling et al. 2019).

Moreover, phytase helps in retaining more nutrients inside the fish body (Shahzad et al. 2020b). Increased digestibility of minerals such as Fe, Mg, Ca, P, K, Zn, Cu, Na, Al, Mn, Sr, Cr, and Pb has been observed in *O. niloticus* (Sarfraz et al. 2020), *C. mrigala* **(**Hussain et al. 2017), *L. rohita* (Roy et al. 2016), *P. fulvidraco* (Cheng et al. 2016), and *I. punctatus* (Chen et al. 2018) after feeding with phytase-supplemented feed. Besides, increased digestibility of protein was noticed in *C. chanos* (Rachmawati et al. 2017), *O. niloticus* (Rachmawati et al. 2018), *P. hypophthalmus* (Rachmawati and Samidjan 2018b) and *C. carpio* (Rachmawati and Samidjan 2018a). Protein as well as P digestibility was higher when dietary protein and phytase were added to the diet of *C. gariepinus* (Kemigabo et al. 2018). Although the apparent digestibility of protein, ash, and P was higher in *P. maxima*, the absorption of minerals

remained unaffected (Danwitz et al. 2016). A positive correlation between phytase and dietary Ca:P ratio was studied in bone mineralization in *C. gariepinus* {Uzezi et al. (2016, 2017}.

Although phytase is produced at a commercial scale by various companies all over the world, there is still a requirement to develop an economical and efficient microbial phytase that meets all the properties of an ideal phytase, including thermostability, resistance to proteases, and broad pH. The use of modern molecular biology, genetic engineering and bioinformatics tools and techniques combined with fundamental approaches will aid in the identification and development of a single novel and ideal phytase suitable as a feed additive to improve the nutrition and growth performance of monogastric animals. Using such microbial phytase could limit phosphate reserves, improve their health and nutrition, and reduce environmental problems. Many studies are underway to work on these aspects but their applicability in aquaculture feed is the target for future research. In addition, due to the diverse pH conditions of the digestive tract of different species of fish, a single phytase cannot be an ideal additive for fish diets, therefore emphasis should be given to investigating potent phytases.

CHAPTER 2

MATERIALS & METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and Chemicals:

Chemicals – Sodium chloride, Luria-Bertani agar, Nutrient agar, Mueller-Hinton agar, Potato dextrose agar, D-glucose, Calcium chloride, Ammonium sulphate, Potassium chloride, Ferrous sulphate, Magnesium sulphate, Manganese sulphate, Sodium phytate, Agar-Agar, Cobalt chloride, Ammonium vanadate, Ammonium molybdate, Glycerol, Potassium dihydrogen phosphate, Trichloroacetic acid, Sulphuric acid, Hydrochloric acid, Sodium hydroxide, Sodium acetate, Soluble starch, Beef extract, Urea, Calcium phytate, Carboxymethylcellulose, Birchwood xylan, Xylose, p-nitrophenyl laurate, Bovine serum albumin, Sodium carbonate, Copper sulphate, Sodium potassium tartrate, 3,5-dinitrosalicylic acid, Sodium sulphite, Barium chloride, Tris-base, Ferric chloride, β-mercaptoethanol (ß-ME), Sodium azide, Sodium molybdate, Ethylenediaminetetraacetic acid (EDTA), Dithiothreitol (DTT), Trypsin, Pepsin, Hydrogen peroxide (H_2O_2), Sodium alginate, Magnesium chloride, Manganese chloride, Zinc chloride, Lysozyme, Sulfosalicylic acid {Hi-Media, India}, Tween-80, Glacial acetic acid, Phenol crystals, Glycine Acetone, Methanol, Ethanol, Butanol, Isopropanol, Dimethyl sulfoxide (DMSO), Tween-20, Tween-60, Sodium dodecyl sulphate, Triton X 100, Guaiacol {Merck Pvt Ltd, India}, Folin-Ciocalteu reagent, Lactophenol cotton blue {Sisco Research Laboratories (SRL), India}, Dialysis tubing, p-nitrophenyl phosphate, Glucose-6-phosphate, Adenosinetriphosphate (ATP), Adenosine diphosphate (ADP), Adenosine monophosphate (AMP) {Sigma-Aldrich, USA}, Wheat bran, Orange, fish feed, soybean, wheat flour {local market}.

Glassware – Petri plates, flasks (150, 250, 500, 1000 ml), test tubes, Eppendorf, slides and coverslip, reagent bottles (100, 250, 500, 1000 ml), falcons, beaker (100, 250, 500, 1000 ml), measuring cylinder (10, 25, 50, 100, 250, 1000 ml), glass pipette, glass rod, round bottom flask.

Instruments – Incubator (Remi), Laminar (Hicon), Autoclave, Shaker, Microscope, Hemocytometer, -20ºC deep freezer, Hot-air oven, pH meter, Centrifuge (Sigma), weighing balance, vortex, UV-VIS spectrophotometer (Shimadzu), water bath, magnetic stirrer, Lyophilizer, Pelletizer.

Miscellaneous items – Inoculation loop, burner, 70% ethanol, L-shaped spreader, parafilm, tissue, distilled water, cotton plug, sealed plastic bags, micropipette, micropipette tips, funnel, muslin cloth, Oakridge tubes, test tube stand, Eppendorf stand, wash bottle, aluminium foil, spatula, Whatman filter paper no. 1, cuvette, dilution tubes, needle, enamel coated metallic tray, wrap roll, magnetic beads, ice box, thread, scissor, cellotape, syringe, pestle and mortar, mixer-grinder

2.2 Methodology

OBJECTIVE 1: Isolation and identification of potent phytase-producing microbes.

2.2.1 Isolation and screening of microbes

The samples were collected from different conditions like soil beneath the decayed leaves, agricultural land, nursery, manure and rotten orange from Delhi and NCR region (Noida) in a sterile polythene bag separately, and processed further in the laboratory.

2.2.2 Qualitative screening of phytate hydrolyzing activity

The 1 g soil sample was added into 100 ml autoclaved normal saline (0.85% NaCl and 0.1% Tween-80) and serial dilution was done. Inoculated 1 ml suspension from the last diluted tube, spread on an autoclaved agar plate of different media (LB, NA, MHA, PDA, Czapek-Dox) and incubated at 30ºC for a few days. Similarly, the microbes from a rotten orange sample were spread on a PDA plate. The cultures obtained on different agar plates were further screened on PSM plates and incubated at 28ºC. The composition of PSM (g/ml) is as follows: D-glucose (2%), $CaCl_2.2H_2O$ (0.2%), $(NH_4)_2SO_4$ (0.5%), KCl (0.05%), $MgSO_4.7H_2O$ (0.05%), $FeSO_4.7H_2O$ (0.001%), $MnSO_4.H_2O$ (0.001%), sodium phytate (0.4%), agar-agar (1.5%) having pH 5.0 (Suleimanova et al. 2015, Jatuwong et al. 2020b). D-glucose and sodium phytate were filter sterilized. A clear zone of hydrolysis at the centre indicates the presence of phytase activity in the microbes. To avoid false selection of phytase-producing microbes, further screening was carried out. For the double confirmation of the hydrolysis zone, PSM plates were

flooded with 2% cobalt chloride solution and kept for 5 min at room temperature and then replaced with equal volumes of 6.25% ammonium molybdate and 0.42% ammonium vanadate solution. The culture plates were incubated for 5 min at room temperature and then observed the hydrolysis zone after decanting the solution (Bae et al. 1999).

2.2.3 Maintenance of microbes

The phytase-producing microbes were maintained on slants and broth at 4ºC and also stored at -20º C in glycerol stock.

2.2.4 Quantitative screening

For quantitative screening, phytase-containing microbes were cultured in 50 ml liquid PSM (without agar) for phytase production. The 1 ml of microbial inoculum was dissolved in PSM and incubated at 28ºC for 3 days on a shaker with 200 rpm. Whatman filter paper No. 1 was used to filter the phytase production media. The supernatant was used to detect the phytase activity through assay.

2.2.5 Enzyme assay

2.2.5.1 Standard curve for inorganic phosphate

The different concentrations of potassium dihydrogen phosphate (KH_2PO_4) were used to create a standard curve of inorganic phosphate. Stock solution of KH_2PO_4 was prepared and then used to make dilution range from 25-250 µg/ml. In the 1 ml of solution, added 1 ml (10% trichloroacetic acid, TCA), 1 ml distilled water and 0.5 ml 9 N H_2SO_4. Tubes were kept for the incubation period of 10 min at room temperature. Thereafter, 0.5 ml of ammonium molybdate (6%) was added and mixed with vortex and kept for 15 min in dark. Further, 0.5 ml freshly made $FeSO_4$ (8% $FeSO_4$ - 2g in 20 ml of distilled water with the addition of 0.5 ml 9 N H_2SO_4 and made the volume up to 25 ml with distilled water) was added. Mixed it properly, kept it

for 30 min in the dark and took the absorbance at 660 nm with a spectrophotometer against blank (Fiske and Subbarow 1925).

2.2.5.2 Quantitative estimation of phytase

Phytase activity was determined by measuring the release of inorganic phosphate from the substrate at 50°C with pH 5.0 through the Fiske and Subbarow (1925) method. One ml reaction mixture contains 0.5 ml substrate dissolved in 0.1 M sodium acetate buffer (pH 5.0) and 0.5 ml enzyme diluted with buffer. Blank and control (substrate and enzyme) were also set during the experiment, and all were performed in triplicate. Substrate and enzyme control represented 0.5 ml substrate with 0.5 ml buffer and 0.5 ml enzyme with 0.5 ml buffer respectively. Kept the tubes for 5 min incubation at 50ºC in a water bath. After that, 1 ml TCA was added to stop the reaction. The quantity of inorganic phosphate was measured by the above-mentioned procedure at 660 nm. Phytase activity was calculated using the regression equation of the standard graph of KH_2PO_4 (Sapna and Singh 2013). One unit of phytase is defined as the amount of enzyme used to liberate 1 nmol of inorganic phosphorus per second under assay conditions.

2.2.6 Selection of the best phytase producer microbe from the isolates

The microbe showing the maximum hydrolysis zone and highest phytase activity among the isolates was selected and used in further experiments for the production of phytase.

2.2.7 Identification of selected microbe

2.2.7.1 Morphological identification

The fungal isolate was cultured on a potato dextrose agar media plate and incubated at 30ºC and the plate was regularly observed by the naked eye. Microscopic features were examined under light microscopy. A liquid droplet of distilled water was put on the glass slide and the

mycelium was kept on it. Then, it was torn apart with the help of a needle and stained with lactophenol cotton blue. Kept the coverslip on it and viewed it under the microscope (100X).

2.2.7.2 Molecular identification

Molecular identification was done by ITS sequencing through the ITCC facility available at the Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi. The obtained sequence was compared through the BLAST program and multiple sequence alignment was done by the MEGA software and a phylogenetic tree was also constructed.

2.2.8 Maintenance and culture conditions of *Penicillium oxalicum* PBG30

Inoculation of the *Penicillium oxalicum* PBG30 was done on the potato dextrose agar media and kept in the incubator for 3 days at 30°C. Further, the fungus was maintained on agar slants and glycerol stock and was stored at 4°C and -20°C respectively.

OBJECTIVE 2. Process optimization for the production of microbial phytase.

2.2.9 Preparation of spore suspension of *P. oxalicum* PBG30

The spores were scrapped from a 3-day-old culture via a sterile loop by adding 25 ml autoclaved normal saline solution containing 0.1% Tween 80. The spore suspension was filtered through a sterile filter assembly, and the spore count was measured by a hemocytometer.

2.2.10 Production by solid-state fermentation

Wheat bran was sieved, washed with tap water, and then distilled water. Dried the sample at 55°C in a hot air oven or sun-dried and packed in a sealed polythene bag. Phytase production was carried out by four fermentation media (pH 5.0) in 250 ml Erlenmeyer flasks (Table 2.1).

5 g wheat bran was used as a substrate, moistened with 10 ml media (1:2), and autoclaved at 121°C for 20 min. After that, cool down the flask at room temperature, inoculated with 1ml of spore suspension (7.7 X 10^7 CFU/ml), and kept in the incubator for 3 days at 30°C without shaking. After 3 days of incubation, the enzyme extraction was done by adding 50 ml of normal saline solution in the flask and shaking at 30°C with 200 rpm for 1 h. The extract was filtered by double-layered muslin cloth, and the filtrate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was used to test the phytase activity by using calcium phytate substrate in the assay, and the fungal biomass was dried in an oven at 60°C and measured as dry mouldy residue (DMR). All the production trials were performed in duplicate.

Table 2.1 Different moistening media used for the phytase production

S. No.	Composition	Reference
Media I	Distilled water	Sapna and Singh 2014
Media II	1.5% Starch, 0.6% Beef extract, 0.01% $FeSO_4.7H_2O$, 0.05% KCl	Sapna and Singh 2014
Media III	0.5% $(NH_4)_2SO_4$, 0.5% $MgSO_4.7H_2O$, 0.03% $FeSO_4.7H_2O$, 0.1% NaCl	Sapna and Singh 2014
Media IV	0.5% Urea, 0.1% $MgSO_4.7H_2O$, 0.1% KCl, 0.1% $FeSO_4.7H_2O$	Mahmood et al. 2021

2.2.11 Optimization of phytase by one variable at a time (OVAT) method

OVAT is an approach to optimize the production of enzymes by considering one variable at a single time and keeping other variables constant. Factors such as wheat bran amount, substrate and moistening media ratio, temperature, pH, and incubation days were studied to know the effect of these parameters on phytase production.

2.2.11.1 Wheat bran concentration

The 5, 10, and 15 g of wheat bran were used in the production media (0.5% Urea, 0.1% $MgSO_4.7H_2O$, 0.1% KCl and 0.1% $FeSO_4.7H_2O$) with a moisture ratio of 1:2. The media was autoclaved and incubated for 3 days at 30°C with pH 5.0 and 1 ml of spore suspension. The

culture filtrate was obtained and centrifuged at 10000 rpm for 10 min at 4°C. The assay determined phytase activity in the supernatant.

2.2.11.2 Ratio of substrate and moistening media

10 g wheat bran was mixed with a moistening agent in different ratios of 1:1, 1:1.5, 1:2, 1:2.5, and 1:3. Autoclaved the media at 121°C for 20 min and kept for cooling at room temperature. Add 1 ml fungal inoculation and kept for incubation at 30°C for 3 days, and phytase assay was performed with supernatant.

2.2.11.3 Different incubation temperature

The 1 ml spore suspension was inoculated in fermentation media, and incubated at different temperatures of 25°C, 30°C, 35°C and 40°C, and the activity of phytase was evaluated from supernatant.

2.2.11.4 Different incubation time

The *P. oxalicum* PBG30 spore suspension (1 ml) was mixed into production media and kept in an incubator at 30°C for 1-7 days and phytase activity was analysed after each time interval.

2.2.11.5 Different pH

The production media was set with varied pH conditions (4-8) and autoclaved. After that, flasks were inoculated with 1 ml of *P. oxalicum* PBG30 spore suspension and incubated at 30°C for 5 days. Later on, the amount of phytase activity was measured.

2.2.12 Presence of other hydrolytic enzymes in the crude extract obtained from *P. oxalicum* PBG30

After the optimization of parameters, the phytase production was carried out by 10 g wheat bran dissolved with 20 ml moistening agent of pH 7.0 in a 1:2 ratio, incubated at 30ºC for 5 days. The extraction was done and different enzyme assay was performed and the activity was mentioned in U/g DMR.

2.2.12.1 Phytase assay

Phytase activity was assessed at 70°C with calcium phytase substrate through the Fiske and Subbarow (1925) method (as mentioned above).

2.2.12.2 Cellulase assay

The reaction mixture includes 0.5 ml crude extract and 0.5 ml carboxymethyl cellulose substrate (1%), both dissolved in buffer and incubated at 70º C for 5 min (Sapna and Singh 2014). The cellulase activity was determined by released reducing sugars measured via dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of activity is defined as the amount of enzyme required to liberate 1 nmol of reducing sugar from glucose per sec under the reaction.

2.2.12.3 Xylanase assay

Xylanase activity was estimated by using birchwood xylan as a substrate dissolved in buffer (Sapna and Singh 2014). Similarly, the reaction was conducted at 70ºC for 5 min. The quantity of liberated sugars was determined by using the DNS method (Miller, 1959). One unit is defined as the quantity of enzyme used in the release of 1 nmol sugars from xylose per sec per ml in the reaction mixture.

2.2.12.4 Amylase assay

The amylase activity was analyzed by incubating crude extract with 1% soluble starch substrate at 70ºC for 5 min (Sapna and Singh 2014). The reducing sugars were quantified by using the DNS method (Miller, 1959). One unit is defined as the quantity of enzyme needed to liberate 1 nmol of reducing sugars from glucose per sec per ml under the reaction conditions.

2.2.12.5 Lipase assay

The lipase assay was assessed by mixing the crude extract with p-nitro phenyl laurate substrate solution (Singh and Satyanarayana 2006) and observing the absorbance at 410 nm every min up to 10 min. One unit is defined as the amount of enzyme required to release 1 nmol of p-nitrophenol from the substrate per sec per ml from the reaction.

2.2.13 Analytical procedures for estimating reducing sugars and protein

2.2.13.1 Soluble protein content

The amount of soluble protein was measured by Lowry et al. (1951) method and Bovine serum albumin (BSA) was used as a reference. In the 0.5 ml reaction sample, 2.5 ml reagent D [98 ml reagent A (2% sodium carbonate dissolved in 0.1 N sodium hydroxide), 1 ml reagent B (0.5% copper sulphate) and 1 ml reagent C (1% sodium potassium tartrate)] was added and kept for 15 min at room temperature. In the dark surroundings, 250 µl reagent E (equal ratio of Folin-Ciocalteu reagent and distilled water) is added and vortexed immediately and kept for 25-30 min at room temperature in the dark. Absorbance was taken at 660 nm with blank.

2.2.13.2 Reducing sugars

Reducing sugars were quantified by the DNS method (Miller, 1959) with a standard curve of glucose. DNS reagent was prepared by mixing 10 g/l of sodium hydroxide, 2 g/l of phenol crystals and 2 g/l of 3,5-dinitrosalicylic acid. Before using, 0.05% (w/v) sodium sulphite was added to it. To 1 ml of sample solution, 1 ml of DNS reagent was added and boiled for 20 min in a water bath set at 100º C. Cooled down the solution and added 200 µl of sodium potassium tartrate (33% w/v). The absorbance was noted at 540 nm along with blank.

2.2.14 Statistical optimization

Statistical optimization includes two steps; the first one is Plackett Burman Design (PBD) and the next one is Response Surface Methodology (RSM).

2.2.14.1 Plackett-Burman Design

The Plackett-Burman method helps identify the significant factors useful for producing phytase. In this design, a total of 11 variables (n) were considered with their high (+) and low (-) concentration values and 12 runs of the experiment (n+1) were performed (Table 2.2). Each experiment has an equal number of positive (n+1/2) and negative (n-1/2) balance. The selected independent factors were wheat bran, pH, incubation days, nitrogen source (ammonium sulphate, urea), carbon source (starch, glucose), metal ions (ferrous sulphate, calcium chloride, magnesium sulphate) and detergent (Tween 80). The difference between the average of one

variable at high and low levels provides the impact of that variable on the production, which was calculated by

$$E(Xi) = \frac{2(\Sigma Pi+ - \Sigma Pi-)}{N} \ldots\ldots\ldots\ldots\ldots\ldots\ldots (Eq.\ 2.1)$$

Here, E(Xi) denotes the impact of each variable, Pi+ and Pi- means high and low values, and N means the total number of runs performed in the experiment. The PB experimental design is represented in Table 2.2. All the runs were carried out in duplicate and phytase activity was calculated, and the critical factors were determined by pareto chart.

Fig. 2.1 Optimization of phytase production by PBD

Table 2.2 Factors studied during the Plackett Burman experiment

Run No	A	B	C	D	E	F	G	H	I	J	K
1	5	5	5	1	2	0	1	0.05	0.0	0.5	0
2	10	5	3	0	2	2	1	0.00	0.2	0.5	0
3	10	8	3	1	2	0	1	0.00	0.0	0.1	1
4	5	8	3	0	0	2	1	0.05	0.0	0.5	1

5	5	8	5	1	0	2	1	0.00	0.2	0.1	0
6	10	5	5	0	0	0	1	0.05	0.2	0.1	1
7	10	8	3	1	0	0	0	0.05	0.2	0.5	0
8	5	5	3	1	2	2	0	0.05	0.2	0.1	1
9	5	8	5	0	2	0	0	0.00	0.2	0.5	1
10	5	5	3	0	0	0	0	0.00	0.0	0.1	0
11	10	5	5	1	0	2	0	0.00	0.0	0.5	1
12	10	8	5	0	2	2	0	0.05	0.0	0.1	0

{A – Wheat bran (g), B – pH, C – Incubation period (days), D – Urea (% w/v), E – Starch (% w/v), F – Glucose (% w/v), G -Ammonium sulphate (% w/v), H – Ferrous sulphate (% w/v), I – Calcium chloride (% w/v), J – Magnesium sulphate (% w/v), K – Tween 80 (% v/v)}

2.2.14.2 Response Surface Methodology

Four independent factors pH (A), incubation days (B), $MgSO_4$ (C), and Tween 80 (D) obtained from PBD, were evaluated further by the RSM experiment. The optimal level and interaction effect of these parameters on phytase production is determined using central composite design (CCD) Version 6.0.7, Stat-Ease and Minneapolis, MN (RSM). In addition, 0.5% ammonium sulphate and 0.01% $FeSO_4$ supplied as a supplement. Five different levels (-α, -1, 0, +1, +α) were assigned to these parameters, and 30 experimental runs were conducted as shown in Tables 2.3 & 2.4.

Table 2.3: Parameters with their assigned level in RSM for phytase production

S. No.	**Parameters**	**Level of parameters**				
		-α	**-1**	**0**	**+1**	**+α**
1.	pH	5	6	7	8	9
2.	Incubation days	3	4	5	6	7
3.	Tween 80 (%)	0.5	1	2	3	3.5
4.	$MgSO_4$ (%)	0.25	0.50	0.75	1.00	1.25

Production of phytase is measured by the following equation:

$$Y = ß_0 + ß_1A + ß_2B + ß_3C + ß_4D + ß_{11}A^2 + ß_{22}B^2 + ß_{33}C^2 + ß_{44}D^2 + ß_1ß_2AB + ß_1ß_3AC + ß_1ß_4AD + ß_2ß_3BC + ß_2ß_4BD + ß_3ß_4CD \quad (Eq.\ 2.2)$$

(Y- phytase production, $ß_0$ - intercept, $ß_1$, $ß_2$, $ß_3$, $ß_4$ - linear coefficients, $ß_{11}$, $ß_{22}$, $ß_{33}$, $ß_{44}$ - squared coefficients, $ß_1ß_2$, $ß_1ß_3$, $ß_1ß_4$, $ß_2ß_3$, $ß_2ß_4$, $ß_3ß_4$ - interaction coefficients, A, B, C, D, AB, AC, AD, BC, BD, CD – variables and their interaction. Statistical software was used to design the experiment and produce 3-D contour plots. In this method, predicted and experimental values were compared and statistical analysis was done by ANOVA.

Fig. 2.2 Optimization of phytase production by CCD of response surface method

Table 2.4 Experimental design for RSM by CCD

Run No	pH	Incubation period (days)	Tween 80 (%)	Magnesium sulphate (%)
1	7	5	2.0	0.75

2	6	4	3.0	0.50
3	7	5	2.0	1.25
4	6	6	1.0	0.50
5	8	6	3.0	1.00
6	6	3	1.0	0.50
7	8	6	3.0	0.50
8	9	5	2.0	0.75
9	7	5	0.5	0.75
10	6	4	1.0	1.00
11	6	6	3.0	1.00
12	6	6	1.0	1.00
13	7	4	2.0	0.75
14	7	4	2.0	0.75
15	8	4	1.0	0.50
16	7	5	2.0	0.75
17	6	4	3.0	1.00
18	7	5	3.5	0.75
19	8	4	3.0	0.50
20	8	4	1.0	1.00
21	8	6	1.0	0.50
22	6	6	3.0	0.50
23	7	5	2.0	0.75
24	7	7	2.0	0.75
25	8	4	3.0	1.00
26	8	6	1.0	1.00
27	5	5	2.0	0.75
28	7	5	2.0	0.75
29	7	5	2.0	0.25
30	7	5	2.0	0.75

2.2.15 Optimization of phytase production on a large scale

The optimized parameters chosen through PBD and RSM were used to enhance the phytase activity and sustainability on a large scale. Phytase production was carried out in flasks (250-1000 ml) and enamel-coated metallic trays (27 cm x 22 cm x 5 cm, 35 cm x 32 cm x 5 cm) containing wheat bran (5, 10, 20, 100, 150 g) moistened with the medium in a 1:2 ratio. The flasks were autoclaved at 121ºC for 20 min and cooled down. Inoculated with a spore suspension of *P. oxalicum* PBG30, mixed with a sterile spatula, covered with a clean wrap for trays, and incubated for 5 days at 30ºC. The samples were collected from different sections of the tray, and extraction was performed, as mentioned earlier.

Fig. 2.3 Phytase production in trays on a large-scale

OBJECTIVE 3. **Purification and characterization of the microbial phytase.**

2.2.16 Phytase purification

The phytase was partially purified by conventional methods and concentrated by salt precipitation, dialysis and lyophilization.

2.2.16.1 Ammonium sulphate precipitation

The crude enzyme solution was taken in the beaker and kept on the magnetic stirrer. The amount of ammonium salt was added slowly to the extract until it reached 80% saturation. The entire procedure was performed at 4°C, followed by overnight incubation to settle down the precipitated enzyme. Centrifuged at 10,000 rpm for 10 min at 4°C and re-suspended the pellet in a minimum volume of 0.1 M sodium acetate buffer (pH 3.0). The phytase activity and soluble protein were calculated from the sample.

2.2.16.2 Lyophilization

The crude extract was concentrated in a powder form in a round bottom flask by lyophilizer. The phytase activity was estimated in the lyophilized sample by dissolving powder in a minimum volume of 0.1 M sodium acetate buffer (pH 3.0).

2.2.16.3 Dialysis

A desired length of the dialysis membrane was cut and activated via pre-treatment. Cooled down the membrane, filled it with precipitated enzyme and tied from both sides. The dialysis bag was dipped in the 20 mM sodium acetate buffer (pH 3.0) and kept over a magnetic stirrer at 4°C. The buffer was changed at least three times and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was used to check the phytase activity and protein content.

2.2.17 Characterization of partially purified phytase enzyme

2.2.17.1 Effect of temperature on phytase activity

The optimum value of reaction temperature was determined by analyzing phytase assay at different temperature ranges from 30°C to 80°C in 0.1 M sodium acetate buffer (pH 3.0) with 1 mM calcium phytate as a substrate.

2.2.17.2 Effect of pH on phytase activity and stability

The optimum value of pH was estimated at 70°C by dissolving phytase in a buffer of different pH range 2.5-8.0 (0.1 M glycine buffer pH 2.5, 0.1 M sodium acetate buffer pH 3.0-6.0, 0.1 M tris buffer pH 7.0-8.0) and the assay was conducted.

2.2.17.3 Thermal stability of phytase enzyme

The thermostability of phytase was evaluated by incubating phytase at 70 and 80 °C in a buffer of pH 3.0 for 2 h. Samples were taken at 0, 5, 10, 30, 60 and 120 min, and the assay was performed at 70°C (pH 3.0).

2.2.17.4 pH stability of phytase enzyme

The pH stability of phytase was assessed by mixing phytase in the buffer of pH (3.0, 5.0) and incubated at 70°C. Samples were taken at 0, 5, 10, 30, 60 and 120 min, and the assay was carried out at 70°C (pH 3.0).

2.2.17.5 Determination of substrate specificity

The specificity of phytase towards different substrates, viz, sodium phytate, calcium phytate, glucose-6-phosphate, p-nitrophenyl phosphate, ATP, ADP and AMP were examined. The phytase assay was performed at 70°C with pH 3.0. Calcium phytate (1 mM) is considered as a reference and its value was taken as 100%.

2.2.17.6 Effect of organic solvents on phytase activity

Different organic solvents (ethanol, methanol, butanol, isopropanol, acetone, DMSO) with concentrations of 2 and 5% were provided to the reaction mixture and a phytase assay was performed.

2.2.17.7 Effect of detergents on phytase activity

The experiment was conducted to determine the influence of detergents (SDS, Tween-20, Tween-60, Tween-80, Triton-X) on the activity. In the reaction mixture, different concentrations of detergent (0.1 and 0.5%) were added and the assay was carried out.

2.2.17.8 Effect of metal ions on phytase activity

The effect of metal ions was studied by incubating phytase with different metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Na^{+}) with 1 mM and 5 mM concentrations in the reaction mixture. The enzymatic assay was performed along with the control.

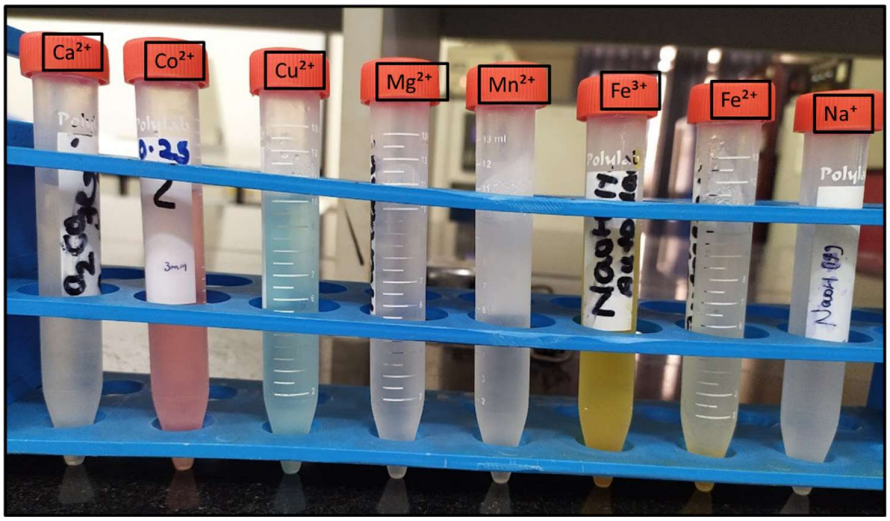


Fig. 2.4 Stock solution of different metal ions

2.2.17.9 Effect of inhibitors on phytase activity

The effect of inhibitors on phytase activity was analyzed by providing different inhibitors (sodium azide, EDTA, sodium molybdate, ß-ME, DTT) in the reaction mixture with 1 mM and 5 mM concentrations and a phytase assay was conducted.

2.2.17.10 Determination of kinetic parameters

The kinetic values (K_m and V_{max}) were evaluated from the Lineweaver-Burk plot by using different concentrations of calcium phytate (0.1-2 mM) and the assay was estimated at 70°C (pH 3.0).

2.2.17.11 Effect of proteolytic enzymes on phytase activity

The phytase enzyme was incubated with 1% of trypsin and pepsin for 2 h at 37°C. The samples were taken at the desired interval (0, 30, 60 and 120 min) and a phytase assay was performed.

2.2.17.12 Effect of storage conditions on phytase activity

Phytase was kept for 6 months in different conditions (room temperature, 4°C, -20°C) and its activity was determined at the desired interval. Activity on the 0^{th} day is considered 100%.

2.2.17.13 Effect of vanadium on the phytase activity

Phytase was dissolved with ammonium metavanadate (5 and 10 µM) and incubated at 30°C for 4 h with 100 rpm. Added H_2O_2 and guaiacol to the solution and observed the absorbance at 470 nm for each minute up to 10 min. The relative activity of phytase was also calculated during the experiment.

OBJECTIVE 4. Immobilization and Application of the phytase in the feed industry.

2.2.18 Immobilization of phytase through the calcium alginate method

Partially purified phytase was mixed with 3% sodium alginate (dissolved in buffer) on a magnetic stirrer. A sterile syringe was used to add the mixture drop by drop into 0.2 M calcium chloride solution (chilled) in the form of beads. The prepared beads were kept in the solution for 1.5 - 2 h, washed with distilled water 3 - 4 times, and then stored in a buffer at 4ºC. The phytase activity was determined by using 5 or 10 beads in a reaction tube.

2.2.19 Reusability of immobilized phytase

For the determination of reusability, the assay was carried out at 70ºC with 10 beads of immobilized phytase in a reaction mixture. The beads were removed and used again in the next reaction and the step was repeated up to 5 cycles. The first cycle is considered to show 100% activity.

2.2.20 Applications of phytase in the feed industry

2.2.20.1 Preparation and hydrolysis of insoluble phytate salts:

Metal phytates (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}) were formed by mixing each metal ion along with sodium phytate. The 100 mM stock solution of metal ions ($CaCl_2.2H_2O$, $CoCl_2.2H_2O$, $FeSO_4.7H_2O$, $MgCl_2.6H_2O$, $MnCl_2.4H_2O$, $ZnCl_2.7H_2O$) and 10 mM of sodium phytate were prepared in distilled water separately (Sapna and Singh 2013). Both were mixed in equal volume and kept at 4°C for overnight incubation. The precipitated salts were centrifuged at 10000 g for 5 min at 4°C. Discard the supernatant, wash the pellet three times with distilled water and finally suspend in 5 ml of 0.1 M Na-acetate buffer (pH 3.0). Each phytate salt was treated with 20 U of phytase and incubated at 37°C and 50°C. Samples were taken at different intervals (3, 6, 12, 24 h) and salts were pelleted by centrifuge. The supernatant was used to determine the amount of released inorganic phosphate at 600 nm.

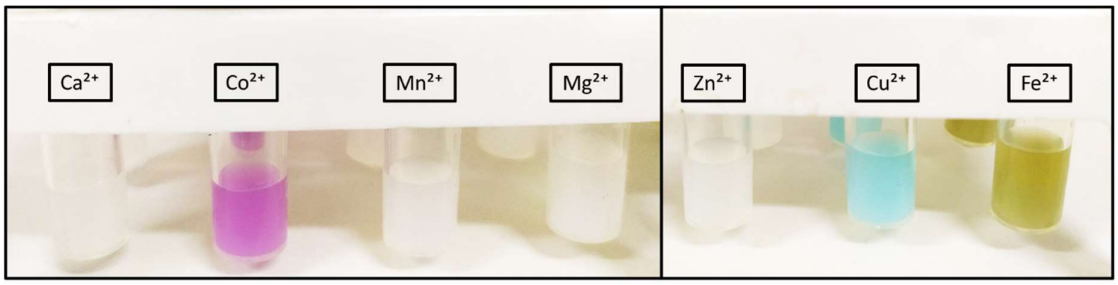


Fig. 2.5 Insoluble salt complex after mixing of phytate with different metal ions

2.2.20.2 Preparation and hydrolysis of insoluble protein-phytate:

The protein-phytate complex was made by adding an equal amount of lysozyme (2.5 mM) in distilled water and sodium phytate (3 mM) in distilled water and kept overnight at 4ºC. Centrifuge the solution at 10000 g for 5 min at 4ºC and discard the supernatant. Washed the pellet three times with distilled water, dissolved in buffer and 20 U of phytase was added into it. The reaction was incubated at 30ºC and samples were collected at defined intervals (0, 5, 10, 30, 60, 120 and 360 min). Centrifuged the sample, and inorganic phosphate was estimated in the supernatant at 600 nm.

2.2.20.3 Dephytinization of fish feed by *P. oxalicum* PBG30 phytase

The commercial fish feed was brought from the local market of Delhi. The feed was ground into fine powder by pestle and mortar and washed with distilled water. Dried the sample at 55°C in a hot air oven and kept it in a sealed polythene bag.

2.2.20.3.1 Effect of phytase in dephytinizing feed at different temperatures

One gram feed was dissolved in 20 ml sodium acetate buffer (0.1 M, pH 3.0) and autoclaved at 121°C for 20 min. Phytase from *P. oxalicum* PBG30 (80 U) was mixed and incubated at 37°C, 50°C, and 70°C with shaking at 100 rpm. Samples were taken at different time intervals, kept at 4°C, and centrifuged at 10,000 rpm for 10 min. The supernatant was used to detect inorganic phosphate, reducing sugars and soluble protein content by Fiske and Subbarow (1926), Miller's DNS method (Miller 1959) and Lowry et al. (1951), respectively.

2.2.20.3.2 Effect of phytase in dephytinizing feed with different doses of phytase

The 1 g of commercial fish feed was dissolved in 20 ml sodium acetate buffer (0.1 M, pH 3.0), mixed with different phytase doses (25-200 U) and incubated at 37°C. Samples were taken at desired intervals for the estimation of inorganic phosphate, reducing sugars and soluble protein content.

2.2.20.3.3 Effect of phytase in dephytinizing feed with different feed amounts

Different amounts of feed varied from 0.5-2 g were dissolved in 20 ml sodium acetate buffer (0.1 M, pH 3.0) with supplementation of 200 U of phytase. The mixtures were kept in an incubator at 37ºC and samples were collected within a definite period. Further, inorganic phosphate, reducing sugars and soluble protein content were measured.

2.2.20.3.4 Effect of incubation time in dephytinizing feed

The 0.5 g of commercial fish feed was dissolved in 20 ml sodium acetate buffer (0.1 M, pH 3.0). Kept for incubation at 37ºC after the addition of phytase. Samples were collected up to 48 h and nutritional by-products were estimated.

Fig. 2.6 Processing of commercially brought feed from market

2.2.20.4 Determination of phytic acid content in fish feed

2.2.20.4.1 Preparation of experimental diets

The formulation of the feed sample is mentioned in Table 2.5 containing 35% protein in the diet. The control diet contains plant protein sources without the supplementation of phytase. Other experimental diets include the composition of the control diet along with different doses of crude phytase (500 FTU/kg, 1000 FTU/kg, 1500 FTU/kg). One FTU is defined as the amount of enzyme used to liberate 1µmol of inorganic phosphorus from calcium phytate under the test conditions. All the feed ingredients were mixed properly and pellets of different experimental diets were formed with the help of a pelletizer and dried overnight at 30ºC. After drying, pellets were kept in a sealed polythene bag and stored at 4°C for further analysis.

Table 2.5 Formulation of experimental feed

Feed ingredients (g)	**Diet I (Control)**	**Diet II**	**Diet III**	**Diet IV**
Soybean flour	181.57	181.57	181.57	181.57
Wheat flour	118.40	118.40	118.40	118.40
Fish oil	3.00	3.00	3.00	3.00
Vitamins and minerals	1.20	1.20	1.20	1.20
Phytase	-	500 FTU/kg	1000 FTU/kg	1500 FTU/kg

2.2.20.4.2 Standard curve of sodium phytate for phytic acid measurement

For the quantification of phytic acid in a sample, a standard of phytic acid needs to be made. Here, the sodium phytate is used as a reference. A stock solution of 1 mg/ml was prepared and further diluted to make a 0.1 mg/ml solution. Next, the dilutions were made from 2.5-25 µg/ml from this solution. In the 1 ml solution of different concentrations, 500 µl Wade reagent was added (mixture of 0.03% ferric chloride and 0.3% sulfosalicylic acid) and kept for incubation of 20 min. The absorbance was taken at 500 nm by UV-VIS spectrophotometer. Distilled water is used as blank. All the experiments were performed in triplicate.

Mixture of feed ingredients

Formation of pellets

By pelletizer

Pellets

Fig. 2.7 Pelleting of feed was done with the help of pelletizer

2.2.20.4.3 Estimation of phytic acid level in prepared fish feed

The determination of phytic acid in the phytase-treated and untreated feed pellet was performed by using Wade reagent colorimetric method (Wade and Morgan 1955). Firstly, the sample was treated with 10 ml of 2.4% HCl and kept on a shaker for 16 h at 220 rpm. Centrifuged at 10,000 rpm for 20 min at 10°C and the obtained supernatant was shifted to falcon consisting of 1 g NaCl. Again, the mixture was shaken at 350 rpm for 20 min so that salts dissolved completely and settled it by keeping at 4°C for 60 min or -20ºC for 20 min. Centrifuge for 20 min at 10,000 rpm. The supernatant was diluted by 25X and used to estimate the phytic acid content by adding 500 µl Wade's reagent and kept for 20 min incubation. The absorbance was taken at 500 nm (Gao et al. 2007). The phytic acid was calculated from the regression equation of the sodium phytate standard.

Fig. 2.8 Pellets of different feed with and without phytase treatment

CHAPTER 3

RESULTS
&
DISCUSSION

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Results

OBJECTIVE 1: Isolation and identification of potent phytase-producing microbes.

3.1.1 Isolation of microbes

The total of 55 isolates collected from the various samples, such as soil beneath the decayed leaves, agricultural land, nursery, manure and rotten orange. These cultures were grown on agar plates of different media, Luria-Bertini (LB), Nutrient agar (NA), Mueller-Hinton agar (MHA), Potato dextrose agar (PDA), and Czapek-Dox, as shown in fig. 3.1.

3.1.2 Qualitative screening for phytase activity

A massive screening was conducted to isolate potent phytase producers. The qualitative screening was done based on the plate assay, and isolates were screened on PSM plates to observe the phytate hydrolyzing activity. Phytase activity was shown by the zone of hydrolysis formed in the surroundings of the isolates (Fig. 3.2). Among isolated microbes, only 8 isolates represented phytase activity. The zone of hydrolysis was also confirmed by double staining method.

Fig. 3.1 Isolation and culturing of microbes on different agar plates

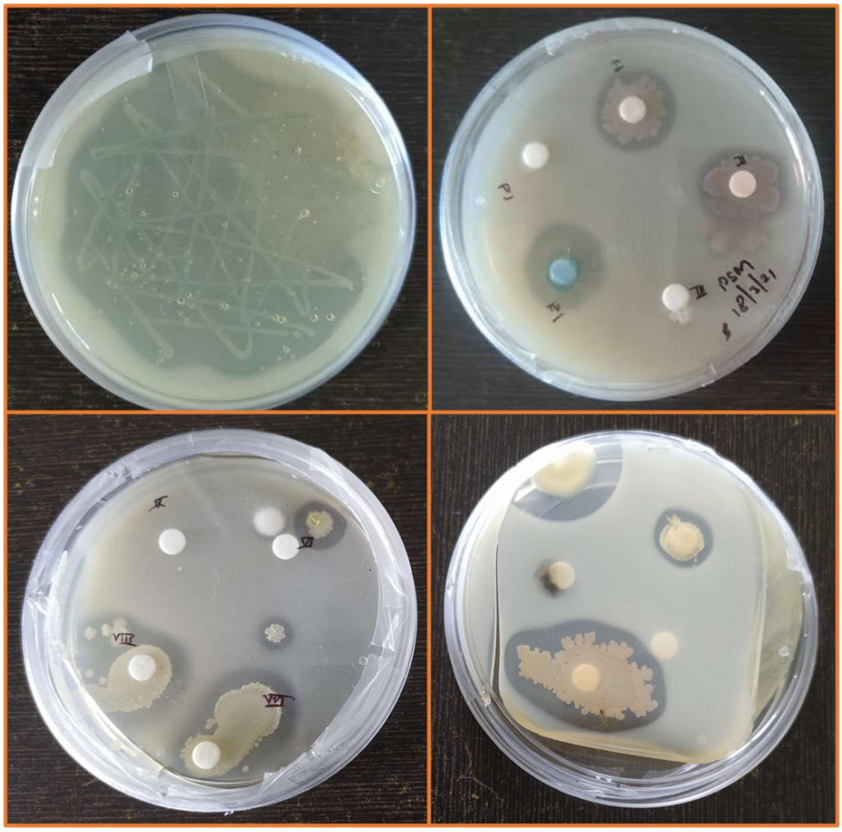


Fig. 3.2 Formation of hydrolysis zone by microbes on PSM plates

3.1.3 Quantitative screening

For the estimation of the quantity of phytase enzyme, these isolates were cultured in the liquid PSM. The supernatant was used to determine the phytase activity through phytase assay. The phytase activity was found in the range from 9.085 – 51.40 U/ml. The highest activity was recorded in case of isolate 4 (51.40 ± 0.05 U/ml), while the lowest is obtained with isolate 8 (9.085 ± 0.08 U/ml), as shown in Table 3.1. Hence, isolate 4 is selected further for phytase production through the solid-state fermentation method.

Table 3.1 Quantitative estimation of phytase production through microbes

Sample	Phytase (U/ml)
Isolate 1	9.635±0.05
Isolate 2	18.237±0.37
Isolate 3	17.087±0.16
Isolate 4	51.40±0.05
Isolate 5	27.005±0.03
Isolate 6	13.669±0.13
Isolate 7	18.054±0.05
Isolate 8	9.085±0.08

3.1.4 Identification of selected fungal isolate

The isolated fungus was grown on a PDA plate for 3 days at 30ºC. Fig. 3.3 shows the morphological growth of the fungus. The fungus was initially white and became dark green on maturation. The texture of the colonies was velvety and had powdery growth. Under the microscope, the fungal hyphae were septate, branched conidiophores and conidia (fungal spores) were found in a chain with conidiophore (Fig. 3.5), a characteristic feature of Penicillium genus.

The isolated fungus was confirmed as *Penicillium oxalicum* by ITS sequencing analysis. The sequence was amplified by PCR and then BLAST and MSA were performed to obtain sequence similarity. The phylogenetic tree was constructed by MEGA software (Fig. 3.6). PBG30 showed 99% similarity with *P. oxalicum* strain WZ-119 (MN856268.1).

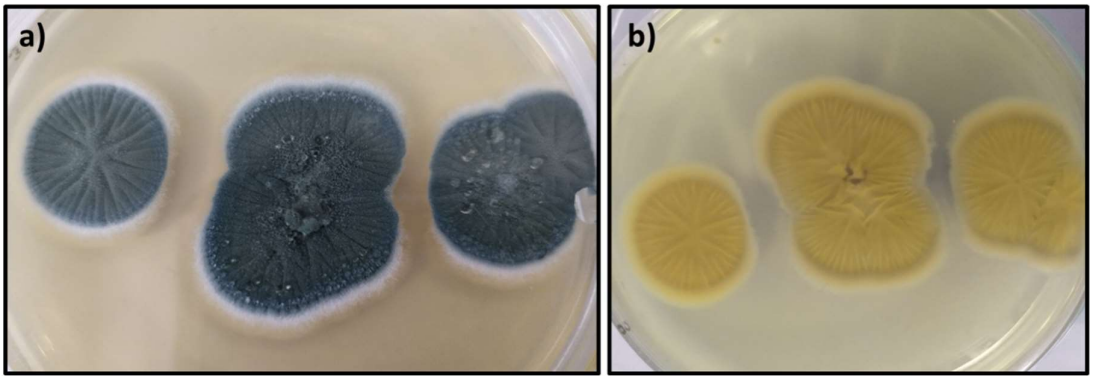


Fig. 3.3 The growth of *Penicillium oxalicum* on PDA plate (a) front side (b) backside

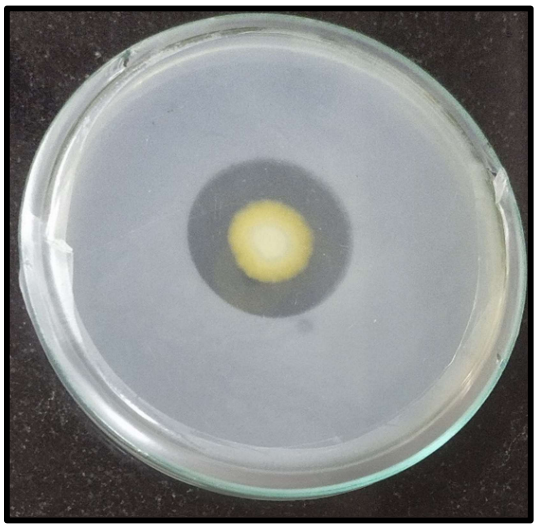


Fig. 3.4 A zone of hydrolysis formed by *Penicillium oxalicum*

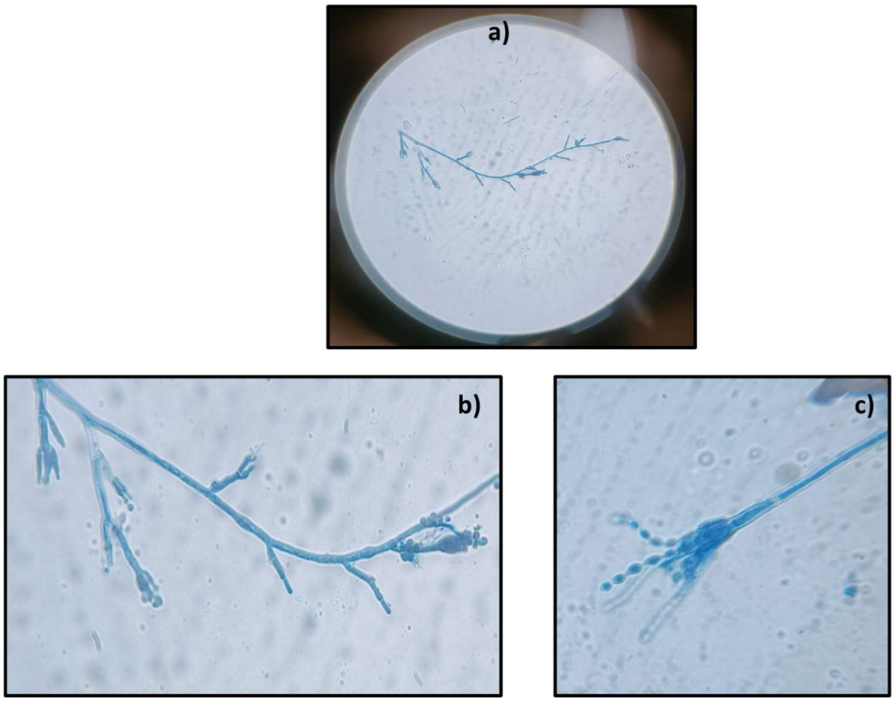


Fig. 3.5 Microscopic view of *P. oxalicum* shows (a) fungal hyphae (b) branched conidiophores (c) chain of conidia

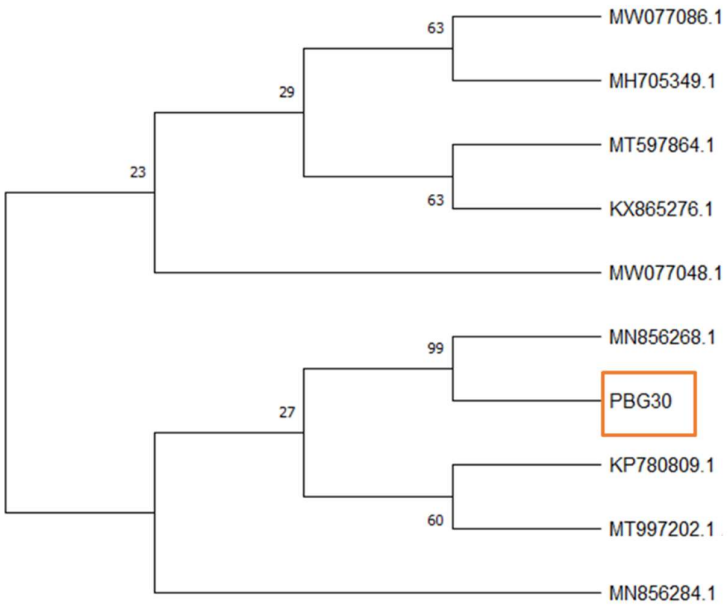


Fig. 3.6 Phylogenetic tree representing the isolated fungus as *P. oxalicum* PBG30

OBJECTIVE 2. Process optimization for the production of microbial phytase.

3.1.5 Phytase production in SSF by using wheat bran

Among the different production media for SSF, media IV (0.5% Urea, 0.1% $MgSO_4.7H_2O$, 0.1% KCl and 0.1% $FeSO_4.7H_2O$) supports the maximum phytase production (84.600 ± 2.54 U/g DMR) with wheat bran, as shown in Fig. 3.7. However, the phytase activity from other moistening media was considerable low.

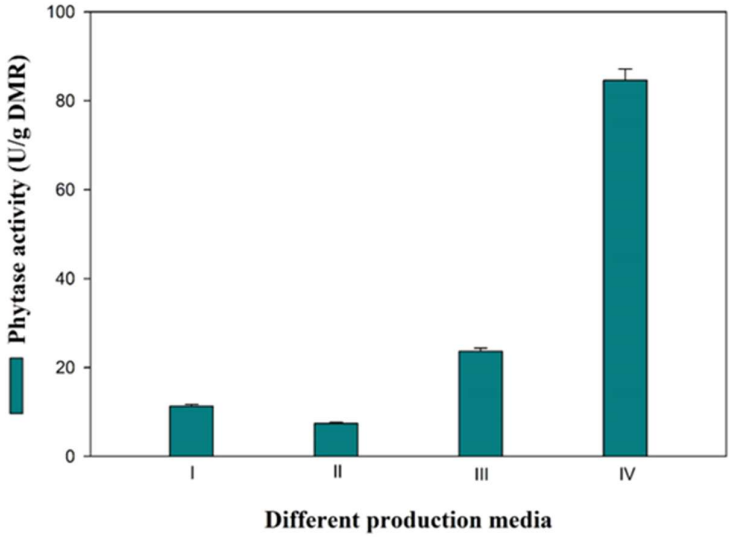


Fig. 3.7 Production of *P. oxalicum* PBG30 phytase on wheat bran by using different production media

3.1.6 Optimization of phytase production by OVAT

3.1.6.1 Effect of wheat bran amount

Production media is supplemented with different amounts of wheat bran (5, 10, 15 g), and the best results come out with 10 g wheat bran (98.85 ± 2.96 U/g DMR). A significant amount of phytase was produced with 5 g wheat bran. However, a high amount of wheat bran leads to a

decline in the production level. As shown in Fig. 3.8, there was a decrease in the phytase activity on applying 15 g of wheat bran.

3.1.6.2 Effect of temperature

Different temperatures (25°C, 30°C, 35°C, 40°C) were given to the production media. The highest yield of phytase was obtained (102.50 ± 3.07 U/g DMR) at 30°C temperature. However, there was a sharp decrease in the production level at higher temperatures (Fig. 3.9). More than 50% activity was reported at 25°C and 35°C, while the lowest production was observed at 40°C.

3.1.6.3 Effect of substrate and moisture ratio

Wheat bran is mixed with a moistening solution in the ratio of 1:1, 1:1.5, 1:2, 1:2.5, 1:3, and the maximum phytase (121.02 ± 3.63 U/g DMR) was reported with 1:2 ratio of substrate and moistening media. Below and above this ratio, there was a reduction in the phytase amount (Fig. 3.10). Approximately, a similar amount of phytase was obtained with a 1:1.5 and 1:3 ratio and the minimum activity was obtained with a 1:1 ratio.

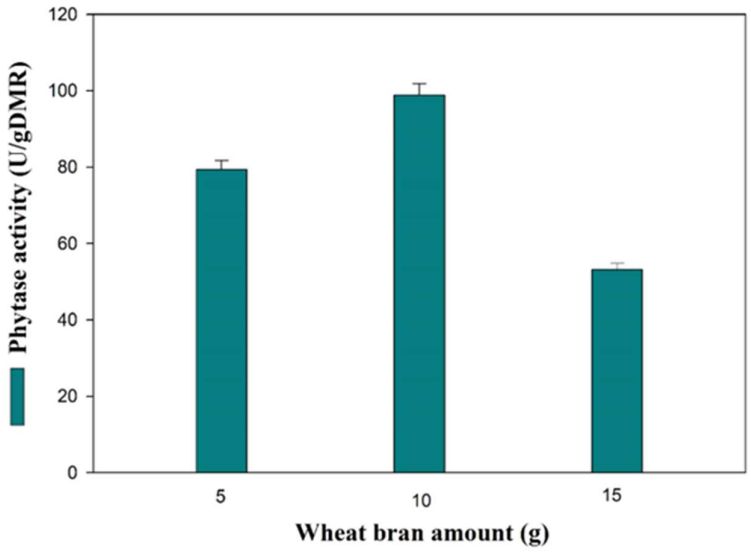


Fig. 3.8 Production of *P. oxalicum* PBG30 phytase with different concentrations of wheat bran

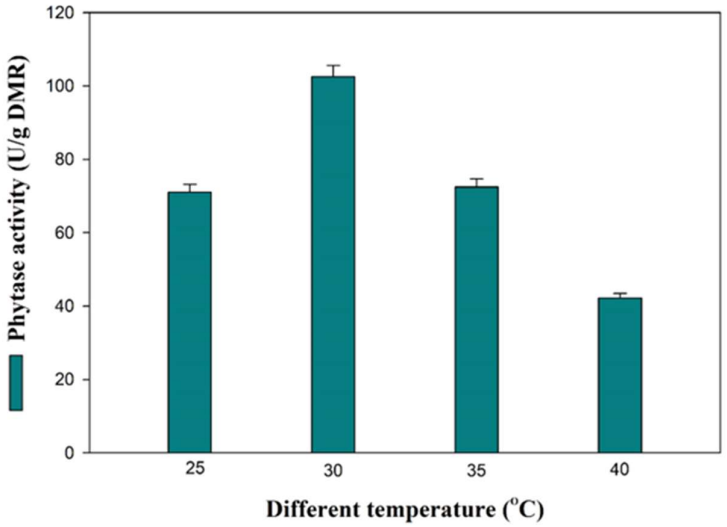


Fig. 3.9 Production of *P. oxalicum* PBG30 phytase with different temperatures

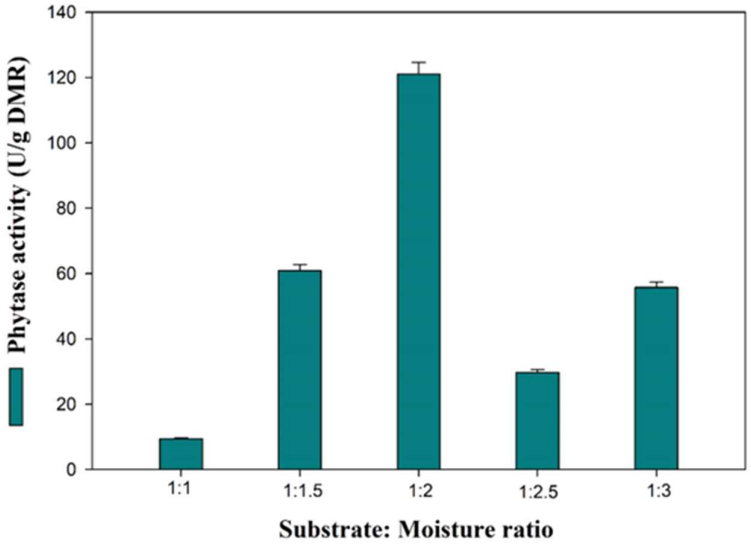


Fig. 3.10 Production of *P. oxalicum* PBG30 phytase with different substrate to moisture ratio

3.1.6.4 Effect of incubation days

The maximum phytase production (153.41 ± 4.60 U/g DMR) was obtained after 5 days of incubation of fermentation media followed by a reduction in the phytase level due to restriction of *P. oxalicum* PBG30 growth in the absence of nutrients. A linear increase in the phytase level was acquired with an increase in the incubation period (Fig. 3.11).

3.1.6.5 Effect of pH

The different pH conditions (4, 5, 6, 7, 8) were provided for the growth of *P. oxalicum* PBG30 and phytase production. The highest yield was obtained at pH 7.0 (200.41 ± 6.01 U/g DMR), depicting the neutral nature of phytase. Also, phytase production was higher with pH 8.0 as compared to the acidic range. More than 50% of activity was reported in the pH range of 6.0-8.0 (Fig. 3.12).

After applying the OVAT approach in SSF, a 2.4-fold enhancement occurred in the production level of phytase. The production level was increased from 84.600 ± 2.54 U/g DMR to 200.41 ± 6.01 U/g DMR.

3.1.7 Presence of other hydrolytic enzymes

The highest amount of phytase production was achieved (200.41 ± 6.01 U/g DMR) with 10 g wheat bran moistened with media (0.5% Urea, 0.1% $MgSO_4.7H_2O$, 0.1% KCl and 0.1% $FeSO_4.7H_2O$) in 1:2 ratio of pH 7.0, incubated at 30°C for 5 days via OVAT. Along with phytase, *P. oxalicum* PBG30 exhibited the presence of other hydrolytic enzymes such as amylase, xylanase, cellulase, and lipase. The activity of cellulase, xylanase, amylase, and lipase were 51.06, 18.05, 86.20, and 7.05 U/g DMR respectively.

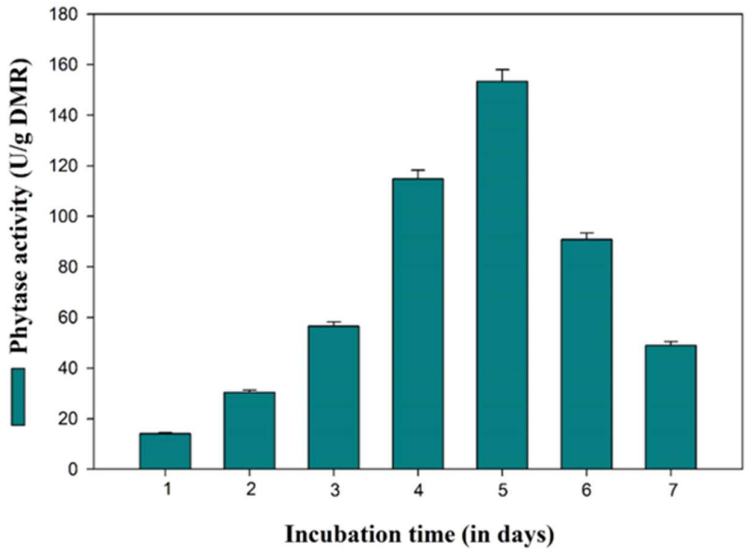


Fig. 3.11 Production of *P. oxalicum* PBG30 phytase with different incubation time

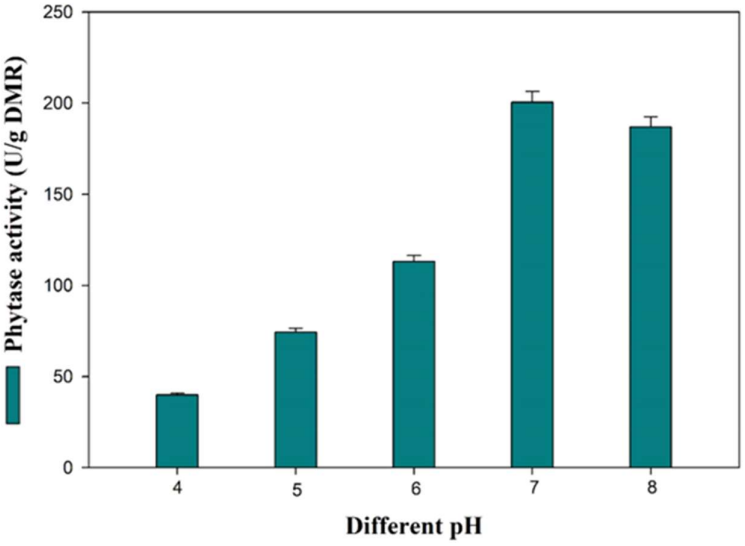


Fig. 3.12 Production of *P. oxalicum* PBG30 phytase with different pH

3.1.8 Statistical optimization of phytase production by PBD

A PBD experiment was performed with 11 variables and 12 experimental runs with their high (+) and low (-) level. Table 3.2 shows the phytase production of 12 experimental runs. A pareto chart was created to determine the impact of variables on phytase production (Fig. 3.13). The positive and negative values in the pareto graph show the importance of individual components in production. Run no. 9 shows the maximum output.

Table 3.2 Factors studied during the Plackett Burman experiment

Run No	A	B	C	D	E	F	G	H	I	J	K	Phytase Activity (U/g DMR ± S.E)
1	5	5	5	1	2	0	1	0.05	0.0	0.5	0	108.56 ± 10.01
2	10	5	3	0	2	2	1	0.00	0.2	0.5	0	24.01 ± 15.51
3	10	8	3	1	2	0	1	0.00	0.0	0.1	1	150.5 ± 31.80
4	5	8	3	0	0	2	1	0.05	0.0	0.5	1	239.81 ± 0.08
5	5	8	5	1	0	2	1	0.00	0.2	0.1	0	97.24 ± 8.84
6	10	5	5	0	0	0	1	0.05	0.2	0.1	1	102.29 ± 3.93
7	10	8	3	1	0	0	0	0.05	0.2	0.5	0	59.36 ± 18.96
8	5	5	3	1	2	2	0	0.05	0.2	0.1	1	97.54 ± 14.40
9	5	8	5	0	2	0	0	0.00	0.2	0.5	1	268.77 ± 0.03
10	5	5	3	0	0	0	0	0.00	0.0	0.1	0	5.75 ± 1.94
11	10	5	5	1	0	2	0	0.00	0.0	0.5	1	102.06 ± 39.92
12	10	8	5	0	2	2	0	0.05	0.0	0.1	0	137.578 ± 1.47

A – Wheat bran (g), B – pH, C – Incubation days, D – Urea (% w/v), E – Starch (% w/v), F – Glucose (% w/v), G -Ammonium sulphate (% w/v), H – Ferrous sulphate (% w/v), I – Calcium chloride (% w/v), J – Magnesium sulphate (% w/v), K – Tween 80 (% v/v)

This experiment concluded that among the 11 factors, pH, incubation days, $MgSO_4$, and Tween 80 are the critical variables that contribute more to the production of *P. oxalicum* PBG30 phytase, and others have low contributions and thus are less significant.

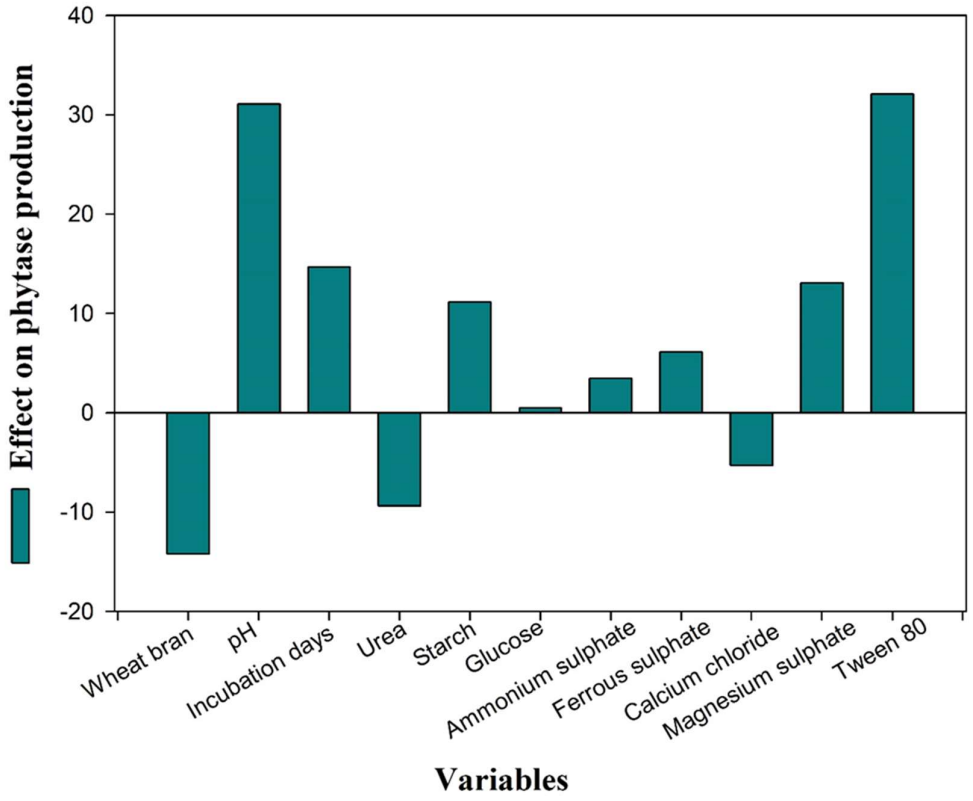


Fig. 3.13 Pareto chart showing the screening of variables through Plackett Burman experiment

3.1.9 Optimization by Response Surface Methodology

The parameters selected through PBD were further analyzed with RSM by the CCD model to determine the optimum level of the variables. A total of 30 experiments were designed via CCD using 5 g wheat bran, 0.5 % ammonium sulphate, and 0.01% ferrous sulphate as fixed parameters and pH, incubation period, magnesium sulphate, and Tween 80 as essential parameters (Table 3.3). The best result of phytase activity (373.32 U/g DMR) was found in the flask containing 5 g of wheat bran moistened with 10 ml of media including 0.5% ammonium sulphate, 0.01% $FeSO_4$, 3.5% Tween 80 and 0.75% $MgSO_4$ with pH 7.0 and 5 days of incubation. The phytase production was elucidated by the following model equation:

$$Y\ (U/g\ DMR) = 117.85 - 3.12\ A - 20.67\ B + 79.26\ C - 7.61\ D - 26.70\ A^2 + 48.85\ C^2 - 9.95\ D^2 + 8.64\ AB - 7.87\ AC - 12.28\ AD - 29.37\ BC + 5.33\ BD - 17.49\ CD \quad (Eq.\ 3.1)$$

ANOVA is used for evaluating the data designed by the RSM experiment as shown in Tables 3.4 & 3.5. The Pred R^2 value is 0.9571 depicting the correlation between the predicted and experimental values. R^2 value, i.e., the coefficient of determination, is found to be 0.99, indicating 99% variability within the model's parameters. Adequate precision gives us a signal-to-noise ratio that should be > 4 for better results (Joshi and Satyanarayana 2015). The ratio in this model comes out to 44.209, delivering more than a sufficient signal to navigate the design space. The significance of the model is determined by the F value, which is 127.35 in our experiment, indicating that the model is significant and Prob>F was less than 0.05, which implies that there is only a 0.01% chance that the occurrence of the F value is due to noise (Pragya et al. 2023a). Significant model terms were B, C, D, A^2, C^2, D^2, AB, AC, AD, BC, BD, and CD which significantly affected the phytase production in SSF.

Table 3.3 Phytase activity of RSM experimental runs

Run No	pH	Incubation Period (days)	Tween 80 (%)	Magnesium sulphate (%)	Phytase activity (U/g DMR)	
					Experimental value	Predicted value
1	7	5	2.0	0.75	114.74 ± 14.70	117.85
2	6	4	3.0	0.50	278.39 ± 17.17	297.11
3	7	5	2.0	1.25	64.48 ± 6.39	62.82
4	6	6	1.0	0.50	12.27 ± 1.85	18.60
5	8	6	3.0	1.00	120.42 ± 7.47	124.90
6	6	3	1.0	0.50	51.28 ± 6.91	50.25
7	8	6	3.0	0.50	177.51 ± 12.47	188.98
8	9	5	2.0	0.75	13.28 ± 1.74	4.82
9	7	5	0.5	0.75	109.57 ± 6.68	108.87
10	6	4	1.0	1.00	60.27 ± 8.02	62.80
11	6	6	3.0	1.00	141.69 ± 6.17	154.13

12	6	6	1.0	1.00	76.52 ± 6.48	73.58
13	7	4	2.0	0.75	144.09 ± 7.34	138.52
14	7	4	2.0	0.75	145.74 ± 8.06	138.52
15	8	4	1.0	0.50	44.36 ± 4.56	45.92
16	7	5	2.0	0.75	114.74 ± 14.70	117.85
17	6	4	3.0	1.00	264.27 ± 18.60	260.82
18	7	5	3.5	0.75	373.32 ± 3.28	346.66
19	8	4	3.0	0.50	278.09 ± 1.28	282.42
20	8	4	1.0	1.00	25.71 ± 8.49	30.45
21	8	6	1.0	0.50	65.12 ± 5.90	69.95
22	6	6	3.0	0.50	172.45 ± 15.59	169.09
23	7	5	2.0	0.75	114.74 ± 20.79	117.85
24	7	7	2.0	0.75	82.73 ± 7.41	76.52
25	8	4	3.0	1.00	191.39 ± 14.52	197.01
26	8	6	1.0	1.00	80.52 ± 9.68	75.81
27	5	5	2.0	0.75	24.21 ± 5.56	17.28
28	7	5	2.0	0.75	114.74 ± 14.70	117.85
29	7	5	2.0	0.25	106.99 ± 7.15	93.25
30	7	5	2.0	0.75	114.74 ± 14.70	117.85

Table 3.4 ANOVA for Response Surface Reduced Quadratic Model

Source	**Sum of Squares**	**dF**	**Mean square**	**F value**	**Prob > F**	
Model	2.121E+005	13	16315.40	127.35	< 0.0001	significant
A	226.28	1	226.28	1.77	0.2025	
B	11156.38	1	11156.38	87.08	< 0.0001	
C	1.245E+005	1	1.245E+005	971.51	< 0.0001	
D	1349.22	1	1349.22	10.53	0.0051	
A^2	20241.05	1	20241.05	157.99	< 0.0001	
C^2	28704.97	1	28704.97	224.05	< 0.0001	

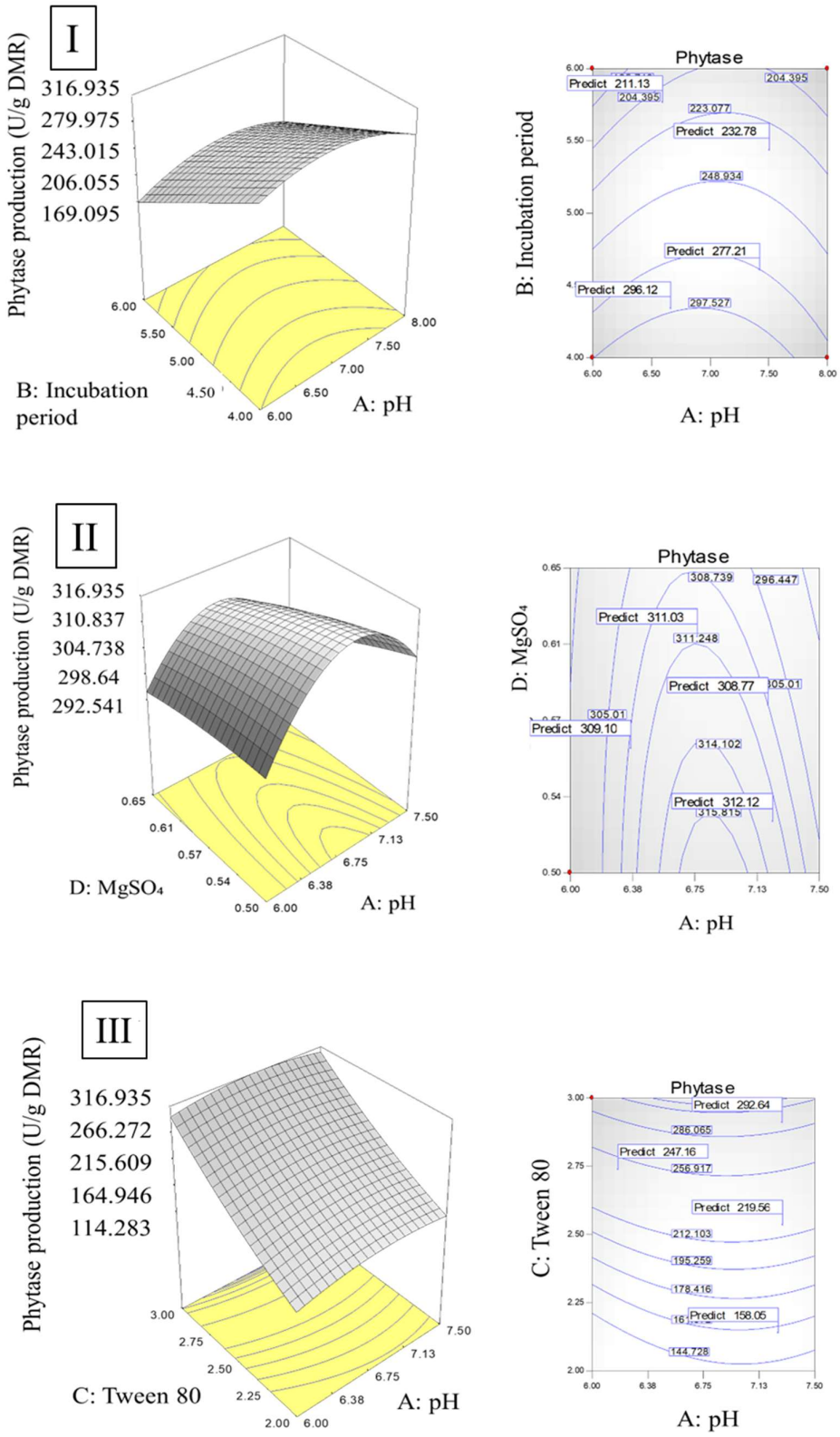
D^2	2813.01	1	2813.01	21.96	0.0002	
AB	1527.12	1	1527.12	11.92	0.0033	
AC	947.85	1	947.85	7.40	0.0151	
AD	2310.40	1	2310.40	18.03	0.0006	
BC	17622.46	1	17622.46	137.55	< 0.0001	
BD	580.75	1	580.75	4.53	0.0491	
CD	4683.10	1	4683.10	36.55	< 0.0001	

Table 3.5 ANOVA regression analysis

Std. Dev.	11.32	R^2	0.9904
Mean	122.61	Adj R^2	0.9827
C.V.	9.23	Pred R^2	0.9571
PRESS	9188.99	Adeq Precision	44.209

Response surface graphs showed the optimum level of parameters and interaction among them on which phytase activity is demonstrated on the Y-axis and influencing parameters on the X-axis and Z-axis (Kumari and Bansal 2021b). The peak denotes phytase production with the optimal value of each variable (Mahmood et al. 2022). Fig. 3.14(I) shows the relation between pH and incubation period. At pH 7.0 and 5 days of incubation, production is higher than below or above the values of both factors. Similarly, the interactions of pH and $MgSO_4$, pH and Tween 80, incubation period and Tween 80, Tween 80 and $MgSO_4$ and incubation period and $MgSO_4$ were shown in 3D graphs and contour plots (Fig. 3.14). The trial showing maximum phytase titre was repeated further to validate the experiment and found to be 339.94 U/g DMR which was closer to the estimated value of 346.66 U/g DMR.

Optimization through statistical methods significantly enhanced phytase production. In our results, the phytase activity showed 4.4-fold enhancement after statistical optimization compared to un-optimized conditions.


I
Phytase production (U/g DMR)
316.935
279.975
243.015
206.055
169.095
B: Incubation period
A: pH
Phytase
Predict 211.13
204.395
223.077
Predict 232.78
248.934
Predict 277.21
Predict 296.12
297.527
B: Incubation period
A: pH
II
Phytase production (U/g DMR)
316.935
310.837
304.738
298.64
292.541
D: MgSO4
A: pH
Phytase
308.739
296.447
Predict 311.03
311.248
Predict 308.77
305.01
Predict 309.10
314.102
Predict 312.12
D: MgSO4
A: pH
III
Phytase production (U/g DMR)
316.935
266.272
215.609
164.946
114.283
C: Tween 80
A: pH
Phytase
Predict 292.64
286.065
Predict 247.16
256.917
Predict 219.56
212.103
195.259
178.416
Predict 158.05
144.728
C: Tween 80
A: pH

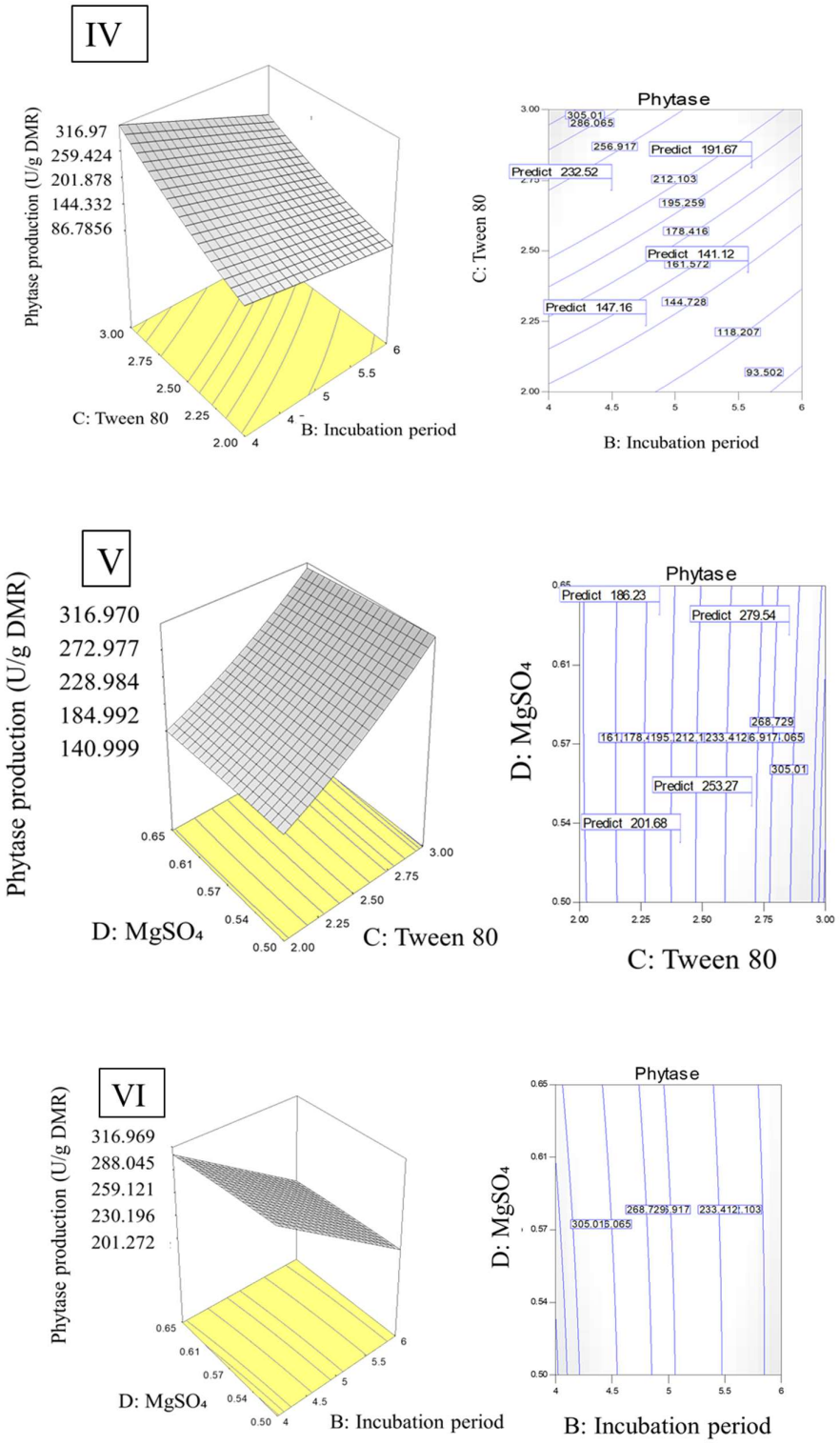


Fig. 3.14 3D graph (left) and Contour plots (right) showing the interaction between I) pH and incubation period II) pH and $MgSO_4$ III) pH and Tween 80 IV) incubation period and Tween 80 V) Tween 80 and $MgSO_4$ VI) Incubation period and $MgSO_4$

3.1.10 Optimization of phytase on large-scale production

The RSM model was validated by performing phytase production in Erlenmeyer flasks (250 ml-1000 ml) and enamel-coated trays on a large scale using 5-150 g of wheat bran, as shown in Table 3.6. The amount of substrate was enhanced from 5 g wheat bran in a 250 ml flask (Fig. 3.15) to 150 g wheat bran in the enamel-coated metallic tray (Fig. 3.16). The phytase production was found sustainable in the range of 394.95 – 475.42 U/g DMR and validated the model at a large scale. Among flasks, the 500 ml flask showed the highest phytase amount (431.25 ± 48.33 U/g DMR), while the tray having 100 g wheat bran exhibited the maximum level (475.42 ± 77.31 U/g DMR). These results uplift the optimization of *P. oxalicum* PBG30 phytase via SSF under a pilot scale. Moreover, phytase production showed an overall 5.6-fold enhancement, making the fermentation process less costly.

Table 3.6 Phytase production on a large scale in flasks and trays under optimized conditions

S.No.	Flasks/Trays	Amount of substrate used (g)	Phytase production (U/g DMR) ± S.E
1.	250 ml	5	394.95 ± 34.63
2.	500 ml	10	431.25 ± 48.33
3.	1000 ml	20	432.81 ± 15.99
4.	Tray (27 cm x 22 cm x 5 cm)	100	475.42 ± 77.31
5.	Tray (35 cm x 32 cm x 5 cm)	150	463.42 ± 92.16

Fig. 3.15 Phytase production on a large scale in different flask (250-1000 ml)

Fig. 3.16 Phytase production on a large scale in enamel-coated trays

Statistical optimization leads to more enhancement fold in the phytase production as compared to the OVAT method. The statistical approach provided a 5.6-fold while OVAT leads a 2.4-fold enhancement in phytase titre over the unoptimized production (Table 3.7).

Table 3.7 Enhancement of phytase production through optimization

S.No.	Conditions	Phytase production (U/g DMR) ± S.E	Fold enhancement
1.	Unoptimized	84.60 ± 2.53	1
2.	OVAT	200.41 ± 6.01	2.4
3.	PBD and RSM	373.32 ± 3.28	4.4
4.	Trays	475.42 ± 77.31	5.6

OBJECTIVE 3. **Purification and characterization of the microbial phytase.**

3.1.11 Partial purification of phytase from *P. oxalicum* PBG30

The *P. oxalicum* PBG30 phytase was partially purified by ammonium sulphate precipitation and dialysis. The total activity, specific activity, and fold enhancement are shown in Table 3.8. The crude sample expressed the lowest specific activity (0.31 U/mg) with a total enzyme activity of 381.57 U. After 80% ammonium sulphate precipitation, the sample showed 357.48 U activity with 1.23 U/mg specific activity. Further, the dialyzed sample exhibited 1.52 U/mg specific activity with total enzyme activity (211.04 U). The enzyme was purified to homogeneity with 4.9-fold from the crude sample having 55.31% yield. Further, purification by column chromatography was omitted to make the process cost-effective. The lyophilized sample exhibited 2.99 U/mg phytase activity (Fig. 3.17).

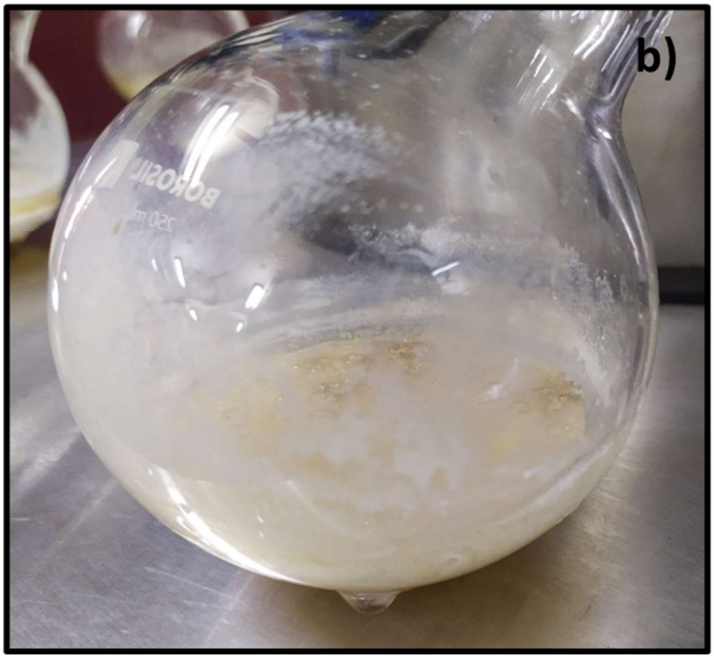


Fig. 3.17 Lyophilization of *P. oxalicum* PBG30 phytase enzyme: a) Lyophilizer b) Lyophilized sample

Table 3.8 Purification table of *Penicillium oxalicum* PBG30 phytase

Sample	Total enzyme activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	381.57	1212.33	0.31	100	1
Ammonium precipitated	357.48	289.98	1.23	93.69	3.97
Dialysed	211.04	138.88	1.52	55.31	4.90

3.1.12 Biochemical characterization of *P. oxalicum* PBG30 phytase

3.1.12.1 Effect of temperature

The *P. oxalicum* PBG30 phytase exhibited a significant amount of activity during the temperature range from 30°-80°C. More than 80% of activity was shown within a range between 55°C to 80°C (Fig. 3.18). The optimal value was found at 70°C showing its survival at high temperature.

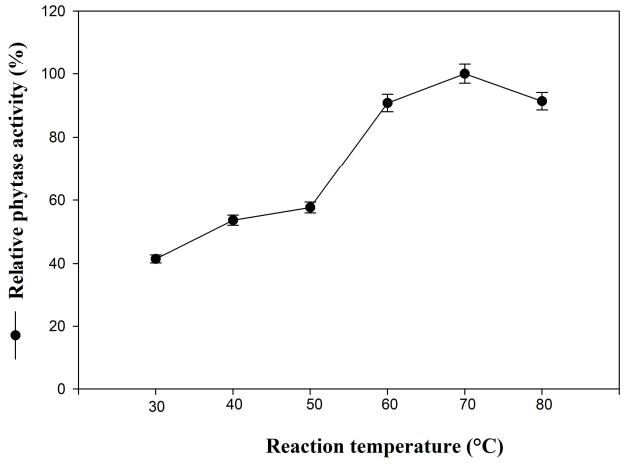


Fig. 3.18 Effect of temperature range (30°C – 80°C) on phytase activity.

3.1.12.2 Effect of pH

The phytase was active in the acidic range (2.5-5.0) and found optimum at pH 3.0 (sodium acetate buffer) during the assay, while the phytase activity declined under alkaline conditions, hence it is considered as an acidic phytase (Fig. 3.19).

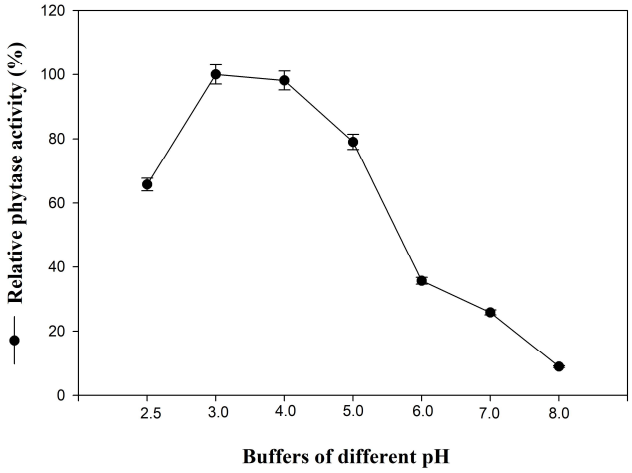


Fig. 3.19 Effect of pH range (2.5 – 8.0) on phytase activity.

3.1.12.3 Thermostability

The thermostability of phytase is studied at 70 and 80 °C as shown in Fig. 3.20. The *P. oxalicum* PBG30 phytase is found thermostable at 70°C with half-life found at 60 min. A loss of 30% activity occurred within 10 min while maintaining 50% activity up to 1 h. At 80°C, $t_{1/2}$ of phytase was 6-7 min and lost 70% activity within 60 min.

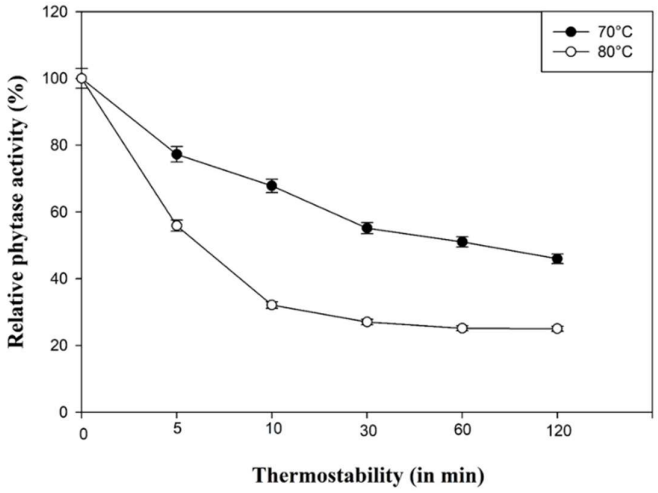


Fig. 3.20 Thermostability of phytase at 70°C and 80°C

3.1.12.4 pH stability

The stability of pH was checked at 3.0 and 5.0. At pH 3.0, 50% phytase activity was retained for up to 60 min, while at pH 5.0, 40% activity was lost within 10 min and maintained 50% activity for up to 40 min. Phytase was found to exhibit a similar response (Fig. 3.21) and found stable at both pH.

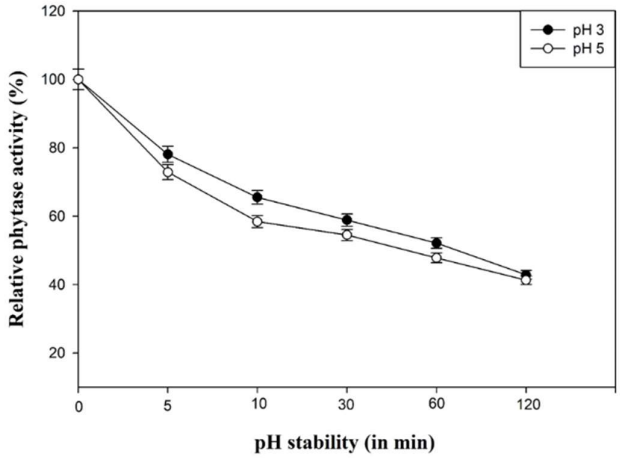


Fig. 3.21 Stability of phytase with pH 3.0 and 5.0

3.1.12.5 Effect of substrate

Phytase exhibited broad substrate specificity as shown in Fig. 3.22. The highest specificity is observed with sodium phytate and considered as best substrate for *P. oxalicum* PBG30 phytase. The order of specificity is sodium phytate> calcium phytate> p-nitrophenyl phosphate> ATP> ADP> Glucose-6-phosphate> AMP.

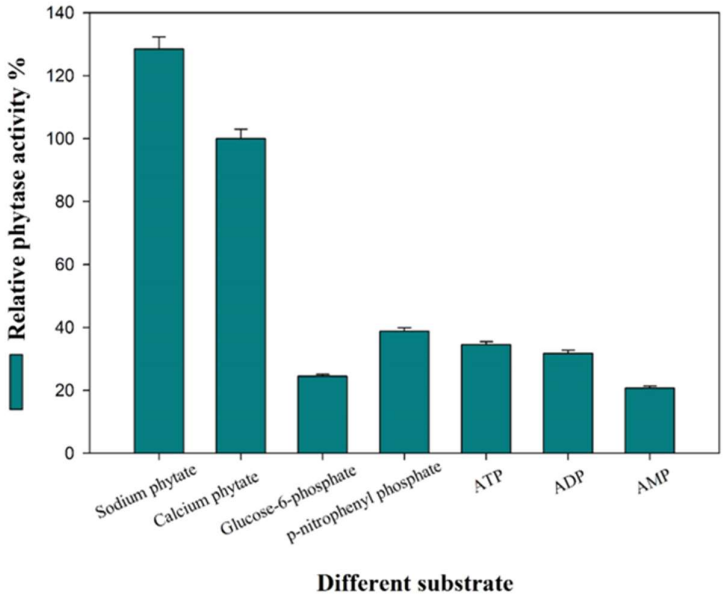


Fig. 3.22 Effect of different substrates on phytase activity

3.1.12.6 Effect of organic solvents

Phytase activity increases in the presence of ethanol, methanol, butanol, isopropanol, acetone, and DMSO (Fig. 3.23). Ethanol showed the maximum stimulatory effect on both 2 and 5% concentrations. Methanol and Propan-2-ol exhibited a similar positive effect on both concentrations. In the case of acetone and butanol, there is a less increment in the phytase activity at 2% and more at 5%. However, in the presence of DMSO, opposite results were obtained.

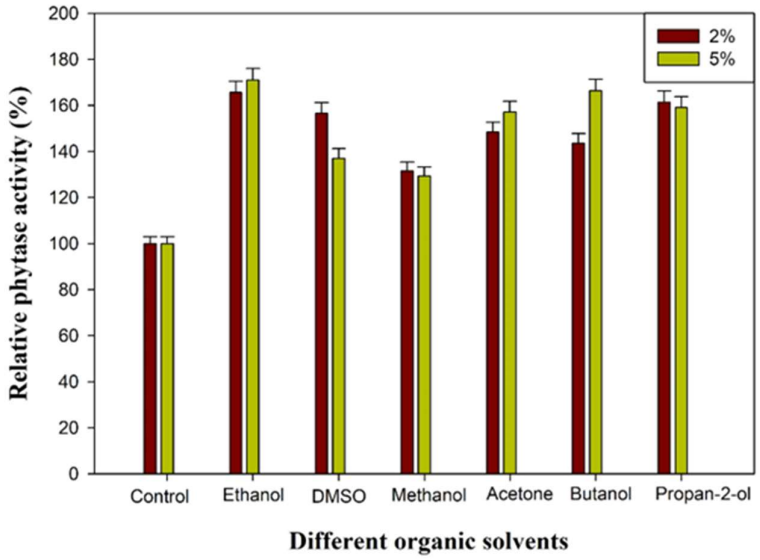


Fig. 3.23 Effect of different organic solvents on phytase activity

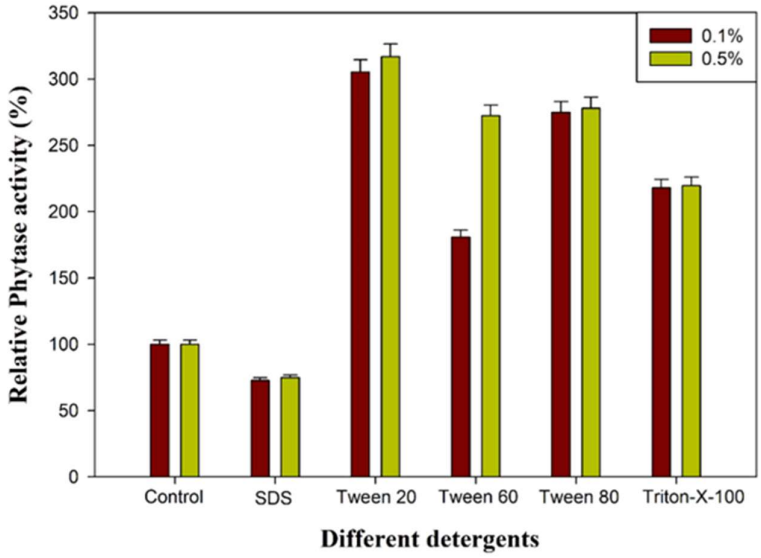


Fig. 3.24 Effect of different detergents on phytase activity

3.1.12.7 Effect of detergents

In the present study, the Tween 20, Tween-60 and Tween-80, Triton-X-100 considerably increased phytase activity, while the SDS decreased its activity (Fig. 3.24). The maximum

activity was obtained with Tween-20 at both concentrations, followed by Tween-80 and Triton-X-100 respectively. However, in the case of Tween 60, the activity of phytase was higher at a concentration of 0.5%, which is at level with Tween 80.

3.1.12.8 Effect of metal ions

The results showed a significant enhancement in the activity of phytase in the presence of Ca^{2+}, Co^{2+}, and Cu^{2+} with highest activity at 1 mM and lowest at 5 mM. The Mg^{2+} represented a slight increment in the activity at both concentrations. At 1mM concentration of Mn^{2+}, phytase activity was stimulated moderately but inhibited at high concentration. No significant effect of Fe^{3+} on phytase activity, while Fe^{2+} inhibited the activity at 1 mM. In fact, the activity of phytase was lower than the control at both the concentrations of Na^{+} ions (Fig. 3.25).

3.1.12.9 Effect of inhibitors

In the present study, except for sodium azide, rest of the inhibitors (EDTA, ß-ME, DTT, sodium molybdate) were involved in suppressing the phytase activity (Fig. 3.26). The maximum inhibition was shown with sodium molybdate. Both DTT and sodium molybdate exhibited the same pattern in inhibiting the phytase activity with high at 1 mM and low at 5 mM. EDTA and ß-ME inhibitors also showed the reduction in activity of phytase at both the concentrations.

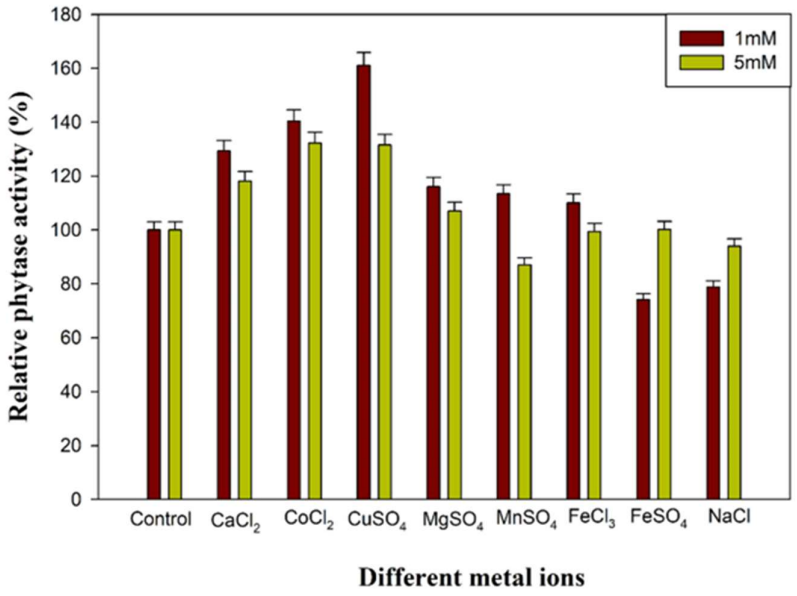


Fig. 3.25 Effect of different metal ions on phytase activity

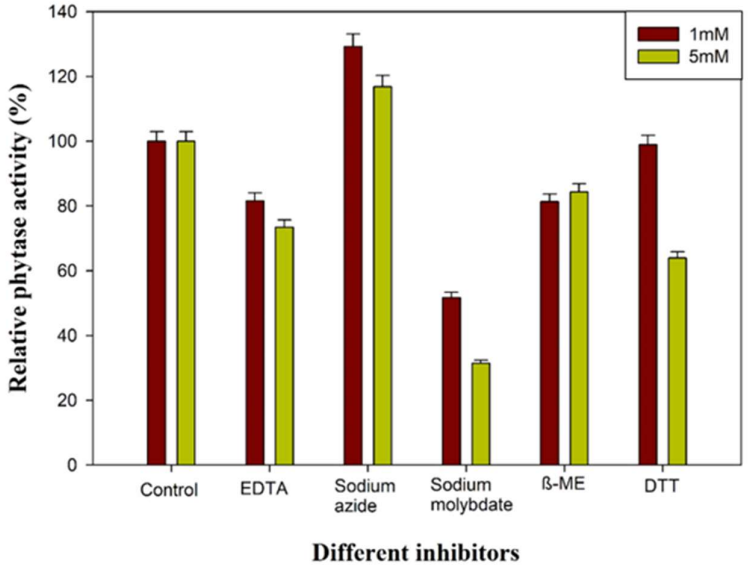


Fig. 3.26 Effect of different inhibitors on phytase activity

3.1.12.10 Determination of kinetic parameters

The K_m and V_{max} were calculated from the Lineweaver-Burk plot and found to be 4.42 mM and 909.1 U/ml respectively (Fig. 3.27).

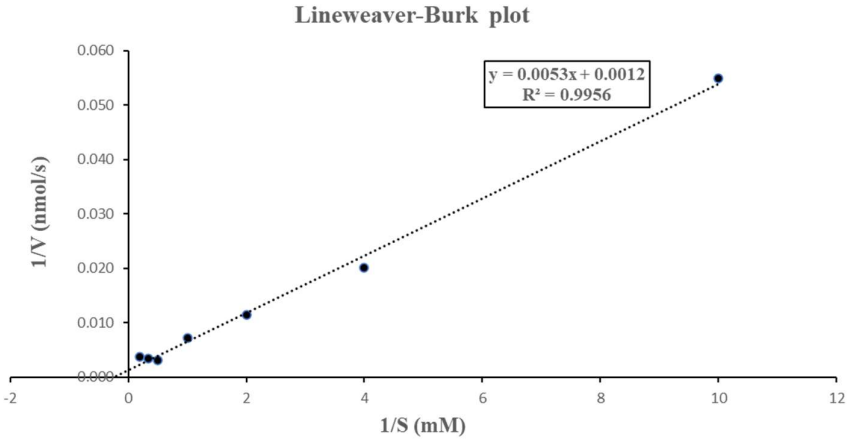


Fig. 3.27 Lineweaver-Burk plot for *P. oxalicum* PBG30 phytase representing K_m and V_{max}

3.1.12.11 Effect of protease

As shown in Table 3.9, there is no significant reduction in the activity of phytase against trypsin and pepsin treatments, substantiating the protease resistant nature of enzyme. Pepsin showed 8 and 12% reductions in the activity of phytase after the incubation of 1 and 2 h respectively while trypsin exhibited 10-20% reduction in the activity of phytase within 1-2 h.

Table 3.9 Effect of proteases on phytase activity

Sample	**Incubation time and phytase activity**			
	0 min	**30 min**	**60 min**	**120 min**
Control	100	95 ± 0.98	92 ± 0.99	89 ± 0.32
Pepsin treated	100	99 ± 0.77	92 ± 0.09	88 ± 0.18
Trypsin treated	100	90 ± 1.24	90 ± 1.40	80 ± 1.31

3.1.12.12 Storage life

The storage condition of *P. oxalicum* PBG30 phytase was analyzed at room temperature, 4°C and -20°C (Fig. 3.28). Phytase kept at room temperature lost 50% activity after 2 months, while samples stored at 4°C and -20°C maintained 50% activity after 4-5 months. 43% of phytase activity is exhibited at -20°C and 38% at 4ºC after 6 months, while 15% of the activity is left with phytase stored at room temperature. The results showed that the stability of *P. oxalicum* PBG30 phytase is higher with lower temperatures (4°C and -20°C) as compared to room temperature.

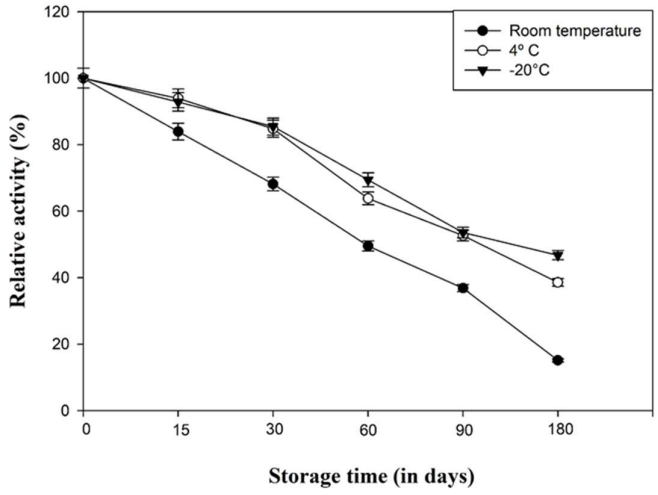


Fig. 3.28 Effect of storage conditions (RT, 4°C and -20°C) on phytase activity

3.1.12.13 Effect of vanadium on the phytase activity

The incubation of *P. oxalicum* PBG30 phytase with ammonium metavanadate modifies the phosphatase nature of phytase into peroxidase at both 5 and 10 µM concentrations. This synthetic haloperoxidase enhances the peroxidase activity while decreasing the phytase activity. The enhancement in peroxidase activity was observed up to 10 min (Fig. 3.30). As shown in Fig. 3.29, phytase activity was reduced with an increase in the incubation period.

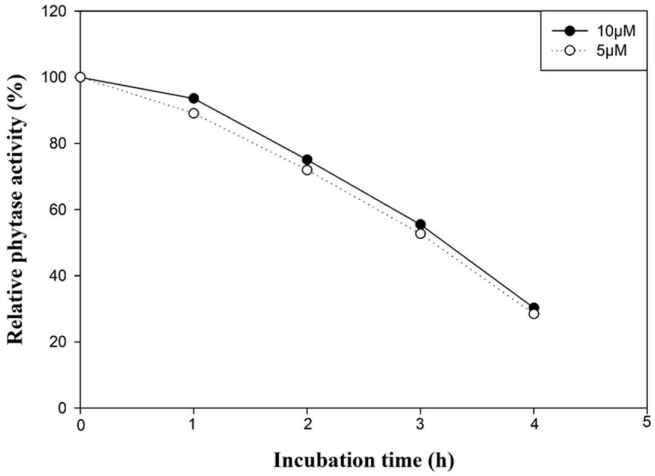


Fig. 3.29 Reduction of phytase activity with time in the presence of vanadium

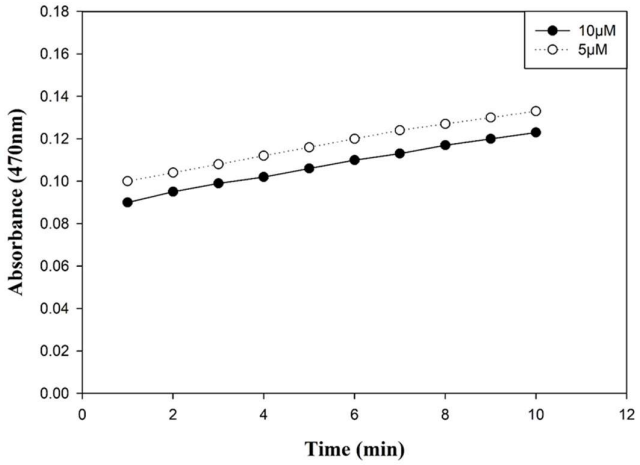


Fig. 3.30 Increase in absorbance with time showing peroxidase behaviour of phytase

OBJECTIVE 4. Immobilization and application of the phytase in the feed industry.

3.1.13 Immobilization of *P. oxalicum* PBG30 phytase

P. oxalicum PBG30 phytase was immobilized in calcium alginate beads by entrapment method using 3% sodium alginate and 0.2 M $CaCl_2$ as shown in fig. 3.31. The reusability of beads was studied by analyzing phytase activity in the continuous cycles. The 30% activity of phytase was lost with the initial two cycles and the 20% activity was left within immobilized beads for up to 5 consecutive cycles.

Fig. 3.31 Immobilization of *P. oxalicum* PBG30 phytase in calcium-alginate beads

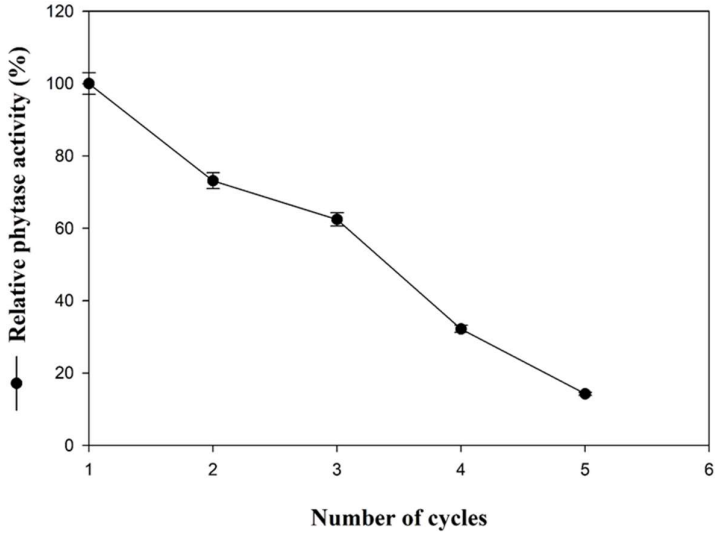


Fig. 3.32 Reusability of *P. oxalicum* PBG30 phytase beads

3.1.14 Applications of *P. oxalicum* PBG30 phytase in the feed industry

3.1.14.1 Hydrolysis of insoluble metal-phytate complex

As shown in Fig. 3.33 and 3.34, *P. oxalicum* PBG30 phytase is capable of hydrolyzing insoluble metal phytate complex at both 37°C and 50°C. It releases inorganic phosphorus from the metal ions complex which increases with time. All the metal ions complex of Ca^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} showed efficient hydrolysis and release of inorganic phosphate except Mg^{2+}, and Fe^{2+}. The maximum liberation of inorganic phosphorus occurred with Co^{2+} after 24 h, was 1022.15 ± 30.66 µg/ml at 37°C and 1192.15 ± 35.76 µg/ml at 50°C.

3.1.14.2 Hydrolysis of insoluble protein-phytate complex

A turbid solution was observed due to the insoluble complex formation between sodium phytate and lysozyme. As the phytase is supplied to the complex, the liberation of inorganic phosphate was observed. This release of inorganic phosphate was increased with time as shown in Fig. 3.35. The amount of released inorganic phosphate was 182.15 ± 5.46 µg/ml at 30°C.

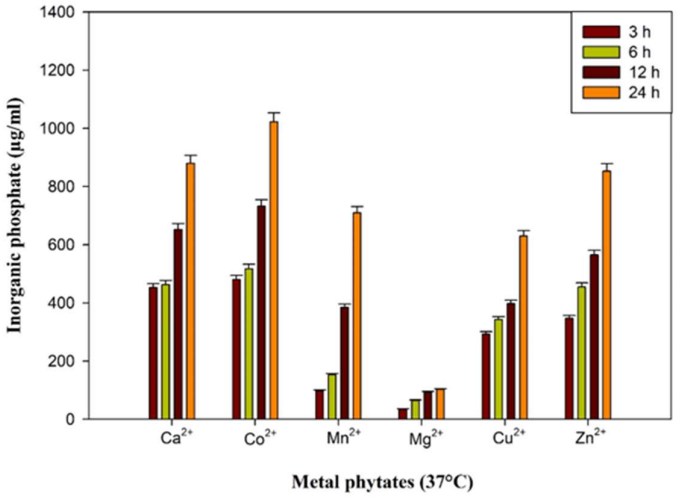


Fig. 3.33 Hydrolysis of insoluble metal phytate by *P. oxalicum* PBG30 phytase at 37°C

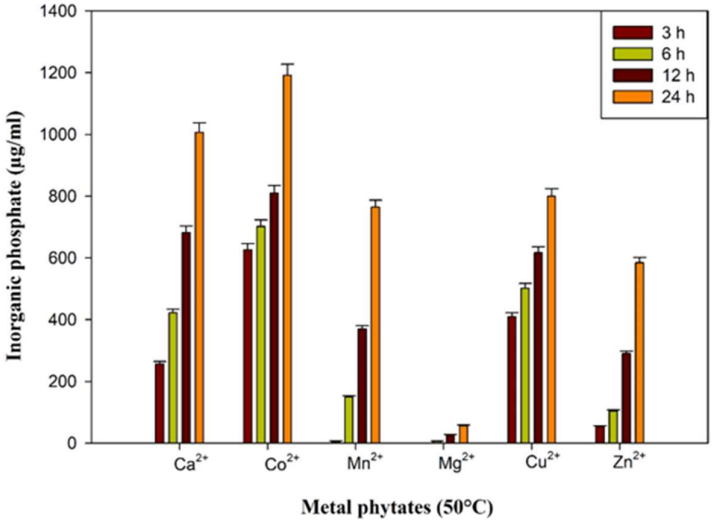


Fig. 3.34 Hydrolysis of insoluble metal phytate by *P. oxalicum* PBG30 phytase at 50°C

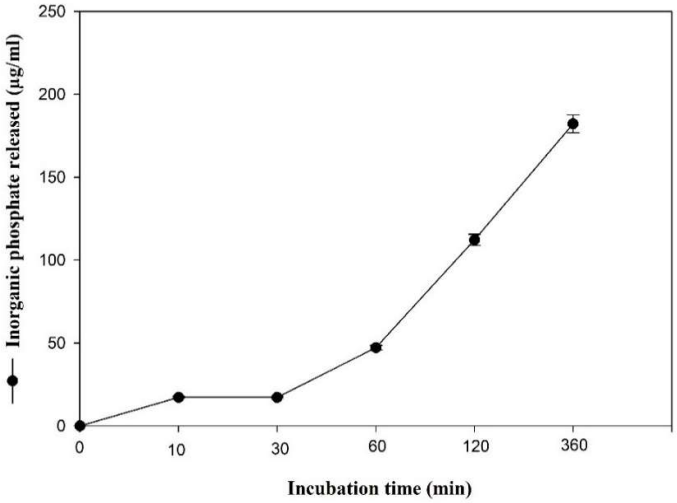


Fig. 3.35 Hydrolysis of lysozyme-phytate complex by *P. oxalicum* PBG30 phytase at 30°C

3.1.14.3 Dephytinization of commercial fish feed

3.1.14.3.1 Dephytinization of feed at different temperatures

The liberation of inorganic phosphorus from the fish feed is observed at 37°C, 50°C, and 70°C with 2, 4, and 6 h by using *P. oxalicum* PBG30 phytase. A high release of inorganic phosphorus (14.993 mg/g feed) is recorded at 37°C during 6 h [Fig. 3.36 (i)]. Also, nutritional by-products, soluble protein (69.08 mg/g feed), and reducing sugars (133.2 mg/g feed) are liberated from the feed [Fig. 3.36 (ii) and (iii)] treated with phytase due to the presence of other hydrolytic enzymes in *P. oxalicum* PBG30. Also, 12.793 mg/g feed of inorganic phosphorus, 128.28 mg/g feed of reducing sugar and 81.48 mg/g feed of soluble protein was observed after 6 h at 50°C. Similarly, at 70°C for 6 h, a low amount of inorganic phosphorus, reducing sugar and soluble protein was reported as 4.293, 0.56, and 6.16 mg/g feed respectively.

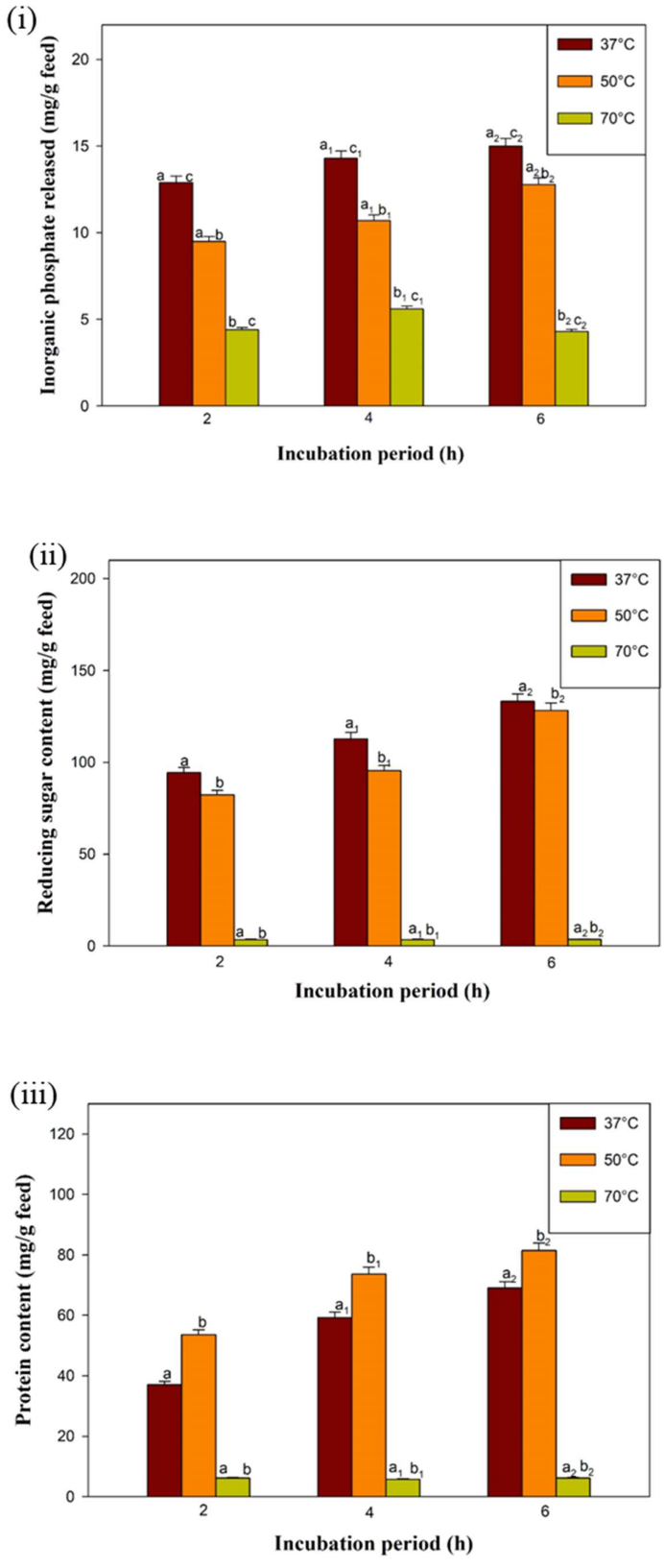


Fig. 3.36 Dephytinization of phytate by *P. oxalicum* PBG30 phytase at different temperatures (37°C, 50°C and 70°C) (i) inorganic phosphorus (ii) reducing sugar (iii) soluble protein

3.1.14.3.2 Dephytinization of feed by using different doses of phytase

The results showed that the liberation of inorganic phosphate enhances as the amount of dose increases. The total amount of inorganic phosphorus secreted after 6 h with phytase doses of 25, 50, 75, 100 and 200 U was 8.79, 11.29, 13.29, 14.99 and 26.19 mg/g feed respectively [Fig. 3.37 (i)]. Inorganic phosphate is found maximum with 200U (26.193 mg/g feed). Along with this, 138.48 mg/g feed (reducing sugars) and 96.5 mg/g feed (protein content) are obtained respectively [Fig. 3.37 (ii) and Fig. 3.37 (iii)].

3.1.14.3.3 Dephytinization effect in varied feed amounts

The effect of phytase on varied amounts of feed (0.5-2 g) was studied and the secreted amount of inorganic phosphate with 0.5 g, 1.0 g, 1.5 g and 2.0 g was 33.986 mg/g feed, 21.693 mg/g feed, 13.325 mg/g feed and 12.596 mg/g feed respectively, as shown in Fig. 3.38 (i). The results concluded that 0.5 g feed releases inorganic phosphate at the rate of 33.986 mg/g feed, at the most. Similarly, reducing sugar is found unaffected (134.4 mg/g feed) and protein is enhanced (115.52 mg/g feed) [Fig. 3.38 (ii) and Fig. 3.38 (iii)].

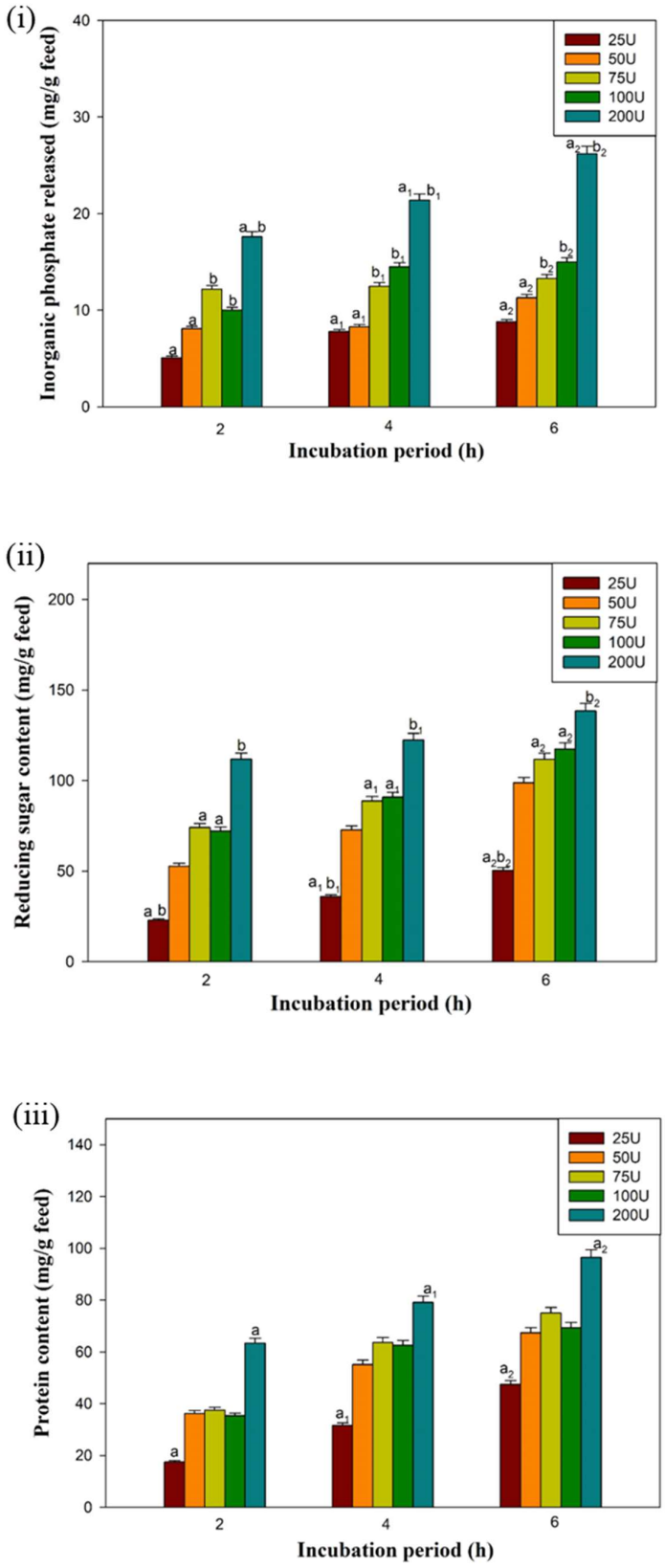


Fig. 3.37 Dephytinization of phytate by *P. oxalicum* PBG30 phytase with different phytase doses (25U, 50U, 75U, 100U, 200U) (i) inorganic phosphorus (ii) reducing sugar (iii) soluble protein

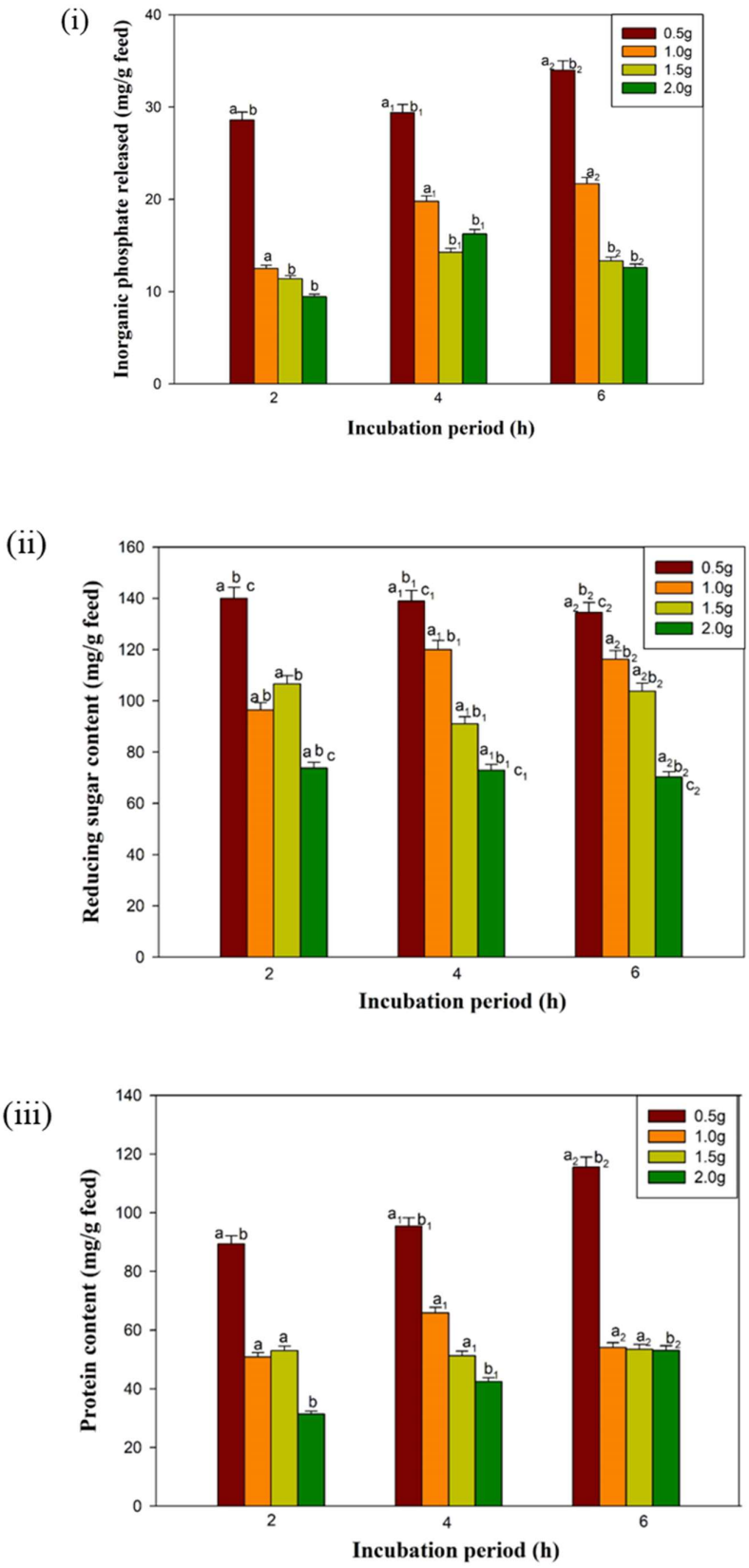


Fig. 3.38 Dephytinization of phytate by *P. oxalicum* PBG30 phytase with different feed amount (0.5g, 1.0g, 1.5g, 2.0g) (i) inorganic phosphorus (ii) reducing sugar (iii) soluble protein

3.1.14.3.4 Dephytinization of feed with incubation period

Further, the dephytinization was performed up to 48 h. The secretion of inorganic phosphate was 26.096, 28.396, 32.896, 32.996, 33.396 and 33.796 mg/g feed with their respective time at 2, 4, 6, 12, 24, and 48 h. the degradation of phytic acid with phytase shows the highest liberation of inorganic phosphate, soluble protein, and reducing sugars at 6 h and maintained up to 48 h (Fig. 3.39).

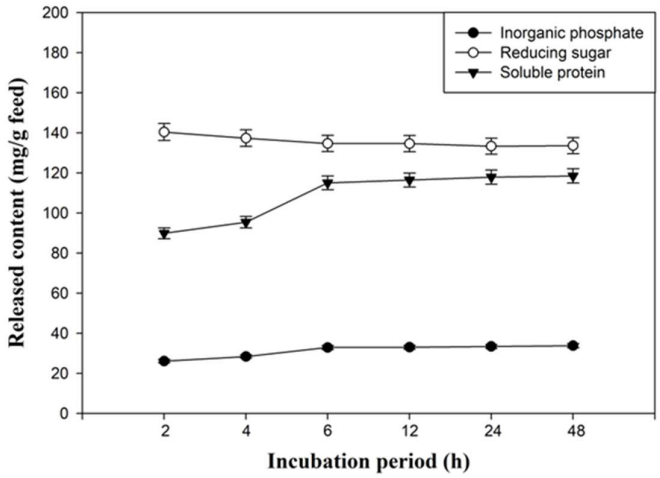


Fig. 3.39 Dephytinization of phytate by *P. oxalicum* PBG30 phytase at 37°C with 200U in feed

The maximum hydrolysis was observed with 200 U of *P. oxalicum* PBG30 phytase added in 0.5 g of feed dissolved in 20 ml buffer and incubated at 37°C for 6 h. The highest level of inorganic phosphorus (33.986 mg/g of feed), reducing sugars (134.4 mg/g of feed), and soluble protein (115.52 mg/g of feed) were found during the experiment.

3.1.14.4 Phytic acid estimation in experimentally prepared feed

As shown in Table 3.10, the control diet (without the addition of phytase) showed 50.43 mg/g phytic acid content while the phytase-added diet exhibited less phytic acid amount. The reduction of phytic acid in the presence of phytase depicts the hydrolysis action of phytase in

the diet. The level of phytic acid decreases as the phytase dosage increases in the diet. The diet containing 500FTU/kg phytase has 33.00 ± 0.13 mg/g feed phytic acid with a 34.6% reduction as compared to the control diet. The sample with 1500 FTU/kg exhibited minimum phytic acid content (26.91 ± 0.83) with a 46.6% reduction of phytic acid level.

Table 3.10 Estimation of phytic acid in experimental feed

Sample	**Phytic content (mg/g feed)**	**Reduction in phytic acid level (%)**
Diet I (Control)	50.43 ± 1.15	100
Diet II (Control + 500 FTU/kg phytase)	33.00 ± 0.13	34.6
Diet III (Control + 1000 FTU/kg phytase)	30.37 ± 0.45	39.8
Diet IV (Control + 1500 FTU/kg phytase)	26.91 ± 0.83	46.6

3.2 Discussion

3.2.1 Isolation of phytase-producing microbes

Phytase is an enzyme that hydrolyses phytate molecules and releases myo-inositol, inorganic phosphate, and other associated elements. Microbes are the best source of phytase, among these, filamentous fungi are excellent in producing extracellular phytases (Singh et al. 2024). Owing to the importance of microbial phytases in improving the nutritional quality of a feed and reducing environmental pollution, due to the accumulation of excessive P, researchers have isolated phytases from various fungi such as *Aspergillus niger* (Niera-Vielma et al. 2018a, Kumari and Bansal 2021a, Nascimento et al. 2022), *A. fumigatus* (Sanni et al. 2019, Thakur et al. 2022), *A. tubingensis* (Qasim et al. 2017, Caliskan-Ozdemir et al. 2021, Mahendran et al. 2022), *A. ficuum* (Shahryari et al. 2018), *A. foetidus* (Ajith et al. 2019), *A. aculeatus* (Saxena et al. 2020), *A. terreus* (Chaudhari and Peshwe 2022), *Penicillium oxalicum* (Kaur et al. 2017), *R. oryzae* (Arora et al. 2017), *Thermomyces lanuginosus* SSBP (Makolomakwa et al. 2017), *Grifola frondosa* (Huang et al. 2018), *Pholiota adiposa* (Jatuwong et al. 2020b), *Thermoascus aurantiacus* SL16W (Tanruean et al. 2021) and *Talaromyces purpureogenus* NSA20 (Ahmed et al. 2022). Also, the fungal phytases possess thermostability, resistance towards protease, acidic pH, and a wide substrate range, making them more feasible to use in the feed industry. Since different species of fish exhibit the diverse pH conditions of the digestive tract, therefore, a single phytase could not be an ideal additive for fish feed, hence it is important to investigate the potent phytases.

3.2.2 Qualitative and Quantitative Screening

In the current study, 55 microbial species were obtained from different samples and cultured on agar plates containing different media. These isolates were further screened on PSM agar plates. Out of a total of 55 isolates, only 8 isolates exhibited a clear zone of hydrolysis against sodium phytate. The zone of hydrolysis of these 8 isolates was confirmed by a two-fold staining method (Bae et al. 1999) to avoid false results that occurred due to acid-producing microbes which also formed a zone around the microbe by secreting organic acids such as acetic acid, citric acid, malic acid, and others. These organic acids reduce the pH and solubilize the phytate

that forms the zone of hydrolysis (Mahendran et al. 2022). Additionally, the quantitative estimation of phytase in these 8 isolates was done and found to be in the range of 9.085 – 51.40 U/ml. The highest activity of phytase was obtained with isolate 4 (51.40 ± 0.05 U/ml) and selected for subsequent experiments. The screening of phytase-producing microbes through qualitative and quantitative methods has been documented in various studies by using different compositions of phytase screening media (Kaur et al. 2017, Puppala et al. 2018, Yaseer et al. 2018, Jatuwong et al. 2020b, Sharma and Shukla 2020, Nagar et al. 2021, Kumari and Bansal 2021a, Chaudhari and Peshwe 2022, Mahendran et al. 2022). Isolate 4 was identified through morphological and microscopic features. The morphology of the fungus revealed its dark green colour, velvety texture, and powdery growth of colonies. At the microscopic level, the septate hyphae, branched conidiophores, and spores were present in a chain, indicating that the fungus belongs to the Penicillium genus. For the identification at the species level, ITS sequencing was performed concluding that the isolated fungus is *Penicillium oxalicum*. However, limited work has been done on phytase production from the *Penicillium* sp. from their original strain (Tseng et al. 2000, Awad et al. 2014, Kim et al. 2015, Lee et al. 2015, Kaur et al. 2017, Kalkan et al. 2020) while most of the studies have been carried out on recombination and engineering of phytase isolated from *Penicillium* sp. (Lee et al. 2007, Zhao et al. 2010, Correa et al. 2015, Ya-nan et al. 2020).

3.2.3 Phytase production in SSF and optimization by OVAT

The solid-state fermentation is an effective method for the production of microbial enzymes and other secondary products. In this process, microbes are grown on a solid substrate in either the absence or a low amount of water (Rizwanuddin et al. 2023a). The agricultural waste or residues are commonly utilized as a solid substrate that makes the production cost-effective and also resolves the issues that occur due to their accumulation. Among the various substrates used for the SSF method, wheat bran is commonly used because it is rich in carbon, nitrogen, amino acids, and a high amount of phytic acid (Suresh and Radha 2015). Due to the presence of phytate, it acts as an inducer for the production of phytase (Chanderman et al. 2016). It is easily available, has a low cost, and provides support to the growth of fungi (Qasim et al. 2017, Kumari and Bansal 2021a). Moreover, optimization is a requisite for the identification of essential components and culture conditions necessary for the fungal growth and production of enzymes. The composition and types of chemicals used for the production are necessary factors

that should be considered in the optimization process as they provide adequate nutrients for the growth of microorganisms (Pragya et al. 2023b). In this experiment, the media used for phytase production from *P. oxalicum* PBG30 contains 0.5% Urea, 0.1% $MgSO_4.7H_2O$, 0.1% KCl, and 0.1% $FeSO_4.7H_2O$. The moistening media including K_2HPO_4 (0.5%), $(NH_4)_2SO_4$ (0.4%), $CaCl_2$ (0.03%), $MgSO_4.7H_2O$ (0.3%), $FeSO_4.7H_2O$ (0.03%) supported maximum production of 5.05 U/g DMR in *A. oryzae* SBS50 (Sapna and Singh 2015), while distilled water (208.30 U/g DMR) suited best for *A. niger* NT-7 phytase (Kumari and Bansal 2021a). Media containing magnesium sulphate (0.5%), ferrous sulphate (0.5%), and ammonium sulphate (0.5%) resulted in 361.88 U/g DMR phytase from *A. oryzae* SBS50 (Pragya et al. 2023b). Similarly, *P. oxalicum* EUFR-3 possess 12.80 U/g phytase with 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl and 0.001% $FeSO_4$ (Kaur et al. 2017).

An adequate amount of wheat bran produces phytase while beyond that it acts as a limiting factor and restricts the production level (Kumari and Bansal 2021a). In the present study, 10 g wheat bran promotes the highest production of phytase, that corroborates the findings of previous studies on phytase production by *A. oryzae* SBS50 (Sapna and Singh 2015), *A. tubingensis* (Qasim et al. 2016), *A. tubingensis* FSS223 (Yasser et al. 2018) and *A. fumigatus* (Mahendran et al. 2022) where applying 10 g wheat bran results into the highest production of phytase. However, 5 g wheat bran has also supported phytase production in other cases such as *P. oxalicum* EUFR-3 (Kaur et al. 2017) and *A. niger* NT-7 (Kumari and Bansal 2021a). Besides wheat bran, other substrates such as soybean meal, rice polish, rice bran, and rice straw have also been used for the production of phytase through SSF, in *A. niger* (Saithi and Tongta 2016), *A. niger* (Mahmood et al. 2021), *T. aurantiacus* SL16W (Tanruean et al. 2021) and *A. awamori* NRC- F18 (Elkhateeb and Fadel 2022) respectively. Further, the use of mixed substrate has also been considered good for phytase production. A mixture of sugarcane bagasse and wheat bran for the production of phytase in *S. thermophile* BJTLR50 (Kumari et al. 2016) and wheat bran and rice straw in *A. oryzae* SBS50 (Pragya et al. 2023b) have been suggested as well.

Temperature is a significant component of the fermentation process. All fungi have an optimum temperature for their growth. Enzymes are generally produced in the exponential phase, however beyond that growth and production are reduced (Qasim et al. 2017). The poor growth and denaturation of enzymes also occurred at high temperatures (Suresh and Radha 2016). It is shown in the results that the production of phytase is maximum at 30°C and decreases at high temperatures, which is in accordance with previous results on phytase

production from *A. oryzae* SBS50 (Pragya et al. 2023b), *A. aculeatus* APF1 (Saxena et al. 2020), *A. tubingensis* (Mahendran et al. 2022), *A. tubingensis SKA* (Qasim et al. 2017), *A. flavus* (Onibokun et al. 2022) and *A. niger* (Nascimento et al. 2022). These studies also showed the maximum production of phytase at 30°C. However, other researchers reported increased phytase production at 32ºC in *A. niger* SBS49 (Singh et al. 2015), 35ºC in *A. niger NT-7* (Kumari and Bansal 2021a), and *A. niger* (Mahmood et al. 2021), and 40ºC in *A. terreus* fsp-4 (Chaudhari and Peshwe 2022).

The level of moistening media affects both the growth of fungus and phytase production. When the moisture level becomes higher than optimum, it leads to a reduction in the porosity, decreases oxygen transfer, and changes in the size of the particle, and formation of the aerial mycelium, thereby influencing the activity of phytase. If the moisture level becomes low, it creates high water tension, low nutrient solubility, and increases its concentration (Gaind & Singh 2015), therefore, an adequate ratio is required. Phytase isolated from *P. oxalicum* PBG30 showed the highest production with a 1:2 ratio. Below and above this ratio, the production level decreases. Similar to the results, the highest production of phytase from *A. oryzae* SBS50 (Sapna and Singh 2015), and *A. niger* NT7 (Kumari and Bansal 2021a) occurred with a 1:2 substrate and moisture level ratio. Opposite to this, a 1:4 ratio was reported in *A. awamori* NRC- F18 (Elkhateeb and Fadel 2022) and *A. oryzae* SBS50 (Pragya et al. 2023b).

The production of enzymes is directly linked to the growth of microbes. Therefore, initially, the level of enzyme is generally low, but gradually reaches its highest level, and after that, decreases due to a low level or deficiency of nutrients (Chaudhari & Peshwe 2022). The present study showed a low level of phytase production at the initial stage, reached maximum at the 5th day of incubation, but decreased beyond that. The maximum production has also been observed at 5 days of incubation in *A. tubingensis* FSS223 (Yasser et al. 2018), *A. niger* NT-7 (Kumari and Bansal 2021a), *A. niger* (Mahmood et al. 2021), *A. tubingensis* (Mahendran et al. 2022) and *A. oryzae* SBS50 (Pragya et al. 2023b). However, the level of phytase production was high after 4th day of incubation in *A. tubingensis* SKA (Qasim et al. 2017), 12 days in *T. aurantiacus* SL16W (Tanruean et al. 2021) and after 6 days of incubation in *A. awamori* NRC-F18 (Elkhateeb and Fadel 2022), *A. terreus* fsp-4 (Chaudhari and Peshwe 2022) and *T. purpureogenus* NSA20 (Ahmed et al. 2022).

The pH has a significant effect on the extracellular enzyme activity and metabolic reactions of microbes, therefore affecting enzyme production (Qasim et al. 2017). *P. oxalicum* PBG30 phytase has shown the highest production of phytase at pH 7.0, which substantiates the findings of Yasser et al (2018). They also suggested pH 7.0 optimal, for the production of phytase from *A. tubingensis* FSS223 (Yasser et al. 2018), while an acidic pH leads to reduced growth and production (Suresh & Radha 2016). Contrary to this, the maximum production of phytase was recorded at pH 5.0 in several fungi such as *A. oryzae* SBS50 (Pragya et al. 2023b), *A. tubingensis* SKA (Qasim et al. 2017), *A. niger* NT-7 (Kumari and Bansal 2021a) and *A. tubingensis* (Mahendran et al. 2022), pH 6.0 in *A. niger* (Mahmood et al. 2021) and *A. awamori* NRC- F18 (Elkhateeb and Fadel 2022) and pH 4.5 in *A. terreus* fsp-4 (Chaudhari and Peshwe 2022).

P. oxalicum PBG30 exhibited the presence of other hydrolytic enzymes such as cellulase, amylase, xylanase, and lipase in the crude enzyme solution along with phytase. Phytase showed the highest activity while others reported a low amount of activity. These enzymes directly or indirectly influence the phytase activity and its effect. In the similar findings, *A. oryzae* SBS50 showed the secretion of amylase, cellulase, pectinase, and xylanase enzymes with phytase (Sapna and Singh 2015). In another study, carboxymethyl cellulase, amylase, acid, and alkaline phosphatase were produced from *A. flavus* ITCC 6720 along with phytase (Gaind and Singh 2015). Kanti and Sudiana (2018) demonstrated the activity of phytase, amylase, and cellulase after the extraction of crude enzyme solution from three different fungi (*A. niger, R. oryzae* and *N. sitophila.* The simultaneous production of phytase, protease, and xylanase from *A. niveus* was reported and their utilization in animal feed was also studied (Simas et al. 2024).

3.2.4 Statistical optimization of phytase production by PBD and RSM

Statistical optimization is preferred over the conventional method (OVAT) due to the fast process, cost-effectiveness, and examination of multiple factors in a single duration with their interaction level. PBD and RSM are the commonly used statistical methods that enhance the production of enzymes via the design of experiments (DoE) approach. The optimization of phytase production by using PBD and RSM has been mentioned in the literature as well (Jatuwong et al. 2020b, Kumari and Bansal 2021b, Javaid et al. 2022, Pragya et al. 2023a). In

the present study, incubation period, pH, $MgSO_4$, and Tween-80 are the significant parameters that were identified through PBD. In other studies, the factors selected through PBD were glucose, moisture content, $MgSO_4$, and dextrin for *A. niger* phytase production (Buddhiwant et al. 2016). Similarly, yeast extract, incubation period, and Tween-80 were found as critical parameters for *S. thermophile* (Kumari et al. 2016), glucose, $FeSO_4$, pH and citric acid for *W. saturnus* (Pable et al. 2019), water hyacinth and moisture content for *P. adiposa* (Jatuwong et al. 2020b), amount of wheat bran, pH, temperature, mannitol, incubation period and ammonium sulphate for *A. niger* NT7 (Kumari and Bansal 2021b), temperature, Tween-40, pH, incubation period and NH_4NO_3 for *A. niger* (Mahmood et al. 2022) and glucose, $MgSO_4$, moisture content and Tween-80 for *A. oryzae* (Pragya et al. 2023a) phytase production through PBD method.

The time of incubation is an important variable in enzyme production. Most of the enzymes are produced in the logarithmic phase. However, a long period of duration decreases the production level of enzymes due to nutrient deficiency and cell lysis (Chaudhari and Peshwe 2022). The results showed maximum production of phytase within 5 days of incubation period, thereafter, the phytase yield decreases due to a deficiency of nutrients for the growth of fungi. Likewise, the 5 days incubation period was also adequate for maximum phytase production from *A. niger* NT7 (Kumari and Bansal 2021b) and *R. oligosporus* (Suresh and Radha 2016). However, in the case of *A. oryzae* SBS50 (Sapna and Singh 2015) and *S. thermophile* (Kumari et al. 2016) only 2 days of incubation period was sufficient for the maximum production of phytase. In contrast, the 6 days incubation period was required for the optimum level of phytase production from *A. niger* (Mahmood et al. 2022). pH is crucial factor for all metabolic processes, and it strongly affects phytase production. The optimum pH for *P. oxalicum* phytase is 7.0. Opposite to this, *A. niger* NT7(Kumari and Bansal 2021b) and *Williopsis saturnus* NCIM 3298 (Pable et al. 2019) require pH 5.0 and *A. niger* requires pH 6.0 (Mahmood et al. 2022) for the maximum production.

Tween-80 is one of the surfactants that significantly affects the production of phytase, as it permeabilizes the cell membrane and releases more protein/enzyme in the medium (Priya et al. 2023b). In the case of *P. oxalicum* PBG30, Tween-80 exhibits its highest effect at 3.5% concentration, whereas in the case of *S. thermophile* (Kumari et al. 2016) and *A. oryzae* (Pragya et al. 2023a) 2.5% concentration of Tween-80 could exhibit its highest effect on phytase production. The metal ions such as magnesium are a requisite for the fungus growth (Pragya et al., 2023a). In this experimental study, 0.75% $MgSO_4$ was involved in increasing the

production of phytase, while 0.4% $MgSO_4$ is found for *A. oryzae* SBS50 (Pragya et al. 2023a) and 1.5% for *A. niger* (Buddhiwant et al. 2016).

The optimization through statistical methods significantly enhanced phytase production. In this study, the level of phytase production increased 4.4-fold after statistical optimization (373.32 U/g DMR) as compared to un-optimized conditions (84.60 U/g DMR). A 36.67-fold enhancement has also been observed in the phytase production from *A. niger* (Buddhiwant et al. 2016), 11.6-fold from *S. thermophile* (Kumari et al. 2016), 2.73-fold from *R. oligosporus* (Suresh and Radha 2016), 3.15-fold from *P. adiposa* (Jatuwong et al. 2020b), 6.8-fold from *A. niger* (Kumari & Bansal 2021b), 1.37-fold from *A. niger* (Mahmood et al. 2022) and 2.29-fold from *A. oryzae* (Pragya et al. 2023a) through statistical approaches.

In this study, for the sustainable production of phytase on a large scale, the optimized conditions were performed in flasks and trays. Increased phytase production in the case of trays indicates that the conditions were suitable for the production of phytase and sustainably useful and feasible for large-scale production. On a large scale, production of phytase in trays is influenced by substrate amount, the presence of oxygen, and uneven allocation of inoculum and bed height (Suresh and Radha 2015, Arora et al. 2017). If the amount of substrate is more than sufficient, oxygen becomes unavailable to fungus, and production decreases (Suresh and Radha 2015). A 1.5-2 cm bed height is considerable for the production, but it is not compulsory. It may vary depending upon the production system, kinetic parameters, and operating conditions above 2 cm, influence on cell biomass and production due to a high level of substrate and less availability of moisture and oxygen. (Arora et al. 2017). The sustainable production of phytase in flasks and trays on a large scale has also been studied in *A. oryzae* SBS50 (Sapna and Singh 2015), *R. oligosporus* MTCC 556 (Suresh and Radha 2015), *S. thermophile* (Kumari et al. 2016), *R. oryzae* (Arora et al. 2017) and *A. niger* 7A-1 (Neira-Vielma et al. 2018a). Moreover, phytase production showed an overall 5.6-fold increase, making the fermentation process less costly. The approach of fermentation in trays can be upgraded by using bioreactors for the production of phytase, which provides constant temperature and moisture (Suresh and Radha 2015).

3.2.5 Partial purification

Purification is a process to purify the enzyme in concentrated form leading to its homogeneity by removing impurities from the crude enzyme extract. It includes a series of steps such as precipitation, dialysis, and chromatographic techniques. Precipitation can be done by either salts or organic solvents. Salt precipitation or more commonly ammonium sulphate precipitation is used for purification. The solubility of protein is affected by ionic salt concentration. At low concentration, solubility enhances (salting-in) while decreases at high concentration (salting-out). During the ammonium salt precipitation method, ammonium ions having a positive charge associated with phytase having a negative charge and aggregate the enzyme (Saxena et al. 2020). *P. oxalicum* PBG30 phytase was partially purified by ammonium sulphate precipitation and dialysis methods. Further, the dialysis was performed with 20 mM acetate buffer by changing the buffer at least three times. The phytase is purified up to 4.9-fold with a 55.31% yield. Gunashree and Venkateswaran (2015) isolated phytase from *Aspergillus niger* CFR 335 which showed a 66.8% yield with 4.1-fold purification after the precipitation and dialysis methods. Similarly, Saxena et al. (2020) reported 1.42-fold purification after the dialysis of *Aspergillus aculeatus* APF1 phytase with a 25.54% yield. Also, the 3.4-fold purification was reported in *Aspergillus fumigatus* with a 40% yield of phytase after ammonium sulphate precipitation (Thakur et al. 2022). Further, a 5.9-fold purification of phytase was observed in *T. purpurogenus* NSA20 after dialysis (Ahmed et al. 2022).

3.2.6 Biochemical characterization of *P. oxalicum* PBG30 phytase

During food and feed processing, enzymes have to face extreme temperatures that influence the enzyme activity by modifying their structure or kinetic energy, therefore they must be resistant to high temperatures. In the present investigation, the optimum temperature for *P. oxalicum* PBG30 phytase was 70°C, which resembles with the optimal temperature of *T. lanuginosus* IMI 096218 phytase (70°C) (Bujna et al. 2016). A decrease in phytase activity could be observed at both extremes due to loss of kinetic energy and collision of molecules at below optimal temperature and denaturation at above (Pragya et al. 2023). The optimal temperature value for fungal phytases has been reported 37ºC in the case of *A. foetidus* MTCC 11682 (Ajith et al. 2019), 40ºC in *A. niger* BIONCL8 (Bhandari et al. 2023), *P. oxalicum* (Kaur

et al. 2017), and *A. fumigatus* (Sanni et al. 2019), 50ºC in *R. oligosporus* MTCC 1116 (Suresh and Radha 2016), *Rhodotorula mucilaginosa* (Yu et al. 2015), *A. aculeatus* APF1 (Saxena et al. 2020), and *A. oryzae* (Pragya et al. 2023a), 55ºC in *A. fumigatus* (Thakur et al. 2022), 56ºC in *A. niger* 7A-1 (Niera-Vielma et al. 2018b) and 60ºC in the case of *A. niger* NT7 (Kumari and Bansal 2021a). The variation in the temperature values might be due to the source of the phytase from which it was isolated.

The pH is a critical parameter because it influences the activity of an enzyme by changing the ionic composition in its microenvironment. As the human and animal stomach is acidic (pH lies from 1.5-3.5), phytases possessing acidic pH are considered to be useful in the food and feed industry. In this study, *P. oxalicum* PBG 30 phytase was found optimally active at pH 3.0. The phytase obtained from *A. aculeatus* APF1 (Saxena et al. 2020), and *A. niger* (Singh et al. 2015) also exhibited the optimum pH at 3.0, thus validating the findings of this work. In contrast, the phytase obtained from *P. oxalicum* EUFR-3 was active at the neutral pH (Kaur et al. 2017). However, phytase activity declined in alkaline conditions due to changes in the ionic state of amino acids. Studies conducted on *P. oxalicum* KCTC6440 (Kim et al. 2015), *R. oligosporus* MTCC 1116 (Suresh and Radha 2016), *A. niger* NT7 (Kumari and Bansal 2021a), *A. niger* BIONCL8 (Bhandari et al. 2023), *A. niger* 7A-1 (Niera-Vielma et al. 2018b) and *A. oryzae* (Sapna and Singh 2017, Pragya et al. 2023a) showed a decrease in activity of phytase within the alkaline region.

It has been suggested that phytase should be thermostable and pH stable for its utilization in the feed industry. The results showed that *P. oxalicum* PBG30 phytase is thermostable and stable in acidic pH. However, the studies carried out on the *A. niger* BIONCL8 showed that it lost 70% phytase activity (Bhandari et al. 2023), *A. fumigatus* lost 86% (Sanni et al. 2019) and *A. niger* CFR 335 lost 80% phytase activity within 1 h at 80°C (Gunashree and Venkateswaran 2015). In the case of *A. niger* 7A-1, only 9.3% phytase activity is left after 5 min of incubation at 80ºC (Niera-Vielma et al. 2018b), and *A. oryzae* retains only 4.2% phytase activity after 1 h (Gampa et al. 2023). A reduction in the phytase activity of *R. mucilaginosa* was observed within 10 min (Yu et al. 2015), and only 20% activity was left in *A. fumigatus* phytase after 1 h (Sanni et al. 2019) at 70ºC. Phytase obtained from *A. fumigatus* showed 90% activity after 20 min while exhibiting more than 50% at 2 h (Thakur et al. 2022). The thermostable half-life of *A. oryzae* phytase was found as 300 min at 50°C (Sapna and Singh 2017), while at 70ºC phytase lost its activity within 100 min (Pragya et al. 2023a). After an incubation period of 60 min and 90 min at 70°C, *A. oryzae* left with 50.2% and 41.6 % phytase

activity (Gampa et al. 2023). In present study, the *P. oxalicum* PBG 30 phytase was found stable at both pH 3.0 and pH 5.0 with 50% activity maintained up to 60 and 30 min respectively. About 80% of phytase activity was retained for 45 min at pH 3.0 and 100 min at pH 5.0 in *A. oryzae* (Sapna and Singh 2017). *A. niger* BIONCL8 phytase exhibited stability at pH 2.0 and 8.0. However, at pH 2.0, phytase showed 80% activity with an incubation period of 24 h, while at pH 8.0, it retained more than 50% activity within the same duration (Bhandari et al. 2023). The phytase obtained from *P. oxalicum* PBG 30 exhibited a broad substrate specificity, a characteristic feature of an enzyme to be used for animal feed. It shows the highest specificity against sodium phytate and has also been reported in the case of phytase obtained from *P. oxalicum* PJ3 (Lee et al. 2015), *R. oligosporus* MTCC 556 (Suresh and Radha 2016), and *A. niger* 7A-1 (Niera-Vielma et al. 2018b), that demonstrated maximum specificity with sodium phytate.

Organic solvents have a positive impact on the activity of phytase enzyme. The impact of hydrophobic residues on the activity of phytase is negligible. All the organic solvents used during the experiment exhibited a stimulatory effect. In the existence of ethanol, methanol, butanol, and acetone, phytase activity was enhanced in *A. oryzae* (Sapna and Singh 2017). The inhibitory effect of ethanol, butanol, isopropanol, and acetone was observed in *A. niger* BIONCL8 (Bhandari et al. 2023). The functional group and molecular structure of organic solvents are accountable for designing the artificial environment around the enzyme, which affects the enzyme structure and its catalytic properties. Depending upon the type of conformational modifications, phytase activity is affected. It can either stimulate or inhibit the enzyme activity (Sapna and Singh 2017). Limited literature is available on the effect of organic solvents on phytase. Due to the presence of organic solvents solubility of hydrophobic residues is enhanced, it avoids reactions with water molecules, makes enzymes more stable and reusable, and protects them from microbial contamination (Caliskan-Ozdemir et al. 2021).

The results of present study showed that although SDS inhibits the phytase activity, but stimulates in the presence of Tweens. In a previous study carried out on *P. oxalicum* PBG 30*,* SDS has been reported as an inhibitor of phytase activity (Kaur et al. 2017). Moreover, SDS inhibits the activity of phytase obtained from *A. oryzae* (Sapna and Singh 2017) and *A. fumigatus* (Sanni et al. 2019). Detergents can also increase or decrease the activity of enzyme, depending upon the type of interacting molecule whether it is anionic, cationic, or non-ionic. It binds with protein molecules and changes the tertiary structure of protein (Sapna and Singh 2017). SDS is an anionic detergent that is not specific and can attach anywhere to the protein

and unfold it. It imposes conformational changes in the active site and thus inactivates the enzyme. Non-ionic detergents such as Tweens and Triton X-100, bind on the surface of the protein and thus either stimulate or do not influence the activity of phytase by making the conformational changes in the enzyme structure.

The involvement of metal ions in the regulation of enzyme catalysis is crucial. The effect of different metal ions was observed on the activity of phytase. The inhibition of phytase activity is possibly due to the association of metal and substrate complex, which restricts the binding of the substrate with enzyme or the modification of enzyme active site due to the binding of ions (Saxena et al. 2020). An enhancement in the phytase activity might be due to the requirement of metal ions in the activation of enzymes. The impact of metal ions on enzyme activity depends on the microbial species that produce the enzyme (Yu et al. 2015). In this study, Ca^{2+}, Co^{2+}, and Cu^{2+} enhance the phytase activity obtained from *P. oxalicum* PBG 30. Similarly, the involvement of Ca^{2+} in increasing the phytase activity of *A. niger* NT7 (Kumari and Bansal 2021a), *A. fumigatus* (Sanni et al. 2019), and *A. oryzae* (Sapna and Singh 2017) has also been reported in literature as well. Also, Co^{2+} was found to stimulate the phytase activity in *A. niger* NT7 (Kumari and Bansal 2021a) and *A. oryzae* (Sapna and Singh 2017), but in contrast, Cu^{2+} inhibited the phytase activity in *A. niger* NT7 (Kumari and Bansal 2021a), and *A. oryzae* (Sapna and Singh 2017). In other reports, Ca^{2+} and Cu^{2+} decrease the activity of phytase isolated from *P. oxalicum* KCTC6440 (Kim et al. 2015) and *P. oxalicum* PJ3 (Lee et al. 2015). Although the effect of Mg^{2+} on phytase activity found positive, but did not show a significant difference. A similar stimulatory effect of Mg^{2+} has been recorded in *R. mucilaginosa* (Yu et al. 2015) and *A. oryzae* (Sapna and Singh 2017). Mn^{2+} showed the variable effects on phytase activity, positive at low and negative at high concentrations. Mn^{2+} decreases the activity of phytase extracted from *A. flavus* (Gaind and Singh 2015). The inhibitory effect of NaCl is observed with *P. oxalicum* phytase, which is in accordance with the study of Kaur et al. (2017) in *P. oxalicum*. In turn, Na^{+} enhances the activity of phytase isolated from *R. mucilaginosa* (Yu et al. 2015).

The results of present study showed that phytase activity was inhibited in the presence of EDTA, sodium molybdate, ß-ME, and DTT, but stimulated in the presence of sodium azide. Similarly, EDTA inhibited the activity of phytase obtained from *P. oxalicum* (Kaur et al. 2017), and *A. tubingensis* TEM 37 (Caliskan-Ozdemir et al. 2021), indicating the requirement of metal ions for maintaining the activity of an enzyme. Inversely, EDTA largely increased the activity of phytase obtained from *Rhodotorula mucilaginosa* (Yu et al. 2015), however, there was no

impact of EDTA on the activity of *A. oryzae* phytase (Sapna and Singh 2017). ß-ME and DTT were also reported to decrease the activity of *A. niger* UFV-1 (Monteiro et al. 2015) and *P. oxalicum* (Kaur et al. 2017) phytase, depicting the importance of sulfhydryl groups in the catalysis of phytase (Monteiro et al. 2015). Sodium molybdate and sodium azide were found to be involved in lowering the activity of *A. oryzae* phytase (Sapna and Singh 2017) and *A. tubingensis* TEM 37 phytase (Caliskan-Ozdemir et al. 2021) respectively.

In this study, the K_m and V_{max} values of *P. oxalicum* PBG 30 phytase were 4.42 mM and 909.1 U/ml respectively. The high K_m indicates a low affinity between substrate and enzyme, and a high concentration of substrate is required to achieve the V_{max}. The K_m and V_{max} values of *A. oryzae* phytase were 1.14 mM and 58.82 µmol/ml/min (Sapna and Singh 2017), *A. aculeatus* APF1 phytase were 3.21 mM and 3.78 U/mg (Saxena et al. 2020), and of *A. fumigatus* phytase were 0.75 mM and 345 µmol/min/mg-protein (Thakur et al. 2022) with the calcium phytate. In other studies, the K_m and V_{max} values of phytase with sodium phytate were 0.545 mM and 600 U/mg in the case of *P. oxalicum* PJ3 (Lee et al. 2015), 0.48 mM and 672 U/mg in *P. oxalicum* KCTC6440 (Kim et al. 2015), 0.815 mM and 1092 U/mg in *R. mucilaginosa* (Yu et al. 2015) and 1.98 mM and 99 U/mg in the case of *A. niger* NT7 (Kumari and Bansal 2022).

Pepsin and trypsin are two important digestive enzymes present in the animals and human beings. Phytase must have the ability to resist the action of both pepsin and trypsin for supplementing animal feed with phytase. The *P. oxalicum* PBG 30 phytase retained 88 and 80% activity after the treatment with pepsin and trypsin up to 2 h, indicating that there is no significant effect of digestive enzymes on phytase. Moreover, the phytase obtained from *P. oxalicum* PBG 30 is protease-resistant. Similarly, 88 and 98 % phytase activity was observed after 120 min in *A. tubingensis* TEM 37 when treated with pepsin and trypsin respectively (Caliskan-Ozdemir et al. 2021). The phytase isolated from *A. niger* UFV-1 exhibited more than 90% activity in the presence of pepsin and trypsin treatment carried out for 1 h (Monteiro et al. 2015). The protease-resistant phytase have also been isolated from other microorganisms such as *S. thermophile* (Ranjan et al. 2015), *R. mucilaginosa* JMUY14 (Yu et al., 2015), *A. niger* NT7 (Kumari and Bansal 2022).

Over the period, the activity of an enzyme gets reduced, therefore it is necessary to study the effect of storage conditions on the activity of enzymes. The stability of an enzyme depends upon the molecule and storage conditions, and thus their shelf-life duration varies from

a few days to a year (Sapna and Singh 2017). When the enzyme is stored in liquid, frozen, or powder forms at different temperatures, it faces stress due to changing micro-environmental conditions, thus conformational modification occurs in the enzyme (Sapna and Singh 2017). The *P. oxalicum* PBG30 phytase maintained its activity for a long time at 4°C and -20°C. Similarly, Sapna and Singh (2017) found 4º C as the most suitable storage condition retaining 63% activity in *A. oryzae* phytase after 6 months while 56% activity remain persists with phytase kept at -20°C. Moreover, *A. niger* CFR 335 showed more than 80 % phytase activity at room temperature after the incubation of 3 months (Gunashree and Venkateswaran 2015) and *P. adiposa* phytase expressed a reduction in its activity after incubation of 24 days at room temperature, 32 days at -20°C and 42 days at 4°C (Jatuwong et al. 2020b).

The results indicated that *P. oxalicum* PBG30 phytase acts as haloperoxidase in the presence of vanadium. The vanadium ion gets incorporated in the active site of phytase and makes a semi-synthetic peroxidase. This peroxidase tends to catalyze the sulfoxidation of thioanisole. Structurally, vanadium chloroperoxidase resembles with acid phosphatases and acts as a substitute for phosphate and binds to phosphoryl transfer enzyme, thus reaching to intermediate state of reaction and decreasing the phytase activity. Vanadate acts as an anion and binds with positively charged amino acids present in the active site of phytase. By interfering with the phosphoesterase activity, it changes the enzyme functionality and develops a vanadate-dependent haloperoxidase. Only HAP phytase behaves as haloperoxidase due to the similarity in its active site (Joshi and Satyanarayana 2015, Singh et al. 2018). The semi-synthetic peroxidase behaviour of phytase has also been studied as well in *S. thermophile* (Singh et al. 2018), *P. anomala* (Joshi and Satyanarayana 2015), and *A. oryzae* (Pragya et al. 2023b). The application of vanadate-dependent haloperoxidase found in the formation of radiolabelled monoclonal antibodies, quantification of chloride amount in samples, diagnostic purposes, antimicrobial work, and immunological studies (Joshi and Satyanarayana 2015, Singh et al. 2018).

3.2.7 Immobilization of *P. oxalicum* PBG30 phytase

Immobilization is a widely adopted technique used to prevent enzymes from harsh environmental conditions and denaturation and provide stability to enzyme activity. The process is beneficial as it is reusable, easy to handle, has no loss of enzyme, easy to carry, and

prevents activity for a long time. Alginate has been commonly utilized as a matrix for the immobilization of enzymes as it provides a long shelf-life and enhances enzyme performance. Matrix is made up of cross-linking polymers of barium chloride, calcium chloride, poly-L-lysine with carboxylic group of α-L-guluronic acid and has no harmful impacts as the enzyme is captured mechanically in the matrix (Qamar et al. 2020).

Selection of a suitable concentration of sodium alginate is necessary for the immobilization. As the high concentration leads to reduced gel porosity and limited diffusion of a substrate, the addition of more than sufficient alginate makes the solution more viscous leading to a difficult entrapment procedure. Furthermore, low concentration developed beads with huge pore sizes, which is responsible for enzyme loss occurring due to leakage from pores (Bilal et al. 2018, Aslam et al. 2021). The *P. oxalicum* PBG30 phytase is immobilized in the form of calcium alginate beads. Sirin et al. (2017) immobilized phytase isolated from *Geobacillus* sp. TF16 via calcium alginate method with 42% binding efficiency rate. In another study, *A. oryzae* phytase showed 53% efficiency through the alginate process (Pragya et al. 2023a). The reusability of calcium alginate beads was studied and found effective up to 5 consecutive cycles. A decrease in phytase activity was found after each consecutive cycles because of enzyme leakage conditions arise due to the washing of beads at the end of each cycle. In the last cycle, beads become fragile and unable to be extracted from the reaction mixture.

3.2.8 Applications of *P. oxalicum* PBG30 phytase in the feed industry

3.2.8.1 Hydrolysis of the insoluble metal-phytate complex:

Phytic acid is associated with various metal ions and is available in the complex form of insoluble metal phytate. These insoluble complexes are unable to be digested by monogastric animals and restrict the assimilation of P and other essential metal ions. In the case of plants, phytic acid is adsorbed on clays or found in the form of insoluble salts of Fe and Al (acidic soils) or salts of Ca (alkaline soils). These insoluble forms of salts are unable to be utilized and, hence must be hydrolyzed before their absorption (Sapna and Singh 2013). The hydrolysis of these insoluble salts could accomplish with the help of phytase enzymes. In animals, these insoluble metal phytate complex leads to malnutrition conditions (Kumari and Bansal 2022).

The hydrolysis of the metal-phytate complex by *P. oxalicum* PBG30 phytase was studied at both 37°C and 50°C and exhibited the highest efficiency at 37°C. The hydrolysis of insoluble salt phytates has also been studied using *A. oryzae* phytase (Sapna and Singh 2013). The hydrolysis action occurred at 50°C was better than the action taken at 25°C and liberation of the inorganic phosphate was enhanced with time (Sapna and Singh 2013). The effect of *S. thermophile* phytase on the hydrolysis of metal phytate was found higher at 60°C in comparison to 26°C (Singh and Satyanarayana 2010). *A. niger* NT-7 phytase was also found capable of releasing metal ions from the insoluble metal phytate complex and showed more liberation of Zn^{2+}> Ca^{2+}> Co^{2+}> Fe^{2+}> Fe^{3+} at 50°C than that of the 37°C (Kumari and Bansal 2022). Phytate complexes of Ca, Mn, Mg, Zn, and Cd were hydrolysed more efficiently by *A. niger* phytase than the complexes of Al and Fe-phytates (Sun et al. 2021).

3.2.8.2 Hydrolysis of insoluble protein-phytate complex:

Phytic acid also makes complexes with proteins, amino acids, and enzymes and restricts their utilization. Plant-based proteins are usually found as insoluble protein-phytate complex forms, therefore, these are incapable of being utilized by monogastric animals. Due to the phytate-protein complex, turbidity occurred in the solution (Tran et al. 2011). The effect of *P. oxalicum* PBG30 phytase in degrading phytate-lysozyme complex was represented by the release of inorganic phosphorus with time. Similar work was performed on the hydrolysis of phytate association with BSA and lysozyme by using the phytase of *A. oryzae*. Further, they described the time-dependent relationship between inorganic phosphorus and the absorbance of the solution. Inorganic phosphorus was increased while absorbance was decreased with time (Sapna and Singh 2013). Also, the hydrolysis of complexes between phytate-lysozyme, phytate-BSA, phytate-calcium, and phytate-lysine was studied by Tran et al. 2011. Among them, the phytate-lysozyme complex was considered as best substrate for the hydrolysis and liberation of inorganic phosphate. They also observed that the release of inorganic phosphate was correlated with the turbidity of complex. As the amount of inorganic phosphate increased, the level of turbidity decreased. Kumari and Bansal (2022) also studied the relation of the absorbance of turbid solution with the release of inorganic phosphate from phytate-lysozyme complex in the presence of *A. niger* NT-7 phytase. A decrease in absorbance value was seen with the hydrolysis action of phytase, indicating that a drop in turbidity level is inversely proportional with the liberation of inorganic phosphate.

3.2.8.3 Dephytinization of commercial fish feed

Agricultural feed additives contain phytic acid that binds minerals, proteins, and other biomolecules and makes phytate complex, which restricts the availability of nutritional components and lowers down the nutritive value of feed. Phytase is effective in phytate hydrolysis and releases inorganic phosphate, soluble protein, and reducing sugar. Monogastric animals like humans, poultry, birds, and fish lack phytase in their gastrointestinal region. Due to this, phytate accumulates in their body and remains undigested and excreted out in manure, runs off through erosion, reaches water bodies, thereby pollutes the aquatic environment. Dephytinization is the best-selected approach to hydrolyze the phytate and release the P and other essential components by supplementing phytase exogenously. The addition of phytase as a feed additive not only ameliorates the feed's nutritive value but also reduces phosphorus pollution (Priya et al. 2023b). The present study demonstrated the impact of *P. oxalicum* PBG30 phytase on hydrolyzing phytate molecules present in the feed. With time, the liberation of inorganic phosphate, reducing sugar, and soluble protein is enhanced due to the supplementation of phytase in the diet (Jain et al. 2018).

In the present study, the effect of *P. oxalicum* PBG30 phytase on dephytinizing feed was studied at 37°C, 50°C, and 70°C. The less difference was observed in the amount of inorganic phosphorus between 37°C and 50°C showing the activeness of phytase enzyme at both temperatures. Further, a low secretion of inorganic phosphorus at 70°C might be due to the denaturation of enzyme for a long period of incubation or disruption of nutritional components of the feed. The reduction of phytic acid in flours has also been studied at 50°C and 37°C by adding phytase isolated from *A. oryzae* and exhibited maximum secretion of inorganic phosphate, soluble proteins, and reducing sugars at 50°C (Pragya et al. 2023b). The dephytinization of feed samples showed maximum liberation of nutritional components at 50°C with *A. oryzae* phytase (Sapna and Singh 2017), and at 55°C with *R. oligosporus* phytase (Suresh and Radha 2015). In another study, the dephytinization of poultry feed was carried out at 37°C using *A. niger* phytase (Buddhiwant et al. 2016). Similarly, inorganic phosphorus was released after the addition of phytase isolated from recombinant *Pichia anomala* in poultry feed at 40°C (Joshi and Satyanarayana 2015). The involvement of phytase obtained from recombinant strain *P. griseoroseum* T73 in dephytinizing pig feed (corn, soybean, and wheat) was evaluated at 37°C. The liberation of inorganic phosphate in corn, soybean, and wheat was recorded as 85%, 67%, and 73% respectively (Correa et al. 2015). Also, the hydrolysis of

commercial pig feed was assessed at 50°C with phytase isolated from *A. niger* UFV-1 that secreted 15.3 μmol phosphate/ml during the experiment (Monteiro et al. 2015). The inorganic phosphate was released from the feedstuffs including rice bran, soybean meal, and corn meal upon treatment with *P. oxalicum* phytase at 55°C (Kim et al. 2015). The dephytinization of feed was also studied at 25°C by phytase extracted from *Morchella importuna*. The inorganic phosphate obtained after degradation of phytate was 3.15 ± 0.29 mg from soybean meal, 2.60 ± 0.32 mg from rapeseed meal, and 2.82 ± 0.31 mg from corn meal (Tan et al. 2017).

During the present investigation, different doses of phytase (25-200 U) were supplied to the feed and evaluated their impact on phytate degradation. Phytase exhibited a dose-response relationship. The secretion of inorganic phosphorus is enhanced as the amount of phytase doses increases and phytase removes an organically bound phosphate associated with the phytate molecule and reaches a plateau when the phytate becomes deficient (Thorsen et al. 2021). Results of the present study showed that there is a simultaneous increase in the inorganic phosphorus with an increase in phytase dose. In contrast, poultry feed treated with different doses of *S. thermophile* showed equivalent nutritional amounts at 50 and 100 U phytase doses (Kumari et al. 2016), while in flours, it was observed that the increment of released components gradually increased with phytase dose and declined after a certain level (Pragya et al. 2023b).

In this study, the highest dephytinization was found with 0.5 g feed. This might be due to the highest binding between active sites of substrate and enzyme in 0.5 g feed. As the amount of feed increases, no more enhancement is observed due to the possibility of no more binding site availability or the presence of substrate in insoluble form. Reduction in the liberation of inorganic phosphorus might be due to negative feedback inhibition of inorganic phosphorus (the hydrolytic end product) on the phytase.

The maximum liberation of nutritional by-products was found within 6 h of incubation period. The release of inorganic phosphorus in poultry feed by rSt-Phy was evaluated with 25, 50, and 75 min, and the highest secretion was found with 50 min (Ranjan and Satyanarayana 2016). At 2.5 h, the pig feed represented the maximum release of inorganic phosphorus with *A. niger* UFV-1 phytase (Monteiro et al. 2015). The supplementation of *A. niger* NT7 phytase in cattle feed secretes the highest inorganic phosphate up to 72 h (Kumari and Bansal 2021a).

The application of immobilized phytase on feed and flours has also been studied, showing the release of inorganic phosphorus, reducing sugars, and soluble proteins with time (Lopes et al. 2021). The comparison effect of free and immobilized phytase was evaluated on

the hydrolysis of a legumes-based diet. The immobilized phytase exhibited more efficiency than free phytase in the hydrolysis of legumes-based diet (Onem et al. 2016). However, in another study, the efficiency of free and immobilized phytase was almost similar (Coutinho et al. 2020). Dahiya et al. (2020), performed the dephytinization of flours by supplementing both phytase and xylanase and showed the highest liberation due to the synergistic effect of both of the enzymes.

3.2.8.4 Phytic acid estimation in experimentally prepared feed

Several methods like high-performance liquid chromatography (HPLC), gas chromatography (GC), fourier transform infrared radiations (FTIR), nuclear magnetic resonance (NMR), spectrophotometry, or colorimetric methods can be applied for the detection of phytic acid in food and feed samples. However, a colorimetric method is preferred due to its cheaper and easy availability. The modified colorimetric method with the use of Wade reagent is commonly utilized for the estimation of the phytic acid amount in food and seeds. In this method, pink colour is developed due to a reaction between phytic acid and Wade reagent (a mixture of ferric chloride and sulphosalicylic acid). In the plant-based feed, phytic acid is present in major amounts. In the present study, the application of phytase in the formulated fish feed is analyzed to determine the reduction of phytic acid in the feed. The extraction was done by 2.4% HCl as it promotes hydrolysis of phytic acid and liberates the phosphate group in an acidic condition. HCl provides H^+ ions to the phosphate, separates it from the phytic acid molecule, and releases minerals. The secreted phosphate ions make monobasic and dibasic phosphate salts with the HCl, which are soluble in solution. After centrifugation, these phosphate salts and hydrolyzed phytic acid accumulated in the supernatant, which reacts further with the Wade reagent (Pragya et al. 2023b).

The phytic acid content was checked in the control and phytase-treated diet. The diet treated with *P. oxalicum* PBG30 phytase showed a reduction in the phytic acid amount, which was further lowered with an increase in the phytase dose. The minimum amount of phytic acid was found with the diet IV (1500 FTU/kg phytase). The reduction of phytic acid content is due to the hydrolysis action of phytase. Similarly, the effect of phytase in the degradation of phytate was evaluated by adding *S. cerevisiae* MTCC 5421 phytase in refined wheat flour and pearl millet flour for the formation of naan and rabadi respectively. A 95% reduction was found in

the case of naan while complete hydrolysis was observed with rabadi (Roopashri and Varadaraj 2015). In another experimental work, bread was formed with the insertion of a recombinant phytase enzyme obtained from *Sporotrichum thermophile* in wheat flour. Naan and tandoori roti were also made from this enzyme. A decrease in phytic acid content was recorded as 70, 62.5, and 75% in bread, naan, and tandoori roti respectively (Ranjan et al. 2015). Also, tandoori and naan were made from the supplementation of phytase isolated from a recombinant strain of *Pichia anomala* showed a decrease in phytic acid amount by 67.5% and 23.2% respectively (Joshi and Satyanarayana 2015).

CHAPTER 4

SUMMARY, CONCLUSION & FUTURE SCOPE

CHAPTER 4

SUMMARY, CONCLUSION AND FUTURE SCOPE

4.1 Summary

The phytic acid is a storage form of phosphorus and are commonly found in plant seeds such as cereals, legumes, oilseed, nuts and pollen grains. Phytate (a complex form of phytic acid) associates with metal ions, proteins, enzymes, and other biomolecules and restricts their absorption. Phosphorus is an essential element for the growth and development of living organisms. It is involved in various metabolic and signalling pathways. Phytase degrades phytic acid and releases inorganic phosphate and lowers esters forms of myo-inositol. Phytases are commonly present in plants, animals and microbes (bacteria, fungi and yeast). Among them, microbial phytase showed excellent phytate degrading activity. Both enzymatic and non-enzymatic methods are involved in the reduction of phytic acid. The non-enzymatic method includes physical and chemical processes that lower the nutritional value of food and feed, therefore, using phytase (an enzymatic way) could be an efficient approach. Phytase is absent in monogastric animals like fish, hence, the supplementation of exogenous phytase in the feed is essential for their nutrition. Phytase liberates phosphate molecules from the phytate-phosphorus and enhances the phosphorus level in the diet. Along with phosphorus, it also increases the availability of other essential nutrients remain limited due to phytate. Phytase has diverse applications in the food and feed industry, pharmaceutical, bioethanol formation, enhancement of plant growth and reducing environmental pollution.

In this research work, 55 microbes were screened through the samples collected from various sources. *Penicillium oxalicum* PBG30 was selected as a potent phytase-producing fungus among all the microbes screened, and used for the phytase production. *P. oxalicum* PBG30 is identified by morphological features and molecular identification via ITS sequencing. *P. oxalicum* PBG30 is cultured on potato dextrose agar medium and incubated at 30°C for 3 days. The morphological description of *P. oxalicum* PBG30 was an initial white colour that became dark green on maturation. The texture of the colonies was velvety and had powdery growth. Under the microscope, the fungal hyphae were septate, branched

conidiophores and conidia (fungal spores) were found in a chain with conidiophore, a characteristic feature of Penicillium species.

Phytase is produced through the SSF method with wheat bran substrate. Wheat bran is commonly used for SSF because it is rich in carbon, nitrogen, and amino acids, and has a high amount of phytic acid. Due to the presence of phytate, it acts as an inducer for the production of phytase. It is easily available at low cost and also provides a matrix for the growth of fungi. The factors such as wheat bran concentration, moisture and substrate ratio, incubation days, pH, and temperature were optimized by OVAT. After applying OVAT, the production of *P. oxalicum* PBG30 phytase was recorded with 10 g wheat bran with production media of pH 7.0 in a 1:2 ratio, incubated at 30°C for 5 days. Production media includes 0.5% Urea, 0.1% $MgSO_4.7H_2O$, 0.1% KCl and 0.1% $FeSO_4.7H_2O$. A 2.2-fold phytase level was increased during OVAT.

The statistical optimization including PBD and RSM was performed after OVAT leading to a 4.4-fold enhancement in phytase production. Incubation period, pH, magnesium sulphate and Tween 80 were found as a significant variables by PBD. The best production was obtained with 5 g wheat bran with 10 ml production media (including 0.5% ammonium sulphate, 0.01% $FeSO_4$, 3.5% Tween 80 and 0.75% $MgSO_4$) of pH 7.0 in 1:2 ratio with incubation at 30°C for 5 days in the RSM experiment. The phytase production was also performed in flasks and trays on a large scale. On a large scale, the production of phytase increases by 5.6-fold. Additionally, *P. oxalicum* PBG30 also produces other hydrolytic enzymes such as cellulase (51.06 U/g DMR), amylase (86.20 U/g DMR), xylanase (18.05 U/g DMR) and lipase (7.05 U/g DMR) along with phytase.

P. oxalicum PBG30 was partially purified by ammonium sulphate precipitation and dialysis. The optimal temperature and pH were 70°C and 3.0, respectively. The thermostable half-life of phytase was 1 h at 70°C. *P. oxalicum* PBG30 exhibited broad substrate specificity and the maximum is shown with sodium phytate. The K_m and V_{max} values were 4.42 mM and 909.1 U/ml. The phytase activity was influenced by organic solvents, detergents, metal ions, and inhibitors. Organic solvents exhibited a positive impact. Except for SDS, other detergents were involved in enhancing the activity. Among inhibitors, EDTA, ß-ME, DTT and sodium molybdate inhibited the activity. *P. oxalicum* PBG30 phytase showed resistance against pepsin and trypsin. The shelf life of *P. oxalicum* PBG30 phytase represented more stability at 4°C and

-20°C compared to room temperature. Besides that, the phytase of *P. oxalicum* PBG30 also possesses the ability to act as haloperoxidase.

The phytase is immobilized into calcium alginate beads by entrapment method. The beads retained phytase activity up to 5 consecutive cycles. *P. oxalicum* PBG30 phytase catalyzes the complex of insoluble phytate with metal and proteins. Except Mg^{2+}, Fe^{2+}, all the metal ions complex of Ca^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+} showed release of inorganic phosphate. The liberation of inorganic phosphate increases with time. A similar result was obtained with the phytate-lysozyme complex. The dephytinization of fish feed was studied at 37°C with the release of inorganic phosphate, reducing sugars and protein. The maximum hydrolysis was observed with 200 U of *P. oxalicum* PBG30 phytase added in 0.5 g of feed dissolved in 20 ml buffer with 6 h of incubation. Moreover, the reduction of phytic acid was examined after mixing different doses of phytase in the ingredient of fish feed and the highest degradation (46.6%) was observed with 1500 FTU/kg feed.

4.2 Conclusion

- Through screening, *P. oxalicum* PBG30 was found as the most suitable fungus for the production of phytase, which was identified based on morphological features and molecular sequencing.

- Wheat bran is a low-cost and easily available agricultural waste commonly used for phytase production. The utilization of wheat bran as a substrate resolves its disposal issues as well as environmental problems.

- The highest secretion of *P. oxalicum* PBG30 phytase (475.42 ± 77.31 U/g DMR) was obtained with 5 g of wheat bran mixed with 10 ml moistening media of pH 7.0 incubated at 30°C for 5 days. Moistening media contains 0.5% ammonium sulphate, 0.01% $FeSO_4$, 3.5% Tween 80 and 0.75% $MgSO_4$.

- Statistical optimization includes PBD and RSM enhanced the production level by 5.6-fold.

- Along with phytase, *P. oxalicum* PBG30 also secretes other hydrolytic enzymes like cellulase (51.06 U/g DMR), amylase (86.20 U/g DMR), xylanase (18.05 U/g DMR) and lipase (7.05 U/g DMR).

- Partially purified phytase of *P. oxalicum* PBG30 exhibited optimum activity at 70°C and 3.0. The phytase obtained from *P. oxalicum* PBG30 is found thermostable and protease-resistant. Also, it has an acidic nature, broad substrate specificity and is stable in storage conditions. Due to these characteristics features, it can be applicable in food and feed industries to overcome the anti-nutritional effect of phytate.

- Phytase behaves as a haloperoxidase in the existence of vanadium ions.

- Phytase of *P. oxalicum* PBG30 is immobilized on calcium alginate beads that retain phytase activity up to 5 cycles.

- *P. oxalicum* PBG30 phytase hydrolyzed insoluble metal phytate and protein phytate complex and gradually released inorganic phosphate with time.

- The dephytinization of fish feed by *P. oxalicum* PBG30 phytase liberates inorganic phosphate, reducing sugar and soluble protein with time. The highest dephytinization was obtained with 200 U of *P. oxalicum* PBG30 phytase added in 0.5 g of feed dissolved in 20 ml buffer with 6 h of incubation.

- The involvement of *P. oxalicum* PBG30 phytase in lowering phytic acid in the fish feed is studied with a 1500 FTU/kg dose of phytase showed the highest reduction (46.6%).

4.3 Future Scope

- The phytase possesses features of an ideal phytase (thermostable, protease-resistant, wide pH range, acidic, broad substrate specificity, long shelf life) and can be applicable in the food and feed industry.

- Production of phytase can be enhanced by using bioreactors or cloning and recombination techniques by inserting phytase gene into a suitable host.

- Recombination and protein engineering are the alternatives that can be chosen to enhance the properties of an ideal phytase.

- Immobilization of phytase by nanomaterials can be used to improve the reusability of phytase.

- The *in-vitro* and *in-vivo* study of phytase impact on monogastric animal nutrition as a feed additive needs more examination.

- Phytase exhibits haloperoxidase behaviour in the presence of vanadium requires further investigation.

REFERENCES

REFERENCES

- Abdulwaliyu, I., Arekemase, S. O., Adudu, J. A., Batari, M. L., Egbule, M. N. & Okoduwa, S. I. R. (2019). Investigation of the medicinal significance of phytic acid as an indispensable anti-nutrient in diseases. *Clinical Nutrition Experimental*, 28, 42–61. https://doi.org/10.1016/j.yclnex.2019.10.002.
- Abd El-Hack, M. E., Alagawany, M., Arif, M., et al. (2018). The uses of microbial phytase as a feed additive in poultry nutrition—a review. *Annals of Animal Science*, 18 (3), 639-658. https://doi.org/10.2478/aoas-2018-0009.
- Adeshina, I., Akpoilih, B. U., Tiamiyu, L. O., Badmos, A. A., Emikpe, B. O. & Abdel-Tawwab, M. (2023). Effects of dietary supplementation with microbial phytase on the growth, bone minerals, antioxidant status, innate immunity and disease resistance of African catfish fed on high soybean meal-based diets. *Journal of Animal Physiology and Animal Nutrition*, 107 (2), 733-745. https://doi.org/10.1111/jpn.13765.
- Afzal, M., Fatima, M., Qamar, A., Farhan, M. & Shah, S. Z. H. (2020). Growth, muscle proximate composition and whole-body nutrient status of *Labeo rohita* fed acidified and phytase pre-treated sunflower meal based diet. *Pakistan Journal of Zoology*, 52 (3), 937-947. https://doi.org/10.17582/journal.pjz/20190408080431.
- Agostinho, A. J., de Souza Oliveira, W., Anunciação, D. S., & Santos, J. C. C. (2016). Simple and sensitive spectrophotometric method for phytic acid determination in grains. Food Analytical Methods, 9, 2087-2096. https://doi.org/10.1007/s12161-015-0387-0.
- Ahmed, N. E., Salem, S. S. & Hashem, A. H. (2022). Statistical optimization, partial purification, and characterization of phytase produced from *Talaromyces purpureogenus* NSA20 using potato peel waste and its application in dyes de-colorization. *Biointerface Research in Applied Chemistry*, 12 (4), 4417-4431. https://doi.org/10.33263/BRIAC124.44174431.
- Ajith, S., Shet, D., Ghosh, J., Awachat, V. B., Bhat, K., Pal, D. & Elangovan, A. V. (2018). Effect of immobilized fungal phytase on growth performance and bone traits of broilers fed with low dietary calcium and phosphorus. *Veterinary World*, 11 (6), 758-764. https://doi.org/10.14202/vetworld.2018.758-764.

- Ajith, S., Ghosh, J., Shet, D., ShreeVidhya, S., Punith, B. D., & Elangovan, A. V. (2019). Partial purification and characterization of phytase from *Aspergillus foetidus* MTCC 11682. *AMB Express*, 9 (3), 1-11. https://doi.org/10.1186/s13568-018-0725-x.
- Ali, S. & Kaviraj, A. (2018). Aquatic weed Ipomoea aquatica as feed ingredient for rearing Rohu, *Labeo rohita* (Hamilton). *Egyptian Journal of Aquatic Research*, 44 (4), 321-325. https://doi.org/10.1016/j.ejar.2018.09.004.
- Alves, N. M., Guimarães, L. H. S., Piccoli, R. H. & Cardoso, P. G. (2016). Production and partial characterization of an extracellular phytase produced by *Muscodor* sp. under submerged fermentation. *Advances in Microbiology*, 6 (1), 23-32. http://dx.doi.org/10.4236/aim.2016.61003.
- Aly, M. M., Sanaa Tork, S. T., Al-Garni, S. M. & Kabli, S. A. (2015). Production and characterization of phytase from *Streptomyces luteogriseus* R10 isolated from decaying wood samples. *International Journal of Agriculture and Biology,* 17 (3), 515-522. https://doi.org/10.17957/IJAB/17.3.14.453.
- Arora, S., Dubey, M., Singh, P., Rani, R. & Ghosh, S. (2017). Effect of Mixing Events on the Production of a Thermo-Tolerant and Acid-Stable Phytase in a Novel Solid-State Fermentation Bioreactor. *Process Biochemistry*, 61, 12–23. https://doi.org/10.1016/j.procbio.2017.06.009.
- Aslam, S., Asgher, M., Khan, N. A. & Bilal, M. (2021). Immobilization of *Pleurotus nebrodensis* WC 850 laccase on glutaraldehyde cross-linked chitosan beads for enhanced biocatalytic degradation of textile dyes. *Journal of Water Process Engineering*, 40, 101971. https://doi.org/10.1016/j.jwpe.2021.101971.
- Attia, Y. A., Bovera, F., Al-Harthi, M. A., El-Din, A. E. R. E. T. & Said Selim, W. (2021). Supplementation of microbial and fungal phytases to low protein and energy diets: Effects on productive performance, nutrient digestibility, and blood profiles of broilers. *Agriculture*, 11 (5), 414. https://doi.org/10.3390/agriculture11050414.
- Awad, G. E. A., Helal, M. M. I., Danial, E. N. & Esawy, M. A. (2014). Optimization of phytase production by *Penicillium purpurogenum* GE1 under solid state fermentation by using Box-Behnken design. *Saudi Journal of Biological Sciences*, 21, 81-88. http://dx.doi.org/10.1016/j.sjbs.2013.06.004.
- Awad, G. E., Esawy, M. A., El-Gammal, E. W., Ahmed, H. M., Elnashar, M. M., & Atwa, N. A. (2015). Comparative studies of free and immobilized phytase, produced

by *Penicillium purpurogenu* GE1, using grafted alginate/carrageenan beads. *Egyptian Pharmaceutical Journal*, 14(2), 87-93. https://doi.org/10.4103/1687-4315.161268.

- Aziz, G., Nawaz, M., Anjum, A. A., Yaqub, T., Nazir, J., Khan, S. U. & Aziz, K. (2015). Isolation and characterization of phytase producing bacterial isolates from soil. *Journal of Animal & Plant Sciences*, 25 (3), 771-776.
- Bae, H. D., Yanke, L. J., Cheng, K. J. & Selinger, L. B. (1999). A novel staining method for detecting phytase activity. *Journal of microbiological methods*, 39 (1), 17-22. https://doi.org/10.1016/s0167-7012(99)00096-2.
- Baruah, K., Norouzitallab, P. & Pal, A. K. (2017). Development of low cost and eco-friendly feed for various candidate species for the sustainability of commercial aquaculture and reduction of aquatic pollution [Chapter 24]. In: Bagchi D, Nair S, eds. *Developing New Functional Food and Nutraceutical Products.* Academic Press, 441-453. https://doi.org/10.1016/B978-0-12-802780-6.00024-9.
- Belho, K. & Kumar Ambasht, P. (2021). Immobilization of phytase from rice bean (*Vigna umbellata* Thunb.) on glutaraldehyde activated chitosan microspheres. *Journal of Scientific Research*, 65 (1), 111–119. https://doi.org/10.37398/jsr.2021.650115.
- Bentes, V. V., Bittencourt, F., Neu, D. H., Signor, A. & Eyng, C. (2020). Replacement of corn by rice grits, with or without phytase supplementation, in diets for silver catfish (*Rhamdia quelen*) juveniles. *Aquaculture Nutrition*, 26 (5), 1584-1591. https://doi.org/10.1111/anu.13104.
- Bhagat, D., Raina, N., Kumar, A., Katoch, M., Khajuria, Y., Slathia, P. S. & Sharma, P. (2020). Probiotic properties of a phytase producing *Pediococcus acidilactici* strain SMVDUDB2 isolated from traditional fermented cheese product, Kalarei. *Scientific Reports*, 10 (1), 1926. https://doi.org/10.1038/s41598-020-58676-2.
- Bhandari, Y., Sonwane, B. & Vamkudoth, K. R. (2023). Isolation and Biochemical Characterization of Acid Phytase from *Aspergillus niger* and Its Applications in Dephytinization of Phytic Acid in Poultry Feed Ingredients. *Microbiology*, 92 (2), 221-229. https://doi.org/10.1134/S0026261722601452.
- Bilal, M., Zhao, Y., Rasheed, T. & Iqbal, H. M. N. (2018). Magnetic nanoparticles as versatile carriers for enzymes immobilization: A review. *International Journal of Biological Macromolecules,* 120, 2530–2544. https://doi.org/10.1016/j.ijbiomac.2018.09.025.

- Bloot, A. P. M., Kalschne, D. L., Amaral, J. A. S., Baraldi, I. J. & Canan, C. (2023). A review of phytic acid sources, obtention, and applications. *Food Review International*, 39 (1), 73-92. https://doi.org/10.1080/87559129.2021.1906697.
- Buddhiwant, P., Bhavsar, K. & Kumar, V. R. (2016). Phytase production by solidstate fermentation of groundnut oil cake by *Aspergillus niger*: a bioprocess optimization study for animal feedstock applications. *Preparative Biochemistry and Biotechnology*, 46, 531–8. http://dx.doi.org/10.1080/10826068.2015.1045606.
- Bujna, E., Rezessy-Szabó, J. M., Nguyen, D. V. & Nguyen, D. Q. (2016). Production and some properties of extracellular phytase from *Thermomyces lanuginosus* IMI 096218 on rice flour as substrate. *Mycosphere*, 7 (10), 1576-1587. https://doi.org/10.5943/mycosphere/si/3b/9.
- Buzek, A., Zaworska-Zakrzewska, A., Muzolf-Panek, M. & Kasprowicz-Potocka, M. (2023). Microbial Phytase in a Diet with Lupine and Extruded Full-Fat Soya Seeds Affects the Performance, Carcass Characteristics, Meat Quality, and Bone Mineralization of Fatteners. *Animals*, 13 (10), 1655. https://doi.org/10.3390/ani13101655.
- Caliskan-Özdemir, S., Önal, S. & Uzel, A. (2021). Partial Purification and Characterization of a Thermostable Phytase Produced by Thermotolerant *Aspergillus tubingensis* TEM 37 Isolated from Hot Spring Soil in Gediz Geothermal Field, Turkey. *Geomicrobiology Journal*, 38 (10), 895-904. https://doi.org/10.1080/01490451.2021.1977432.
- Cantas, I. B. & Yildirim, O. (2020). Supplementation of microbial phytase with safflower meal in rainbow trout (*Oncorhynchus mykiss*): the effects on growth, digestibility, environmental, and serum biological parameters. *Journal of Chemistry*, 8, 1-8. https://doi.org/10.1155/2020/4634796.
- Capusoni, C., Serra, I., Donzella, S. & Compagno, C. (2021). Screening for yeast Phytase leads to the identification of a new cell-bound and secreted activity in *Cyberlindnera jadinii* CJ2. *Frontiers in bioengineering and biotechnology*, 9, 662598. https://doi.org/10.3389/fbioe.2021.662598.
- Chanderman, A., Puri, A. K., Permaul, K. & Singh, S. (2016). Production, Characteristics and Applications of Phytase from a Rhizosphere Isolated *Enterobacter* sp. ACSS. *Bioprocess and Biosystems Engineering*, 39, 1577–1587. https://doi.org/10.1007/s00449-016-1632-7.

- Chatraei, N. & Emtiazi, G. (2019). Immobilization of phytase producing probiotics in shrimp chitosan cross-linked by zinc oxide nanoparticles and assay its antibacterial activity. *Applied Food Biotechnology*, 6 (3), 139-150. http://dx.doi.org/10.22037/afb.v6i3.23398.
- Chaudhari, S. B. & Peshwe, S. (2022). Exploiting *Aspergillus terreus* for the production of thermotolerant phytase. *Journal of Biodiversity*, 13 (1-2), 1-13. https://doi.org/10.31901/09766901.2022/13.1-2.107.
- Chen, C. C., Cheng, K. J., Ko, T. P. & Guo, R. T. (2015). Current progresses in phytase research: Three-dimensional structure and protein engineering. *ChemBioEng Reviews,* 2, 76–86. https://doi,org/10.1002/cben.201400026.
- Chen, A., Liu, X., Cui, C. et al. (2018). An evaluation of phytase for channel catfish (*Ictalurus punctatus*) fed all plant-protein diet: growth performance, nutrient utilization and P equivalency value. *Aquaculture Nutrition*, 25 (1), 215-224. https://doi.org/10.1111/anu.12845.
- Cheng, N., Chen, P., Lei, W., Feng, M. & Wang C. (2016). The sparing effect of phytase in plant-protein-based diets with decreasing supplementation of dietary NaH2PO4 for juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquaculture Research*, 47 (12), 3952-3963. https://doi.org/10.1111/are.12845.
- Chu, Z. J., Yu, D. H., Dong, G. F. & Gong, S. Y. (2016). Partial replacement of fish meal by soybean meal with or without methionine and phytase supplement in diets for juvenile Chinese sucker, *Myxocyprinus asiaticus*. *Aquaculture Nutrition*, 22 (5), 989-996. https://doi.org/10.1111/anu.12318.
- Coban, H. B. & Demirci, A. (2017). Phytase as a diet ingredient: from microbial production to its applications in food and feed industry [Chapter 2]. *Microbial production of food ingredients and additives*, 33-55. http://dx.doi.org/10.1016/B978-0-12-811520-6.00002-7.
- Correa, T. L. R., Queiroz, M. V. & Araújo, E. F. (2015). Cloning, recombinant expression and characterization of a new phytase from *Penicillium chrysogenum*. *Microbiological Research*, 170, 205–212. http://dx.doi.org/10.1016/j.micres.2014.06.005.
- Correa, T. L. R. & de Araújo, E. F. (2020). Fungal phytases: from genes to applications. *Brazilian Journal of Microbiology*, 51 (3), 1009-1020. https://doi.org/10.1007/s42770-020-00289-y.

- Coutinho, T. C., Tardioli, P. W. & Farinas, C. S. (2020). Phytase immobilization on hydroxyapatite nanoparticles improves its properties for use in animal feed. *Applied Biochemistry and Biotechnology*, 190, 270–92. https://doi.org/10.1007/s12010-019-03116-9.
- Czech, A., Samolińska, W., Tomaszewska, E., Muszyński, S. & Grela, E. R. (2022). Effect of microbial phytase on ileal digestibility of minerals, plasma and urine metabolites, and bone mineral concentrations in growing–finishing pigs. *Animals*, 12 (10), 1294. https://doi.org/10.3390/ani12101294.
- Dahiya, S., Kumar, A. & Singh, B. (2020). Enhanced endoxylanase production by *Myceliophthora thermophila* using rice straw and its synergism with phytase in improving nutrition. *Process Biochemistry*, 94, 235–42. https://doi.org/10.1016/j.procbio.2020.04.032.
- Danwitz, A. V., van Bussel, C. G., Klatt, S. F. & Schulz, C. (2016). Dietary phytase supplementation in rapeseed protein based diets influences growth performance, digestibility and nutrient utilisation in turbot (*Psetta maxima* L.). *Aquaculture*, 450, 405-411. http://dx.doi.org/10.1016/j.aquaculture.2015.07.026.
- Das, P. & Ghosh, K. (2015). Improvement of nutritive value of sesame oil cake in formulated diets for rohu, *Labeo rohita* (Hamilton) after bioprocessing through solid state fermentation by a phytase-producing fish gut bacterium. *International Journal of Aquatic Biology*, 3 (2), 89-101. https://doi.org/10.22034/ijab.v3i2.52.
- Dersjant-Li, Y., Awati, A., Schulze, H. & Partridge, G. (2015). Phytase in nonruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *Journal of the Science of Food and Agriculture,* 95(5), 878-896. https://doi.org/10.1002/jsfa.6998.
- Dersjant-Li, Y., Wealleans, A. L., Barnard, L. P. & Lane, S. (2017). Effect of increasing Buttiauxella phytase dose on nutrient digestibility and performance in weaned piglets fed corn or wheat based diets. *Animal Feed Science and Technology*, 234, 101–109. https://doi.org/10.1016/j.anifeedsci.2017.09.008.
- Dutta, N., Raj, D., Biswas, N., Mallick, M. & Omesh, S. (2017). Nanoparticle assisted activity optimization and characterization of a bacterial phytase immobilized on single layer graphene oxide. *Biocatalysis and Agricultural Biotechnology*, 9, 240–247. https://doi.org/10.1016/j.bcab.2017.01.008.

- Elkhateeb, Y. A. & Fadel, M (2022). Bioinformatic Studies, Experimental Validation of Phytase Production and Optimization of Fermentation Conditions for Enhancing Phytase Enzyme Production by Different Microorganisms under Solid-State Fermentation. *The Open Microbiology Journal*, 16, 1–11. https://doi.org/10.2174/18742858-v16-e2202160.
- Englmaierova, M., Skřivan, M., Skřivanová, E., Bubancova, I., Čermák, L. & Vlčková, J. (2015). Effects of a low-phosphorus diet and exogenous phytase on performance, egg quality, and bacterial colonisation and digestibility of minerals in the digestive tract of laying hens. *Czech Journal of Animal Science*, 60 (12), 542-549. https://doi.org/10.17221/8596-CJAS.
- Food and Agriculture Organization of the United Nations (via World Bank) – processed by Our World in Data (2023). "Aquaculture production (metric tons)" [dataset]. https://ourworldindata.org/grapher/aquaculture-farmed-fish-production?tab=map. Accessed on 27th May 2024.
- Filippovich, S. Y., Isakova, E. P., Gessler, N. N. & Deryabina, Y. I. (2023). Advances in immobilization of phytases and their application. *Bioresource Technology*, 379, 129030. https://doi.org/10.1016/j.biortech.2023.129030.
- Fiske, C. H. & Subbarow, Y. (1925). The Colorimetric Determination of Phosphorus. *Journal of Biological Chemistry,* 66 (2), 375–400. https://doi.org/10.1016/S0021-9258(18)84756-1.
- Fries, E. M., Oxford, J. H., Godoy, A. C. et al. (2020). Phytase on the digestibility of plant protein feed for silver catfish, *Rhamdia voulezi*. *Aquaculture,* 528, 735528. https://doi.org/10.1016/j.aquaculture.2020.735528.
- Gaind, S. & Singh, S. (2015). Production, purification and characterization of neutral phytase from thermotolerant *Aspergillus flavus* ITCC 6720. *International Biodeterioration & Biodegradation*, 99, 15-22. http://dx.doi.org/10.1016/j.ibiod.2014.12.013.
- Gampa, M., Nagar, S., Kumari, K., Tanwar, E., Goyal, S., Kumar, V. & Singh, B. (2023). Cyclic extraction of phosphate from soybean meal using immobilized *Aspergillus oryzae* SBS50 phytase. *Bioprocess and Biosystems Engineering*, 47 (1), 39-55. https://doi.org/10.1007/s00449-023-02943-9.

- Gangoliya, S. S., Gupta, R. K. & Singh, N. K. (2015). Phytase production through response surface methodology and molecular characterization of *Aspergillus fumigatus* NF191. *Indian Journal of Experimental Biology*, 53, 350-355.
- Gao, Y., Shang, C., Maroof, M. S., Biyashev, R. M., Grabau, E. A., Kwanyuen, P., Burton, J. W. & Buss, G. R. (2007). A modified colorimetric method for phytic acid analysis in soybean. *Crop science*, 47 (5), 1797-1803. https://doi.org/10.2135/cropsci2007.03.0122.
- Gessler, N. N., Serdyuk, E. G., Isakova, E. P. & Deryabina, Y. I. (2018). Phytases and the prospects for their application. *Applied Biochemistry and Microbiology*, 54 (4), 352-360. https://doi.org/10.1134/S0003683818040087.
- Goswami, G., Bora, S. S., Parveen, A., Boro, R. C. & Barooah, M. (2017). Identification and functional properties of dominant lactic acid bacteria isolated from Kahudi, a traditional rapeseed fermented food product of Assam, India. *Journal of Ethnic Foods*, 4 (3), 187-197. https://doi.org/10.1016/j.jef.2017.08.008.
- Gowthami, G. A. & Gunashree, B. S. (2023). Isolation, characterization and optimization of *Cystobasidium minutum* for phytase production. *Biomedicine*, 43 (01), 329-334. https://doi.org/10.51248/.v43i01.2654.
- Greiling, A. M., Tschesche, C., Baardsen, G., Kröckel, S., Koppe, W. & Rodehutscord M. (2019). Effects of phosphate and phytase supplementation on phytate degradation in rainbow trout (*Oncorhynchus mykiss* W.) and Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 503, 467-474. https://doi.org/10.1016/j.aquaculture.2019.01.035.
- Gunashree, B. S. & Venkateswaran, G. (2015). Extracellular phytase from *Aspergillus niger* CFR 335: purification and characterization. *Journal of food science and technology*, 52 (7), 4558-4564. https://doi.org/10.1007/s13197-014-1304-z.
- Handa, V., Sharma, D., Kaur, A. & Arya, S. K. (2020). Biotechnological applications of microbial phytase and phytic acid in food and feed industries. *Biocatalysis and Agricultural Biotechnology*. 25, 101600. https://doi.org/10.1016/j.bcab.2020.101600.
- Hellstrom, A., Qvirist, L., Svanberg, U., Veide Vilg, J. & Andlid, T. (2015). Secretion of non-cell-bound phytase by the yeast *Pichia kudriavzevii* TY13. *Journal of applied microbiology*, 118 (5), 1126-1136. https://doi.org/10.1111/jam.12767.
- Hidayatullah, M. E., Sajidan, Susilowati, A., Mkumbe, B. S. & Greiner, R. (2020). Biochemical characterization of recombinant phytase enzyme (phyk) from *Klebsiella*

sp. ASR1 encapsulated with alginate. *Jurnal Bioteknologi & Biosains Indonesia*, 7, 105-113. https://doi.org/10.29122/jbbi.v7I1.2997.

- Holloway, C. L., Dean Boyd, R., Koehler, D., Gould, S. A., Li, Q. & Patience, J. F. (2019). The impact of "super-dosing" phytase in pig diets on growth performance during the nursery and grow-out periods. *Translational Animal Science*, 3, 419–428. https://doi.org/10.1093/tas/txy148.
- Huang, S. J., Chen, C. H. & Tsai, S. Y. (2018). Phytase production by *Grifola frondosa* and its application in inositol-enriched solid-state fermentation brown rice. *International Journal of Food Engineering*, 4 (4), 263-267. https://doi.org/10.18178/ijfe.4.4.263-267.
- Hussain, S. M., Hameed, T., Afzal, M. et al. (2017). Growth performance and nutrient digestibility of *Cirrhinus mrigala* fingerlings fed phytase supplemented sunflower meal based diet. *Pakistan Journal of Zoology*, 49 (5), 1713-1724. https://doi.org/10.17582/journal.pjz/2017.49.5.1713.1724.
- Hussain, S. M., Hanif, S., Sharif, A., Bashir, F. & Iqbal, H. M. N. (2021). Unrevealing the sources and catalytic functions of phytase with multipurpose characteristics. *Catalysis Letter*, 152, 1358-1371. https://doi.org/10.1007/s10562-021-03752-z.
- Jain, J., Sapna & Singh, B. (2016). Characteristics and biotechnological applications of bacterial phytases. *Process Biochemistry*, 51 (2), 159-169. https://doi.org/10.1016/j.procbio.2015.12.004.
- Jain, J., Kumar, A., Singh, D. & Singh, B. (2018). Purification and kinetics of a protease-resistant, neutral, and thermostable phytase from *Bacillus subtilis* subsp. *subtilis* JJBS250 ameliorating food nutrition. *Preparative Biochemistry and Biotechnology*, 48 (8), 718-724. https://doi.org/10.1080/10826068.2018.1487848.
- Jatuwong, K., Suwannarach, N., Kumla, J., Penkhrue, W., Kakumyan, P. & Lumyong, S. (2020a). Bioprocess for production, characteristics, and biotechnological applications of fungal phytases. *Frontiers in Microbiology*, 11, 493778. https://doi.org/10.3389/fmicb.2020.00188.
- Jatuwong, K., Kumla, J., Suwannarach, N., Matsui, K. & Lumyong, S (2020b). Bioprocessing of Agricultural Residues as Substrates and Optimal Conditions for Phytase Production of Chestnut Mushroom, *Pholiota adiposa*, in Solid State Fermentation. *Journal of Fungi*, 6 (4), 384. https://doi.org/10.3390/jof6040384.

- Javaid, A., Yasinzai, M. & Kiyani, K. S. (2022). Enhanced phytase production from indigenous *Bacillus subtilis* KT004404 by response surface methodology to be used as poultry feed supplement. *Pakistan Journal of Zoology*, 1–12. https://doi.org/10.17582/journal.pjz/20210910100924.
- Jing, M., Zhao, S., Rogiewicz, A., Slominski, B. A. & House, J. D. (2021). Effects of phytase supplementation on growth performance, plasma biochemistry, bone mineralisation and phosphorus utilisation in pre-lay pullets fed various levels of phosphorus. *Animal Production Science*, 61 (6), 568-576. https://doi.org/10.1071/AN20265.
- Joshi, S. & Satyanarayana, T. (2015). Bioprocess for efficient production of recombinant *Pichia anomala* phytase and its applicability in dephytinizing chick feed and whole wheat flat Indian breads. *Journal of Industrial Microbiology and Biotechnology*, 42 (10), 1389-1400. https://doi.org/10.1007/s10295-015-1670-1.
- Joudaki, H., Aria, N., Moravej, R., Rezaei Yazdi, M., Emami-Karvani, Z. & Hamblin, M. R. (2023). Microbial Phytases: Properties and Applications in the Food Industry. *Current Microbiology*, 80 (12), 374. https://doi.org/10.1007/s00284-023-03471-1.
- Kalkan, S. O., Bozcal, E., Hames Tuna, E. E. & Uzel, A. (2020). Characterisation of a thermostable and proteolysis resistant phytase from *Penicillium polonicum* MF82 associated with the marine sponge Phorbas sp. *Biocatalysis and Biotransformation,* 38, 469-479, https://doi.org/10.1080/10242422.2020.1785434.
- Kalsi, H. K., Singh, R., Dhaliwal, H. S. & Kumar, V. (2016). Phytases from *Enterobacter* and *Serratia* species with desirable characteristics for food and feed applications. *3 Biotech*, 6, 1–13. https://doi.org/10.1007/S13205-016-0378.
- Kamaci, U. D. & Peksel, A. (2022). Poly(vinyl alcohol)-based electrospun nanofibers: characterization and phytase immobilization. *Biointerface Research in Applied Chemistry*, 12 (6), 7573–7583. https://doi.org/10.33263/briac126.75737583.
- Kammoun, R., Farhat, A., Chouayekh, H., Bouchaala, K. & Bejar, S. (2012). Phytase production by *Bacillus subtilis* US417 in submerged and solid state fermentations. *Annals of microbiology*, 62, 155-164. https://doi.org/10.1007/s13213-011-0240-7.
- Kanti, A. & Sudiana, I. M. (2016). Comparison of *Neurospora crassa* and *Neurospora sitophila* for phytase production at various fermentation temperatures. *Biodiversitas Journal of Biological Diversity*, 17 (2). https://doi.org/10.13057/biodiv/d170253.

- Kanti, A. & Sudiana, I. M. (2018). Production of Phytase, Amylase and Cellulase by *Aspergillus, Rhizophus* and *Neurospora* on Mixed Rice Straw Powder and Soybean Curd Residue. *IOP Conference Series: Earth and Environmental Science*, 166, https://doi.org/10.1088/1755-1315/166/1/012010.
- Kanti, A., Idris, I. & Sudiana, I. M. (2020). *Aspergillus niger* Str 3 and *Neurospora sitophila* for phytase production on coconut oil cake supplemented with rice brand in solid-state fermentation. *IOP Conference Series. Earth and Environmental Science*, 439, 12020. https://doi.org/10.1088/1755-1315/439/1/012020.
- Kaur, R., Saxena, A., Sangwan, P., Yadav, A. N., Kumar, V. & Dhaliwal, H. S. (2017). Production and characterization of a neutral phytase of *Penicillium oxalicum* EUFR-3 isolated from Himalayan region. *Nusantara Bioscience*, 9 (1), 68-76. https://doi.org/10.13057/nusbiosci/n090112.
- Kaur, P., Vohra, A. & Satyanarayana, T. (2021). Multifarious applications of fungal phytases. *Encyclopedia of Mycology*. https://doi.org/10.1016/B978-0-12-819990-9.00028-7.
- Kemigabo, C., Abdel-Tawwab, M., Lazaro, J. W., Sikawa, D., Masembe, C. & Kang'Ombe, J. (2018). Combined effect of dietary protein and phytase levels on growth performance, feed utilization, and nutrients digestibility of African catfish, *Clarias gariepinus* (B.), reared in earthen ponds. *Journal of Applied Aquaculture,* 30 (3), 211-226. https://doi.org/10.1080/10454438.2018.1439425.
- Kim, J. H., Pitargue, F. M., Jung, H., Han, G. P., Choi, H. S. & Kil, D. Y. (2017). Effect of superdosing phytase on productive performance and egg quality in laying hens. *Asian-Australasian Journal of Animal Sciences*, 30 (7), 994. https://doi.org/10.5713/ajas.17.0149.
- Kim, B. H., Lee, J. Y. & Lee, P. C. (2015). Purification, sequencing and evaluation of a divergent phytase from *Penicillium oxalicum* KCTC6440. *The Journal of General and Applied Microbiology*, 61 (4), 117-123. https://doi.org/10.2323/jgam.61.117.
- Klosowski, G., Mikulski, D. & Jankowiak, O. (2018). Extracellular phytase production by the wine yeast *S. cerevisiae* (Finarome Strain) during submerged fermentation. *Molecules,* 23, 848. https://doi.org/10.3390/molecules23040848.
- Kumar, A., Singh, B., Raigond, P., Sahu, C., Mishra, U. N., Sharma, S. & Lal, M.K. (2021). Phytic acid: Blessing in disguise, a prime compound required for both plant and

human nutrition. *Food Research International*, 142, 110193. https://doi.org/10.1016/j.foodres.2021.110193.

- Kumari, A., Satyanarayana, T. & Singh, B. (2016). Mixed substrate fermentation for enhanced phytase production by thermophilic mould *Sporotrichum thermophile* and its application in beneficiation of poultry feed. *Applied Biochemistry and Biotechnology*, 178, 197–210. https://doi.org/10.1007/s12010-015-1868-8.
- Kumari, N. & Bansal, S. (2021a). Production and characterization of a novel, thermotolerant fungal phytase from agro-industrial byproducts for cattle feed. *Biotechnology Letters*, 43, 865-879. https://doi.org/10.1007/s10529-020-03069-8.
- Kumari, N. & Bansal, S. (2021b). Statistical modeling and optimization of microbial phytase production towards utilization as a feed supplement. *Biomass Conversion and Biorefinery,* 1–11. https://doi.org/10.1007/s13399-021-01672-x.
- Kumari, N. and Bansal, S. (2022). Production, immobilization and characterization of fungal phytase and its utilization in food and feed industry. (Doctoral thesis)
- Kryukov, V. S., Glebova, I. V. & Zinoviev, S. V. (2021). Reevaluation of phytase action mechanism in animal nutrition. *Biochemistry*, 86 (1), S152-S165. https://doi.org/10.1134/S000629792114011X.
- Lee, J., Choi, Y., Lee, P. C., Kang, S., Bok, J. & Cho, J. (2007). Recombinant production of *Penicillium oxalicum* PJ3 phytase in Pichia pastoris. *World Journal of Microbiology and Biotechnology*, 23, 443-446. https://doi.org/10.1007/s11274-006-9236-z.
- Lee, S. H., Cho, J., Bok, J., Kang, S., Choi, Y. & Lee, P. C. (2015). Characterization, gene cloning, and sequencing of a fungal phytase, PhyA, from *Penicillium oxalicum* PJ3. *Preparative Biochemistry and Biotechnology*, 45 (4), 336-347. https://doi.org/10.1080/10826068.2014.923446.
- Lee, S. A., Lupatsch, I., Gomes, G. A. & Bedford, M. R. (2020). An advanced *Escherichia coli* phytase improves performance and retention of phosphorus and nitrogen in rainbow trout (*Oncorhynchus mykiss*) fed low phosphorus plant-based diets, at 11°C and 15°C. *Aquaculture*, 516, 734549. https://doi.org/10.1016/j.aquaculture.2019.734549.

- Li, G. E., Kong, W. L., Wu, X. Q. & Ma, S. B. (2021). Phytase-producing *Rahnella aquatilis* JZ-GX1 promotes seed germination and growth in corn (*Zea mays* L.). *Microorganisms*, 9, 1647. https://doi.org/10.3390/microorganisms9081647.
- Lopes, M. M., Coutinho, T. C., Malafatti, J. O. D. et al (2021). Immobilization of phytase on zeolite modified with iron (II) for use in the animal feed and food industry sectors. *Process Biochemistry*, 100, 260–271. https://doi.org/10.1016/j.procbio.2020.10.017.
- Lowry, O., Rosebrough, N., Farr, A. & Randall, R. J. (1951). Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry,* 193, 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6.
- Mahendran, S., Sankaralingam, S., Maheswari, P., Dhivya, R. R., Kathiresan, D., Karthikeyan, S., Ramya, S. S., Seethapathy, P., Harinathan, B. & Palpperumal, S. (2022). Production, Characterization, and Feed Supplement Applications of Phytase Enzyme from *Aspergillus tubingensis* Isolated from Western Ghats Soil. *Biomass Conversion and Biorefinery*, 1–11. https://doi.org/10.1007/s13399- 022-02894-3.
- Mahmood, S., Shahid, M. G. & Nadeem, M. (2021). Screening of Phytate Degrading Fungi and Optimization of Culture Conditions for Phytase Synthesis Using Agro-Industrial by-Products. *Pakistan Journal of Botany*, 53 (2), 763–770. https://doi.org/10.30848/PJB2021-2(12).
- Mahmood, S., Shahid, M. G., Nadeem, M., Nelofer, R. & Irfan, M. (2022). Application of Statistical Design for the Economical Production of Phytase by *Aspergillus niger* Using Solid State Fermentation. *Journal of Animal and Plant Sciences*, 32 (1), 238–246. https://doi.org/10.36899/JAPS.2022.1.0419.
- Makolomakwa, M., Puri, A. K., Permaul, K. & Singh, S. (2017). Thermo-acid-stable phytase-mediated enhancement of bioethanol production using *Colocasia esculenta*. *Bioresource Technology*, 235, 396–404. https://doi.org/10.1016/j.biortech.2017.03.157.
- Marolt, G., and Kolar, M. (2020). Analytical methods for determination of phytic acid and other inositol phosphates: A review. *Molecules,* 26, 174. https://doi.org/10.3390/molecules26010174.
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426–428.

- Miller, V., Reedy, J., Cudhea, F., Zhang, J., Shi, P., Erndt-Marino, J., ... & Lopez-Jaramillo, P. (2022). Global, regional, and national consumption of animal-source foods between 1990 and 2018: findings from the Global Dietary Database. The Lancet Planetary Health, 6(3), e243-e256. https://doi.org/10.1016/S2542-5196(21)00352-1.
- Molina, D. C., Novelli Poisson, G. F., Kronberg, F. & Galvagno, M. A. (2021). Valorization of an Andean crop (yacon) through the production of a yeast cellbound phytase. *Biocatalysis and Agricultural Biotechnology*, 36, 102116. https://doi.org/10.1016/j.bcab.2021.102116.
- Monteiro, P. S., Guimarães, V. M., Melo, R. R. D. & Rezende, S. T. D. (2015). Isolation of a thermostable acid phytase from *Aspergillus niger* UFV-1 with strong proteolysis resistance. *Brazilian Journal of Microbiology*, 46 (1), 251-260. http://dx.doi.org/10.1590/S1517-838220120037.
- Muslim, S. N., Ali, A. N. M., Al-Kadmy, I. M., Khazaal, S. S., Ibrahim, S. A. et al. (2018). Screening, nutritional optimization and purification for phytase produced by *Enterobacter aerogenes* and its role in enhancement of hydrocarbons degradation and biofilm inhibition. *Microbial pathogenesis*, 115, 159-167. https://doi.org/10.1016/j.micpath.2017.12.047.
- Mullaney, E. J. & Ullah, A. H. J. (2006). Phytases: Attributes, catalytic mechanisms and applications. *Inositol Phosphates: Linking Agriculture and the Environment*, 97–110. https://doi.org/10.1079/9781845931520.0097.
- Nagar, A., Kamble, A. & Singh, H. (2021). Preliminary screening, isolation and identification of microbial phytase producers from soil. *Environmental and Experimental Biology*, 19 (1), 11-22. http://doi.org/10.22364/eeb.19.03.
- Naghshbandi, M. P. & Moghimi, H. (2020). Stabilization of phytase on multi-walled carbon nanotubes via covalent immobilization [Chapter 18]. *Methods in Enzymology*, 630, 431–451. https://doi.org/10.1016/bs.mie.2019.10.013.
- Nascimento, J. C. dos S., Ribeiro, A. G., Pessoa, R. A. S., Rabello, C. B. V., Venâncio, A. et al. (2022). Effect of pH and temperature on phytase and biomass production by submerged fermentation with *Aspergillus niger* var. *phoenicis* URM 4924. *Research Society and Development*, 11 (6), e41311628994--e41311628994. https://doi.org/10.33448/rsdV11I6.2899.

- Naseem, N., Abdullah, S. & Aziz, S. (2021). Effect of phytase supplemented diet on whole body proximate composition of *Labeo rohita* fingerlings. *Pakistan Journal of Zoology*, 54 (3), 1479-1482. https://doi.org/10.17582/journal.pjz/20200928150918.
- Nassar, M., Nassar, R., Maki, H., Al-Yagoob, A., Hachim, M., et al. (2021). Phytic acid: Properties and potential applications in dentistry. *Frontiers in Materials,* 8, 638909. https://doi.org/10.3389/fmats.2021.638909.
- Neira-Vielma, A. A., Aguilar, C. N., Ilyina, A., Contreras-Esquivel, J. C., Carneiro-da-Cunha, M. D. G., Michelena-Álvarez, G., & MartÃnez-Hernandez, J. L. (2018a). Optimized production of phytase by solid-state fermentation using triticale as substrate and source of inducer. African Journal of Biotechnology, 17(4), 81-90. https://doi.org/10.5897/AJB2017.16267.
- Neira-Vielma, A. A., Aguilar, C. N., Ilyina, A., Contreras-Esquivel, J. C., das Graça Carneiro-da-Cunha, M., Michelena-Álvarez, G. & Martínez-Hernández, J. L. (2018b). Purification and biochemical characterization of an *Aspergillus niger* phytase produced by solid-state fermentation using triticale residues as substrate. *Biotechnology reports*, 17, 49-54. https://doi.org/10.1016/j.btre.2017.12.004.
- Nie, X. Z., Chen, S., Zhang, X. X., Dai, B. Y. & Qian, L. C (2017). Effects of neutral phytase on growth performance and phosphorus utilization in crucian carp (*Carassius auratus*). *Journal of Zhejiang University Science B*, 18 (10), 886-896. https://doi.org/10.1631/jzus.B1600280.
- Norag, M. A. A., El-Shenawy, A. M., Fadl, S. E. et al. (2018). Effect of phytase enzyme on growth performance, serum biochemical alteration, immune response and gene expression in Nile tilapia. *Fish Shellfish Immunology*, 80, 97-108. https://doi.org/10.1016/j.fsi.2018.05.051.
- Ogunremi, O. R., Agrawal, R. & Sanni, A. (2020). Production and characterization of volatile compounds and phytase from potentially probiotic yeasts isolated from traditional fermented cereal foods in Nigeria. *Journal of Genetic Engineering and Biotechnology*, 18 (1), 16. https://doi.org/10.1186/s43141-020-00031-z.
- Onem, H., Cicek, S. & Nadaroglu, H. (2016). Immobilization of a thermostable phytase from Pinar melkior (*Lactarius piperatus*) onto magnetite chitosan nanoparticles. *CyTA – Journal of Food*, 14 (1), 74–83. https://doi.org/10.1080/19476337.2015.1045942.

- Onibokun, E. A., Eni, A. O. & Oranusi, S. U. (2022). Purification and characterization of phytase from a local poultry isolate of *Aspergillus flavus* MT899184. *Green Energy Technology*, 99–112. https://doi.org/10.1007/978-3-030-96721-5.
- Outchkourov, N. & Petkov, S. (2019). Phytases for feed applications [Chapter 3]. In: Vogel A, May O, eds. *Industrial enzyme applications*, Wiley-VCH Verlag GmbH & Co. KGaA, 255-285. https://doi.org/10.1002/9783527813780.ch3_3.
- Pable, A. A., Shah, S., Ravi Kumar, V. & Khire, J. M. (2019). Use of Plackett–Burman Design for Enhanced Phytase Production by *Williopsis saturnus* NCIM 3298 for Applications in Animal Feed and Ethanol Production. *3 Biotech,* 9, 237. https://doi.org/10.1007/s13205-019-1764-y.
- Pires, E. B. E., Freitas, A. J. De, Souza, F. F. E., Salgado, R. L., Guimarães, V. M. et al. (2019). Production of fungal phytases from agroindustrial byproducts for pig diets. *Scientific Reports*. https://doi.org/10.1038/s41598-019-45720-z.
- Pontes, T. C., Brito, J. M. D., Wernick, B. et al. (2021). Top-sprayed phytase enhances the digestibility of energy, protein, amino acids and minerals, and reduces phosphorus output in Nile tilapia fed all-vegetable diets. *Aquaculture Research*, 00, 1-9. https://doi.org/10.1111/are.15527.
- Puppala, K. R., Naik, T., Shaik, A., Dastager, S., Kumar, R., Khire, J. & Dharne, M. (2018). Evaluation of *Candida tropicalis* (NCIM 3321) extracellular phytase having plant growth promoting potential and process development. *Biocatalysis and agricultural biotechnology*, 13, 225-235. https://doi.org/10.1016/j.bcab.2017.12.013.
- Prado, D. Z. D., Okino-Delgado, C. H., Zanutto-Elgui, M. R., da Silva, R. B. G., Pereira, M. S. et al. (2019). Screening of *Aspergillus, Bacillus* and *Trichoderma* strains and influence of substrates on auxin and phytases production through solid-state fermentation. *Biocatalysis and agricultural biotechnology*, 19, 101165. https://doi.org/10.1016/j.bcab.2019.101165.
- Pragya, Sharma, K. K., Kumar, A., Singh, D., Kumar, V. & Singh, B. (2021). Immobilized phytases: an overview of different strategies, support material, and their applications in improving food and feed nutrition. *Critical Reviews in Food Science and Nutrition*, 1, 23. https://doi.org/10.1080/10408398.2021.2020719.
- Pragya, Sharma, K. K., Kumar, S., Manisha, Singh, D., Kumar, V. & Singh, B. (2023a). Enhanced production and immobilization of phytase from *Aspergillus oryzae*: a safe

and ideal food supplement for improving nutrition. *Letters in Applied Microbiology*, 76 (2), ovac077. https://doi.org/10.1093/lambio/ovac077.

- Pragya, Sharma, K. K. & Singh, B. (2023b). Phytase from *Aspergillus oryzae* SBS50: Biocatalytic reduction of anti-nutritional factor and exhibiting vanadium-dependent haloperoxidase activity. *Biocatalysis and Agricultural Biotechnology*, 52, 102840. https://doi.org/10.1016/j.bcab.2023.102840.
- Pragya, Sharma, K. K. & Singh, B. (2023). Phytase production and dephytinization of food samples using free and immobilized phytases. (Doctoral thesis).
- Priya, Virmani, I., Pragya, Goswami, R. K., Singh, B., Sharma, J. G. & Giri, B. (2023a). Role of Microbial Phytases in Improving Fish Health. *Reviews in Aquaculture*, 15 (4), 1480–1500. https://doi.org/10.1111/raq.12790.
- Priya, Singh, B., Sharma, J. G., & Giri, B. (2023b). Optimization of phytase production by *Penicillium oxalicum* in solid-state fermentation for potential as a feed additive. *Preparative Biochemistry & Biotechnology*, 1-11. https://doi.org/10.1080/10826068.2023.2297688.
- Qamar, S. A., Asgher, M., and Bilal, M. (2020). Immobilization of alkaline protease from Bacillus brevis using ca-alginate entrapment strategy for improved catalytic stability, silver recovery, and dehairing potentialities. Catal. Letter.s 150, 3572–3583. https://doi.org/10.1007/S10562-020-03268.
- Qasim, S. S., Shakir, K. A., Al-Shaibani, A. B. & Walsh, M. K. (2017). Optimization of culture conditions to produce phytase from *Aspergillus tubingensis* SKA. *Food and Nutrition Sciences*, 08, 733–745. https://doi.org/10.4236/fns.2017.87052.
- Rachmawati, D., Istiyanto, S. & Maizirwan, M. (2017). Effect of phytase on growth performance, diet utilization efficiency and nutrient digestibility in fingerlings of *Chanos chanos* (Forsskal 1775). *Philippine Journal of Science*, 146 (3), 237-245.
- Rachmawati, D., Samidjan, I. & Elfitasari, T. (2018). Effect of the phytase enzyme addition in the artificial feed on digestibility of feed, feed conversion ratio and growth of Gift Tilapia Saline Fish (*Oreochromis niloticus*) Nursery Stadia I. *IOP Conference Series: Earth and Environmental Science*, 116 (1), 012009. https://doi.org/10.1088/1755-1315/116/1/0120.
- Rachmawati, D. & Samidjan, I. (2018a). Performance efficiency of feed utilization, relative growth rate, and survival rate of common carp (*Cyprinus carpio*) through the

addition of phytase in the feed. *IOP Conference Series: Earth and Environmental Science*, 137 (1), 012027. https://doi.org/10.1088/1755-1315/137/1/012027.

- Rachmawati, D. & Samidjan, I. (2018b). Effect of phytase enzyme on growth, nutrient digestibility and survival rate of catfish (*Pangasius hypothalamus*) fingerlings. *Pertanika Journal of Tropical Agricultural Science*, 41 (2), 865-878.
- Ranjan, B., Singh, B. & Satyanarayana, T. (2015). Characteristics of recombinant phytase (rSt-Phy) of the thermophilic mold *Sporotrichum thermophile* and its applicability in dephytinizing foods. *Applied biochemistry and biotechnology*, 177, 1753-1766. https://doi.org/10.1007/s12010-015-1851-4.
- Ranjan, B. & Satyanarayana, T. (2016). Recombinant HAP Phytase of the Thermophilic Mold *Sporotrichum thermophile*: expression of the Codon-Optimized Phytase Gene in *Pichia pastoris* and Applications. *Molecular Biotechnology*, 58, 137–147. https://doi.org/10.1007/s12033-015-9909-7.
- Rebello, S., Jose, L., Sindhu, R. & Aneesh, E. M. (2017). Molecular advancements in the development of thermostable phytases. *Applied Microbiology and Biotechnology*, 101, 2677-2689. https://doi.org/10.1007/s00253-017-8195-7.
- Rizwanuddin, S., Kumar, V., Naik, B., Singh, P., Mishra, S., Rustagi, S. & Kumar, V. (2023a). Microbial phytase: Their sources, production, and role in the enhancement of nutritional aspects of food and feed additives. *Journal of Agriculture and Food Research*, 12, 100559. https://doi.org/10.1016/j.jafr.2023.100559.
- Rizwanuddin, S., Kumar, V., Singh, P., Naik, B., Mishra, S., et al. (2023b). Insight into phytase-producing microorganisms for phytate solubilization and soil sustainability. *Frontiers in microbiology*, 14, 1127249. https://doi.org/10.3389/fmicb.2023.1127249.
- Rodrigues, M. L., Souza dos Santos Sanchez, M., Ernzen Pessini, J. et al. (2020). Replacement of corn by sorghum and phytase supplementation in silver catfish (*Rhamdia quelen*) diets: growth performance, physiological variables and bone mineralization. *Journal of Applied Animal Research*, 48 (1), 142-150. https://doi.org/10.1080/09712119.2020.1750411.
- Roopashri, A. N. & Varadaraj, M. C. (2015). Functionality of phytase of *Saccharomyces cerevisiae* MTCC 5421 to lower inherent phytate in selected cereal flours and wheat/pearl millet-based fermented foods with selected probiotic attribute. *Food Biotechnology*, 29 (2), 131-155. https://doi.org/10.1080/08905436.2015.1026602.

- Roy, T., Dan, S. K., Banerjee, G., Nandi, A., Ghosh, P. & Ray, A. K. (2016). Comparative efficacy of phytase from fish gut bacteria and a commercially available phytase in improving the nutritive value of sesame oilseed meal in formulated diets for fingerlings of rohu, *Labeo rohita* (Actinopterygii:Cypriniformes: Cyprinidae). *Acta Ichthyologica et Piscatoria*, 46 (1), 9-23. https://doi.org/10.3750/AIP2016.46.1.02.
- Sadaf, N., Haider, M. Z., Iqbal, N., Abualreesh, M. H. & Alatawi, A. (2022). Harnessing the phytase production potential of soil-borne fungi from wastewater irrigated fields based on eco-cultural optimization under shake flask method. *Agriculture*, 12, 103. https://doi.org/10.3390/agriculture12010103.
- Saithi, S. & Tongta, A. (2016). Phytase production of *Aspergillus niger* on soybean meal by solid-state fermentation using a rotating drum bioreactor. *Agriculture and Agricultural Science Procedia*, 11, 25-30. https://doi.org/10.1016/j.aaspro.2016.12.005.
- Saleh, A. A., Elsawee, M., Soliman, M. M., Elkon, R. Y., Alzawqari, M. H. et al. (2021). Effect of bacterial or fungal phytase supplementation on the performance, egg quality, plasma biochemical parameters, and reproductive morphology of laying hens. *Animals*, 11 (2), 540. https://doi.org/10.3390/ani11020540.
- Salmon, D. N. X., Fendrich, R. C., Cruz, M. A., Montibeller, V. W., Vandenberghe, L. P. S., Soccol, C. R. & Spier, M. R. (2016). Bioprocess for phytase production by *Ganoderma* sp. MR-56 in different types of bioreactors through submerged cultivation. *Biochemical engineering journal*, 114, 288-297. http://dx.doi.org/10.1016/j.bej.2016.07.015.
- Salman, A. H. & Alkhafaji, K. A. (2020). Use of treated lentil *Lens culinaris* L. with phytase enzyme in diets of common carp *Cyprinus carpio* L. *Plant Archives*, 20 (2), 941-944.
- Sandhya, A., Sridevi, A., Suvarnalatha Devi, P. & Narasimha, G. (2015). Production and optimization of phytase by *Aspergillus niger*. *Der Pharmacia Lettre*, 7, 148–153.
- Sanni, D. M., Lawal, O. T. & Enujiugha, V. N. (2019). Purification and characterization of phytase from *Aspergillus fumigatus* Isolated from African Giant Snail (*Achatina fulica*). *Biocatalysis and agricultural biotechnology*, 17, 225-232. https://doi.org/10.1016/j.bcab.2018.11.017.
- Sapna & Singh, B. (2013). Improved production of protease-resistant phytase by *Aspergillus oryzae* and its applicability in the hydrolysis of insoluble phytates. *Journal*

of Industrial Microbiology and Biotechnology, 40 (8), 891-899. https://doi.org/10.1007/s10295-013-1277-3.

- Sapna & Singh B. (2014). Phytase production by *Aspergillus oryzae* in solid-state fermentation and its applicability in dephytinization of wheat ban. *Applied Biochemistry and Biotechnology*, 173, 1885–95. https://doi.org/10.1007/s12010-014-0974-3.
- Sapna & Singh, B. (2015). Biocatalytic potential of protease-resistant phytase of *Aspergillus oryzae* SBS50 in ameliorating food nutrition. *Biocatalysis and Biotransformation*, 33 (3), 167–174. https://doi.org/10.3109/10242422.2015.1076215.
- Sapna & Singh, B. (2017). Purification and characterization of a protease-resistant phytase of *Aspergillus oryzae* SBS50 whose properties make it exceptionally useful as a feed supplement. *International journal of biological macromolecules*, 103, 458-466. http://dx.doi.org/doi:10.1016/j.ijbiomac.2017.05.077.
- Sarfraz, Q., Hussain, S. M., Sharif, A., Selamoglu, Z., Bashir, F. & Ahsan, S. (2020). Potential of phytase supplemented Moringa oleifera leaf meal based diet on mineral digestibility of *Oreochromis niloticus* fingerlings. *Journal of Survey in Fisheries Sciences*, 6 (2), 65-77. https://doi.org/10.18331/SFS2020.6.2.8.
- Sato, V. S., Jorge, J. A. & Guimarães, L. H. S. (2016). Characterization of a thermotolerant phytase produced by *Rhizopus microsporus* var. *microsporus* biofilm on an inert support using sugarcane bagasse as carbon source. *Applied biochemistry and biotechnology*, 179, 610-624. https://doi.org/10.1007/s12010-016-2018-7.
- Saxena, A., Verma, M., Singh, B., Sangwan, P., Yadav, A. N., Dhaliwal, H. S. & Kumar, V. (2020). Characteristics of an acidic phytase from *Aspergillus aculeatus* APF1 for dephytinization of biofortified wheat genotypes. *Applied Biochemistry and Biotechnology*, 191, 679-694. https://doi.org/10.1007/s12010-019-03205-9.
- Shah, P. C., Kumar, V. R., Dastager, S. G. & Khire, J. M. (2017). Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides. *AMB express*, 7, 1-11. https://doi.org/10.1186/s13568-017-0370-9.
- Shahryari, Z., Fazaelipoor, M. H., Setoodeh, P., Nair, R. B., Taherzadeh, M. J. & Ghasemi, Y. (2018). Utilization of wheat straw for fungal phytase production. *International Journal of Recycling of Organic Waste in Agriculture*, 7, 345–355. https://doi.org/10.1007/S40093-018-0220.

- Shahzad, M. M., Hussain, S. M., Hussain, M. et al (2020a). Improvement in overall performance of *Catla catla* fingerlings fed phytase included low cost plant by products-based diet. *Saudi Journal of Biological Sciences*, 27 (8), 2089-2096. https://doi.org/10.1016/j.sjbs.2020.06.012.
- Shahzad, M. M., Hussain, S. M., Akram, A. M. et al. (2020b). Improvement in nutrient digestibility and growth performance of *Catla catla* fingerlings using phytase in Moringa oleifera leaf meal based diet. *Pakistan Journal of Zoology*, 52 (1), 157-168. https://doi.org/10.17582/journal.pjz/2020.52.1.157.168.
- Shahzad, M. M., Bashir, S., Hussain, S. M. et al (2021a). Effectiveness of phytase pre-treatment on growth performance, nutrient digestibility and mineral status of common carp (*Cyprinus carpio*) juveniles fed Moringa by-product based diet. *Saudi Journal of Biological Sciences*, 28 (3), 1944-1953. https://doi.org/10.1016/j.sjbs.2020.12.046.
- Shahzad, M. M., Rafique, T., Hussain, S. M. et al. (2021b). Effect of phytase supplemented Moringa by-products based diets on the performance of *Oreochromis niloticus* fingerlings. *Journal of Animal and Plant Sciences*, 31 (1), 288-295. https://doi.org/10.36899/JAPS.2021.1.0216.
- Sharma, B. & Shukla, G. (2020). Isolation, identification, and characterization of phytase producing probiotic lactic acid bacteria from neonatal fecal samples having dephytinization activity. *Food Biotechnology*, 34, 151–171. https://doi.org/10.1080/08905436.2020.1746332.
- Sharma, A., Ahluwalia, O., Tripathi, A. D., Singh, G. & Arya, S. K. (2020). Phytases and their pharmaceutical applications: Mini-review. *Biocatalysis and Agricultural Biotechnology,* 23, 101439. https://doi.org/10.1016/j.bcab.2019.101439.
- Simas, A. L. D. O., de Alencar Guimarães, N. C., Glienke, N. N., Galeano, R. M. S., de Sá Teles, J. S. et al. (2024). Production of Phytase, Protease and Xylanase by *Aspergillus niveus* with Rice Husk as a Carbon Source and Application of the Enzymes in Animal Feed. *Waste and Biomass Valorization*, 1-13. https://doi.org/10.1007/s12649-024-02455-x.
- Singh, B. & Satyanarayana, T. (2006). Phytase production by thermophilic mold *Sporotrichum thermophile* in solid-state fermentation and its application in dephytinization of sesame oil cake. *Applied Biochemistry and Biotechnology*, 133, 239-250. https://doi.org/10.1385/abab:133:3:239.

- Singh, B. & Satyanarayana, T. (2010). Plant growth promotion by an extracellular HAP-phytase of a thermophilic mold *Sporotrichum thermophile*. *Applied Biochemistry and Biotechnology*, 160 (5), 1267–1276. https://doi.org/10.1007/s12010-009-8593-0.
- Singh, N. K., Joshi, D. K. & Gupta, R. K. (2013). Isolation of phytase producing bacteria and optimization of phytase production parameters. *Jundishapur Journal of Microbiology*, 6 (5), e6419. https://doi.org/10.5812/jjm.6419.
- Singh, N., Kumari, A., Gakhar, S. K. & Singh, B. (2015). Enhanced cost-effective phytase production by *Aspergillus niger* and its applicability in dephytinization of food ingredients. *Microbiology*, 84 (2), 219-226. https://doi.org/10.1134/S0026261715020149.
- Singh, B., Sharma, K. K., Kumari, A., Kumar, A. & Gakhar, S. K. (2018). Molecular modeling and docking of recombinant HAP-phytase of a thermophilic mould *Sporotrichum thermophile* reveals insights into molecular catalysis and biochemical properties. *International Journal of Biological Macromolecules*, 115, 501–508. https://doi.org/10.1016/j.ijbiomac.2018.04.086.
- Singh, B., Boukhris, I., Pragya, Kumar, V., Yadav, A. et al. (2020). Contribution of microbial phytases to the improvement of plant growth and nutrition: A review. *Pedosphere*, 30 (3), 295–313. https://doi.org/10.1016/S1002-0160(20)60010-8.
- Singh, B., Pragya, Tiwari, S. K., Singh, D., Kumar, S. & Malik, V. (2024). Production of fungal phytases in solid state fermentation and potential biotechnological applications. *World Journal of Microbiology and Biotechnology*, 40 (1), 22. https://doi.org/10.1007/s11274-023-03783-1.
- Sirin, Y., Akatin, M. Y. & Colak, A. et al. (2017). Dephytinization of food stuffs by phytase of *Geobacillus* sp. TF16 immobilized in chitosan and calcium-alginate. *International Journal of Food Properties*, 20 (12), 2911–2922. https://doi.org/10.1080/10942912.2016.1261151.
- Sivakumaran, K., and Kothalawala, S. (2018). An overview of the analytical methods for food phytates. *International Journal of Chemical Studies*, 6(1), 2016–2020
- Soccol, C. R., Costa, E. S. F. da, Letti, L. A. J., Karp, S. G., Woiciechowski, A. L. & Vandenberghe, L. P. de S. (2017). Recent developments and innovations in solid state fermentation. *Biotechnology Research and Innovation*, 1, 52–71. https://doi.org10.1016/j.biori.2017.01.002.

- Soman, S., Kumarasamy, S., Narayanan, M. & Ranganathan, M. (2020). Biocatalyst: Phytase production in solid state fermentation by ovat strategy. *Biointerface Research in Applied Chemistry*, 10, 6119–6127. https://doi.org/10.33263/briac105.61196127.
- Song, H. Y., El Sheikha, A. F. & Hu, D. M. (2019). The positive impacts of microbial phytase on its nutritional applications. *Trends in Food Science and Technology*, 86, 553-562. https://doi.org/10.1016/j.tifs.2018.12.001.
- Suleimanova, A. D., Danilova, Y. V., Greiner, R. & Sharipova, M. R. (2013). A new intracellular phytase of enterobacteria: isolation and characterization. *Russian Journal of Bioorganic Chemistry*, 39, 378-383. https://doi.org/10.1134/S1068162013040146.
- Suleimanova, A. D., Beinhauer, A., Valeeva, L. R., Chastukhina, I. B., Balaban, N. P. et al. (2015). Novel glucose-1-phosphatase with high phytase activity and unusual metal ion activation from soil bacterium *Pantoea* sp. strain 3.5. 1. *Applied and environmental microbiology*, 81 (19), 6790-6799. https://doi.org/10.1128/AEM.01384-15.
- Sumengen, M., Dincer, S. & Kaya, A. (2012). Phytase production from *Lactobacillus brevis*. *Turkish Journal of Biology*, 36 (5), 533-541. https://doi.org/10.3906/biy-1111-2.
- Sun, M., He, Z. & Jaisi, D. P. (2021). Role of metal complexation on the solubility and enzymatic hydrolysis of phytate. *PloS one*, 16 (8), e0255787. https://doi.org/10.1371/journal.pone.0255787.
- Suresh, S. & Radha, K. V. (2015). Effect of a mixed substrate on phytase production by *Rhizopus oligosporus* MTCC 556 using solid state fermentation and determination of dephytinization activities in food grains. *Food Science and Biotechnology*, 24 (2), 551-559. https://doi.org/10.1007/s10068-015-0072-5.
- Suresh, S. & Radha, K. V. (2016). Statistical optimization and mutagenesis for high level of phytase production by *Rhizopus oligosporus* MTCC 556 under solid state fermentation. *Journal of Environmental Biology*, 37 (2), 253.
- Suzuki, U., Yoshimura, K., Takaishi, M. (1907). Ueber ein enzym “phytase” das “anhydro-oxy-methylen diphosphorsaure” spaltet. *Bull. Coll. Agric. Tokyo Imper. Univ*, 7, 503–512.
- Tan, H., Tang, J., Li, X., Liu, T., Miao, R. et al. (2017). Biochemical characterization of a psychrophilic phytase from an artificially cultivable morel *Morchella importuna*.

Journal of Microbiology and Biotechnology, 27(12), 2180-2189. https://doi.org/10.4014/jmb.1708.08007.

- Tanruean, K., Penkhrue, W., Kumla, J., Suwannarach, N. & Lumyong, S. (2021). Valorization of lignocellulosic wastes to produce phytase and cellulolytic enzymes from a thermophilic fungus, *Thermoascus aurantiacus* SL16W, under semi-solid state fermentation. *Journal of Fungi,* 7, 286. https://doi.org/10.3390/jof7040286.
- Terrey, D. E., Braidi, D. A. & Serwata, R. (2023). Effect of a microbial phytase on the growth performance, digestibility and retention in a high plant meal inclusion diet for Atlantic salmon (*Salmo salar*). *Aquaculture International*, 1-16. https://doi.org/10.1007/s10499-023-01295-1.
- Thakur, N., Patel, S. K., Kumar, P., Singh, A., Devi, N., Sandeep, K., Pandey, K., & Chand, D. (2022). Bioprocess for hyperactive thermotolerant *Aspergillus fumigatus* phytase and its application in dephytinization of wheat flour. *Catalysis Letters*, 152 (11), 3220-3232. https://doi.org/10.1007/s10562-021-03886-0.
- Thorsen, M., Nielsen, L. A., Zhai, H. X., Zhang, Q., Wulf-Andersen, L. & Skov, L. K. (2021). Safety and efficacy profile of a phytase produced by fermentation and used as a feed additive. *Heliyon*, 7, e07237. https://doi.org/10.1016/j.heliyon.2021.e07237.
- Thyagarajan, R., Namasivayam, S. K. R. & Kumar, G. N. (2014). Optimization of medium components for phytase production by *Hypocrea lixii* SURT01 using response surface methodology. *Journal of Pure and Applied Microbiology*, 8 (3), 2485-2490.
- Tian, M. & Yuan, Q. (2016). Optimization of phytase production from potato waste using *Aspergillus ficuum*. *3 Biotech*, 6, 256. https://doi.org/10.1007/S13205-016-0573-9.
- Tirunagari, H., Basetty, S., Rode, H. B. & Fadnavis, N. W. (2018). Crosslinked enzyme aggregates (CLEA) of phytase with soymilk proteins. *Journal of Biotechnology*, 282, 67–69. https://doi.org/10.1016/j.jbiotec.2018.07.003.
- Tran, T. T., Hatti-Kaul, R., Dalsgaard, S. & Yu S. (2011). A simple and fast kinetic assay for phytases using phytic acid–protein complex as substrate. *Analytical Biochemistry*, 410, 177–184. https://doi.org/10.1016/j.ab.2010.10.034.
- Tripathi, P., Garg, S., Panwar, D., Kaira, G. S., Kumar, R. & Kapoor, M. (2017). Phytase from *Citrobacter koseri* PM-7: Cost-Effective Production Using Agro-Industrial Residues, Biochemical Characterization and Application in de-Phytinization.

Waste and biomass valorization, 8, 1105-1118. https://doi.org/10.1007/s12649-016-9662-6.

- Trouillefou, C. M., Le Cadre, E., Cacciaguerra, T., Cunin, F., Plassard, C. & Belamie, E. (2015). Protected activity of a phytase immobilized in mesoporous silica with benefits to plant phosphorus nutrition. *Journal of Sol-Gel Science and Technology*, 74, 55–65. https://doi.org/10.1007/S10971-014-3577.
- Tsai, T. C., Dove, R., Bedford, M. R., Michael, J. & Azain, M. J. (2020). Effect of phytase on phosphorous balance in 20-kg barrows fed low or adequate phosphorous diets. *Animal Nutrition*, 6, 9–15. https://doi.org/10.1016/j.aninu.2019.11.002.
- Tseng, Y. H., Fang, T. J. & Tseng, S. M. (2000). Isolation and characterization of a novel phytase from *Penicillium simplicissimum*. *Folia Microbiologica*, 45, 121–127. https://doi.org/10.1007/BF02817409.
- United Nations (2022). https://www.un.org/en/global-issues/population. Accessed on 28th March 2024.
- Uzezi, A. B., Ajani, E. K. & Omitoyin, B. O. (2016). Optimum dietary Ca/P ratio and phytase for growth and bone mineralization in juvenile *Clarias gariepinus* fed soya bean-based diet. *Journal of Aquaculture Research and Development*, 7 (2), 403. https://doi.org/10.4172/2155-9546.1000403.
- Uzezi, A. B., Omitoyin, B. O. & Ajani, E. K. (2017). Phosphorus utilization in juvenile *Clarias gariepinus* fed phytase-supplemented diets based on soya bean (oil-extracted) and full-fat (roasted): a comparison. *Journal of Applied Aquaculture,* 29 (2), 126-151. https://doi.org/10.1080/10454438.2016.1276000.
- Uzezi, A. B., Kolawole, A. E. & Oluwarotimi, O. B. (2019). Effect of microbial phytase on nutrient availability and growth of juvenile *Clarias gariepinus* fed soyabean and groundnut-based diets. *Annual Research & Review in Biology*, 31 (4), 1-21. https://doi.org/10.9734/ARRB/2019/v31i430057.
- Vasudevan, U. M., Jaiswal, A. K., Krishna, S., & Pandey, A. (2019). Thermostable phytase in feed and fuel industries. *Bioresource technology*, 278, 400-407. https://doi.org/10.1016/j.biortech.2019.01.065.
- Wade, H. E. & Morgan, D. M. (1955). Fractionation of phosphates by paper ionophoresis and chromatography. *Biochemical Journal*, 60, 264. https://doi.org/10.1042/BJ0600264.

- Wang, Z. H., Dong, X. F., Zhang, G. Q., Tong, J. M., Zhang, Q. & Xu, S. Z. (2017). Waste vinegar residue as substrate for phytase production. *Waste Management & Research: The Journal for a Sustainable Circular Economy*, 29, 1262–1270. https://doi.org/10.1177/0734242X11398521.
- Wisniewska, Z., Nollet, L., Lanckriet, A., Vanderbeke, E., Petkov, S., Outchkourov, N., Kasprowicz-Potocka, M., Za-WorskaZakrzewska, A. & Kaczmarek, S. A. (2020). Effect of phytase derived from the *E. coli* appa gene on weaned piglet per-formance, apparent total tract digestibility and bone mineralization. *Animals,* 10, 121. https://doi.org/10.3390/ani10010121.
- Woyengo, T. A. & Wilson, J. (2019). Enhancing nutrient utilization in maize for broiler chickens by superdosing phytase. *Animal feed science and technology*, 252, 34-40. https://doi.org/10.1016/j.anifeedsci.2019.04.005.
- Xie, Z., Fong, W. P. & Tsang, P. W. K. (2020). Engineering and optimization of phosphate-responsive phytase expression in *Pichia pastoris* yeast for phytate hydrolysis. *Enzyme and microbial technology*, 137, 109533. https://doi.org/10.1016/j.enzmictec.2020.109533.
- Xin, C., Wang, X., Liu, L. L., Yang, J., Wang, S. & Yan, Y. (2020). Rational design of monodisperse mesoporous silica nanoparticles for phytase immobilization. *ACS Omega,* 5, 30237–30242. https://dx.doi.org/10.1021/acsomega.0c04696.
- Ya-nan, L., Li-hong, Y., Xin-mei, C., Hao-meng, Y. & Huo-qing, H. (2020). Expression and characterization of aquatic neutral phytase gene from *Penicillium* sp. C1 in *Pichia pastoris*. *Biotechnology Bulletin*, 36, 134–141. https://doi.org/10.13560/j.cnki.biotech.bull.1985.2019-0704.
- Yasser, B., Hassan, E. & Yasser, A. (2018). Screening of phytase producing fungi isolated from Syrian soil. *Journal of Bio Innovation*, 7 (3), 462-474.
- Yu, P., Wang, X. T. & Liu, J. W. (2015). Purification and characterization of a novel cold-adapted phytase from *Rhodotorula mucilaginosa* strain JMUY14 isolated from Antarctic. *Journal of Basic Microbiology*, 55 (8), 1029-1039. https://doi.org/10.1002/jobm.201400865.
- Yudhiyanto, T., Suminto, S. & Rachmawati, D. (2017). The effect of dietary phytase supplementation on digestibility and growth of Asian seabass *Lates calcarifer*. *Omni-Akuatika,* 13 (2), 144-154. https://doi.org/10.20884/1.oa.2017.13.2.263.

- Zeng, Z., Li, Q., Tian, Q., Zhao, P., Xu, X., Yu, S. & Piao, X. (2015). Super high dosing with a novel buttiauxella phytase continuously improves growth performance, nutrient digestibility, and mineral status of weaned pigs. *Biological Trace Element Research*, 168, 103–109. https://doi.org/10.1007/s12011-015-0319-2.
- Zhao, Q., Liu, H., Zhang, Y. & Zhang, Y. (2010). Engineering of protease-resistant phytase from *Penicillium* sp.: high thermal stability, low optimal temperature and pH. *Journal of bioscience and bioengineering*, 110 (6), 638-645. https://doi.org/10.1016/j.jbiosc.2010.08.003.
- Zhou, Y., Anoopkumar, A. N., Tarafdar, A., Madhavan, A., Binoop, M. et al. (2022). Microbial engineering for the production and application of phytases to the treatment of the toxic pollutants: A review. *Environmental Pollution*, 308, 119703. https://doi.org/10.1016/j.envpol.2022.119703.

PUBLICATIONS

- Priya, Virmani, I., Pragya, Goswami, R. K., Singh, B., Sharma, J. G. and Giri, B. (2023). Role of Microbial Phytases in Improving Fish Health. *Reviews in Aquaculture*. 15, 1480–1500. DOI: 10.1111/raq. 12790. Impact factor – 10.4

- Priya, Singh, B., Sharma, J. G. and Giri, B. (2023). Optimization of phytase production by *Penicillium oxalicum* in solid-state fermentation for potential as a feed additive. *Preparative Biochemistry & Biotechnology*. DOI: 10.1080/10826068.2023.2297688. Impact factor – 2.9

CONFERENCES

- Participated and Presented a Poster entitled "**Optimization of phytase production from *Penicillium oxalicum* and its utilization as a feed supplement**" in Annual International conference of the Association of Microbiologists of India "Microbial Technologies for Sustainable Biosphere (Microbio Tech- 2023)", organized by Department of Microbiology at Maharshi Dayanand University, Rohtak, Haryana from 2nd February 2023-4th February 2023.

- Participated and Presented a Poster entitled "**Partial purification and characterization of phytase from *Penicillium oxalicum* for feed industry**" in International Conference on "Microbial Bioprospecting Towards Sustainable Development Goals", organized by AMI-LPU Unit and Society of Chemical and Synthetic Biology at Lovely Professional University, Punjab from 24th November 2023-25th November 2023.

- Participated and Presented a Poster entitled "***In-vitro* application of *Penicillium oxalicum* phytase in the aquafeed industry**" in International Conference on "Strategies for global food and nutritional security, sustainability and wellness (NUTRI-2023)", held in Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana from 4th December 2023-6th December 2023.

- Attended International Conference on Innovations in Biotechnology and Life Sciences (ICIBLS), held in Department of Biotechnology, Delhi Technological University from 18th December-20th December 2020.

PAPER NAME

full thesis.docx

WORD COUNT	CHARACTER COUNT
42554 Words	**238787 Characters**
PAGE COUNT	FILE SIZE
190 Pages	**25.9MB**
SUBMISSION DATE	REPORT DATE
Jun 30, 2024 7:42 AM GMT+5:30	**Jun 30, 2024 7:45 AM GMT+5:30**

● 7% Overall Similarity

The combined total of all matches, including overlapping sources, for each database.

- 5% Internet database
- Crossref database
- 5% Publications database
- Crossref Posted Content database

● Excluded from Similarity Report

- Submitted Works database
- Quoted material
- Small Matches (Less then 10 words)
- Bibliographic material
- Cited material
- Manually excluded sources

Summary

PRIYA
Qualification: Ph.D. Biotechnology
Area of Specialization: Microbial enzyme
Delhi Technological University
Shahbad Daulatpur, Main Bawana Road
Delhi-110042
Email:- priya19968@gmail.com
Phone:-9310467601

Education

Course	Discipline	College/School	University/ Board	Passing Year	Percentage %
Biotechnology	Ph.D.	Department of Biotechnology	Delhi Technological University	2024	-
Botany	M.Sc.	Department of Botany	Maharshi Dayanand University, Rohtak	2018	71.8%
Botany (H)	B.Sc.	Kalindi College	University of Delhi	2016	80.5%

TITLE OF RESEARCH WORK:

Process optimization for the production of microbial phytase and its application in feed industry.

PROJECT WORK:

Worked as a Junior Project fellow in the project entitled: "Conservation of Threatened Plants in Indian Himalayan Region: Recovery and Capacity Building" for six months from January 2019 to July 2019 at Botanic Garden of Indian Republic, Botanical Survey of India.

DISSERTATION:

Phytochemical analysis, antioxidant and antibacterial activity of *Tephrosia purpurea* (L.) Pers.

INNOVATION PROJECT:

Worked in the Innovation Project entitled: 'Inventory and Creation of Butterfly Conservatory in an Urban Setup' for eight months from October 2015 to May 2016 at Kalindi College, Delhi University.

TRAINING PROGRAMME:

Worked as trainee in the course entitled: 'Management of Small Botanical Gardens under the Green Skill Development Programme of the MOEF&CC, Govt. of India, New Delhi at Botanic Garden of Indian Republic from October 2018 to December 2018.

ACHIEVEMENTS:

- Qualified Gratitude Aptitude Test in Engineering (GATE) in 2018.
- Qualified NET in 2018 organized by CSIR-UGC NET (LIFE SCIENCE) DEC-2017
- Qualified JRF in 2019 organized by CSIR-UGC NET (LIFE SCIENCE) DEC-2018

PUBLICATIONS:

- Priya, Virmani, I., Pragya, Goswami, R. K., Singh, B., Sharma, J. G. and Giri, B. (2023). Role of Microbial Phytases in Improving Fish Health. Reviews in Aquaculture. 15, 1480–1500. DOI: 10.1111/raq. 12790. IF - 10.4
- Priya, Singh, B., Sharma, J. G. and Giri, B. (2023). Optimization of phytase production by *Penicillium oxalicum* in solid-state fermentation for potential as a feed additive. Preparative Biochemistry & Biotechnology. DOI: 10.1080/10826068.2023.2297688. IF - 2.9
- Rao, A. S., Yadav, S. S., Priya, Nandal, A., Singh, N., Ganaie, S. A., Yadav, N., Kumar, R., Bhandoria, M. S., and Bansal, P. (2020). A comprehensive review on ethnomedicine, phytochemistry, pharmacology, and toxicity of *Tephrosia purpurea* (L.) Pers. Phytotherapy Research. DOI: 10.1002/ptr.6657. IF – 5.8

RESEARCH EXPERIENCE

- Microbial enzymes
- Purification of enzymes
- Medicinal plants (Phytochemical analysis)
- Antioxidant and antibacterial work
- Immobilization through sodium alginate method and magnetic iron oxide nanoparticles
- Bioinformatics - BLAST, MSA, and construction of phylogenetic tree

HANDLING INSTRUMENTS & TECHNIQUES

- Soxhlet apparatus
- Autoclave & Laminar flow
- Lyophilizer
- Nitrogen analyzer
- UV-Vis Spectrophotometer
- Column chromatography and SDS-PAGE

CONFERENCES/ SEMINARS/ SYMPOSIUMS:

- Participated and Presented a Poster entitled “Optimization of phytase production from *Penicillium oxalicum* and its utilization as a feed supplement” in Annual International conference of the Association of Microbiologists of India “Microbial Technologies for Sustainable Biosphere (Microbio Tech- 2023)”, organized by Department of Microbiology at Maharshi Dayanand

University, Rohtak, Haryana from 2nd February 2023-4th February 2023.

- Participated and Presented a Poster entitled "Partial purification and characterization of phytase from *Penicillium oxalicum* for feed industry" in International Conference on "Microbial Bioprospecting Towards Sustainable Development Goals", organized by AMI-LPU Unit and Society of Chemical and Synthetic Biology at Lovely Professional University, Punjab from 24th November 2023-25th November 2023.
- Participated and Presented a Poster entitled "*In-vitro* application of *Penicillium oxalicum* phytase in the aquafeed industry" in International Conference on "Strategies for global food and nutritional security, sustainability and wellness (NUTRI-2023)", held in Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana from 4th December 2023-6th December 2023.
- Attended International Conference on Innovations in Biotechnology and Life Sciences (ICIBLS), held in Department of Biotechnology, Delhi Technological University from 18th December-20th December 2020.
- Attended online talk "Forward Genetics to understand pathogenesis in *Botrytis cinerea*- A Broad Host Range Fungal Necrotroph" by Prof. Rupam Kapoor, in Fungal Awareness Talk Series, organized by Department of Botany and Microbiology, Swami Shraddhanand College, University of Delhi on 1st October 2021.
- Attended and presented a poster in International Conference on "Sustainable Agriculture, Energy, Environment and Technology" organized by Environment Sustainability Management Cell in collaboration with Society for Sustainable Agriculture and Resource Management at Maharshi Dayanand University, Rohtak on 24-25th February 2018.
- Participated in workshop organized by Environment Sustainability Management Cell, Maharshi Dayanand University, Rohtak (Haryana) on 6th February 2018.
- Attended UGC Sponsored National Seminar on "Biodiversity: Status and Significance" organized by Department of Botany, Maharshi Dayanand University, Rohtak (Haryana) on 21st March 2017.
- Awarded second prize in collage making competition in the Second National Symposium on Environment: Greener Future & Awareness held at Deshbandhu College, University of Delhi on 19th March 2016.

References

Prof. Bhoopander Giri
Professor
Department of Botany,
Swami Shraddhanand College,
University of Delhi
Phone No. – 9540077805
Email – bhoopg@yahoo.com

Prof. Jai Gopal Sharma
Professor
Department of Biotechnology
Delhi Technological University
Phone No. - 9811090186
Email - sharmajaigopal@dce.ac.in

Dr. Sandeep K. Chauhan
Scientist-F
Botanic Garden of Indian Republic
BSI, MOEF & CC
Phone No. – 9910797114
Email - sandeep.chauhan@rediffmail.com

Prof. Bijender Singh
Professor
Department of Biotechnology
Central University of Haryana
Phone No. - 9996983652
Email – ohlanbs@gmail.com

Received: 31 August 2022 | Revised: 9 January 2023 | Accepted: 10 January 2023

DOI: 10.1111/raq.12790

REVIEW

REVIEWS IN Aquaculture

Role of microbial phytases in improving fish health

Priya[1] | Ishita Virmani[2] | Pragya[3] | Ravi Kumar Goswami[4] | Bijender Singh[3,5] | Jai Gopal Sharma[1] | Bhoopander Giri[6]

[1]Industrial and Environmental Biotechnology Laboratory, Department of Biotechnology, Delhi Technological University, Delhi, India

[2]RECETOX, Masaryk University, Brno, Czech Republic

[3]Laboratory of Bioprocess Technology, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana, India

[4]Department of Zoology, Deen Dayal Upadhyaya College, University of Delhi, Delhi, India

[5]Department of Biotechnology, Central University of Haryana, Mahendargarh, Haryana, India

[6]Department of Botany, Swami Shraddhanand College, University of Delhi, Delhi, India

Correspondence
Bhoopander Giri, Department of Botany, Swami Shraddhanand College, University of Delhi, Delhi, India.
Email: bhoopg@yahoo.com

Funding information
University Grants Commission (UGC); the Ministry of Education, Youth and Sports, Grant/Award Number: LM2018121; Operational Programme Research Development and Innovation-project CETOCOEN Excellence, Grant/Award Number: CZ.02.1.01/0.0/0.0/26617_043/0009632; European Union's Horizon 2020, Grant/Award Number: 857560

Abstract

Fishmeal is the most preferred protein source in aquafeed industry due to its balanced amino acid and fatty acid profile, but its limited global supply and high price value, necessitates researchers to focus on sustainable alternative sources of protein and plant-based feed, to meet the growing demand of fish feed. Phytate is a reservoir of phosphorus present in the plants; however, its chelating ability makes it anti-nutritional. Phytate forms complexes with minerals, proteins, lipids and carbohydrates, confining their accessibility in fish feeds, hence, additional dietary nutrients are required in the fish feed to overcome nutritional deficiencies. The elimination of phytate-phosphorus through animal faeces and its pass on to aquatic bodies leads to enrichment of nutrients, eutrophication and algal blooms. Microbial phytase segregates phosphorus, minerals, proteins and lower inositol phosphates from phytate. The supplementation of fish feed with microbial phytase enhances the digestibility of minerals, due to which less nutrients are excreted in the faeces; therefore, minimizing pollution in water bodies. Monogastric and agastric animals inherently exhibit the deficiency of phytase; hence, supplementation of dietary material with phytase has been an emerging choice to make dietary nutrients available for fish. Moreover, microbial phytases have a broad pH range and are thermostable, which makes them an excellent choice for commercial fish feed production. Additionally, microbial phytase improves fish growth as these enzymes enhance nutrients bioavailability and their digestion. This review article presents the application of microbial phytases as a feed additive, and their role in enhancing fish growth.

KEYWORDS
fish feed, growth performance, nutrients digestibility, phosphorus, phytase, phytate

1 | INTRODUCTION

The United Nations projected that the current world population will rise to 9.7 billion by the end of the year 2050[1]; therefore, meeting the food requirement of growing human population is a challenging task. Food of animal origin is necessary for global food security as it accounts for 25% of protein and 18% of calorie intake around the world.[2] Fish is the only source of protein, accounting for almost 16% of the animal protein consumed by the world's population. This is mainly a vital source of protein in countries where farm animals are rare. Fish provides up to 10% of the protein of animal origin caught on the continents of Europe and North America, 17% on the African continent, 22% in China and 26% in the rest of Asia.[3] In addition, fish is a good source of essential amino acids (EAAs), polyunsaturated fatty acids (PUFAs), especially omega-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Being an important source of dietary food, fish should be healthy and nutritious as well.

The aquaculture industry is considered as the rapidly growing food-producing sector in the world and has raised expectations of bridging the gap between fish demand and supply.[4] The development

of aquaculture industry mainly depends on the supply of high-quality feed for farmed fish.[5] Fishmeal is a commonly used protein source in aquafeeds because of its high protein content, excellent amino acid and fatty acids profile, vitamins, minerals, digestibility, palatability and low carbohydrate content.[6] However, due to excessive use, rising fish production costs (40%–60%) and limited global supply of fishmeal, it must be replaced with a sustainable alternative source of protein such as the protein-rich plants.[7,8] Among various plants, legumes and oilseeds are chosen as substitutes for protein sources because they contain an enormous amount of protein.[9–11] Plant-based feed ingredients are rich in phytate content, which act as an anti-nutritional factor. Phytate masks the absorption of minerals and other nutrients in the fish digestive tract; therefore, having a poor or meagre influence on fish growth.[12]

Phytic acid or phytate (when in salt form) is the phosphorylated derivative of myo-inositol, chemically termed myo-inositol hexakis-phosphate (IP6), which accounts for 50%–85% of total phosphorus (P) in plants.[13] P is a vital element for the growth and development of living organisms and regulates various metabolic processes.[14] It exists as phytate salts, stored inside the legume seeds, cereals, oilseeds, nuts, pollens, spores and vegetative tissues (root, stem, leaves).[15] Phytate has anti-nutritional properties due to its binding nature with minerals, proteins and other biomolecules.[16–18] Therefore, agastric or monogastric fish either cannot utilize these minerals or absorbs them partially, thus having a negative impact on their health. The breakdown of phytic acid occurs enzymatically and non-enzymatically. The non-enzymatic breakdown occurs through various chemical and physical processes, reducing the nutritional value of feed. Indeed, the enzymatic breakdown of phytic acid, using enzyme like phytase, is an eco-friendly and sustainable approach. Phytase releases P and other essential nutrients hence ameliorating the nutritive value of feed.[19] Phytase-producing fungi and bacteria are present in the gut of ruminant animals; but absent in monogastric animals like fish.[15] Such animals depend on the supplementation of their diet to acquire adequate nutrition; hence, their diet is often fortified with inorganic P to achieve their optimal growth and development. The addition of inorganic P leads to the accumulation of dietary P inside the body of fish, which is released out in manure or excreta, causing algal blooms, hypoxia, death of aquatic species and nitrous oxide production.[13,20] Due to the lack of adequate amount of phytase in digestive tract of agastric and monogastric fish, phytate-P is also excreted in aquatic bodies leading to environmental problems such as eutrophication and P pollution.

The application of exogenous phytase is a requisite to release P from phytate rather than supplying inorganic P in their feed. Indeed, the supplementation of fish feed with microbial phytases can improve the utilization of P from phytate-P, it avoid the use of inorganic P in feed, minimize P discharge in water bodies, decrease aquatic pollution and preserve the aquatic environment (Figure 1).[21]

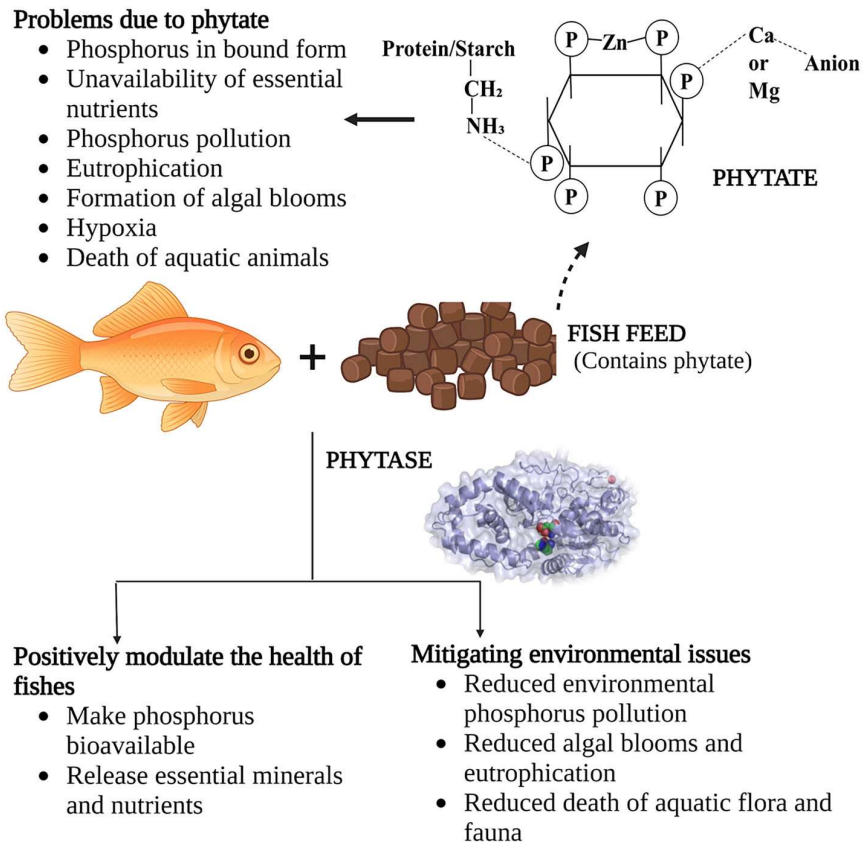


FIGURE 1 A diagram illustrating the effects of phytase addition to fish feed

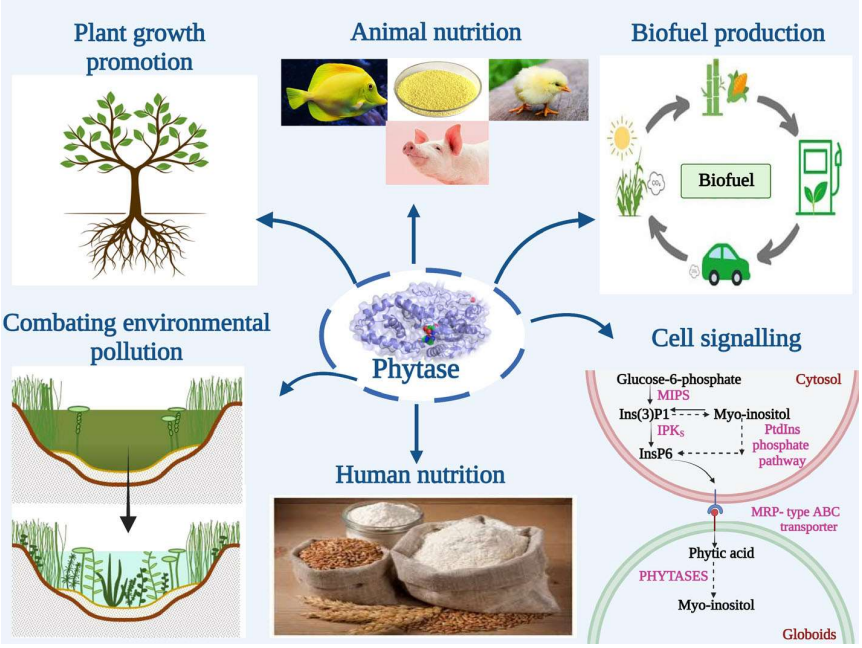


FIGURE 2 Multifarious applications of phytases

Although, exogenous phytase provides P from phytate, it enhances the bioavailability of minerals such as magnesium (Mg), iron (Fe), calcium (Ca), copper (Cu), zinc (Zn), manganese (Mn) and increases the protein accessibility, amino acids and energy. Hence, researchers recommend phytase for feed industry to fulfil the requirement of P and to improve the nutritional value of dietary components by hydrolyzing phytate. Besides, phytase has been applied in various fields (Figure 2). Although phytases can be obtained from several sources such as animals, plants, and microbes, the latter are considered to be a potent source of phytases.[22–24] On a commercial scale, microbes can be easily propagated, and the phytase produce by them is stable and usable under different aquaculture conditions.[25] Over the last decades, considerable progress has been made to understand the effect of microbial phytases as feed additive and their impact on fish growth performance.[23,26,27] The role of phytases in improving the growth performances and nutrition in aquaculture has been documented earlier.[23,28–30] However, these review articles emphasized on the effect of phytate in fish, phytase and its source, dephytinization methods, factors affecting phytase activity, phytase impact on cold water and warm water species, effect on growth, nutrients and mineral digestibility but lacking information including hydrolysis pathway of phytate in the gastrointestinal tract, impact of phytase with other additives in improving fish nutrition and role of immobilized phytase. This review article describes the role of phytate in the growth and digestive enzymes of fish, factors affecting the role of phytase in fish feed and nutrients bioavailability using phytases in fish feed. It also describes the role of immobilized phytases in improving fish nutrition along with current status, applications and future technologies in this field.

2 | IMPACT OF PHYTATE ON GROWTH AND DIGESTIVE ENZYMES OF FISH

Phytate is a significant reserve of P and myo-inositol found in plant seeds. It exists in free form (without metal) and complex structure (with metal) at acidic and neutral pH.[18,19] Phytate isolated from the plants is called phytin. The amount of phytate differs among plant seeds. Cereal grains contain 0.04%–3.3%, nuts 0.2%–9.4% and oilseeds 0.4%–5.7%,[13,31] legumes 1.0%–1.5% (soybean), rapeseeds 5.0%–7.5% and sesame seeds possess 2.4% of phytate.[29] Phytate hinders essential minerals and amino acids and negatively affects the absorption of fish nutrients.[32] Several elements (viz., P, Mg, Zn, Ca, Fe, Cu, K, Mn, Na, Al, Ni and Cr) have been found to be associated with phytate, therefore, not readily available for fish.[33] Besides mineral nutrients, phytate also restricts metabolism of lipids and proteins consequently affecting their weight gain (WG), feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER) and specific growth rate (SGR; Figure 3).[32] The amount of phytate in feed variably affects growth parameters of fish. The SGR of rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) significantly reduced when phytate was more than 1% of total diet.[34] Similarly, growth parameters such as WG, FI, PER, SGR significantly decreased in Japanese Flounder (*Paralichthys olivaceus*),[35] turbot (*Psetta maxima*),[36] gilthead sea bream (*Sparus aurata*)[37] and Nile Tilapia (*Oreochromis niloticus*)[38] due to the formation of phytate complex. Likewise, 2.6% of phytate in the feed of chinook salmon (*Oncorhynchus tshawytscha*) reduced its overall growth performance. The presence of 0.5% or 1.0% phytate content in the feed of agastric common carp (*Cyprinus carpio*), significantly decreased growth and nutritional value of feed, and 2.2% of phytate content had a

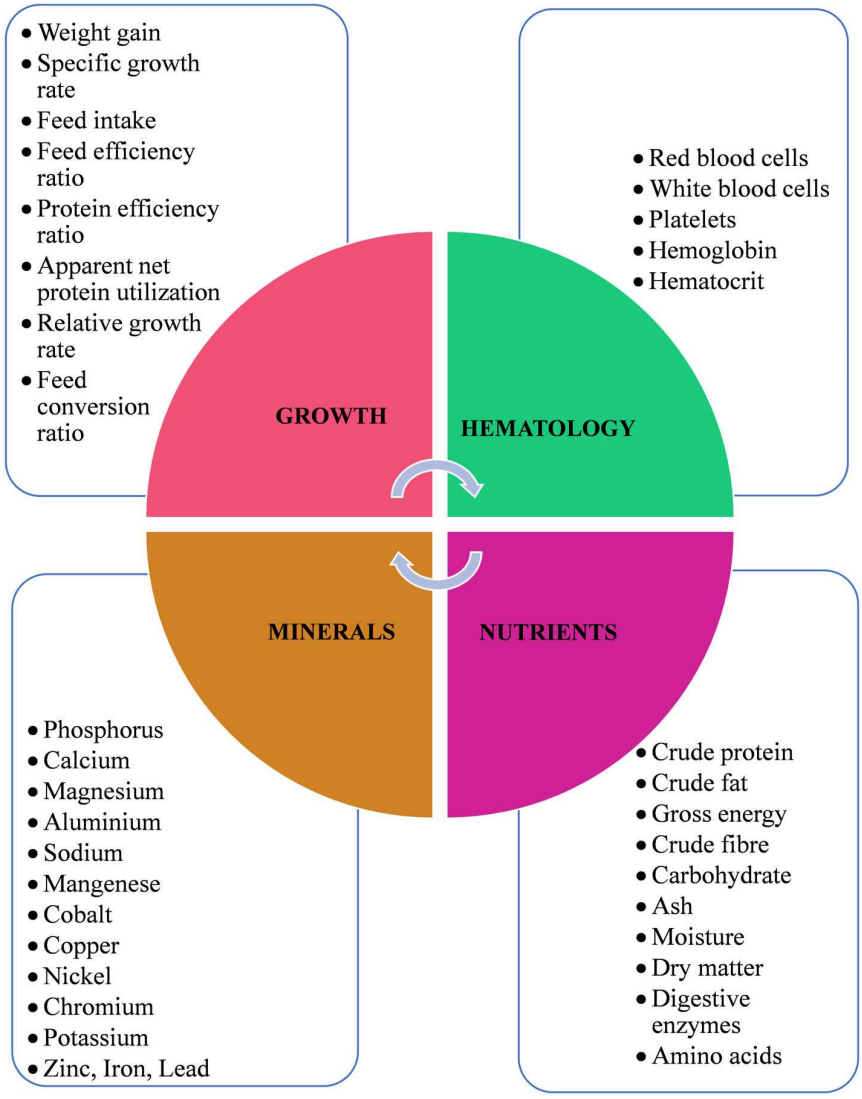


FIGURE 3 Various growth and nutritional parameters affected by supplementation of phytase in fish feed

significant reduction in WG, feed efficiency, and Zn level in channel catfish (*Ictalurus punctatus*).[29] The diet of Atlantic salmon (*Salmo salar*) containing 2.07% phytate content significantly reduced the growth performance, availability and utilization of minerals (P, Ca, Zn and Mg).[20,39] The reduced availability of nutrients may be due to the formation of insoluble complex on interaction of phytate with nutrients in the upper small intestine, where absorption of minerals usually takes place. The formation of phytate-nutrients complex attributes to restricted absorption of these nutrients, therefore, affecting growth and development of fish. Further, declined growth performance and lower mineral absorption were reported in striped bass (*Morone saxatilis*) in the presence of high content of phytate (1.31%).[40] A reduction in Ca and Zn absorption, but not of Mg, Cu and Fe was reported in the plasma of *C. carpio* when its feed was supplemented with more than 1% of phytate,[20,29] which may be due to more affinity of Ca and Zn ions towards phytate than with the Mg, Cu and Fe ions, hence preventing their availability to fish utilization. Phytate has been found to reduce reabsorption of endogenous Zn and availability of dietary Zn too.[41]

As phytate is a negatively charged molecule, pH is a crucial parameter for the interaction of phytate with proteins. During acidic conditions, phytate binds with the amino group, imidazole group and guanidyl group of proteins in the stomach of *O. niloticus*[42] and makes a complex with protein in the digestive system of *S. salar*.[43] Whereas, at the alkaline pH of intestine, the phytate–protein association makes a ternary complex with cations, restricting protein digestibility.[42] There is no direct effect of phytic acid on the digestibility of protein, rather it is a consequence of amino acid digestibility affected due to enhancement in loss of endogenous amino acid in the intestine.[27] The digestibility of protein and the growth of rainbow trout (*Oncorhynchus mykiss*) remarkably decreased on the supplementation of feed with 0.5% phytate, however, feed containing 0.8% phytate though affects

protein digestibility of *S. salar*, does not affect its growth.[44] On interacting with lipid molecules, phytate restricts energy utilization, as has been demonstrated in *C. mrigala* and triggers low-fat content.[29] Phytate largely affects digestive enzymes of fish, therefore, these digestive enzymes do not act properly, as has been revealed in the case of *S. salar* feed, where 2.07% phytate content drastically reduced trypsin activity.[39] Further, Khan and Ghosh[45] discussed the inhibitory effect of phytate on digestive enzymes in major Indian carps and found a severe effect of phytate on the activity of protease as compared with amylase. The activity of amylase and protease were reported low in the intestine of *O. niloticus* due to phytate content.[38] The effect of phytate on digestive enzymes was studied in vitro. Phytate make a binary complex with pepsin and affects its action on the digestibility of dietary proteins. Similarly, it makes a ternary complex (phytate-Ca-trypsin) and inhibits trypsin activity.[20]

3 | MICROBIAL PHYTASES WITH IDEAL PROPERTIES AS FISH FEED ADDITIVE

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are a class of phosphatases (EC 3.1.3) that breaks phytic acid into orthophosphates and esters of myo-inositol phosphates, therefore, rendering P available for absorption. Depending upon the hydrolysis position, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) classified phytases into two classes: 3-phytase and 6-phytase, but other phytases such as 2-phytase, 4-phytase and 5-phytase are also mentioned in the literature.[19] The 3-phytase can be obtained from a wide array of microorganisms and releases P from the third carbon of the inositol ring, whereas the 6-phytase is obtained from plants and removes P from the sixth carbon of phytate.[13,15] However, Bhavsar and Khire[46] detected 3-phytase in soybean and 6-phytase in *Escherichia coli*. A 5-phytase was extracted from lily pollen grains and the anaerobic ruminant bacterium *Selenomonas ruminantium*.[47] Based on the pH tolerance, phytases are classified as acidic and alkaline. The optimum pH of acidic phytase is around 5.0 or lesser. They exhibit a broader specificity towards phytate free from metals, whereas the optimum pH for alkaline phytase is about 8.0. Phytases exhibit specificity strictly towards metal-bound phytate.[28,48] Four phytases are categorized based on the catalytic mechanisms, histidine acid phosphatase (HAP), β-propeller phytase (BPP), cysteine phosphatase (CP) and purple acid phosphatase (PAP).[13]

Phytase is absent in monogastric and agastric fish; therefore, its application as a feed additive in fish nutrition is highly recommended.[16] Due to its importance, more than 22 countries are using this enzyme as a feed additive with the approval of the Food and Drug Administration (FDA).[46,49] It contributes more than 80% of feed enzymes in the global market share.[50] A few examples of microbial phytases used in fish feed have been compiled and summarized in Table 1. It has been suggested that 500–1000 units of phytase could replace about 1 g of inorganic P and decrease 30%–50% excreted P in manure or excreta.[94] Phytases are present in plants, animals and microorganisms.[46] Among plant species, wheat, rye, barley, maize, rice, pea and soybean have been explored as well for the phytase production,[13,23] while animal phytases are extracted from the calf liver and lower vertebrates' blood, including birds, sea turtles and reptiles.[13,95] Generally, the efficiency of animal phytase is negligible compared to plants and microorganisms. As far as microbes are concerned, phytase has been extracted from *Bacillus* spp., *Anabaena*, *Gloeobacter*, *Streptomyces*, *Flavobacterium*, *Prosthecochloris*, *Desulfuromonas*, anaerobic rumen bacteria, *Pseudomonas*, *Azospirillum*, *Raoultella* spp., *E. coli*, *Citrobacter braakii*, and *Enterobacter* spp.[19,96,97] The most commonly studied fungal species for phytase production are *Aspergillus niger*, *A. oryzae*, *A. terreus*, *A. ficuum*, *A. carbonarius*, *A. fumigatus*, *Myceliophthora thermophila*, *Thermomyces lanuginosus*, *Rhizopus oligosporus*, *Chaetomium thermophilum*, *Rhizomucor miehi*, *Rhizomucor pusillus*, *Thermoascus aurantiacus*, *Agaricus bisporus*, *Ceriporia* spp., *Ganoderma stipitatum*, *Agrocybe pediades*, *Lentinula edodes*, *Grifola frondose*, *Schizophyllum commune*, *Peniophora lycii*, *Pleurotus cornucopiae*, *Trametes pubescens*, *Cladosporium* spp., *Trichoderma* spp., *Penicillium* spp, *Mucor* spp, *Schwanniomyces castellii*, *Pichia anomala* and *Saccharomyces cerevisiae*.[18,22–24]

Indeed, numerous microbes are utilized for phytase production, but only a few are producing potent marketable phytases. The reasons behind their failure could be the high temperature during extrusion processing, limited pH range, high price and storage instability.[98] Studies revealed that phytase produced from bacteria possesses an optimum pH range between neutral and alkaline.[98] In contrast, fungal phytase have an optimum pH range from 2.0 to 6.0.[23,24] Due to their properties, such as heat tolerance, chitinase activity, and extracellular nature, fungal phytases are preferred over bacterial phytases.[12] Among fungi, *A. niger* is the most preferred fungus for phytase production due to its biochemical properties like acidic nature and heat stability. Bacterial phytases are highly substrate-specific, least sensitive toward proteolysis and have better catalytic efficiency.[18] Usually, fungal phytases are extracellular, whereas most bacterial phytases are cell-associated, except *Lactobacillus amylovorus*, *Bacillus subtilis* and *Enterobacter* sp. 4.[19] In comparison to plants, microorganisms exhibit better phytase activity, which might be due to their ability to produce phytases that are stable at a broad pH range (even below 3.0 and above 8.0); however, plant phytases show stability at a narrow pH range (4.0–7.5). Plant phytases undergo pre-treatment with high production costs.[98] The broad pH range, thermal stability, high specificity, genetic modification and large-scale production make microbial phytases an ideal choice for feed additives in order to improve nutritional quality.[12,18,99]

FDA has approved phytase as generally recognized as safe (GRAS) status.[100] The first commercial phytase, Natuphos produced from *A. niger* became available in 1991 by Gist Brocades. It was introduced as a first-generation phytase.[28,46] The OptiPhos is referred to as a second-generation phytase, which was extracted from *E. coli* and expressed in *Pichia pastoris*, exhibiting greater catalytic efficiency and resistant to pepsin.[47] Besides, Finase from Alko Co. (Finland), Allzyme from Altech (USA), granulated BioFeed® Ronozyme from Novo Nordisk and Quantum™ phytase from Syngenta Animal Nutrition, Inc. (USA) are also available in the market.[21] These enzymes are used as a

TABLE 1 A table showing the variety of microbial phytases used in fish feed

Microbes/commercial phytase	Fish species	References
AxtraPHY	*Oreochromis niloticus*	51,52
Quantum Blue	*Oncorhynchus mykiss*	44
	Oreochromis niloticus	53
Natuphos	*Labeo rohita*	54
	Rhamdia quelen	55,56
	Clarias gariepinus	57–59
	Psetta maxima	36
	Oncorhynchus mykiss	60
	Salmo salar	61
	Rhamdia voulezi	62,63
	Chanos chanos	64
	Pangasius hypothalamus	65
	Oreochromis niloticus	66
	Ictalurus punctatus	67
	Ctenopharyngodon idella	68
Phyzyme XP	*Cirrhinus mrigala*	69
	Catla catla	70,71
	Labeo rohita	72,73
Biophos-TS	*Labeo rohita*	74
Bacillus subtilis subsp. *subtilis*	*Labeo rohita*	75
Lactobacillus plantarum (LP20)	*Seriola dumerili*	76
Bacillus licheniformis	*Labeo rohita*	77
Ronozyme	*Cyprinus carpio*	78,79
	Oreochromis niloticus	80
	Piaractus mesopotamicus	81,82
	Pagrus major	83
	Oncorhynchus mykiss	84
	Salmo salar	84
Ronozyme HiPhos	*Oreochromis niloticus*	85
	Oncorhynchus mykiss	
CIBENZA® PHOS	*Pelteobagrus fulvidraco*	86
Stenotrophomonas maltophilia strain KUAKSP1	*Labeo rohita*	87
Pedobacter sp. MJ11	*Myxocyprinus asiaticus*	88
Escherichia coli	*Pelteobagrus fulvidraco*,	89
	Oncorhynchus mykiss	90
S. cerevisiae	*Ctenopharyngodon idella*	68
Pichia pastoris	*Oncorhynchus mykiss*	91
Phytase Aska-25	*Cyprinus carpio*	78
SMIZYME	*Oreochromis niloticus*	92
OPTIPHOS®	*Oncorhynchus mykiss*	93
	Salmo salar	

powder, granular or in liquid form with the feed during post-pelleting or pre-treatment.[28] Though various commercial phytases are available in the market, there is still a need for a more efficient phytase to meet the required condition of an ideal phytase. The characteristics of an ideal phytase include heat tolerance, protease resistance, low pH tolerance, broad pH range and cost-effectiveness. The selection of the phytase source plays a critical role for its production. Besides this, the processing of phytase is also cumbersome.

Cost-effective phytase production requires upstream (choice of strain, improvement of strain, bioreactor designing and fermentation) and downstream processing (separation methods, purification, formulation of end product). The selection of production method depends upon culture conditions, strain type, substrate nature and nutrients availability, as production yield is essentially affected by these factors.[101] Since, the yield of phytase from naturally occurring strains is meagre, improvement is needed for their production. Phytase activity can be increased by improving strains through genetic approaches such as changes at the gene level, transgenic approaches, protoplast fusion to develop hybrid strains, and of course, direct evolution, statistical methodology for optimization and immobilization techniques.[46] Many approaches require researchers' attention, like low-temperature active phytase for aquaculture, engineering a suitable phytase based on 3D structure and engineering of protein refolding pathways.[98]

4 | FACTORS AFFECTING PHYTASE EFFICACY IN FISH FEED

4.1 | pH value

Among different factors, pH is one of the crucial factors affecting phytase activity and efficacy as fish feed additive. Microbial phytases require an optimum pH for maximum efficiency; however, it varies among species.[20,21] Broadly, there are two fish categories—gastric and agastric. Gastric fish such as *O. niloticus*, *I. punctatus*, have acidic stomach, and agastric fish like *L. rohita*, thaila (*Catla catla*) are stomachless having neutral to alkaline pH in their digestive system. However, the activity of commercial microbial phytase is in the range of 2.5–5.5,[51] which is suitable only for gastric fish as reported in *I. punctatus* having stomach pH between 2.0–4.0.[102] The lower gastrointestinal pH in gastric fish favours efficient breakdown of phytate by the microbial phytases in such species. While common carps possess pH 6.5–8.4 in their digestive system, which is not suitable for phytase action.[28] The large difference of pH between optimal phytase activity and digestive tract of agastric fish may be a possible cause of the poor efficacy of phytase in the stomachless fish.[29] For the action of phytase in agastric fish, either organic acids, neutral phytase or pre-treated phytase can be used as feed additives.[19,28] The efficiency of phytase and its utilization increased on the supplementation of citric acid, fumaric acid, lactic acid and formic acid in the feed of agastric fish as these organic acids lower the feed acidity, reduce the pH of digestive tract and amplify the activity of supplied phytase.[29,103]

4.2 | Effect of temperature

Temperature is another critical factor affecting the phytase efficacy in fish gastrointestinal tract as well as during feed pelleting. The optimum temperature range of microbial phytase is 45–60°C. During the feed extrusion process, phytase faces high temperatures (>80°C), which denatures phytase activity. The high temperature during extrusion process leads to the partial destruction of intrinsic phytase in feed ingredients and thereby limiting the availability of P and trace elements present in plant-based feed. Therefore, phytase is generally applied to the feed before treatment or after the feed processing method.[28,29] The phytases retaining activity at high temperature are considered desirable for feed industry as feed pelleting involves a brief exposure to higher temperature (up to 85°C). Vohra and Satyanarayana[104] purified phytases from *Schwanniomyces castellii* and *Arxula adeninivorens* which were remain active at the high temperature (up to 80°C). Some phytases are found to be more efficient at higher water temperatures as compared to lower water temperature.[30,81,105] Also, the effect of phytase on cold-water fish species is different from warm water species because the warm water species provide better environment for phytase action. In cold water species, lower temperature influences the phytase activity and reduces its efficacy[44,106] and phytase shows better activity at ≥37°C.[78,107,108]

4.3 | Effect of calcium:phosphorus ratio

Both Ca and P are crucial for maintaining the optimal development of bone and different growth stages of fish. Since Ca and P have vital role in bone development, it is of utmost importance to take in to account the ratio of Ca and P in the fish diet. However, a high level of Ca:P ratio affects phytase activity in many ways. Ca either bound to phytate and makes a more complex insoluble structure, or acts as phytase inhibitor, which blocks the phytase active site in phytate, or it can increase the pH and disturbs the phytase activity.[28,29,109] Similarly, a high level of P also hampers phytase activity by end-product inhibition. Phytases can perform efficiently only when the amount of non-phytate P is less than the amount of required P in fish.[28] The excessive Ca:P ratio in fish severely affects bone development by interfering with their bone metabolism and disturbing the bone mineral homeostasis. Therefore, it is recommended that Ca to P ratio should be maintained in the range of 1.1–1.4:1 for better phytase performance as fish feed additive.[28,29,109,110]

4.4 | Feed processing methods

As described earlier, extrusion is the commonly used method during fish feed manufacturing. In this method, heat treatment denatures the phytase added during the pelleting process. Once the enzyme is denatured, it loses its activity. Hence, phytase is incorporated into feed to pass through the heat without inactivation. Either pre-treatment of feed or dephytinization can be performed before the formation of pellets, or phytase can be sprayed onto pellets, or liquid formulation of phytase can also be applied after the formation of pellets.[28,111] Coating of phytase with fat or salt does not influence the specific activity of phytase but has a less effect in improving thermostability.[47]

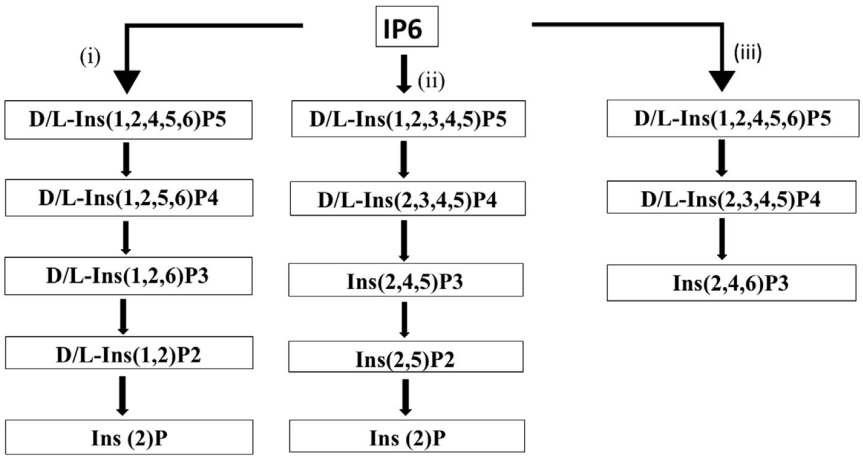


FIGURE 4 Degradation pathway of phytic acid by (i) 3-phytase, (ii) 6-phytase and (iii) β-propeller phytase

Spraying phytase on the top of pellets is a better approach used nowadays to retain phytase efficacy.[27,112]

5 | HYDROLYSIS OF PHYTATE BY PHYTASES

The degradation of phytate by phytase takes place sequentially. Phytase dissociates the entire IP6 into IP5 (inositol pentaphosphate) and then breaks down into lower inositol phosphates. In the in vivo conditions, hydrolysis occurs partially; therefore, it provides a mixture of lower ester forms, that is, IP5, IP4 and IP3. The dissociation of phytate complex initialized during diet preparation, and then further hydrolysis occurs in the fish stomach.[28,113] Phytase has been found to degrade phytate in the stomach region of *S. salar*.[43] During the first few hours, hydrolysis of IP6 to lower inositols (IP3–IP5) takes place in the stomach region.[102] Similarly, in *S. aurata*, most of the degradation of phytate complex occurred in the stomach after 1–2 h of ingestion of feed by the fish. Phytate is more soluble at lower pH, and after feeding, the stomach pH declines, making phytase more active for the hydrolysis of solubilized phytate.[114] The mechanism behind the phytate degradation in the fish gastrointestinal tract is still unknown. However, phytases are involved in the stepwise breakdown of phytates, releasing intermediate products of myo-inositol phosphates, inorganic phosphate and minerals. Phytate degradation occurs via different pathways among fish species, depending on the types of phytases. The mechanism of phytate hydrolysis by histidine acid phytases (HAPs) occurs in two steps. Initially, the histidine residue of the conserved sequence (RHGXRP) attack at the phosphate group formed a phosphohistidine bond, which was further released by the aspartate residue of the C terminal motif (HD). The degradation pathway is shown in the Figure 4, and found that Ins(2)P_1 is the final product in both cases of 3-phytases and 6-phytases.[115] Whereas, β-propeller phytases provide inositol triphosphate (IP3) as the end product. The breakdown of phytate has been observed in *O. mykiss* and *S. salar*, whereas *O. mykiss* followed the 3-phytase pathway and *S. salar* followed the 6-phytase pathway.[84] The IP3 acts as an intracellular secondary messenger in calcium signalling. It increases the proliferation of the intestinal mucosa in fish and improves the absorption of nutrients. It also increases the activity of digestive enzymes such as chymotrypsin, amylase and lipase, thereby improving fish growth and health. IP3 plays a role in osmoregulation, regulates antioxidant activity and protects fish from stressful conditions.[116]

6 | ROLE OF PHYTASES IN GROWTH PERFORMANCE OF FISH

The effect of phytase on fish growth performance can be determined by studying various parameters including WG, FI, FCR, SGR, PER and apparent net protein utilization (ANPU) as shown in Table 2. Except for FCR, other variables increase in the presence of phytase. Mahmoud et al.[117] observed enhanced body weight, SGR, and length of *O. niloticus*. The WG in the body might be due to digestive enzymes, which are also responsible for protein and lipid digestibility and making more P available from phytate. Relative growth, PER, FI, and FCR increased after the addition of phytase.[53,66,80] Besides, the supplementation of feed with phytase has shown improvement in the growth performance of several fish such as African catfish (*Clarias gariepinus*),[57] *L. rohita*,[87] *C. carpio*,[124] *C. mrigala*,[129] *C. catla*,[32,131] *O. mykiss*,[11] *P. maxima*,[36] yellow catfish (*Pelteobagrus fulvidraco*),[89] *I. punctatus*,[67] milkfish (*Chanos chanos*),[64] grass carp (*Ctenopharyngodon idella*),[132] red sea bream (*Pagrus major*),[83] *S. salar*,[61] catfish (*Pangasius hypophthalmus*),[65] and Asian sea bass (*Lates calcarifer*).[137] The effect of commercial and non-commercial phytases were compared in *L. rohita*[74] and *C. carpio*[126] and increased growth was obtained in both the cases. The inclusion of phytase in a methionine-supplemented diet showed a significant improvement in the growth of Chinese sucker (*Myxocyprinus asiaticus*).[88] A positive effect of neutral phytase was recorded on the growth performance, nutrients and minerals

TABLE 2 A table showing the effect of phytase on growth and performance of fish

S. No	Fish species	Phytase dose/kg	Feed	Effect	References
1.	*Oreochromis niloticus*	1000 U	Corn–soy	WG, Length ↑	117
		500 mg	Soy protein concentrate	No improvement, FCR ↑	80
		500 U	Soybean meal	WG, SGR, PER↑ FCR↓	53
		1000 U	Soybean–maize yellow corn meal	WG, SGR, length↑	118
		1000 FTU	Mixed diet	RGR, PER↑ FCR↓	66
2.	*Clarias gariepinus*	250 FTU	Soybean	WG, PER↑ FCR↓	57
			Groundnut diets	WG, SGR, FI↑ FCR↓	57
		2000 FTU	Mixed diet	WG, SGR↑ FCR↓	59
		250 FTU	Soybean meal	WG, FI↑ FCR↓	58,110
		1000 FTU	Plant-based diet	WG, SGR↑	119
3.	*Labeo rohita*	750 FTU	Corn gluten meal	WG↑ FCR ↓	120
			Sunflower meal	WG, FI↑ FCR↓	121
			Cottonseed meal	WG, FI↑ FCR↓	122
			Canola meal	WG, FI, SGR↑ FCR↓	123
		Stenotrophomonas maltophilia (KUAKSP1)	25% fermented leaf of Ipomoea aquatica	WG, SGR, PER↑ FCR↓	87
		Bacillus licheniformis (LF1 and LH1)	30% Fermented sesame oilseed meal	WG, SGR, PER, ANPU↑ FCR↓	77
		Bacillus licheniformis LF1 (40 FTU)	Sesame oilseed	WG, SGR, PER↑ FCR↓	74
		30 FTU	Sesame oilseed	WG, SGR↑ FCR↓	74
		Bacillus subtilis subsp. *subtilis* (40%)	Fermented sesame meal	WG, SGR, PER, ANPU↑ FCR↓	75
4.	*Pelteobagrus fulvidraco*	1000 IU + 10 g NaH_2PO_4	Plant-based diet	Protein retention efficiency↑ FCR↓	89
5.	*Ictalurus punctatus*	1500 FTU	Plant-based diet	WG, SGR, PER↑ FCR, FI↓	67
6.	*Cyprinus carpio*	950 FTU	Moringa seed + leaf meal	WG, SGR↑ FCR↓	124
		4000 IU	Commercial feed	WG, SGR, FCE↑ FCR↓	125
		–	Lentil	WG, FI, FCE, RGR, SGR↑ FCR↓	126
		1000 U	Mixed feed	RGR, PER↑ FCR↓	127
7.	*Oncorhynchus mykiss*	2000 U	20% safflower meal	WG, SGR↑ FCR↓	11
			Fishmeal + MCP	WG, SGR↑ FCR↓	85
8.	*Psetta maxima*	2000 FTU	Rapeseed protein concentrate	No improvement	36
9.	*Cirrhinus mrigala*	1000 FTU	Soybean meal	WG↑ FCR↓	128
			Sunflower meal	WG↑ FCR↓	129
10.	*Chanos chanos*	1000 FTU	Mixed feed	RGR↑ FCR↓	64
11.	*Catla catla*	900 FTU	Moringa seed meal	WG, FI, SGR↑ FCR↓	71
			Moringa seed + leaf meal	WG, FI, SGR↑ FCR↓	32,130
			Moringa leaf	WG, FI, SGR↑ FCR↓	131
12.	*Ctenopharyngodon idella*	1500 U	Plant-based feed	WG, SGR, FI↑ (pre-treated phytase); PER↑ FCR↓ (spraying)	132
		500 FTU + 1.5% MCP	Plant-based feed	WG, FI, SGR, PER↑ FCR↓	133
13.	*Pagrus major*	2000 FTU + 5 g NaH_2PO_4	Soybean meal	WG, SGR↑ FCR↓	83
14.	*Salmo salar*	250 U	Soy Protein Concentrate	WG↑	61
15.	*Cromileptes altivelis*	500 FTU	Artificial feed	RGR↑ FCR↓	134

(Continues)

TABLE 2 (Continued)

S. No	Fish species	Phytase dose/kg	Feed	Effect	References
16.	*Piaractus mesopotamicus*	4001.1 ± 160.0 PU	Corn meal	No effect	81
17.	*Rhamdia quelen*	1500 FTU	Sorghum feed	No significant interaction	55
			Rice grits	No effect	56
18.	*Carassius auratus gibelio*	500 U	Plant meal	No improvement	135
19.	*Scophthalmus maximus*	30% phytase treated	Soybean meal	No significant result	136
20.	*Lates calcarifer*	1000 FTU	Soybean meal	RGR, PER↑	137
21.	*Pangasius hypophthalmus*	750 FTU	30% Cottonseed meal	WG, length, SGR↑ FCR↓	138
		300 FTU	Mixed diet	SGR, EFU, PER↑	65
22.	*Myxocyprinus asiaticus*	1500 FTU	Soybean meal + methionine	WG, SGR, PER↑ FCR↓	88
23.	*Oreochromis mossambicus*	500 FTU	Plant-based diet	No effect	139

Abbreviations: ANPU, apparent net protein utilization; EFU, efficiency of feed utilization; FCE, food conversion efficiency; FCR, feed conversion ratio; FI, feed intake; PER, protein efficiency ratio; RGR, relative growth rate; SGR, specific growth rate; WG, weight gain.

digestibility of goldfish (*Carassius auratus*).[140] Rodrigues et al.[55] stated that silver catfish (*Rhamdia quelen*) acquires high FCR in a sorghum feed with 1500 FTU/kg of phytase. In contrast, an insignificant growth performance of pacú (*Piaractus mesopotamicus*),[81] turbot (*Scophthalmus maximus*)[136] and *Oreochromis mossambicus*[139] has also been recorded after the addition of phytase to the fish feed. The effect of phytase was also insignificant on fish growth and body composition.[56] Likely, there has been no effect of neutral phytase (500 U/kg) on the growth parameters of crucian carp (*Carassius auratus gibelio*)[135] and *C. idella*,[133] substantiating the fact that efficacy of microbial phytases varies among different fish species.

7 | NUTRIENTS DIGESTIBILITY

Phytate restricts the absorption of minerals, proteins, amino acids, lipids, and carbohydrates in fish species. Inversely, phytase supplementation in the feed enhances nutrients and minerals accumulation in the bone, plasma and whole-body of fish. The assimilation of crude protein (CP), crude fat, gross energy (GE) and minerals can be determined by calculating the apparent digestibility coefficient (ADC). The digestibility of CP, crude fat, GE and minerals improved significantly due to the supplementation of fish diet with phytases (Table 3). The involvement of phytase in increasing protein digestibility in fish is contradictory as in some cases it increases, decreases or remains unaffected.[150] The involvement of phytase in increasing protein digestibility is not direct, in fact, it is an outcome of phytase action on the digestibility of amino acid.[48] Phytase helps in retaining more nutrients inside the fish body.[32,70,71,131] Increased digestibility of minerals such as Fe, Mg, Ca, P, K, Zn, Cu, Na, Al, Mn, Sr, Cr and Pb has been observed in *O. niloticus*,[8,146] *C. mrigala*,[128,129] *C. carpio*,[124] *L. rohita*,[74,144] *P. fulvidraco*,[89] *S. salar*[61] and *I. punctatus*[67] after feeding with phytase-supplemented feed. Besides, increased digestibility of protein was noticed in *C. chanos*,[64] *O. niloticus*,[66] *P. hypophthalmus*,[65] *C. carpio*[127] and *P. major*.[83] Protein as well as P digestibility was higher when dietary protein and phytase were added to the diet of *O. mykiss*[60] and *C. gariepinus*.[119] Although, the apparent digestibility of protein, ash, and P was more in *P. maxima*, the absorption of minerals remained unaffected.[36] A positive correlation between phytase and dietary Ca:P ratio was studied on bone mineralization.[110,148] The positive effect of neutral phytase (500 U/kg) was further recorded on the digestibility of CP and DM in *C. auratus gibelio*.[135] Moreover, the bioavailability of amino acids such as His, Leu, Ile, Phe, Lys, Val, Asp, Thr, Glu, Ala, Pro, Gly and Ser increased by spraying phytase on the top of pellets.[27] In contrast, protein digestibility in *C. carpio*,[126] crude protein in *C. idella*[68] and the digestibility of DM and CP in *S. maximus* did not show a significant change in the supplementation of phytase to the fish feed.[136]

8 | PHYTATE DIGESTION AND BIOAVAILABILITY OF PHOSPHORUS

P is an essential element for fish, which can be absorbed by skin, gills or fins, but the level of P is low in water and thus fulfilment of P can be done by feed. Feed contains phytate-P which remains undigested by fish.[150] Therefore, supplementation of phytase is a cost-effective solution for the bioavailability of P by degrading phytate in the feed. The digestion of phytate is a stepwise removal of orthophosphate and lower forms of inositol ester.[46] It relies on two factors—the supply of adequate phytase in the feed, and the amount of phytate accessible to react with phytase in the feed.[48] Since monogastric fish lack phytase, the inclusion of microbial phytase in the fish feed is essential. The phytate complex dissociates and makes P available for fish nutrition.[11] The bioavailability of P is determined by how much phytate-bound P is digestible in the fish feed. It depends upon many factors such as rearing phase, fish species, pH, gastrointestinal condition, meal type, dietary inorganic P content, phytate quantity, feed additives supplementation and phytase dosage. The accessibility of P can also be indicated by the declining P level in the waste. In the fishmeal, consumable P is 60%, whereas, in plant-based meals, only 0%–20% P is available due to phytate; therefore, exogenous microbial phytase is required in the diet to utilize adequate amount of P.[151]

TABLE 3 A table showing the effects of phytase on nutrients and minerals digestibility of fish

S. No	Fish species	Phytase dose/kg	Diet	Effect	References
1.	*Labeo rohita*	750 FTU	Cottonseed meal	Ca, P, Na, K, Fe, Cu, Zn, Mn↑ CP, crude fat, GE↑	72,122
			Sunflower meal	Ca, P, Mg, Na, K, Fe, Cu, Mn↑	121
			Canola meal	CP, crude fat, GE↑	123,141
			Corn gluten meal	Crude fat, GE↑	120,142
				Ca, P, Mg, Na, K, Fe, Cu, Zn↑	
			Dry bread meal	Ca, P, Na, K, Fe, Cu, Zn, Mn↑	143
		500 FTU	Distiller's dried grains with solubles (DDGS)	CP↑ Moisture↓	144,145
		40 FTU (*Bacillus licheniformis*, LF1), 30 FTU (commercial)	Sesame	CP, crude fat, ash↑ P, Ca, Mn, Cu, Fe↑	74
		Bacillus licheniformis (LF1 and LH1)	30% Fermented sesame oilseed meal	CP, crude fat, GE, P, Ca, Mn, Cu, Fe↑	77
2.	*Catla catla*	900 FTU	Moringa leaf	CP, GE↑	70,131
			Moringa products	Ca, Na, Fe, Cu, Mn, P↑	32,130
			Moringa seed	CP, crude fat, GE↑	33,71
3.	*Cyprinus carpio*	950 FTU	Moringa products	CP, crude fat, GE; Ca, K, P, Mn↑	124
		1000 FTU	Mixed diet	CP, P↑	127
4.	*Oreochromis niloticus*	950 FTU	Moringa products	CP, crude fat, GE↑ ash, CF, moisture↓	8
		600 FTU	Moringa leaf	Mg, Na, K, Cu, Zn, Fe, Cr, Ca↑	146
		1000 FTU	Mixed diet	CP, P↑	66
		2000 U	Fishmeal + MCP	P↑	85
5.	*Cirrhinus mrigala*	1000 FTU	Sunflower meal	CP, crude fat, GE↑	129
			Soybean	CP, crude fat, GE; Ca, P, Na, Fe, Cu↑	128,147
6.	*Clarias gariepinus*	1000 FTU	Soybean	CP, P↑ crude fat, GE↓	57
		500 FTU	Groundnut	CP, GE, crude fat↑	57
		4000 FTU	Mixed diet	CP, P↑ crude fat, GE↓	59
		1250 FTU	Plant-based diet	CP, P↑	119
		250 FTU	soybean diet	CP, Ash, Moisture↑ P, Ca, Zn↑	110,148,149
7.	*Chanos chanos*	1000 FTU	Mixed diet	CP, P↑	64
8.	*Pangasius hypophthalmus*	300 FTU	Mixed diet	CP, P↑	65
9.	*Ctenopharyngodon idella*	550 FTU (both microbial strain and commercial enzyme	Defatted rice bran	P, Mg, Fe, CP↑ (strain); DM, lipid, ash↑ moisture↓ (enzyme)	68
10.	*Psetta maxima*	2000 FTU	Rapeseed	No improvement	36
11.	*Rhamdia voulezi*	1500 FTU	Corn, sorghum, wheat bran	CP↑ (wheat bran), DM, GE↑ (Sorghum), P↑ (corn)	62
			Different meal	CP, crude fat, GE, DM↑ (Soybean), ash↑ (canola)	63
12.	*Oncorhynchus mykiss*	2000 U	20% Safflower meal	Protein and P digestibility rate↑ CP, crude fat, DM↑	11
		4000 FTU	Mixed diet	Ash, CP, P, Ca↑	60
		2000 U	Fishmeal + MCP	P↑	85
13.	*Pelteobagrus fulvidraco*	1000 IU + 10 g NaH_2PO_4	Plant based diet	CP, P↑	89
14.	*Ictalurus punctatus*	1500 FTU	Plant-based diet	CP, Cu, Fe↑	67

(Continues)

TABLE 3 (Continued)

S. No	Fish species	Phytase dose/kg	Diet	Effect	References
15.	*Piaractus mesopotamicus*	0.2 g	Different meal (fishmeal, plant-based meal)	Fe, Zn, P, CP↑ (high in plant meal)	82
16.	*Scophthalmus maximus*	30% phytase treated	Soybean meal	DM, CP↑	136
17.	*Salmo salar*	250 U	Soy protein concentrate	Zn, Ca↑	61
18.	*Carassius auratus gibelio*	500 U	Plant meal	CP, P, DM↑	135
19.	*Pagrus major*	2000 FTU + 5 g NaH_2PO_4	soybean meal	CP, P, Mg↑	83
20.	*Lates calcarifer*	1000 FTU	Soybean meal	CP, P↑ crude fat↓	137
21.	*Myxocyprinus asiaticus*	500 FTU	Soybean meal + methionine	CP↑	88

Abbreviations: CF, crude fibre; CP, crude protein; DM, dry matter; GE, gross energy.

The addition of phytase in the fish feed declines the phytate level and gradually generates P, indicating the digestion of phytate.[84] In the *C. idella* diet, about 95% of the P becomes accessible on addition of phytase.[68] In common carps about 20%–40% of P was liberated on addition of exogenous phytase.[150] The digestibility of P increases in *C. gariepinus*,[57] *M. asiaticus*[88] and *L. calcarifer* by supplementing their feed with phytase[137]; however, the P digestibility further improved in *M. asiaticus* using phytase (1500 FTU/kg) in the methionine supplemented feed.[88] Even at the low water temperatures (11°C and 15°C), phytase efficiently produces P in *O. mykiss*[44] and increase its utilization,[11,152] which is eventually lowered the secretion of P in the faecal matter.[98] Studies revealed that the dietary intake of P and its excretion decreases with a rise in the phytase dose, but their retention level increased.

9 | EFFECTS OF PHYTASES AND OTHER ADDITIVES IN IMPROVING FISH FEED NUTRITION

The effect of phytase in improving fish nutrition is significant, but the combination with other substances enhances its efficacy in fish (Table 4).

9.1 | Organic acids

The addition of organic acids in the fish feed decreases the intestinal pH and improves the solubility of phytate-P thereby provides more P to fish. They act as a chelating agent, bind minerals along the intestine and enhances the absorption of these minerals. They also improve absorption by prompting proliferation of epithelial cell in the gastrointestinal mucosa,[29,155] and provide more time for the action of phytase.[161] The positive effect of the combination of citric acid (CA) and phytase on the nutrients bioavailability and growth performance in terms of WG, SGR, survival rate (SR) and digestibility of CP, crude fat and GE's and in the FCR value has been observed in *C. mrigala* fingerlings.[153–155] Interaction between phytase and CA influenced the composition of the whole body with improved levels of crude protein, ash, P, and Ca and low total lipid content.[52] The favourable influence of CA and phytase in boosting the growth of *O. mykiss* was studied by Singh et al.[168] Increased activities of digestive enzymes such as amylases, lipases and proteases were recorded in *L. rohita* after addition of CA and phytase in its feed.[39] The CA and phytase enhanced P consumption in *C. carpio*.[79] However, a few researchers observed no influence on the growth and digestibility of DM, protein and P in *P. fulvidraco*[86] and in *L. rohita*[161] when organic acid was supplemented along with phytase in their feed.

9.2 | Vitamin D

Vitamin D influences the growth, development, bone mineralization and maintain Ca & P homeostasis in fish.[171] Vitamin D_3 (cholecalciferol) and its compounds might be involved in improving phytase response in fish. It increases Ca absorption and restricts it from making phytate-Ca complex which indirectly stimulates the breakdown of phytate and enhances the utilization of hydrolyzed P.[28,29,82] As reported in *O. mykiss*, providing low level of vitamin D_3 with phytase in soy protein concentrate feed enhances WG.[28,29] Opposite to this, there is no effect of supplementing vitamin D_3 on *P. fulvidraco* along with phytase.[171]

9.3 | Enzymes cocktail

The digestibility of nutrients has been improved when phytase was added in combination with other hydrolytic enzyme like xylanases, cellulases, proteases and pectinases in feed of fish. The enzyme cocktail in the feed supplemented with phytase could increase WG, SGR, FI, PER and decrease FCR in *O. mossambicus*.[170] A significant improvement in the growth of *O. niloticus* was recorded upon supplementation of its feed with phytase and xylanase. Maas et al.[163] compared the combined effect of phytase and xylanase on different feeds (wheat bran, sunflower meal, citrus pulp). They inferred that the efficacy of enzymes is related to dietary components. The combined and

TABLE 4 A table showing the effects of phytase combination with other compound in fish health

S. No	Fish species	Dosage	Diet	Effect	References
1.	*Cirrhinus mrigala*	2.5% CA 750 FTU/kg	50% Canola meal	WG, SGR↑ FCR↓ CP, crude fat, GE↑	153
		5% CA 500 FTU/kg	30% Corn gluten	DM, CP, crude fat, GE, WG↑ FCR↓ Ca, P, Na, K, Mg, Fe, Cu, Mn, Zn↑	69,154
		2.5% CA 1000 FTU/kg	Guar based meal	WG↑ FCR↓ DM, CP, crude fat, GE↑ Ca, P, Na, K, Mg, Fe, Cu, Mn, Zn↑	155
2.	*Labeo rohita*	3% CA 1000 FTU/kg	Sunflower meal	Ca, P↑ WG, SGR↑ FCR↓ DM, crude fat, GE↑ P, Ca, Mn, Zn, Fe, Na, K↑	156,157
			Canola meal	Ca, P, Mg, K, Na↑	73
			Soybean diet	Crude fat, GE↑ P, Ca, K, Fe, Cu↑	112
		4% CA 400 FTU/kg	Cottonseed meal	Ca, P, Na, K, Mg, Fe, Cu, Mn, Zn↑	158
		2% CA 1000 FTU/kg	Sunflower meal	SGR↑ FCR↓ DM, crude fat, Crude ash↑ Ca, Cu, Fe, P, Na, K↑	159,160
				No significant effect	161
			Soybean meal	SGR↑ FCR↓	162
3.	*Oreochromis niloticus*	3% CA 1000 FTU/kg	Soybean diet	WG, SGR, FI, PER↑ FCR↓	52
		Phytase (1000 FTU/kg) Xylanase (4000 U/kg)	Different diet	FCR↓ Ash, Energy, P, Ca↑	163
		Phytase (660 FTU/kg) Xylanase (6000 FTU/kg)	Different diet	No significant effect	164
		Phytase (1000 FTU/kg) Xylanase (6000 FTU/kg) *Bacillus amyloliquefaciens* (60 mg/kg)	Mixed diet	No significant effect	51
		Phytase (1000 FTU/kg) Xylanase (4000 XU/kg) β-glucanase (500 BGU/kg)	Mixed diet	No significant effect	165
		Phytase (300 mg/kg) Protease (200 mg/kg) Carbohydrase (300 mg/kg)	–	No combinatory effect	166
		3% papain 0.1% phytase	Commercial feed	Protein retention rate↑	92
		Natuzyme (1.5 g/kg)	soybean meal	P and N retention rate↑	167
4.	*Oncorhynchus mykiss*	1.5% CA 1200 FYT	Fishmeal	WG, SGR, PER↑ FCR↓	168
		10% sodium diformate 4000 FTU/kg	Plant-based diet	WG↑	90
		Phytase and protease (1–2 g/kg)	Soybean	No significant effect	169
		Phytase, cellulase and pectinase (0.33 g/kg)	Canola meal	Not effective	91
5.	*Cyprinus carpio*	0.22% CA 750 FYT/kg	Soybean meal	P↑	79
		Phytase (0.3%) Pepsin (0.6%)	Different meal	No combinatory effect	78

(Continues)

TABLE 4 (Continued)

S. No	Fish species	Dosage	Diet	Effect	References
6.	*Pelteobagrus fulvidraco*	4 g/kg 1000 IU/kg	Mixed diet	No significant effect	86
7.	*Oreochromis mossambicus*	Natuzyme50 (0.50 g/kg)	Kikuyu-based diet	WG, FI, SGR, PER↑ FCR↓ CP↑ protease, cellulase, amylase, lipase↑	170

Abbreviations: CP, crude protein; DM, dry matter; FCR, feed conversion ratio; FI, feed intake; GE, gross energy; PER, protein efficiency ratio; SGR, specific growth rate; WG, weight gain.

separate effects of the enzymes (phytase, xylanase) and probiotics were examined on the growth, body composition and nutrients absorption. This cocktail of enzymes and probiotics showed greater performance of *O. niloticus*. Moreover, the recent studies carried out by Maas et al.[51,165] revealed that growth and nutrients assimilation was remained unchanged with the supplementation of phytase, xylanase and β-glucanase in the feed of *O. niloticus*. The effect of phytase was found to be independent of the feed quality (either high quality or low quality) in regulating growth and nutrition.[164] Although, the combination of phytase, protease and carbohydrase did not alter the growth performance of *O. niloticus*,[166] the rate of protein retention increased significantly due to the incorporation of phytase at the rate of 0.1% and papain 3% in the feed of *O. niloticus*,[92] and there was no change in the energy retention. The retention rate of P was maximum in *O. niloticus* after adding Natuzyme in soybean meal, indicating more accessibility of P, and less discharge in water bodies.[167] On the other hand, an insignificant response in the growth, body composition, and nutrients digestibility in *O. mykiss* was noticed on adding phytase and proteases[169] as well as phytase, cellulase and pectinase in its feed.[91]

10 | IMMOBILIZED PHYTASES IN FISH FEED

As described earlier, during feed processing, enzymes often face extreme conditions such as high temperature, causing their denaturation. To overcome the problem, immobilization process is adopted to maintain enzyme activity and its stability[99] that not only preserves enzyme from inactivation but also provides a support to survive in the acidic pH and resists the enzyme from proteolytic degradation.[172] Immobilization can be performed by various approaches such as adsorption, entrapment, cross-linking, covalent bonding and encapsulation.[99] Vandenberg et al.[173] investigated the effect of free and encapsulated phytase on the excretion of P. The activity of encapsulated phytase was low for liberating P due to limited interaction between entrapped phytase and phytate. The effects of immobilized phytase and free phytase were compared in vitro according to gastrointestinal condition of *O. niloticus*. Both free and immobilized phytase were incubated for 1 h at pH 2 and 3. Free phytase retains its activity on both pH values but immobilized phytase lost its 40% activity in these acidic conditions.[26] Likewise, Coutinho et al.[172] analysed the impact of free and immobilized phytase by incubating them at pH 2, 2.5 and 3 for 1 h followed by 1 h incubation at pH 7. They concluded that both phytases lost its activity up to some extent but remains effective in the intestine (pH 7). Also, the immobilized phytase was found to be protease resistant when it was treated with pepsin and trypsin.

11 | CURRENT STATUS, APPLICATION AND NOVEL TECHNOLOGIES IN USING PHYTASE

Phytase is an important enzyme used in the feed market worldwide.[174] Its demand has increased up to a market contribution of US $592.5 million in 2021,[175] which is projected to provide a net worth of more than USD 1.0 billion by 2025 with 6.3% compound annual growth rate (CAGR).[176,177] Though phytase has been majorly utilized in the animal feed industry, mainly in the poultry and piggery feed for decades, its necessity is increasing in the aquaculture industry as it helps in mitigating P pollution and conserving the limited P reserves, which makes it vital for the environmental protection agency. Besides, the food, beverages and pharmaceutical industries also stimulate phytase growth in the market. The global phytase market is the leading market and is classified on the basis of type, application and region. Based on type, phytases are available in the form of granulated, liquid, powder and thermostable. Among them, powder and granular forms share more than 60% of the feed market. In upcoming years, the requirement for liquid phytase may further increase as it avoids the heat treatment process of pelleting.[178] A new water-soluble form of phytase has been created by Huvepharma.[177] Another criterion of application categorized phytase into swine, poultry, aquaculture and pharmaceuticals in which the poultry section has the largest contribution. North America is the largest producer of phytase and the Asia Pacific comes under the fastest-growing region for the phytase market. Various companies are manufacturing phytases. Among them, Cargill, Incorporated, BASF SE, DuPont, Bluestar Adisseo Co., Ltd, DSM, BASF, AB Vista and Danisco Animal Nutrition (DuPont) are the leading firms involved in the production of phytase at the commercial scale. Recently, Danisco introduced a novel phytase "Axtra PHY GOLD" in Malaysia, Thailand and Australia on 15 July 2021.[175]

Although phytase is produced at commercial scale by various companies all over the world, there is still requirement for an ideal phytase suitable for the application in a specific field like aquaculture. Therefore, there is an utmost demand to improve the

performances of phytases using newer technologies to develop a cost-effective ideal phytase such as immobilization, gene cloning, site-directed mutagenesis, random mutagenesis, protein engineering and structural modifications. These technologies can be utilized to enhance phytase properties such as thermal stability, catalytic efficiency, pH range and resistivity against protease. Many studies are underway to work on these aspects but their applicability in aquaculture feed is the target for future research.

12 | CONCLUSIONS AND FUTURE PROSPECTS

Finding fishmeal alternatives for fish nutrition is the utmost need for sustainable production in aquaculture. Plant-based feed contains phytate, an anti-nutritional factor; hence need supplementation of phytate-degrading enzymes during or after pelleting of the feed as a green solution for mitigating environmental problem caused by the excess deposition of P in aquatic environments. Phytase supplementation has improved the digestibility of nutrients and minerals, resulting in fewer nutrients being excreted in the faeces reaching water bodies, eventually minimizing the pollution. Phytase further reduces the use of additional inorganic P in the feed, thereby decreasing the stress on phosphate reserves and reducing the overall cost of fish feed by avoiding the use of dietary supplements. However, the concentration of phytase in the fish feed have been a matter of concern in the fish growth. In addition, the interaction of phytase with other compounds also affects the health of fish species.

The mechanism of phytate hydrolysis through phytase in the gastrointestinal tract of fish needs to be evaluated, the impact of phytase on cold water species is still unclear, limited studies have been conducted with neutral phytase, and thus it is remaining an area to be explored. Mechanisms behind the involvement of phytase in improving amino acids or protein digestibility, the cause and effect relation between minerals and protein digestibility, the interaction of phytase with dietary components, effect on physiological, endocrine, immunology and hormone levels need to be investigated. The optimum dosage requirement for fish feed needs to be standardized. Transgenic fish can be created so that it can produce its own digestive phytase. In future, it will be vital to develop an economical and efficient microbial phytase meeting all the properties of an ideal phytase, including thermostability, resistance to proteases and broad pH. The use of modern molecular biology/genetic engineering and bioinformatics tools and techniques combined with fundamental approaches will aid in the identification and development of a single novel and ideal phytase suitable as feed additive to improve nutrition and growth performance of fish. Using such microbial phytase will limit phosphate reserves, improve fish health and nutrition and reduce environmental problems. In addition, due to diverse pH conditions of digestive tract of different species of fish, a single phytase cannot be an ideal additive for fish diets, therefore emphasis should be given to investigate potent phytases.

AUTHOR CONTRIBUTIONS

* **Priya:** Writing – original draft; writing – review and editing. **Ishita Virmani:** Writing – review and editing; formal analysis; investigation. * **Pragya:** Writing – review and editing; investigation. **Ravi Kumar Goswami:** Writing – review and editing. **Bijender Singh:** Writing – review and editing; conceptualization; supervision. **Jai Gopal Sharma:** Conceptualization; supervision; writing – review and editing. **Bhoopander Giri:** Conceptualization; supervision; writing – review and editing; investigation.

ACKNOWLEDGEMENTS

The author (Ms. Priya) gratefully acknowledges the financial assistance as Junior/Senior research fellowship from the University Grants Commission (UGC), New Delhi, India, during the course of writing this review. Miss Virmani thanks the Research Infrastructure RECETOX RI (No. LM2018121) financed by the Ministry of Education, Youth and Sports, and the Operational Programme Research Development and Innovation-project CETOCOEN Excellence (No. CZ.02.1.01/0.0/0.0/26617_043/0009632) for supportive background. This work was supported by the European Union's Horizon 2020 research and innovation programme under grant agreement 857560. This publication reflects only the author's view and the European Commission is not responsible for any use that may be made of the information it contains.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ETHICS STATEMENT

No animal was used for the development of this article.

CONSENT TO PARTICIPATE

Not Applicable.

HUMAN AND ANIMAL ETHICS

Not Applicable.

CONSENT FOR PUBLICATION

All authors consented to the publication.

ORCID

Bhoopander Giri https://orcid.org/0000-0002-5510-4021

REFERENCES

1. United Nations. World Population Prospects 2019. Accessed February 15, 2020. https://population.un.org/wpp/.
2. FAO STAT. *Food and Agriculture Data 2016*. Food and Agriculture Organization of the United Nations (FAO); 2016.
3. FAO. *The State of World Fisheries and Aquaculture*. FAO; 2000.

4. FAO. *The State of Food Security and Nutrition in the World 2018. Building Climate Resilience for Food Security and Nutrition*. Food and Agriculture Organization; 2018 Accessed September 29, 2021. http://www.fao.org/3/i9553en/i9553en.pdf
5. Goswami RK, Sharma JG, Shrivastav AK, et al. Effect of Lemna minor supplemented diets on growth, digestive physiology and expression of fatty acids biosynthesis genes of *Cyprinus carpio*. *Sci Rep*. 2022; 12:3711. doi:10.1038/s41598-022-07743-x
6. National Research Council (NRC). *Nutrient Requirements of Fish and Shrimp*. The National Academic Press; 2011.
7. Lech GP, Reigh RC. Plant products affect growth and digestive efficiency of cultured Florida pompano (*Trachinotus carolinus*) fed compounded diets. *PLoS One*. 2012;7(4):e34981. doi:10.1371/journal.pone.0034981
8. Shahzad MM, Rafique T, Hussain SM, et al. Effect of phytase supplemented Moringa by-products based diets on the performance of *Oreochromis niloticus* fingerlings. *J Anim Plant Sci*. 2021;31(1):288-295. doi:10.36899/JAPS.2021.1.0216
9. Gatlin DM III, Barrows FT, Brown P, et al. Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquacult Res*. 2007;38(6):551-579. doi:10.1111/j.1365-2109.2007.01704.x
10. Hardy RW. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquacult Res*. 2010;41(5):770-776. doi:10.1111/j.1365-2109.2009.02349.x
11. Cantas IB, Yildirim O. Supplementation of microbial phytase with safflower meal in rainbow trout (*Oncorhynchus mykiss*): the effects on growth, digestibility, environmental, and serum biological parameters. *J Chem*. 2020;8:1-8. doi:10.1155/2020/4634796
12. Song HY, El Sheikha AF, Hu DM. The positive impacts of microbial phytase on its nutritional applications. *Trends Food Sci Technol*. 2019;86:553-562. doi:10.1016/j.tifs.2018.12.001
13. Gupta RK, Gangoliya SS, Singh NK. Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *J Food Sci Technol*. 2015;52(2):676-684. doi:10.1007/s13197-013-0978-y
14. Menezes-Blackburn D, Greiner R. In: Cirillo G, Gianfranco Spizzirri U, Lemma F, eds. *Functional Polymers in Food Science*. Scrivener Publishing LLC; 2015:47-73.
15. Shanmugam G. Characteristics of phytase enzyme and its role in animal nutrition. *Int J Curr Microbiol App Sci*. 2018;7(3):1006-1013. doi:10.20546/ijcmas.2018.703.120
16. Vohra A, Kaur P, Satyanarayana T. Production, characteristics and applications of the cell-bound phytase of *Pichia anomala*. *Antonie Leeuwenhoek*. 2011;99(1):51-55. doi:10.1007/s10482-010-9498-1
17. Balaban NP, Suleimanova AD, Valeeva LR, et al. Microbial phytases and phytate: exploring opportunities for sustainable phosphorus management in agriculture. *Am J Mol Biol*. 2017;7:11-29. doi:10.4236/ajmb.2017.71002
18. Singh B, Boukhris I. Pragya et al. contribution of microbial phytases in improving plants growth and nutrition: a review. *Pedosphere*. 2020;30(3):295-313. doi:10.1016/S1002-0160(20)60010-8
19. Singh B, Satyanarayana T. Fungal phytases: characteristics and amelioration of nutritional quality and growth of non-ruminants. *J Anim Physiol Anim Nutr*. 2015;99(4):646-660. doi:10.1111/jpn.12236
20. Morales GA, Marquez L, Hernández AJ, Moyano FJ. In: Walk CL, Kuhn I, Stein HH, Kidd MT, Rodehutscord M, eds. *Phytate Destruction-Consequences for Precision Animal Nutrition*. Wageningen Academic Publishers; 2016:129-166. doi:10.3920/978-90-8686-836-0_9
21. Abd El-Hack ME, Alagawany M, Arif M, et al. The uses of microbial phytase as a feed additive in poultry nutrition—a review. *Ann Anim Sci*. 2018;18(3):639-658. doi:10.2478/aoas-2018-0009
22. Jatuwong K, Suwannarach N, Kumla J, Penkhrue W, Kakumyan P, Lumyong S. Bioprocess for production, characteristics, and biotechnological applications of fungal phytases. *Front Microbiol*. 2020;11:188. doi:10.3389/fmicb.2020.00188
23. Hussain SM, Hanif S, Sharif A, Bashir F, Iqbal HMN. Unrevealing the sources and catalytic functions of phytase with multipurpose characteristics. *Catal Lett*. 2021;152:1358-1371. doi:10.1007/s10562-021-03752-z
24. Kaur P, Vohra A, Satyanarayana T. Multifarious applications of fungal phytases. *Encycl Mycol*. 2021;2:358-369. doi:10.1016/B978-0-12-819990-9.00028-7
25. Rocky-Salimi K, Hashemi M, Safari M, Mousivand M. Valorisation of untreated cane molasses for enhanced phytase production by *Bacillus subtilis* K46b and its potential role in dephytinisation. *J Sci Food Agric*. 2017;97(1):222-229. doi:10.1002/jsfa.7716
26. Lopes MM, Coutinho TC, Malafatti JOD, Paris EC, de Sousa CP, Farinas CS. Immobilization of phytase on zeolite modified with iron (II) for use in the animal feed and food industry sectors. *Process Biochem*. 2021;100:260-271. doi:10.1016/j.procbio.2020.10.017
27. Pontes TC, Brito JMD, Wernick B, et al. Top-sprayed phytase enhances the digestibility of energy, protein, amino acids and minerals, and reduces phosphorus output in Nile tilapia fed all-vegetable diets. *Aquacult Res*. 2021;00:1-9. doi:10.1111/are.15527
28. Cao L, Wang W, Yang C, et al. Application of microbial phytase in fish feed. *Enzyme Microb Technol*. 2007;40(4):497-507. doi:10.1016/j.enzmictec.2007.01.007
29. Kumar V, Sinha AK, Makkar HPS, De Boeck G, Becker K. Phytate and phytase in fish nutrition. *J Anim Physiol Anim Nutr*. 2012;96(3):335-364. doi:10.1111/j.1439-0396.2011.01169.x
30. Lemos D, Tacon AGJ. Use of phytases in fish and shrimp feeds: a review. *Rev Aquac*. 2015;9:1-17. doi:10.1111/raq.12138
31. Moroni RF, Cazzaniga E, Palestini P, Sculati M. The feasibility of a diet which enhances inositol availability. *Prog Nutr*. 2021;23(2):e2021078. doi:10.23751/pn.v23i2.10356
32. Shahzad MM, Hussain SM, Hussain M, et al. Improvement in overall performance of *Catla catla* fingerlings fed phytase included low cost plant by products-based diet. *Saudi J Biol Sci*. 2020;27(8):2089-2096. doi:10.1016/j.sjbs.2020.06.012
33. Shahzad MM, Hussain SM, Jabeen F, et al. Improvement in mineral digestibility and whole body composition of *Catla catla* fingerlings fed phytase supplemented MOSM based diet. *Pak J Zool*. 2018;50(5):1909-1920. doi:10.17582/journal.pjz/2018.50.5.1909.1920
34. Usmani N, Jafri AK. Influence of dietary phytic acid on growth, conversion efficiency and carcass composition of mrigala *Cirrhinus mrigala* (Hamilton) fry. *J World Aquacult Soc*. 2002;33(2):199-204. doi:10.1111/j.1749-7345.2002.tb00495.x
35. Laining A, Traifalgar RF, Thu M, Komilus CF, Kader MA. Influence of dietary phytic acid on growth, feed intake, and nutrient utilization in juvenile Japanese flounder, *Paralichthys olivaceus*. *J World Aquac Soc*. 2010;41(5):746-755. doi:10.1111/j.1749-7345.2010.00416.x
36. Av D, Bussel CG, Klatt SF, Schulz C. Dietary phytase supplementation in rapeseed protein based diets influences growth performance, digestibility and nutrient utilisation in turbot (*Psetta maxima* L.). *Aquaculture*. 2016;450:405-411. doi:10.1016/j.aquaculture.2015.07.026
37. Kokou F, Sarropoulou E, Cotou E, et al. Effects of fish meal replacement by a soybean protein on growth, histology, selected immune and oxidative status markers of gilthead sea bream, *Sparus aurata*. *J World Aquac Soc*. 2015;46(2):115-128. doi:10.1111/jwas.12181
38. Kumar V, Makkar HPS, Devappa RK, Becker K. Isolation of phytate from *Jatropha curcas* kernel meal and effects of isolated phytate on growth, digestive physiology and metabolic changes in Nile tilapia (*Oreochromis niloticus* L.). *Food Chem Toxicol*. 2011;49(9):2144-2156. doi:10.1016/j.fct.2011.05.029

39. Shah SZH, Afzal M, Hussain SM, et al. Supplementation of citric acid and phytase improves the digestive enzymes activities in *Labeo rohita* fingerlings. *Biologia*. 2015;6(1):63-68.
40. Papatryphon E, Howell RA, Soares JH. Growth and mineral absorption by striped bass *Morone saxatilis* fed a plant feedstuff based diet supplemented with phytase. *J World Aquacult Soc*. 1999;30(2):161-173. doi:10.1111/j.1749-7345.1999.tb00863.x
41. Hardy RW. Phytate. *Aquac Mag*. 1998;11/12:77-80.
42. Riche M, Trottier NL, Ku PK, Garling DL. Apparent digestibility of crude protein and apparent availability of individual amino acids in tilapia (*Oreochromis niloticus*) fed phytase pretreated soybean meal diets. *Fish Physiol Biochem*. 2001;25(3):181-194. doi:10.1023/A:1022222431004
43. Sajjadi M, Carter CG. Dietary phytase supplementation and the utilisation of phosphorus by Atlantic salmon (*Salmo salar* L.) fed a canola-meal-based diet. *Aquaculture*. 2004;240(1–4):417-431. doi:10.1016/j.aquaculture.2004.07.003
44. Lee SA, Lupatsch I, Gomes GA, Bedford MR. An advanced *Escherichia coli* phytase improves performance and retention of phosphorus and nitrogen in rainbow trout (*Oncorhynchus mykiss*) fed low phosphorus plant-based diets, at 11°C and 15°C. *Aquaculture*. 2020;516:734549. doi:10.1016/j.aquaculture.2019.734549
45. Khan A, Ghosh K. Phytic acid-induced inhibition of digestive protease and α-amylase in three Indian major carps: an in vitro study. *J World Aquac Soc*. 2013;44(6):853-859. doi:10.1111/jwas.12084
46. Bhavsar K, Khire JM. Current research and future perspectives of phytase bioprocessing. *RSC Adv*. 2014;4(51):26677-26691. doi:10.1039/c4ra03445g
47. Outchkourov N, Petkov S. In: Vogel A, May O, eds. *Industrial Enzyme Applications*. Wiley-VCH Verlag GmbH & Co. KGaA; 2019:255-285. doi:10.1002/9783527813780.ch3_3
48. Kryukov VS, Glebova IV, Zinoviev SV. Reevaluation of phytase action mechanism in animal nutrition. *Biochemistry*. 2021;86(1):S152-S165. doi:10.1134/S000629792114011X
49. Megha PM, Nishaa S. In: Tripathi RB, Vijaya P, Priya Senan V, Sona A, eds. *Recent Advancements and Research in Biological Sciences*. Thanuj International Publishers; 2021:210-216.
50. Thakur N, Patel SK, Kumar P, et al. Bioprocess for hyperactive thermotolerant *aspergillus fumigatus* phytase and its application in dephytinization of wheat flour. *Catal Lett*. 2022;152:1-13. doi:10.1007/s10562-021-03886-0
51. Maas RM, Verdegem MC, Debnath S, Marchal L, Schrama JW. Effect of enzymes (phytase and xylanase), probiotics (*B. amyloliquefaciens*) and their combination on growth performance and nutrient utilisation in Nile tilapia. *Aquaculture*. 2021;533:736226. doi:10.1016/j.aquaculture.2020.736226
52. Mahmoud N, Eid AE, Ali BA, Wahdan A, Enany ME, Abd El-Nab AS. Effect of phytase and citric acid on growth performance, feed utilization and its antibacterial activity against fish pathogens of Nile tilapia fingerlings. *Egypt J Aquat Res*. 2019;9(4):35-53. doi:10.21608/EJA.2019.47194
53. Norag MAA, El-Shenawy AM, Fadl SE, et al. Effect of phytase enzyme on growth performance, serum biochemical alteration, immune response and gene expression in Nile tilapia. *Fish Shellfish Immunol*. 2018;80:97-108. doi:10.1016/j.fsi.2018.05.051
54. Baruah K, Pal AK, Sahu NP, Debnath D, Nourozitallab P, Sorgeloos P. Microbial phytase supplementation in rohu, *Labeo rohita*, diets enhances growth performance and nutrient digestibility. *J World Aquac Soc*. 2007;38(1):129-137. doi:10.1111/j.1749-7345.2006.00081.x
55. Rodrigues ML. Souza dos Santos Sanchez M, Ernzen Pessini J, et al. Replacement of corn by sorghum and phytase supplementation in silver catfish (*Rhamdia quelen*) diets: growth performance, physiological variables and bone mineralization. *J Appl Anim Res*. 2020;48(1):142-150. doi:10.1080/09712119.2020.1750411
56. Bentes VV, Bittencourt F, Neu DH, Signor A, Eyng C. Replacement of corn by rice grits, with or without phytase supplementation, in diets for silver catfish (*Rhamdia quelen*) juveniles. *Aquacult Nutr*. 2020;26(5):1584-1591. doi:10.1111/anu.13104
57. Uzezi AB, Kolawole AE, Oluwarotimi OB. Effect of microbial phytase on nutrient availability and growth of juvenile *Clarias gariepinus* fed soyabean and groundnut-based diets. *Annu Res Rev Biol*. 2019;31(4):1-21. doi:10.9734/ARRB/2019/v31i430057
58. Uzezi AB, Kolawole AE, Oluwarotimi OB. Dietary phytase improves growth and water quality parameters for juvenile *Clarias gariepinus* fed soyabean diet-based diets. *Int J Aquacult*. 2015;5(41):1-20. doi:10.5376/ija.2015.05.0041
59. Olugbenga O, Falaye AE, Kareem OK. Effect of phytase supplementation on the growth, mineral composition and phosphorus digestibility of African catfish (*Clarias gariepinus*) juveniles. *Anim Res Int*. 2017;14(2):2741-2750.
60. Vandenberg GW, Scott SL, de la Noüe J. Factors affecting nutrient digestibility in rainbow trout (*Oncorhynchus mykiss*) fed a plant protein–based diet supplemented with microbial phytase. *Aquacult Nutr*. 2012;18(4):369-379. doi:10.1111/j.1365-2095.2011.00901.x
61. Carter CG, Sajjadi M. Low fishmeal diets for Atlantic salmon, *Salmo salar* L., using soy protein concentrate treated with graded levels of phytase. *Aquac Int*. 2011;19(3):431-444. doi:10.1007/s10499-010-9358-z
62. Signor A, Lewandowski V, Silva RAD, Fries EM, Schuller JM. Effect of phytase on digestibility of corn, sorghum and wheat bran by silver catfish (*Rhamdia voulezi*). *Acta Sci Anim Sci*. 2016;38(4):355-359. doi:10.4025/actascianimsci.v38i4.32054
63. Fries EM, Oxford JH, Godoy AC, et al. Phytase on the digestibility of plant protein feed for silver catfish, *Rhamdia voulezi*. *Aquaculture*. 2020;528:735528. doi:10.1016/j.aquaculture.2020.735528
64. Rachmawati D, Istiyanto S, Maizirwan M. Effect of phytase on growth performance, diet utilization efficiency and nutrient digestibility in fingerlings of *Chanos chanos* (Forsskal 1775). *Philipp J Sci*. 2017;146(3):237-245.
65. Rachmawati D, Samidjan I. Effect of phytase enzyme on growth, nutrient digestibility and survival rate of catfish (*Pangasius hypothalamus*) fingerlings. *Pertanika J Trop Agric Sci*. 2018;41(2):865-878.
66. Rachmawati D, Samidjan I, Elfitasari T. Effect of the phytase enzyme addition in the artificial feed on digestibility of feed, feed conversion ratio and growth of Gift Tilapia Saline Fish (*Oreochromis niloticus*) Nursery Stadia I. *IOP Conference Series: Earth and Environmental Science*. 2018;116(1):012009. doi:10.1088/1755-1315/116/1/012009
67. Chen A, Liu X, Cui C, et al. An evaluation of phytase for channel catfish (*Ictalurus punctatus*) fed all plant-protein diet: growth performance, nutrient utilization and P equivalency value. *Aquacult Nutr*. 2018;25(1):215-224. doi:10.1111/anu.12845
68. Ries EF, Ferreira CC, Goulart FR, et al. Improving nutrient availability of defatted rice bran using different phytase sources applied to grass carp (*Ctenopharyngodon idella*) diet. *An Acad Bras Cienc*. 2020;92(3):e20190201. doi:10.1590/0001-3765202020190201
69. Hussain SM, Ahmad N, Javid A, Shahzad MM, Hussain M, Arsalan MZUH. Effects of phytase and citric acid supplemented corn gluten (30%) meal-based diets on the mineral digestibility of *Cirrhinus mrigala* fingerlings. *Turkish J Fish Aquat Sci*. 2018;18(4):501-507. doi:10.4194/1303-2712-v18_4_01
70. Shahzad MM, Hussain SM, Jabeen F, et al. Carcass composition and hematological study of *Catla catla* fingerlings fed on phytase supplemented *Moringa oleifera* leaf meal (MOLM) based diet. *J Biodivers Environ Sci*. 2016;9(1):57-68.
71. Hussain SM, Shahzad MM, Aslam N, et al. Use of phytase at graded levels for improving nutrient digestibility, growth and hematology of *Catla catla* fingerlings fed *Moringa oleifera* seed meal (MOSM) based diet. *Indian J Fish*. 2017;64(2):48-57. doi:10.21077/ijf.2017.64.2.62418-08

72. Hussain SM, Afzal M, Javid A, et al. Role of phytase supplementation in improving nutrient digestibility in *Labeo rohita* (Hamilton, 1822) fingerlings fed on cottonseed meal based diet. *Indian J Fish*. 2015; 62(1):78-84.
73. Habib RZ, Afzal M, Shah SZH, Fatima M, Bilal M, Hussain SM. Potential of phytase and citric acid treated canola meal based diet to enhance the minerals digestibility in *Labeo rohita* fingerlings. *Pak J Zool*. 2018;50(6):2045-2050. doi:10.17582/journal.pjz/2018.50.6.2045.2050
74. Roy T, Dan SK, Banerjee G, Nandi A, Ghosh P, Ray AK. Comparative efficacy of phytase from fish gut bacteria and a commercially available phytase in improving the nutritive value of sesame oilseed meal in formulated diets for fingerlings of rohu, *Labeo rohita* (Actinopterygii: Cypriniformes: Cyprinidae). *Acta Ichthyol Piscat*. 2016;46(1):9-23. doi:10.3750/AIP2016.46.1.02
75. Das P, Ghosh K. Improvement of nutritive value of sesame oil cake in formulated diets for rohu, *Labeo rohita* (Hamilton) after bioprocessing through solid state fermentation by a phytase-producing fish gut bacterium. *Int J Aquat Biol*. 2015;3(2):89-101.
76. Dawood MA, Koshio S, Ishikawa M, Yokoyama S. Effects of partial substitution of fish meal by soybean meal with or without heat-killed *lactobacillus plantarum* (LP20) on growth performance, digestibility, and immune response of amberjack, *Seriola dumerili* juveniles. *Biomed Res Int*. 2015;2015:514196. doi:10.1155/2015/514196
77. Roy T, Banerjee G, Dan SK, Ghosh P, Ray AK. Improvement of nutritive value of sesame oilseed meal in formulated diets for rohu, *Labeo rohita* (Hamilton), fingerlings after fermentation with two phytase-producing bacterial strains isolated from fish gut. *Aquac Int*. 2014; 22(2):633-652. doi:10.1007/s10499-013-9691-0
78. Watanabe T, Tanemura N, Sugiura S. Effects of in vitro enzymatic digestion of rapeseed meal, soybean meal, macrophyte meal, and algal meal on *in vivo* digestibilities of protein and minerals evaluated using common carp *Cyprinus carpio*. *Aquac Sci*. 2016;64(2):215-222.
79. Phromkunthong W, Nuntapong N, Gabaudan J. Interaction of phytase RONOZYME®P(L) and citric acid on the utilization of phosphorus by common carp (*Cyprinus carpio*). *Songklanakarin J Sci Technol*. 2010;32(6):547-554.
80. Xue Y. *The Effect of Dietary Phytase Supplementation and Incubation in Soy Protein Concentrate Based Diet Fed to Nile Tilapia [Dissertation]*. Norwegian University of Life Sciences; 2014.
81. Bacchetta C, Rossi AS, Cian RE, Cazenave J, Drago SR. Impact of a plant-based diet supplemented with phytase on growth, tissue energy and bone mineral status of juvenile pacú (*Piaractus mesopotamicus*). *J Appl Aquac*. 2020;33(4):300-314. doi:10.1080/10454438.2020.1742267
82. Cian RE, Bacchetta C, Cazenave J, Drago SR. In vitro assays predicts mineral retention and apparent protein digestibility of different fish feed measured using a juvenile *P. mesopotamicus* model. *Aquacult Res*. 2018;49(6):2267-2277. doi:10.1111/are.13687
83. Laining A, Ishikawa M, Koshio S, Yokoyama S. Dietary inorganic phosphorus or microbial phytase supplementation improves growth, nutrient utilization and phosphorus mineralization of juvenile red sea bream, *Pagrus major*, fed soybean-based diets. *Aquacult Nutr*. 2011; 18(5):502-511. doi:10.1111/j.1365-2095.2011.00914.x
84. Greiling AM, Tschesche C, Baardsen G, Kröckel S, Koppe W, Rodehutscord M. Effects of phosphate and phytase supplementation on phytate degradation in rainbow trout (*Oncorhynchus mykiss* W.) and Atlantic salmon (*Salmo salar* L.). *Aquaculture*. 2019;503:467-474. doi:10.1016/j.aquaculture.2019.01.035
85. Verlhac-Trichet V, Vielma J, Dias J, et al. The efficacy of a novel microbial 6-phytase expressed in *Aspergillus oryzae* on the performance and phosphorus utilization of cold-and warm-water fish: rainbow trout, *Oncorhynchus mykiss*, and Nile tilapia, *Oreochromis niloticus*. *J World Aquac Soc*. 2014;45(4):367-379. doi:10.1111/jwas.12133
86. Zhu Y, Qiu X, Ding Q, Duan M, Wang C. Combined effects of dietary phytase and organic acid on growth and phosphorus utilization of juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquaculture*. 2014; 430:1-8. doi:10.1016/j.aquaculture.2014.03.023
87. Ali S, Kaviraj A. Aquatic weed *Ipomoea aquatica* as feed ingredient for rearing Rohu, *Labeo rohita* (Hamilton). *Egypt J Aquat Res*. 2018; 44(4):321-325. doi:10.1016/j.ejar.2018.09.004
88. Chu ZJ, Yu DH, Dong GF, Gong SY. Partial replacement of fish meal by soybean meal with or without methionine and phytase supplement in diets for juvenile Chinese sucker, *Myxocyprinus asiaticus*. *Aquac Nutr*. 2016;22(5):989-996. doi:10.1111/anu.12318
89. Cheng N, Chen P, Lei W, Feng M, Wang C. The sparing effect of phytase in plant-protein-based diets with decreasing supplementation of dietary NaH2PO4 for juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquacult Res*. 2016;47(12):3952-3963. doi:10.1111/are.12845
90. Morales GA, Denstadli V, Collins SA, Mydland LT, Moyano FJ, Øverland M. Phytase and sodium diformate supplementation in a plant-based diet improves protein and mineral utilization in rainbow trout (*Oncorhynchus mykiss*). *Aquacult Nutr*. 2015;22(6):1301-1311. doi:10.1111/anu.12340
91. Yigit NO, Keser E. Effect of cellulase, phytase and pectinase supplementation on growth performance and nutrient digestibility of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) fry fed diets containing canola meal. *J Appl Ichthyol*. 2016;32(5):938-942. doi:10.1111/jai.13088
92. Saifulloh A, Santanumurti MB, Lamid M, Lokapirnasari WP. Combination of papain enzyme and phytase enzyme in commercial feed and the protein and energy retention of tilapia *Oreochromis niloticus*. *IOP Conference Series: Earth and Environmental Science*. 2019;236(1): 012069. doi:10.1088/1755-1315/236/1/012069
93. Rychen G, Aquilina G, Azimonti G, et al. Safety and efficacy of OPTIPHOS®(6-phytase) as a feed additive for finfish. *EFSA J*. 2017;15(4): e04763. doi:10.2903/j.efsa.2017.4763
94. Vashishth A, Ram S, Beniwal V. Cereal phytases and their importance in improvement of micronutrients bioavailability. *3 Biotech*. 2017;7(1):42. doi:10.1007/s13205-017-0698-5
95. Cangussu ASR, Aires Almeida D, Aguiar RWDS, et al. Characterization of the catalytic structure of plant phytase, protein tyrosine phosphatase-like phytase, and histidine acid phytases and their biotechnological applications. *Enzyme Res*. 2018;2018:1-12. doi:10.1155/2018/8240698
96. Sharma V. Microbial phytases: restoration of phosphorus balance in ecosystem. *EC Microbio*. 2017;6(4):113-126.
97. Gessler NN, Serdyuk EG, Isakova EP, Deryabina YI. Phytases and the prospects for their application. *Appl Biochem Microbiol*. 2018;54(4): 352-360. doi:10.1134/S0003683818040087
98. Handa V, Sharma D, Kaur A, Arya SK. Biotechnological applications of microbial phytase and phytic acid in food and feed industries. *Biocatal Agric Biotechnol*. 2020;25:101600. doi:10.1016/j.bcab.2020.101600
99. Pragya, Sharma KK, Kumar A, Singh D, Kumar V, Singh B. Immobilized phytases: an overview of different strategies, support material, and their applications in improving food and feed nutrition. *Crit Rev Food Sci Nutr*. 2021;1-23. doi:10.1080/10408398.2021.2020719
100. Krishna C, Nokes SE. Predicting vegetative inoculum performance to maximize phytase production in solid-state fermentation using response surface methodology. *J Ind Microbiol Biotechnol*. 2001; 26(3):161-170. doi:10.1038/sj.jim.7000103
101. Kammoun R, Farhat A, Chouayekh H, Bouchaala K, Bejar S. Phytase production by *Bacillus subtilis* US417 in submerged and solid state fermentations. *Ann Microbiol*. 2012;62(1):155-164. doi:10.1007/s13213-011-0240-7
102. Yan W, Reigh RC, Xu Z. Effects of fungal phytase on utilization of dietary protein and minerals, and dephosphorylation of phytic acid in the alimentary tract of channel catfish *Ictalurus punctatus* fed an

all-plant-protein diet. *J World Aquac Soc*. 2002;33(1):10-22. doi:10.1111/j.1749-7345.2002.tb00473.x
103. Baruah K, Pal AK, Sahu NP, Jain KK, Mukherjee SC, Debnath D. Dietary protein level, microbial phytase, citric acid and their interactions on bone mineralization of *Labeo rohita* (Hamilton) juveniles. *Aquacult Res*. 2005;36:803-812. doi:10.1111/j.1365-2109.2005.01290.x
104. Vohra A, Satyanarayana T. Purification and characterization of a thermostable and acid-stable phytase from *Pichia anomala*. *World J Microbiol Biotechnol*. 2002;18:687-691.
105. Forster I, Higgs DA, Dosanjh BS, Rowshandeli M, Parr J. Potential for dietary phytase to improve the nutritive value of canola protein concentrate and decrease phosphorus output in rainbow trout (*Oncorhynchus mykiss*) held in 11°C fresh water. *Aquaculture*. 1999;179(1–4):109-125. doi:10.1016/S0044-8486(99)00156-8
106. Dauda AB, Ajadi A, Tola-Fabunmi AS, Akinwole AO. Waste production in aquaculture: sources, components and managements in different culture systems. *Aquac Fish*. 2019;4(3):81-88. doi:10.1016/j.aaf.2018.10.002
107. Sugiura SH, Gabaudan J, Dong FM, Hardy RW. Dietary microbial phytase supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout [*Oncorhynchus mykiss* (Walbaum)] fed soybean meal-based diets. *Aquacult Res*. 2001;32(7):583-592. doi:10.1046/j.1365-2109.2001.00581.x
108. Yoo GY, Wang X, Choi S, Han K, Kang JC, Bai SC. Dietary microbial phytase increased the phosphorus digestibility in juvenile Korean rockfish *Sebastes schlegeli* fed diets containing soybean meal. *Aquaculture*. 2005;243(1–4):315-322. doi:10.1016/j.aquaculture.2004.10.025
109. Hassaan MS, Soltan MA, Agouz HM, Badr AM. Influences of calcium/phosphorus ratio on supplemental microbial phytase efficiency for Nile tilapia (*Oreochromis niloticus*). *Egypt J Aquat Res*. 2013;39(3):205-213. doi:10.1016/j.ejar.2013.09.001
110. Uzezi AB, Ajani EK, Omitoyin BO. Optimum dietary Ca/P ratio and phytase for growth and bone mineralization in juvenile *Clarias gariepinus* fed soya bean-based diet. *J Aquac Res Dev*. 2016;7(2):403. doi:10.4172/2155-9546.1000403
111. Denstadli V, Storebakken T, Svihus B, Skrede A. A comparison of online phytase pre-treatment of vegetable feed ingredients and phytase coating in diets for Atlantic salmon (*Salmo salar* L.) reared in cold water. *Aquaculture*. 2007;269:414-426. doi:10.1016/j.aquaculture.2007.02.033
112. Shah SZH, Afzal M, Fatima M, Hussain SM, Ahmed T. Nutrient digestibility in *Labeo rohita* fingerlings fed citric acid acidified phytase sprayed diet. *Pak J Zool*. 2021;53(2):459-465. doi:10.17582/journal.pjz/20150923011725
113. Dersjant-Li Y, Awati A, Schulze H, Partridge G. Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *J Sci Food Agric*. 2015;95(5):878-896. doi:10.1002/jsfa.6998
114. Morales GA, Márquez L, Saenz de Rodrigañez M, Bermúdez L, Robles R, Moyano FJ. Effect of phytase supplementation of a plant-based diet on phosphorus and nitrogen bioavailability in sea bream *Sparus aurata*. *Aquacult Nutr*. 2014;20(2):172-182. doi:10.1111/anu.12063
115. Balaban NP, Suleimanova AD, Shakirov EV, Sharipova MR. Histidine acid phytases of microbial origin. *Microbiology*. 2018;87(6):745-756. doi:10.1134/S0026261718060024
116. Shirmohammad F, Mehri M, Joezy-Shekalgorabi S. A review on the role of inositol in aquaculture. *Iran J Fish Sci*. 2016;15(4):1388-1409.
117. Mahmoud AH, Hussein SY, Abdelnabi MA, Essa NM. Effect of supplemental microbial phytase on growth performance of Nile tilapia (*Oreochromis niloticus*). *Minia J Agric Res Dev*. 2014;34(1):123-135.
118. Khaled AA. Effects of dietary protein and phytase on growth performance and body composition of Nile tilapia, *Oreochromis niloticus* L. *Middle East J Appl Sci*. 2019;9(2):474-482.
119. Kemigabo C, Abdel-Tawwab M, Lazaro JW, Sikawa D, Masembe C, Kang'Ombe J. Combined effect of dietary protein and phytase levels on growth performance, feed utilization, and nutrients digestibility of African catfish, *Clarias gariepinus* (B.), reared in earthen ponds. *J Appl Aquac*. 2018;30(3):211-226. doi:10.1080/10454438.2018.1439425
120. Hussain S, Afzal M, Rana SA, Javid A, Iqbal M. Effect of phytase supplementation on growth performance and nutrient digestibility of *Labeo rohita* fingerlings fed on corn gluten meal-based diets. *Int J Agric Biol*. 2011;13(6):916-922.
121. Hussain SM, Afzal M, Nasir S, et al. Effect of phytase supplementation on growth performance and mineral digestibility in *Labeo rohita* (Hamilton, 1822) fingerlings fed on sunflower meal based diet. *Indian J Fish*. 2015;62(4):71-79.
122. Hussain SM, Afzal M, Javid A, et al. Efficacy of phytase supplementation on growth performance and mineral digestibility of *Labeo rohita* fingerlings fed on cottonseed meal based diet. *Pak J Zool*. 2015;47(3):699-709.
123. Hussain SM, Afzal M, Nasir S, et al. Role of phytase supplementation in improving nutrient digestibility and growth performance for *Labeo rohita* fingerlings fed on canola meal-based diet. *J Appl Anim Res*. 2017;45(1):15-21. doi:10.1080/09712119.2015.1091331
124. Shahzad MM, Bashir S, Hussain SM, et al. Effectiveness of phytase pre-treatment on growth performance, nutrient digestibility and mineral status of common carp (*Cyprinus carpio*) juveniles fed Moringa by-product based diet. *Saudi J Biol Sci*. 2021;28(3):1944-1953. doi:10.1016/j.sjbs.2020.12.046
125. Fadhal AA, Mustafa SA. Influence of phytase enzyme on growth performance and survival rate challenged with saprolegnia spp. in common carp. *Iraq J Agric Sci*. 2020;51(5):1458-1465. doi:10.36103/ijas.v51i5.1156
126. Salman AH, Alkhafaji KA. Use of treated lentil *Lens culinaris* L. with phytase enzyme in diets of common carp *Cyprinus carpio* L. *Plant Arch*. 2020;20(2):941-944.
127. Rachmawati D, Samidjan I. Performance efficiency of feed utilization, relative growth rate, and survival rate of common carp (*Cyprinus carpio*) through the addition of phytase in the feed. *IOP Conference Series: Earth and Environmental Science*. 2018;137(1):012027. 10.1088/1755-1315/137/1/012027
128. Hussain SM, Shahzad MM, Jabeen F, et al. Growth performance and nutrient digestibility of *Cirrhinus mrigala* fingerlings fed on soybean meal-based diet supplemented by phytase. *Int J Biosci*. 2014;5(12):212-221. doi:10.12692/ijb/5.12.212-221
129. Hussain SM, Hameed T, Afzal M, et al. Growth performance and nutrient digestibility of *Cirrhinus mrigala* fingerlings fed phytase supplemented sunflower meal based diet. *Pak J Zool*. 2017;49(5):1713-1724. doi:10.17582/journal.pjz/2017.49.5.1713.1724
130. Shahzad MM, Hussain SM, Javid A, Hussain M. Role of phytase supplementation in improving growth parameters and mineral digestibility of *Catla catla* fingerlings fed *Moringa* by-products based test diet. *Turkish J Fish Aquat Sci*. 2018;18(4):557-566. doi:10.4194/1303-2712-v18_4_07
131. Shahzad MM, Hussain SM, Akram AM, et al. Improvement in nutrient digestibility and growth performance of *Catla catla* fingerlings using phytase in *Moringa oleifera* leaf meal based diet. *Pak J Zool*. 2020;52(1):157-168. doi:10.17582/journal.pjz/2020.52.1.157.168
132. Liu L, Zhou Y, Wu J, et al. Supplemental graded levels of neutral phytase using pretreatment and spraying methods in the diet of grass carp, *Ctenopharyngodon idellus*. *Aqua Res*. 2013;45(12):1932-1941. doi:10.1111/are.12145
133. Liu LW, Luo YL, Hou HL, Pan J, Zhang W. Partial replacement of monocalcium phosphate with neutral phytase in diets for grass carp, *Ctenopharyngodon idellus*. *J Appl Ichthyol*. 2012;29(3):520-525. doi:10.1111/jai.12021

134. Samidjan I, Rachmawati D. Engineering technology of fish farming floating nets cages on Polka Dot Grouper (*Cromileptes altivelis*) used artificial feed enriched phytase enzyme. *IOP Conference Series: Earth and Environmental Science*. 2018;116(1):012010. doi:10.1088/1755-1315/116/1/012010
135. Liu LW, Su J, Luo Y. Effect of partial replacement of dietary monocalcium phosphate with neutral phytase on growth performance and phosphorus digestibility in gibel carp, *Carassius auratus gibelio* (Bloch). *Aquacult Res*. 2011;43(9):1404-1413. doi:10.1111/j.1365-2109.2011.02944.x
136. Zhang B, Gen H, Wang X, Pi X, Mai K, Zhou H. Effects of replacing fish meal with soybean meal or fermented and phytase-treated soybean meal respectively, on growth performance, feed utilization, and apparent digestibility in juvenile turbot (*Scophthalmus maximus* L.). *Isr J Aquac*. 2016;68:1-8. doi:10.46989/001c.20814
137. Yudhiyanto T, Suminto S, Rachmawati D. The effect of dietary phytase supplementation on digestibility and growth of Asian seabass *Lates calcarifer*. *Omni-Akuatika*. 2017;13(2):144-154. doi:10.20884/1.oa.2017.13.2.263
138. Yaqoob T, Khan N, Rasool F, et al. Effect of phytase supplementation in formulated feed for *Pangasius hypophthalmus* to increase the use of cotton seed meal by replacing fish meal. *Pak J Zool*. 2018;13:36-41.
139. Dechavez RB, Serrano AE Jr. Evaluation of phytases of three *Bacillus spp*. in the diet of sex-reversed *Oreochromis mossambicus* fingerlings on growth, feed efficiency and mineral deposition. *Ann Biol Res*. 2012;3(9):4584-4592.
140. Nie XZ, Chen S, Zhang XX, Dai BY, Qian LC. Effects of neutral phytase on growth performance and phosphorus utilization in crucian carp (*Carassius auratus*). *J Zhejiang Univ Sci B*. 2017;18:886-896. doi:10.1631/jzus.B1600280
141. Hussain SM, Afzal M, Nasir S, et al. Efficacy of phytase supplementation in improving mineral digestibility in *Labeo rohita* fingerlings fed on canola meal-based diets. *Iran J Fish Sci*. 2016;15(2):645-661.
142. Hussain SM, Rana SA, Afzal M, Shahid M. Efficacy of phytase supplementation on mineral digestibility in *Labeo rohita* fingerlings fed on corn gluten meal (30%) based diets. *Pak J Agric Sci*. 2012;48(3):237-241.
143. Marjan S, Afzal M, Shah SZH, Hussain SM, Mubarik MS. Role of phytase supplementation in improving mineral digestibility of dry bread meal based diet fed to *Labeo rohita* fingerlings. *Pak J Life Soc Sci*. 2014;12(3):150-154.
144. Naseem N, Abdullah S, Aziz S. Effect of phytase supplemented diet on whole body proximate composition of *Labeo rohita* fingerlings. *Pak J Zool*. 2021;54(3):1479-1482. doi:10.17582/journal.pjz/20200928150918
145. Akhtar S, Zahoor I, Yahya S, et al. Effect of phytase supplementation on *Labeo rohita* fingerlings that are fed by phytase supplemented ddgs based diet. *Int J Aquac Fish Sci*. 2020;6(3):61-67. doi:10.17352/2455-8400.000058
146. Sarfraz Q, Hussain SM, Sharif A, Selamoglu Z, Bashir F, Ahsan S. Potential of phytase supplemented *Moringa oleifera* leaf meal based diet on mineral digestibility of *Oreochromis niloticus* fingerlings. *J Surv Fish Sci*. 2020;6(2):65-77. doi:10.18331/SFS2020.6.2.8
147. Hussain SM, Shahzad MM, Afzal M, et al. Efficacy of phytase enzyme for increasing mineral digestibility of *Cirrhinus mrigala* fingerlings fed on soybean meal-based diet. *Pak J Zool*. 2015;47(6):1807-1816.
148. Uzezi AB, Omitoyin BO, Ajani EK. Phosphorus utilization in juvenile *Clarias gariepinus* fed phytase-supplemented diets based on soya bean (oil-extracted) and full-fat (roasted): a comparison. *J Appl Aquac*. 2017;29(2):126-151. doi:10.1080/10454438.2016.1276000
149. Uzezi AB. Serum biochemistry, body composition and bone mineralization of juvenile *Clarias gariepinus* fed phytase supplemented soya bean-based diets. *J Aquat Sci*. 2015;30(2):407-425. doi:10.4314/jas.v30i2.12
150. Baruah K, Norouzitallab P, Pal AK. Development of low cost and eco-friendly feed for various candidate species for the sustainability of commercial aquaculture and reduction of aquatic pollution [Chapter 24]. In: Bagchi D, Nair S, eds. *Developing New Functional Food and Nutraceutical Products*. Academic Press; 2017:441-453. doi:10.1016/B978-0-12-802780-6.00024-9
151. Mustapha A, El Bakali M. Phosphorus waste production in fish farming a potential for reuse in integrated aquaculture agriculture. *Int J Environ Agric Res*. 2021;7(1):5-13. doi:10.5281/zenodo.4482931
152. Biswas A, Araki H, Sakata T, Nakamori T, Takii K. Optimum fish meal replacement by soy protein concentrate from soymilk and phytase supplementation in diet of red sea bream, *Pagrus major*. *Aquaculture*. 2019;506:51-59. doi:10.1016/j.aquaculture.2019.03.023
153. Arsalan M, Hussain SM, Ali S, Ahmad B, Sharif A. Use of phytase and citric acid supplementation on growth performance and nutrient digestibility of *Cirrhinus mrigala* fingerlings fed on canola meal based diet. *Braz J Biol*. 2021;83:e246568. doi:10.1590/1519-6984.246568
154. Hussain SM, Nisar A, Farhat J, et al. Effects of citric acid and phytase supplementation on nutrient digestibility and growth performance of *Cirrhinus mrigala* fingerlings fed on corn gluten (30%) meal based diets. *Int J Biosci*. 2015;6(7):82-91. doi:10.12692/ijb/6.7.82-91
155. Hussain SM, Ahmad N, Shahzad MM, et al. Efficacy of phytase enzyme and citric acid on growth performance, nutrients and mineral digestibility of *Cirrhinus mrigala* fingerlings fed guar meal-based diet. *Iran J Fish Sci*. 2020;19(3):1573-1588. doi:10.22092/ijfs.2018.117462
156. Bano N, Afzal M. Combined effect of acidification and phytase supplementation on calcium and phosphorus digestibility and body composition of Rohu (*Labeo rohita*). *Pak J Zool*. 2017;49(6):2093-2101. doi:10.17582/journal.pjz/2017.49.6.2093.2101
157. Bano N, Afzal M. Synchronized effect of citric acid and phytase supplementation on growth performance and nutrient digestibility of *Labeo rohita*. *Aquacult Nutr*. 2017;24(2):786-792. doi:10.1111/anu.12607
158. Hussain SM, Ahmad S, Shahzad MM, et al. Mineral digestibility of *Labeo rohita* fingerlings fed on cottonseed meal based diets supplemented with citric acid and phytase enzyme. *Int J Biosci*. 2016;8(2):25-35. doi:10.12692/ijb/8.2.25-35
159. Afzal M, Fatima M, Qamar A, Farhan M, Shah SZH. Growth, muscle proximate composition and whole-body nutrient status of *Labeo rohita* fed acidified and phytase pre-treated sunflower meal based diet. *Pak J Zool*. 2020;52(3):937-947. doi:10.17582/journal.pjz/20190408080431
160. Saeed L, Afzal M, Shah SZH, Fatima M, Liaquat I, Hussain SM. Effects of phytase and citric acid supplementation on scale mineralization in *Labeo rohita* juveniles fed sunflower meal based diet. *Pak J Life Soc Sci*. 2017;15(1):1-6.
161. Rabia S, Afzal M, Shah SZH. Nutrient digestibility performance by rohu (*Labeo rohita*) juveniles fed acidified and phytase pre-treated sunflower meal-based diet. *J Appl Anim Res*. 2017;45(1):331-335. doi:10.1080/09712119.2016.1190731
162. Afzal M, Sultana N, Hassan A, et al. Effectiveness of acidification and phytase pretreatment on growth performance, muscle proximate composition and nutrient digestibility of Rohu (*Labeo rohita*, Hamilton 1822) juveniles fed soybean meal based diet. *Pak J Zool*. 2019;51(5):1741-1750. doi:10.17582/journal.pjz/2019.51.5.1741.1750
163. Maas RM, Verdegem MC, Schrama JW. Effect of non-starch polysaccharide composition and enzyme supplementation on growth performance and nutrient digestibility in Nile tilapia (*Oreochromis niloticus*). *Aquacult Nutr*. 2019;25(3):622-632. doi:10.1111/anu.12884

164. Maas RM, Verdegem MC, Stevens TL, Schrama JW. Effect of exogenous enzymes (phytase and xylanase) supplementation on nutrient digestibility and growth performance of Nile tilapia (*Oreochromis niloticus*) fed different quality diets. *Aquaculture*. 2020;529:735723. doi:10.1016/j.aquaculture.2020.735723
165. Maas RM, Verdegem MC, Lee CN, Schrama JW. Effects and interactions between phytase, xylanase and β-glucanase on growth performance and nutrient digestibility in Nile tilapia. *Anim Feed Sci Technol*. 2021;271:114767. doi:10.1016/j.anifeedsci.2020.114767
166. Adeoye AA, Jaramillo-Torres A, Fox SW, Merrifield DL, Davies SJ. Supplementation of formulated diets for tilapia (*Oreochromis niloticus*) with selected exogenous enzymes: overall performance and effects on intestinal histology and microbiota. *Anim Feed Sci Technol*. 2016;215:133-143. doi:10.1016/j.anifeedsci.2016.03.002
167. Moustafa YT, Amer TN. Toward more environmentally friendly aquaculture: effect of dietary exogenous enzymes (NATUZYME®) on nitrogen and phosphorus retention and excretion in *Nile tilapia* (*Oreochromis niloticus*). Paper presented at the 1st International Conference (Central Laboratory for Aquaculture Research in Cooperation with Worldfish); November 20–22, 2017; Cairo, Egypt, 2: 478–501.
168. Singh SK, Bhandari MP, Timalsina P. Effect of dietary inclusion of citric acid with phytase as supplement on growth responses of rainbow trout. *Turkish JAF Sci Tech*. 2020;8(11):2355-2360. doi:10.24925/turjaf.v8i11.2355-2360.3574
169. Yigit NO, Bahadir Koca S, Didinen BI, Diler I. Effect of protease and phytase supplementation on growth performance and nutrient digestibility of rainbow trout (*Oncorhynchus mykiss*, Walbaum) fed soybean meal-based diets. *J Appl Anim Res*. 2018;46(1):29-32. doi:10.1080/09712119.2016.1256292
170. Hlophe-Ginindza SN, Moyo NA, Ngambi JW, Ncube I. The effect of exogenous enzyme supplementation on growth performance and digestive enzyme activities in *Oreochromis mossambicus* fed kikuyu-based diets. *Aqua Res*. 2015;47(12):3777-3787. doi:10.1111/are.12828
171. Zhu Y, Ding Q, Chan J, Chen P, Wang C. The effects of concurrent supplementation of dietary phytase, citric acid and vitamin D3 on growth and mineral utilization in juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquaculture*. 2015;436:143-150. doi:10.1016/j.aquaculture.2014.11.006
172. Coutinho TC, Tardioli PW, Farinas CS. Phytase immobilization on hydroxyapatite nanoparticles improves its properties for use in animal feed. *Appl Biochem Biotechnol*. 2020;190(1):270-292. doi:10.1007/s12010-019-03116-9
173. Vandenberg GW, Scott SL, Sarker PK, Dallaire V, de la Noüe J. Encapsulation of microbial phytase: effects on phosphorus bioavailability in rainbow trout (*Oncorhynchus mykiss*). *Anim Feed Sci Technol*. 2011;169(3–4):230-243. doi:10.1016/j.anifeedsci.2011.07.001
174. https://www.marketsandmarkets.com/Market-Reports/feed-enzyme-market-1157.html
175. https://precisionbusinessinsights.com/market-reports/feed-phytase-market/
176. Kumari N, Bansal S. Production and characterization of a novel, thermotolerant fungal phytase from agro-industrial byproducts for cattle feed. *Biotechnol Lett*. 2021;43(4):865-879. doi:10.1007/s10529-020-03069-8
177. https://www.acumenresearchandconsulting.com/phytase-market
178. https://www.gminsights.com/industry-analysis/animal-feed-phytase-market

How to cite this article: Priya, Virmani I, Pragya, et al. Role of microbial phytases in improving fish health. *Rev Aquac*. 2023; 1-21. doi:10.1111/raq.12790

PREPARATIVE BIOCHEMISTRY & BIOTECHNOLOGY
https://doi.org/10.1080/10826068.2023.2297688

Taylor & Francis
Taylor & Francis Group

Optimization of phytase production by *Penicillium oxalicum* in solid-state fermentation for potential as a feed additive

Priya[a], Bijender Singh[b,c], Jai Gopal Sharma[a], and Bhoopander Giri[d]

[a]Environmental and Industrial Biotechnology Laboratory, Department of Biotechnology, Delhi Technological University, Delhi; [b]Laboratory of Bioprocess Technology, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana; [c]Department of Biotechnology, Central University of Haryana, Mahendargarh, Haryana; [d]Department of Botany, Swami Shraddhanand College, University of Delhi, Delhi

ABSTRACT

The study aims to statistically optimize the phytase production by *Penicillium oxalicum* PBG30 in solid-state fermentation using wheat bran as substrate. Variables viz. pH, incubation days, $MgSO_4$, and Tween-80 were the significant parameters identified through the Plackett-Burman design (PBD) that majorly influenced the phytase production. Further, central composite design (CCD) method of response surface methodology (RSM) defined the optimum values for these factors i.e., pH 7.0, 5 days of incubation, 0.75% of $MgSO_4$, and 3.5% of Tween-80 that leads to maximum phytase production of 475.42 U/g DMR. Phytase production was also sustainable in flasks and trays of different sizes with phytase levels ranging from 394.95 to 475.42 U/g DMR. Enhancement in phytase production is 5.6-fold as compared to unoptimized conditions. The *in-vitro* dephytinization of feed showed an amelioration in the nutritive value by releasing inorganic phosphate and other nutrients in a time-dependent manner. The highest amount of inorganic phosphate (33.986 mg/g feed), reducing sugar (134.4 mg/g feed), and soluble protein (115.52 mg/g feed) was achieved at 37 °C with 200 U of phytase in 0.5 g feed for 48 h. This study reports the economical and large-scale production of phytase with applicability in enhancing feed nutrition.

KEYWORDS

Dephytinization; fish feed; large-scale; *P. oxalicum*; phytase; statistical optimization; wheat bran

Introduction

Phytic acid (myo-inositol hexakis dihydrogen phosphate) is a reserve of organic phosphorus and exists as phytate, i.e., the more complex form of phytic acid. Phytate binds cations and other essential biomolecules like amino acids, lipids, proteins, digestive enzymes, and carbohydrates and acts as an anti-nutritional factor for monogastric animals.[35] Besides, it causes severe phosphorus pollution due to the enrichment of nutrients and algal bloom formation in water bodies leading to hypoxia and the death of aquatic animals.[29]

Phytase (myo-inositol hexakisphosphate phosphohydrolases) is a significant enzyme in the food and feed industry that makes phytate-restricted nutrients accessible for utilization. It catalyzes the hydrolysis of phytic acid sequentially and liberates inorganic phosphate and lower esters of myo-inositol. It also removes metal ions, proteins, enzymes, and other biomolecules associated with the phytate complex. Plants, animals, and microbes produce phytases in intracellular and extracellular forms. Microbial phytases are preferable over other phytase sources because of their thermostability, wide pH range, and resistance toward proteases.[16] It has applications in animal nutrition, human nutrition, pharmaceutical, plant growth, bioethanol production, environmental issues, and transgenic plant formation.[29]

Both solid-state (SSF) and submerged fermentation (SmF) are applicable for phytase production, but acceptance of SSF over SmF is more due to its simple process, low cost, and yield of more end product.[36] In SSF, microbes are grown on solid material in the absence of oxygen and require adequate moisture for the growth,[20] and agricultural residues are used as a substrate for the fermentation process because being lignocellulosic, they stimulate the growth of microorganisms and are less costly.[12] One variable at a time (OVAT) approach analyses the factors one by one, which is more time-consuming, laborious, and costly. At the same time, the statistical methods (PBD and RSM via CCD) involve an experimental design that examines various parameters in a single duration and their relation with the help of mathematics and statistics. Furthermore, it creates two- and three-dimensional contour plots to demonstrate the optimization of parameters and interaction among them.[16] Previous literature also supported the use of statistical methods in enhancing phytase production via SSF using *Aspergillus niger*,[2] *A. oryzae* SBS50,[36] *Sporotrichum thermophile*,[17] *Rhizopus oligosporus*,[40] *Pholiota adiposa*,[12] *A. niger* NT7,[16] *A. tubingensis*,[20] *A. niger*.[22]

The plant-based feed contains anti-nutritional factors (ANFs) like phytic acid, oxalates, saponins, and tannins which deprive the nutrients in the feed. Up to 80% of phosphorus in the plant feed exists as phytate-phosphorus (P).

CONTACT Bhoopander Giri bhoopg@yahoo.com Department of Botany, Swami Shraddhanand College, University of Delhi, Delhi.

Due to the lack of phytase in monogastric animals, phytate-P remains undigestible; thus, additional inorganic phosphate (Pi) is requisite in the diet to fulfill the demand of P, which affects the finite P reserve stock. Also, unutilized phosphorus in the form of phytate-P and Pi excreted in feces causes P pollution in aquatic bodies.[16] Moreover, the phytate-metal complex inhibits the activity of digestive enzymes in the gastrointestinal region, thus regulating the digestibility of protein, lipids, and starch. Supplementation of phytase is the practical approach that degrades phytic acid, makes nutrients available for utilization, improves the quality of feed, and reduces P pollution.[29]

The phytases involved in the fish feeding are mostly commercial and obtained by SmF while, this work is utilizing SSF for phytase production from a new fungal isolate, *P. oxalicum*. SSF is economical cheaper method than SmF and makes a cost-effective process for phytase production. The phytase is useful in removing ANFs, mainly phytate and makes the feed healthy and nutritious. Initial degradation of phytate is started in the formulated feed. The determination of phytate hydrolysis is necessary to understand the impact of phytase in the feed before fish feeding. Therefore, *in-vitro* analysis of plant-based feed is requisite. Most of the investigation is done on the fish growth and its performance (*in-vivo*) by adding phytase to their diet.[9,29] But limited research is available on the dephytinization of fish feed at *in-vitro* level. Dephytinization is a process of phytic acid degradation and liberation of inorganic phosphorus on the addition of phytase in the diet. Besides, it also releases reducing sugars and soluble protein. These products enhance the nutritional value of diet.

The study investigates the extracellular phytase production from *Penicillium oxalicum* in SSF using wheat bran as a substrate and its enhancement through statistical optimization. Here, the study also evaluates the production of cost-effective phytase on large scale. The *in-vitro* effect of partially purified phytase in dephytinizing plant-based fish feed and ameliorating its nutritional value was analyzed. Optimization of various factors (temperature, dose, amount of feed, incubation time) were performed to know the best conditions for the operation of phytase in fish feeding. This research indicates the potential of *P. oxalicum* phytase as a supplement in feed industry.

Materials and methods

Microorganism and culture conditions

The fungus was isolated from the rotten orange peel collected from the local market of Delhi. The mold was identified as *Penicillium oxalicum* PBG30 on the basis of morphological features as well as ITS sequencing (data not shown). The mold was cultured on the potato dextrose agar media at 30 °C and stored at 4 °C and at −20 °C in glycerol stock.[33]

The fungal spores were scrapped from 3-day-old culture via a sterile loop by adding 25 ml normal saline solution containing 0.1% Tween 80. The spore suspension was filtered through a sterile filter assembly, and the spore count was measured by a hemocytometer.[36]

Phytase production in solid-state fermentation

Phytase production was carried out using four different moistening media (pH 5.0) in 250 ml Erlenmeyer flasks (Table 1). Wheat bran is used as a substrate (5 g) with 10 ml moistening media (1:2), and autoclaved at 121 °C for 20 min. After cooling at room temperature, inoculated with 1 ml of spore suspension (7.7×10^7 CFU/ml) and kept in the incubator for 3 days at 30 °C. After 3 days of incubation, the enzyme extraction was done by adding the 50 ml normal saline solution with 0.1% Tween 80 in the flask and shaking at 30 °C with 200 rpm for 1 h. The extract was filtered by double-layered muslin cloth and centrifuged the filtrate at 10,000 rpm for 10 min at 4 °C. The supernatant was used to test the phytase activity, and the residual substrate was dried at 60 °C for 24 h.[36]

Phytase assay

Phytase activity was determined by measuring the release of inorganic phosphate from the calcium phytate substrate at 70 °C with 0.1 M sodium acetate buffer (pH 3.0) through[8] method. Blank and control (substrate and enzyme) were also set during the experiment, and all were performed in triplicate. Phytase activity was calculated using the regression equation of the standard graph of inorganic phosphate (KH_2PO_4). The phytase activity is expressed as unit per gram dry moldy residue (U/g DMR).[36] One unit of phytase is defined as the amount of enzyme used to liberate 1 nmol of inorganic phosphorus per sec under assay conditions.[33]

Optimization of phytase production by statistical approach

Plackett-Burman design

The optimization of phytase production from *P. oxalicum* includes two steps. In the first step, the Plackett-Burman method helps to identify the significant factors useful for production.[28] In this design, a selection of 11 variables (n) with their high (+) and low (-) concentration values and 12 runs of the experiment (n + 1) was set in an equal manner

Table 1. Different moistening media used for the phytase production.

S. No.	Composition of moistening media	Reference
Medium I	Distilled water	[37]
Medium II	Starch – 1.5% (w/v), Beef extract – 0.6% (w/v), $FeSO_4.7H_2O$ – 0.01% (w/v), KCl – 0.05% (w/v)	[37]
Medium III	$(NH_4)_2SO_4$ – 0.5% (w/v), $MgSO_4.7H_2O$ – 0.5% (w/v), $FeSO_4.7H_2O$ – 0.03% (w/v), NaCl – 0.1% (w/v)	[37]
Medium IV	Urea – 0.5% (w/v), $MgSO_4.7H_2O$ – 0.1% (w/v), KCl – 0.1% (w/v), $FeSO_4.7H_2O$ – 0.1% (w/v)	[21]

Table 2. Factors studied during Plackett Burman experiment.

Run No	A	B	C	D	E	F	G	H	I	J	K	Phytase Activity (U/g DMR ± S.E)
1	5	5	5	1	2	0	1	0.05	0.0	0.5	0	108.56 ± 10.01
2	10	5	3	0	2	2	1	0.00	0.2	0.5	0	24.01 ± 15.51
3	10	8	3	1	2	0	1	0.00	0.0	0.1	1	150.5 ± 31.80
4	5	8	3	0	0	2	1	0.05	0.0	0.5	1	239.81 ± 0.08
5	5	8	5	1	0	2	1	0.00	0.2	0.1	0	97.24 ± 8.84
6	10	5	5	0	0	0	1	0.05	0.2	0.1	1	102.29 ± 3.93
7	10	8	3	1	0	0	0	0.05	0.2	0.5	0	59.36 ± 18.96
8	5	5	3	1	2	2	0	0.05	0.2	0.1	1	97.54 ± 14.40
9	5	8	5	0	2	0	0	0.00	0.2	0.5	1	268.77 ± 0.03
10	5	5	3	0	0	0	0	0.00	0.0	0.1	0	5.75 ± 1.94
11	10	5	5	1	0	2	0	0.00	0.0	0.5	1	102.06 ± 39.92
12	10	8	5	0	2	2	0	0.05	0.0	0.1	0	137.578 ± 1.47

A – Wheat bran (g), B – pH, C – Incubation period (days), D – Urea (% w/v), E – Starch (% w/v), F – Glucose (% w/v), G -Ammonium sulfate (% w/v), H – Ferrous sulfate (% w/v), I – Calcium chloride (% w/v), J – Magnesium sulfate (% w/v), K – Tween 80 (% v/v).

(Table 2). The selected independent factors were A) wheat bran, B) pH, C) incubation days, D) ammonium sulfate, E) urea, F) starch, G) glucose, H) ferrous sulfate I) calcium chloride, J) magnesium sulfate, and H) Tween 80. The difference between the average of one variable at its high and low levels provides the impact of that variable on the production, which was calculated by

$$E(Xi) = \frac{2(\Sigma Pi+ \; - \; \Sigma Pi-)}{N}$$

Here, E(Xi) denotes the effect of each variable, Pi+, and Pi- means high and low values, and N means the total number of runs performed in the experiment.[36] All the runs were performed in triplicates, phytase activity was calculated, and the critical factors were determined by a pareto chart.[42]

Response surface methodology

In the second step, four independent factors, pH (A), incubation time (B), $MgSO_4$ (C), and Tween 80 (D) obtained from PBD, were evaluated further by the RSM experiment. The optimal level and interaction effect of these parameters on phytase production is determined using central composite design (CCD Version 6.0.7, Stat-Ease, and Minneapolis, MN). In addition, 0.5% ammonium sulfate and 0.01% $FeSO_4$ supplied as a supplement. Five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) were assigned to each parameter, and 30 experimental runs were designed as shown in Tables 3 and 4 respectively.

Production of phytase is measured by the following equation:

$$\begin{aligned} Y = {} & ß_0 + ß_1A + ß_2B + ß_3C + ß_4D + ß_{11}A^2 \\ & + ß_{22}B^2 + ß_{33}C^2 + ß_{44}D^2 + ß_1ß_2AB + ß_1ß_3AC \\ & + ß_1ß_4AD + ß_2ß_3BC + ß_2ß_4BD + ß_3ß_4CD \end{aligned}$$

(Y- predicted phytase production, $ß_0$ - intercept, $ß_1$, $ß_2$, $ß_3$, $ß_4$ - linear coefficients, $ß_{11}$, $ß_{22}$, $ß_{33}$, $ß_{44}$ - squared coefficients, $ß_1ß_2$, $ß_1ß_3$, $ß_1ß_4$, $ß_2ß_3$, $ß_2ß_4$, $ß_3ß_4$ - interaction coefficients, A, B, C, D, AB, AC, AD, BC, BD, CD – variables and their interaction.[33] Statistical software was used to set up the experiment and created 3-D contour plots.[11] In this method, predicted and experimental values were compared, and statistical analysis was done by analysis of variance (ANOVA).[42]

Table 3. Parameters with their assigned level in CCD method of RSM for phytase production.

S. No.	Parameters	Level of parameters				
		$-\alpha$	-1	0	$+1$	$+\alpha$
1.	pH	5	6	7	8	9
2.	Incubation period	3	4	5	6	7
3.	Tween 80 (%)	0.5	1	2	3	3.5
4.	$MgSO_4$ (%)	0.25	0.50	0.75	1.00	1.25

Phytase production in flasks and trays

Phytase production was carried out in flasks (250-1000 ml) and enamel-coated metallic trays (27 cm × 22 cm × 5 cm, 35 cm × 32 cm × 5 cm) containing wheat bran moistened with medium (5, 10, 20, 100, 150) in a 1:2 ratio. The flasks were autoclaved at 121 °C for 20 min and cooled down.[36] Inoculated with a spore suspension of *P. oxalicum*, mixed with a sterile spatula, covered with a clean wrap for trays, and incubated for 5 days at 30 °C. Samples were collected from different sections of the tray, and extraction was performed, as mentioned earlier.[17]

Dephytinization of feed by P. oxalicum phytase

In-vitro hydrolysis of phytate was performed by adding partially purified phytase to the feed and analyzing the quantity of released inorganic phosphate, reducing sugar and protein content. Partial purification of phytase is done by salt precipitation and dialysis method. One gram of commercial fish feed was dissolved in 20 ml sodium acetate buffer (0.1 M, pH 3.0) and autoclaved at 121 °C for 20 min. Phytase from *P. oxalicum* (80 U/g) was mixed and incubated at 37 °C, 50 °C, and 70 °C with shaking at 100 rpm. Samples were taken at different time intervals, kept at 4 °C, and centrifuged at 10,000 rpm for 10 min. The supernatant was used to detect inorganic phosphate, reducing sugars and soluble protein content by the[8,19,23] respectively. Further experiments with different doses of phytase (25-200 U/g), feed amount (0.5-2 g), and incubation time (2-48 h) were performed at 37 °C.[38] All the experiments were conducted in triplicates and statistical analysis was done by showing

Table 4. Phytase activity of RSM experimental runs designed by CCD method.

Run No	pH	Incubation Period (days)	Tween 80 (%)	Magnesium sulphate (%)	Phytase activity (U/g DMR ± S.E) Experimental value	Predicted value
1	7	5	2.0	0.75	114.74 ± 14.70	117.85
2	6	4	3.0	0.50	278.39 ± 17.17	297.11
3	7	5	2.0	1.25	64.48 ± 6.39	62.82
4	6	6	1.0	0.50	12.27 ± 1.85	18.60
5	8	6	3.0	1.00	120.42 ± 7.47	124.90
6	6	3	1.0	0.50	51.28 ± 6.91	50.25
7	8	6	3.0	0.50	177.51 ± 12.47	188.98
8	9	5	2.0	0.75	13.28 ± 1.74	4.82
9	7	5	0.5	0.75	109.57 ± 6.68	108.87
10	6	4	1.0	1.00	60.27 ± 8.02	62.80
11	6	6	3.0	1.00	141.69 ± 6.17	154.13
12	6	6	1.0	1.00	76.52 ± 6.48	73.58
13	7	4	2.0	0.75	144.09 ± 7.34	138.52
14	7	4	2.0	0.75	145.74 ± 8.06	138.52
15	8	4	1.0	0.50	44.36 ± 4.56	45.92
16	7	5	2.0	0.75	114.74 ± 14.70	117.85
17	6	4	3.0	1.00	264.27 ± 18.60	260.82
18	7	5	3.5	0.75	373.32 ± 3.28	346.66
19	8	4	3.0	0.50	278.09 ± 1.28	282.42
20	8	4	1.0	1.00	25.71 ± 8.49	30.45
21	8	6	1.0	0.50	65.12 ± 5.90	69.95
22	6	6	3.0	0.50	172.45 ± 15.59	169.09
23	7	5	2.0	0.75	114.74 ± 20.79	117.85
24	7	7	2.0	0.75	82.73 ± 7.41	76.52
25	8	4	3.0	1.00	191.39 ± 14.52	197.01
26	8	6	1.0	1.00	80.52 ± 9.68	75.81
27	5	5	2.0	0.75	24.21 ± 5.56	17.28
28	7	5	2.0	0.75	114.74 ± 14.70	117.85
29	7	5	2.0	0.25	106.99 ± 7.15	93.25
30	7	5	2.0	0.75	114.74 ± 14.70	117.85

significant differences ($p < 0.05$) via using one-way ANOVA and Tukey's post hoc tests. GraphPad Prism software version 10.0 (GraphPad Software, San Diego, CA, USA) is used for the statistical analysis. Sigma plot (version 10.0) is used for the creating the graphs.[34]

Results and discussion

Phytase production in SSF

Among the different production media for SSF, media IV (84.60 ± 2.54 U/g DMR) supports the maximum phytase production, as shown in Figure 1. Different fungi were studied for producing phytase through SSF by using wheat bran as substrate.[15,20,30,32,36] Wheat bran is commonly used for SSF because it is rich in carbon, nitrogen, and amino acids and high amount of phytic acid. Due to presence of phytate, it acts as inducer for the production of phytase.[3] It also provides support for the growth of fungi, easily available, and low cost.[15] The production of phytase via wheat bran as substrate has been reported in *A. oryzae* SBS50 (5.05 U/g DMR) using 10 g substrate in 1:2 ratio of moistening media incubated at 30 °C for 4 days.[36] Similarly, *R. oligosporus* MTCC 556 produced 15.8 U/gds,[39] 208.30 U/gds by *A. niger* NT7[15] and 225.6 U/g by *A. tubingensis*[20] using wheat bran as substrate. [7] studied the phytase production with different fungal strains on wheat bran and reported that *A. oryzae* F-923, *A. oryzae* F-937, *A. niger* F-258, *A. awamori* NRC- F18, *A. fumigatus* NRCF-113, *Trichoderma viride* NRCF-107 and *Saccharomyces cerevisiae* F-307

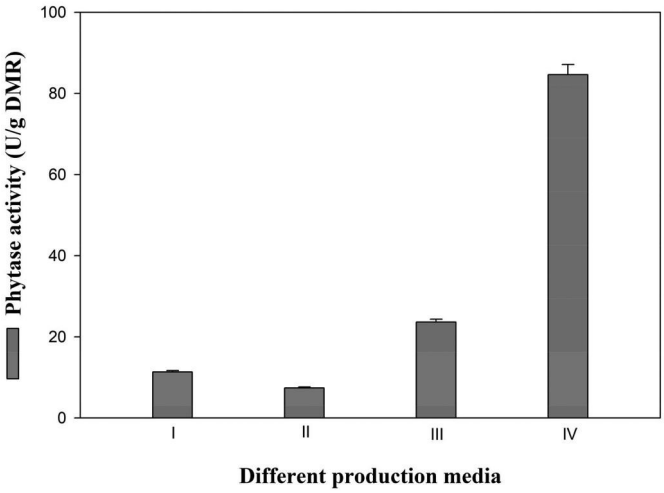


Figure 1. The graph shown the involvement of different media in the production of phytase.

secreted phytase as 66.56, 58.19, 110.62, 143.67, 60.24, 54.41 and 64.86 IU/g, respectively.

Screening of critical components through PB design

Plackett-Burman is a majorly adopted statistical method used for studying several factors together, which consumes less time and provides significant factors that influence the production of phytases. The factors showing a high impact on phytase production are considered critical or essential parameters. A PB design was performed with 11 variables and 12 experimental runs with their high (+) and low (-)

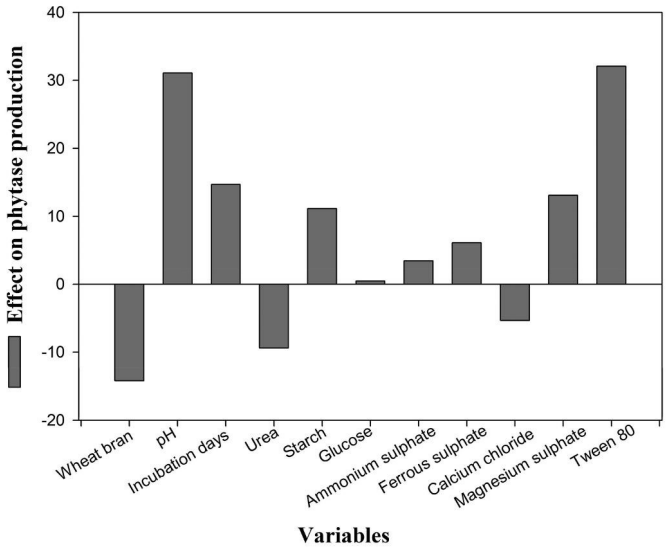


Figure 2. Pareto chart showing the effect of different variables on phytase production.

Table 5. ANOVA for response surface reduced quadratic model.

Source	Sum of Squares	dF	Mean square	F value	Prob > F	
Model	2.121E + 005	13	16315.40	127.35	<0.0001	significant
A	226.28	1	226.28	1.77	0.2025	
B	11156.38	1	11156.38	87.08	<0.0001	
C	1.245E + 005	1	1.245E + 005	971.51	<0.0001	
D	1349.22	1	1349.22	10.53	0.0051	
A^2	20241.05	1	20241.05	157.99	<0.0001	
C^2	28704.97	1	28704.97	224.05	<0.0001	
D^2	2813.01	1	2813.01	21.96	0.0002	
AB	1527.12	1	1527.12	11.92	0.0033	
AC	947.85	1	947.85	7.40	0.0151	
AD	2310.40	1	2310.40	18.03	0.0006	
BC	17622.46	1	17622.46	137.55	<0.0001	
BD	580.75	1	580.75	4.53	0.0491	
CD	4683.10	1	4683.10	36.55	<0.0001	

Table 6. ANOVA regression analysis.

Std. Dev.	11.32	R^2	0.9904
Mean	122.61	Adj R^2	0.9827
C.V.	9.23	Pred R^2	0.9571
PRESS	9188.99	Adeq Precision	44.209

level. Table 2 shows the phytase production of 12 experimental runs. A pareto chart was created to determine the impact of variables on phytase production (Figure 2). The positive and negative values in the pareto graph showed the importance of individual components in phytase production. Run no. 9 showed the maximum output in term of phytase production. This experiment concluded that among the 11 factors, pH, incubation time, $MgSO_4$, and Tween 80 are the critical variables that contribute more to the production of *P. oxalicum* phytase, and others have low contributions and thus are less significant. Surfactants like Tweens and Triton X-100 seem to affect the fungus growth and enzyme production and hence, involved in the enhancement of enzyme yield. Surfactants solubilize proteins on the membrane that increases cell permeability and more liberation of enzymes.[17] Tween-80 has been reported for enhancing the phytase production by *S. thermophile*[17] and *A. oryzae* SBS50.[33] Triton X-100 enhanced phytase production by *A. oryzae* SBS50[36] and Tween-40 by *A. niger*[22] in SSF.

Moisture content is also one of the factors that affect the phytase production. At low moisture content, substrate becomes dry and at high level, the substrate air content is decreased. So, an optimum moisture is required for the growth.[33] It has been mentioned as important component in the production of phytase from *A. oryzae* SBS50,[36] *A. niger*,[2] *P. adiposa*[12] and *A. oryzae* SBS50.[33] Metal ions like $MgSO_4$, are necessary for the growth of fungi but have no influence on phytase activity.[33] Many studies showed the importance of $MgSO_4$ in phytase production from *A. oryzae* SBS50,[36] *A. niger*[2] and *A. oryzae*.[33]

The impact of incubation period and pH occurs on the metabolic process during the fungal growth and they also help in stabilizing the enzymes.[15] Incubation period is shown as a major component for production of phytase in *A. oryzae* SBS50,[36] *S. thermophile*,[17] *A. niger* NT7[16] and *A. niger*.[22] Similarly, pH is found necessary in the phytase production from *Williopsis saturnus* NCIM 3298,[27] *A. niger* NT7[16] and *A. niger*.[22] Other useful factors involved in the production of phytase were selected through PBD like glucose and dextrin for *A. niger*,[2] yeast extract for *S. thermophile*,[17] glucose, $FeSO_4$, citric acid for *W. saturnus*,[27] water hyacinth for *P. adiposa*,[12] amount of wheat bran, temperature, mannitol and ammonium sulfate for *A. niger* NT7,[16] temperature and NH_4NO_3 for *A. niger*,[22] glucose for *A. oryzae*.[33]

Optimization by response surface methodology

A total of 30 experiments were designed via CCD using 5 g wheat bran, 0.5% ammonium sulfate, and 0.01% ferrous sulfate as fixed parameters and pH, incubation period, magnesium sulfate, and Tween 80 as varying parameters. The best result of phytase activity (373.32 U/g DMR) was found in the flask containing 5 g of wheat bran moistened with 10 ml of media including 0.5% ammonium sulfate, 0.01% $FeSO_4$, 3.5% Tween 80 and 0.75% $MgSO_4$ with pH 7.0 and 5 days of incubation. The phytase production was elucidated by following model equation:

$$\begin{aligned} Y\ (\mathrm{U/g\ DMR}) = {} & 117.85 - 3.12\ A - 20.67\ B \\ & + 79.26\ C - 7.61\ D - 26.70\ A^2 + 48.85\ C^2 - 9.95\ D^2 \\ & + 8.64\ AB - 7.87\ AC - 12.28\ AD - 29.37\ BC \\ & + 5.33\ BD - 17.49CD \end{aligned}$$

ANOVA is used for evaluating the data designed by the RSM experiment as shown in Tables 5 and 6 The Pred R^2 value is 0.9571 depicting the correlation between the predicted and experimental values. R^2 value, i.e., the coefficient of determination, is found to be 0.99, indicating 99% variability within the model's parameters. Adequate precision gives us a signal-to-noise ratio that should be > 4 for better results.[13] The ratio in this model comes out to 44.209, delivering more than a sufficient signal to navigate the design space. The significance of the model is determined by the F value, which is 127.35 in our experiment, indicating

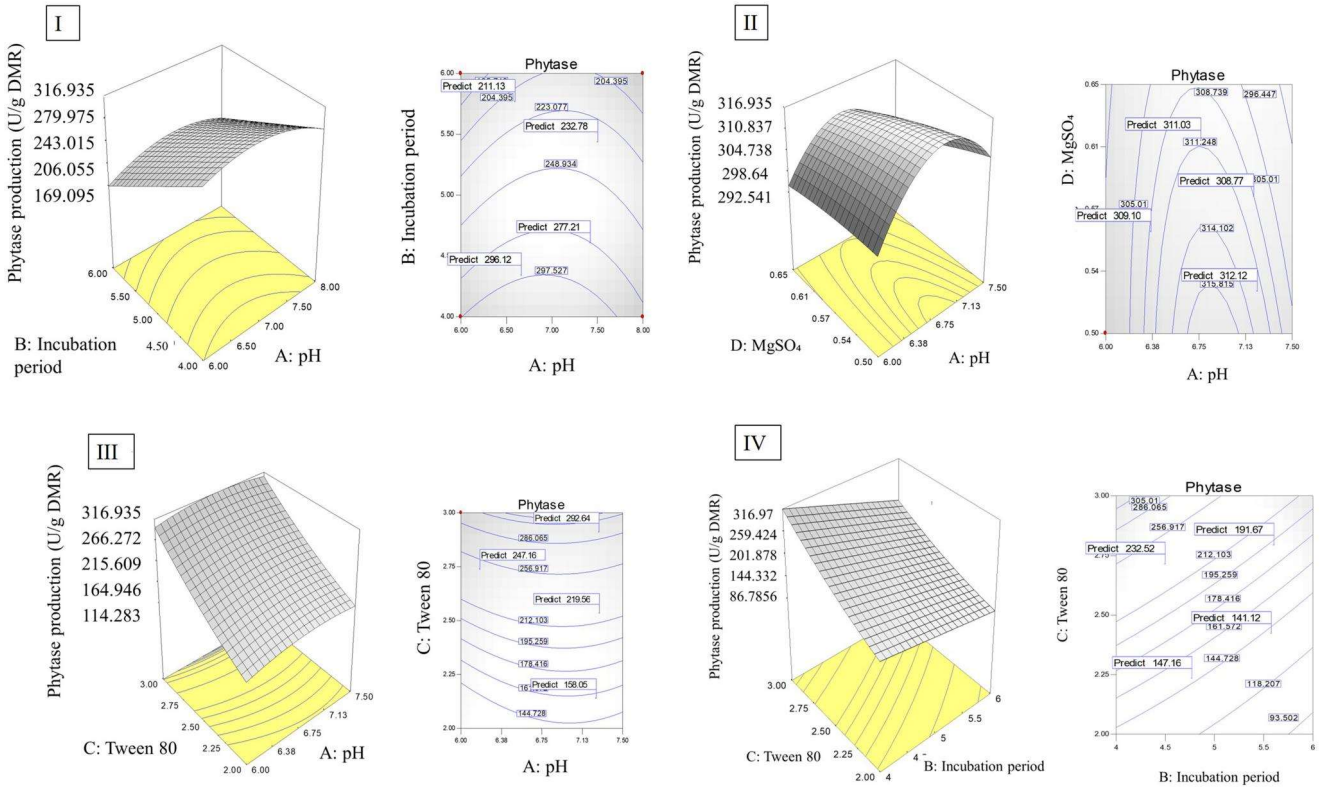


Figure 3. 3D response surface plot showing interaction between I) pH and incubation period II) pH and $MgSO_4$ III) pH and Tween 80 IV) incubation period and Tween 80.

that the model is significant and Prob > F was less than 0.05, which implies that there is only a 0.01% chance that the occurrence of the F value is due to noise.[33] Significant model terms were B, C, D, A^2, C^2, D^2, AB, AC, AD, BC, BD, and CD that significantly affected phytase production in SSF.

Response surface graphs showed the optimum level of parameters and interaction among them on which phytase activity is demonstrated on the y-axis and influencing parameters on the x-axis and z-axis.[16] The peak denotes the phytase production with the optimal value of each variable.[22] Figure 3(I) shows the relation between pH and the incubation period. At pH 7.0 and 5 days of incubation, production is higher than below or above the values of both factors. The trial showing maximum phytase titer was repeated further to validate the experiment and found to be 339.94 U/g DMR which was closer to the estimated value of 346.66 U/g DMR.

Optimization is requisite for the identification of essential components and culture conditions necessary for the fungal growth and production of enzymes. Incubation time is a significant influencing variable in enzyme production. In the logarithmic phase, most of the enzymes are produced. A long time period decreases the production level due to a deficiency of nutrients and cell lysis.[36] The results showed maximum production within 5 days of incubation; after that, phytase yield decreases due to a deficiency of nutrients for the growth of fungi. Likewise, the incubation period was also found 5 days for *A. niger* NT7[16] and *R. oligosporus.*[40] In contrast, 2 days were reported for *A. oryzae* SBS50[36] and *S. thermophile*[17] and 6 days for *A. niger.*[22]

Medium pH is crucial for all metabolic processes and strongly affects phytase production. The optimum pH for *P. oxalicum* is 7.0, i.e., neutral pH. Opposite to this, *A. niger* NT7 requires pH 5.0,[16] and *A. niger* requires pH 6.0[22] for high phytase production. Tween-80 is one of the surfactants that significantly affects the production level as it permeabilizes the cell membrane and releases more proteins/enzymes in the medium.[36] At 3.5% concentration, Tween-80 depicts its highest effect. Tween-80 (2.5%) was found optimum for phytase production from *S. thermophile*[17] and *A. oryzae* SBS50.[33] Metal ions such as magnesium are requisite for the fungus growth but barely affect the phytase production.[33] In our results, 0.75% $MgSO_4$ was involved in increasing the production of phytase, while 0.4% $MgSO_4$ is found for *A. oryzae* SBS50[36] and 1.5% for *A. niger.*[2]

Optimization through statistical methods enhanced phytase production significantly. In our results, the phytase activity showed 4.4-fold enhancement after statistical optimization compared to unoptimized conditions. Similarly, 3.35-fold enhancement has been mentioned in the phytase production from *A. oryzae* SBS50,[36] 36.67-fold in *A. niger,*[2] 11.6-fold in *S. thermophile,*[17] 2.73-fold in *R. oligosporus,*[40] 3.15-fold in *P. adiposa,*[12] 6.8-fold in *A. niger,*[16] 1.37-fold by *A. niger*[22] and 2.29-fold in *A. oryzae* SBS50[33] through statistical approaches.

Optimization of phytase on large scale production

The RSM model was validated by performing phytase production in Erlenmeyer flasks and trays on a large scale using 5-150 g of wheat bran, as shown in Table 7. The amount of substrate was enhanced from 5 g wheat bran in a 250 ml flask to 150 g wheat bran in the enamel-coated metallic tray. The phytase production was found sustainable in the range of 394.95 – 475.42 U/g DMR and validated the model at large scale. These results uplift the optimization of *P. oxalicum* phytase via SSF under pilot scale. On a large scale, trays production is influenced by substrate amount, presence of oxygen and unevenly allocation of inoculum and bed height.[1] If the amount of substrate is more than sufficient level, oxygen becomes unavailable to fungus, and production decreases.[39] A 1.5-2 cm bed height is considerable for the production, but it is not compulsory. It may varies depending upon the production system, kinetic parameters and operating conditions Above than 2 cm, cell biomass and production are affected due to high level of substrate and less availability of moisture and oxygen.[1] Similarly, sustainable production of phytase in flasks and trays on a large scale was studied in *A. oryzae* SBS50,[38] *R. oligosporus* MTCC 556,[39] *S. thermophile,*[17] *R. oryzae*[1] and *A. niger* 7 A-1.[25]

Table 7. Phytase production on large scale in flasks and trays.

S.No.	Flasks/Trays	Amount of substrate used (g)	Phytase production (U/g DMR) ± S.E
1.	250ml	5	394.95 ± 34.63
2.	500ml	10	431.25 ± 48.33
3.	1000ml	20	432.81 ± 15.99
4.	Tray (27cm x 22cm x 5cm)	100	475.42 ± 77.31
5.	Tray (35cm x 32cm x 5cm)	150	463.42 ± 92.16

Moreover, phytase production showing overall 5.6-fold enhancement makes the fermentation process less costly. The approach of fermentation in trays can be upgraded by using bioreactors for the production of phytase which provides constant temperature and moisture.[39]

Application of phytase in dephytinization of feed

Agricultural feed additives contain phytic acid that binds minerals, proteins, and other biomolecules and makes phytate complex which restricts the availability of nutritional components and lowers downs the nutritive value of feed. Phytase is effective in phytate hydrolysis and releases inorganic phosphate, soluble protein, and reducing sugar. Monogastric animals like humans, poultry, birds, and fishes lack phytase enzyme in their gastrointestinal region. Due to this, phytate accumulates in the body and remains undigested and excreted out in manure, runs off through erosion reaches water bodies, and pollutes the environment. Dephytinization is the best-selected approach to hydrolyze the phytate and release the P and other essential components by supplementing the phytase exogenously. The addition of phytase as a feed additive not only ameliorates the feed's nutritive value but also reduces phosphorus pollution.[29] The study demonstrated the impact of *P. oxalicum* phytase on hydrolyzing phytate present in the feed. With time, the liberation of inorganic phosphate, reducing sugar, and soluble protein is enhanced due to the supplementation of phytase in the diet.[10]

In this experiment, the effect of *P. oxalicum* phytase in dephytinizing fish feed is performed at different temperatures (37 °C, 50 °C, and 70 °C). The liberation of inorganic

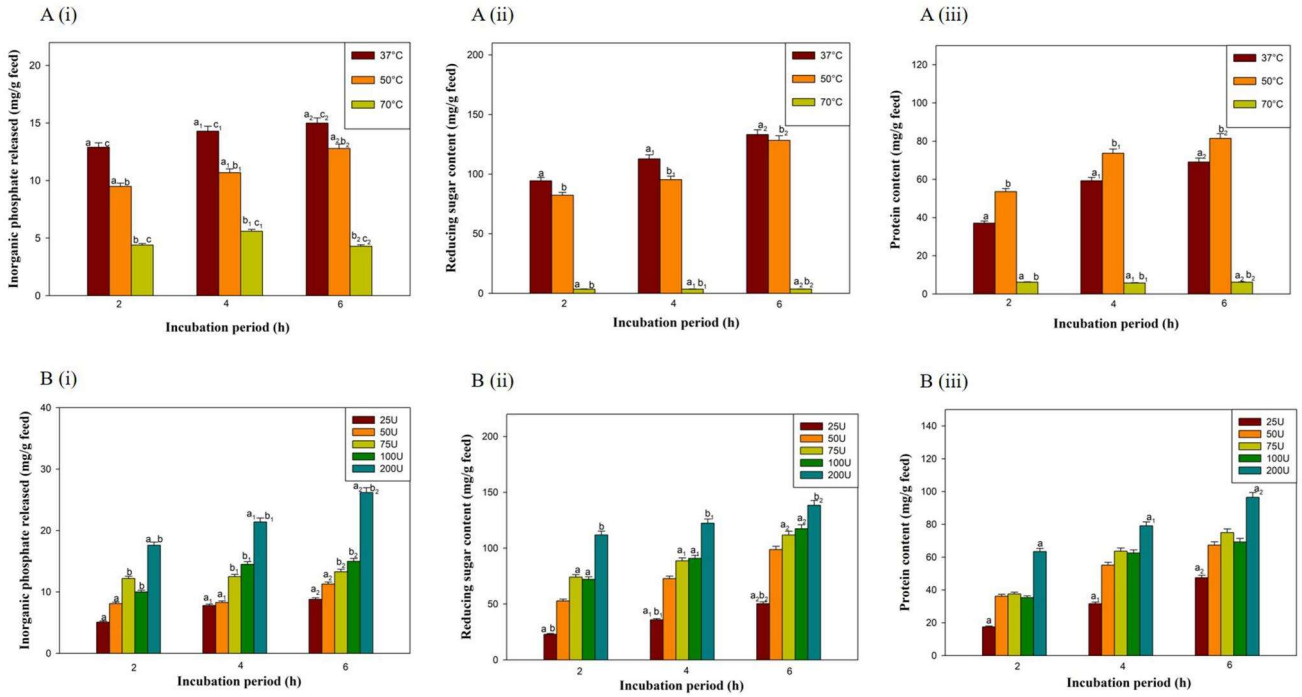


Figure 4. Dephytinization of feed by phytase from *P. oxalicum* A) at different temperature (37 °C, 50 °C, 70 °C), B) with different dose (25 U, 50 U, 75 U, 100 U, 200 U): i) inorganic phosphate ii) reducing sugar iii) protein content. The data showed as means ± standard deviation. Samples were studied in triplicates. Means with different letters represent values significantly different ($p < 0.05$).

phosphorus from the fish feed is observed at 37 °C, 50 °C, and 70 °C with 2, 4, and 6 h by using *P. oxalicum* phytase. A high release of inorganic phosphorus (14.993 mg/g feed) is seen at 37 °C (Figure 4A). Less difference is found in the amount of inorganic phosphorus between 37 °C and 50 °C showing the activeness of phytase enzyme at both temperatures. But, at 70 °C, there is almost a low secretion of inorganic phosphorus might be due to the denaturation of enzyme for a long period of incubation or disruption of nutritional components of feed. Also, nutritional by-products, soluble protein (69.08 mg/g feed), and reducing sugars (133.2) are liberated from the feed treated with phytase due to the presence of other hydrolytic enzymes in *P. oxalicum*. The amount of soluble protein is found more at 50 °C (81.48 mg/g feed). A significant variation ($p < 0.05$) was observed between feed sample treated at 37 °C and 70 °C after the supplementation of phytase.

Likewise, the reduction of phytic acid in flours was studied at 50 °C and 37 °C by adding phytase isolated from *A. oryzae* and exhibited maximum secretion of inorganic phosphate, soluble proteins and reducing sugars at 50 °C.[34] Dephytinization of feed samples was analyzed at 50 °C with *A. oryzae* phytase,[38] and at 55 °C with *R. oligosporus* phytase[39] and poultry feed at 37 °C by *A. niger* phytase[2] showed maximum liberation of nutritional components. Similarly, the release of inorganic phosphorus after the addition of phytase is studied in poultry feed by rPPHY at 40 °C,[13] animal feed by *P. griseoroseum* T73 at 37 °C,[4] pig feed by *A. niger* UFV-1 at 50 °C,[24] feedstuffs by *P. oxalicum* at 55 °C,[14] fish feed by *Morchella importuna* at 25 °C.[41]

Also, different phytase doses (25-200 U/g) were supplied to the feed and evaluated their impact on phytate degradation (Figure 4B). Phytase exhibits a dose-response relationship. The secretion of inorganic phosphorus is enhanced as the amount of phytase doses increases as the phytase removes an organically bound phosphate associated with the phytate molecule and reaches a plateau when the phytate becomes deficient.[43] The results showed that the liberation of inorganic phosphate enhances as the amount of dose increases. At 25 U/g (8.79 mg/g feed), 50 U/g (11.29 mg/g feed), 75 U/g (13.29 mg/g feed), 100 U/g (14.99 mg/g feed), and 200 U/g (26.19 mg/g feed) of inorganic phosphate released. Inorganic phosphate is found at maximum with

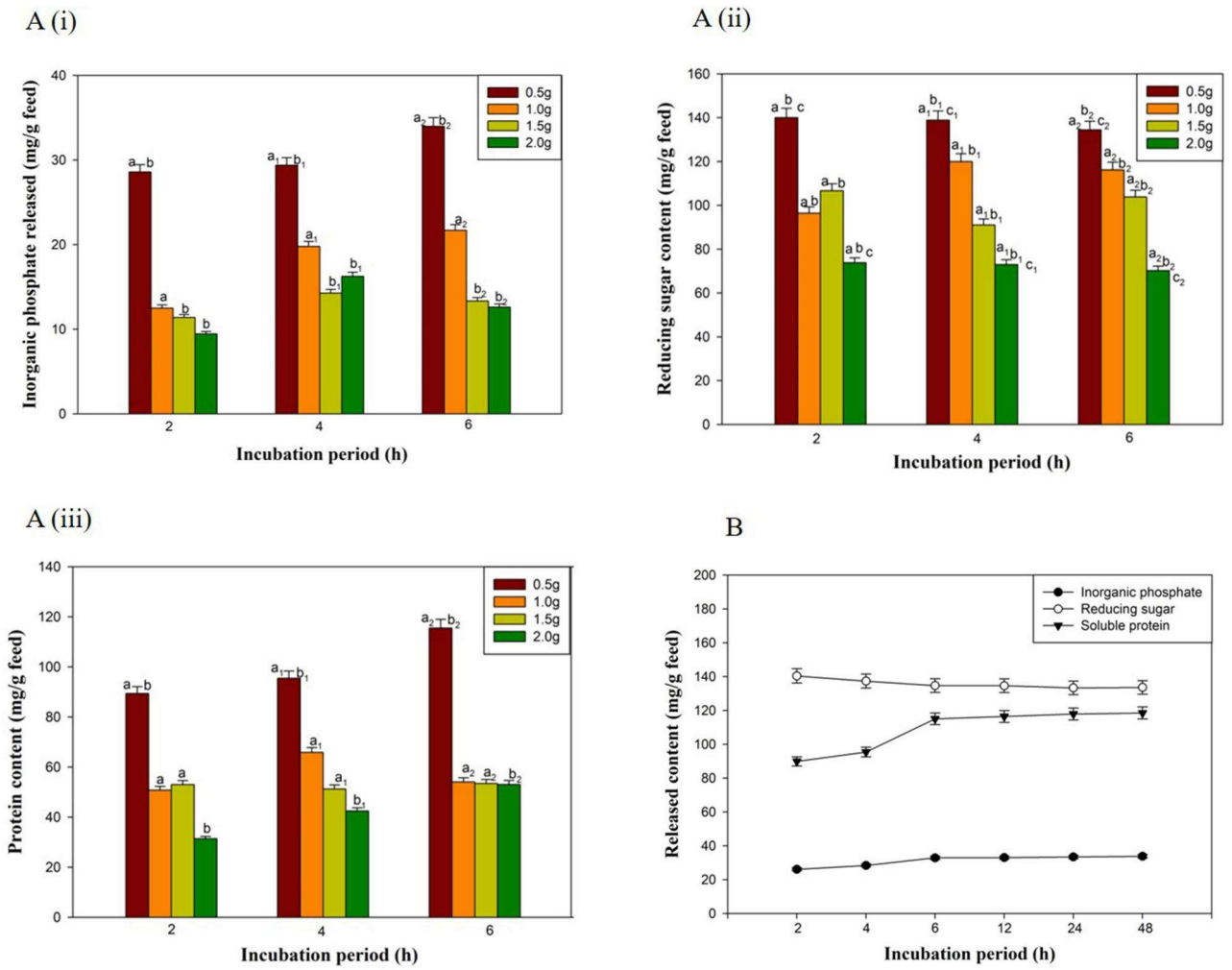


Figure 5. Dephytinization of feed by phytase from *P. oxalicum* A) with varied amount of feed (0.5 g, 1.0 g, 1.5 g, 2.0 g): i) inorganic phosphate ii) reducing sugar iii) protein content, B) with different incubation period. The data showed as means ± standard deviation. Samples were studied in triplicates. Means with different letters represent values significantly different ($p < 0.05$).

200 U/g (26.193 mg/g feed). Along with this, 138.48 mg/g feed and 96.5 mg/g feed amount of reducing sugar and protein content are obtained respectively. Reducing sugar is not much affected by increasing the phytase dosage, whereas soluble protein is enhanced. In contrast, poultry feed treated with different doses of *S. thermophile* showed equivalent nutritional amounts at 50 and 100 U,[17] while in flours it was observed that increment of these released components is gradually increased with phytase dose and declined after a certain level.[34]

The effect of phytase on varied amounts of feed (0.5-2 g) concluded that 0.5 g feed releases inorganic phosphate (33.986 mg/g feed) at the most (Figure 5A). This might be due to highest binding between active sites of substrate and enzyme in 0.5 g feed. As the amount of feed increases, no more enhancement is observed due to the possibility of no more binding sites availability or presence of substrate in insoluble form. Reduction in liberation of inorganic P might be due to negative feedback inhibition of inorganic P (the hydrolytic end product) on the phytase. Similarly, reducing sugar is found unaffected (134.4 mg/g feed) and protein is enhanced (115.52 mg/g feed).

Further, the dephytinization was performed for up to 48 h. Degradation of phytic acid with phytase shows the highest liberation of inorganic phosphate, soluble protein, and reducing sugars at 6 h and maintained up to 48 h, as shown in Figure 5B. Likewise, the release of inorganic phosphorus in poultry feed by rSt-Phy was evaluated on 25 min, 50 min and 75 min, with 50 min exhibited highest secretion.[31] At 2.5 h, pig feed represented the maximum release from *A. niger* UFV-1 phytase.[24] Supplementation of *A. niger* NT7 phytase in cattle feed secretes the highest inorganic phosphate up to 72 h.[15]

Maximum hydrolysis was observed with 200 U of *P. oxalicum* phytase added in 0.5 g of feed dissolved in 20 ml buffer and incubated at 37 °C for 6 h. The highest inorganic phosphorus (33.986 mg/g of feed), reducing sugars (134.4 mg/g of feed), and soluble protein (115.52 mg/g of feed) were found during the experiment.

Also, the application of immobilized phytase on feed and flours has been studied showing the release of inorganic phosphorus, reducing sugars and soluble proteins with time.[18] The comparison effect of free and immobilized phytase was evaluated on the hydrolysis of a legumes-based diet and concluded that immobilized phytase hydrolyzed more efficiently[26] while the efficiency of free and immobilized phytase is observed similar.[5] [6] performed the dephytinization of flours by supplementing both phytase and xylanase and showed the highest liberation due to the synergistic action of both enzymes.

Conclusion

In the present study, phytase production was enhanced by 5.6-fold by *Penicillum oxalicum* PBG30 as a result of statistical optimization in SSF using wheat bran as substrate. The maximum phytase production (475.42 U/g DMR) was obtained with 5 g wheat bran mixed with moistening media in a 1:2 ratio at 30 °C, pH 7.0, and 5 days of incubation. pH, incubation period, $MgSO_4$, and Tween 80 are essential components that majorly influence phytase production. Phytase production was sustainable in shake flasks and trays of different sizes. In addition, partially purified phytase is applied in the feed to determine the hydrolysis of phytate. It effectively degrades phytate and liberates inorganic phosphate from the fish feed. Also, it lessens the requirement for additional dietary inorganic phosphate in the feed. Therefore, cost-effective phytase from *P. oxalicum* can be produced at industrial scale and can be considered an excellent additive for fish feed in aquaculture and other industries.

Acknowledgement

The author acknowledges the Department of Biotechnology, Delhi Technological University, Delhi and Department of Microbiology, Maharshi Dayanand University, Rohtak for supporting this research work.

Authors contributions

The original draft was prepared and edited by Ms. Priya and all the experiments were performed by her. Data were analyzed by Priya, Dr. Bijender Singh, Dr. Bhoopander Giri and Dr. Jai Gopal Sharma. The draft was critically revised and improved under the supervision of Dr. Bijender Singh, Dr. Bhoopander Giri and Dr. Jai Gopal Sharma. All authors read and approved the final manuscript.

Disclosure statement

The authors declare no conflicts of result.

Financial support

The author (Ms. Priya) gratefully acknowledges the financial assistance as Junior/Senior research fellowship from the University Grants Commission (UGC), New Delhi, India.

ORCID

Bijender Singh http://orcid.org/0000-0002-2193-2910
Bhoopander Giri http://orcid.org/0000-0002-5510-4021

Data availability statement

Data will be made available on request.

References

[1] Arora, S.; Dubey, M.; Singh, P.; Rani, R.; Ghosh, S. Effect of Mixing Events on the Production of a Thermo-Tolerant and Acid-Stable Phytase in a Novel Solid-State Fermentation Bioreactor. *Process. Biochem.* **2017**, *61*, 12–23. DOI: 10.1016/j.procbio.2017.06.009.

[2] Buddhiwant, P.; Bhavsar, K.; Kumar, V. R.; Khire, J. M. Phytase Production by Solid-State Fermentation of Groundnut Oil Cake by *Aspergillus niger*: A Bioprocess Optimization Study for Animal Feedstock Applications. *Prep. Biochem. Biotechnol.* **2016**, *46*, 531–538. DOI: 10.1080/10826068.2015.1045606.

[3] Chanderman, A.; Puri, A. K.; Permaul, K.; Singh, S. Production, Characteristics and Applications of Phytase from a Rhizosphere

Isolated *Enterobacter* sp. ACSS. *Bioprocess Biosyst. Eng.* **2016**, *39*, 1577–1587. DOI: 10.1007/s00449-016-1632-7.

[4] Corrêa, T. L. R.; de Queiroz, M. V.; de Araújo, E. F. Cloning, Recombinant Expression and Characterization of a New Phytase from *Penicillium chrysogenum*. *Microbiol. Res.* **2015**, *170*, 205–212. DOI: 10.1016/j.micres.2014.06.005.

[5] Coutinho, T. C.; Tardioli, P. W.; Farinas, C. S. Phytase Immobilization on Hydroxyapatite Nanoparticles Improves Its Properties for Use in Animal Feed. *Appl. Biochem. Biotechnol.* **2020**, *190*, 270–292. DOI: 10.1007/s12010-019-03116-9.

[6] Dahiya, S.; Kumar, A.; Singh, B. Enhanced Endoxylanase Production by *Myceliophthora thermophila* Using Rice Straw and Its Synergism with Phytase in Improving Nutrition. *Process. Biochem.* **2020**, *94*, 235–242. DOI: 10.1016/j.procbio.2020.04.032.

[7] Elkhateeb, Y. A.; Fadel, M. Bioinformatic Studies, Experimental Validation of Phytase Production and Optimization of Fermentation Conditions for Enhancing Phytase Enzyme Production by Different Microorganisms under Solid-State Fermentation. *TOMICROJ.* **2022**, *16*, 1–11. DOI: 10.2174/18742858-v16-e2202160.

[8] Fiske, C. H.; Subbarow, Y. The Colorimetric Determination of Phosphorus. *J. Biol. Chem.* **1925**, *66*, 375–400. DOI: 10.1016/S0021-9258(18)84756-1.

[9] Hussain, S. M.; Hanif, S.; Sharif, A.; Bashir, F.; Iqbal, H. M. N. Unrevealing the Sources and Catalytic Functions of Phytase with Multipurpose Characteristics. *Catal. Lett.* **2021**, *152*, 1358–1371. DOI: 10.1007/s10562-021-03752-z.

[10] Jain, J.; Kumar, A.; Singh, D.; Singh, B. Purification and Kinetics of a Protease-Resistant, Neutral, and Thermostable Phytase from *Bacillus subtilis* Subsp. subtilis JJBS250 Ameliorating Food Nutrition. *Prep. Biochem. Biotechnol.* **2018**, *48*, 718–724. DOI: 10.1080/10826068.2018.1487848.

[11] Jain, J.; Singh, B. Phytase Production and Development of an Ideal Dephytinization Process for Amelioration of Food Nutrition Using Microbial Phytases. *Appl. Biochem. Biotechnol.* **2017**, *181*, 1485–1495. DOI: 10.1007/s12010-016-2297-z.

[12] Jatuwong, K.; Kumla, J.; Suwannarach, N.; Matsui, K.; Lumyong, S. Bioprocessing of Agricultural Residues as Substrates and Optimal Conditions for Phytase Production of Chestnut Mushroom, *Pholiota adiposa*, in Solid State Fermentation. *J. Fungi (Basel)* **2020**, *6*, 384. DOI: 10.3390/jof6040384.

[13] Joshi, S.; Satyanarayana, T. Bioprocess for Efficient Production of Recombinant *Pichia anomala* Phytase and Its Applicability in Dephytinizing Chick Feed and Whole Wheat Flat Indian Breads. *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 1389–1400. DOI: 10.1007/s10295-015-1670-1.

[14] Kim, B. H.; Lee, J. Y.; Lee, P. C. Purification, Sequencing and Evaluation of a Divergent Phytase from *Penicillium oxalicum* KCTC6440. *J. Gen. Appl. Microbiol.* **2015**, *61*, 117–123. DOI: 10.2323/jgam.61.117.

[15] Kumari, N.; Bansal, S. Production and Characterization of a Novel, Thermotolerant Fungal Phytase from Agro-Industrial Byproducts for Cattle Feed. *Biotechnol. Lett.* **2021**, *43*, 865–879. DOI: 10.1007/s10529-020-03069-8.

[16] Kumari, N.; Bansal, S. Statistical Modeling and Optimization of Microbial Phytase Production towards Utilization as a Feed Supplement. *Biomass Conv. Bioref.* **2021**, *13*, 8339–8349. DOI: 10.1007/s13399-021-01672-x.

[17] Kumari, A.; Satyanarayana, T.; Singh, B. Mixed Substrate Fermentation for Enhanced Phytase Production by Thermophilic Mould *Sporotrichum thermophile* and Its Application in Beneficiation of Poultry Feed. *Appl. Biochem. Biotechnol.* **2016**, *178*, 197–210. DOI: 10.1007/s12010-015-1868-8.

[18] Lopes, M. M.; Coutinho, T. C.; Malafatti, J. O. D.; Paris, E. C.; de Sousa, C. P.; Farinas, C. S. Immobilization of Phytase on Zeolite Modified with Iron (II) for Use in the Animal Feed and Food Industry Sectors. *Process. Biochem.* **2021**, *100*, 260–271. DOI: 10.1016/j.procbio.2020.10.017.

[19] Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R. J. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. DOI: 10.1016/S0021-9258(19)52451-6.

[20] Mahendran, S.; Sankaralingam, S.; Maheswari, P.; Dhivya, R. R.; Kathiresan, D.; Karthikeyan, S.; Ramya, S. S.; Seethapathy, P.; Harinathan, B.; Palpperumal, S. Production, Characterization, and Feed Supplement Applications of Phytase Enzyme from *Aspergillus tubingensis* Isolated from Western Ghats Soil. *Biomass Convers. Biorefinery.* **2022**, 1–11. DOI: 10.1007/s13399-022-02894-3.

[21] Mahmood, S.; Shahid, M. G.; Nadeem, M. Screening of Phytate Degrading Fungi and Optimization of Culture Conditions for Phytase Synthesis Using Agro-Industrial by-Products. *Pak. J. Bot.* **2021**, *53*, 763–770. DOI: 10.30848/PJB2021-2(12).

[22] Mahmood, S.; Shahid, M. G.; Nadeem, M.; Nelofer, R.; Irfan, M. Application of Statistical Design for the Economical Production of Phytase by *Aspergillus niger* Using Solid State Fermentation. *J. Anim. Plant Sci.* **2022**, *32*, 238–246. DOI: 10.36899/JAPS.2022.1.0419.

[23] Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. DOI: 10.1021/ac60147a030.

[24] Monteiro, P. S.; Guimarães, V. M.; Melo, R. R. D.; Rezende, S. T. D. Isolation of a Thermostable Acid Phytase from *Aspergillus Niger* UFV-1 with Strong Proteolysis Resistance. *Braz. J. Microbiol.* **2015**, *46*, 251–260. DOI: 10.1590/S1517-838220120037.

[25] Neira-Vielma, A. A.; Aguilar, C. N.; Ilyina, A.; Contreras-Esquivel, J. C.; Carneiro-da-Cunha, M. D. G.; Michelena-Álvarez, G.; MartÃnez-Hernandez, J. L. Optimized Production of Phytase by Solid-State Fermentation Using Triticale as Substrate and Source of Inducer. *Afr. J. Biotechnol.* **2018**, *17*, 81–90. DOI: 10.5897/AJB2017.16267.

[26] Onem, H.; Cicek, S.; Nadaroglu, H. Immobilization of a Thermostable Phytase from Pinar Melkior (*Lactarius piperatus*) onto Magnetite Chitosan Nanoparticles. *CYTA J. Food.* **2016**, *14*, 74–83. DOI: 10.1080/19476337.2015.1045942.

[27] Pable, A. A.; Shah, S.; Ravi Kumar, V.; Khire, J. M. Use of Plackett–Burman Design for Enhanced Phytase Production by *Williopsis saturnus* NCIM 3298 for Applications in Animal Feed and Ethanol Production. *3 Biotech.* **2019**, *9*, 237. DOI: 10.1007/s13205-019-1764-y.

[28] Plackett, R. L.; Burman, J. P. The Design of Optimum Multi-Factor Experiments. *Biometrika.* **1946**, *33*, 305–325. DOI: 10.1093/biomet/33.4.305.

[29] Priya; Virmani, I.; Pragya; Goswami, R. K.; Singh, B.; Sharma, J. G.; Giri, B. Role of Microbial Phytases in Improving Fish Health. *Rev Aquac* **2023**, *15*, 1480–1500. DOI: 10.1111/raq.12790.

[30] Qasim, S. S.; Shakir, K. A.; Al-Shaibani, A. B. Isolation, Screening and Production of Phytate Degrading Enzyme (Phytase) from Local Fungi Isolate. *Iraqi J. Agric. Sci.* **2016**, *47*, 121–128.

[31] Ranjan, B.; Satyanarayana, T. Recombinant HAP Phytase of the Thermophilic Mold *Sporotrichum thermophile*: expression of the Codon-Optimized Phytase Gene in *Pichia pastoris* and Applications. *Mol. Biotechnol.* **2016**, *58*, 137–147. DOI: 10.1007/s12033-015-9909-7.

[32] Saxena, A.; Verma, M.; Singh, B.; Sangwan, P.; Yadav, A. N.; Dhaliwal, H. S.; Kumar, V. Characteristics of an Acidic Phytase from *Aspergillus aculeatus* APF1 for Dephytinization of Biofortified Wheat Genotypes. *Appl. Biochem. Biotechnol.* **2020**, *191*, 679–694. DOI: 10.1007/s12010-019-03205-9.

[33] Sharma, Krishna Kant; Kumar, Sandeep; Singh, Davender; Kumar, Vijay; Singh, Bijender; Pragya; Manisha. Enhanced Production and Immobilization of Phytase from *Aspergillus oryzae*: A Safe and Ideal Food Supplement for Improving

Nutrition. *Lett. Appl. Microbiol.* **2023**, *76*(2), ovac077. DOI: 10.1093/lambio/ovac077.

[34] Sharma, Krishna Kant; Singh, Bijender; Pragya. Phytase from *Aspergillus oryzae* SBS50: Biocatalytic Reduction of Anti-Nutritional Factor and Exhibiting Vanadium-Dependent Haloperoxidase Activity. *Biocatal. Agric. Biotechnol.* **2023**, *52*, 102840. DOI: 10.1016/j.bcab.2023.102840.

[35] Singh, Bijender; Boukhris, Ines; Kumar, Vinod; Yadav, Ajar Nath; Farhat-Khemakhem, Ameny; Kumar, Anil; Singh, Davender; Blibech, Monia; Chouayekh, Hichem; Alghamdi, Othman A.; Pragya. Contribution of Microbial Phytases to the Improvement of Plant Growth and Nutrition: A Review. *Pedosphere*. **2020**, *30*(3), 295–313. DOI: 10.1016/S1002-0160(20)60010-8.

[36] Singh, Bijender; Sapna. Biocatalytic Potential of Protease-Resistant Phytase of *Aspergillus oryzae* SBS50 in Ameliorating Food Nutrition. *Biocatal. Biotransform.* **2015**, *33*(3), 167–174. DOI: 10.3109/10242422.2015.1076215.

[37] Singh, Bijender; Sapna. Phytase Production by *Aspergillus oryzae* in Solid-State Fermentation and Its Applicability in Dephytinization of Wheat Bran. *Appl. Biochem. Biotechnol.* **2014**, *173*, 1885–1895. DOI: 10.1007/s12010-014-0974-3.

[38] Singh, Bijender; Sapna. Purification and Characterization of a Protease-Resistant Phytase of *Aspergillus oryzae* SBS50 Whose Properties Make It Exceptionally Useful as a Feed Supplement. *Int. J. Biol. Macromol.* **2017**, *103*, 458–466. DOI: 10.1016/j.ijbiomac.2017.05.077.

[39] Suresh, S.; Radha, K. V. Effect of a Mixed Substrate on Phytase Production by *Rhizopus oligosporus* MTCC 556 Using Solid State Fermentation and Determination of Dephytinization Activities in Food Grains. *Food Sci. Biotechnol.* **2015**, *24*, 551–559. DOI: 10.1007/s10068-015-0072-5.

[40] Suresh, S.; Radha, K. V. Statistical Optimization and Mutagenesis for High Level of Phytase Production by *Rhizopus oligosporus* MTCC 556 under Solid State Fermentation. *J. Environ. Biol.* **2016**, *37*, 253.

[41] Tan, H.; Tang, J.; Li, X.; Liu, T.; Miao, R.; Huang, Z.; Wang, Y.; Gan, B.; Peng, W. Biochemical Characterization of a Psychrophilic Phytase from an Artificially Cultivable Morel *Morchella importuna*. *J. Microbiol. Biotechnol.* **2017**, *27*, 2180–2189. DOI: 10.4014/jmb.1708.08007.

[42] Thakur, N.; Patel, S. K.; Kumar, P.; Singh, A.; Devi, N.; Sandeep, K.; Pandey, D.; Chand, D. Bioprocess for Hyperactive Thermotolerant *Aspergillus fumigatus* Phytase and Its Application in Dephytinization of Wheat Flour. *Catal. Lett.* **2022**, *152*, 3220–3232. DOI: 10.1007/s10562-021-03886-0.

[43] Thorsen, M.; Nielsen, L. A.; Zhai, H. X.; Zhang, Q.; Wulf-Andersen, L.; Skov, L. K. Safety and Efficacy Profile of a Phytase Produced by Fermentation and Used as a Feed Additive. *Heliyon.* **2021**, *7*, e07237. DOI: 10.1016/j.heliyon.2021.e07237.

MAHARSHI DAYANAND UNIVERSITY ROHTAK
(A+ GRADE UNIVERSITY ACCREDITED BY NAAC)

Azadi ka Amrit Mahotsav

63rd Annual International Conference of
Association of Microbiologists of India (AMI)

Microbial Technologies for Sustainable BIOSPHERE

Microbio Tech- 2023

(2nd-4th February, 2023)

Certificate of Appreciation

This is to certify that Mr./Ms./Mrs./Dr. Priya participated/presented a Poster/~~Oral/Invited/Lead Lecture~~ entitled Optimization of phytase production ~~-----~~ utilization as a feed supplement at 63rd Annual International Conference of Association of Microbiologists of India conducted by Maharshi Dayanand University Rohtak (Haryana) on 2nd to 4th February 2023.

Prof. R. C. Kuhad	Prof. Praveen Rishi	Prof. Namita Singh	Dr. K. K. Sharma
Conference Chairperson	President AMI	General Secretary AMI	Organizing Chairperson

Reg. No. : 1398

INTERNATIONAL CONFERENCE
ON
STRATEGIES FOR GLOBAL FOOD AND NUTRITIONAL SECURITY, SUSTAINABILITY AND WELLNESS
(NUTRI-2023)

DECEMBER 04-06, 2023

Certificate of Participation

This certificate is awarded to Ms. PRIYA, Delhi Technological University, Delhi for Participation and Poster presentation on "In-vitro application of Penicillium oxalicum phytase in the aquafeed industry" in the International Conference on Strategies for Global Food and Nutritional Security, Sustainability and Wellness (NUTRI-2023) held at CCS Haryana Agricultural University, Hisar (Haryana) India.

Dr. Rajesh Gera
Co-Organizing Secretary-cum-
Additional Director of Research

NAHEP

Dr. Jeet Ram Sharma
Organizing Secretary-cum-
Director Research

ICMBSDG
Microbial Bioprospecting Towards Sustainable Development Goals

LOVELY PROFESSIONAL UNIVERSITY
Transforming Education Transforming India

NAAC GRADE A++

Certificate No. 299006

Certificate of Participation

This is to certify that **Prof./Dr./Mr./Ms. Priya** of **Delhi Technological University** has Participated/Presented a Poster Presentation entitled **Partial purification and characterization of phytase from Penicillium oxalicum for feed industry** in International Conference on **"Microbial Bioprospecting Towards Sustainable Development Goals"** held on **24th- 25th November 2023** organized by Association of Microbiologist of India-LPU Unit and Society of Chemical and Synthetic Biology at Lovely Professional University, Punjab.

Date of Issue : 12-12-2023
Place : Phagwara (Punjab), India

Prepared by
(Administrative Officer-Records)

Dr. Arun Karnwal
President
AMI-LPU Unit

Dr. Karthik Loganathan
President
SCBS

Dr. Ashish Vyas
Organizing Secretary
AMI-LPU Unit

Dr. Neeta Raj Sharma
Senior Dean
SBEB, LPU

DEPARTMENT OF BIOTECHNOLOGY
MINISTRY OF SCIENCE & TECHNOLOGY, GOVERNMENT OF INDIA

ICIBLS/PC/344

CERTIFICATE

OF PARTICIPATION

This certificate is awarded to

Priya

for participating in International Conference on Innovations in Biotechnology and Life Sciences, 2020 (ICIBLS) from December 18th to 20th, 2020

Prof. Jai Gopal Sharma
Patron
Head, Dept. of Biotechnology, DTU

Dr. Asmita Das
Convenor
Dept. of Biotechnology