PRODUCTION, IMMOBILIZATION AND CHARACTERIZATION OF FUNGAL PHYTASE AND ITS UTILIZATION IN FOOD AND FEED INDUSTRY

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IN

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BY

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Dedicated to my loving family

"Love opens the most impossible gate; love is the gate to all the secrets of the universe"

Swami Vivekananda

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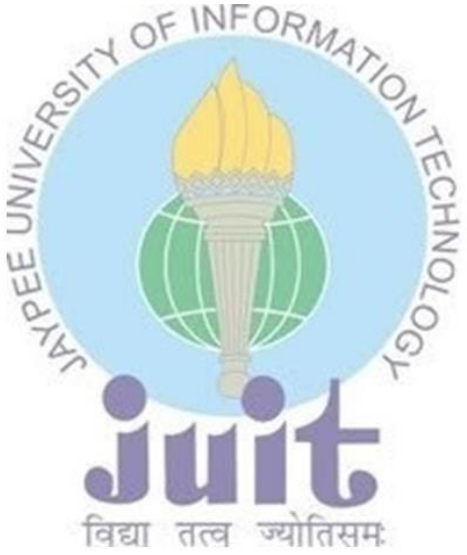
DECLARATION BY THE SCHOLAR

I, **Neha Kumari** hereby declare that the work reported in the Ph.D. thesis entitled **"Production, Immobilization and Characterization of Fungal Phytase and its Utilization in Food and Feed Industry"** submitted at **Jaypee University of Information Technology, Waknaghat, India** is an authentic record of my work carried out under the guidance of **Dr. Saurabh Bansal**. This work has not submitted elsewhere for any other degree or diploma. I am fully accountable for the contents of my Ph.D. thesis.



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SUPERVISOR'S CERTIFICATE

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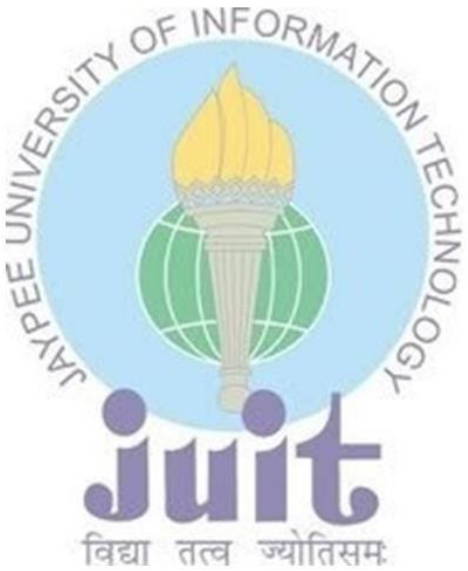

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ABSTRACT

ABSTRACT

The phytases hydrolyze the phytic acid to release the bound phosphorus and other vital nutrients in the feed of domestic animals, plummeting the requirement for extra subjection to feed. However, its short half-life owing to less stability and the high production cost due to the unavailability of cheaper sources for phytase production limited its application in developing countries. Also, fewer studies have been reported on phytases usage in humans. The use of phytase as a food additive can overcome the issue of malnutrition and silent hunger in developing countries. Besides this, using phytase in the animal feed not only reduces the cost of the livestock industry but also deals with the concern of phosphorus pollution (eutrophication) up to a level. Therefore, in the current study, a fungal culture, *Aspergillus niger* NT7, was isolated from the soil sample of the agriculture field and identified as a potential phytase producer. The *A. niger* NT7 phytase production was done using an economical substrate, wheat bran, an agricultural waste, via solid-state fermentation (SSF). The enhanced phytase production was obtained through optimizing different physiochemical parameters by two approaches; one variable at a time (OVAT) and statistical Response Surface Methodology (RSM). The optimized parameters obtained by the OVAT are the moistening agent (distilled water), inoculum age (3-days) and level (15×10^7 spores/ml), pH (5.0), temperature (30 °C) and different supplements of biochemical such as nitrogen (ammonium sulphate), sugar (mannitol), phosphorous (potassium dihydrogen phosphate) and detergent (Tween 80) source. The optimization procedures through OVAT (208 ± 0.22 U/gds) enhanced the overall output by around 2.7-fold compared to unoptimized culture conditions (76.34 ± 0.99 U/gds).

Since the OVAT does not study the interaction between multiple factors concurrently and the approach is time-consuming and laborious, the parameters for phytase production were sub-optimized. In contrast, through response surface methodology (RSM), various factors significantly affecting phytase production in SSF can be studied concurrently through statistical and mathematical models. Therefore, a statistical RSM approach was used to optimize further six significant parameters identified from the OVAT approach to enhance the additional phytase production. After five days of SSF, when carried out at 35 °C with 5 g of wheat bran, supplemented with 2% mannitol and 0.5% ammonium sulfate maintaining a pH of 4.3, resulting in the improved phytase (521 ± 28.16 U/gds) production. Compared

with the unoptimized culture conditions and the OVAT method, the phytase yield (521 ± 28.16 U/gds) after statistical optimization increased by 6.8 and 2.5-times, respectively. The crude phytase exhibited the proficient and persistent discharge of proteins, sugars (>60 h) and principally inorganic phosphorus from the wheat and maize bran. As per our best knowledge, this is the first report that shows maize bran nutritional enhancement and ICP-MS analysis of released minerals using crude enzyme *A. niger* NT7 preparation. The current work effectively indicates the possible use of *A. niger* NT7 phytase in feed supplementation to diminish the antinutrient character of phytate molecules.

For the application of phytase for humans, the phytase was purified to homogeneity using ammonium sulphate precipitation and anion exchange chromatography. The purified phytase was found to be proteolytic resistant and has optimal activity in acidic pH (5.0) and at a pretty high temperature (50 °C). The physiochemical properties of purified phytase make it a possible option to be utilized as a food additive. The purified phytase was immobilized on chemically synthesized ZnO nanoparticles to improve the stability and other physiochemical properties further. The immobilized phytase on ZnP (Phy-ZnP) has shown improved thermostability, protease resistance, higher optimal temperature (70 °C) and low pH (4.0) compared to free phytase (Phy). Phy-ZnP can be used effectively for up to 10 catalytic cycles.

The application of purified phytase on sorghum flour improves its nutritional content in simulated digested conditions. The release of the nutrients (phosphorous, reducing sugar, proteins and free amino acid) were higher after the test gastric phase treatment than the test intestinal phase. The observed release of Zn and Fe metal ions was higher in the stomach phase, but Mn and Ca were more released in the intestinal phase. This is the first study on sorghum flour to show such results. Another study on maize flour for dephytinization has shown that Phy-ZnP dephytinizes maize flour more efficiently than Phy, resulting in improved nutrient release. The current prevailing study efficiently explains the potential application of fungal phytase as feed and food additive.

Keywords: *A. niger* NT7; Phytase; Solid-state fermentation; Optimization; Response surface methodology; Purification; Immobilization; Zinc oxide nanoparticles; Dephytinization; Mineral release

LIST OF SYMBOLS & ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µg/ml	Micrograms per milliliter
µmol	Micromole
ANOVA	Analysis of variance
BME	β-mercaptoethanol
BPPs	β-propeller phosphatases
BSA	Bovine Serum Albumin
CCD	Central composite design
cm	Centimeter
DEAE	Diethylaminoethyl
EC	Enzyme commission
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FESEM	Field emission electron microscope
FTIR	Fourier transform infrared spectroscopy
FTU	Phytase unit
g	Gram
g/l	Grams per liter
GI	Gastrointestinal tract
GRAS	Generally recognized as safe
h	Hours
H_2SO_4	Sulfuric acid

HAP	Histidine acid phosphatase
HCl	Hydrochloric acid
HNO_3	Nitric acid
ICP-MS	Inductive coupled plasma-mass spectrophotometry
KDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium iodide
l	Liter
M	Molar
mg/ml	Milligrams per milliliter
min	Minutes
mm	Millimeter
ml	Milliliter
mM	Millimolar
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NH_4NO_3	Ammonium nitrate
nm	Nanometer
OVAT	One variable at a time
P	Phosphorous
PAGE	Polyacrylamide gel electrophoresis
PAPs	Purple acid phosphatases

PDA	Potato dextrose agar
Phy	Phytase
Phy-ZnP	Phytase immobilized on zinc oxide nanoparticles
Pi	Inorganic phosphorous
PSM	Phytase screening media
rpm	Revolutions per minute
RSM	Response surface methodology
SDS	Sodium dodecyl sulphate
SmF	Submerged fermentation
SSF	Solid state fermentation
sSSF	Semi-solid state fermentation
TEMED	Tetramethylethylenediamine
U	Unit
UV-VIS	Ultraviolet-visible
w/v	Weight by volume
XRD	X-ray diffraction
ZnP	Zinc oxide (ZnO) nanoparticles
$MnCl_2.4H_2O$	Manganese (II) chloride tetrahydrate
$FeSO_4.7H2O$	Iron (II) sulfate heptahydrate
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate
$(NH_4)_2SO_4$	Ammonium sulphate
$CaCl_2$. $7H_2O$	Calcium chloride dehydrate
$MgSO_4$. $7H_2O$	Magnesium sulfate heptahydrate

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CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

In this period, we are part of a culture that is becoming increasingly health-conscious, where wellness is of utmost importance. Currently, environmental pollution and waste management are extremely important, and food safety is of paramount importance. Animal feed plays an important part in producing safe food, and recent events have highlighted its influence on public health, feed and food commerce, and food security. New feed products, particularly biotechnology products, are being developed as a result of economic and technical advancements. The enzymes are currently being utilized on the industrial scale for enhancing the nutritional values of various food formulations [1]. New innovative development for using biocatalyst is very prevalent in the food sector. Over the last two decades, phytases (EC 3.1.3.8 and 3.1.3.26) have become outstandingly important enzymes for ameliorating nourishment and ecological protection, with a significant impact on human health [2, 3]. Phytases cleave orthophosphate groups from the phytic acid molecules, releasing bound nutrients (Figure 1.1). In cereals, legumes, and other plant-based oilseeds, the inositol core of phytate is the most common phosphorus storage form (>80%) [4, 5]. However, only a few organisms can hydrolyze phytic acid and use the released phosphorus. Bacteria, yeast, and fungi can hydrolyze phytate as they possess the phytase enzyme [6, 7]. In contrast, monogastric animals lack the phytase enzyme and thus cannot digest phytate molecules, resulting in malnutrition. In addition, unconsumed phytate causes eutrophication and phosphorus pollution when discharged into the ecosystem as excreta [8, 9]. Under normal physiological circumstances, phytic acid forms complexes with essential minerals, including calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{2+}), and zinc (Zn^{2+}) (Figure 1.1). It also inhibits digestive juices by adhering to the bioactive peptides [4, 5].

Dephosphorylation of phytic acid during food processing generates only partially phosphorylated derivatives of *myo*-inositol products. For complete dephosphorylation, phytases should be used in the food industry to develop quality foodstuffs with decreased phytic acid content and enhanced nutritional status (soluble protein, reducing sugar and inorganic phosphorus; Pi) [10, 11]. The cationic metal ions, proteins, amino acids, essential minerals, and other components chelated with phytic acid are released by phytase, along with the release of Pi and *myo*-inositol (Figure 1.1).

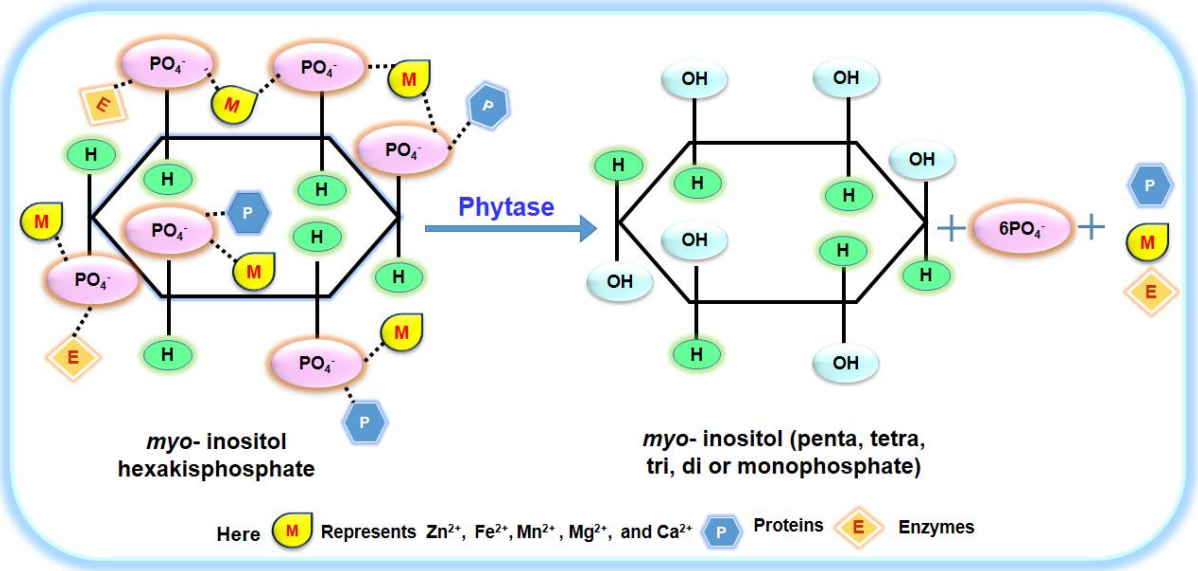


Figure 1.1 Hydrolysis of phytic acid with phytase treatment to release conjugated nutrients (cationic metal ions, proteins and enzymes) along with free *myo*-inositol.

Incorporating phytase into pig, fish, and poultry feed reduces or eliminates the need for supplementing Pi in their diets [12, 13]. It improves nutrient utilization efficiency, diminishes phosphorus efflux, indeed reduces phosphorus pollution and facilitates the maintenance of human and ecosystem health. Countries like Netherland have strict phosphorous management rules, so enzyme expenses counterbalance the heavy fines imposed for disposing of untreated waste (mainly phosphorous) [14, 15]. Phytase hydrolysis improves nutritional quality in plant-based meals by increasing proteins and minerals bioavailability. The by-products of phytate degradation, i.e., *myo*-inositol phosphates, are used to treat Multiple sclerosis, Alzheimer's, and Parkinson's disease. Some *myo*-inositol phosphates may have medicinal benefits, such as antioxidative properties, anti-cancerous, lowering cholesterol and lipids levels in serum, minimizing kidney calculi by mineral deposition, and diabetic mitigation [7, 14-17].

1.2 Scope of the current study

The traditional approaches (soaking, cooking, roasting, fermentation and addition of vitamin-C) opted for phytic acid reduction are less efficient and time-consuming. Phytase treatment can completely hydrolyse and remove all the phytic acid without compromising the nutritional status of feed and food formulations [18, 19]. Phytase has high nutritional market value globally as well as in the Indian market. In the Indian scenario, the growth rate of the phytase industry is increasing at a rate of 10-12 % per annum [14].

The available phytases are unstable and exhibit inconsistent behavior in the gastrointestinal tract of monogastric animals. The production costs associated with them also limit their applicability. As a result, there is an immense need for novel phytase-producing microbial strains with lower production costs [7, 8, 20-22].

Another limiting factor is that commercially available phytase cannot withstand the pelleting temperature of feed treatment. Phytase should have strong pH and proteolysis resistance to remain catalytically active in the digestive tract of monogastric animals. Bacterial phytases are active in the alkaline range only. Furthermore, phytase becomes inactivated as the pH shifts from the alkaline to the acidic range during fermentation. The shortcomings associated with bacterial and plant phytases can be overcome by fungal phytases [3, 22].

The rationale for utilizing fungal sources for phytase is that

- Fungal sources are already known as efficient producers of various extracellular enzymes.
- Fungal sources can be used in transforming waste into value-added products, which helps to reduce costs and ease downstream processing.
- Fungal phytases are active over an acidic range and can withstand the harsh conditions of the gastrointestinal tract.
- The fungal phytases are thermostable, i.e., the prerequisite condition required for industrial application.
- Besides this, fungal biomass can directly be utilized as animal feed.

Improving the catalytic properties of phytase through immobilization is a more fascinated and active field in enzyme research. Immobilization on zinc oxide nanoparticles (ZnP; having high chemical stability, surface-to-volume ratio, catalytic efficiency, biocompatibility, and non-toxic) enhances the operational stability (thermostability and reusability) of the enzyme [23].

1.3 Objectives and outlines of the thesis

In view of the aforementioned discussion and the lack of a suitable and ideal phytase with desirable features such as active at acidic pH, protease resistance, and thermostable, the current study was planned and carried out with the following aims:

- To optimize the solid-state fermentation conditions for phytase production from isolated *A. niger* NT7

- Modeling and optimization of phytase production through response surface methodology and its application in the feed industry
- Purification, immobilization, and characterization of fungal phytase
- Applications of fungal phytase in the food industry

In the current study, phytase was produced from *Aspergillus niger* NT7 using agricultural residues as a substrate for solid-state fermentation. Agricultural leftovers are trash with little utility in modern culture. They cause significant economic losses as well as environmental degradation by producing harmful gases as a result of natural fermentation. Bioconversion of these wastes to a commercially relevant enzyme such as phytase is promising in terms of waste value addition, bioprocess economics, waste disposal, and environmental pollution reduction. Therefore, the current study aims to enhance the phytase production using agricultural waste (wheat bran) by optimizing various physiochemical parameters using the One Variable at A Time approach (OVAT) and a statistical approach through Response Surface Methodology (RSM). Another objective of the current study was the analysis of the utilization of crude phytase preparation for wheat bran and maize bran dephytinization so that the same can be used as a feed additive.

For the utilization of phytase for human usage, the enzyme must be purified and well characterized. The operational stability of the phytase is also very important for its economic feasible production and usability in the industry. Therefore, the next objective of the study was to purify the phytase and immobilize it on zinc oxide nanoparticles (ZnP). The soluble enzyme and the immobilized phytase were then characterized and compared. The final and last objective of the current study is to establish its applicability for human usage. Therefore, the purified phytase was used in a simulated human gastric and intestinal batch model for the dephytinization of sorghum flour. The free and immobilized phytase were also used for maize flour dephytinization to show further its application in human food. Moreover, phytase immobilization on ZnP enhances the enzyme's operational stability.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Phytic acid

Phytic acid (*myo*-inositol hexakisphosphate) is a ubiquitous molecule found in the form of calcium/magnesium salt of inositol hexaphosphoric acid. In 1855-1856, phytic acid was discovered in seeds as a small round biomolecule [1, 2]. Phytic acid is commonly present in all plant-derived foods and feeds [3, 4] (Table 2.1). Naturally occurring plant-based fibrous foods, grains, oilseeds, legumes and nuts store 80% phosphorous in this bounded form [5, 6]. Green leafy vegetables and fruits contain phytic acid as a phosphorous reservoir [7]. Phytic acid serves as an energy source as well as its derived *myo*-inositol also plays an imperative role in plant physiological functionality. Studies have shown that low phytate mutants have poor germination rate, low survival rate, poor agronomic performance and are more sensitive to environmental stresses [8, 9]. Phytic acid is a naturally occurring antioxidant as it protects seeds and foods from oxidative stresses as well as has a preventive role in curing various human diseases [10]. Phytic acid generally disguises in its functional role as it is regarded as an anti-nutrient in food and feed formulations; however, it also acts as an antioxidant, anticarcinogenic and hypoglycemic agent [11, 12].

Table 2.1 List of common plant-based foods with phytic acid content.

Plant-Based Food Formulation	Phytic Acid Content (%)
Oil seed-based food	
Dried sesame seed	1.44-5.36
Soybean	1.0-2.22
Dried Linseed (Flax) seed	2.15-3.69
Watermelon seed	1.36
Pumpkin embryo	4.08
Sunflower embryo	2.10
Dried mustard seed	2.0
Legumes	
Lentils	0.27-1.51
Dried cowpeas	0.37-2.90
Dried beans	1.41
Cocoa beans	1.04
Broad beans	0.51-1.77
Kidney beans	0.61-2.38
Chickpea	0.28-1.60
Peas	0.22-1.22

Cereal based food	
Dried sorghum grain	0.57-3.35
Barley	0.38-1.16
Maize	6.39
Oats	0.42-1.16
Rice	0.06-1.08
Wheat germ	1.14-3.91
Nut based food	
Pine nut	0.20
Hazelnut	0.23-0.92
Peanut	1.70
Pistachio	1.38
Cashew nut	0.19-4.98
Walnut	0.20-6.69
Almond	0.35-9.42
Brazil nut	0.29-6.34
Agro-industrial byproduct	
Maize bran	0.72-2.22
WB	2.1-7.3
Rice bran	2.56-8.7
Others	
Kiwi fruit	1.34
Eggplant seed	1.42
Tomato seed	1.66
Cucumber Seed	1.07

Sources: Adapted from [4, 13]

Phytic acid is chemically composed of a core inositol ring with six phosphate groups that has a resilient electrostatic interaction with positively charged biomolecules (metal ions, digestive proteins, amino acids and carbohydrates) [14] (Figure 2.1). The chelating property of polyanionic phytic acid is desirable for treating tumor proliferation as cationic ions (Fe^{3+}, Zn^{2+} and Cu^{2+}) are detrimental to their progression [15]. Positive aspects showed that phytase has antitumor, antidiabetic (binds with starch results in reduced glycemic index) properties, also balances the cholesterol level and has therapeutic effects on Parkinson's and Alzheimer's disease [16-20]. Phytic acid also inhibits aortic and renal calcification by altering the bioavailability of ions such as Zn^{2+} and Ca^{2+} by chelating them [7]. Phytic acid has the potential

to prevent kidney stones by inhibiting the formation of calcium-oxalate complexes [11, 21] (Figure 2.2).

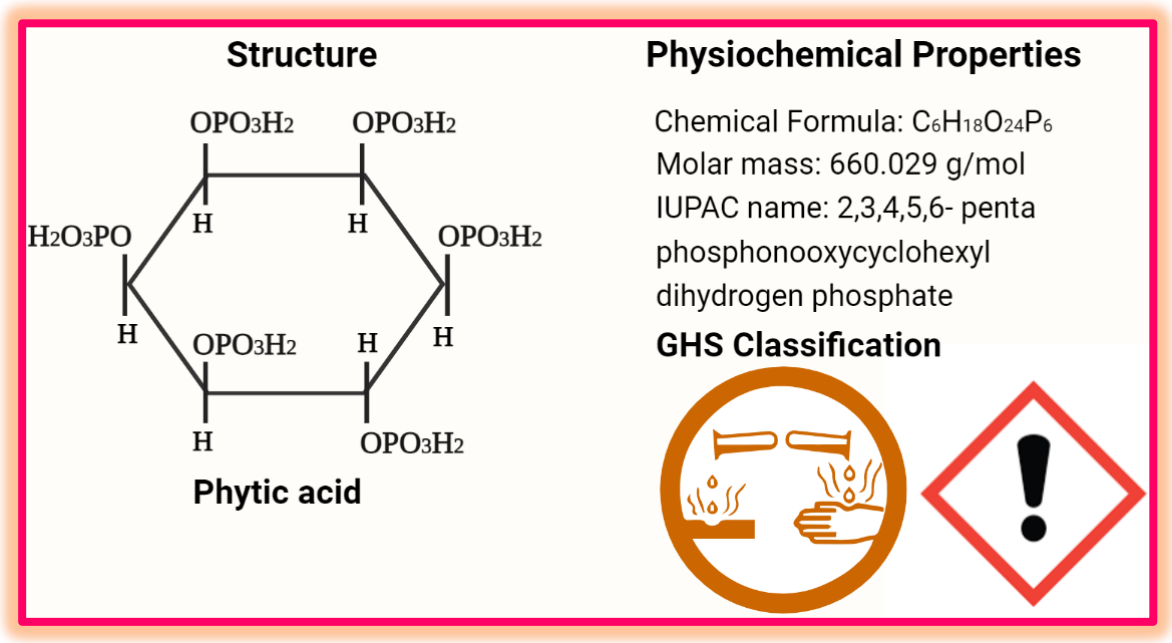


Figure 2.1 The structure and physicochemical properties of phytic acid.

Phytic acid contributes negatively to the health of monogastric animals, which rely mainly on plant-based fibrous food formulations [12]. Phytic acid forms a stable ternary complex as the metal-protein-phytic acid forms a sandwich-like structure that remains unaffected and non-soluble at the physiological pH of the digestive system of monogastric animals [22]. Phytic acid further makes the conditions more complicated as it hinders the absorption of metal ions (Fe^{3+}, Fe^{2+}, Ca^{2+}, $Zn^{2+}$and Cu^{2+}) and thus reduces the bioaccessibility of essential minerals and nutrients [11, 12]. Phytic acid notoriety affects the functionality and solubility of proteins as it forms complexes with digestive enzymes (lysozyme), making them catalytically inactive (Figure 2.2). Nutritionists have evidenced that phytic acid has a negative impact on feed formulation due to the phytate-protein complex [12, 23]. As most of the population consumes phytate-rich cereals, this is the most common cause of malnutrition and anaemia like conditions in developing countries (Asians) [24, 25]. Phytic acid is arduous to the digestive process of monogastric animals due to its unavailability or poor expression of synthetic machinery to digest and absorb the highly phosphorylated form of phytic acid [13]. Therefore, phytic acid is regarded as an anti-nutrient factor. Extra supplementation of inorganic phosphorous (Pi) is done to meet its requirements, which adds an extra burden to sustain the livestock industry. Moreover, the Pi, which remains unabsorbed by monogastric animals, will pass into the

environment through their faecal matter. This faecal matter can run off to contaminate water reservoirs, leading to algal blooms (eutrophication) and hypoxia conditions in aquatic animals [26].

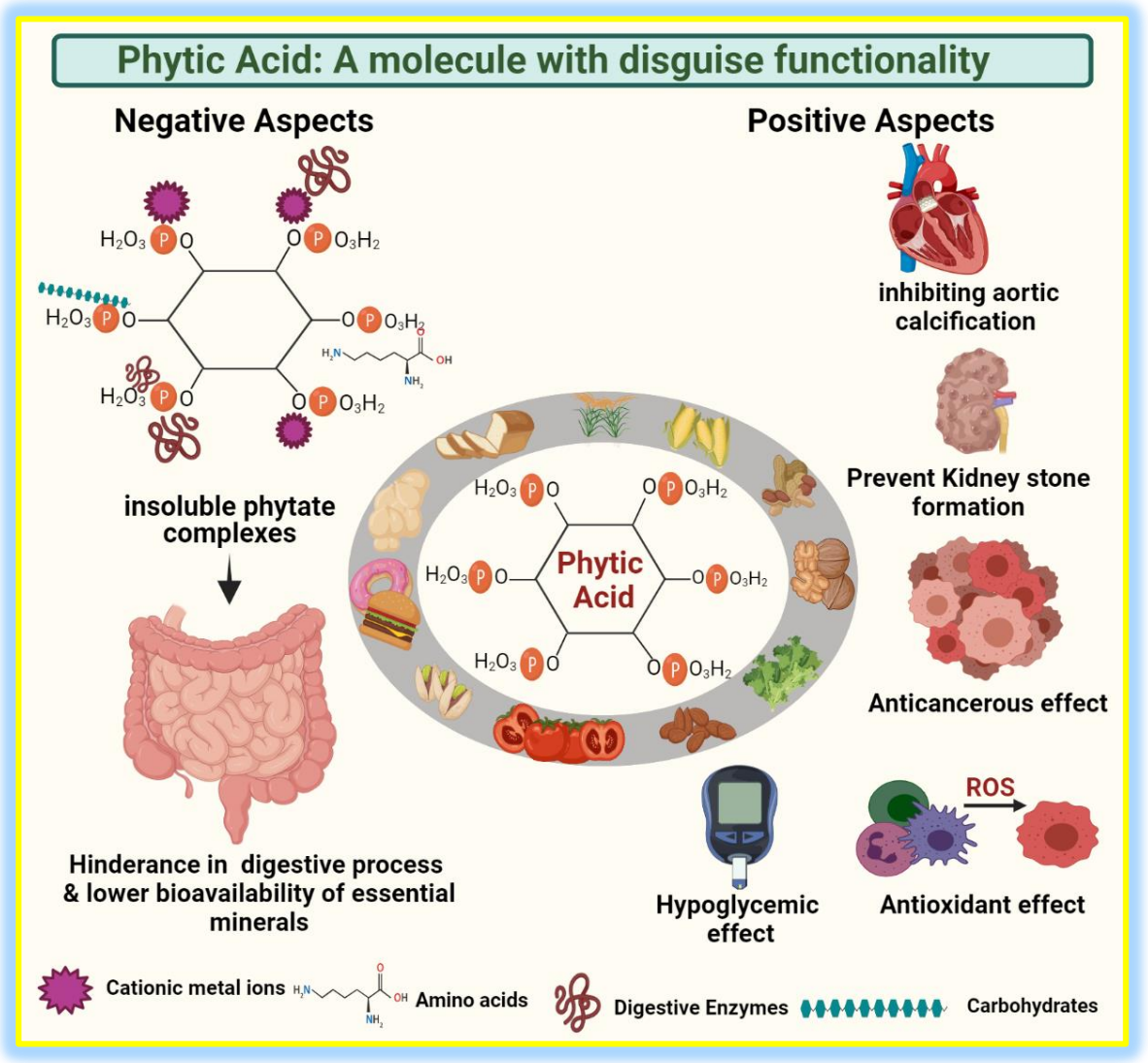


Figure 2.2 Phytic acid molecule has disguised functionality in food-based formulations: showing its negative as well as positive impacts on human health.

2.2 Approaches for removal of phytic acid

Conventionally, phytic acid could be removed by roasting, soaking, germination, cooking, vitamin C supplementation and fermentation. The soaking process, either for short (10-30 min) or long (overnight) duration, facilitates the removal of phytic acid content from legumes (soakable foods only) as the former is water-soluble. Cooking also results in only a small loss of phytic acid as phytic acid salts are heat-stable [27, 28]. During germination, legumes and cereal seeds use the Pi stored as phytic acid by catalyzing it intrinsically using dephosphorylating

enzymes [29]. The fermentation approach ameliorates the nutritional value of food by degrading phytic acid content as associated microbial strains (*Lactobacillus, Bifidobacterium*) have phytase activity. Prior studies have reported that ascorbic acid (50-80 mg) can combat the anti-nutrient effect of phytic acid salts [30, 31]. Through conventional methods, complete phytic acid removal is not possible, so one needs to combine one or more traditional methods for complete hydrolysis of phytic acid [13] (Figure 2.3).

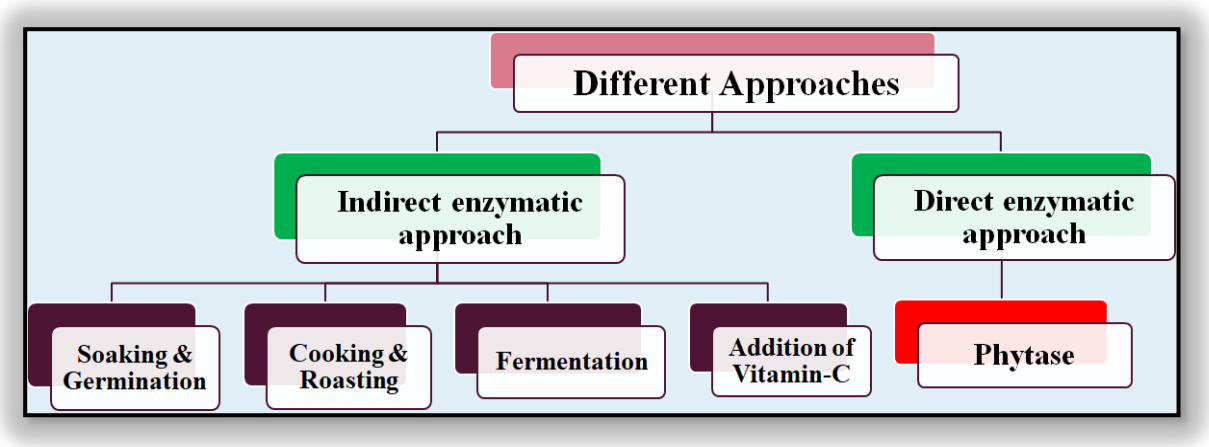


Figure 2.3 Different approaches opted for the removal of phytic acid.

2.3 Phytase

Phytase enzyme (EC 3.1.3.8 and EC 3.1.3.26) (Phosphohydrolase; *myo*-inositol-hexakis-phosphate) is a dephosphorylating enzyme that releases Pi and other complexed nutrients from phytic acid and phytate complexes. Approximately 70% of Pi remains bonded in food-based formulation (Cereals, legumes, nuts, oilseeds and green fruits and vegetables). Phytase releases the bound Pi and other minerals from phytic acid stepwise and converts it to a less phosphorylating form, *myo*-inositol, after complete hydrolysis [32].

2.4 Sources of phytase

Phytases are ubiquitously present in microorganisms, plants, algae and animals. Microbial sources such as bacteria, fungus, and yeast are appropriate for phytase production on a commercial scale [33-35] (Table 2.2).

2.4.1 Animal sources

Animal phytase was initially discovered in calf liver and blood by McCollum and Hart (1908) [36]. Phytase activity has also been found in the blood of lower vertebrates (fish, sea turtles, reptiles) to the digestive system (intestine) of higher organisms like birds, rats, cows, sheep,

and pigs [37, 38]. In mammals gut, nonspecific alkaline phosphatases are responsible for phytate hydrolysis. In monogastric animals, intestinal phytate hydrolyzing activity does not impart a substantial role in phytate breakdown; instead, a dietetic phytate degrading enzymes play the role of dephytinization [39].

2.4.2 Plant sources

Phytases are mainly expressed during germination because they are associated with the aleuronic layer of grains and cotyledons, consequently assisting in seedling and better plant growth. Phytate degrading enzymes are expressed in grains (rice, rye, barley, soybeans, and wheat), legumes (peas, beans), veggies (lettuce, spinach), nuts, oilseeds and pseudo-grains (buckwheat, amaranth) [40]. The first time, phytase from plant origin was isolated from rice bran. Phytase purified from wheat and sorghum showed maximum activity at an acidic pH (4.5-5) and temperature of 50 °C [41, 42]. Phytase from plant sources is not preferable for commercial applications since the approaches associated with their isolation, production and purification are troublesome, expensive and time-demanding. Moreover, the production yield is very low and the phytase is heat-labile. Plant phytases are not ideal for the dephytinization of feed and foodstuffs as they are heat sensitive and only active between pH 4-7 [39].

2.4.3 Algal sources

A sea alga, *Dunaliella tertiolecta,* has been used for expressing various recombinant phytases of *A. niger*, *E. coli*, *Caulobacter crescentus* and *Bacillus subtilis* [43]. The thermotolerant blue-green algae *Chroococcidiopsis thermalis Geitler, Synechococcus bigranulatus Skuja, S. lividus* DSK74 and *S. lividus Copeland* SKP50 isolated from a hot spring of northern Thailand also produce intracellular phytase [44]. For the first time, the highly active phytase, PhyAsr, which belongs to the tyrosine phosphatase class, was recently successfully produced in the chloroplast of *Chlamydomonas reinhardtii* (green microalga) derived from the ruminant-harboring bacterium *Selenomonas ruminantium*. According to the findings, *C. reinhardtii* is an appropriate host for producing the PhyAsr phytase, which might be utilized as a feed additive in monogastric animal diets [45].

2.4.4 Microbial sources of phytase

2.4.4.1 Fungal sources

Filamentous fungi and molds are most commonly utilized commercially to produce extracellular phytase. Fungal phytases belong to Histidine Acid Phosphatases (HAPs) and are optimally active over a broad range of temperatures. Phytases of fungal origin are active over an acidic pH (pH 2.0-5.0) range, making them more suitable for feed application as they can withstand the harsh conditions of the gastric pH of monogastric animals [46-51]. The filamentous fungi *Aspergillus* [51-56], and moulds *Rhizopus* [57-60], *Penicillium* [61, 62], *Sporotrichum Thermophile* [63-65], *Humicola* [66] have been studied extensively as fungal phytase producers (Table 2.2).

Both solid-state and submerged fermentation opted for phytase production from fungal sources. Fungal sources are preferred for commercial phytase production due to their inherent thermostability, and acidic pH tolerant potential [42, 67].

2.4.4.2 Yeast sources

Yeast sources express both extracellular as well as cell-bound phytases. Yeast phytase not only helps to overcome the chelating potential of phytic acid but also has probiotic potential, which enhances the nutritional quality of functional foods [68]. Back in the 19th century, most conventional yeast, *Saccharomyces cerevisiae*, was used for phytase extraction, which was active at acidic pH and optimal temperature 45℃ [69]. Afterwards, other strains of *Saccharomyces* were also studied for phytase production [68, 70–72]. Other potential yeast phytase producers are *Aexula adeninivorans* [73], *Candida krusei* [74], *Pichia anomala* [75], *Candida tropicalis* [76] and *Wickerhamomyces anomalus* [77] (Table 2.2).

2.4.4.3 Bacterial sources

Bacterial phytase on a commercial scale is mainly produced by *Bacillus* species, which catalyzes the hydrolysis of phytic acid at an alkaline pH (7.0-7.5) range and temperature optima at 50 ℃ [78, 79]. Phytases have been found in numerous bacteria, including *Aerobacter aerogenes* [80], *E. Coli* [81, 82], *Klebsiella* spp. [83, 84], *Enterobacter* spp. [85], *Pseudomonas* spp. [86, 87], *Bacillus* spp. [88-92], *Bifidobacterium* spp. [93], *Lactobacillus* spp. [94], *Citrobacter braakii* [95], *Megasphaera elsdenii* [96], *Mitsuokella* spp. [97], *Prevotella* spp. [96], and *Selanomonas ruminantium* [98] (Table 2.2). Bacterial sources produce phytase intracellularly except *Bacillus subtilis*, which synthesizes phytase extracellularly [88]. Bacterial

phytases are highly specific and act optimally at the alkaline pH range. Bacterial phytases require Ca^{2+} for catalysis and have strong proteolysis resistance potential to withstand the GI tract of monogastric animals [79, 99].

Table 2.2 Different microbial sources of phytases.

Various microbial sources of phytases	
Bacterial sources	**References**
Aerobacter aerogenes	[80]
B. subtilis	[88]
Bacillus spp.	[88, 89, 91, 92]
Bifidobacterium spp.	[93]
Citrobacter braakii	[95]
E. coli	[81, 82, 100]
Enterobacter spp.	[85]
Klebsiella spp.	[83, 84]
Lactobacillus spp.	[94]
Megasphaera elsdenii	[96]
Mitsuokella spp.	[97]
Pediococcus acidilactici BNS5B	[101]
Prevotella spp.	[96]
Pseudomonas spp.	[86, 87]
Ruhnella aquatilis JZ-GX1	[102]
Selanomonas ruminantium	[98]
Serratia marcescens	[103]
Yeast Sources	
Aexula adeninivorans	[73]
Candida krusei	[74]
Candida tropicalis	[76]
Pichia anomala	[75]
Saccharomyces cerevisiae	[69]
Schwanniomyces occidentalis	[104]
Wickerhamomyces anomalus	[77]
Fungal Sources	
A. ficuum	[105]
A. ficuum SGA 01, *A. niger* CFR 335	[106]

A. *flavus*	[48]
A. niger 7A-1	[107]
A. niger NCIM 612	[108]
A. niger NRF 9	[109]
A. niger NT7	[51]
A. oryzae SBS50	[110]
A. tubingensis SKA	[111]
Agrocybe pediades	[112]
A. Carbonarim	[113]
Mucor racemosus	[114]
Muscodor spp.	[115]
Myceliopthora thermophile Lomy 713	[116]
Neurospora sitophila	[117]
R. oligosporus MTCC 556	[118]
Rhizomucor pusillus	[58]
Rhizopus oryzae	[60]
Sporotrichum thermophile	[119]
Talaromyces purpureogenus NSA20	[120]
Thermoascus aurantiacus (TUB F 43)	[121]
Thermomyces lanuginosus	[122]
Thermomyces lanuginosus IMI 096218	[123]

2.5 Classification of phytases

Phytase belongs to the phosphomonoesterase, which hydrolyzes the phytate to the lowest phosphorylated form. The classification of phytase can be categorized based on pH optima, catalytic action and site of action (Table 2.3).

2.5.1 Based on pH optima

Acid, alkaline and neutral phytases are categorized based on their pH optima at which they are catalytically active. Fungal phytases are primarily active at acidic pH, whereas some bacteria and plants express alkaline/ neutral phytases [79, 124].

2.5.2 Based on structural and catalytic mode of action

Phytases are classified as β-propeller phosphatases (BPPs), Histidine Acid Phosphatases (HAPs), and phosphatases/protein tyrosine like phosphatases having dual-specificity

phosphatases (DSPs) and purple acid phosphatases (PAPs) based on their sequence properties and catalytically active 3D structures [124] (Table 2.3).

HAPs are mainly reported in fungi like *Aspergillus*, *Cladosporium* spp., *Fusarium oxysporum* and *Debaryomyces castellii* [67, 79]. HAPs have pH optima over the acidic range (2.5-5.5) and are expressed mainly in fungi, bacteria and yeast. The HAPs have a unique conserved active site hepta-peptide motif RHGXRXP and a conservative catalytically active dipeptide HD residues.The conserve motifs and residues are involved in substrate binding, thermostability and product release from the active site of phytase. HAPs catalysed the phytate degradation in two steps, mainly by the nucleophilic attack of an active site conserved residue, His, on the phosphomonoester bond, finally leading to deprotonation and forming *myo*-inositol and monophosphate [124, 125].

The configuration of BPP is similar to a six-bladed propeller folded structure. The BPP requires calcium ions for catalysis and preferably forms *myo*-inositol triphosphate. They are protease-resistant and very specific about their substrate. They are primarily active in alkaline, and neutral pH ranges by bacteria like *Bacillus* spp. [79, 99]. Recently, fungal phytase obtained from *Arthrobotrys oligospora* possesses catalytic activity in the alkaline range and is utilized for feed processing [126].

PAPs are acid phosphatases that act on phosphomonoesters optimally at a pH of less than 7.0. They are expressed in lower strata (bacteria, fungi) as well as higher eukaryotic groups (plants and animals) [124]. They have five conserved motifs: DXG, GDXXY, GNH (D/E), VXXH, GHXH and a binuclear metal centre (usually iron) and usually show much higher expression in plants. PTLP, also known as cysteine phytase, has an HCXXGXXR conserved motif, mainly expressed by the ruminant anaerobic bacterium, *Selenomonas ruminantium* [127].

2.5.3 Based on site of action

Phytases have been classified as 3-, 5-, and 6-phytases based on hydrolytic site [79, 128] (Table 3). The 3-phytases (EC 3.1.3.8), mainly expressed in microbes, first hydrolyzed the Pi group from the C-3 position of ringed structured phytic acid to form *myo*-inositol-2-phosphate as the end product. While the 6-phytases (EC 3.1.3.26), mainly expressed in plants, first start cleaving the bond at the C-6 position and completely dephosphorylate the phytic acid to *myo*-inositol [46, 129]. 5-Phytases (EC 3.1.3.72), which remove the Pi on the 5^{th} position of carbon, have been discovered in *Selenomonas lacticifex* bacterium [124, 130].

Table 2.3 Classification of phytases on a different basis.

Classification criteria for phytases				
Based on the structural and catalytic mechanism				
Class	**Abbreviation**	**pH range**	**Features**	**References**
Histidine Acid Phosphatases	HAPs	2.5 - 5.5	The HAPs have conserved heptapeptide motif (RHGXRXP) and conservative catalytic active dipeptide (HD) residue, which involves in substrate binding, thermostability and release of end product *myo*-inositol and monophosphate	[79, 124, 125, 127]
β-propeller phosphatases	BPPs	7.0 - 8.0	BPPs have a six-bladed propeller folded structure which requires calcium ions for catalysis and preferably forms *myo*-inositol triphosphate	
Cysteine or protein tyrosine like phosphatases	PTLPs	4.5 - 6.5	It has HCXXGXXR conserved motif and forms *myo*-inositol monophosphate as the end product	
Purple acid phosphatases	PAPs	4.0 - 5.5	They have five conserved motifs: DXG, GDXXY, GNH (D/E), VXXH, GHXH and have a binuclear metal centre (usually iron)	
Based on the site of dephosphorylation at the carbon atom				
Class	**EC number**		**Features**	
3-Phytases	EC 3.1.3.8		Mainly expressed in microbes, first hydrolyzed the Pi group from the C-3 position of ringed structured phytic acid to form *myo*-inositol-2-phosphate as the end product	[46, 124, 129, 130]
6-Phytases	EC 3.1.3.26		Mainly expressed in plants, first start cleaving the bond at the C-6 position and completely dephosphorylate the phytic acid to *myo*-inositol	
5-Phytases	EC 3.1.3.72		Remove Pi on the 5^{th} position of carbon and have been discovered in *Selenomonas lacticifex* bacterium	

Based on pH optima		
Class	**Features**	
Acid phytases	Fungal phytases (*Aspergillus* spp.) are primarily active at acidic pH	[79, 124]
Alkaline phytases	Bacteria (*Bacillus* spp.) and plants (wheat, soybean) express alkaline/ neutral phytases	
Neutral phytases	Some bacteria and plants express neutral phytases	

2.6 Phytase production

Three approaches are producing phytase, i.e., solid-state fermentation (SSF), semi-solid fermentation (sSSF), and submerged fermentation (SmF).

2.6.1 Semi-solid fermentation (sSSF)

sSSF is a kind of SSF that has increased water activity and offers better nutrient availability and controlled fermentation parameters for fungal growth. sSSF might be utilized to alleviate some of the limitations of SSF operated at high temperatures by using a different set of parameters. Through the sSSF approach, phytase from *Thermoascus aurantiacus* strain SL16W resulted in higher phytase activity (58.6 U/g substrate) by utilizing rice bran as a cheap lignocellulosic substrate [131].

2.6.2 Submerged fermentation (SmF)

SmF is the major productive approach opted for enzyme production where nutrients are either dissolved or suspended and fermentation is carried out in enclosed containers. Various biological factors like the type of microbial strain, substrate and nutritional status of culture conditions affect the efficacy of bio products (phytase) production in SmF [132, 133]. SmF is mainly opted for bacterial cultures, providing enough moisture and water activity for growth and production. Some prior studies also suggested that fungal strains like *Agrocybe pediades* [112], *A. niger* [106], *Sporotrichum thermophile* [119] and *Thermoascus aurantiacus* [121] were capable of producing efficient phytase in SmF (Table 2.4).

2.6.3 Solid-state fermentation (SSF)

Solid-state fermentation employs an inert solid substrate that supports the growth of phytase producing microbial strains on solid physical support either without or with a minimal amount of available free water while maintaining enough moisture content to sustain the microbial growth. SSF utilizes low-cost agricultural and food waste as a substrate for the growth of microorganisms and enzyme production [134]. The SSF process not only helps in tackling waste management but also transforms low-value waste into high-value products. The SSF operational cost is less than SmF and produces a high enzyme yield with minimum maintenance cost and microbial contamination. Moreover, enzymes can be extracted using an inexpensive medium like water, making this process more economical and less time-consuming [51, 135].

Agricultural waste like Wheat Bran (WB), maize bran, citrus peel, wheat straw, rice bran, oil cake, soybean meal, corn cob, and husk is used as a substrate for SSF.

Prior studies have established that SSF is preferred for fungal and yeast sources as it mimics the natural habitat of fungus [39] (Table 2.4). Bacterial cultures are not preferred as they require high water activity for their growth. It has been postulated in previous studies that the production yield of products (enzyme production) is higher in SSF, probably as these conditions are optimal and behave as the natural habitat of microbial strains. One of the significant shortcomings associated with SSF is handling heat-mass transfer during fermentation. The heat produced by the metabolic activities of microbial strains remained entrapped in the solid substrate as it has poor conductivity for heat transfer, which could lead to the denaturation of the enzyme [135].

2.7 Cultural parameters influencing the phytase production

Phytase production conditions by different microbial cultures in SSF are affected by various physiochemical and nutritional parameters. Optimization of each condition is mandatory to carry out efficient and high phytase production in SSF. Various physical parameters (pH, temperature, moisture content, fermentation time), biological parameters (inoculum age and level) and nutritional parameters (substrate, carbon, nitrogen, Pi source and surfactant addition) play an imperative role in determining the most efficient phytase production in SSF [135, 136] (Table 2.4).

2.7.1 Selection of substrate

The solid substrate utilized for SSF plays a key role in enzyme production as this insoluble solid matrix provides not only physical support for microbial growth but also nutritional content for their survival. Phytase production from various agri-waste includes the mixed substrate oil cake (linseed oil cake) with WB used by *R. oryaze* [50], WB used by *Aspergillus* spp. [51, 54, 109], potato waste used by *A. ficuum* [137], oil cakes used by *Mucor racemosus* [114] and *S. thermophile* [64], and triticale waste used by *A. niger* [107] were found to be optimal in prior studies (Table 2.4). Several previous reports suggested that phytase production could be efficiently achieved in SSF by using bacterial cultures as well. WB and corn flour efficiently enhanced phytase production by *E. coli* and *Bacillus* spp. T4, respectively [138, 139].

2.7.2 Inoculum age and size

Inoculum age directly affects phytase production as young microbial cells are metabolically more active than older cells. Thus, young microbial cells are used for phytase production as they can proliferate quickly and produce the phytase enzyme. With a three-day-old inoculum, *A. flavus* ITCC 6720 and *A. niger* NT 7 give optimal phytase production [48, 51]. Several prior studies show that *A. niger* CFR 335, *A. niger* SGA 01 and *A. niger* NRF9 produce phytase optimally with 6-day-old and five-day-old inoculums, respectively [106, 109].

Inoculum size defines the initial microbial cells utilized to carry out fermentation. An inoculum containing a spore suspension (12×10^7 spores/ml) was recently used by *A. niger* NT7 for phytase production [51], whereas a higher spore suspension (7.2×10^6) was used by *A. flavus* ITCC 6720 [48]. Too high inoculum level could lead to diminished phytase production as a high initial microbial population will utilize all the available nutrition from the fermentation medium, making them limited [135, 140]. An inoculum size of 1×10^7 spores/ml gives optimal phytase production in *A. niger* NRF9 [109]. A low inoculum is usually not recommended for filamentous fungi because it can form pellets in the fermentation medium, resulting in a low phytase enzyme yield [129].

2.7.3 Fermentation time

The time required by microbial cells varies with the type of source and strain used for phytase production. Fungal sources require a long fermentation time as compared to bacterial cultures. Phytase extracted from the moldy medium after five days of SSF resulted in enhanced phytase production in *Mucor racemosus* [114], *A. oryzae* SBS50 [54] and *A. niger* NT7 [51]. Going beyond the optimal fermentation time may lead to lower phytase yield due to exhausted nutritional status, accumulation of toxic end products and feedback inhibition caused by the formed product in the fermentation medium.

2.7.4 pH

pH is the most important factor as it alters the metabolic activity of microbial strain. Phytase production from fungus and yeast preferably occurs over a broad acidic pH range which is advantageous as it blocks bacterial contamination. Bacterial culture prefers a neutral to alkaline pH range for phytase production. Bacterial cultures like *Bacillus* and *Lactobacillus* synthesize phytases over a broad alkaline pH range (6.0-8.0) [99, 141]. Several studies show that fungal strains *R. oligosporus* [57], *A. niger* NRF9 [109] and *A. oryzae* SBS50 [54] produce phytase

optimally at acidic pH 5.0. Certain fungal strains, *S. Commune* [142] and *A. flavus* ITCC 6720 [48], produce phytase at pH 6.0 and neutral pH (7.0).

2.7.5 Temperature

Temperature is the chief parameter in SSF as it directly affects the growth of microbial cultures, spore generation and secondary metabolite production. Thermophilic molds showed high phytase production at high temperatures and could be commercially utilized for feed additive applications. The mesophilic fungi *A. niger*, *Rhizopus* spp. produce the phytase efficiently at 30 °C [52, 106], whereas thermophilic molds such as *S. thermophile*, *Rhizomucor pusillus*, *M. thermophile* Lomy 713 and *T. lanuginosus* produce the phytase optimally at 45 °C [143]. *R. pusillus* yields high phytase production at much higher temperatures, i.e., 50 °C [58] (Table 2.4).

Table 2.4 Different cultural requirements of fungal cultures for phytase production in SSF and SmF (Here WB stands for wheat bran).

Fungal strain	Process	Nutritional requirements		Biochemical Factors		References
		Carbon source	Nitrogen source	Opt pH	Opt temp.	
A. carbonarim	SSF	Canola meal	-	-	30	[113]
A. ficuum	SSF	WB, glucose	$(NH_4)_2SO_4$	-	30	[105]
A. ficuum SGA 01, *A. niger* CFR 335	SSF	WB, rice bran, groundnut Cake	-	4.5	30	[106]
A. ficuum SGA 01, *A. niger* CFR 335	SmF	Potato dextrose	-	4.5	30	[106]
A. flavus	SSF	Mustard cake	Malt extract	6	37	[48]
A. niger	SSF	WB	Full-fat soybean meal	-	30	[144]
A. niger	SmF	Glucose, starch	NH_4NO_3	6.5	30	[145]
A. niger	SSF	Sugarcane bagasse, starch	NH_4NO_3	4.0	32	[146]
A. niger	SmF	Cane Molasses	NH_4NO_3	4.0	32	[146]
A. niger	SSF	Rice bran,	NH_4NO_3	5	30	[147]

		Glucose				
A. niger 7A-1	SSF	triticale waste, dextrose	NH_4NO_3	-	28±1	[107]
A. niger NCIM 612	SSF	Rice straw, Glucose	NH_4NO_3	-	30	[108]
A. niger NRF 9	SSF	$NaNO_3$	$NaNO_3$	4.5	30	[109]
A. niger NT7	SSF	WB, Mannitol	$(NH_4)_2SO_4$	5	35	[51]
A. niger SBS50	SSF	WB, Sucrose	Beef extract	5.0	30	[54]
A. oryzae SBS50	SmF	Starch	Beef extract	5.0	35	[110]
A. tubingensis SKA	SSF	WB, glucose	$(NH_4)_2SO_4$	5	30	[111]
Agrocybe pediades	SmF	Malt dextrin, soya flour	Peptone	5.5	26	[112]
Mucor racemosus	SSF	WB, sesame oilcake, Starch	$NaNO_3$	5.5	30	[114]
Muscodor spp.	SmF	WB	Yeast extract, $NaNO_3$	5	30	[115]
Myceliopthora thermophila	SmF	Glucose	$NaNO_3$	5.5	45	[148]
Myceliopthora thermophile Lomy 713	SSF	Sugarcane bagasse	$NaNO_3$	6	45	[116]
Neurospora sitophila	SSF	Rice straw powder and soybean curd residue	-	-	-	[117]
R. oligosporus MTCC 556	SSF	WB, mustard oil	$(NH_4)_2SO_4$	5.5	30	[118]

		cake, maltose				
R. oryzae	SSF	Linseed oil Cake, WB, mannitol	$(NH_4)_2SO_4$	7.6	30	[60]
R. oryzae	SSF	WB, Linseed oil cake, Mannitol	$(NH_4)_2SO_4$	5.6	30	[50]
R. oryzae	SSF	Deoiled rice bran	-	-	28±1	[149]
R. pusillus	SSF	WB	Asparagine and corn steep liquor	8	50	[58]
S. thermophile	SSF	Sesame oilcake, glucose	$(NH_4)_2SO_4$	5	45	[64]
S. thermophile	SmF	Starch, Glucose	Peptone	5	45	[119]
T. aurantiacus	SSF	Sugarcane bagasse	$NaNO_3$	6	45	[116]
Talaromyces purpureogenus	SmF	Potato peel	-	5.5	30	[120]
Thermoascus aurantiacus	SmF	Starch, Glucose, WB	Peptone	5.5	45	[121]
Thermomyces lanuginosus	SSF	WB	$NaNO_3$	5.5	45	[122]
Thermomyces lanuginosus IMI 096218	SmF	Rice flour	$NaNO_3$	-	47	[123]

2.7.6 Moisture content and water activity (a_w)

The water activity parameter determines which microbial strain could be utilized in SSF with potential applicability as it determines the transfer of mass, heat and nutrients between microbes and solid substrates. Moisture content is also a major factor, as its low content could lead to lower microbial culture growth due to low accessibility to nutrients available in SSF. In contrast, high moisture content could diminish porosity in the substrate for oxygen and heat transfer. It could also lead to particle agglomeration and cross-contamination of SSF due to bacterial load. Fungi requires less moisture as compared to bacteria (≥70%) [135, 138]. Previous studies have shown that higher moisture content, 60%, improved phytase production in *A. flavus* ITCC 6720 [48] and *S. Commune* [142]. Distilled water is one of the most economic moistening agents used in solid-state fermentation by *A. niger* NCIM 563, *A. niger* CFR 335 [52, 106, 150]. Distilled water infused with various salts is also utilized for improved phytase production.

2.7.7 Effect of nutritional factors

Nutritional factors like carbon and nitrogen source play an imperative role in SSF. The carbon source acts as an energy source for microbial culture. Extraneous supplementation of nutritional factors is mandatory as the fermentation progression takes place; one or more nutrients may become exhausted during the process. Microbial strains can utilize simple to complex sugar for phytase production. An assimilated monosaccharide glucose supported the phytase production in *S. thermophile* [119], *A. niger* NCIM563 [47], *A. niger* NCIM612 [151], *Penicillium purpurogenum* GE1 [61], and *A. flavus* ITCC6720 [48]. Previous studies have also shown that sugar alcohol mannitol has been utilized for phytase production in *R. oryzae* [50] and *A. niger* NT7 [51]. Sucrose addition leads to a high phytase titer by *A. niger* CFR335 [106] and *S. commune* [142]. Starch addition in the fermentation medium caused improved phytase titer by *A. ficuum* NRRL3135 [136].

Nitrogen sources help in the maintenance of the cell's various metabolic activities as well as protein synthesis [140]. The supplementation of both organic as well as inorganic sources of nitrogen supports phytase production in the fermentation medium. Previous studies reported that the ammonium sulphate supplementation enhances phytase production in *R. oryzae* [50], [60] and *A. niger* NT7 [51]. Ammonium nitrate supplementation improves phytase production from *T. lanuginosus* TL-7 [122], *A. niger* NRF9 [109], and *A. niger* 7A-1 [107]. *Rhizomucor*

pusillus showed high phytase secretion when the medium was supplemented with the industrial by product corn steep liquor and the amino acid asparagine [58]. Other previous studies have suggested that complex nitrogen sources such as beef extract and malt extract can be utilized by fungi like *A. oryzae* SBS50 and *A. flavus,* respectively, for enhancing phytase production in SSF [48, 54].

Pi supplementation in the fermentation medium is believed to enhance the expression of phytase. Prior reports show that the supplementation of potassium dihydrogen Pi at a low concentration improves phytase production in *A. niger* NCIM 612 [151], *A. niger* van Teigham [145], and *A. niger* NT7 [51].

2.7.8 Effect of surfactant

Surfactant application is believed to enhance the permeability of fungal cells to release the cell-bound metabolites. Previous studies have shown that surfactant enhances the phytase production in SSF [121, 152]. Tween 80 positively enhances the phytase titer from *S. thermophile* [64], *A. niger* NRF9 [109], and *A. niger* NT7 [51]. Triton X-100 showed its positive impact on phytase production in *A. oryzae* SBS50 [54], whereas SDS showed its deleterious effects on addition to phytase production medium [51, 143].

2.8 Optimization approach for phytase production

The optimization of fermentation is of utmost importance to attain maximum phytase production. Two approaches are adopted for phytase production, namely, one variable at a time (OVAT) and a statistical approach through software. Optimization through both approaches resulted in upgraded phytase production. The OVAT techniques used a methodology that optimized one parameter at a time while leaving all other parameters constant. This approach is promising for enhancing phytase production, yet very tedious, time-consuming and error-prone. OVAT approach previously enhanced phytase titer in *A. oryzae* SBS50 [54] and *A. niger* NT7 [51] (Table 2.5). On the other hand, mathematical and statistical procedures achieve phytase optimization through response surface methodology (RSM). This approach is less laborious, fast and reliable for enhancing phytase production. Multiple factors can be optimized at once through this approach [153, 154]. Previous studies found that the statistical approach augmented phytase production using SSF methodology [56, 120]. RSM resulted in a 3.08-fold increase by *A. niger* NCIM 563 [155], a 10.83-fold increase by *Thermomyces lanuginosus* [156], and a 6.8-fold increase by *A. niger* NT7 [56] (Table 2.5).

Table 2.5 Optimization of phytase production using OVAT and statistical approach.

Microorganism	Substrate	Optimization approach	Phytase production (U/gds)	References
A. ficuum	Wheat straw	Box Behnken design	16.46	[157]
A. ficuum NRRL 3135	Wheat-bran	Plackett-Burman and CCD	25	[136]
A. ficuum NTG-23	Waste vinegar	Plackett-Burman and CCD	8.72	[158]
A. ficuum PTCC 5288	WB, Glucose	Plackett-Burman and CCD	25.6	[159]
A. niger	Cowpea meal	OVAT	56.5	[160]
A. niger FS3	Citric pulp bran	OVAT	51.53	[161]
A. niger NCIM563	WB	Box Behnken and Plackett-Burman Design	154	[47]
A. niger NRF9	WB	Plackett-Burman Design	112.1	[67]
A. niger NT7	WB	OVAT	208.30	[51]
A. niger NT7	WB	Minitab and CCD	521.29	[56]
A. oryzae SBS50	WB	Taguchi design	185.75	[162]

A. oryzae SBS50	WB	OVAT	145	[54]
P. purpurogenum GE1	Corn cob, corn bran	Box Behnken	444	[61]
Pholiota adiposa	Water hyacinth	OVAT	53.66	[67]
R. pusillus	Wheatbran	Box Behnken	8.82	[58]
S. thermophile	Sesame oil cake	Plackett-Burman Design and CCD	1881.56	[65]
S. thermophile TLR50	Sesame oil cake	OVAT	260	[64]
T. lanuginosus	Rice bran	Minitab and CCD	3.24	[156]
T. lanuginosus TL-7	Wheat-bran	Box-Behnken	32.19	[122]
Thermomyces lanuginosus TL-7	Wheat-bran	OVAT	13.26	[122]

2.9 Purification of phytase and characterization of phytase

Purification of enzymes is one of the utmost significant components for commercial use and understanding structural-functional relationships. However, animal feed formulation requires a crude form of phytase but to explore the potential of phytase as a food additive and medical agent; purified phytase is the primary requirement. *Bacillus* and *Aspergillus* spp. are commonly used for phytase production as they are Generally Regarded As Safe (GRAS). The first step for phytase purification is to concentrate the crude enzyme, which can be accomplished by salt-induced precipitation, organic solvents-induced precipitation, ultrafiltration and lyophilization. The concentrated protein is further purified to homogeneity by chromatographic techniques like ion exchange and gel permeation chromatography (Figure 2.4). Some previous studies have shown that more than one chromatographic technique may be opted to get highly purified enzyme [163, 164] (Table 2.6).

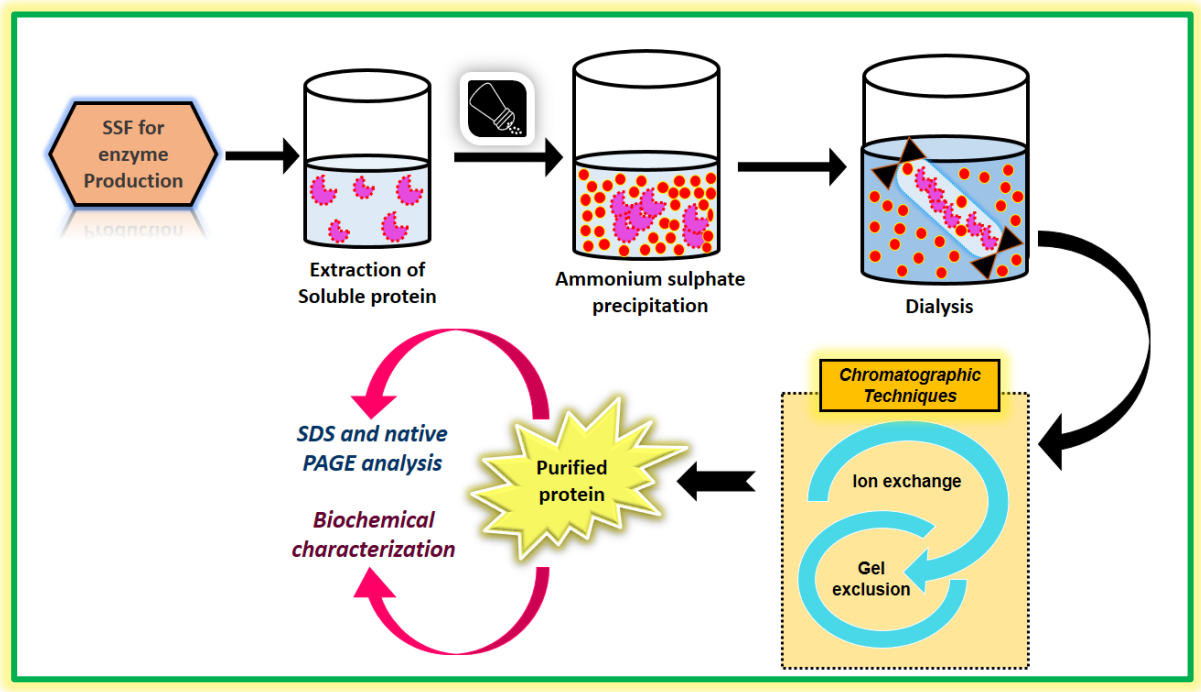


Figure 2.4 Generalized pictorial presentation for enzyme purification.

Phytase activity is calculated as the quantity of inorganic phosphorus (Pi) liberated from the substrate in one min. under standard ambient pH and temperature assay conditions. Phytase catalytic properties depend upon their structure as well as the reaction conditions. They are influenced by several biochemical parameters like optimal pH, temperature, substrate specificity, thermostability profile, proteolysis resistance, and catalytic determinants (K_M and V_{max}). Microbial phytases have strong specificity for phytic acid and phytate, which they

hydrolyze to a low phosphorylated form or *myo*-inositol. However, some plant phytases (*Zea mays* and *Corylus avellana*) [165, 166] and *A. fumigatus* phytase have broad substrate specificity [125].

The bacterial origin phytases (*Bacillus* spp.) exhibit pH optima in the neutral or alkaline range as it belongs to BPPs [89, 99]. In contrast, fungal phytases possess an optimal pH range from acidic to neutral and belong to the HAP family. The fungus *A. niger* phytase has two optimal pH levels (2.5 and 5.5) [107, 167]. *A. ficcum* NTG-2375 phytase has intrinsically strong optima at a much lower acidic pH of 1.3 [168]. The phytase of *A. foetidus* MTCC 11682, *A. aculeatus* APF1 and *A. tubingensis* TEM 37 showed catalytic activity at low pH of 3.5, 3.0 and 2.0, respectively [164, 169, 170].

The microbial phytases are catalytically active over a broad temperature range of 30-60 ºC. These phytases showed promising results in the swine and poultry industries, where temperatures usually varied from 37-40 ºC. Thermostable phytases are very promising as feed additives as they remain catalytically active when exposed to the very high temperatures of pelleting around 90-100 ºC [125, 171] (Table 2.6).

Earlier studies have shown that the phytase of *A. niger* is catalytically active at pH 2.5 and 55 ºC [145]. Strong proteolysis resistance against digestive enzymes is the most desirable feature for the commercial application of phytase for feed and food additives. *A. oryzae* SB50 remarkably showed high protease resistance against digestive enzymes [110], whereas *A. fumigatus* phytase showed no sensitivity against pepsin treatment [172].

Table 2.6 Different purification approaches and biochemical characteristics of purified phytase from various *Aspergillus* spp.

Fungal Strain	Purification approach	Opt pH	Opt Temp.	K_M	V_{max}	MW	References
A. aculeatus APF1	Ammonium sulphate precipitation, SP-Sepharose, lyophilization of fractions and loaded on gel filtration chromatography (BioGel P-60)	3	50	3.21 mM	3.78 U/mg	25-35	[164]
A. ficuum NTG 2375	DEAE cellulose and CM-cellulose chromatography followed by FPLC-gel filtration on Superdex	1.3	65.5	0.295 mM	55.9 nmol	67	[168]
A. flavus ITCC 6720	Acetone precipitation, Macroprep high Q column	6.5	45	-	-	30	[48]
A. foetidus MTCC 11682	Precipitation by ammonium sulphate followed by gel filtration on Sephacryl S-200HR	3.5,5.5	37	-	-	90.5	[169]
A. fumigatus	Ammonium sulphate, dialysis and ultrafiltration by millipore, 10 KDa,	5.5	55	0.114 mM	0.114 U/mg	88	[172]

	DEAE sepharose chromatography (ion exchange)						
A. niger	Ultrafiltration, then ion exchange and gel permeation chromatography	2.5	55	606 μM	1,074 IU/mL	353	[167]
A. niger	Ultrafiltration on PS 50 membrane and Sephadex G-100 followed by chromatography on DEAE-Sepharose	2.6,5.0	55,58	0.929 μM	52.36 nkat/cm^3	39	[173]
A. niger 11T53A9	Ammonium sulphate, DEAE sepharose, CM-sepharose followed by gel filtration (sephacryl S-200HR)	5.0	55	54 μmol	-	85	[174]
A. niger 7A-1	Crude enzyme concentrated with microfiltration, ultrafiltration followed by DEAE-Sepharose column	5.3	56 °C	220 μM	25 μM/min	89	[107]

A. niger UFV-1	Ultrafiltration, DEAE-Sepharose CL-6B column, Sephacryl S-300 HR	2.0	60	30.9 mM	7.48 µmol/min	81	[175]
A. niger NCIM 563	Concentration by Rotavapor, followed by column chromatography (Phenyl-Sepharose)and gel filtration(Sephacryl S-200)	2.5,5.0	55 5018, 1671	2.01, 0.145 mM	5018, 1671 µmol/min/ mg	66, 264, 150 and 148	[176]
A. oryzae SBS50	Precipitation by ammonium sulphate, SP-Sepharose ion exchange matrix followed by gel filtration chromatography (P-60 matrix)	5.0	50	1.14 mM	58.82 µmol/ml/min	80	[49]
A. tubingensis TEM 37	-	2.0,5.0	45	-	-	45	[170]
*A. niger*ATCC 9142	Ultrafiltration step, ion exchange, gel filtration and chromato-focusing	5.0	65	100 µM	7 nmol/s	84	[177]

2.10 Cloning and expression of phytase

Wild strains of filamentous fungi produce phytase enzymes in limited amounts, which cannot match the industrial demand. For industrial purposes, genetic engineering approaches have been applied to develop transgenic strains in which a high yield of enzymes has been achieved [67, 178, 179]. In order to enhance phytase production and its desirable biochemical characteristics, genes are isolated from wild strains and expressed in various expression systems. The fungal phytase genes are expressed in *Pichia pastoris* and *Saccharomyces cerevisiae*. These recombinant microbial strains expressed and secreted phytase enzymes extracellularly, eased downstream processing [180] (Table 2.7). The cloning and gene expression technology improved the enzyme's operational stability over its wild strain enzyme-producing counterpart. The *A. fumigatus* phytase (*phyA*) gene was cloned and expressed in yeast *P. pastoris* showed high thermostability and better protease (pepsin) resistance potential [172, 181]. The PhyA gene from *A. niger* expressed in the bacterial system (*Kluyveromyces lactis*) showed two pH optima (2.0 and 5.5) and its optimal temperature at 55 ºC owing to its conserved Asn residue at 345 position [182]. Another study has shown the ability of recombinant phytase (*S. thermophile* (St-Phy) gene expressed in *E. coli*) for dephytinzation of wheat bread (tandoori naan) [183].

Table 2.7 *Aspergillus spp.* phytase expressed in different expression hosts.

Host	Expression Host	References
A. aculeatus RCEF 4894	*P. pastoris*	[184]
A. awamori (PhyA), A. fumigatus	*A. awamori*	[185]
A. ficuum	*S. cerevisiae*	[186]
A. fumigatus	*P. pastoris*	[181]
A. fumigatus	*A. niger*	[187]
A. japonicas, A. niger	*P. pastoris*	[188]
A. niger	*S. cerevisiae*	[189]
A. niger	*P. pastoris*	[190]
A. niger	*P. pastoris* GS115	[172]
A. niger	*E. coli*	[191]
A. niger XP	*P. pastoris*	[192]
A. niger NII 08121	*Kluyveromyces lactis*	[182]
A. niger SK-57	*P. pastoris*	[193]

2.11 Structure and catalytic mechanism of fungal phytase

The structural information of phytase determines the folding and biophysical properties of the enzyme. Therefore their structural information is vital to decipher their functionality and provides insight for possible modification to meet the requirement of the ideal phytase. The crystal structures of the microbial communities *E. coli*, *B. amyloliquefaciens* and *A. niger* have already been elucidated in prior literature [194, 195]. The crystal structure of *A. niger* revealed that it belongs to the histidine acid phosphatase family, which has two α/β domains with the conserved domain RHGXRXP and another α domain. HAP family member hydrolyze phosphomonoesters in two phases, the first of which involves the creation of a phosphohistidine intermediate via a nucleophilic reaction trailed by hydrolysis of the phosphamide intermediate. The catalytic active site residues (Histidine and Aspartic Acid) reside between these two domains. The histidine (H) residue forms a phosphohistidine intermediate, while aspartic acid (D) deprotonates electrons to the oxygen of the phosphomonoester bond of phytic acid, thus releasing phosphate residues [196] (Figure 2.5).

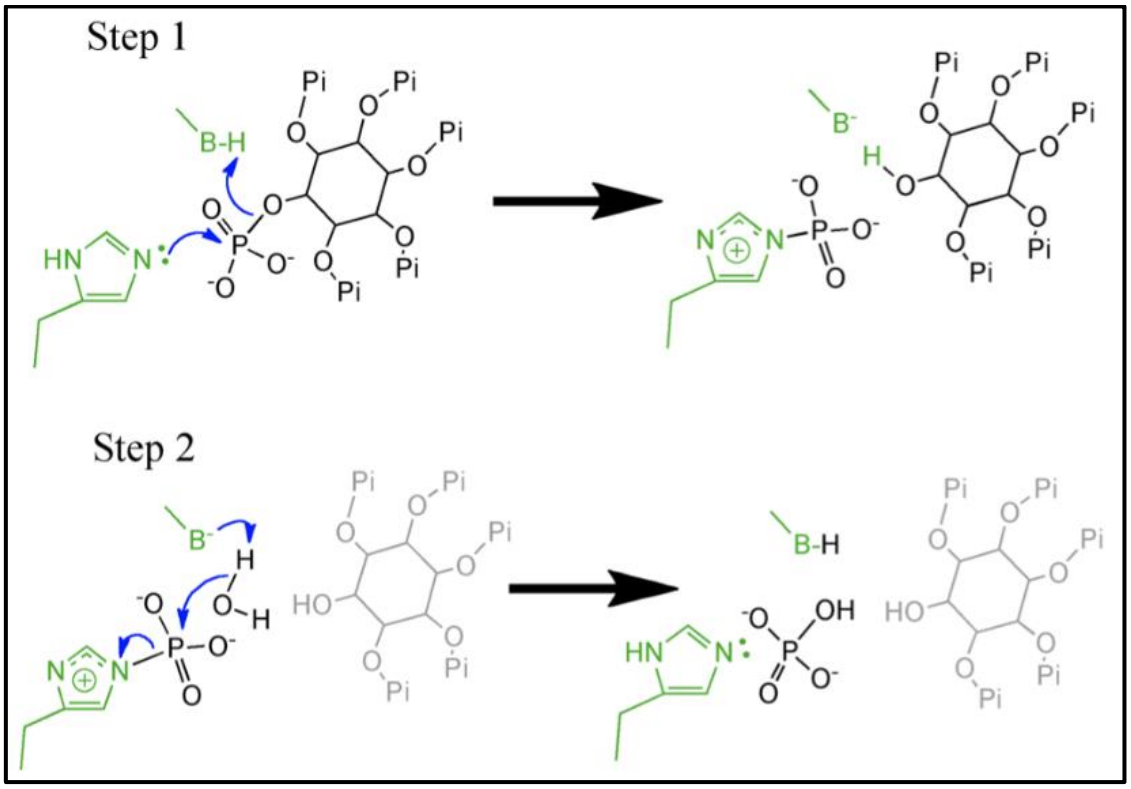


Figure 2.5 Mechanism of fungal phytase (HAP) catalysis; In step one, the conserved RHGXRXP motif's histidine residue behaves as a nucleophilic attack moiety, forming a phosphohistidine intermediary. In step two, after the water molecule attacks the phospho group of the phosphohistidine, the intermediary is cleaved, resulting in a liberating free phosphate group.

Based on pH optima, fungal HAPs belong to two subtypes, phyA and phyB. The phyA is monomeric, acidic and specific to phytic acid (Figure 2.6). The structure has and N-terminal extension, α/β domains, and an α domains. It has glycosylated N-acetylglucosamine residues attached to four residues located at N82, N184, N316 and N353. The catalytic histidine residue (H59) lies between loop and helix. The residues R58, R62, R14, and D339 involved and interact with substrate through H-bond and salt bridges and histidine residues brings about catalysis [197]. The phyB is tetrameric, and less specific toward phytic acid. The crystal structure of *A. niger* phytase at pH 2.5 was elucidated at 2.4 Å resolution, depicting that the *A. niger* phytase has a tetramer structure built up by two dimers (chain a and b which are identical) (Figure 2.6). The Arg62, His63, Arg66, Arg156, His318 and Asp319 are conserved catalytic residues from catalytic centre and the two amino acid residues having acidic nature, Asp75 and Glu272, involves in substrate specificity. The catalytic active site has uniform charge distribution, making phytase active at low pH (2.5) [195]. The *A. fumigatus* thermostable phytase structure at 1.5 Å resolution showed sequence similarity with *A. niger*. The previous have some variation in structural elements like the presence of polar amino acids and helical capping at the C-terminal, which contributes to improved thermostability at high temperatures [198].

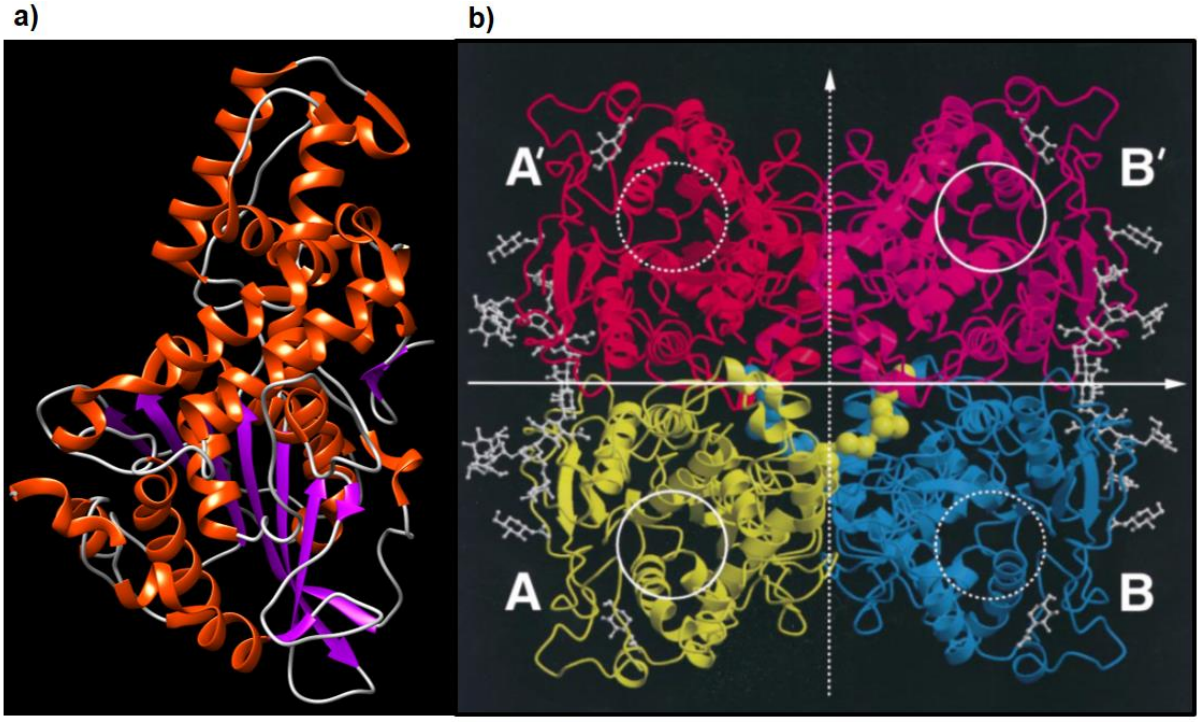


Figure 2.6 Structure of a) phyA (PDB ID: 3K4Q) having monomeric unit b) phyB (PDB ID: 1QFX) has two tetramer structure form from two identical A and B chains. White circle showing catalytic site (Figure 2.6b adapted from Kostrewa et al. [195]).

2.12 Protein engineering of microbial phytase

In microbial systems, protein engineering for improved enzymatic qualities such as thermostability may result from increased folding complexity [199]. Superior phytase producing strains were concurrently developed to obtain the desired amounts of recombinant protein for industrial operations [200]. The phytase activity and heat stability of *E. coli* phytase expressed in *P. pastoris* were improved by site-directed mutagenesis. The replacement of C200N in the mutant (C200N/D207N/S211N) appears to abolish the disulfide link between the G helix and the loop GH in the alpha-domain of the protein, according to the crystal structure of *E. coli* phytase. This alteration might affect the enzyme's domain flexibility, modulating the catalytic efficiency and thermostability potential [201].

When *E. coli* phytase mutants (Q349N, Q258N) were expressed in *P. pastoris*, they demonstrated improved glycosylation and thermostability. The insertion of numerous N-glycosylation sites boosted the mutants thermostability [202]. The mutant V89T showed a 17.5 percent upsurge in catalytic activity compared to the other mutants [203]. N-glycosylation can change the structure and activity of the enzyme.

The HAP from *Yersinia kristensenii* (YkAPPA) and *Yersinia rohdei* (YrAPPA) possess an N-glycosylation motif and show the pepsin-resistant potential. HAP phytase from *Yersinia enterocolitica* is pepsin-sensitive. The mutants created by changing the N-glycosylation state of each enzyme using site-directed mutagenesis were produced in *P. pastoris*. Pepsin resistance was imparted by introducing an N-glycosylation site into YkAPPA, YeAPPA, and YrAPPA, which moved the optimum pH and enhanced stability at acidic pH. The YkAPPA and YrAPPA expressed in *P. pastoris* showed augmented pepsin digestion resistance owing to replacing pepsin hydrolyzing sites, L197 and L396 in the N-glycosylation motif. N-glycosylation may thereby increase resistance to pepsin digestion by increasing stability at acidic pH and decreasing pepsin approachability to peptic hydrolytic sites [204].

To enhance the expression of *E. coli* phytase in *P. pastoris*, a multi-strategy approach was used. The α-factor secretion signal was shown to be the best signal peptide for phytase secretion. The phytase enzyme yield was enhanced by 234.35% when the number of gene copies was amplified to four. Extracellular phytase production was improved with PDI overexpression and Pep4 gene deletion [205]. Using the random mutation approach, two *Penicillium* spp. mutants were generated with better heat stability and ideal pH and temperature. By deciphering its

structural analysis, it was found that the substitutions of G56E, Q144H, L65F and L151S increased the protein's stability to the heat treatment by creating new hydrogen bonds amongst the adjacent secondary structures. Another mutation, N354D, induces the change in the mutant's pH profile by shifting the pKa values of the active center by reducing the interaction with the adjacent chain of D353 [206].

Site-directed mutagenesis enhances the pH optima of *A. niger* phytase [128]. The site-directed mutation of Gln27 to leucine increased the specific phytase activity of *A. fumigatus* [207]. Disulphide bond also confers thermostability to *A. niger* phytase. Deleting the disulfide bridge resulted in a loss of catalytic activity of the phytase mutant (PhyA) of *A. niger* when expressed in *P. pastoris*. The other disulfide mutants confer catalytic features, such as a lower optimal temperature for HAP [208].

2.13 Immobilization of phytase

The immobilization technique confined the enzyme into the matrix in such a way to retain its catalytic activity and could be utilized for multiple enzymatic reactions. The immobilization technique provides stability and enhances the thermostability of the phytase enzyme to resist a high-temperature treatment during pelleting feed additives. The phytase immobilization also improves their pH tolerance to withstand the acidic atmosphere of the gastric tract of monogastric animals. Enzyme immobilization using electrostatic interaction, covalent bonding, encapsulation, entrapment and cross-linked enzyme aggregates (CLEA) techniques have already been reported in previous literature [209, 210] (Figure 2.7). In recent years, numerous novel carriers and methods have been employed to improve the classic immobilization techniques of enzymes, aiming to enhance the enzyme's loading, activity, and stability to reduce the overall cost of the biocatalysts in industrial applications. These comprise CLEA, microwave-assisted immobilization, mesoporous carriers, click chemical techniques, and, more recently, nanoparticle-based enzyme immobilization.

The cross-linked gelatin particles of *A. ficcum* phytase have an optimum pH 5.0 and a temperature 50 ºC [211]. The immobilized soybean sprout phytase on a polymethacrylate-based polymer retained 64.7% enzyme activity with potential application in phytate degradation in soymilk [212]. The phytase of *E. coli* adsorbed on food grade microbial spores of *Bacillus polyfermenticus* enhanced the half-life by three to ten folds at high temperatures [213]. The *E. coli* phytase fused with maltose-binding protein, immobilized on starch agar bead, showed 10% enhanced phosphorus release from soymilk than the free phytase enzyme (maltose infused

phytase) [214]. After 6 h of incubation, phytase CLEA was produced using soymilk as a protein feeder and demonstrated 100% phytase activity up to 50 °C, and at 70 °C, retained activity was 70%. CLEA has activity over 1-6 pH, retains 80% activity upon proteolytic hydrolysis and can be reused up to the 5th cycle [215].

Nanomaterial provide a greater advantage for the physical adsorption of phytase enzymes as they provide a large surface area for attachment without altering their chemical composition [216-218]. Earlier studies have also demonstrated that purified phytase of *Lactarius quietus* immobilized on iron nanoparticles can dephytinize green legumes [219]. Phytase was covalently attached to functionalized multi-walled carbon nanotubes (F-MWNT) with a 62% immobilization effectiveness. The bounded phytase enzyme showed 33% and 51% retained activity after 2 min at 90 and 80 °C [220].

Purified phytase from cowpea immobilized on polyvinyl alcohol (PVA)-chitosan Nano fibers using an electrospun technique showed enhanced pH (6.0) and temperature (65 °C) than the free enzyme (optimum pH 5.0 and temperature 45 °C) [221]. Hydroxyapatite (HA) nanoparticles, a nontoxic calcium phosphate salt, acts as physical support for the immobilization of *A. niger* phytase by forming an ionic bond between calcium on of HA and the carboxyl group of the enzyme. The immobilized phytase showed high thermostability, pH tolerance and enhanced hydrolysis of soybean meal [222]. Phytase immobilized in monodisperse mesoporous silica nanoparticles by physical adsorption showed activity under an acidic atmosphere [223]. Phytase immobilized on zeolite modified with iron (II) ions showed that bound enzyme could be used for successive up to the seventh cycle while retaining 80% catalytic activity. Bounded phytase could be used to hydrolyze phytate from soymilk and can be reused up to the 6th cycle while retaining 50% of phytase activity [224].

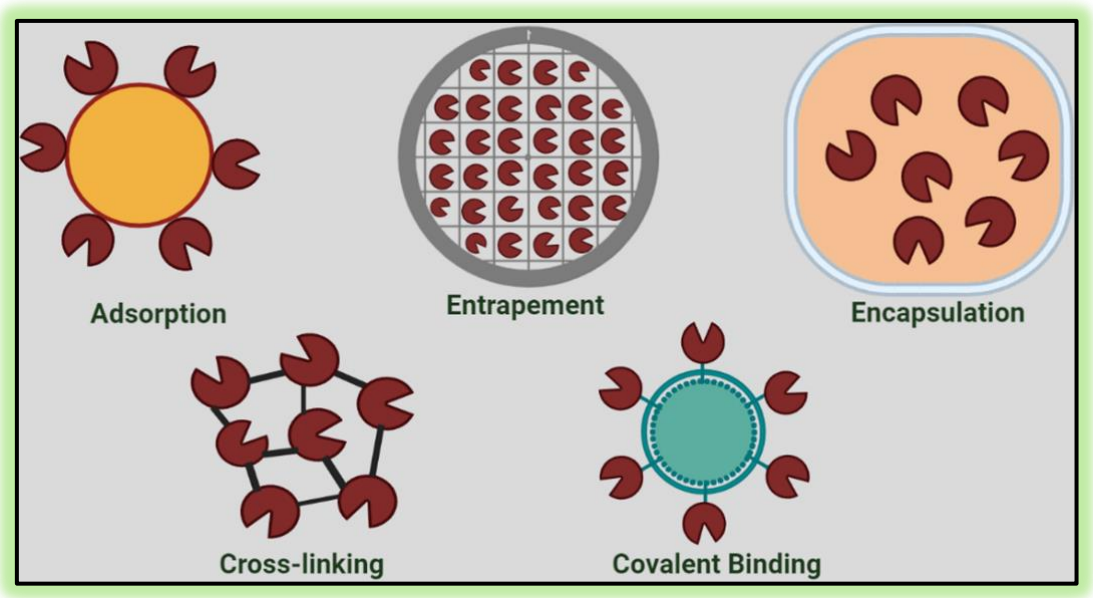


Figure 2.7 Schematic representation of different modes opted for enzyme immobilization.

2.13.1 Synthesis and ZnO nanoparticles and phytase immobilization

The integration of nanoparticles in various innovative biotechnological applications has emerged from the coupling of nanoparticle's distinctive chemical, optical, physical, and electrical capabilities with the catalytic properties of biomolecules. They have been used several times to immobilize industrial enzymes with enhanced properties. Because of the elevated surface-to-volume ratio provided by nanoparticles, the concentration of the immobilized item was significantly greater. Enzymes immobilized on nanoparticles have a wider pH and temperature range than natural, free enzymes, as well as better thermal stability [225]. Nanostructure materials demonstrated intriguing features such as a high surface-to-volume ratio, high catalytic efficiency, high surface reaction activity and excellent adsorption capacity, making them eye-catching potential candidate materials for enzyme immobilization. The high surface area of nanomaterial's offered a superior carrier for enzyme immobilization, resulting in greater protein loading per unit mass of nanoparticles. Furthermore, the multiple-point attachment of enzyme molecules to the nanomaterial surface constrains protein unfolding, resulting in increased stability of the enzyme bound to the outer surface of nanoparticles [226-228].

Biocompatibility, non-toxicity, low cost, large surface area and chemical stability are the benefits of zinc oxide (ZnO) nanoparticles over other nanometal oxides [229]. Also, the ZnO has already been approved as a food additive by FDA (Food and Drug Administration). Antimicrobial nanomaterial's, such as nano ZnO, are being used in food. It was experimentally

elucidated that it can be utilized as packaging and food contact materials in such a way that it functions as an antibacterial agent [230]. These characteristics facilitate the use of nano ZnO as biomimetic support for adsorbing and modifying proteins and enzymes. Various interactions, such as hydrogen bonding, hydrophobic, electrostatic, ionic, and van der Waals, occur between the enzyme and the (ZnO) carrier molecules [231]. The 3-D construction using ZnO nanorods enabled direct electron transmission of uricase while demonstrating an outstanding thermal stability profile and anti-interference capabilities. Such effective enzyme immobilization processes may lead to a unique approach to biosensor fabrication [232]. ZnO nanoparticles act as bioaffinity support to develop a distinctive and effectual method for immobilizing *A. oryzae* galactosidase. According to AFM (Atomic force microscopy), the produced matrix delivers an ideal microenvironment and a high surface area appropriate for attaching a substantial quantity of the enzyme. Zinc oxide nanoparticles retained 84 % of the enzymatic activity after immobilization. The pH optimum for soluble and immobilized galactosidase was 4.5, whereas the temperature suitable for the immobilized enzyme was elevated from 50 to 60 °C. When the free enzyme was treated at 60 °C for 2 h, 81% of its activity was lost, while the immobilized enzyme retained 60% of its activity under similar experimental conditions [233]. The α-amylase was effectively immobilized on the ZnO porous nanosheet's surface. The chemical nature of the enzyme adsorption indicates chemisorption [234].

2.14 Current potential biotechnological applications of phytase

Microbial phytases have a number of potential applications in feed and food supplementation (Figure 2.8). The prerequisite for phytase for potential applicability in feed and supplementation should be active in the digestive system and stable during storage and food processing. It has also been explored as a plant growth promoter. Phytases have been used in the food and feed processing industries, as well as in the peroxidase synthesis, plant growth stimulation, phosphorus pollution control, and the manufacture of various medicinally important inositol phosphates [67, 99, 143, 196] (Figure 2.8). Earlier studies have shown that phytase ameliorates the nutritional status of staple foods. Because inorganic phosphate is a non-renewable resource, it will be exhausted on the planet in the next 50 years [235]. As a result, phytase is a crucial enzyme for the long-term supply of natural phosphorus. Plant phytate may be transformed into usable phosphorus, which monogastric animals can then consume. Many plants, such as alfalfa and soybean, have been shown to express endogenous phytases. Adding phytase to the diet can improve digestibility and the effectiveness of minerals and other nutrients. Microbial phytases

added to monogastric diets improved copper, zinc, and magnesium availability in fish, pigs, poultry, pigs, and other monogastric animals [23, 33, 99].

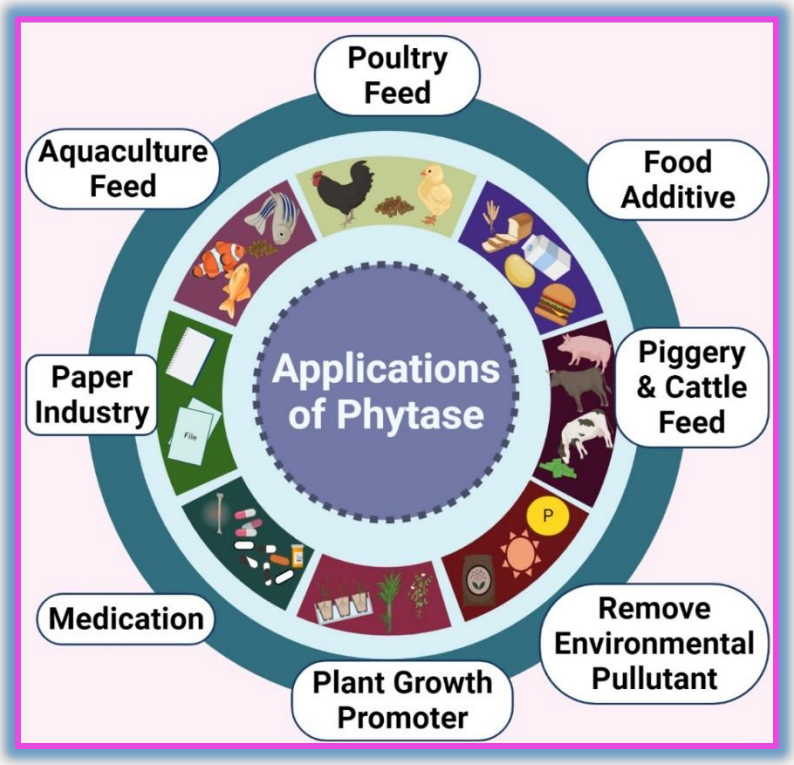


Figure 2.8 Various biotechnological applications of microbial phytase.

2.14.1 Feed additive

Animal feed is mainly derived from plant-based formulations rich in anti-nutrient phytic acid. Ruminant animals can only partially digest phytic acid as intrinsic microflora expresses phytases to a limited extent. An extraneous supply of Pi is required to meet the desired requirement, which adds extra cost constraints to the livestock industry. Phytase is being utilized as the chief ingredient to release the bound Pi and other minerals. For commercial utilization of phytase, it should be produced economically in an eco-friendly manner. Feed is pretreated at high temperatures to decrease the chances of infection caused by pathogenic microbes like *Salmonella*. The pretreatment temperature goes up to 95 °C. Thus, adding enzyme during preprocessing, feed treatment would not be effective as the enzyme would denature at such a high temperature. Thermostable enzymes are much more desirable in such scenarios. At the same time, adding phytase enzymes active at low temperatures after feed treatment needs special equipment, time-consuming and extra cost to feed formulation.

Moreover, liquid formulations of phytase are less stable and need stabilizers (polyols, ions, polymers and ethylene glycol) which adds additional cost constraints [171, 236]. It should have

high thermostability profiling to withstand the pelleting temperature of feed pretreatment and processing [67, 99, 237]. It should have acidic pH tolerance potential to withstand the harsh conditions of digestive juices (pepsin, trypsin, papain, pancreatic juices). It should have a proteolytic resistance mechanism to remain unaffected by the catalytic activities of the digestive enzymes of monogastric animals. It has been found that substrate-specific and broad range pH active phytases are more suitable for animal feed applications.

Most commonly, HAPs are being utilized commercially for releasing bound minerals (calcium, Pi, zinc, iron) and biomolecules (carbohydrates, proteins and digestive enzymes) [46, 238]. Phytase derived from *A. niger* was the first commercial source for phytase production in 1999. Afterwards, new advancements in strain development and enzyme engineering further improved phytase production and catalytic properties [125]. There are very few reports where multienzyme diet administration results in improved growth performance in monogastric animals. Broiler chickens, when administered with a corn-soybean rich diet along with *E. coli* phytase and *Penicillium funiculosum* carbohydrases (xylanase and glucanase), resulted in better growth and bone mineralization [239]. When broiler chickens were fed with a corn/soybean meal regime supplemented with phytase and a cocktail of enzymes (XAP: xylanase, amylase, and protease), it resulted in 14% more weight gain than those who were fed with each enzyme alone [240]. The nutrient digestibility was enhanced in broilers from 43 to 54.3% when their diet was supplemented with citric acid along with multi-carbohydrase and phytase enzymes [241]. Another study showed that dietary Pi requirement could be lowered without affecting milk supply in cows when faba beans were utilized as the predominant source of protein along with supplementary phytase. This supplementation not only improves Pi digestibility but also decreases its excretion in the faecal matter [242].

2.14.1.1 Poultry

Phytase has been utilized since the 19th century in poultry farming. Earlier studies carried out by Nelson et al., showed that broilers fed with soybean meal and corn soya diet along with *A. niger* phytase resulted in reduced Pi release in faecal matter. The Pi availability was enhanced by up to 60%, while body weight was enhanced for both male and female chickens with a P deficient diet [243]. Earlier reports established that phytase administration in poultry farming resulted in a 56% reduced Pi release along with enhanced body weight of chicken when fed on a P deficient diet with a phosphorolytic enzyme (phytase + acid phosphatase) cocktail [244]. Adding *P. anomala* phytase to chick feed increased development and phosphorus retention

while lowering phosphorus excretion [245]. The dephosphorylating phytate content in animal feed was caused by *A. niger* van Teighem's phytase [246].

Improved growth performance and mineral utilization potential of corn-soybean meal diet of chicken feed showed a synergistic effect of phytase with multi-carbohydrase [241]. Different doses of *A. niger* phytase result in better digestibility of calcium and Pi with better egg quality and eggshell. The probiotic *Lactobacillus* spp. increased in ileum when phytase was administrated with a regular mixed diet (maize, soybean meal, wheat, rapeseed oil) of hens [247-249]. Adding *S. thermophile* phytase to poultry feed lowered phytic acid levels while simultaneously improving the bioavailability of minerals and lowering the feed rate [65]. Super dosing phytase to egg-laying hens positively affects the productive parameters while egg quality remains unaffected [250]. Calcium and Pi are two important nutrients required for the cellular process, and bone formation is required in feed formulation. Dietary super dosage addition of phytase enhances the energy utilization and digestibility of proteins and P in the maize diet [251]. Recent studies have shown that an exogenous high dose of phytase resulted in better energy and mineral utilization with weight gains without adversely affecting growth and performance [252, 253]. Excluding the increased shell weight and thickness ($P \leq 0.05$), dietary supplementation of bacterial and fungal phytases had no impact on growth performance or egg quality attributes. In treated hens, serum hepatic functioning biomarkers and serum lipids were unaffected, although calcium and Pi levels were higher ($P \leq 0.05$) than in control systems [254].

2.14.1.2 Piggery

Phytase supplementation in pig's diet has similar utility as in poultry farming. The phytase should be active in the stomach and small intestinal part of a pig's digestive system to show a similar effect. A study has shown a better catalytic activity of *A. niger* phytase in the stomach than in the intestinal phase of digestion [255]. Organic acids and *A. niger* phytase increase animal growth and nutritional digestion [256]. The microbial phytase was added to improve the apparent digestibility of mineral deposits such as phosphorus, zinc, calcium, iron and amino acids. The supplementation also reduces the nitrogen and Pi content in the faecal matter of pigs [257]. Revy et al. found that adding microbial phytase to pig's soybean meal-maize-based diets enhanced plasma alkaline phosphatase activity, along with zinc concentrations in bone and plasma [258]. A previous study has shown that phytase with xylanase have better Pi retention in all ages of pigs [259]. Adding phytase to the diets of piggery animals improved the digestibility of phosphorus and amino acids [260]. Another study showed that pigs fed on a

wheat-based diet with phytase supplementation showed enhanced digestibility of P and calcium [261]. Studies have shown that fungal phytase addition to the pig diet reduces the P content in excreta while improving Pi retention with better nutrition digestibility without affecting growth and performance [262].

2.14.1.3 Aquaculture

Fish cannot hydrolyze phytate to release Pi as they lack intrinsic cellular mechanisms. Thus, extraneous P is added to extensive fish farming to meet the P requirement, posing a major environmental problem as unutilized organic phosphorous is excreted in the faecal matter. Phytase supplementation results in the limited requirement of mineral addition with less release of organic phosphorous and a better aquaculture setting, thus relieving pressure on tackling P pollutants [263, 264]. Approximately 60% reduced Pi excretion was observed in fish culture when catfish (*Ictalurus punctatus)* were fed a phytase-rich diet. Phytase supplementation proved more economical than adding Pi to the feed diet [265]. Dietary supplementation of fish with phytase and plant-based diets like soybean meal, wheat and corn middling improves Pi accessibility for absorption along with better utilization of released proteins and minerals.

Previous studies have shown that ash and mineral contents (calcium, Pi and manganese) were higher in fish feed with a phytase supplemented diet as compared to the control (without phytase) group [266, 267]. The dephytinization process is completed in the stomach, and efficacy depends upon the dose of phytase supplementation [267]. Prior studies have shown that feeding a rohu (*Labeo rohita*) with sesame oilcake processed through solid-state fermentation by *B. subtilis* significantly enhanced minerals, free amino acids and fatty acid content along with the reduction in phytic acid, tannins and trypsin inhibitors [108]. A study has established that β-propeller phytase is more beneficial for aquaculture application, but more elaborated studies need to be conducted to decipher this structure-function relationship [268]. An ideal phytase for aqua specific applicability should be catalytically active at low temperatures and have low pH optima. Recently, a study has shown that *Bacillus aryabhaltai* RS1 isolated from the rhizosphere of *Cicer arietinum* (chickpea) has an active phytase catalytically efficient at low temperatures [269]. Transgenic algal species and aquatic animals could efficiently utilize Pi, but this approach is impractical owing to extensively listed ethical obligations. The ideal level of phytase to produce an inexpensive and environmentally friendly diet for enhanced development of *Cyprinus carpio* fingerlings is 950 FTU kg^{-1} (phytase unit) since it reduces mineral and nutritional outflow into the aquatic environment [270].

2.14.2 Applicability in the Food industry

Phytase from different microbial sources resulted in dephytinization of food, converting inaccessible Pi to a soluble and accessible form by reducing the phytic acid content. The steady upsurge in the liberation of inevitable nutrients (Pi, reducing sugar, protein and metal ions) during dephytinization occurs in a time-dependent manner and at optimal temperature and pH [51, 54, 56, 64, 68]. Phytase supplementation dramatically enhances the nutritional quality of plant-based staple foods. This supplementation is highly recommended for childbearing women, growing children, and people who follow vegetarian and vegan diets as it can overcome the major deficiency caused by minerals like zinc, iron and calcium [271].

One of the most widely studied applications of phytase is in bread making as phytase addition during dough fermentation extensively enhances the quality and nutritional properties of bread by reducing anti-nutrient elements, i.e., phytic acid, and enhances the availability of reducing sugar to carry out fermentation [4, 272]. In vitro studies have shown that phytase can release iron from wheat bread and enhance its digestibility with various doses of citric acid [82]. Studies have shown that strains of *Bifidobacterium (B. catenulatum*, *B. breve*, and *B. longum*), when used as a starter culture for dough fermentation, express phytase, which in turn reduces the phytic acid content with the progression of the fermentation process. Approximately 44 and 67% reduced phytate content was observed after treating whole wheat bread and bread augmented with bran separately. The texture quality has also improved compared to the control sample [273, 274]. Lactic acid bacteria (*Lactobacillus sanfranciscensis* and *L. plantarum*) assisted fermentation also brings down the phytic acid content with the metal ions (Ca^{2+}, Zn^{2+}, and Mg^{2+}) released from gluten-free bread and enhances the physical as well as shelf-life of bread [275, 276]. The phytase activity of *Bifidobacterium pseudocatenulatum* ATCC 27919 positively impacts on mineral availability for high fiber whole rye bread [277]. The exogenous phytase supplementation improves the protein content, quality, and texture of the bread [132]. The phytase of *A. niger* and *S. cerevisiae* reduce the phytic acid content by 45.55%, along with improving the minerals release and bread attributes (texture, appearance, taste) [278, 279]. Another study showed a phytic acid content reduction of up to 70% in bread by a recombinant phytase of *Sporotrichum thermophile* [183]. Recently, one study showed that the dialyzability of key minerals Fe and Zn increased by 21.9 to 48% and 39.5 to 96% with APF1 phytase (fungal phytase) treatment respectively in contrast to untreated samples. Similarly with wheat phytase Fe and Zn increased from 6.10 to 30% and 23.2 to 81% respectively, when compared with the

untreated samples. Phytase supplementation improves soluble protein levels and Pi in treating foodstuff [280].

2.14.3 Plant growth promoter

Phosphorus (P) is the most important macronutrient for plant development and growth [281]. Both organic and Pi are present in the soil, and phytate contributes 10-50% of the total organic Pi available in the soil. Plants can't get phosphorus from the soil as the phytate forms insoluble complexes with the minerals. Thus, P deficiency is a major concern worldwide for agricultural production. Microbial phytase plays an important role in converting inaccessible P into an available form for plant growth [99]. In the rhizosphere, various plant growth-promoting bacteria (PGPB) mobilize and solubilize the unavailable organic P. Bacteria like *Enterobacter* have been isolated as phytase producers from the rhizosphere of legume plants [85]. Two major groups of bacteria, phosphate solubilizing bacteria (PSB) and phytate mineralizing bacteria (PMB), belong to the genera *Enterobacter, Pseudomonas, Klebsiella, Citrobacter, Pantoea, Advenella* and *Cellulosimicrobium* spp. for plant growth promotion [282, 283]. Phytase producer *Bacillus megaterium* A6 is utilized as a biofertilizer for oilseed rape [284]. Under low and sufficient P conditions, expressing PvPHY1 (phytase gene) from *Pteris vittata* in tobacco boosted its growth and enhanced P retention by 10-50%. It improves soil P usage efficiency, thus ultimately lowering the need for P fertilizer in agricultural production [285].

2.14.4 *Myo*-inositol phosphate formulation

The key functions of lower *myo*-inositol phosphate derivatives are in cell signalling and calcium ion mobilization from inside cellular regions of cells [286]. The antioxidant properties of inositol-2,3,4,5-tetrakisphosphate and inositol-2,4,5-triphosphate were studied by Hawkins et al. and Phillipy and Graf [287, 288]. *Myo*-inositol phosphates have also been found to have health advantages, such as preventing kidney stone formation [289], heart disease [290], and some malignancies [291, 292]. Siren postulated that inositol triphosphates might be used as an analgesic [293]. Another study reported that inositol triphosphate esters have a significant inhibitory effect on retroviral infections, including HIV [294]. Immobilized phytases have been employed to make different *myo*-inositols [295].

2.14.5 Microbial phytases for peroxidases synthesis

Microbial phytases and haloperoxidases have similar active sites. After including vanadium ions in their active site, they are changed into peroxidase [296, 297]. Vanadium

chloroperoxidases have structural similarities to acid phosphatases and are effective in identifying organic compounds, antigen quantification, liquid chloride estimation, and protein iodination [297, 298]. The enantioselective oxidation of prochiral sulphides was performed by the vanadium-incorporated phytase from *A. niger* NRRL3135. When phytases from *A. nidulans*, *A. fumigatus*, and *A. ficuum* were combined with vanadium, they catalyzed enantioselective oxygen transfer processes. Vanadium inclusion in the active site of a cross-linked enzyme aggregate of 3-phytase turned into peroxidase [299]. The integration of the vanadate ions transformed the *P. anomala* phytase into a haloperoxidase. Similarly, *S. thermophile* recombinant phytase is transformed into a peroxidase on vanadate addition [297]. This transformation from phytase to peroxidase is owing to a common binding site shared by vanadate and phytic acid, as demonstrated by molecular docking experiments [300].

Intensive livestock farming releases a large amount of excreta loaded with unutilized Pi. Extraneous Pi supplementation has been chosen for monogastric animals to meet their P needs since they lack the synthetic machinery to utilize Pi from the phytate rich diet. The leaching out of Pi used as fertilizer in agricultural fields along with un-utilized P from monogastric animals to the water bodies poses a major environmental threat. It leads to eutrophication, resulting in hypoxia conditions for aquatic animals, altering the balanced ecological conditions [99, 128]. Microbial phytase supplementation in animal feed will reduce the extra need for P addition and thus reduce the overall P pollution. Prior studies have shown that mice expressing the appA phytase gene from *E. coli* in the parotid salivary gland have reduced P in their faecal matter than control ones. Thus, phytase helps in P management ecofriendly. Transgenic pigs with phytase expression in their salivary gland, when fed with a P deficient diet, showed normal growth with better Pi utilization and about 75% reduced P in their excreta [301]. *Lactobacillus reuteri* phytase suspension in bird feed also combats P release in an environment with better utilization of their diet [302].

2.14.6 Phytase as an important ingredient for bioethanol production from starch-based substrate

Phytic acid is a major component of cereal/agricultural based starchy raw material, making arduous complexes with polyvalent cations, protein and starch, resulting in diminished availability of fermentable sugar and active enzymes required for alcoholic fermentation [303]. Liberating starch and other bound minerals by phytase application is one of the most feasible approaches to tackle the problem. Phytase results in improved ethanol yield and better tolerance

of yeast during fermentation. Studies have shown that phytase applicability enhances the functionality of lignocellulosic hydrolyzing enzymes as more polyvalent ions are available for their catalytic activities [304]. Phytase can be added either during the liquefaction of starchy raw material, where the temperature could rise to 105 ℃ or during yeast fermentation. Thermostable enzymes are more preferred ones at this stage. Prior studies have shown that phytase addition at this stage resulted in better solubility of contents and activity of alpha-amylase and efficient hydrolysis of starchy material with improved ethanol concentration at the end [305, 306]. Recombinant thermostable *Buttiauxiella* phytase added during the processing of starch results in a low level of phytic acid at the end of the process [307]. Yeast recombinant strain (PHY) expressed phytase on its surface, resulting in increased free Pi up to 142% and an 18% higher fermentation rate along with the reduced release of pollutants (P) comparable to stranded stain *S. cerevisiae* [308]. Another study showed that thermostable phytase from *Thermomyces lanuginosus* SSBP resulted in enhanced ethanol production by reducing the phytic acid content of the tropical plant *Colocasia esculenta* or Taro from 1.43 to 0.05 mg/ml during fermentation [309].

2.14.7 Phytases as an antioxidant

Food with a high level of unsaturated fatty acids and iron can cause oxidation, resulting in loss of organoleptic (flavor and colour) properties and nutritional contents of the food. Adding antioxidants can prevent heft loss mainly due to oxidation in food. Phytase acts as the chief naturally existing and nontoxic antioxidant, preventing nutritive degradation caused in oxidation-prone food [310].

2.14.8 Production of transgenic plants

Low phytate expressing crops provide a new alternative technology to overcome hurdles caused by anti-nutrient factors present in plant-based food formulations. Transgenic plants expressing phytases in crops to produce seeds with substantial phytase content have received much attention. The microbial origin gene directly expressed in crop plants produced in bioreactors provides a direct means for phytase production [42]. Phytase expressing bioengineered plants also have diminished phytate content with ameliorated nutritional content. Microbial sources are usually preferred for transgenic crops due to the broad pH and temperature range [311]. In plant crops, prokaryotic (bacteria) and eukaryotic (fungi) genes are expressed. SrPf6 from *S. ruminantium* and the appA phytase gene from *E. coli* were expressed in transgenic rice

seedlings using an inducible α-amylase gene promoter and α-amylase signal peptide sequence to guide extracellular phytase secretion. When phytase activity was measured in transgenic rice, it was 46–60 times higher than in non-transformant rice, with no adverse effects on seed production or plant growth [312]. Bacterial origin appA gene from *E. coli* was expressed in rice, and phyA gene from *B. subtilis* 168 in tobacco [312, 313]. Overexpression of the bacterial US417 phytase gene in *Arabidopsis* resulted in a drop in phytic acid content of the seed and an upsurge in inorganic phosphate and sulphate content. This resulted in considerable remobilization of free iron without impacting seed germination [314]. *A. niger* gene is successfully expressed in wheat, canola and maize to make transgenic crops [315]. A phytase gene (afphyA) from *A. ficcum* successfully expressed in soy roots assimilates more phosphate than wild control plants [186].

2.14.9 Phytase in the paper industry

Lignocellulosic materials need to be pretreated in the paper and pulp industry to eradicate lignin and phytic acid. The application of phytase assists in removing unwanted residues and improves the quality and brightness of paper. Enzymatic phytic acid elimination produces no harmful byproducts or end products, making the procedure more appealing. Phytase application helps overcome the hazardous, carcinogenic effects of chemical bleaching agents and provides an eco-friendly green technology [99].

2.15 Commercially available phytase in market

Approximately 70% of the animal feed enzymes industry market is only covered by phytase enzyme alone [4]. Several commercial phytases are procured from various sources and sold with different trade names by other manufacturers. For example, 3-phytase from *A. niger* is commercially manufactured by a German company under the trade name ‘Natuphos’. *Peniophora lycii* phytase expressed in *A. niger* was used for the commercial manufacturing of 6- phytase under the brand name Ronozyme. Commercial microbial phytases of fungal and bacterial origin have been used in feed and food formulations. Several commercially available phytases have been listed in Table 2.8.

Table 2.8 Most commercially available phytases of microbial origin.

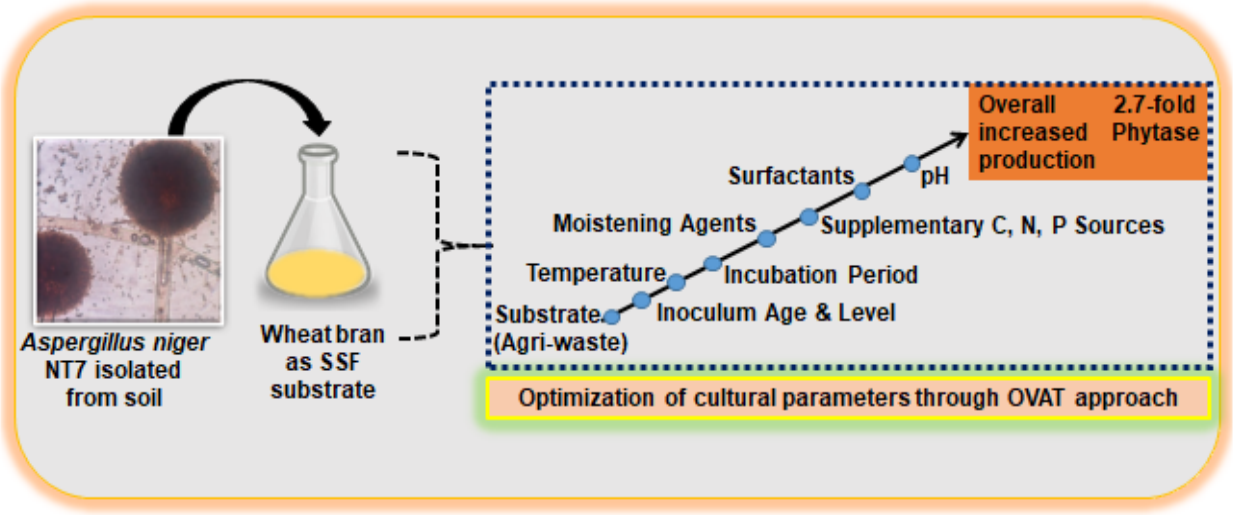
Trade Name	Producer Country/Company	Microbial Origin	Key Point
Fungal Origin Phytases			
Allzyme®	Alltech/ USA	*A. niger*	Produced by SSF and utilized as feed for livestock and Aquaculture
Natuphos®	TMBASF/ Germany	*PhyA* gene from *A. niger* NRRL 3135 cloned in a system named PluGBug®	Catalytically active over an acidic pH range (6.5-5.5)
Ronozyme®	TMP Novo Nordisk/ Denmark	*Peniophora lycii* phytase expressed in *A. niger*	Cost-effective thermotolerant phytase was produced
Bacterial Origin Phytases			
Optiphos®	JBS/ USA	*E. coli*	Produced up to 500 FTU/g with excellent stability, thermotolerant, high catalytic activity, flowability, and mixability
Phyzyme®	XP Danisco, Brabrand/Denmark	*E. coli* phytase expressed in *S. pombe*	Patented technology for superlative thermotolerant potential
Finase® EC	AB Vista	6-Phytase from *E. coli*	Specifically being utilized for swine and poultry
Quantum® phytase XT	Nutrivet	*E. coli*	Utilized as a feed additive
Others			
VemoZyme®	F NTP	Natural Product	Active over pH range 2-6 and stable at high temperature (90-95 °C)

Adapted from [4, 67]

CHAPTER 3

TO OPTIMIZE THE SOLID-STATE FERMENTATION CONDITIONS FOR PHYTASE PRODUCTION FROM ISOLATED *A. NIGER* NT7

GRAPHICAL OUTLINE OF CHAPTER



HIGHLIGHTS

- Potential phytase producing fungal isolates selected based on primary and secondary screening
- *A. niger* NT7 selected as potential phytase producing fungal strain
- Wheat bran as the most appropriate agri-waste to carry out cost effective solid state fermentation
- Overall 2.7-fold enhanced phytase production attained

3.1 INTRODUCTION

Animal feed provides a vast quantity of vitamins, proteins, minerals, and energy. This stuff is primarily comprised of leguminous plants and food grains such as wheat, triticale, oats, soybeans, corn and oil cakes. Phosphorus is generally present in feed as phytate, which is nutritionally unprofitable for monogastric animals. Phytate is recognized as an anti-nutritive factor owing to its ability to form complexes with vital digestive enzymes, cationic metal ions (Zn^{2+}, Fe^{2+}, Ca^{2+} and Mg^{2+}) and nutrients. Thus, these nutrients become inaccessible for absorption, resulting in silent hunger, loss of nutrition, and anemia-like conditions in monogastric animals. Since cattle feed is primarily based on phytate-rich formulations, anti-nutrient components in feed make them more vulnerable to nutritional deficiencies [1, 2]. The supplementation of phosphorus in animal feed makes matters worse as it not only raises the cost of the feed but also negatively impacts on the environment when excreted through faeces. The excreted phosphorus and phytic acid (unabsorbed) from the bodies of monogastric animals (chicken, pigs, and swine) runoff from disposed sites to cause soil and water pollution [2, 3]. The animal farming industry relies heavily on feed consistency, which adds the extra cost of supplementation [4]. Therefore, incorporating phytase enzymes in the feed facilitates not only the liberation of nutrients and phosphorus but also reduces the need for additional supplementation.

Phytases (EC 3.1.3.8) are hydrolase enzymes that hydrolyze phytate to produce less phosphorylated *myo*-inositol derivatives while releasing inorganic phosphate [2, 5]. The dephytinization facilitates the revitalization of the nutritional grade of the feed, as well as the removal of organophosphorus pesticides and the reduction of environmental risk, i.e., eutrophication [6, 7].

According to a report, the market value of phytase is rising at a rate of 6.3% yearly, and it will reach a worldwide value of more than $1 billion by the end of 2025 [8, 9]. The property of phytase not only makes it a profitable enzyme in the animal feed industry but also acts as a massive value in biofuel production. In comparison with other traditional enzymatic methods, the phytases augmented the bioethanol formation from the lignocellulosic and starchy biomass [10]. To date, there are still studies underway to achieve efficient phytase production with excellent thermostability and steady functioning in the gastrointestinal tract of animals with protease resistance potential [11, 12].

The different microbial sources contributed to the extensively high yield of commercial phytase production [12, 13]. Solid-State Fermentation (SSF) is favored over submerged fermentation for fungus cultivation. The SSF requires fewer nutrients for fungal growth and enzyme production. Low-priced feedstock like agri-waste, which requires less water, are a cost-effective SSF substrate. The advantages of SSF include a higher quantity of enzyme production with ease in downstream processing, which can be attained with less equipment expenditure, and less water requirement [14, 15]. Using agri-waste and co-products as SSF substrate helps in waste management and reduces the production of massive amounts of waste [16]. The wide varieties of economical substrates of the agricultural waste and other co-products are utilized for the production of phytase through SSF. The substrate consists of the mixed substrate such as wheat bran with linseed oil cake (*Rhizopus oryzae*) [17], wheat bran (*A. niger* NRF9) [18], deoiled rice bran (*R. oryzae*) [15], potato squander (*A. ficuum*) [19]. These were earlier employed as potent substrates for phytase production. The wheat bran also acts as a valuable and prosperous blend of minerals and nutrients and is utilized as an SSF substrate for fungal growth [20, 21]. To achieve maximum enzyme production conventional approach One Variable at A Time (OVAT) is efficient one as it is simpler one. In the approach, one parameter is varied over a range while keeping all the other understudy parameters constant [22].

The current research was accomplished to discover a novel thermo-tolerant phytase from the fungal resource and its characterization and production for the animal feed formulation. Agricultural fields are bestowed with diverse sources of microbial cultures; thus, soil samples were collected for the isolation of fungal cultures. In this study, phytase was isolated from the fungus *Aspergillus niger* NT7. Furthermore, the OVAT methodology was used to optimize its cultivation conditions to obtain an economical and feasible SSF using Wheat bran as a substrate. Various physiochemical and nutritional parameters were optimized by the OVAT approach to achieve the maximum phytase production in SSF.

3.2 MATERIALS AND EXPERIMENTAL PROCEDURES

3.2.1 Chemical reagents

All the reagents used for the current study were of analytical grade. The chemicals and other components were purchased from the HiMedia laboratories, SRL, and MERCK (India). The agri-waste was collected locally from the Hamirpur and Solan districts of Himachal Pradesh, India.

3.2.2 Selection of phytase producing fungus isolates

3.2.2.1 Sample collection and media enrichment

The samples were collected from the different agricultural fields of rice (*Oryza sativa*), black gram (*Vigna mungo*), maize (*Zea mays*), and wheat (*Triticum aestivum***)** and also from the chicken farms of various areas of Himachal Pradesh. The soil of the uppermost layers of these fields and farms was collected in sterile containers and stored at 4 °C until it was further enriched in a medium and processed. These samples (1g) were then nutritionally supplemented in 100 ml Phytase Screening Medium (PSM) (Figure 3.1). The composition of the phytase screening medium is represented in Table 3.1.

Table 3.1 Components of PSM media.

Media components	**Volume (g/l) (W/V)**
Ammonium nitrate	5
Potassium chloride	0.5
Ferrous sulfate	0.01
Magnesium sulfate	0.5
Manganese sulfate	0.01
Calcium chloride	0.01
Sodium phytate	4
Glucose	15
pH- 5.5	

Furthermore, after enrichment, the samples were serially diluted in 0.9% NaCl (normal saline). The 100 ul sample from 10^{-2}, 10^{-4} and 10^{-6} dilutions were spreaded over the Potato Dextrose Agar plates (PDA) and kept at 30 °C in an incubator for visible growth of fungi. These isolated fungal cultures were preserved at 4 °C in the PDA slants to carry out further experimentation.

3.2.2.2 Primary screening of potential phytase producer fungal strains

The primary screening of microbial cultures with phytase-producing potential was done by the methodology where growth on the Phytase Screening Agar (PSA) was observed after inoculating fungal spores [23]. There was the development of a transparent region near the fungus culture against the opaque background of PSA. The growth zone diameter is subtracted from the total clear disc diameter to estimate the region of hydrolysis. The hydrolysis zone was formed by the action of phytase, which is responsible for clearing the metal-phytate complex.

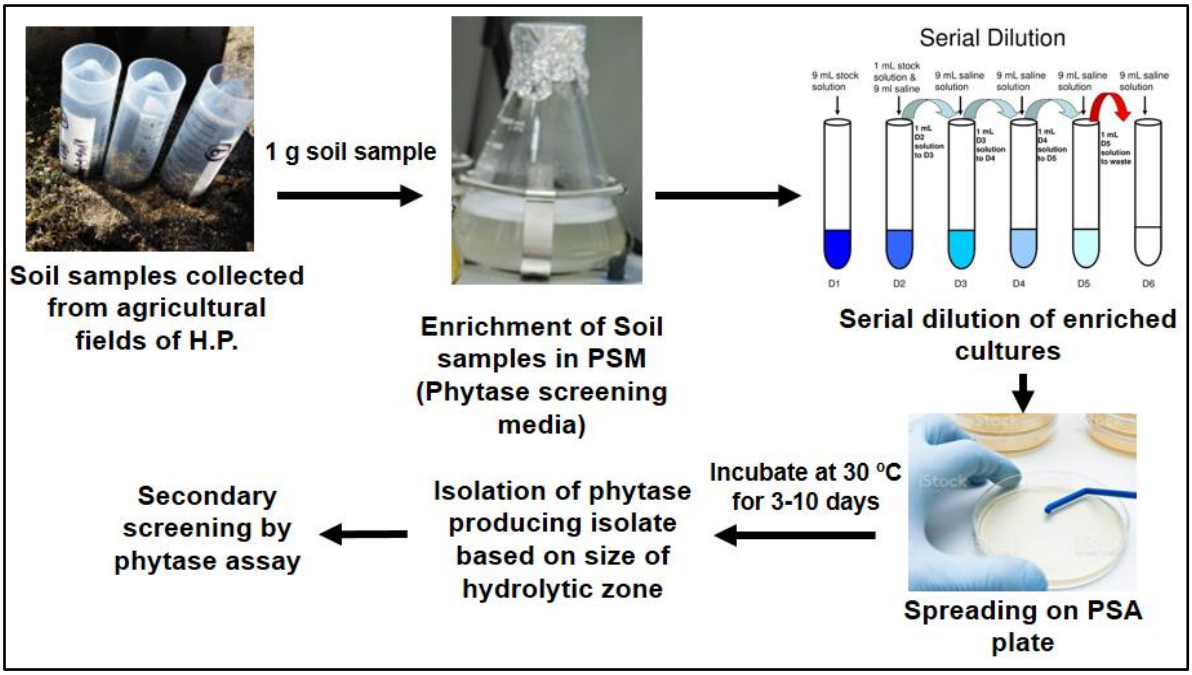


Figure 3.1 Pictorial illustration of isolation and screening of potential phytase-producing fungal isolates from various environmental soil samples.

3.2.2.3 Secondary (quantitative) screening of potential isolates

Qualitatively chosen phytase-producing fungal isolates were tested in a liquid medium for phytase assay. The PSM-grown culture was strained using Whatman No.1 filter paper to remove fungal mycelium growth, and the clear solution was employed for the phytase assay (Figure 3.1) to determine the phytase activity [24].

3.2.2.4 Phytase assay

For the standard curve, potassium dihydrogen phosphate (KH_2PO_4) was employed as the standard stock solution. From the stock solution, a 1 ml dilution series ranging from 50 to 500 µg/ml of KH_2PO_4 was prepared. The 250 µl of 10% Trichloroacetic acid (TCA) was added to a 1 ml solution, followed by the addition of freshly prepared 1 ml colouring reagent (distilled water acidified with 5% sulphuric acid followed by adding 1% ammonium molybdate and 7.20% ferrous sulphate). The mixture was vortexed and allowed to develop a blue-coloured complex in the dark for 10 min. At 750 nm, absorbance was measured in a spectrophotometer against a blank.

The phytase activity was determined using the Fiske and Subbarow method [24] with some modification. The phytase assay was carried out with 0.9 ml of acetate buffer (0.1 M; pH 5.0) containing 2.0 mM sodium phytate as a substrate along with 0.1 ml enzyme. The reaction was

incubated at 50 °C for 30 min and then the reaction was stopped by adding 250 µl of 10% TCA. 1 ml colouring reagent was then added to develop the colour followed by 10 min incubation in dark. The absorbance was measured at 750 nm spectrophotometrically. The amount of liberated free phosphate was then calculated using a KH_2PO_4 standard curve (Annexure A.2).

Under assay conditions, the enzyme required to release one micromole of inorganic phosphorus (Pi) in one min is defined as one unit of phytase activity. The phytase activity in SSF is measured in units per gram dry substrate (U/gds).

3.2.3 Identification and characterization of phytase-producing strains

3.2.3.1 Colony morphology

Fungal culture was inoculated on a PDA plate and incubated at 30 ºC for 72 h before being examined for morphological features (colony colour and texture) [25].

3.2.3.2 Staining of fungal culture

The fungal isolate was morphologically distinguished through Lactophenol Cotton Blue (LCB) staining. The fungal mycelium was put on a glass slide with a droplet of distilled water. With a needle, the mycelium was disseminated and coloured with an LCB stain. Under a microscope, the mycelium was covered with a clean coverslip and examined (100X).

3.2.3.3 Identification using molecular techniques

The fungal culture was identified using the 18s rRNA sequencing through common ITS (Internal Transcribed Spacer) primers [26, 27]. The nucleotide BLAST (Basic Local Alignment Search Tool) algorithm was used to evaluate the sequence obtained from 18s rRNA. This sequence was submitted to the GenBank, and a unique GenBank ID was obtained.

3.2.4 SSF Experimental setup for phytase production from *A. niger* NT7

3.2.4.1 Inoculum preparation

The spores were collected in the normal saline, and the suspension was made from the 96 h old PDA plates. The spore count was done through a hemocytometer. Primarily, the 15×10^7 spores suspension/ml was employed as an inoculum for phytase production.

3.2.4.2 Selection of SSF substrate

Different types of agricultural waste can be utilized as the substrate for the growth of fungus and enzymatic production. These agri-wastes initially passed through the 600 µ pore size sieve (BSS 25 IS46-1962) to attain the uniform particle size. After refining, the substrates were dried

at 55 °C in an oven to get rid of any retained moisture content. The 5 g of dried-intermediate size substrate are moisturized with distilled water (10 ml). Table 3.2 represents the different types of agri-waste screened for selecting an appropriate SSF substrate for phytase production. The moistened substrate is subjected to sterilization in an autoclave for 25 min at 121 °C at 15 psi pressure. The inoculum of 15×10^7 spores/ml (4 days old) was added to these sterilized, moisturized SSF agri-waste substrates and incubated at 30 °C for four days.

Table 3.2 Different types of agriculture waste.

Agri-waste	Symbolic representation
Maize bran	MB
Banana peel	BP
Wheat bran	WB
Fruit Pulp	FP
Coconut fibers	CF
Orange peel	OP
Wheat straw	WS
Mustard oil cake	MOC
Sugarcane bagasse	SB

3.2.4.3 Selection of various physiochemical and nutritional supplement parameters by OVAT approach

After selecting the appropriate agri-waste, SSF affecting physiochemical parameters such as the age of inoculum (2-9 days old), amount of inoculum ($1\text{-}24\times10^7$ spores/ml), optimal temperature (ranging from 20-45 °C at an interval of 5 °C), fermentation period (2-10 days), moistening agents (composition of moistening agents listed in Table 3.3), a ratio of moisture content, and pH of fermentation media were also investigated.

Table 3.3 Type of different moistening agents examined for phytase production.

Moistening solution	Symbolic representation	Composition of moistening agents (g/L)
Solution 1	SI	0.5 g KCl; 0.5 g $MgSO_4$; 0.05 g $CaCl_2$; 0.01g $FeSO_4$; 0.01 g $MnSO_4$
Solution 2	SII	Distilled water (DW)
Solution 3	SIII	8.9 g NaCl
Solution 4	SIV	30 g Sucrose; 2 g $NaNO_3$; 0.01 g $FeSO_4$; 0.5 g $MgSO_4$; 1 g K_2HPO_4; 0.5 g KCl
Solution 5	SV	Potato Dextrose Broth (PDB)

Solution 6	SVI	5 g $NaNO_3$; 0.5 g $MgSO_4$; 1 g NaCl
Solution 7	SVII	Tap water

Other supplemental nutritional (carbon, nitrogen, and phosphorous) sources and surfactants and their effects (stimulatory or inhibitory) were also evaluated for the phytase yield. The various supplemental sources studied are tabulated in Table 3.4.

Table 3.4 Different supplemented biochemical sources affecting phytase production optimized by OVAT parameters.

Sources	Concentration	Name
Carbon	1%	Glucose, Fructose, Sucrose, Galactose, Lactose, Sorbitol, Starch, Cellulose, and Mannitol
Mannitol concentration	0.025 to 3% w/w	
Nitrogen	1% W/W	Yeast extract, Sodium nitrate, Peptone, Arginine, Malt extract, Ammonium nitrate, Asparagine, Ammonium sulfate [AS; $(NH_4)_2SO_4$], and Urea
$(NH_4)_2SO_4$ concentration	0.025-3% W/W	
Phosphorus	1% W/W	Sodium dihydrogen phosphate, Potassium dihydrogen phosphate (KH_2PO_4), Orthophosphoric acid, Sodium phytate, and Ammonium dihydrogen phosphate
KH_2PO_4	0.025-3% W/W	
Surfactants	1% V/W	Tween 80, SDS, Tween 20, and Triton X-100
Tween 80 concentration	0.5-3% V/W	

3.2.5 Extraction of phytase

The fermentation was followed by extracting crude phytase at 30 °C under agitating conditions for 1 h with 50 ml distilled water blended with Tween 80 (0.1%). Afterwards, it was passed through muslin cloth to remove the moldy substrate and centrifuged the crude phytase at 4 °C and 8000 rpm for 20 min to remove spores. The collected clear supernatant was assayed for phytase activity [28]. The WB with a luxuriant growth of fungus dried at 50 °C in a hot-air oven until a steady weight was achieved.

3.2.6 Statistical studies

All the experiments were carried out three times, and the average values along with their standard deviations are shown in graphs. A one-way ANOVA was used to determine the significance of every parameter in SSF for phytase production. Through Tukey's T-test (*Post-hoc)*, the significance is estimated at the 95% confidence level and the P-value at 0.05. Values less than 0.05 are supposed to show a significant effect on phytase production. The similar bar/lines and alphabetic letters in the graphs suggest the values are similar and have no major significant variance.

3.3 RESULTS AND DISCUSSION

3.3.1 Selection and phenotypically characteristics of a phytase-producing fungal isolate

In PSA media, the isolate from maize rhizosphere (X6) of the agricultural field was selected based on the primary screening as it forms a larger zone of hydrolysis, as shown in Figure 3.2.

Figure 3.2 Isolate X6 displaying primary screening on PSA showing its hydrolysis zone.

Further secondary screening of isolates by phytase assay showed isolate X6 as the most potent phytase producer, with phytase activity of 0.201 U/ml (Table 3.5).

Table 3.5 Primary and secondary screening (phytase activity: U/ml) of fungal isolates.

S. No.	Isolate	Zone of hydrolysis (mm) on PSA	Phytase activity (96 h) (U/ml)
1	X1	15	0.191
2	X2	12	0.134
3	X3	13	0.178
4	X4	14	0.153
5	X5	16	0.165
6	X6	18	0.201

The X6 isolate featured a spherical, large, globose fruiting body with brown to dark-black conidial cap under the microscope. Conidiophores had a spherical form and were connected in chains (Figure 3.3). According to McClenny 2005, the physical traits of isolate X6 are similar to those of previously identified *A. niger* strains [29]. The 18s rRNA sequence was applied for the BLASTn run, and through multiple sequence alignment, the isolate was finally identified as the *Aspergillus niger* NT7 strain. The obtained sequence was deposited to the GenBank (GenBank ID: MN337269; Annexure A.1).

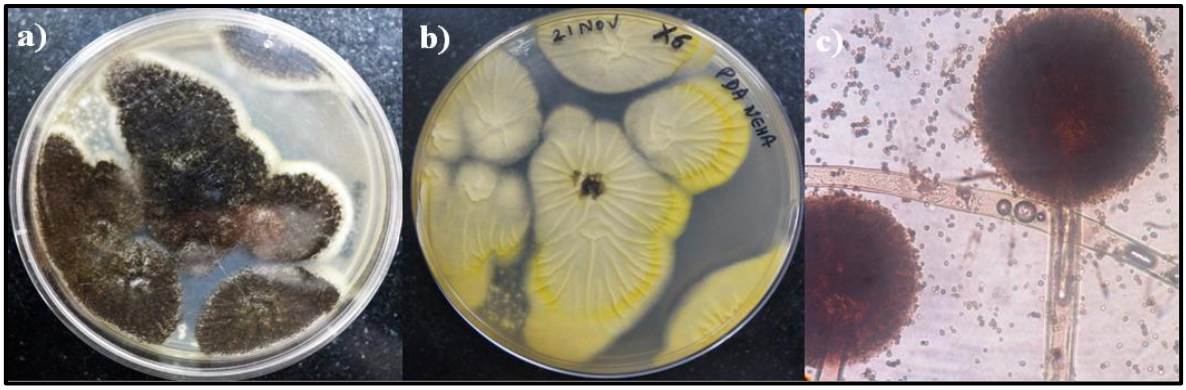


Figure 3.3 Morphological features of isolate X6 **(a)** Top view *A. niger* NT7 growing on PDA plate showing dark brown to the black conidial body, **(b)** Down view showing white mat surrounded by light yellow colour, **(c)** LCB staining shows large black conidial heads with round-shaped conidia.

3.3.2 Optimization of cultural parameters for phytase production in SSF

3.3.2.1 Selection of substrate and SSF parameters for production of phytase

The substrate is one of the utmost important aspects of SSF because it nourishes the fungus and provides a physical surface area for fungal growth [14, 30]. Agro-industrial leftovers (WB, rice bran, maize bran, sugar cane bagasse, straws of wheat and rice, banana waste, coconut coir pith, tea and coffee wastes, mustard oil cake, fruit and vegetable wastes) are generally acknowledged to be the ideal substrates for fermentation technology and enzyme synthesis since they provide the necessary nutrients for microbial development [31]. Statistical analysis (Tukey's T-test) indicates that types of substrates play a major and significant role in the production of phytase (p-value <0.05). Compared with other agricultural waste for phytase production, Maize Bran (MB) and Wheat Bran (WB) have the highest phytase yields, about 71.94 ± 5.09 U/gds and 76.34 ± 0.99 U/gds, respectively. In addition to MB and WB, Sugarcane Bagasse (SB) also showed a noteworthy phytase yield (45.94 ± 2.03) (Figure 3.4). Previous studies suggested that

phytase production from *Streptococcus thermophilus* and *Mucor racemosus* was improved by adding oil cake as substrates [28, 32]. In the present study, mustard oil cake was not a suitable substrate as phytase production was very low, i.e., 4.58 ± 0.05 U/gds. The mixture of linseed oil cake and WB used for the proliferation and production of phytase from *Rhizopus oryzae* seems more suitable for accomplishing efficient SSF [17]. Our study showed no good substrate mixture, so WB was selected to produce phytase because of its economic accessibility and high yield. The WB has an optimal level of carbon and nitrogen to sustain enzyme production (Annexure A.3). In 2018, India produced a large amount (796,000 tons) of WB [33]. The WB is filtered through a sieve BSS 25 (IS 46-1962) to obtain a consistent size in the SSF. These similar particle sizes provide a better physical surface for fungal growth expansion and gas and heat exchange spaces for efficient mass transfer [34].

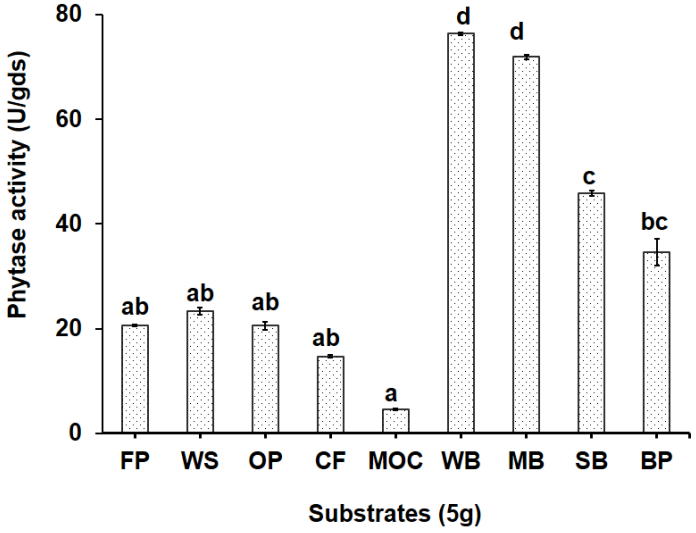


Figure 3.4 Selection of substrate for *A. niger* NT7 phytase production in SSF (FP, WS, OP, CF, MOC, WB, MB, SB, and BP). 5 g of each agri-waste, moisturized with 10 ml distilled water (1:2; WB: distilled water). SSF was operated at 30 °C for four days using a four-day-old inoculum containing $15x10^7$ spores/ml. According to the Tukey's T-test (*Post-hoc*), the significance threshold is 0.05 %, i.e., P-value ≤ 0.05 is significant. The data set with similar alphabets showed no significant difference among them. The values are represented as mean ± S.D (n = 3).

3.3.2.2 Inoculum age and level selection

The growth and metabolic rate of microorganisms are significantly affected by the age and quantity of the inoculum [30, 35]. Statistical studies also showed that the inoculum's age and its level have a significant influence on the production of phytase (P-value <0.05). The 3-days aged inoculum with 15×10^7 spores/ml showed the largest phytase production, which was about

96.36 ± 1.23 U/gds (Figure 3.5a) and afterwards declined with older inoculum. The neutral phytase produced from *A. flavus* ITCC 6720 showed improved phytase production with three-day-old inoculum similarly [36]. The conclusion drawn from this study is that the metabolism of the young inoculum is more vigorous and active than that of the old inoculum. According to other studies, the 6-days-old inoculum showed higher phytase production in *A. niger* SGA 01 and *A. niger* CFR 335 [37].

The phytase production increased with the increase in inoculum level to an optimal level. The 3-day-old inoculum (12×10^7 spores suspension/ml) showed the maximum phytase production, about 116.16 ± 2.32 U/gds (Figure 3.5b). The inoculum having a low spore suspension (7.2×10^6 spores/ml) showed improved phytase production in *A. flavus* ITCC 6720 [36]. A rise in the inoculum concentration above an optimal level may result in a sharp drop in phytase production, possibly due to competition for existing physical support and the limitation of nutrients accessible for the growth of over-proliferating fungi [30], [38].

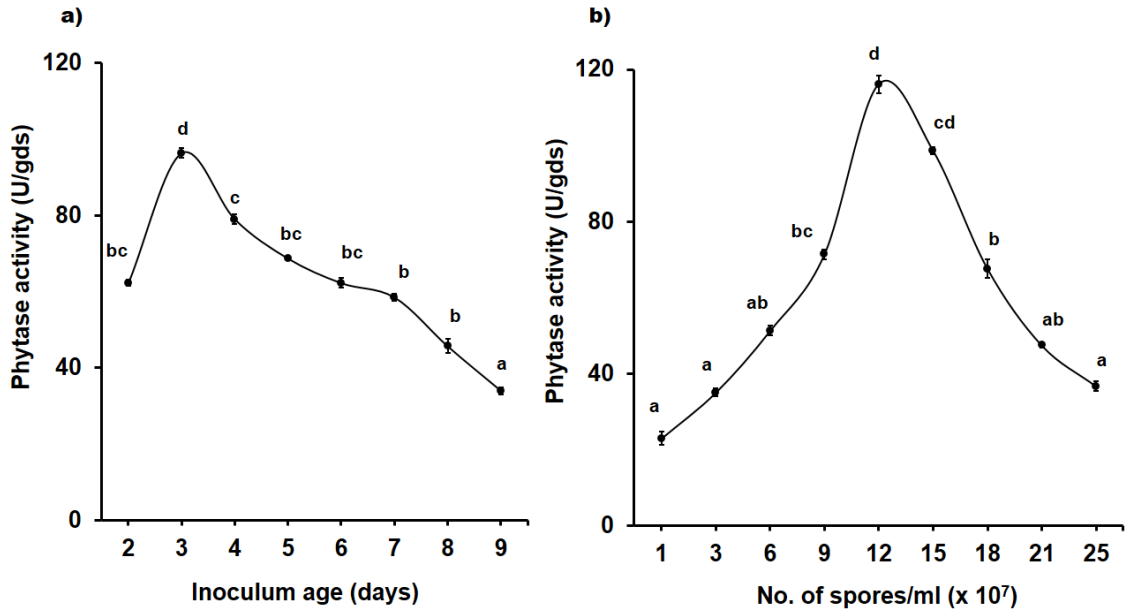


Figure 3.5 a) Inoculum age selection; SSF was performed for four days at 30 °C with a variety of inoculum ages (2-9 days old) with a size of 15×10^7 spores/ml. and **b)** Inoculum level; 3day-old inoculum of varying levels (spores/ml) was used while maintaining all the previously described conditions for SSF. The values are represented as mean ± S.D (n=3).

3.3.2.3 Selection of temperature and fermentation time

Besides the duration of SSF incubation, the temperature is an important SSF affecting parameter because it impacts metabolic activity during the development phase and contributes to the

enzyme's stability [28, 39]. Both temperature and incubation time of SSF are determinants in increasing phytase synthesis and are observed to exert a significant effect (P-value 0.05) on enhancing phytase yield. The temperature of the process is crucial in SSF as it regulates microbial activity, spore production and propagation, and product synthesis. At higher temperatures, *A. niger* NT7 grew fastidiously and achieved its maximal phytase production at 30 °C (120.95 ± 1.25 U/gds) and 35 °C (121.44 ± 1.17 U/gds) (Figure 3.6a). In the prior studies, *A. niger* NRF9 [18], *A. niger* NCIM563 [40], *A. oryzae* SBS50 [41], and *Rhizopus* spp. [42] showed phytase production in an identical range of temperature. Another study showed *A. ficuum* produces phytase at room temperature, 22 °C [19]. High phytase production can also be achieved at much higher temperatures for a few thermophilic molds. *Thermomyces lanuginosus* TL-7, for example, has an optimal temperature at 45 °C [43], while *R. pusillus* [44] thrives at 50 °C.

Furthermore, the current study discovered that phytase synthesis increased with incubation time and attained its peak value after the 4th (127.29 ± 1.58 U/gds) and 5th (132.74 ± 0.27 U/gds) days of fermentation. However, after the 5th day, phytase synthesis began to decline, which might be due to nutrient consumption, hazardous end product buildup, water loss, or changes in the medium's pH. (Figure 3.6b). This corroborates with prior studies carried out on *M. racemosus* [28], *A. oryzae* SBS50 [41], and *A. oryzae* AK 9 [45].

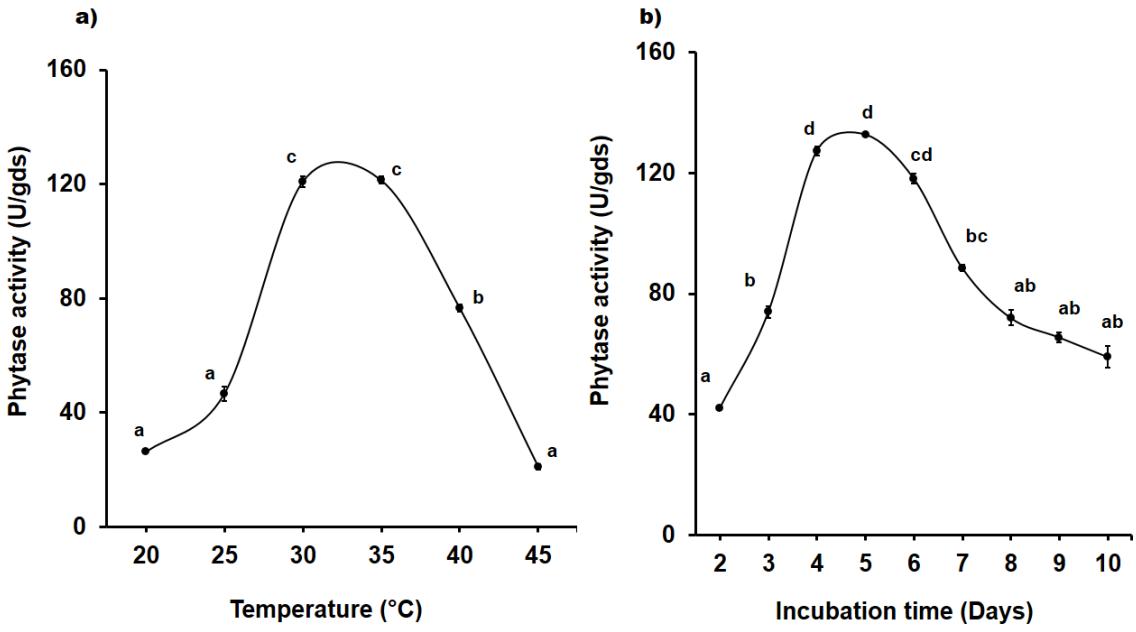


Figure 3.6 Selection of optimal **a)** Temperature; SSF was performed using a three-day-old inoculum containing 12x10^7 spores/ml at different temperatures (20-45 °C with a 5-degree interval) for four days, and **b)** Incubation duration of SSF; SSF was carried out with a 3-day-old inoculum having 12x10^7

spores/ml at 30 °C for different incubation days from 2 to 10 days. The values are represented as mean ± S.D (n=3).

3.3.2.4 Choice of moistening agent and ratio

Another key parameter for efficient enzyme synthesis in SSF is the ingredients of the moistening agent and its ratio (solid: liquid), as it fosters propagation and offers humidity for synthetic activities during fermentation. In SSF, fungal growth and phytase synthesis occur along the SSF substrate surface, having a low-moisture level. The ideal moisture level varies depending on the SSF substrate, size, and fungal strain utilized to carry out SSF. The optimal solid-liquid ratio is critical for nutrient transfer and mass exchange during the SSF process; it is a need for maximal biotransformation of agricultural waste [34, 38, 46]. Distilled water was identified as the best acceptable moistening agent for the phytase production out of all the moistening agents examined (133.32 ± 1.29 U/gds) (Figure 3.7a). Comparable outcomes where distilled water was the most appropriate moistening agent have been observed for *A. niger* F3 [47], *A. niger* NCIM 563 [40], and *Schizophyllum commune* [48]. The statistical analysis revealed no noteworthy differences in phytase production between solutions SI, SII, and SIII (normal saline) (Figure 3.7a). As a result, distilled water was preferred as the cost-effective agent for moistening with the least variation.

The moisture ratio in SSF affects oxygen transfer (aeration), cell growth rate, breakdown rate, and fungal metabolite synthesis [49, 50]. In this current study, the 1:2 ratio (substrate: moistening agent) (134.37 ± 1.17 U/gds) resulted in the maximum enzymatic output (Figure 6b). Going below this ratio has a detrimental effect on phytase production owing to reduced nutrient transport, fungal growth, enzyme stability, and substrate swelling [31]. An increase in the subsequent moisture ratio beyond 1:2 resulted in reduced enzyme synthesis. This might be attributed to flooding, poor aeration and substrate assembly, which negatively affect the mass transfer of nutrients and gases during SSF [14]. Similar consequences of the moisture ratio were also reported in *A. oryzae* SBS50 [41] and *A. niger* NRF9 [18] for phytase production.

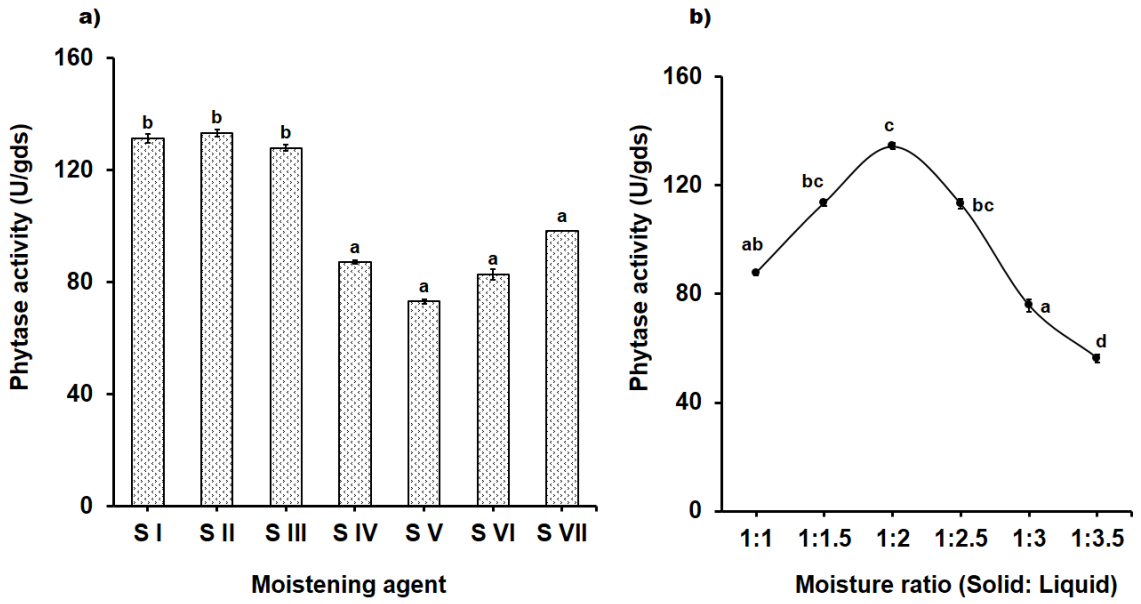


Figure 3.7 a) Moistening agent selection; S denotes the various categories of moistening agents listed in Table 3.3, and **b)** varied moisture ratio of moistening agent (WB: DW). The SSF was carried out with pre-optimized settings: 3-day-old inoculum with $12x10^7$ spores/ml; 4 days fermentation time; 30 °C temperature. The values are represented as mean ± S.D (n=3).

3.3.2.5 Selection of an appropriate source of carbon

During SSF, WB offers all of the nutrition for fungal growth, but subsequently, as the SSF progresses, it may become a restraining parameter for enzyme synthesis. Carbon is the fuel source that will be accessible for fungal growth. It might be a simple monosaccharide like glucose or a complex molecule like cellulose or starch [31]. Both 1% mannitol and 1% starch bring about 156.82 ± 3.64 U/gds and 146.21 ± 2.27 U/gds improved phytase synthesis, respectively. Phytase synthesis by *A. niger* NT7 was inhibited by various carbohydrates such as glycerol, glucose, galactose, sorbitol, fructose, cellulose, and lactose (1% w/w) (Figure 3.8a). The predilection for diverse carbon might be explained by the expression of catabolite repressor proteins for carbon that can be interpreted through transcriptome investigation [51, 52]. Similar inhibitory effects of carbon sources were reported by Tian and colleagues for *A. ficuum* phytase [19]. In other studies, phytase production was augmented with the supplementation of glucose and sugar alcohol, i.e., mannitol, in *A. flavus* ITCC 6720 [36]. Furthermore, 2.84% mannitol was employed to conduct SSF for phytase synthesis in *R. oryzae* [17]. On the other hand, the current study found that varied mannitol concentration (0.025-2.0%) had no significant influence on phytase production (Figure 3.9b).

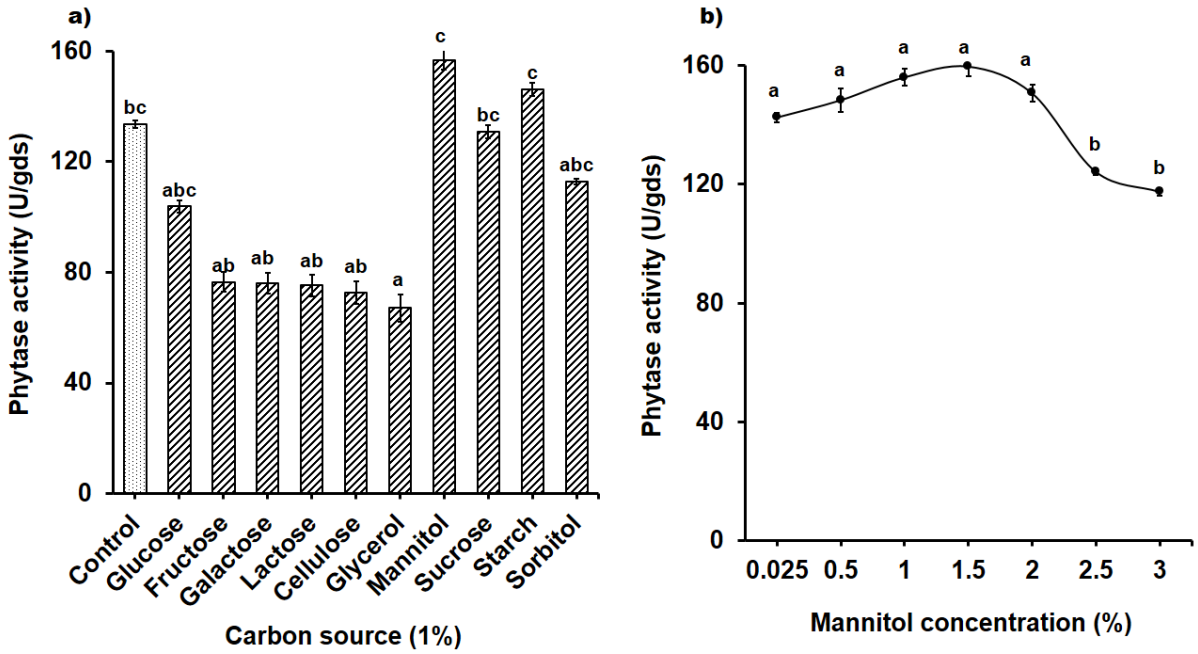


Figure 3.8 a) Carbon source supplementation (1%) against control run (C) without any supplemented carbon source, and **b)** Varied mannitol concentration (0.025-3%). SSF was performed with pre-optimized settings: 5 g WB moistened with 1:2 ratio distilled water; 3-day-old inoculum with 12×10^7 spores/ml incubated at 30 °C temperature for four days while medium pH was maintained at 5.0. The values are represented as mean ± S.D (n=3).

3.3.2.6 Selection of nitrogen source

The supplementation of nitrogen sources (organic and inorganic) in the fermentation medium is found to be significant for phytase synthesis (P-value <0.05). In our study, the supplemented nitrogen sources exhibited a comparable escalating effect on phytase synthesis, with the exception of amino acids arginine and asparagine, which indicated a negative inhibitory impact. Overall, ammonium sulphate (169.05 ± 1.17 U/gds) was shown to induce the most phytase synthesis in our case, trailed by another nitrogen source, ammonium nitrate (166.32 ± 1.03 U/gds) (Figure 3.8a). The present findings are consistent with a previous study of phytase in *R. oryzae* [17, 53]. Ammonium nitrate has also been shown to increase phytase synthesis in *A. niger* NRF9 [18], *T. lanuginosus* TL-7 [43], *A. ficuum* NRRL3135 [50] and *Rhizopus* spp. [54]. Another investigation on *R. pusillus* phytase production found that asparagine is the best nitrogen source [44]. Statistical analysis revealed a 0.5% (174.55 ± 2.5 U/gds) concentration of supplemented ammonium sulphate substantially influenced phytase synthesis. Beyond this level of ammonium sulphate, there is a progressive decrease in phytase production (Figure 3.8b); this might be related to a C/N ratio imbalance throughout SSF [28].

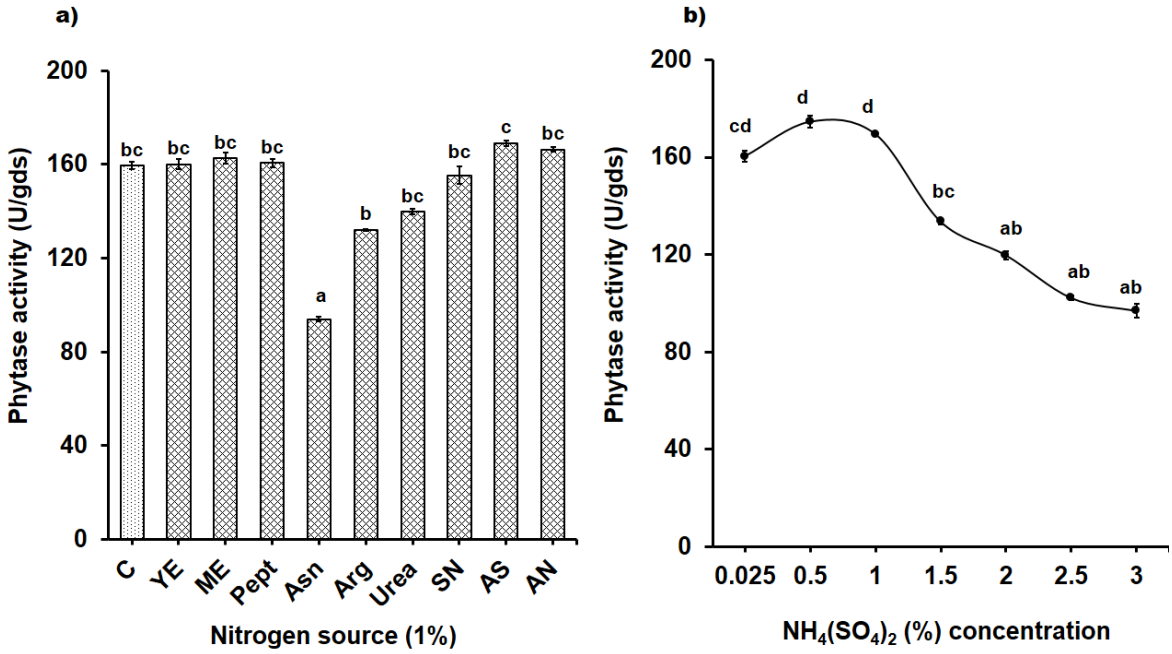


Figure 3.9 Supplementation of **a)** of nitrogen source, Here; C: Control (without any nitrogen source); YE: Yeast Extract; ME: Malt Extract; Pept: Peptone; Asn: L-Asparagine; Arg: L-Arginine; SN: Sodium Nitrate; AS: Ammonium Sulphate; and AN: Ammonium Nitrate, and **b)** Varied Concentration of $(NH_4)_2SO_4$ (0.025-3%). SSF was performed with pre-optimized settings: 5 g WB supplemented with 1% mannitol, moistened with 1:2 ratio distilled water; 3-day-old inoculum with 12×10^7 spores/ml incubated at 30 °C temperature for 4 days while medium's pH maintained at 5.0. The values are represented as mean ± S.D (n=3).

3.3.2.7 Selection of extraneous phosphorous

Due to the stimulating property of phosphorous sources for phytase synthesis, different phosphorous sources have been proven to have a considerable impact on phytase production in current as well as in previous research [53, 55]. Among the various phosphorous sources supplemented to the fermentation medium (Figure 3.9a), 0.5% KH_2PO_4 proved to be the superlative for enhanced phytase synthesis (182.20 ± .06 U/gds) (Figure 3.9b).

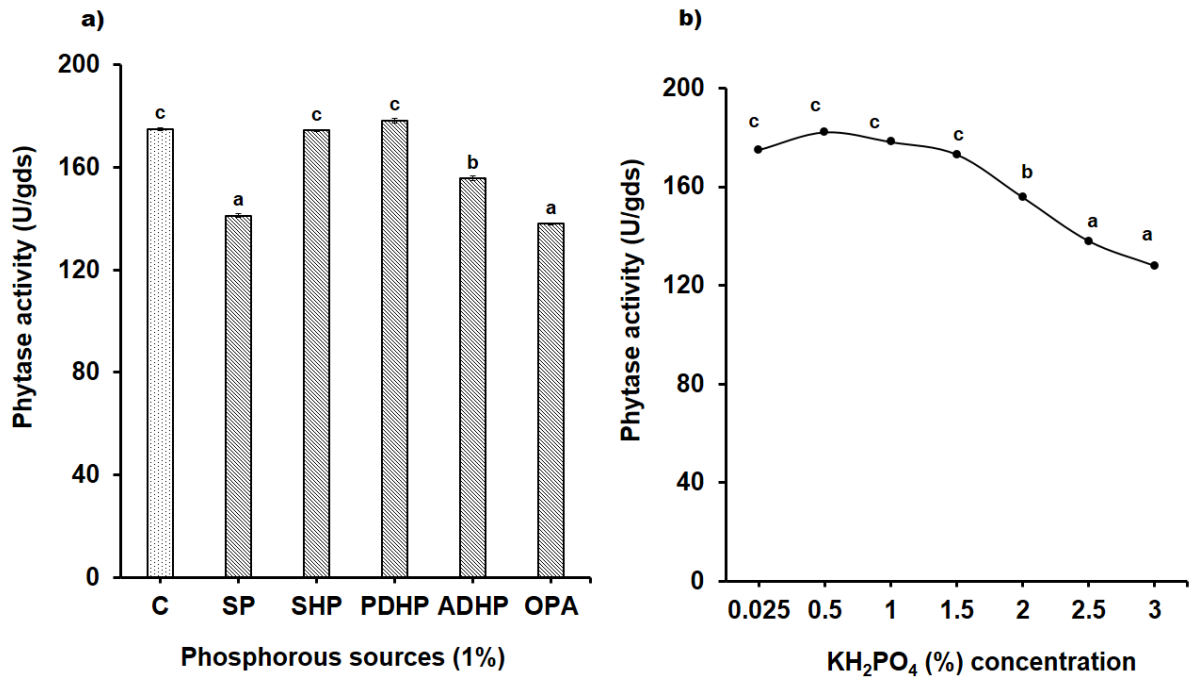


Figure 3.10 a) Supplementation of phosphorous sources (0.025-3%); Here, C: Control; SP: Sodium phytate; SHP: Sodium hydrogen phosphate; PDHP: Potassium dihydrogen phosphate; ADHP: Ammonium dihydrogen phosphate, OPA: Orthophosphoric Acid, and **b)** Varied concentration of KH_2PO_4. SSF was performed with pre-optimized settings: 5 g WB (supplemented with 1% mannitol and 0.5% ammonium sulphate) moistened with 1:2 ratio distilled water; 3-day-old inoculum with 12×10^7 spores/ml incubated at 30 °C temperature for 4 days while fermentation medium's pH maintained at 5.0. The values are represented as mean ± S.D (n=3).

3.3.2.8 Supplementation of surfactants

Surfactants increase membrane permeability, promoting enzyme synthesis and facilitating extracellular secretion [32, 41]. Surfactants, if supplemented at a high level, sharply decline enzyme production due to their unfavorable influence on the organism's physiology and can also disrupt enzyme stability. All non-ionic surfactants, such as Tween 80, Triton X-100, and Tween 20, utilized in this study, had a prompting impact on enzymatic titer. In contrast, SDS, an anionic surfactant, had a detrimental consequence on phytase synthesis, most likely due to disrupting enzyme structure and stability (Figure 3.10a). The 2% (w/v) Tween 80 (204.42 ± 0.55 U/gds) produced the highest phytase yield, which afterwards fell owing to partial phytase degradation (Figure 3.10b). Tween 80 stimulates the phytase titer in *A. niger* NRF9 [18] and *S. thermophile* [32]. Other surfactants, such as Triton X-100, induce phytase synthesis in *A. oryzae* SBS50 [41].

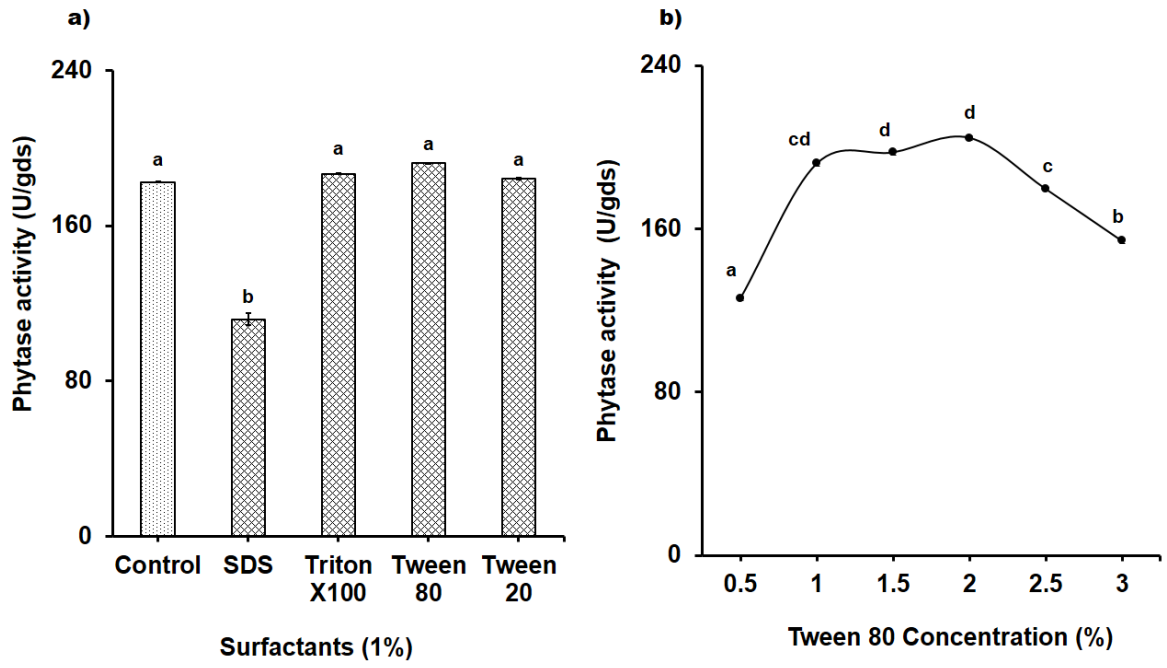


Figure 3.11 a) Supplementation of additional surfactants; Here Control is deprived of surfactant addition; C), and **b)** Varied concentration of Tween 80. SSF was performed with pre-optimized settings: 5 g WB (supplemented with 1% mannitol, 0.5% ammonium sulphate and 0.5% potassium dihydrogen phosphate) moistened with 1:2 ratio distilled water; 3-day-old inoculum with 12×10^7 spores/ml incubated at 30 °C temperature for 4 days while maintaining pH 5.0. The values are represented as mean ± S.D (n=3).

3.3.2.9 Selection of media pH

One of the most significant parameters is the fermentation medium's pH, which regulates and changes numerous organism's metabolic processes while also assisting in the stability of enzymes released in SSF. According to statistical analysis, pH had a substantial influence on phytase synthesis in our study (P-value 0.05). In our case, the highest phytase titer was found at pH 5.0 (208.30 ± 0.05 U/gds), with a sharp reduction in production observed as the pH approaches the alkaline range (Figure 3.11). The pH 5.0 was also reported to be best for the phytase production from *A. niger* NRF9 [18], *A. oryzae* AK9 [45], *A. oryzae* SBS50 [41], and *R. oligosporus* [56]. At pH 6, 7, and 8, filamentous molds such as *A. flavus* ITCC 6720 [36], *S. commune* [48], and *Rhizomucor pusillus* [44] attained the most phytase production.

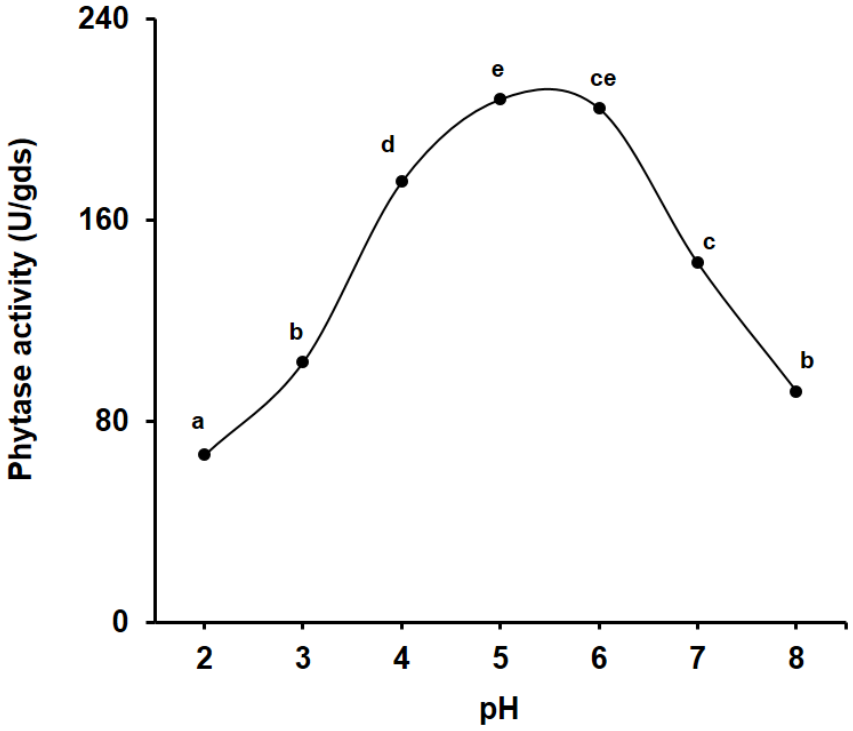


Figure 3.12 Selection of SSF medium's pH for phytase synthesis using *A. niger* NT7. SSF was performed with pre-optimized settings: 5 g WB (supplemented with 1% mannitol, 0.5% ammonium sulphate and 0.5% potassium dihydrogen phosphate) moistened with 1:2 ratio distilled water; 3-day-old inoculum with 12×10^7 spores/ml incubated at 30 °C temperature for 4 days while pH of SSF medium varied from 2-8. The values are represented as mean ± S.D (n=3).

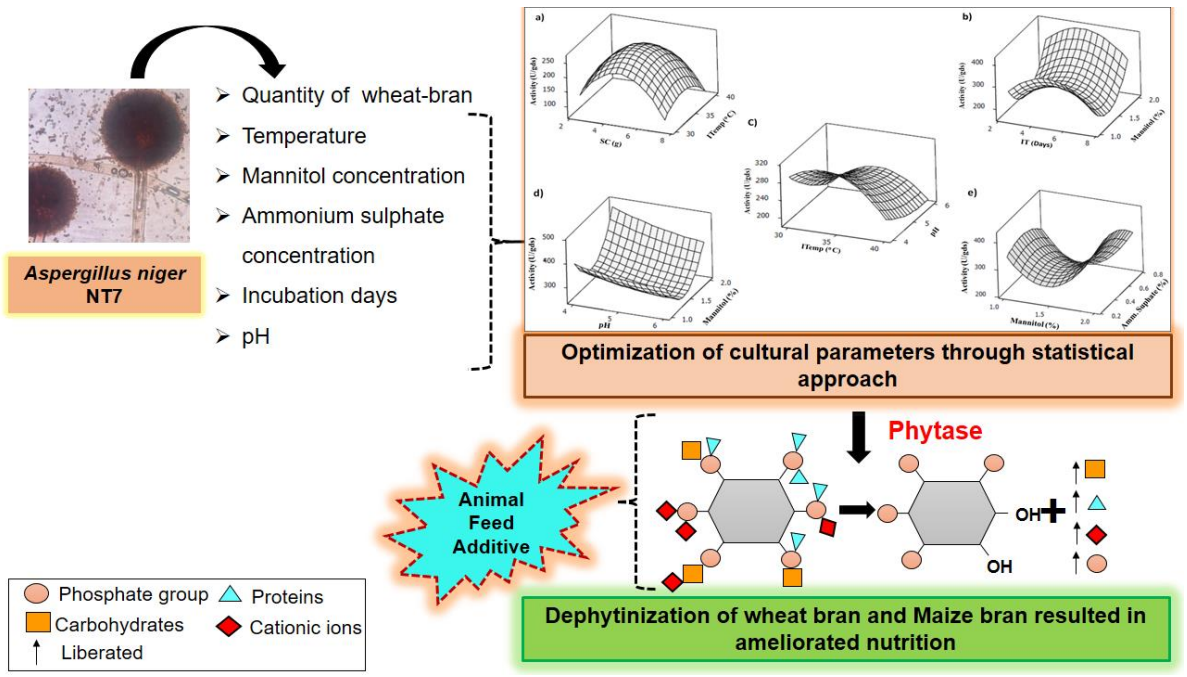
3.4 CONCLUSIONS

We have found that *A. niger* NT7 isolated from the soil of the agricultural field of Himachal Pradesh can be a promising candidate for phytase synthesis. WB was found to be the most suitable agri-waste and supports the luxuriant growth of *A. niger* NT7 for cost-effective phytase production in SSF. WB enriched with mannitol and ammonium sulphate, when inoculated with a three-day-old inoculum having a size of $12x10^7$ spores/ml at pH 5.0, resulted in high phytase yields when SSF was carried out for four days at 30 °C. The *A. niger* NT7 strain has attained the maximum phytase production rate by optimizing the various physiochemical and nutritional parameters using the OVAT approach. Overall, 2.7-fold enhanced phytase production was achieved with OVAT when compared to unoptimized culture conditions.

CHAPTER 4

MODELING AND OPTIMIZATION OF PHYTASE PRODUCTION THROUGH RESPONSE SURFACE METHODOLOGY AND ITS APPLICATION IN THE FEED INDUSTRY

GRAPHICAL OUTLINE OF CHAPTER



HIGHLIGHTS

- 2.5-fold enhanced phytase production attained by RSM
- Crude phytase active at acidic pH range and thermotolerant in nature
- Efficient dephytinization of wheat and maize bran resulted in release of bound nutrients
- Crude phytase has the potential to be utilized as feed additive

4.1 INTRODUCTION

The natural ability of microbial phytases (EC 3.1.3.8) to hydrolyze the phosphate monoester bond present in phytic acid makes them a promising candidate for removing anti-nutrients from plant-based feed and food [1-3]. Various microbial sources are being utilized commercially for phytase production. From the industrial point of view, *Aspergillus* is a major producer of phytase with GRAS (Generally Recognized As Safe) status and has a vast perspective in the animal feed industry [4-8]. Various fermentation approaches (Solid State Fermentation; SSF, SmF: Submerged Fermentation, and sSSF: Semi-Solid Fermentation) can be opted for achieving efficient phytase production from microbial sources. SSF is an economic option as it utilizes cheap agri-waste and can attain maximum enzymatic production with less operational cost [9, 10].

The phytase production in SSF is affected by various parameters and optimization studies aid in accomplishing the maximum achievable production rate. To achieve the most efficient and maximum phytase production, affecting cultural parameters can be optimized by OVAT and statistical approaches. The OVAT (One Variable at A Time) approach improves phytase production in a stepwise manner. The major advantages of the OVAT method are that it primarily helps in the screening of appropriate significant cultural parameters and their operating ranges that affect enzyme production. The selected parameters by the OVAT approach can be further employed for statistical analysis. Previous reports have also advocated the reliability of the OVAT approach for phytase production [6, 8]. The OVAT approach investigates various variables influencing the process independently and does not study their interactions. But laborious steps and time-consuming functions will always remain related to the OVAT [11, 12]. The data obtained from this approach is utilized for the statistical modeling of optimization studies.

On the other hand, statistical-based modeling and optimization are ideal for increasing output through the Design of Experiment (DOE), model development, and analytical skills. In addition, statistical methods include the execution of mathematical and statistical models for experimental verification of the parameters selected for optimization studies. Response Surface Methodology (RSM) is a term that encompasses all three above mentioned concepts together for process design and refinement of optimization techniques. RSM has been successfully used for multivariate optimization of different biological processes to increase enzyme production. The 2-D and 3-D surface plots can be used to study optimization and multiple interactions can

be analyzed simultaneously in less time [11, 13-15]. Previous studies have also reported that statistical methods have led to an increase in the production of extracellular phytases in SSF [4, 16, 17]. RSM approach enhanced the phytase production of *A. niger* NCIM 563 by 3.08-fold [18], *Thermomyces lanuginosus* by 10.83-fold [19], and *Sporotrichum thermophile* by 11.6-fold [20].

Previously, various cultural, physiochemical and nutritional parameters affecting SSF were optimized by the OVAT approach. But multivariate interactions among them cannot be studied at once; that's why the study was further extended for optimization by a statistical approach. This work aimed to use a software-based statistical technique to increase extracellular phytase synthesis from *A. niger* NT7 in SSF by utilizing wheat bran as an SSF substrate.

From an industrial feed perspective, the biochemical characterization of crude phytase is very critical. The biochemical characterization included studying the effects of varied pH, temperature, metal ions, and thermostability. Due to the microorganism's GRAS status, the phytase enzyme directly in a crude form might be used for feed supplementation. The dephytinization capacity against wheat bran and maize bran was also examined to determine its primary applicability as a feed additive.

4.2 MATERIALS AND METHODS

4.2.1 Raw material and chemical

A local vendor from Solan and Hamirpur, Himachal Pradesh (H.P.) India provided the wheat bran. The sodium phytate substrate was purchased from HiMedia Private Limited Mumbai, India. The rest of the chemicals and reagents were of analytical quality.

4.2.2 Microorganism and inoculum preparation

A. niger NT7 was previously discovered as a potential phytase producer after being isolated from the agricultural field of *Zea mays* (Section 3.3.1). The culture was maintained on PDA slants at 4 °C and sub cultured many times. For conducting the fermentation, pre-optimized conditions for inoculum preparation, i.e., three-day-old inoculum having $12x10^7$ spores/ml, was utilized (Section 3.3.2.2) [8].

4.2.3 Response Surface Methodology (RSM) to optimize phytase production

The OVAT approach had previously been used to optimize the phytase production in SSF using wheat bran. The OVAT assisted in identifying the most significant parameters affecting the phytase production in SSF, such as wheat bran quantity, temperature, incubation time, pH,

mannitol concentration, and ammonium sulphate [$(NH_4)_2SO_4$] concentration [8]. These six parameters were then further optimized using Response Surface Methodology (RSM) for additional enhancement of phytase production. Based on RSM's Central Composite Design (CCD), three distinct levels were chosen for the design of the experiment (DOE) in this study: lower, mid, and higher (Table 4.1). The Minitab®16 version was used to obtain the expected values of phytase activities for this DOE (Table 4.2) and to develop a response surface model.

Table 4.1 Levels of specified parameters for the construction of Central Composite Design (CCD).

	The optimal level of parameters		
Variables	**Low level (-1)**	**Mid-level (0)**	**High level (+1)**
Wheat bran (g)	2.5	5	7.5
Temperature (°C)	30	35	40
Incubation time (days)	2.5	5	7.5
pH	4	5	6
Mannitol (%)	1	1.5	2
Ammonium sulphate (%)	0.25	0.5	0.75

4.2.4 Model validation and optimization

The regression equation was created from the software, and R^2 (coefficient of determination) and altered R^2 values (correlation coefficient) were used to check for the lack of fit and adequacy of the generated model. Individual, square, and interaction effects between several experimental data sets were analyzed using statistical significance tests and Analysis of Variance (ANOVA). A P-value less than 0.005 is considered significant at a 95% confidence level. The Minitab®16 software was used to create 3D surface plots based on the interactions between numerous factors affecting the SSF under study. The response optimizer feature of Minitab®16 software created the ideal phytase production conditions, which were then employed for experimental testing for validation.

4.2.5 Biochemical characterization of crude phytase preparation

The effect of pH on the crude phytase activity was evaluated by assaying its activity at different pH buffers ranging from 1.0 to 10.0 at 50 ℃ for 30 min. The buffers (100 mM) of various pH ranges such as KCl-HCl (pH-1.0), glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-5.0), Tris HCl (pH 6.0-8.0) and glycine-NaOH (pH 9.0-10.0) were used. The pH values of each buffer were measured at 25±2 ℃.

The optimum temperature for maximal activity of the crude phytase was determined by assaying its activity at varying temperatures ranging from 30-80 ℃ at pH 5.0.

Thermostability of the crude phytase enzyme was monitored by performing an activity assay at 60°C, pH 5.0, after 300 min incubation of the enzyme at different temperatures, 50, 60 and 70 °C. The residual phytase activity after each 60 min incubation was evaluated to calculate the half-life ($t_{1/2}$) of the enzyme.

The effect of metal ions on phytase activity was studied by incubating the enzyme with different metal ions like Mn^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+} and Co^{2+} at two different concentrations 1 and 5 mM at 60 ℃ for 30 min. Under affirmed reaction state, the control samples in above all experiments signify the enzymes devoid of any treatment. The relative activity of diverse reaction conditions mentioned above was evaluated by taking the control value as 100%.

4.2.6 Dephytinization activity assessment of crude *A. niger* NT7 phytase

4.2.6.1 Amelioration of wheat bran by crude phytase

To analyze the capability of crude *A. niger* NT7 phytase, a dephytinization reaction of wheat bran was set up. For the same, 10 g of wheat bran in 100 ml 0.1 M acetate buffer, pH 4.8, was treated with 10 U of crude phytase at 50 ℃ in an agitating state. The control (omitted with phytase) setup was also assessed simultaneously in the same reaction conditions. The release of nutrients from phytate complexes such as Pi, sugar and total proteins were measured by withdrawing a 2 ml sample at regular time intervals. The release of Pi was estimated using the phytase assay (Section 3.2.2.4), the protein content by Bradford Reagent's method (Section 4.2.7.1), and reducing sugar by Miller's DNSA method (Section 4.2.7.2).

4.2.6.2 Amelioration of maize bran by crude phytase

For dephytinization, 5 g maize bran in 100 ml of 0.1 M sodium acetate buffer, pH 4.8, was incubated with 10 U of crude phytase at 50 ℃ with agitation. A reaction set up without an enzyme was taken as a control. The release of nutrients such as Pi, reducing sugar and proteins from the maize bran was analyzed at regular intervals by withdrawing two ml samples. These nutrients were analyzed similarly to the wheat bran sample (Section 4.2.6.1). The release of amino acid was also estimated using the ninhydrin reagent (Section 4.2.7.3).

Dephytinization also led to the release of metal ions, which was measured by ICP-MS (Inductively Coupled Plasma Mass Spectrometry). One gram of untreated maize bran was oven-dried and then treated with an acidic (HNO_3:$HClO_4$, i.e., 1:4) solution at 120 ℃ until a clear

solution formed. The untreated maize bran is considered as a control and treated as 100%. Test samples (dephytinize maize bran) were taken for metal ion measurement after a 24 h period. All experiments were conducted in triplicate, and the mean values and S.D (Standard Deviation) are shown in graphs.

4.2.7 Analytical assays

4.2.7.1 Bradford's reagent method for protein estimation

The protein concentration in the samples was estimated by the method given by Bradford (1976) [21]. In this method, 0.1 ml of protein sample (diluted with distilled water whenever required) was mixed with 1 ml of Bradford working reagent (Annexure B.1). The absorbance of the blue-coloured reaction mixture was recorded at 595 nm against blank after 10 min incubation in dark conditions. The protein concentration was calculated from the standard plot of Bovine Serum Albumin (BSA) (Annexure B.1).

4.2.7.2 Miller's DNSA reagent method for reducing sugar estimation

The reducing sugar was estimated based on the reduction of 3, 5- dinitrosalicylic acid (DNSA) to 3-amino-5-nitrosalicylic acid [22]. 3 ml DNSA reagent (Annexure B.2) was added to a 0.1 ml sample and incubated in a boiling water bath for 10 min for colorimetric detection of reducing sugar. The absorbance of the orange-coloured reaction mixture was recorded at 540 nm against a blank (sodium acetate buffer). The reducing sugar concentration was calculated from the standard plot of glucose (Annexure B.2).

4.2.7.3 Ninhydrin reagent method for amino acid estimation

Amino acid undergoes oxidative deamination when the amino group of a free amino acid reacts with ninhydrin (1% reagent prepared in ethanol) and releases CO_2, NH_3, an aldehyde and a hydrindantin (a reduced form of reagent ninhydrin). The released ammonia reacts with another ninhydrin molecule and reduces the reagent to generate diketohydrin (also known as Ruhemann's complex), which is quantified by taking absorbance at 570 nm [23]. 1 ml ninhydrin reagent was added to a 0.1 ml sample and incubated in a boiling water bath for 5 min for colorimetric detection of released amino acids.

4.3 RESULTS AND DISCUSSIONS

4.3.1 Optimization of phytase production using RSM in SSF

A. niger NT7 previously optimized in chapter 3 by the OVAT approach, produced 208.30 ± 0.22 U/gds of phytase (5 g wheat bran, moistening agent is distilled water (1:2), 1% mannitol,

and 0.5% $(NH_4)_2SO_4$ after four days SSF at 30 °C, pH 5.0. Since multiple interacting parameters cannot be studied simultaneously through the OVAT approach, a statistical approach, Response Surface Methodology (RSM), was used to understand the interaction between the different physiochemical parameters as well as to enhance the phytase production further. RSM is more effective than a traditional design in establishing the association between several process factors and obtaining maximum responses (phytase activity).

To establish a design of experiments (DOE), a series of 53 experiments were carried out based on CCD. The six process factors of SSF were utilized as input variables, including the amount of wheat bran (g), the incubation period (days), temperature (°C), pH, mannitol and $(NH_4)_2SO_4$ concentrations (% w/w).

Table 4.2 represents the experimental outcomes, i.e., phytase activity (U/gds) from the dataset, as well as the predicted values. The RSM model's reliability was assessed by the strong agreement between experimental results and predicted phytase activity values. The highest phytase production (438.35 U/gds) was recorded with run order number 14, which used 5 g wheat-bran as the SSF substrate along with 2% (w/w) mannitol and 0.5 % (w/w) $(NH_4)_2SO_4$ and was incubated at 35 °C for five days with pH 5.0 of the fermentation medium.

Table 4.2 DOE for RSM representing experimental and predicted phytase production by *A. niger* NT7.

Run order	Wheat bran (g)	IT (Day)	pH	Temp (°C)	Mannitol (%)	$(NH_4)_2SO_4$ (%)	Phytase activity (U/gds)	
							Experimental	Predicted
1	2.5	7.5	6	30	2	0.75	49.02	59.68
2	5	5	5	40	1.5	0.5	178.34	193.71
3	5	5	5	35	1.5	0.5	253.23	265.76
4	5	5	5	35	1.5	0.5	256.24	265.76
5	2.5	2.5	4	30	1	0.25	178.03	174.58
6	2.5	2.5	6	40	2	0.75	33.12	29.61
7	5	5	5	35	1.5	0.5	230.17	265.76
8	5	5	5	35	1.5	0.5	250.89	265.76
9	7.5	2.5	6	30	2	0.75	76.02	95.67
10	2.5	7.5	4	40	2	0.75	87.34	87.93
11	7.5	7.5	6	40	1	0.25	49.37	44.85
12	7.5	2.5	4	40	2	0.75	145.89	133.14
13	2.5	2.5	6	30	1	0.75	55.34	57.58

14	5	5	5	35	2	0.5	438.34	419.16
15	7.5	2.5	4	30	1	0.75	130.37	130.39
16	2.5	2.5	4	40	1	0.75	34.29	47.59
17	5	5	5	35	1.5	0.75	234.21	208.41
18	7.5	7.5	6	30	2	0.25	78.98	65.97
19	2.5	7.5	6	40	1	0.75	53.26	43.01
20	5	5	4	35	1.5	0.5	321.07	310.72
21	5	5	5	35	1.5	0.5	289.02	265.76
22	7.5	7.5	4	30	1	0.25	56.02	59.81
23	5	5	5	35	1	0.5	345.02	359.67
24	7.5	5	5	35	1.5	0.5	134.89	135.33
25	7.5	7.5	4	30	2	0.75	189.93	188.11
26	5	5	5	35	1.5	0.5	289.12	265.76
27	5	5	5	35	1.5	0.5	288.07	265.76
28	2.5	7.5	4	30	2	0.25	234.09	237.81
29	2.5	2.5	4	40	2	0.25	189.32	199.85
30	7.5	7.5	6	30	1	0.75	23.02	12.77
31	5	7.5	5	35	1.5	0.5	150.02	162.60
32	5	5	5	35	1.5	0.5	267.02	265.76
33	7.5	7.5	6	40	2	0.75	28.34	32.07
34	2.5	7.5	6	40	2	0.25	134.02	134.29
35	7.5	2.5	4	30	2	0.25	234.04	244.56
36	7.5	2.5	6	40	1	0.75	32.09	28.66
37	7.5	7.5	4	40	1	0.75	56.78	73.18
38	2.5	2.5	4	30	2	0.75	215.08	219.89
39	2.5	2.5	6	30	2	0.25	219.98	203.87
40	5	5	5	35	1.5	0.5	245.45	265.76
41	5	5	6	35	1.5	0.5	239.49	245.31
42	7.5	2.5	6	30	1	0.25	39.98	39.67
43	7.5	2.5	4	40	1	0.25	78.18	67.81
44	2.5	7.5	4	30	1	0.75	130.03	126.45
45	7.5	7.5	4	40	2	0.25	134.31	132.35
46	2.5	5	5	35	1.5	0.5	170.28	165.31
47	2.5	2.5	6	40	1	0.25	123.27	125.38
48	2.5	7.5	6	30	1	0.25	79.45	92.48
49	5	5	5	35	1.5	0.25	230.37	251.64

50	2.5	7.5	4	40	1	0.25	130.78	111.40
51	5	5	5	30	1.5	0.5	250.83	230.92
52	5	2.5	5	35	1.5	0.5	205.89	188.78
53	7.5	2.5	6	40	2	0.25	118.9	122.76

Data was fed into the Minitab®16 software version, which generates a polynomial response surface model to examine these responses (phytase activity). A regression equation was found for experimental data that predicts optimal phytase production with the six parameters under study. The non-linear regression equations (uncoded form) listed below for phytase activity were created by considering all the individual linear, square, and interacting parameters of SSF variables.

Phytase Activity (U/gds) (Y) = -1476.66 + 152.930 x SC (g) + 134.686 x ITemp (^oC) + 125.387 x IT (Day) -198.445 x pH-1144.00 x Mannitol (%) + 782.743 x Amm. Sulphate (%) -18.4703 x SC (g) * SC (g) - 2.13776 x ITemp (^oC) x ITemp (^oC)-14.4111 x IT (Day) x IT (Day) + 12.2509 x pH x pH + 494.608 x Mannitol (%) x Mannitol (%) -571.810 x Amm. Sulphate (%) x Amm. Sulphate (%) + 0.477802 x SC (g) x ITemp (^oC) - 0.441245 x SC (g) x IT (Day) - 1.59156 x SC (g) x pH + 4.07652 x SC (g) x Mannitol (%) + 26.2063 x SC (g) x Amm. Sulphate (%) + 0.568593 x ITemp (^oC) x IT (Day) + 2.88293 x ITemp (^oC) x pH - 3.64606 x ITemp (^oC) x Mannitol (%) - 5.88287 x ITemp (^oC) x Amm. Sulphate (%) - 0.216263 * IT (Day) x pH - 5.08617 x IT (Day) x Mannitol (%) + 9.00875 x IT (Day) x Amm. Sulphate (%) - 22.0579 x pH x Mannitol (%) - 31.0886 x pH x Amm. Sulphate (%) - 74.7557 x Mannitol (%) x Amm. Sulphate (%)

Here, SC = substrate (wheat bran) concentration, ITemp = Incubation temperature, IT = Incubation Time, Amm. Sulphate = Ammonium Sulphate.

4.3.2 Results of Analysis of variance (ANOVA)

The results of the model's Analysis of Variance (ANOVA) are shown in Table 4.3. The P-value of 0.000 for the current model demonstrates its importance and usefulness for optimization studies. The P-value suggests that the generated model is significant. The model's adequacy and aptness are also indicated by the coefficient of determination (R^2) of 98.07% and the correlation coefficient (adjusted R^2) of 95.99%. As the R^2 value approaches 100%, the model's ability to forecast optimization parameters improves. This demonstrated the viability of our hypothesis and proposed that our experimental data set is following the same trend as expected by the predicted one. Except for the square interaction of pH, all the individual linear and square

interactions appeared effective ($P \leq 0.05$). The significant associations of specified parameters on phytase production were investigated using three-dimensional surface curves, and the optimal level of each variable was established to attain maximal phytase activity.

Table 4.3 Analysis of Variance (ANOVA) and regression analysis for *A. niger* NT7 phytase activity (U/gds) (Minitab®16 version).

Source	DF	Adj MS	Adj SS	F-Value	P-Value
Model	27	17829.3	481392	47.06	0.000
Linear	6	17929.2	107575	47.33	0.000
pH	1	36365.2	36365	95.99	0.000
SC wheat bran (g)	1	7638.4	7638	20.16	0.000
IT (Day)	1	5824.9	5825	15.38	0.001
ITemp (°C)	1	11770.2	11770	31.07	0.000
Amm. Sulphate (%)	1	15888.1	15888	41.94	0.000
Mannitol (%)	1	30088.5	30088	79.42	0.000
Square	6	56486.3	338918	149.10	0.000
ITemp(°C)*ITemp (°C)	1	6811.9	6812	17.98	0.000
IT (Day)*IT (Day)	1	19347.5	19347	51.07	0.000
Mannitol (%)*Mannitol (%)	1	36464.5	36464	96.25	0.000
pH*pH	1	357.9	358	0.94	0.340
SC wheatbran(g)*SCwheat bran (g)	1	31781.5	31781	83.89	0.000
Amm. Sulphate (%)*Amm. Sulphate (%)	1	3046.0	3046	8.04	0.009
2-Way Interaction	15	2326.6	34899	6.14	0.000
SC wheat bran(g)*IT (Day)	1	243.4	243	0.64	0.430
SC wheat bran(g)*ITemp (°C)	1	1141.5	1141	3.01	0.095
SC wheat bran(g)*Mannitol (%)	1	830.9	831	2.19	0.151
SC wheat bran(g)*Amm. Sulphate (%)	1	8584.7	8585	22.66	0.000
SC wheat bran(g)*pH	1	506.6	507	1.34	0.258
ITemp (°C)*pH	1	6649.0	6649	17.55	0.000
ITemp (°C)*IT (Day)	1	1616.5	1616	4.27	0.049
ITemp (°C)*Amm. Sulphate (%)	1	1730.4	1730	4.57	0.043
ITemp (°C)*Mannitol (%)	1	2658.8	2659	7.02	0.014
IT (Day)*Mannitol (%)	1	1293.5	1293	3.41	0.077
IT (Day)*Amm. Sulphate (%)	1	1014.5	1014	2.68	0.114
IT (Day)*pH	1	9.4	9	0.02	0.876
pH*Amm. Sulphate (%)	1	1933.0	1933	5.10	0.033

pH*Mannitol (%)	1	3892.4	3892	10.27	0.004
Mannitol (%)*Amm. Sulphate (%)	1	2794.2	2794	7.38	0.012
Error	25	378.8	9471		
Lack-of-Fit	17	340.9	5795	0.74	0.714
Pure Error	8	459.6	3677		
Total	52		490863		

S	**R-sq**	**R-sq (pred)**
19.464	98.07%	91.45%

Here SC = Substrate (wheat bran) concentration, ITemp = Incubation temperature, IT = Incubation Time, Amm. Sulphate = Ammonium Sulphate

The interaction between various factors was shown using three-dimensional graphs (3-D plots) (Figure 4.1 a-e), with phytase activity represented on the y-axis and two independent variables plotted on the x-axis and z-axis. The form and peak of 3-D plots reflect the maximal attainable phytase production for two interacting parameters.

The positive interaction impact of substrate content (wheat-bran) with incubation temperature is shown in Figure 4.1a, exhibiting an intermediate peak with the highest phytase production. The maximum enzymatic production was obtained at 35 °C with 5 g wheat bran (Figure 4.1a). These optimal values boost phytase production within the range, but when going beyond these values, phytase production sharply declines. Wheat bran offers physical support and contains all of the essential elements necessary for efficaciously SSF process. Because there may be competition for nutrients and physical support when fermenting, the appropriate substrate amount is critical [10, 24, 25].

Increased mannitol concentration with adequate incubation duration resulted in increased enzymatic activity due to their significant interaction (Figure 4.1b). Because of the interplay between pH and incubation temperature, the up-surged phytase production was achieved at 35 °C and pH 5.0. Furthermore, exceeding these parameter's ideal values resulted in a decreased phytase production output (Figure 4.1c). The interaction of mannitol and pH implies that the maximum phytase production is obtained at a maintained low pH of the fermentation medium and a higher carbon source, i.e., 2% mannitol concentration (Figure 4. 1d). The significant interaction between high mannitol (2%) and low ammonium sulphate (0.5%) resulted in an ideal C/N ratio for maximum phytase synthesis. (Figure 4.1e). An earlier study of optimizing phytase in *P. anomala* suggested that 2 % glucose and 0.5 % beef extract concentrations improved the enzymatic yield [26].

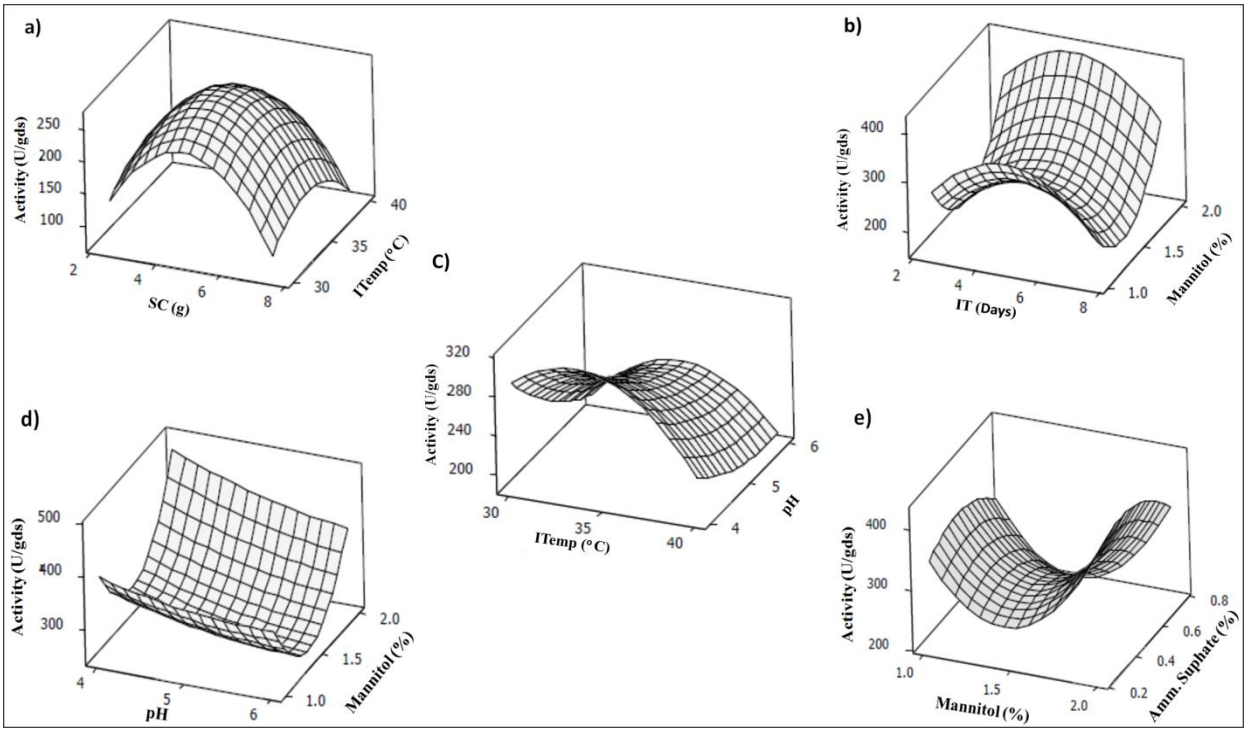


Figure 4.1 Response surface plots of phytase activity (U/gds) with phytase activity on the y-axis and two independent variables on the x- and z-axis, respectively: **a)** SC (g) and ITemp (ºC), **b)** IT (Day) and Mannitol (%), **c)** ITemp (ºC) and pH, **d)** pH and mannitol (%), **f)** Mannitol (%) with Ammonium Sulphate (%).

4.3.3 Enhanced phytase production by optimizing SSF Variables

Using the SSF variables of 5 g wheat-bran, supplemented with 1.97% (w/w) mannitol and 0.44 % (w/w) ammonium sulphate, pH 4.38 at 35 °C for five days, the response optimizer projected predicted phytase production of 442.50 U/gds. For the simplification, the nearest round up values of SSF variables (5 g wheat bran, 2% (w/w) mannitol, 0.5% (w/w) ammonium sulphate, pH 4.3 at 35 °C for five days) were used for conducting the laboratory experiments in triplicates to demonstrate the response optimizer's accuracy. Experimental authentication yielded a phytase activity of 521.29 ± 28.16 U/gds phytase production. The optimization attained by the statistical method resulted in a 2.5-fold improvement when compared to the OVAT approach (208.30 ± 0.22 U/gds) and a 6.8-fold increase in phytase productivity when compared to unoptimized culture conditions (76.34 ± 0.99 U/gds) [8].

In a prior study, four SSF parameters (culture temperature, pH, aeration area, and age of seeded culture) were optimized by CCD of RSM, resulting in 10.38-fold increased phytase production in *Thermomyces lanuginosus* [19]. The selected three parameters (glucose concentration, ammonium sulphate concentration, and moisture content) affecting phytase production of *A.*

ficuum optimized by RSM significantly increased the production level of the enzyme [27]. Bhavsar et al. reported that an RSM resulted in a 3.08-fold increase in phytase production while utilizing 10 g wheat bran as SSF substrate supplemented with 3 g glucose, 1.25 g dextrin, 0.2 g $NaNO_3$, and 0.3 g $MgSO_4$ moistened with 20 ml distilled water and fermentation carried out for four-days [28]. Phytase synthesis was increased 7.34 times through a statistical approach in *A. ficuum* NTG-23 when waste vinegar was used as an energy source for phytase production [29]. Another study found that using wheat bran as a substrate, *A. niger* NRF9 produced 2.9 times more phytase in SSF than in the traditional method [30]. Other recent studies have also employed this approach not only for phytase enzyme production but also for optimizing the production of other hydrolytic enzymes from *A. niger* [31]. Other possible phytase-producing candidates studied using the RSM technique include *Penicillium purpurogenum* GE1, *Sporotrichum thermophile*, and *Williopsis saturnus* NCIM 3298, which resulted in 2.6, 11.6, and 5.8-fold increases, respectively when compared to the original unoptimized culture conditions [20, 32, 33]. Recent research suggested that water hyacinth can be utilized as a cheap agricultural substrate for phytase production from *Pholiota adiposa*. Its statistical optimization resulted in an overall 3.15-fold enhanced enzymatic production in SSF [34].

4.3.4 Biochemical characterization of crude *A. niger* NT7 phytase

Feed enzymes should resist the harsh environments of animal's GI areas and pelleting temperatures used in feed treatment [3, 35]. The biochemical characterization (pH, temperature, and metal ion effects) of crude phytase was studied by the OVAT approach to achieve these required features for its potential applicability as a feed additive.

4.3.4.1 Effect of pH

pH is a critical chemical property which is directly linked with the enzyme's ability to maintain catalytic activity by changing the ionic strength around its surroundings. *A. niger* NT7 crude phytase preparation has shown catalytic activity over a wide acidic pH range (Figure 4.2). Surprisingly, the crude phytase has shown maximal phytase activity at two different pH, 2.0 of Glycine-HCl and 5.0 of sodium acetate buffer. The finding suggests the presence of two isoforms of phytase in the crude preparation. Our findings are in accordance with earlier deciphered crystal structure of *A. niger* phytase. These isoforms have different pH optima i.e., phyA and phyB has optima at 2.5 and 5.5 respectively [36, 37].

An earlier study of phytase from *A. niger* NCIM563 has also shown the optimal activity at two different pHs, 2.5 and 5.0 [5, 38]. From an application point of view, phytase should be active

over a broad range of acidic pH conditions. As digestive pH conditions in chicken varied from 2.5-5.5, phytase should withstand over this range [39]. The current study supports that phytase has the potency to be utilized in feedstuff owing to its ability to withstand the acidic pH range of monogastric animals [40]. The current work shows that phytase is fairly active at acidic pH, but when the pH rises into the alkaline range, phytase activity drops dramatically, possibly due to fluctuations in the ionization conditions of catalytically essential amino acid. Earlier, *A. niger* [41], *A. niger* CFR 335 [42], *A. oryzae* SBS50 [43], and *A. foetidus* MTCC 11682 [44] have all shown similar results for acidic pH optima for phytase activity.

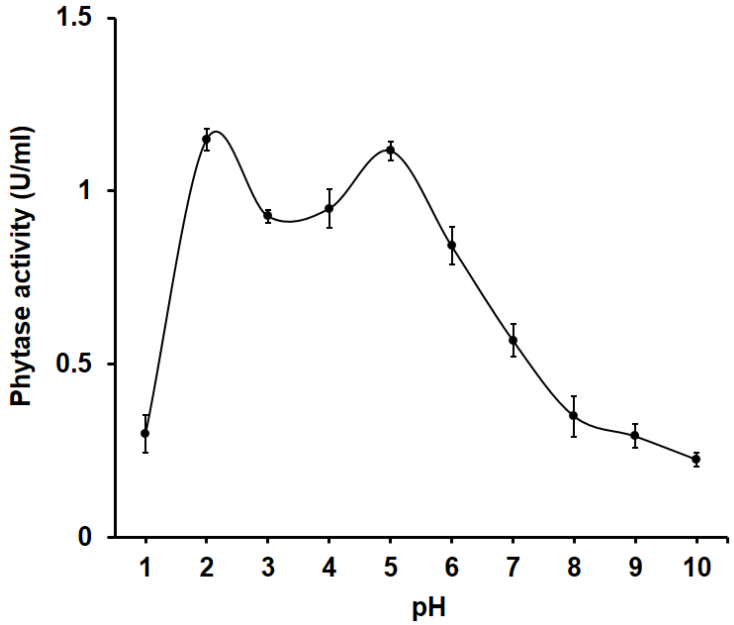


Figure 4.2 Determination of optimum pH [Buffer system pH 1 (KCl-HCl), pH 2-3 (Glycine-HCl), pH 4-5 (Sodium acetate), pH 6-8 (Tris-HCl) and pH 9-10 (Glycine-NaOH). Phytase assays were conducted at 50 °C for 30 min. All experiments were conducted in triplicate and represented as mean ± S.D.

4.3.4.2 Optimum temperature and thermotolerant profiling

Phytase should be able to withstand high pelleting temperatures of 65-95 °C during the processing of feed formulation [3]. The activity of *A. niger* NT7 crude phytase was demonstrated over a wide temperature range (varied from 40-80 °C at the interval of 10 °C), suggesting its applicability in feedstuff and aquaculture [3, 45]. The optimal temperature was found to be 60 °C (Figure 4.3a). Denaturation of the enzyme leads to decreased enzymatic activity when the temperature is raised above the optimal range. Previous results also showed that 60 °C is the optimal temperature for the phytase of *A. niger* UFV-1 and *A. niger* NCIM563, which is consistent with our findings [5, 46].

Thermal stability is indeed one of the requirements for phytase enzymes for industrial utilization. With a half-life (the period when enzyme activity drops to 50% of total activity) of 273.15 min., phytase was more stable at 50 °C. At 60 °C, a $t_{1/2}$ of 72.19 min., while at 70 °C, a $t_{1/2}$ of 36.49 min was perceived (Figure 4.3b). The enzyme can retain more than 50% activity at 60 °C even after 1 h of incubation, representing the thermotolerant nature of our enzyme. Inclusively, the phytase of *A. niger* NT7 outperformed previously investigated phytases from *Aspergillus* spp. in terms of thermostability [42, 47].

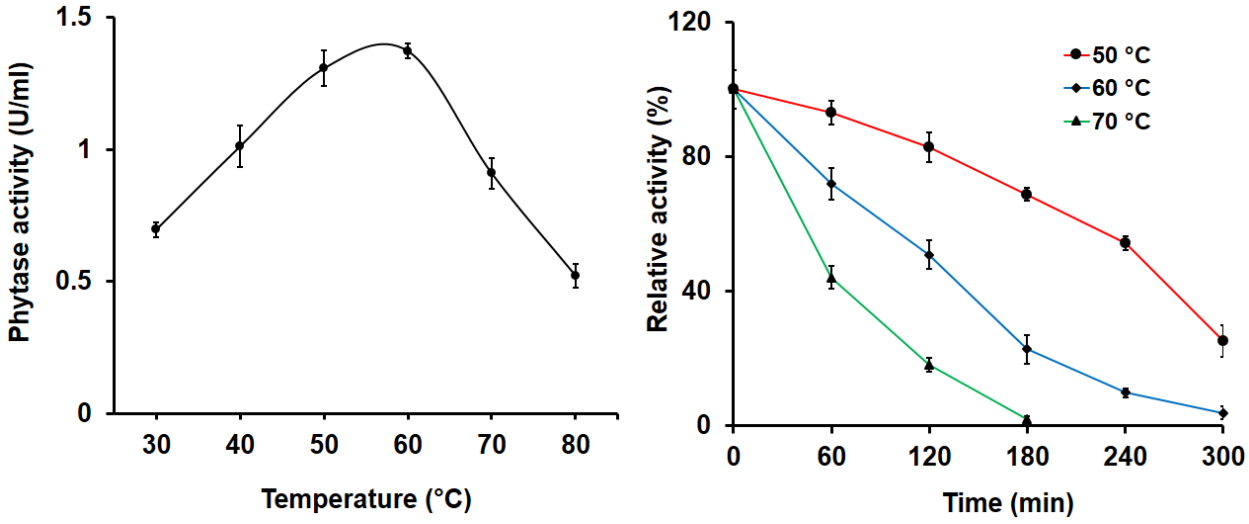


Figure 4.3 a) Determination of optimum temperature and **b)** Thermo-stability profile. The untreated sample is considered as a control for calculating relative activity after heat treatment. All experiments were conducted in triplicate and represented as mean ± S.D.

4.3.4.3 Effect of metal ions

From a feed perspective, it's critical to understand the impact of different metal ions on the catalytic function of phytase. Since the metal ions are the byproduct of the enzyme's dephytinization activity, releasing these metal ions may adversely or positively affect the enzyme activity. Metal ions, such as Ca^{2+}, Zn^{2+}, and Co^{2+} (1 mM and 5 mM), were found to increase phytase activity significantly (Figure 4.4). The favorable impact of metal ions may be attributed to an improved substrate interface with the active site residues of the enzyme [48]. The phytase of *Sporotrichum thermophile* [49] and *A. oryzae* SBS50 [43] displayed comparable activity augmentation in the presence of Ca^{2+} and Co^{2+}. In contrast, other metal ions, including Fe^{2+}, Mn^{2+}, Mg^{2+}, and Cu^{2+}, inhibited phytase activity in our case (Figure 4.4). This inhibition

may be caused by the formation of metal complexes at the enzyme's catalytic site, preventing sodium phytate catalysis [43, 49].

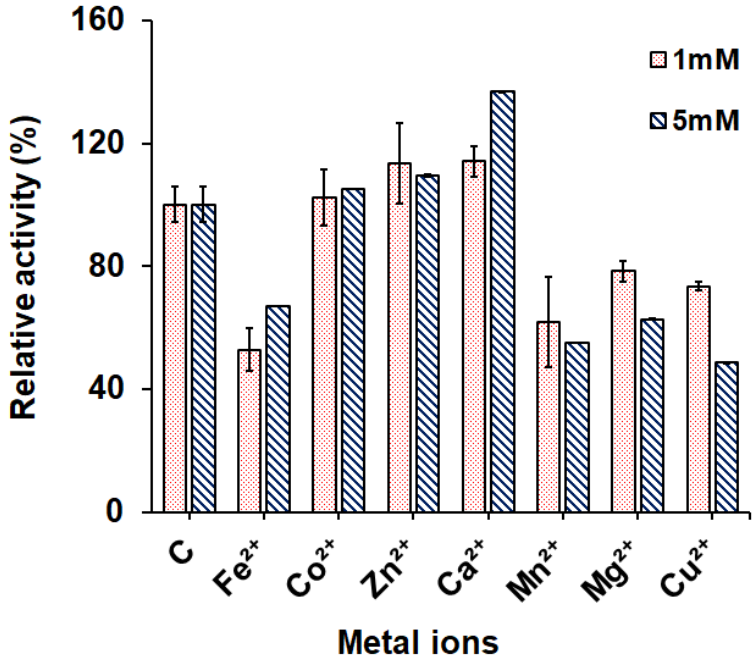


Figure 4.4 Effects of various metal ions on phytase activity. The reaction carried out without any metal ion treatment is control (C) and considered as 100% for calculating the relative residual activity left after the respective metal ions treatment. All experiments were conducted in triplicate and represented as mean ± S.D.

4.3.5 Evaluation of dephytinization activity

4.3.5.1 Amelioration of wheat bran by crude phytase

The phytase acts on phytic acid and releases inorganic phosphorus (Pi) and other nutrients (total soluble proteins and reducing sugar). The dephytinization activity was investigated using wheat bran, a less expensive and frequently used animal feed. The progressive upsurge in the discharge of Pi (2460.53 ± 102.11 µg/ml) and total proteins (491.07 ± 20.21 µg/ml) till 72 h incubation was observed; afterwards, a fast downfall in the release was observed attributed to inhibition of released end-product, substrate constraint, and denaturation of the enzyme (Figure 4.5) [6, 50]. As a result, our findings imply that the discovered phytase effectively catalyzed the breakdown of proteins-phytate complexes. Furthermore, the total reducing sugars release (2019.45 ± 61.07 µg/ml) continuously increased till 60 h. Thus, our findings suggested that the crude phytase from *A. niger* NT7 can effectively catalyze the breakdown of phytate complexes in wheat bran. The current findings were supported by earlier reports of dephytinization of wheat bran using *A. oryzae* SBS50 and *A. niger* NCIM 563 phytases [6, 28].

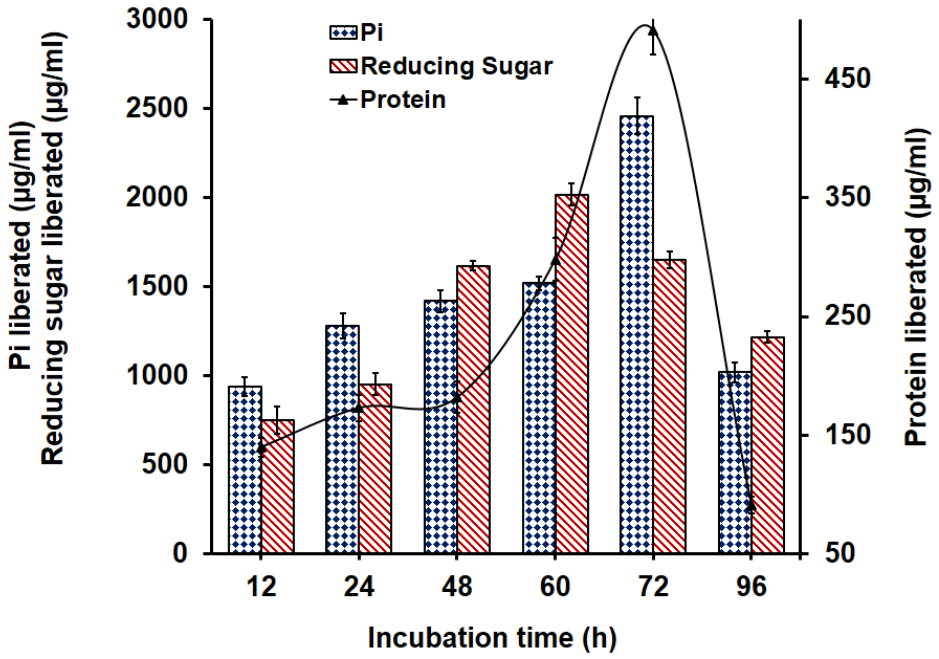


Figure 4.5 Release of nutritional components from wheat bran after dephytinization using crude phytase preparation (Experimental conditions: 0.1 M Acetate buffer, pH 4.8 and reaction carried out at 50 °C). All experiments were conducted in triplicate and represented as mean ± S.D.

4.3.5.2 Amelioration of maize bran by crude phytase

The main waste created after processing maize grains is being utilized as feed (maize bran). The phytic acid content of maize bran is successfully hydrolyzed by *A. niger* NT7 phytase. The procedure resulted in the liberation of Pi, which peaked after 24 h of treatment (1723.04 ± 45.32 µg/ml). It is then progressively declined, most likely due to protein denaturation or end product inhibition/inhibitory effects [17]. Previous research also showed the adequate dephytinization capacity of phytase from *A. niger* NCIM 563 [51], *A. niger* NT7 [8] against wheat bran, and *A. oryzae* phytase against maize bran resulted in the liberation of Pi [6]. After 24 h of treatment, the release of bounded reducing sugar (3210.9 ± 56.35 µg/ml), total proteins (956.4 ± 4.30 µg/ml), and amino acids (250.87 ± 10.04 µg/ml) from phytate attained its maximum value (Figure 4.6). Following that, a gradually falling phase was observed due to the inhibitory effects of reaction end products and denaturation of released proteins and amino acids [20]. Previous studies on phytases of *R. oligosporus* MTCC 556 [52], *A. aculeatus* APF1 [53], and recombinant phytase (rSt-Phy) of thermophilic mold *Sporotrichum thermophile* [54] also showed the dephytinization of food grains (wheat, soybean, corn and rapeseed flour), biofortified wheat, and wheat bread (tandoori and naan), respectively to release the Pi, total reducing sugar, and total protein.

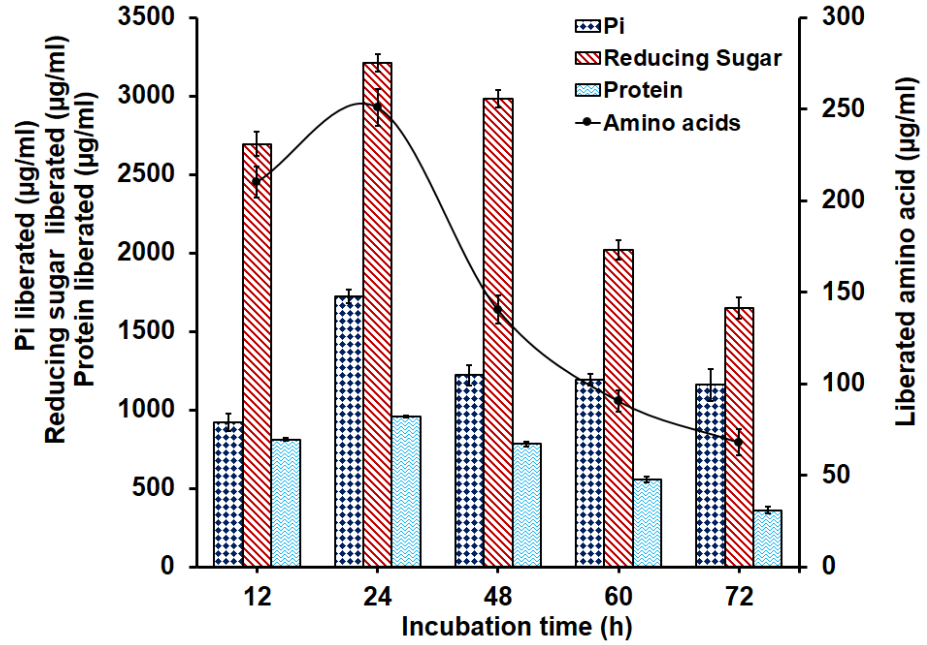


Figure 4.6 Dephytinization of maize bran using crude phytase preparation (Experimental conditions: 0.1M Acetate buffer, pH 4.8 and reaction carried out at 50 °C. All experiments were conducted in triplicate and represented as mean ± S.D.

On comparing untreated maize bran to dephytinized maize bran, ICP-MS analysis revealed that dephytinization results in significant mineral release in the ascending order of Ca < Zn < Mn < Mg < P < Fe as compared to untreated maize bran (Figure 4.7). Mg, P, and Fe were released more than 100%, but Mn and Zn were released less than 50% compared to untreated maize bran. The Ca ions were released the lowest in the current study. The phytase from *A. niger* NCIM 563 extracts more than half of the minerals from soybean flour [7]. The dephytinization activity of *A. niger* NT7 crude phytase resulted in releasing all bound nutrients, decreasing phytic acid levels. The findings mentioned above demonstrate the suitability of *A. niger* NT7 crude phytase for improving the nutrition release from maize bran and evidence its candidature as a promising feed additive on a commercial scale.

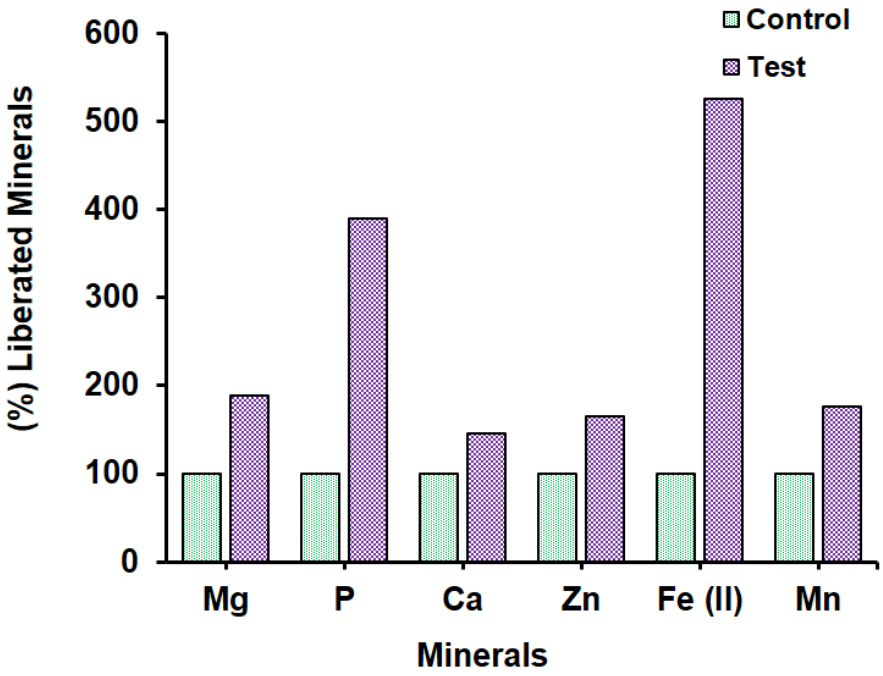


Figure 4.7 ICP-MS analysis of minerals released after 24 h of maize bran dephytinization.

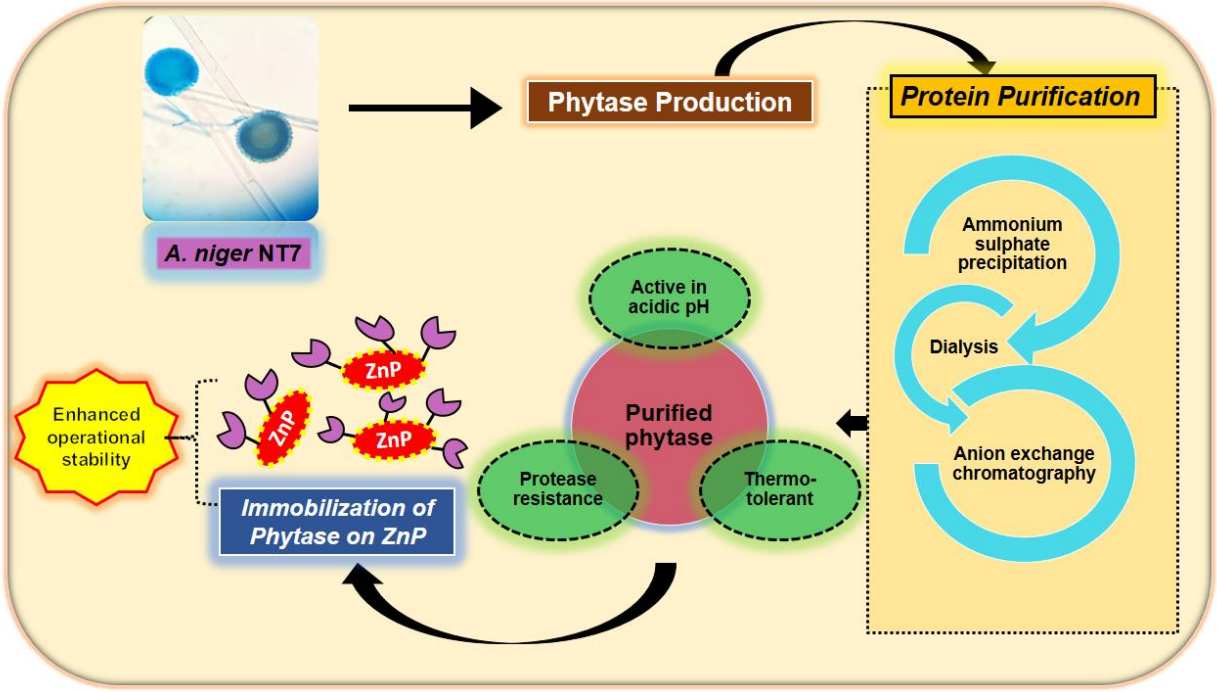
4.4 CONCLUSIONS

The statistical approach successfully achieved the enhanced phytase production in SSF using wheat bran. Compared to OVAT and unoptimized cultural settings, the RSM approach improved the phytase production by 2.5 and 6.8-fold, respectively. Crude phytase preparation has shown catalytic activity at a wide acidic pH range (2-7) and a temperature range (40-70 °C); thus, it can withstand the harsh gastric conditions of the stomach and pelleting temperature of feed treatment. Adding *A. niger* NT7 crude enzyme preparation to maize and wheat bran resulted in a considerable upsurge in releasing reducing sugars, soluble proteins, and numerous minerals, including phosphorus. Furthermore, because the crude formulation does not require any purification, the expense of feed additives can be kept to a minimum level. As a result, the *A. niger* NT7 crude phytase preparation has proven to be a potential feed additive for enhancing nutritional qualities.

CHAPTER 5

PURIFICATION, IMMOBILIZATION, AND CHARACTERIZATION OF FUNGAL PHYTASE

GRAPHICAL OUTLINE OF CHAPTER



HIGHLIGHTS

- Purified phytase of *A. niger* NT7 is active at acidic pH range, protease resistance and thermotolerant in nature
- Chemically synthesized ZnO nanoparticles are crystalline in nature (XRD analysis), smooth, and spherical in shape having 40-45 nm size (FESEM imaging)
- FTIR analysis showed successful immobilization of phytase on ZnO nanoparticles
- Immobilization enhances the operational stability (optimal pH, temperature, thermostability and reusability) of phytase

5.1 INTRODUCTION

Phytate is the biggest pool for phosphorus (P) content in plants, i.e., 60-80% of P content is in phytic acid or phytate form [1]. Phytase catalyzes the phytate hydrolysis and produces intermediate derivatives and finally inositol monophosphate with the release of inorganic phosphorous (Pi). The digestive system of monogastric animals lacks phytase, so they cannot metabolize phytic acid. Phytic acid and phosphate substances are excreted with feces, leading to soil contamination and algal bloom. Phytase is a digestive enzyme that liberates the Pi from phytic acid [2, 3]. Phytase being utilized as a feed supplement improves animal nourishment, resulting in 33% diminished Pi release in the waste matter, ultimately reducing the environmental pollution. The foremost restrictions to utilizing phytase enzyme from the highly nutritious and ecological point of view are heavy market prices and low levels of phytase titer and thermal stability [4, 5].

Phytases from various microorganisms have been reported, and their useful properties have been characterized [6-8]. However, an ideal phytase is still needed to be discovered so that it can be used as a supplement for foodstuff and feed. Purification and biochemical characterization of enzymes must continue to find a novel phytase with the appropriate features. Purification steps for phytase include the salt-induced precipitation method to concentrate crude enzyme and ion exchange (charge basis), gel filtration chromatography (size basis), and combinations of both methods. Although phytase from various fungal sources have been purified, none of them is ideal with all the required features for commercial exploration by food and feed industries. Therefore, there is an increasing demand for new industrial phytases with better and consistent performance in the GI tract of monogastric animals [9-12]. Microorganisms that produce phytase include filamentous fungi *Aspergillus* and bacteria *Bacillus.* Various studies have found that fungal sources express the most active phytase in extracellular locations along with the most desirable characteristics of being active at acidic pH value and stable at higher temperatures. Therefore, *Aspergillus* is mainly utilized at the industrial-scale production of phytase [8, 13-15].

Enzyme immobilization refers to confining molecules within matrix/particles while maintaining their catalytic properties intact. The enzyme immobilization approach augments the thermal stability, reusability, physiochemical properties, enzymatic activity and specificity. The immobilization approach has been categorized into five kinds, namely adsorption, cross-linking, covalent binding, encapsulation and entrapment [16]. Phytase adsorbed on various inert

matrices like glass microsphere, entrapped on cellulose, agars beads, starch agar beads and alginate/polyvinyl alcohol has shown the reduction in the phytic acid content of soymilk [17, 18]. CLEA (cross-linked enzyme aggregates) preparation of phytase with low-cost protein feeder soymilk protein has resulted in highly porous material [19]. The shortcomings associated with the entrapment method, like large beads size, lead to resistance in mass transfer. Moreover, CLEA preparation required very concentrated protein for achieving efficient immobilization. All these associated shortcomings could be certainly overcome by utilizing nanostructures.

Nanostructures offer a significant advantage over conventionally used matrices as they have a huge surface area accessible for binding owing to their nano size. Nanostructured particles (metal oxides), nanowires, nanosheets, nanofibres, nanotubes and nanofilms have been previously studied for enzyme immobilization potential [20-22]. Nanostructure materials have intriguing qualities: a high surface-to-volume ratio, surface reaction activity, catalytic efficiency, and excellent adsorption capacity, making them ideal applicant materials for enzyme immobilization [23-27]. Nanomaterials with a significant surface area resulted in higher enzyme loading per unit mass of particles. Furthermore, the multipoint attachment of enzyme molecules to the surface of nanomaterial hinders protein unfolding, consequential increasing the stability of enzyme bound to the nanoparticle surface [28, 29].

Phytase immobilization greatly improves the enzyme's thermostability, which is the most desirable feature for its exploration for feed supplementation. Previous studies demonstrated that thermal stability and proteolytic resistance of phytase from *A. niger* and *E. coli* improved by its immobilization on iron-coated allophane [30]. Earlier studies have shown zinc oxide nanoparticles (ZnP) as efficient support for enzyme immobilization galactosidase of *Aspergillus oryzae* [23] and phytase of *P. decumbens* [24].

The current study is focused on the purification and biochemical characterization of *A. niger* NT7 phytase and enhancing its operational stability by immobilization on zinc oxide nanoparticles.

5.2 MATERIALS AND METHODS

5.2.1 Phytase production in SSF

The phytase was produced by setting up an SSF using a 5 g wheat bran with 10 ml distilled water [1 (wheat bran): 2 (moistening agent)] supplemented with 2% mannitol and 0.5% ammonium sulphate in a 250 ml Erlenmeyer flask at 35°C, with a pH of 4.3 for five days. The medium was inoculated by 3-day-old inoculum having 12×10^7 spores/ml (previously

optimized). Crude extracellular phytase was extracted from moldy wheat bran after completion of SSF, as was previously mentioned in chapter 3 (section 3.2.5) and further utilized for enzyme purification [31].

5.2.2 Purification of *A. niger* NT7 phytase

Phytase was purified using the traditional approach by concentrating the crude enzyme followed by the chromatographic technique.

5.2.2.1 Enzyme concentration by salt-induced precipitation

The mycelium-free enzyme extract was concentrated by salt-induced precipitation. The ammonium sulphate salt was added until 85% saturation was achieved at 4 °C, followed by incubating the solution overnight at 4 °C. The precipitated protein (phytase) was pelleted down using centrifugation at 4 °C for 20 min at 8000 rpm. The pellet was redissolved in 0.1 M sodium acetate buffer, pH 5.5, and then desalted by dialysis with 0.1 M sodium acetate buffer, pH 5.5 at 4 °C. The denatured protein debris was removed from the dialyzed sample by centrifugation, and the clean supernatant was utilized for the next purification step. The dialyzed protein sample was further concentrated using 10 KDa amicon (Millipore).

5.2.2.2 Ion exchange chromatography

The dialyzed protein sample was then purified by column chromatography on a DEAE (Diethylaminoethyl)-sepharose (10 x 1.2 cm with a bed volume of 6.5 ml) column. Firstly, the column was equilibrated with 0.1 M sodium acetate buffer, pH 5.5. The concentrated dialyzed protein sample was loaded on the pre-equilibrated column and allowed to bind over it for one hour. After loading, unbounded proteins were removed by washing the column with 0.1 M acetate buffer, pH 5.0. Bounded phytase was eluted by a 0-1 M NaCl salt gradient in the 2 ml of fractions. The eluted fractions (2 ml) were collected, and the presence of protein was detected by measuring its absorbance at 280 nm and assayed for phytase activity [32]. The fractions showing phytase activity were pooled and quantified using the Bradford method. The purified protein was then assayed for biochemical characterization.

5.2.2.3 Purity check and molecular weight determination

The fractions collected after ion exchange chromatography were analyzed for purity using SDS-PAGE and Native-PAGE (Composition and preparation explained in Annexure C.1). SDS-PAGE and zymography were also used to estimate the molecular weight of the purified phytase.

For zymography, native PAGE gel was incubated with 1% sodium phytate solution for 5 h at 55 °C. After incubation, the native gel was dipped in a 10% calcium chloride solution to form a transparent zone against opaque background [10].

5.2.3 Biosynthesis of ZnO Particles (ZnP)

ZnP were synthesized by the chemical precipitation method [33]. Zinc nitrate aqueous solution (2 mM) was prepared in deionized water and kept at 60 °C with constant stirring. NaOH (1M) solution was added slowly until the solution turned milky white, which confirmed the formation of ZnP [34]. The solution was allowed to stir for another 2 h at the same temperature; afterwards, nanoparticles were collected by centrifugation. The formation of ZnP was confirmed by monitoring spectra taken from 300-700 nm by UV-visible spectrophotometer (Thermo Scientific™ Evolution 201 spectrophotometer). The synthesized nanoparticles were washed with ethanol and repeatedly with Milli-Q water. After washing, the pellet was dried overnight to collect a fine powdered form of formed ZnP.

5.2.4 Characterization of synthesized ZnP

5.2.4.1 Morphological analysis using FESEM

The nanoparticles morphology (shape and size) was determined using a Field Emission Scanning Electron Microscope (FESEM) imaging. Through Image J software, the size of each particle was observed, and a histogram was plotted to determine the average particle size of ZnP.

5.2.4.2 X-Ray Diffraction (XRD)

XRD provided an analysis to decipher the crystal structure and size of formed ZnP. XRD analysis was carried out using Cu-Kα radiations at a constant voltage of 20 kV and a current of 15 mA. The crystallite size (D) was calculated from Scherrer's formula and its extrapolation through Williamson-Hall (WH) plot.

$$\boldsymbol{D = \frac{0.9\lambda}{\beta COS\theta}}$$

Where λ is the wavelength of irritated X-ray, β is the width at half maximum of the ZnP (101) line, and θ is a diffraction angle. The obtained XRD data was further used to calculate the grain (crystallite) size of synthesized ZnP.

5.2.5 Immobilization of phytase on Zinc nanoparticles (Phy-ZnP)

The purified enzyme (Phy) (1mg) was incubated for 2 h at 37 °C with synthesized ZnP (10 mg in 10 ml 0.1 M acetate buffer) under constant shaking conditions [23]. The immobilized phytase-zinc nanoparticles (Phy-ZnP) were collected by centrifugation at 10000 rpm for 15 min.

5.2.6 Characterization of Phy-ZnP

5.2.6.1 Chemical characterization using Fourier Transform Infrared (FTIR)

When an enzyme interacts with the ZnP (immobilization matrix) during immobilization, several interactions between them have been established. Such interactions between phytase and ZnP during immobilization were studied using FTIR spectroscopy (Agilent Technologies) by recording the transmittance spectra in the range of 650-4000 cm^{-1}. FTIR spectrum of ZnP was also used to decipher their nature and purity level.

5.2.6.2 Determination of immobilization efficiency and yield

Immobilization efficiency was calculated to assess the enzyme binding capacity of the ZnP using the below-mentioned formula

$$\boldsymbol{Immobilization\ Efficiency}\ (\%) = \left(\frac{\boldsymbol{E_0 - E_1}}{\boldsymbol{E_0}}\right) * \mathbf{100}$$

Where E_0 is the total protein loaded before immobilization; E_1 is the amount of protein left in the supernatant after immobilization. The enzyme was quantified by the Bradford method [35]. The immobilization yield or the ratio of the specific activity of the immobilized phytase to the free enzyme was calculated as follows

$$\boldsymbol{Immobilization\ Yield}\ (\%) = \frac{\boldsymbol{Y_1}}{\boldsymbol{Y_0}} * \mathbf{100}$$

Where Y_1 is the specific activity of phytase after immobilization; Y_0 is the specific activity before immobilization.

5.2.7 Biochemical Characterization of free purified phytase (Phy) and immobilized phytase (Phy-ZnP)

5.2.7.1 Effect of pH on phytase activity

The optimum pH of Phy and Phy-ZnP at 50 °C was determined using different buffer systems of varying pH ranges (1 -10). The buffers (100 mM) of various pH ranges such as KCl-HCl-

pH 1, Glycine-HCl- pH 2-3, Sodium acetate-pH 4-5, Tris-HCl- pH 6-8 and Glycine-NaOH- pH 9-10 were used to evaluate the phytase activity.

5.2.7.2 Effect of temperature on phytase activity

The optimum temperature for Phy and Phy-ZnP were determined by carrying out phytase assays within a temperature range of 30-80 ℃ (intervals of 10 ℃).

5.2.7.3 Thermal stability

The thermal stability profiles of the Phy and Phy-ZnP were recorded at 50 ºC, 60 ºC, and 70 ºC temperatures for five hours in a water bath. The phytase assay was performed at 1 h intervals (till 5 h) to determine residual phytase activity. The thermodynamic parameters were calculated using the experimental data in the equation to obtain the inactivation rate

$$\boldsymbol{ln}\left(\frac{\mathbf{A}}{\mathbf{A_o}}\right) = \mathbf{K_d t}$$

Where A_o is the initial activity obtained without pre-incubation, A is the residual activity after heat treatment, K_d is the thermal inactivation rate constant (h^{-1}), and t is the exposure time (h). The following relationship determines the half-life of phytase ($t_{1/2}$, h):

$$\mathbf{t_{1/2}} = \frac{\mathbf{ln\,2}}{\mathbf{K_d}}$$

5.2.7.4 Determination of kinetic parameters

The kinetic parameters, i.e. K_M, V_{max}, K_{cat}, K_{cat}/K_M of the Phy and Phy-ZnP, were determined as a function of sodium phytate concentration (2, 5, 7, 10, 15, 20 and 25 mM) under optimal conditions. For Phy, phytase assays were performed with a 0.1 M sodium acetate buffer pH 5.0 at 50 ºC for 30 min, whereas for Phy-ZnP, with a 0.1 M sodium acetate buffer pH 4.0 at 70 ºC for 30 min**.** The Lineweaver-Burk (LB) plot was used to estimate the K_M and V_{max} values.

5.2.7.5 Effect of metal ions and chemical agents

Additionally, the influence of metal ions (Fe^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+}, Ca^{2+}, $Mn^{2+}$and Cu^{2+}), chemical inhibitors (EDTA, sodium molybdate; BME), and chaotropic agents (Urea and potassium iodide; KI) at 1 mM concentrations were assessed. The Phy and Phy-ZnP were incubated with metal ions and chemical agents under study for 30 min. and then evaluated for residual phytase activity. The control without any treatment was considered 100% for computing the relative activity after treatment of metal ions and chemical agents.

5.2.7.6 Effect of protease treatment

To assess the proteolytic resistance, the Phy and Phy-ZnP were treated with 0.5% trypsin (trypsin prepared in 100 mM Tris-HCl buffer pH 7.5) and pepsin (pepsin prepared in100 mM Glycine-HCl buffer pH 2.5) for 120 min at 37 °C. At an interval of 30 min, the samples were assessed for residual phytase activity. After treatment, residual phytase activity was compared with activity recorded at 0 min (control; 100%) to check its resistance against digestive enzyme treatment.

5.2.7.7 Reusability of Phy-ZnP

Phy-ZnP was reused for multiple rounds of the phytase assay to determine reusability until it lost 50% of its initial activity. The Phy-ZnP was separated from the reaction mixture by centrifugation at 10,000 rpm for 20 min after each cycle. The recovered Phy-ZnP was washed with 100 mM acetate buffer (pH 4.0) before being utilized in the next catalytic cycle. In the first cycle, the phytase activity was assumed to be 100% relative to the activity calculated in consecutive runs.

5.3 RESULTS AND DISCUSSION

5.3.1 Purification of *A. niger* NT7 phytase

5.3.1.1 Phytase concentration

Mycelium-free crude phytase was concentrated by adding 85% ammonium sulphate at 4 °C. The specific activity of phytase was increased from 3.04 to 25.56 U/mg with a 17.47 % yield (Table 5.1). Earlier studies have also shown that ammonium sulphate precipitation results in efficient phytase concentration with maximum yield [10].

5.3.1.2 Ion (anion) exchange chromatography

The dialyzed sample retained 40.25 mg protein with specific phytase activity of 25.56 U/mg and purified using anion exchange chromatography. The maximum phytase activity and protein concentration were observed in fractions 5 and 6 (2 ml) when eluted with 0.3 M NaCl (Annexure C.2). 11.48 purification fold was achieved with a 3.93% phytase yield (Table 5.1) after anion exchange chromatography. Phytases from *A. niger* and *T. lanuginosus* TL-7 were also purified earlier by ion exchange and gel filtration chromatography [36, 37]. The earlier study has also shown that *Aspergillus oryzae* phytase was purified to a 10.68-fold purification with a 7.9 % yield using ammonium sulphate precipitation and chromatographic (ion exchange and gel filtration using SP Sepharose P-60) techniques [10]. A combined chromatographic

approach, anion exchange (DEAE-cellulose), cation exchange (CM-cellulose), and Superdex-75-based gel exclusion chromatography, was used to purify *A. ficuum* NTG-23 phytase with a phytase recovery rate of 23.8% [38].

Table 5.1 Purification summary of phytase of *A. niger* NT7.

Purification Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	5943.52	1953.21	3.04	100	1
Dialyzed	1038.68	40.25	25.56	17.47	8.40
DEAE Sepharose CL-6B	234.08	6.7	34.93	3.93	11.48

5.3.1.3 Purity Check and Molecular weight determination

After ion exchange chromatography, the phytase was checked for purity by SDS-PAGE and Native-PAGE, which showed the enzyme was purified to homogeneity. Further analysis of SDS-PAGE and the combined result of Native-PAGE and zymography determines the molecular weight of purified monomeric phytase as ~70 KDa (Figure 5.1).

A previous study has also shown that purified phytase of *A. ficuum* NTG-23 has a molecular weight of 67 KDa [38]. The phytase of *A. oryzae* has an 80 KDa molecular weight on 12% SDS-PAGE [10]. The crystal structure previously submitted in Protein Data Bank (PDB ID: 3K4Q) also revealed that phyA (Phytase) of *A. niger* is monomeric and acidic in nature which is in accordance with our obtained experimental data [39].

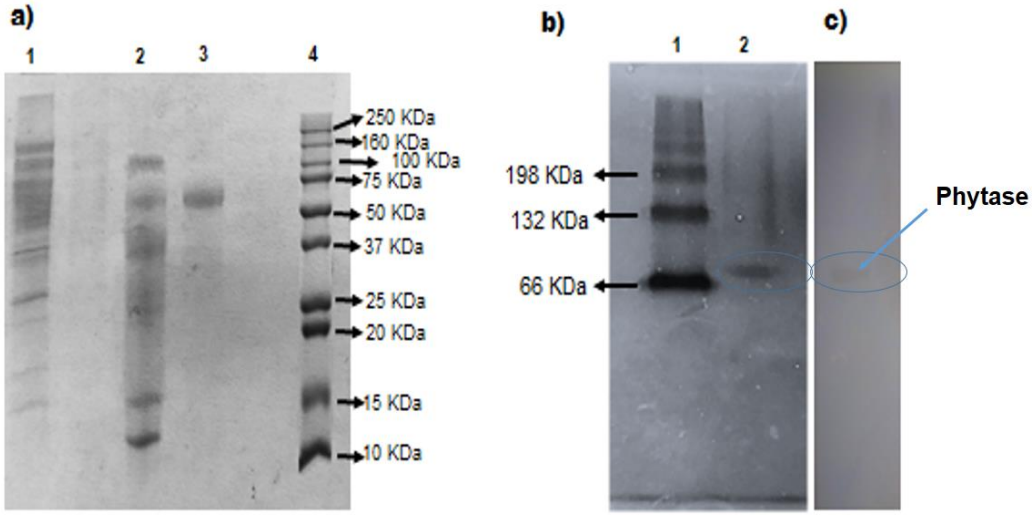


Figure 5.1 Purity check and molecular weight determination using a) 12% SDS-PAGE; lane 1: unpurified phytase, lane 2: dialyzed sample, lane 3: purified enzyme, and lane 4: marker **b)** Native

page showing in lane 1: BSA protein as marker and lane 2: purified phytase, and **c)** Zymogram (the phytase activity represented as hydrolysis zone against an opaque background shown by arrow).

5.3.2 Synthesis and characterization of ZnP

ZnP particles were synthesized using a chemical approach where sodium hydroxide was used as a reducing agent. A change in colour from transparent to opaque milky white visualized the reaction progression within 2 h of reaction time (Figure 5.2a). Furthermore, a maximum absorption spectrum at 352 nm also confirmed the ZnP synthesis (Figure 5.2b). The earlier reports also showed the optical properties of ZnP between 350-390 nm [40, 41].

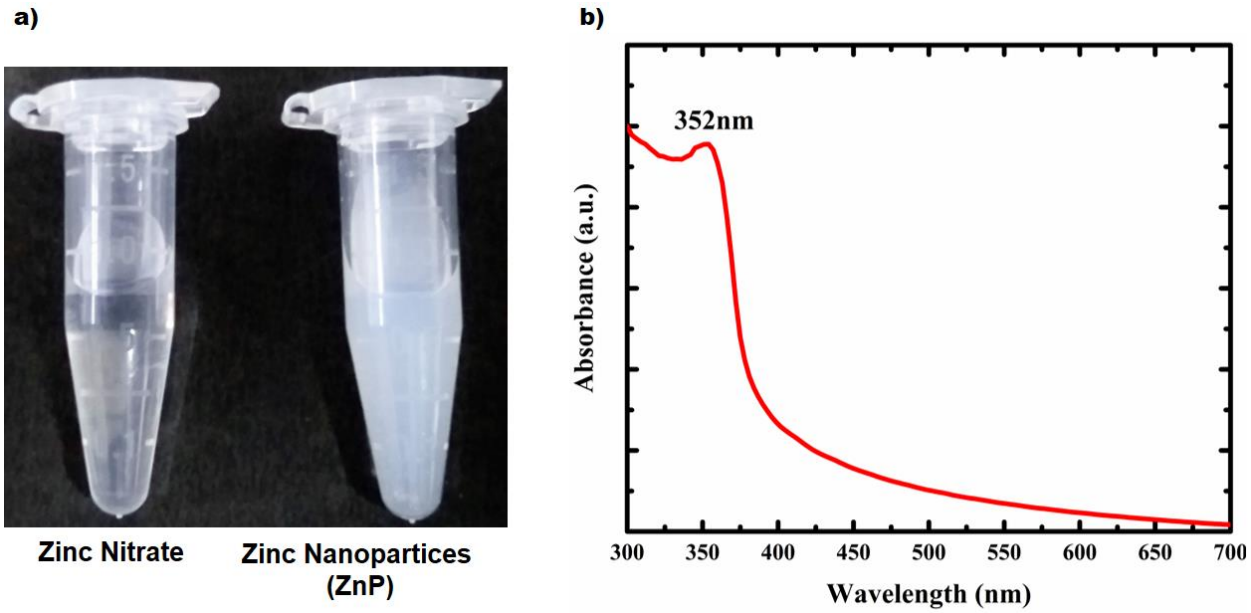


Figure 5.2 a) Visualization of zinc nitrate (transparent solution) and synthesized ZnP (milky white), **b)** Absorption spectra of ZnP by UV-Vis spectroscopy (spectra range from 300-700 nm).

5.3.3 Characterization of synthesized ZnP

5.3.3.1 FESEM of ZnP

The FESEM image analysis confirmed the spherical shape with a smooth surface of the ZnP (Figure 5.3a). The histogram plot also showed that the average particle size lies in the nanorange of 40-45 nm (Figure 5.3b).

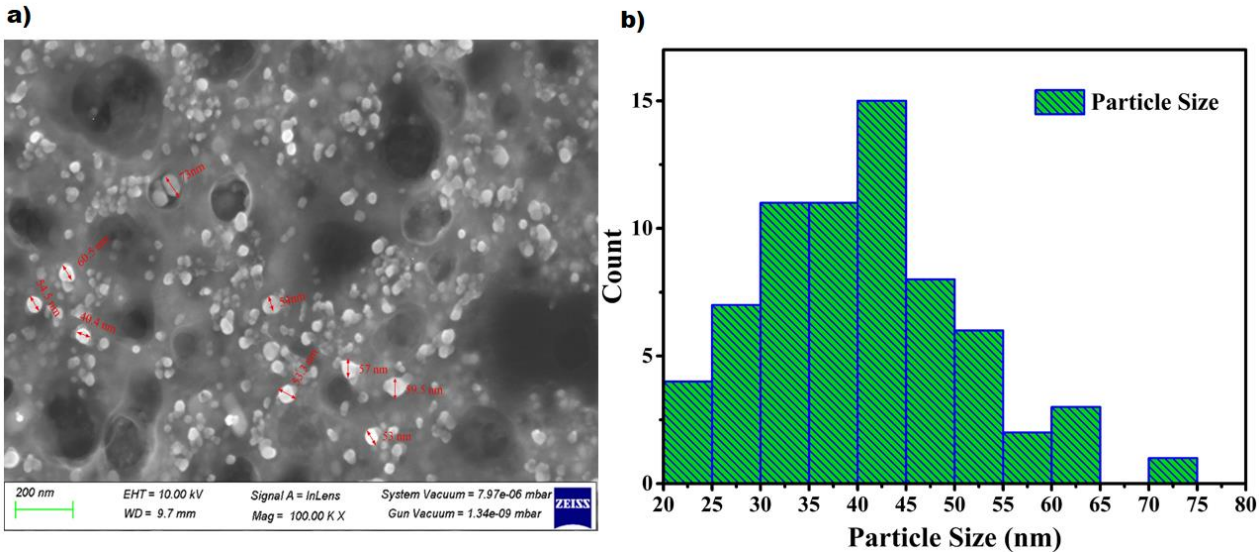


Figure 5.3 a) FE-SEM imaging of ZnP showing spherical morphology (with a scale of 200 nm), **b)** Histogram plot for average size (40-45 nm) determination of synthesized ZnP.

5.3.3.2 XRD analysis of ZnP

XRD analysis of ZnP at room temperature showed that particles are crystalline. XRD pattern of ZnP displayed Bragg's reflections at 31.6°, 34.3°, 36.2°, 47.4°, 56.5°, 62.7°, 67.9° and were corresponding to 100, 002, 101, 102, 110, 103, 112 planes (Figure 5.4a). The crystallite size calculated from Scherrer's equation is 14.28 nm, which also advocated that the synthesized ZnPs are in nanoscale size (Figure 5.4b).

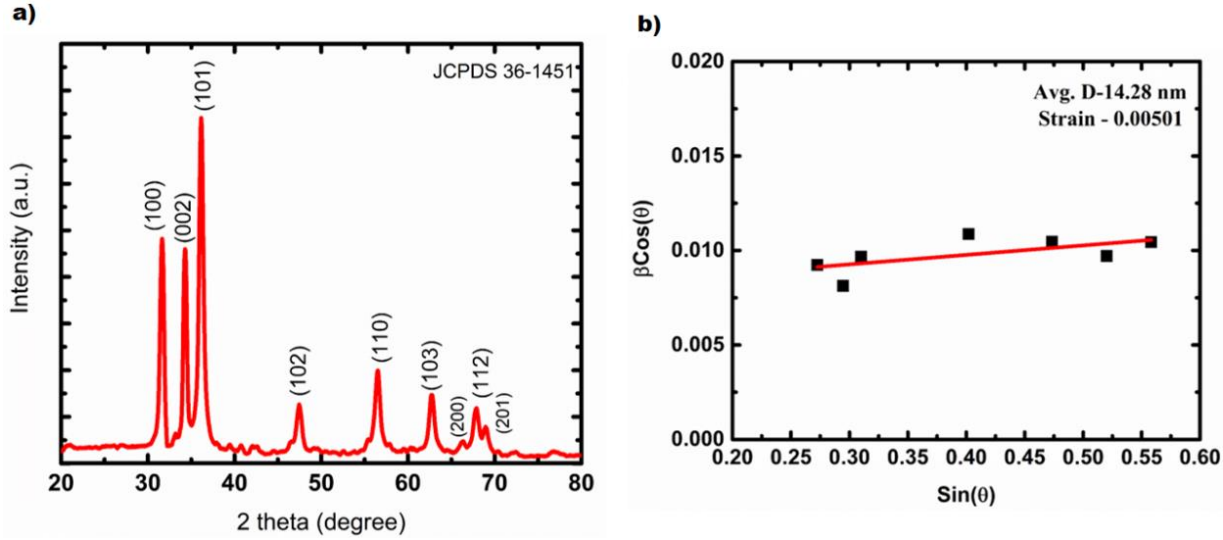


Figure 5.4 a) XRD analysis of synthesized ZnP. XRD pattern of ZnP was recorded at room temperature with Cu-Kα radiation (λ is 1.5418 A°), in 2 theta (°) angle ranging from 20°- 80° and, **b)** Williamson-Hall (WH) plot for crystallite size (14.28 nm) determination.

5.3.4 Characterization of Phy-ZnP

5.3.4.1 FTIR analysis

The adsorption of phytase on ZnP resulted in aberrations in surface morphology. The adsorption resulted in the clustering of phytase on its surface, which led to agglomeration. The FTIR range of metal oxides is generally observed at low values owing to the vibrational energy between atoms. The peak observed in ZnP FTIR at 3328 cm^{-1} corresponds to the stretching vibration of the –OH group (Figure 5.5). Another peek at 1632 cm^{-1} corresponds to the vibration of an asymmetric C=O bond. The free phytase has characteristic peaks at 3257 cm^{-1} and 1020 $cm^{-1,}$ which reflect the β- sheets of polypeptide chains interacting with sugar moieties [42]. The shifting of peaks from 3328 to 3330 cm^{-1} and 1632 to 1636 cm^{-1} corresponded to the interaction of the –C=O group of phytase with ZnP [23]. Also, the amide bond peak of free phytase at 1471, 1020 and 850 cm^{-1} shifted to 1300, 1040 and 820 cm^{-1} due to conformational changes and the active participation of the amide bond for phytase immobilization on ZnP.

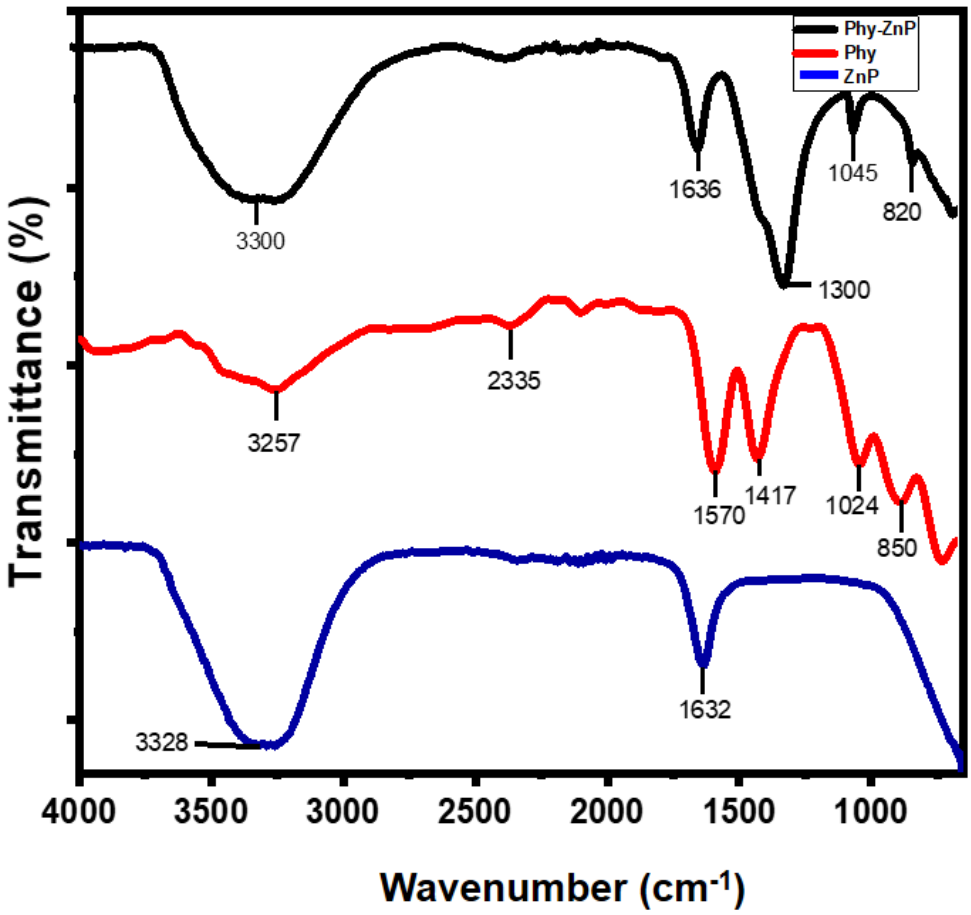


Figure 5.5 FTIR spectra of ZnO nanoparticles (ZnP shown in blue line), free phytase enzyme (Phy shown in red line) and phytase immobilized on ZnP (Phy-ZnP shown in black colour).

5.3.4.2 Immobilization efficiency and yield

The efficiency of the immobilization method using ZnP was calculated as 72.9%. The reduction in specific activity of Phy-ZnP was measured as immobilization yield; the phytase retained 67.01% activity after immobilization. The enzymatic activity reduction could be attributed to the diffusional limitation caused by immobilization.

5.3.5 Biochemical Characterization of Phy and Phy-ZnP

5.3.5.1 Effect of pH on phytase activity

The substrate-enzyme interaction and the subsequent product release are greatly influenced by the pH of the surrounding medium as the pH critically affects the enzyme activity by changing the ionization state of amino acid residues at the active site.

The Phy of *A. niger* NT7 showed optimal pH at 5.0 and a drop in activity as it approached the neutral and alkaline pH range (Figure 5.6). When the pH of the reaction rises, some of the enzymes charged amino acid residues deprotonate, causing the protein to become more negatively charged and, thus, overall ionic potential altered. Earlier studies have also shown similar pH optima at pH 5.0 for phytases from *A. niger* ATCC 9142 [43], *A. niger* [11, 44], *A. oryzae* [10], *A. niger* NCIM 563 [13], and *A. tubingensis* TEM 37 [45].

The Phy-ZnP has an optimal phytase activity at pH 4.0 (Figure 5.6) and showed a sharp dip in phytase activity when moving away from this optimal pH value. The observed reduced phytase activity in the alkaline pH range (7-9) may be due to the fact that solvent molecules are strongly bonded at places equivalent to the oxygen atoms of the phosphate groups of the substrate (sodium phytate). The water molecules act as the substitutes for the substrate/product binding site, preventing its binding and hence lowering the phytase activity [29, 39].

Phy-ZnP showed broader relative activity than Phy, probably due to the secondary interaction between enzyme and immobilizing matrix, changing the physical and chemical properties of the enzyme [19]. Earlier studies have also shown that ZnO nanoparticles bound to *A. oryzae* galactosidase enzyme retained more activity at acidic and alkaline ranges compared to free soluble enzymes [23]. Phytase from *A. niger* (Natuphos®) has an isoelectric point of 4.5; in our study also, phytase is active in the acidic pH range [46].

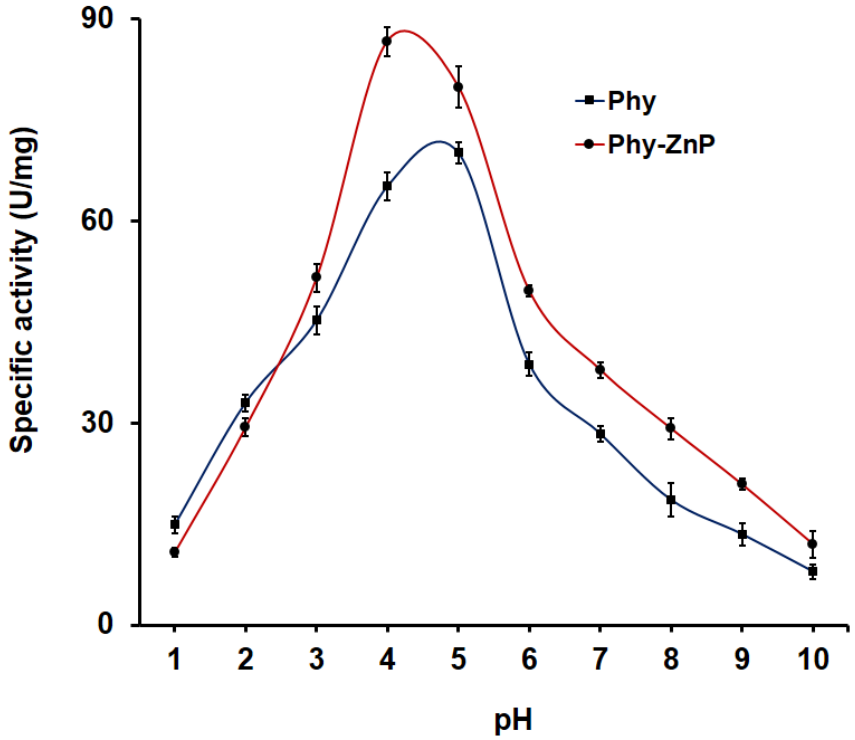


Figure 5.6 Effect of pH [100 mM each; KCl-HCl (pH 1), glycine-HCl (pH 2-3), sodium acetate (pH 4-5), Tris-HCl (pH 6-8) and glycine-NaOH (pH 9-10)] on activities of Phy and Phy-ZnP assayed at 50 °C for 30 min. All experiments were conducted in triplicate and represented as mean ± S.D.

5.3.5.2 Effect of temperature on phytase activity

Temperature is a crucial factor for any reaction to be carried out. It affects the enzyme activity either by enhancing the kinetic energy of the reaction or by modulating the enzyme structure. The purified phytase showed an increasing trend of activity till 60 ºC and had comparable and maximal activity at both the temperatures 50 ºC (86.26 U/mg) and 60 ºC (85.86 U/mg) (Figure 5.7). At a higher temperature above optimal, the reduction in activity is due to the enzyme's denaturation. Previously reported phytases from *A. aculeatus* APF1 [47] and *A. oryzae* SBS50 [10] also showed optimum phytase activity at 50 ºC, whereas *A. niger* UFV-1 showed at 60 ºC [48]. In contrast, the Phy-ZnP has a higher optimum temperature at 70 ºC (Figure 5.7). The immobilization provides resistance to thermal denaturation at a high temperature which is in accordance with the previous studies [18, 49].

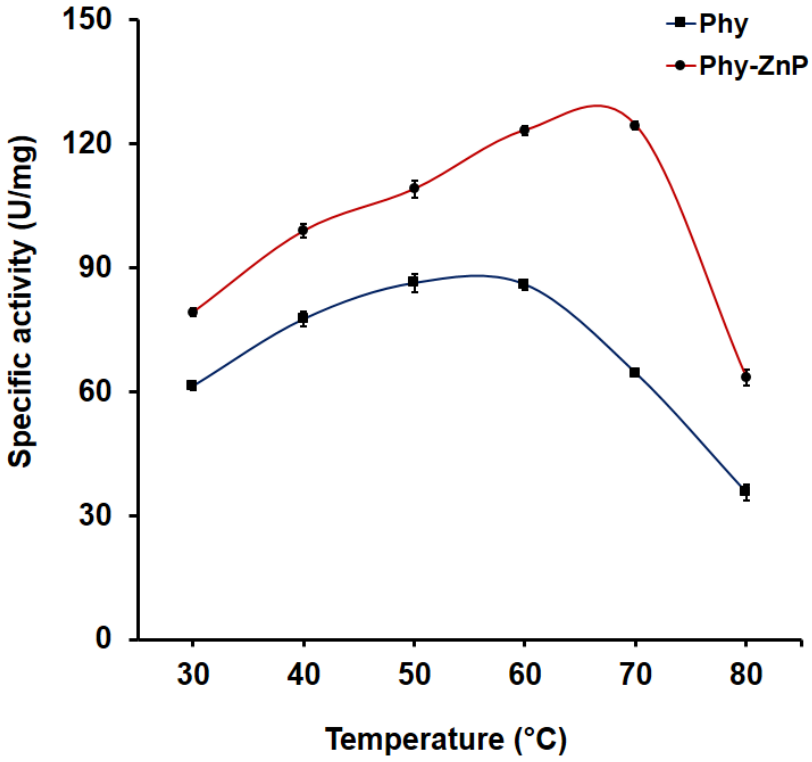


Figure 5.7 Effect of temperature on activities of Phy and Phy-ZnP assayed at pH 5.0 and 4.0 (0.1M sodium acetate buffer), respectively, and the reaction was carried out for 30 min at varied temperatures (30-80 ºC). All experiments were conducted in triplicate and represented as mean ± S.D.

5.3.5.3 Thermal stability

Among the most essential operational characteristics for phytase utilization in feed industry sectors is their thermal stability. The phytase should withstand the high temperature treatment during feed processing. The immobilization technique alters the structural integrity and operational stability of the enzyme for thermal treatment [50].

Phy was found reasonably stable at 50 ºC ($t_{1/2}$ = 288.75 min) and 60 ºC ($t_{1/2}$ = 106.61 min) (Figure 5.8a). Similar to our finding, the purified phytase of *A. oryzae* had a half-life ($t_{1/2}$) of 300 min at 50 ºC [51]. In contrast, Phy-ZnP was further found reasonably stable at 50 ºC ($t_{1/2}$ = 330 min), 60 ($t_{1/2}$ = 256.7 min) and 70 ºC ($t_{1/2}$ = 144.4 min), which are far more than Phy (Figure 5.8b, Table 5.2). The most probable reasons for this enhanced thermostability after immobilization on nanoparticles may be the reduced activation energy and more rigidity in the three-dimensional structure for phytase catalysis [52]. A recent study has also shown that ZnO-phytase nanoparticles have improved the thermo-tolerance potential of phytase [24]. Another study has also shown the improved thermotolerant potential of uricase when immobilized on ZnO nanorods [53].

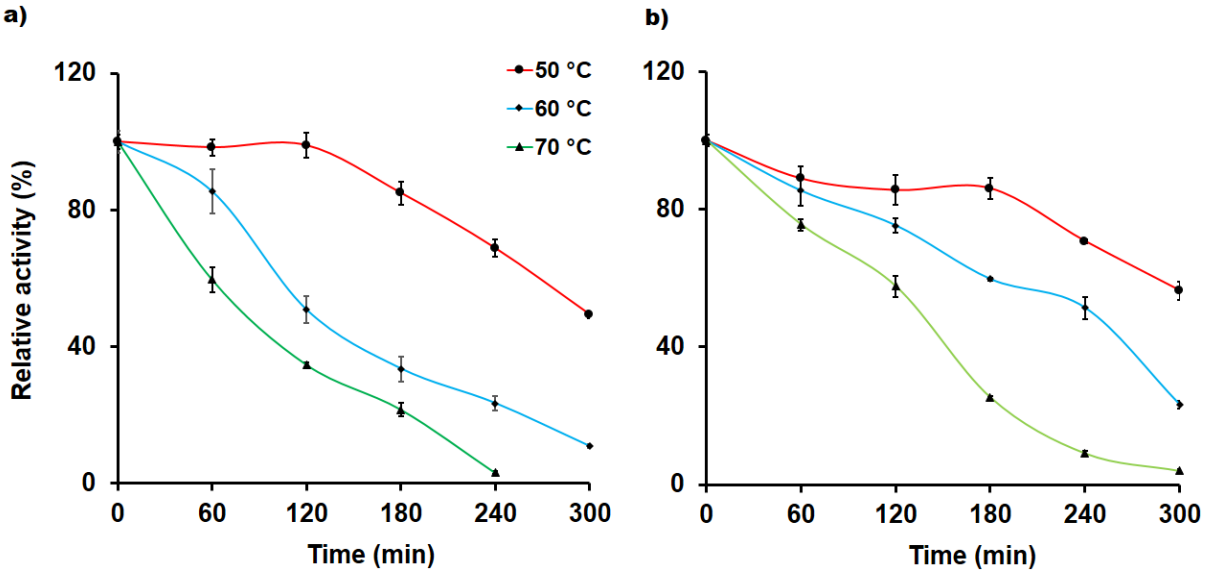


Figure 5.8 Thermostability profile of **a)** Phy and **b)** Phy-ZnP at 50, 60 and 70 °C. The enzyme activity obtained without incubation was taken as control (100%) for calculating the relative activity remained after heat treatment at 50, 60 and 70 °C (till 5 h). All experiments were conducted in triplicate and represented as mean ± S.D.

Table 5.2 Half-life ($t_{1/2}$) of Phy and Phy-ZnP at 50, 60 and 70 °C.

Temp. (°C)	**$t_{1/2}$ (min)**	
	Phy	**Phy-ZnP**
50	288.75	330
60	106.61	256.67
60	52.90	144.37

5.3.5.4 Determination of kinetic parameters

The kinetic parameters (K_M and V_{max}) of Phy and Phy-ZnP were estimated as a function of sodium phytate concentration at optimal conditions using the Lineweaver-Burk plot (Annexure C.3). The K_M and V_{max} for *A. niger* NT7 phytase (Phy) were 1.98 mM and 99 U/mg, respectively, for sodium phytate. In an earlier report, *A. niger* Van Teighem phytase has a K_M value of 0.660 mM and a V_{max} of 1,074 IU/mL [36]. The K_M and V_{max} of partly purified *A. aculeatus* APF1 phytase were 3.21 mM and 3.78 U/mg protein, respectively [47].

The observed turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_M) for Phy were 260.07 sec^{-1} and 1.31 x 10^5 M^{-1} $sec^{-1,}$ respectively (Table 5.3). A recent report for purified phytase of *A. fumigatus* showed a K_{cat} of 357 min^{-1} and catalytic efficiency (K_{cat}/K_M) of 476 mM^{-1} min^{-1} [42]. In contrast, Phy-ZnP showed an enhanced Michaelis constant (K_M) and a decreased

turnover number (K_{cat}) than Phy (Table 5.3). However, Phy-ZnP also showed an altered catalytic efficiency than the Phy, which may be due to combined effect of reduced K_M value and high activation energy of the enzyme. After immobilization, the increase in K_M value shows that the affinity between phytase and phytic acid gets reduced. This reduced enzyme-substrate interaction is most likely due to lessened free movement of enzyme on the solid ZnP support, either due to steric hindrance caused by ZnP on the active site of phytase, or due to reduced flexibility of enzyme required for substrate binding, or a combination of both. All these combinatorial factors resulting in the mass transfer resistance of sodium phytate into immobilized phytase on nanoparticles [52].

Table 5.3 Kinetic parameters of Phy and Phy-ZnP.

Kinetic Parameters	**Phy**	**Phy-ZnP**
K_M (mM)	1.98	4.87
V_{max} (U/mg)	99	96.15
K_{cat} (sec^{-1})	260.07	190.85
K_{cat}/K_M x 10^5 (M^{-1} sec^{-1})	1.31	0.39

5.3.5.5 Effect of metal ions and chemical inhibitors

Metal ions are reported to have a stimulatory effect on phytase activity as well as enhance thermal stability. Zinc, iron, and calcium, which are all abundantly present in animal feed and food matrix, could have a significant impact on supplemented phytase activity. Moreover, feed and food formulations contain various micro and macro metal ions in their matrix, so it's important to study their synergistic and inhibitory actions when supplemented with phytase enzyme [36, 38].

The present study has shown that Co^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+} metal ions have stimulatory effects on phytase activity, whereas slight inhibitory effects were observed with Fe^{2+}, Cu^{2+} and Mn^{2+} for Phy. Approximately 30% of phytase activity was reduced due to the inhibitory effect of Fe^{3+} ions in the reaction mixture (Figure 5.9a). The purified phytase of *A. oryzae* also showed stimulatory effects with Co^{2+}, Zn^{2+}, and Ca^{2+} [10]. Metal ions, such as Mn^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+} and Mg^{2+} do not have any significant stimulatory effect on *A. ficuum* NTG-23 phytase at 1.25 mM concentration. Only EDTA had slightly stimulatory action on phytase activity [38]. The development of metal complexes at the active site may be the origin of this inhibition, preventing substrate catalysis. The binding of these ions to the active site changes the conformational orientation of the phytase, thereby inhibiting the enzyme activity. Another

reasonable explanation for negative regulation may be that the metal ion directly interacts with the substrate phytic acid, and the resulting insoluble metal-phytate complex interferes with the action of the enzyme [7, 10, 47, 54]. The present study demonstrated that Phy-ZnP showed enhanced phytase activity in the presence of Co^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+} metal ions compared to Phy.

Studies have suggested that EDTA had a stimulatory effect on phytase activity owing to its chelating properties, which prevent the agglomeration of the substrate with the metal ion at the enzyme's catalytic site [36, 38]. Present studies have also shown the positive effect of EDTA on the phytase activity of Phy-ZnP, whereas Phy has no such stimulatory effect. Urea slightly, whereas KI strongly inhibits the phytase activity owing to their capability to disrupt intermolecular interactions (H-bond). Previously, KI addition has also shown similar inhibitory effects on phytases of *A. oryzae* [10], *S. thermophile* [54] and *A. aculeatus* [47]. Less inhibitory effects were observed for Phy-ZnP when treated with BME (β-mercaptoethanol), urea and KI than Phy (Figure 5.9b), suggesting immobilization helps in stabilization and has protecting action on inter and intramolecular structural integrity of enzyme. The maximum inhibitory effect for both Phy and Phy-ZnP was observed with sodium molybdate (NaMo). Previous studies also showed that immobilized phytase diminished the inhibitory effect of metal ions [55]. Several non-covalent interactions, such as van der wall force and H-bond, enable the enzyme to regain its active shape. The change in enzyme activity of phytase following regulated exposure to chaotropic substances indicates the impact of non-covalent forces on their structure and conformation [10, 36, 47].

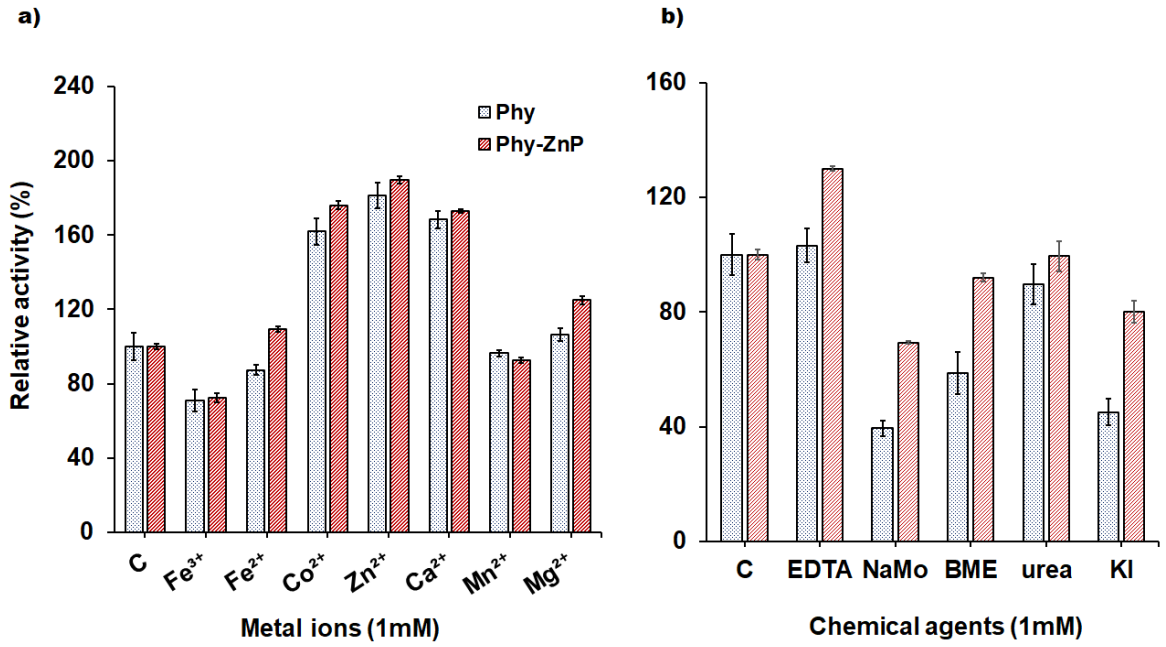


Figure 5.9 a) Effect of metal ions and **b)** chemical inhibitors on Phy and Phy-ZnP. Here C: control; EDTA: Ethylenediaminetetraacetic acid; NaMo: sodium molybdate; BME β-mercaptoethanol; KI: potassium iodide. The enzyme activity obtained without any treatment was taken as control (100%) for calculating the relative activity of the enzyme after treatment with various metal ions and chemical agents. All experiments were conducted in triplicate and represented as mean ± S.D.

5.3.5.6 Effect of protease on phytase activity

Phytase is an essential animal feed additive, it must be resistant to the actions of trypsin and pepsin, which are found in animal digestive systems. The phytase immobilized on ZnP has better performance than the free phytase enzyme on treatment with proteolytic enzymes under the same conditions. After 120 min treatment of pepsin and trypsin, Phy retained 70.12% and 52.35% residual phytase activity, respectively (Figure 5.10a, b).

Previous studies also showed that the soluble phytases of *S. thermophile* BJTLR50 and *A. oryzae* AK9 have protease resistance potential [56, 57]. Another study of *A. oryzae* phytase showed that after 120 min of trypsin (1%) treatment, 27% of activity was lost, while pepsin treatment resulted in only a 2.43% loss in phytase activity. This suggested that pepsin had no noteworthy hydrolytic effect on phytase activity [51]. In contrast, Phy-ZnP had 83.13% and 65% residual phytase activity when treated with pepsin and trypsin for 120 min, respectively (Figure 5.10a, b). This suggests that immobilization enhances the protease resistance against pepsin and trypsin treatment.

Immobilization enhances the protease resistance profile of the phytase enzyme by reducing the exposure of aromatic and basic amino acids or by forming a rigid three-dimensional structure [19, 58]. In terms of applicability, proteolysis resistance towards digestive enzymes is crucial for the phytase to remain functionally active in extreme acidic gastric conditions of monogastric animals.

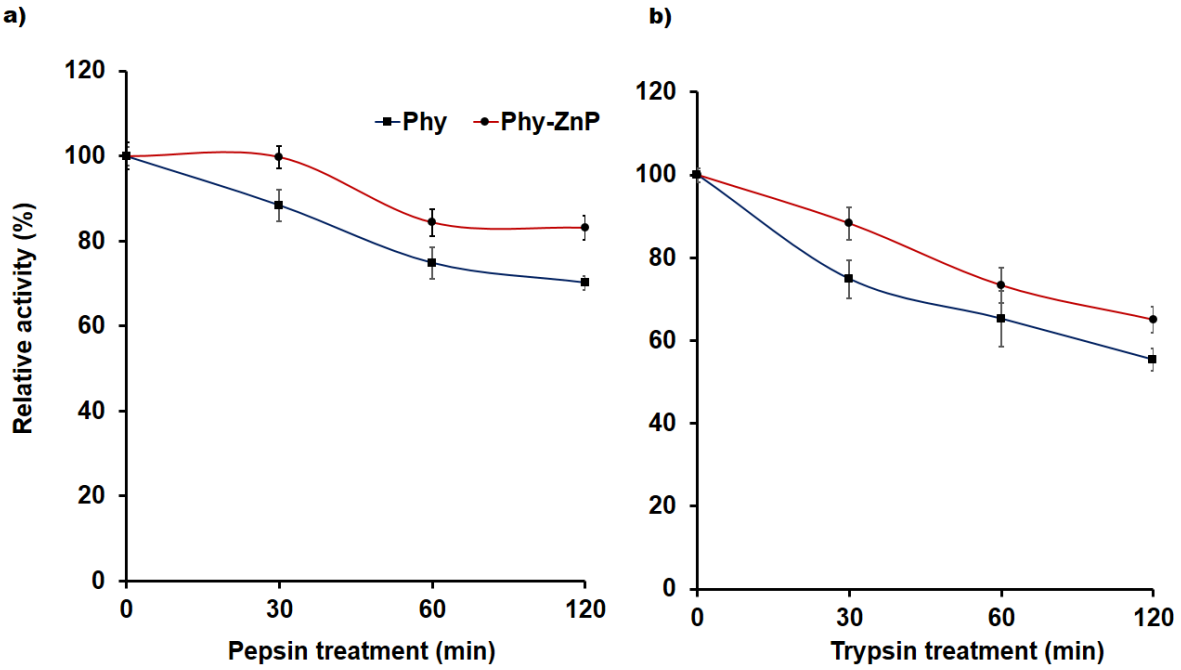


Figure 5.10 Protease resistance potential of Phy and Phy-ZnP against **a)** pepsin and **b)** trypsin treatment. The phytase activity at 0 min. i.e., before enzyme treatment (pepsin and trypsin) is considered as 100% for calculating relative activity after enzymatic treatment.

5.3.5.7 Reusability of immobilized phytase (Phy-ZnP)

The relative phytase activity of Phy-ZnP goes on decreasing with the increasing catalytic runs. The phytase immobilized on ZnO can be utilized up to the 10^{th} (retained 50% of relative phytase activity) successive cycle of phytase assay (Figure 5.11). The phytase assay after subsequent cycles did not show any sharp decrease in phytase activity; rather, a gradual fall was observed. This might be due to the insufficient retrieval of immobilized phytase from the apparatus at the end of catalytic reaction and nanoparticles lost during washing processes [59]. Phytase of *A. niger* immobilized on gold nanoparticles showed retained phytase activity up to 60% after the 10^{th} enzymatic run [52]. After successive enzyme activity up to the seven cycles to galactosidase of *A. oryzae* bound ZnO showed 75% retained activity [23].

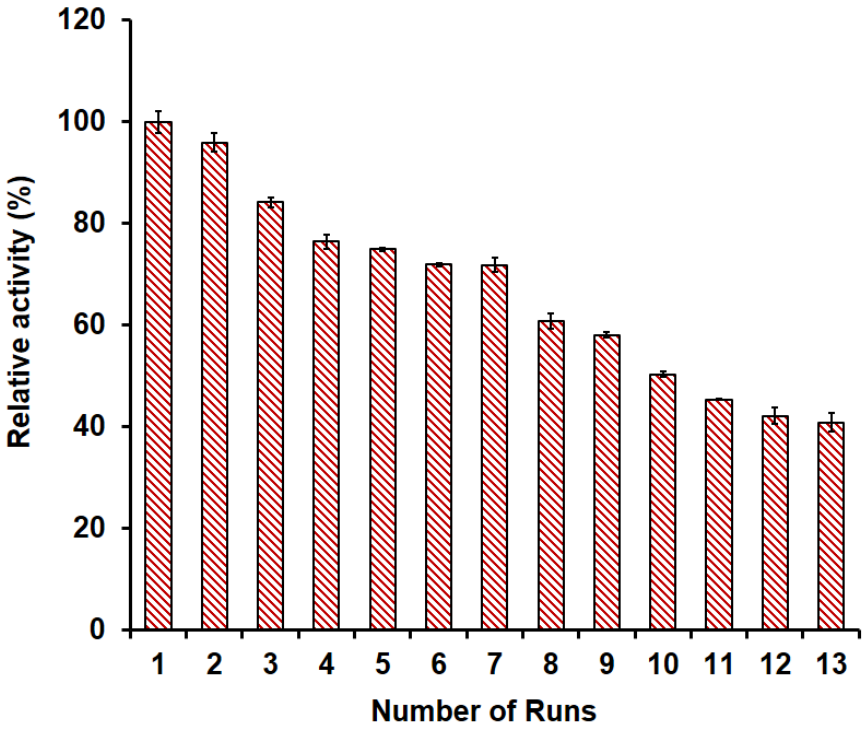


Figure 5.11 Reusability of phytase immobilized on ZnP; the phytase activity recorded after 1st cycle was considered as 100% for calculating relative enzymatic activity after successive cycles. Data are presented as mean ± S.D.

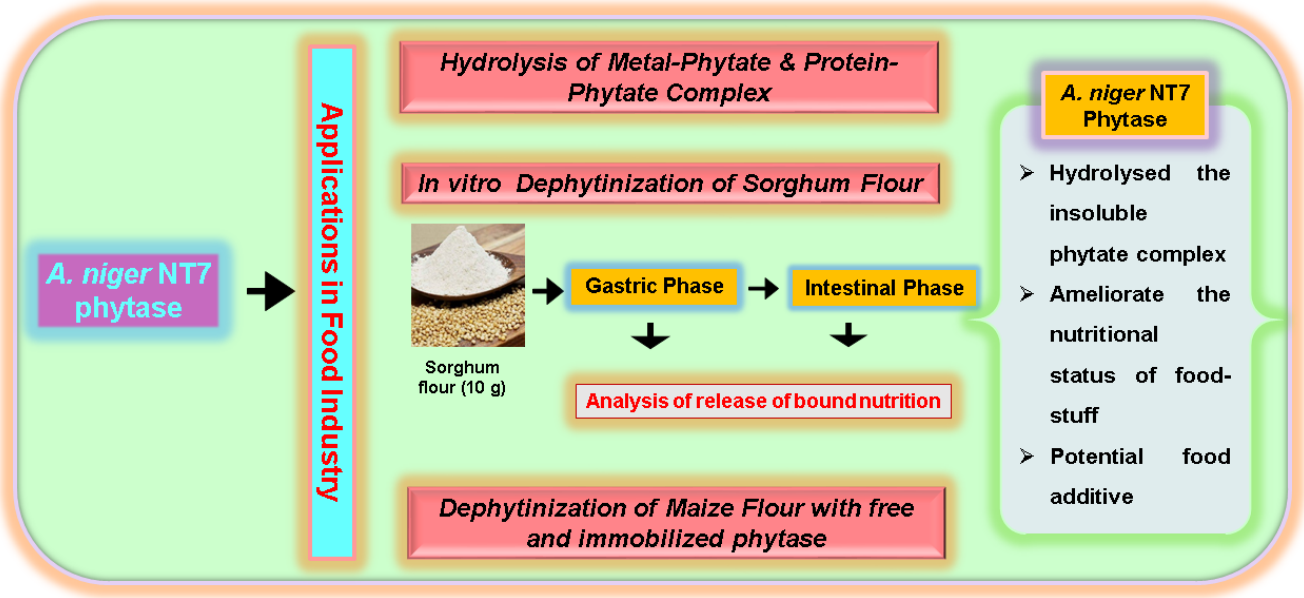
5.4 CONCLUSIONS

The purified phytase of *A. niger* NT7 has approximately 70 KDa molecular weight. The activity of purified phytase was utmost at 50 °C and pH 5.0. The enzyme was found to be heat resistant and protease-resistant. Furthermore, the overall operational stability (optimum reaction conditions; pH, temperature, thermotolerance, and reusability) of the phytase enzyme was increased when it was immobilized on zinc oxide nanoparticles. The phytase is immobilized via the interaction between the OH groups of ZnP and the involvement of other functional (amide, carbonyl) groups of Phy, as evidenced by the shift in the FTIR pattern. Additional bands appear in the spectrum due to the presence of distinctive groups of phytase. The significant features of the phytase enzyme, including optimal pH (pH 4.0), temperature (70 °C), thermostability and protease resistance potential, were immensely improved after immobilization on ZnP. The Phy-ZnP can be utilized efficiently up to 10 catalytic cycles. In a nutshell, the improved biochemical properties and the enhanced operational stability make the Phy-ZnP a promising candidate for the food and feed industries.

CHAPTER 6

APPLICATIONS OF FUNGAL PHYTASE IN THE FOOD INDUSTRY

GRAPHICAL OUTLINE OF CHAPTER



HIGHLIGHTS

- *A. niger* NT7 phytase was shown to be capable of hydrolyzing a variety of insoluble phytate more efficiently at 50 and 37 °C
- Reported first time the *in vitro* efficacy of phytase for dephytinization of sorghum flour
- Immobilized phytase first time showed the efficient hydrolysis of phytic acid of maize flour and ameliorates its nutritional status
- Free and immobilized phytase both have potential for food additive applicability

6.1 INTRODUCTION

Plant-based foods, mainly beans and flour, are a rich source of proteins and essential amino acids in a vegan diet [1]. The primary constraint of plant-based food materials is the existence of antinutritional factors, mainly in the form of phytic acid, oxalate, polyphenols, saponins, and enzyme inhibitors [2]. Phytic acid, which accounts for about 5% of the dry weight of lentils and grain products, is the most prominent storehouse of inositol and phosphorus [1]. Phytic acid interacts strongly with the Proteins, digestive enzymes, carbohydrates, and metals such as magnesium, calcium, zinc, and iron and forms complexes, making them inaccessible for absorption. Also, the GI of monogastric animals such as pigs, chickens, swine, and humans lacks phytate hydrolytic enzymes [3]. Therefore, monogastric animals cannot hydrolyze phytic acid and its complex components. As a result, phytic acid is considered an antinutritional factor [4].

Phytases catalyze the conversion of phytic acid to phosphoric acid and *myo*-inositol. Furthermore, it has significant multifunctional applications in human nutrition [5]. Due to the beneficial properties of phytase and its role as an additive in non-ruminant animal's diets, these enzymes occupy an important position in the application of the biotechnology sector. Phytase application shrinks the phytate and phytic acid levels in feed and commercial foods. Phytase being utilized as a feed additive should be able to effectively release phosphate from phytic acid. They must be proven effective at digestion levels and withstand the pH and temperature conditions of the digestive system.

In the underdeveloped world, iron and zinc deficiencies are prevalent. Inadequate dietary intake is a common cause of iron and zinc deficiency, but besides this, antinutrients present in plant-based foodstuffs play an imperative role in deficient minerals availability [4, 6]. The antinutritional factors in food are one of the leading causes of malnutrition and anaemia in growing children and women (pregnant and lactating) in developing countries [7]. To a limited extent, traditional procedures such as germination, fermentation, soaking, and combination approaches can only eliminate phytic acid and phytate levels [8].

In developing nations, sorghum is a typical staple crop. Whole grain sorghum is often consumed. While sorghum bran has the highest iron content, it also includes phytate, a powerful antinutrient for nonheme iron and zinc absorption [6, 9]. While fermentation is efficient in lowering phytate concentration, fermentation is not employed in many traditional sorghum

culinary applications [6, 9-11]. Sorghum food items with phytase added before or after processing have been demonstrated to have lower phytate content [5].

In the current study, *A. niger* NT7 phytase was efficiently utilized to hydrolyze insoluble phytate complexes (metal-phytate and protein-phytate) to accessible form along with the release of Pi. The present study successfully demonstrates the *in vitro* potential of phytase for hydrolysis of antinutrient of sorghum flour. Furthermore, immobilized phytase was also successfully used for the maize flour dephytinization to release the various nutritional components. The current study is the first report on ameliorating the nutritional value of sorghum and maize flour with fungal phytase.

6.2 MATERIAL AND METHODS

6.2.1 Application of purified phytase in insoluble complexes hydrolysis

6.2.1.1 Hydrolysis of prepared metal-phytate insoluble complexes

The 100 mM stock solutions of metal ions (Zn^{2+}, Fe^{2+}, Ca^{2+}, Co^{2+}, and Fe^{3+}) were generated by dissolving salts of $ZnCl_2.7H_2O$, $FeSO_4.7H_2O$, $CoCl_2.2H_2O$, $CaCl_2.2H_2O$, and $FeCl_3$ in distilled water. 10 mM working metal salt solutions and 10 mM sodium phytate were combined in equal quantities (1:1) and incubated overnight at 4 ºC for the formation of the insoluble metal-phytate complexes [12, 13]. The precipitated salts were centrifuged for 5 minutes at 8,000 rpm at 4 ºC. The precipitated salts were rinsed three times with distilled water before being suspended in 10 ml 100 mM acetate buffer , pH 5.0 [13]. The hydrolysis of the metal-phytate complex was performed at 37 and 50 ºC by incubating each metal-phytate salt with a 1U purified phytase enzyme for 24 h. The controls were set up without metal-phytate complex (enzyme control) and phytase (substrate control). At certain intervals (after every 6 h), aliquots of the mixture were collected and centrifuged to pelletize them, the released Pi in the supernatant was quantified by previously described method (section 3.2.24). The final Pi content was calculated by subtracting the control values from the test samples.

6.2.1.2 Hydrolysis of insoluble and turbid protein-phytate complexes

For making the phytate-protein complexes, lysozyme was used as a model protein. The 5 mM sodium phytate and (2.5 mM) lysozyme (2:1) in distilled water was mixed and incubated at 4 ºC to make the protein-phytate complex [14]. The formation of lysozyme-phytate complexes created a turbid solution. The insoluble lysozyme-phytate (LP) complexes were collected by centrifugation for 5 min at 8000 rpm (4 ºC). The collected insoluble complexes were suspended

in a 10 ml acetate buffer (100 mM; pH 5.0) and, then treated with 2 U phytase to hydrolyze the LP complex. Simultaneously, a control without phytase was also set up. Both test and control samples incubated at 37 ºC and 50 ºC. The aliquots of the mixture were withdrawn at regular time intervals to observe the reduction in turbidity by measuring the absorbance at 600 nm using a UV–Vis spectrophotometer. The samples were tested for Pi estimation as previously described (section 3.2.24).

6.2.2 Efficacy of purified phytase (Phy) for dephytinization of sorghum flour in simulated gastric and small intestine digestion conditions

The Sorghum flour was digested in a stepwise manner, firstly with HCl and pepsin and then with pancreatin and bile salts from the small intestine. For stomach phase digestion, 10 g of sorghum flour was added to 100 mL gastric phase fluid containing 8.0 g/L NaCl (Merck, Mumbai, India) and 1.2 g/L porcine pepsin (MP Biomedicals, California, USA). The pH of gastric phase digestion was maintained at 2.5 with concentrated HCl. The 50 U of purified phytase was added to the gastric phase digestion test sample (Figure 6.1). A control setup was run separately using identical processing conditions without adding phytase enzyme. The digestion was conducted for 2.5 h at 37°C with 120 rpm agitation.

For small intestine phase digestion, the stomach phase (50 ml) samples were diluted with 80 ml of 0.1 M phosphate buffer, pH 7.5 containing 0.175 g/L oxgall bile and 1.1 g/L porcine pancreatin, and digestion was carried out for 2.5 h at 37 °C under shaking conditions at 120 rpm (Figure 6.1). The digested samples were lyophilized and employed to further analyze nutrient (Pi, total protein, reducing sugar, and free amino acid) release [15, 16] by the previously described methods.

The amount of phytic acid content reduction was determined by a colorimetric assay using Wade reagent (0.03% $FeCl_3.H_2O$ + 0.3% sulfosalicylic acid) [17]. The amount of metal released by enzyme treatment was quantified by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) analysis. The percentage of metal release under control conditions (without any phytase treatment) was taken as 100% compared to dephytinization by phytase enzyme under simulated gastric and intestinal test phase digestion.

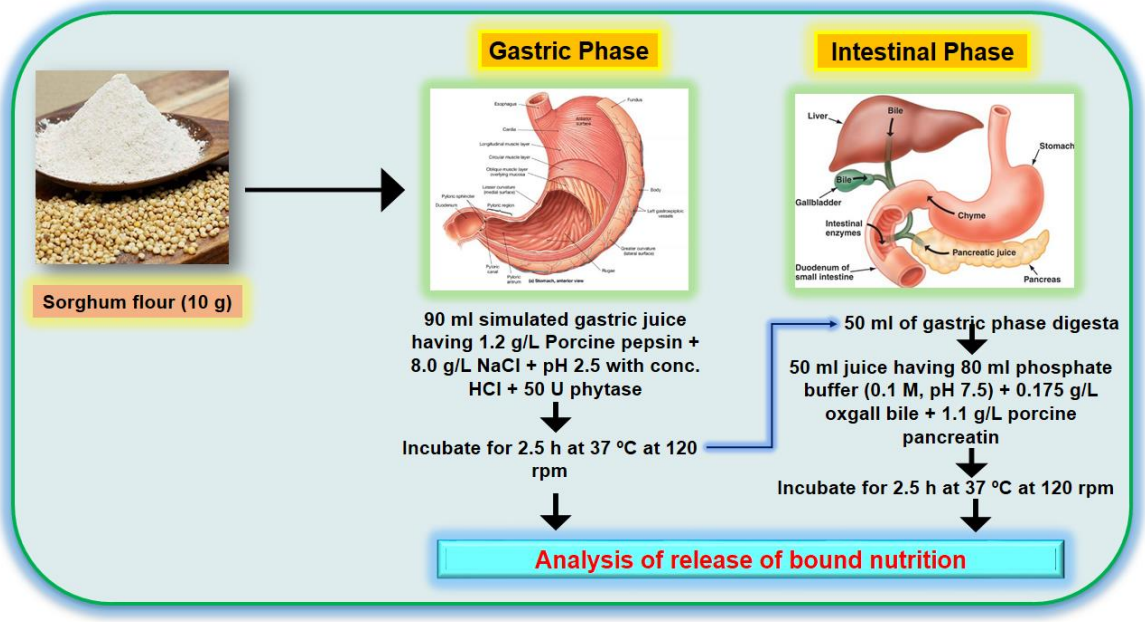


Figure 6.1 Pictorial representation of *in vitro* efficacy of *A. niger* NT7 phytase for dephytinization of sorghum flour.

6.2.3 Dephytinization of maize flour using Phy-ZnP

Maize flour is one of the major foodstuffs with high phytic acid content. However, heat treatment (roasting, sun-dried, boiling, cooked) can reduce phytic acid content but only to a smaller extent (≥50%). Complete hydrolysis is not achieved with these traditional approaches [18]. However, phytase treatment could achieve the highest hydrolysis of the phytic acid content of the foodstuff. 10% maize flour was treated with free soluble (Phy) and immobilized phytase (Phy-ZnP) (10 U/ g of flour) in 100 ml of 0.1 M sodium acetate buffer, pH 4.8 at 70 °C with constant shaking. Similarly, a control reaction was set up without any enzyme. The samples were taken at regular intervals to analyze nutritional release caused by dephytinization. The total proteins, Pi, reducing sugar, amino acid, and phytic acid contents were estimated by previously mentioned colorimetric methods.

The release of divalent metal ions by dephytinization was measured by ICP-MS. One gram of untreated maize bran was oven-dried and then treated under an acidic (HNO_3: $HClO_4$, i.e., 1:4) condition at 120 °C until a clear solution formed. The untreated maize flour has been taken as 100% for elucidating the % metal release by phytase treatment. Test samples were taken from the above-mentioned dephytinization setup for metal ions measurement after a 24 h period.

6.2.4 Statistical analysis

Data were analyzed by SPSS (Statistical Package for the Social Science) through Tueky's T-test (*Post-hoc*) (P-value <0.05) for the *in vitro* dephytinization study of sorghum flour. All tests were conducted in triplicate, and the average values and S.D are shown in graphs.

6.3 RESULTS AND DISCUSSION

6.3.1 Application of purified phytase for hydrolysis of insoluble complexes

6.3.1.1 Hydrolysis of insoluble metal-phytate complexes

Most of the metal-phytate complexes (except for magnesium and calcium phytate) are insoluble at the normal physiological pH of monogastric animals, making them more prone to malnutrition conditions imposed by mineral deficiency [4]. The current study showed that *A. niger* NT7 phytase could hydrolyze various insoluble phytates to varying degrees. The rate of hydrolysis of insoluble phytates was greater at 50 ºC than at 37 ºC, indicating that the enzyme was at its peak activity at optimal temperature. It released Pi in the following order from the respective metal (Zn^{2+} > Ca^{2+}> Co^{2+} > Fe^{2+} > Fe^{3+})-phytate complexes at 50 and 37 ºC after 24 h treatment (Figure 6.2a, b). As the optimal temperature of purified phytase is 50 ºC thus, the observed metal-phytate hydrolysis is much more efficient than the normal physiological temperature (37 ºC). The degradation of metal-phytate took place in a time-dependent manner.

Tang et al. found that fungal, bacterial, and wheat phytases hydrolyze insoluble metal phytate complexes at comparable but slower rates [19]. Phytase from *S. thermophile* was also reported to successfully hydrolyze several insoluble metal-phytates, with a greater hydrolysis rate at 60 ºC than at 26 ºC. It unconstrained Pi more efficiently from Ca^{2+}, Mg^{2+}, and Co^{2+}-phytate complexes than Al^{3+}, Fe^{2+}, Fe^{3+}, and Zn^{2+}-phytate complexes [20]. *A. oryzae* phytase released Pi more effectively from Ca^{2+}, Fe^{2+}, and Co^{2+} at 50 ºC than at 25 ºC [13]. At pH 2.5 and 5.0, the sodium phytate complex was entirely hydrolyzed in the presence of *Aspergillus* phytase (a 3-phytase enzyme) [21].

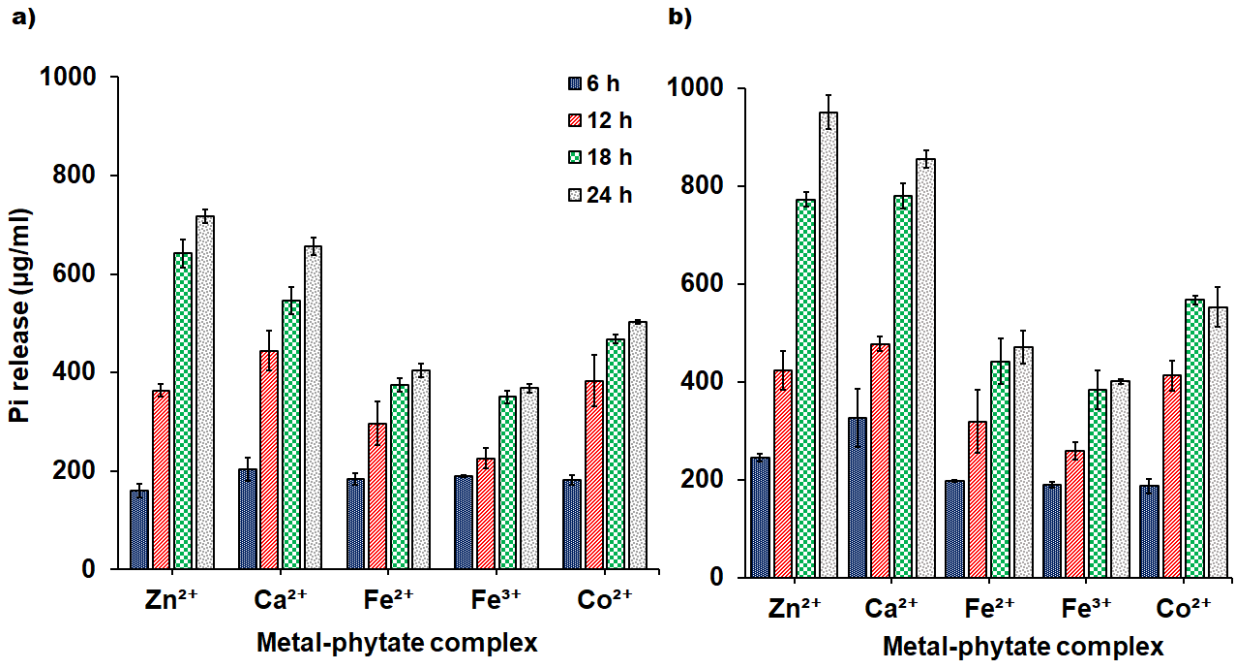


Figure 6.2 a) Hydrolysis of the metal-phytate complex at 37 ºC and **b)** at 50 ºC by purified phytase. All values are the mean of three replicates ± S.D.

6.3.1.2 Hydrolysis of a protein-phytate insoluble complex

In 2019, the marketplace for plant-based protein formulations was about $5.5 billion, with an expected increase to $8 billion by the end of 2025 [4]. However, most plant-derived proteins, such as those from soybeans, peanuts, and cottonseed, form complexes with phytic acid and make them inaccessible for absorption in the GI tract of monogastric animals. The protein-phytate complex is insoluble; therefore, it exhibits turbidity in a wide pH range, from 5.0-8.5. Due to the formation of an insoluble complex, the protein-phytate complex (lysozyme and phytic acid) is a turbid solution. The phytase activity hydrolyzed the protein–phytate complex, resulting in a drop in absorbance [14]. The absorbance of the complex at 600 nm shows a change from 1.13 to 0.362 at 37 ºC after 60 min treatment with the purified phytase (Figure 6.3a). At 50 ºC shift in absorbance from 1.13 to 0.342 was observed after 20 min treatment only (Figure 6.3b). The complete hydrolysis of the lysozyme-phytate complex was observed after 60 min at 50 ºC, relatively earlier than 37 ºC (140 min). Thus, the current study showed that more efficient hydrolysis was observed at optimal temperature (50 ºC) in a time-dependent manner. The release of Pi further supports this hydrolysis mode. With increasing reaction time, the quantity of released Pi rose linearly. Tran et al. also made similar observations. They showed the hydrolysis of the phytate complexes made with lysozyme, BSA and trypsin [14]. Under the

action of *A. oryzae* phytase, the lysozyme-phytate complex also undergoes time-dependent hydrolysis with the release of Pi [13].

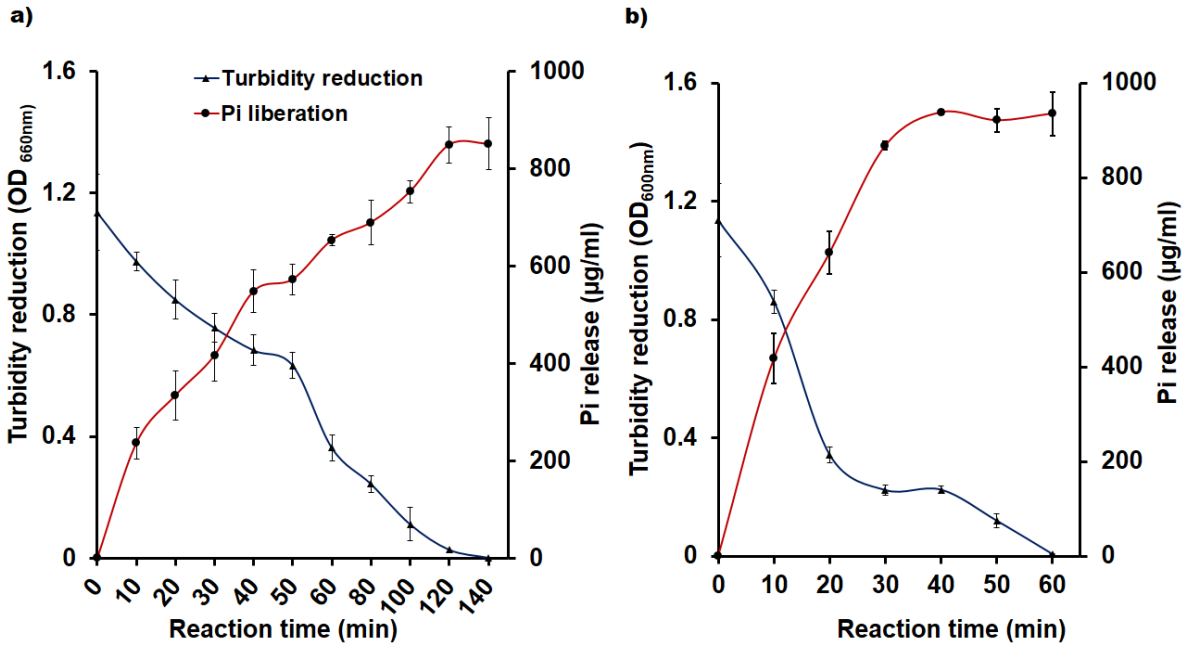


Figure 6.3 a) Hydrolysis of the protein-phytate complex at 37 ºC and **b)** at 50 ºC using purified phytase. All values are the mean of three replicates ± S.D.

6.3.2 Efficacy of purified phytase for sorghum flour dephytinization in simulated gastric and small intestine digestion conditions

The current study successfully showed that the phytase application releases more bound nutrients than without phytase supplementation. Phytase supplementation resulted in the liberation of Pi, proteins, reducing sugars, and free amino acids. Phytic acid content was decreased significantly in test samples of simulated gastric (1.94 ± 0.06 µg/ml) and intestinal phase (0.30 ± 0.04 µg/ml) as compared to their control counterparts. The phytase application resulted in efficient dephytinization of sorghum flour in the simulated gastric phase compared to the control one (without phytase). A prior study has also shown a significant reduction in phytate content by fermentation of sorghum flour by LAB bacteria [22]. Another study also showed that the additional phytase treatment reduces the phytic acid content of seabuckthorn seed protein from 22.46 to 13.27 g/kg, with higher protein solubility and in-*vitro* digestibility [23].

The observed Pi, reducing sugar, proteins, and free amino acid release after test gastric phase (TGP) treatment was 10463.2 ±13.694 µg/ml, 7926.707 ± 27.71 µg/ml, 1121.4 ± 38.33 µg/ml and 678.58 ± 4.53 µg/ml respectively (Table 6.1). The observed release of each nutrient in the

TGP was more than that of the control test phase. Furthermore, the conjugated nutrient release was also observed in the test intestinal phase (TIP) along with phytic acid reduction. After TIP treatment, the observed Pi, reducing sugar, proteins, and free amino acid residues released was 13406.80 ± 105.84 µg/ml, 13213 ± 37.21 µg/ml, 1677.15 ± 37.21 µg/ml, and 1557.50 ± 17.97 µg/ml, respectively. The release of bound nutrients was significantly higher in the TIP than in the TGP (Table 6.1), which could be linked to an elevation in pH (from 2.5 to 7.5), affecting the solubilization in the system. This differential solubilization is attributed to adding bile salt and intestinal juices during the intestinal stage [24, 25]. Similar to our finding, Schons et al. observed that phytase treatment enhances the release of inorganic phosphorous from sorghum flour [26]. The fermentation of sorghum flour also reduces the phytic acid content and improves the in vitro digestibility of protein [27]. A recent study has shown that *Cinnamomum camphora* seed kernel protein isolate (CPI) containing phytic acid was lowered with phytase treatment. The phytase treatment affected the structural and functional features of CPI as well as its in vitro digestibility. *In vitro* gastrointestinal digestion was improved by lowering the phytate content, and CPI bioavailability was dramatically increased [25].

Table 6.1 Nutritional release (Phosphorous, sugar, protein, amino acid release, and reduction in phytic acid) by dephytinization of sorghum flour in simulated gastric and intestinal phase. Here, small alphabetical letters (a-d) on superscripts across the column show the significant difference among them. The significance level test is measured by Tukey's T-test (*Post-hoc*) test.

Treatment phase		Pi release (µg/ml)	Total sugar (µg/ml)	Total protein (µg/ml)	Free amino acids (µg/ml)	Phytic acid (µg/ml)
Control gastric phase	CGP	6072±36.91[a]	4853.88±12.83[a]	648±37.09[a]	452.66±8.17[a]	2.73±0.05[a]
Test gastric phase	TGP	10463.2±13.694[b]	7926.707±27.71[b]	1121.4±38.33[b]	678.58±4.53[b]	1.94±.06[b]
Control intestinal phase	CIP	9587.6±39.50[c]	6657.173±94.23[b]	811.8±40.46[ac]	753±3.45[c]	0.52±.002[c]
Test intestinal phase	TIP	13406.8±105.84[d]	13213±60.40[c]	1677.15±37.21[d]	1557.5±17.97[d]	0.30±.04[d]

The minerals released from TGP are in the increasing order of Zn> Fe> Mn >Ca >Mg, and from TIP are in the order of Mn> Ca >Fe >Zn >Mg (Figure 6.4a, b). Zn and Fe metal ions release more in the gastric phase, whereas the Mn and Ca ions are liberated more in the intestinal phase. These differential mineral's release is attributed to the pH difference between the two phases, affecting the complexes solubility. The phytase treatment resulted in a remarkably high release of conjugated metal ions in the test sample of gastric and intestinal phases compared to their control counterparts. Earlier studies have shown that dephytinization of the seed coat of ragi (finger millet) enhances the Zn bioavailability. Phytase-active *Lactobacillus pentosus* CFR3 can dephytinize the seed coat of ragi, increasing Zn bioavailability and decreasing phytate content to 56.70, 66.65, and 87.85 %, respectively, from native (non-processed), malted, and hydrothermally processed finger millets after 24 h of treatment [16]. An earlier report also showed that when infant cereals were reconstituted with water, followed by dephytinization with exogenous phytase (*A. oryzae*) resulted in improved iron and zinc availability in a Caco-2 cell line [28].

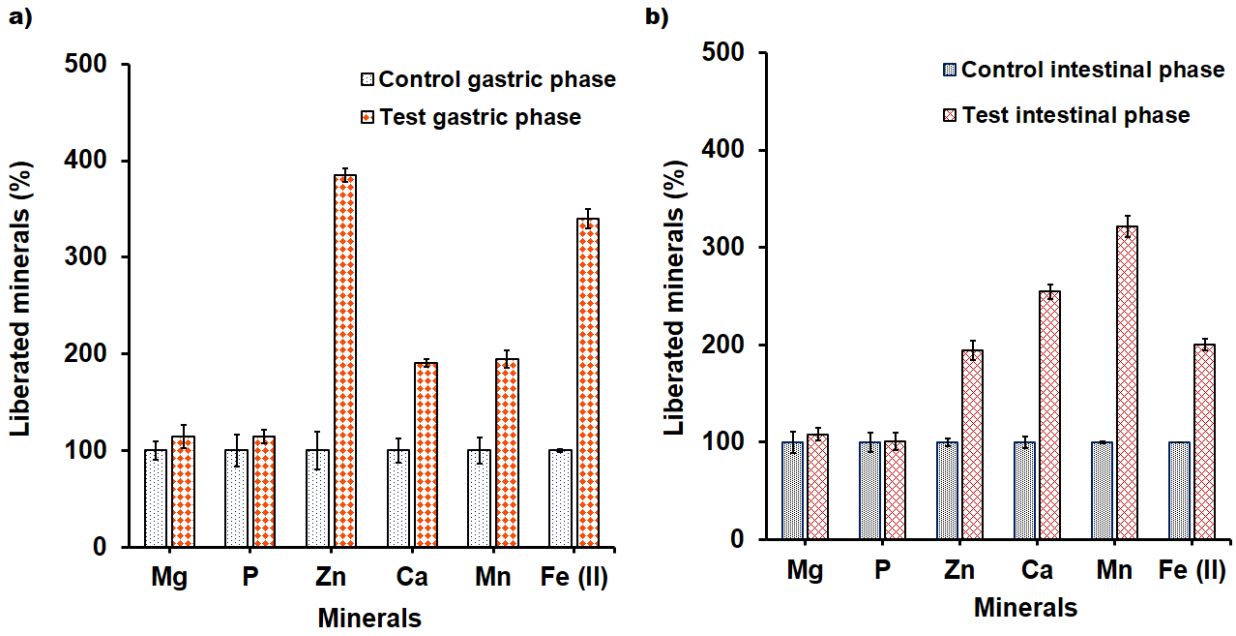


Figure 6.4 Analysis of minerals released after dephytinization of sorghum flour in **a)** simulated gastric and **b)** intestinal phase using ICP-MS [Here, control represents the experiments carried out without the addition of enzyme, whereas tests (gastric and intestinal phase) with 50 U of purified phytase enzyme. The control is taken as 100% for calculating the percentage of minerals released after phytase treatment]. All the experiments were carried out in triplicates.

6.3.3 Amelioration of maize flour nutrition by Phy and Phy-ZnP

Maize flour is one of the key staple food for human consumption. The phytate content in maize varied from 11.5-14.2 mg/g dry matter. The development of insoluble cation-phytate complexes at physiological pH is expected to be the leading cause of nutrient deficiencies [29]. Therefore to check whether the enzyme under study can efficiently hydrolyze the phytate of maize flour with the release of crucial nutrients, the maize flour was treated with Phy and Phy-ZnP enzymes. The results were very appreciating showing the Phy-ZnP releases the Pi more efficiently (6478 ± 326.83 µg/ml) from maize flour than the Phy (5478 ± 546.78 µg/ml) after 12 h of incubation. Beyond 12 h, both the enzymes have shown no further increase in the release of the Pi from the maize flour (Figure 6.5a). A prior study has also demonstrated the Pi release from soybean in a time-dependent manner and attain saturation after 50 min when treated with free and immobilized *A. niger* phytase on hydroxyapatite (HA) nanoparticles [30].

Similarly, the total soluble protein content was improved by Phy (3456 ± 234.32 µg/ml) up to 12 h of treatment at 70 ºC; afterwards, a gradual drop was observed owing to the instability of proteins due to high-temperature exposure. In contrast, Phy-ZnP can withstand high temperature-time regime treatment; thus, a gradual increase in protein concentration was observed up to 24 h (4234 ± 231.23 µg/ml) treatment (Figure 6.5b). Both Phy (765.56 ± 48.34 µg/ml) and Phy-ZnP (798.34 ± 51.2 µg/ml) resulted in the release of bounded free amino acids after 18 h of treatment which is closely comparable (Figure 6.5c). The 18 h treatment resulted in the maximum release of total reducing sugar for both Phy (9345 ± 347.76 µg/ml) and Phy-ZnP (9645 ± 283.56 µg/ml) (Figure 6.5d). The phytic acid content was reduced to 0.23 ± 0.03 µg/ml and 0.13 ± 0.02 µg/ml by Phy and Phy-ZnP, respectively, at the end of dephytinization (Figure 6.5e). This reduction is equivalent to 94.84% and 96.99% of the initial value of phytic acid content. Earlier studies have also shown the hydrolysis of phytic acid of whole maize grain with its intrinsic phytase activity [31].

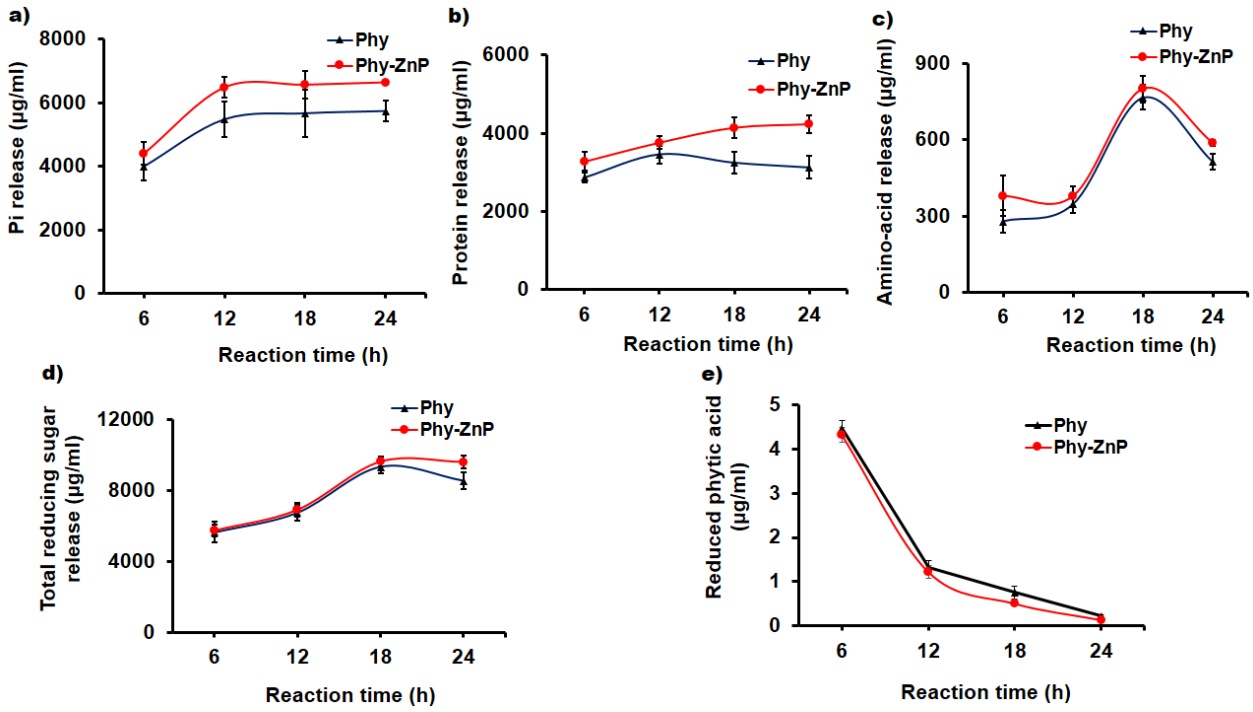


Figure 6.5 Dephytinization of maize flour resulting in the release of **a)** Phosphorous, **b)** Soluble proteins, **c)** Free amino acids, and **d)** Reducing sugar by free (Phy) and immobilized (Phy-ZnP) *A. niger* phytase. The reaction was performed at 70 ºC with pH 4.8.

Mineral deficiency nowadays is regarded as a major malnutrition concern around the world. It is much more common in regions where grain consumption is high. Although minerals present in the diet may be abundant, their bioavailability is crucial to their absorption. Minerals are known to be inhibited by phytic acid. Pregnant and lactating women, infants, and adolescents have higher mineral requirements than adults, putting them at danger of major nutritional deficiencies. If the food's minerals are bioavailable and efficiently absorbed by the digestive system, the mineral deficiency issues can be overcome greatly [32]. For this purpose, the current study analyzed the minerals release from the maize flour after the dephytinization by Phy and Phy-ZnP using ICP-MS.

The Phy treatment on maize flour released the metal ions in the following order of P< Mn <Ca <Zn Mg <Fe. In contrast, Phy-ZnP treatment resulted in metal ions release in increasing order of Mn <P <Mg <Fe <Zn <Ca (Figure 6.6). The Phy-ZnP significantly higher release the minerals than the Phy treatment. Phy-ZnP liberated three essential metal ions, Ca, Zn and Fe, more than twice than released by the Phy treatment.

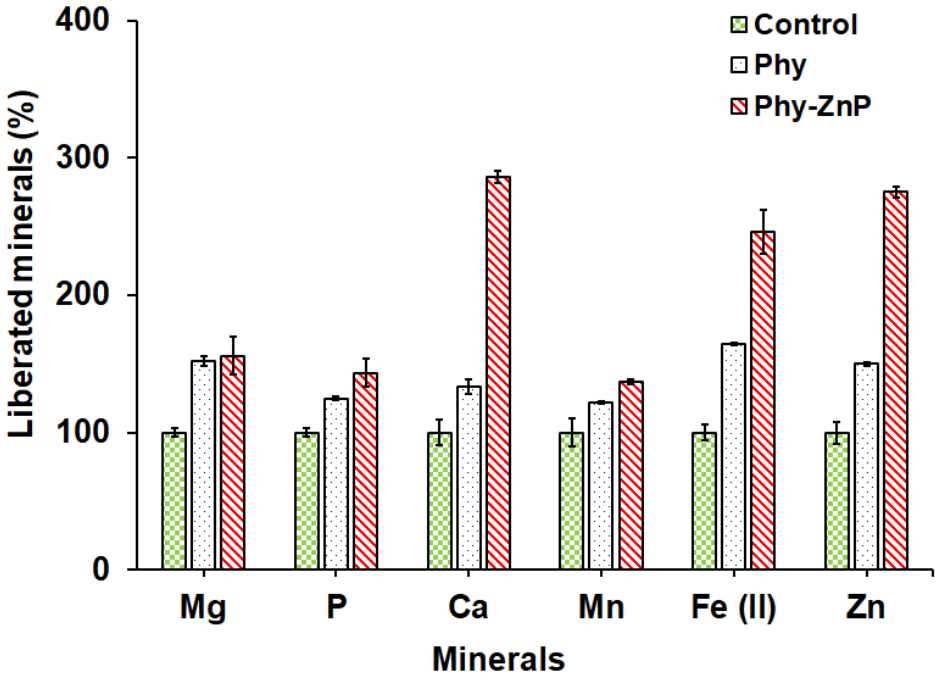


Figure 6.6 ICP-MS analysis for mineral released by maize flour dephytinization. The control (without enzyme) is regarded as 100% for calculating the percentage of minerals released after Phy and Phy-ZnP treatment. All the experiments were conducted in triplicate.

6.4 CONCLUSIONS

The current objective aims to establish the utilization of Phy and Phy-ZnP for food applications. For the same, two different flours, sorghum flour and maize flour, were used to analyze the applicability of the enzyme to reduce the phytate content and release various nutrients. The current study showed the free soluble purified phytase of *A. niger* NT7 (Phy) hydrolyzed the insoluble phytate complex [metal-phytate and protein [(lysozyme)-phytate complex] efficiently. The Phy is effectively functional at both physiological (37 °C) and a relatively higher temperature (50 °C). The phytase hydrolyzes and releases the bound nutrients from sorghum flour in batch *in vitro* conditions making the Phy more reliable for functional food formulations. The immobilized enzyme could dephytinize the maize flour and release the bound minerals with better operational capabilities than the free enzyme. Phytate level and phytic acid proportions in many cereal food products are likely to adversely affect the bioavailability of zinc, iron, and other essential mineral ions in the gastrointestinal tract. Dephytinization with phytase could lower phytate levels in cereal-based diets while improving overall micronutrient absorption in monogastric animals.

SUMMARY AND FUTURE SCOPE

SUMMARY

In the current study, the phytase enzyme from *Aspergillus niger* NT7 was used to improve the release of minerals from plant-based foods by reducing the amount of mineral chelating anti-nutritional phytic acid. In the current investigation, *A. niger* NT7 was chosen among various fungal strains isolated from soil samples taken from different agricultural fields of Himachal Pradesh, India. Based on morphological and molecular traits, the selected fungal isolate was identified as *A. niger* NT7, which grows fast on potato dextrose agar medium. Mature colonies have a pale yellow tint around them and have a powdery texture of brown or black spores. The isolate has a spherical, large, globose fruiting body with a brown to the dark black conidial cap under the microscope.

The foregoing are the study's key significant findings:

- *A. niger* NT7 was identified as the most potential candidate for phytase production in SSF.
- Wheat bran was found to be the most suitable agri-waste for phytase production in SSF.
- The parameters which were optimized during SSF by the OVAT approach include moistening agent (distilled water), inoculum age (3 days), inoculum level (12×10^7 spores/ml), incubation time (4 days), pH (5.0), temperature (30 °C) and different nutritional supplements such as nitrogen (ammonium sulphate), carbon (mannitol), phosphorous (potassium dihydrogen phosphate) and detergents (Tween 80) sources.
- The OVAT approach resulted in 2.7-fold enhanced phytase production (208.30 ± 0.22 U/gds) compared to unoptimized cultural conditions (76.34 ± 0.99 U/gds).
- The main parameters selected by the OVAT methodology were further utilized for statistical modeling. After five days of fermentation, at 35 °C, adding 5 g of wheat bran augmented with 2% carbon (mannitol), 0.5% nitrogen (ammonium sulphate) while maintaining medium pH 4.3 increased the phytase production (521 ± 28.16 U/gds).
- Statistical optimization using RSM (521 ± 28.16 U/gds) resulted in 2.5-fold enhanced phytase production compared to the OVAT approach.

- Crude phytase has shown catalytic activity at a wide acidic pH range (2-7) and temperature range (40-70 °C). Crude phytase has thermo-tolerant nature with a half-life of 273.15 min at 50 °C.
- The metal ions Co^{2+}, Zn^{2+}, and Ca^{2+} increase the enzyme activity and others such as Mg^{2+}, Fe^{3+}, Cu^{2+}, Fe^{2+} and Mn^{2+} hinder the enzyme activity in the crude phytase preparation.
- Crude phytase preparation dephytinizes the wheat bran successfully and releases a significant amount of proteins (419.07 ± 20.21 µg/ml) and Pi (2460.53 ± 102.11 µg/ml) after 72 h and reducing sugar (2019.45 ± 61.07 µg/ml) after 60 h.
- The action of crude phytase on maize bran results in the release of proteins (956.50 ± 4.30 µg/ml), reducing sugars (3210.9 ± 4.30 µg/ml), amino acids 250.87 ± 10.04 µg/ml) and Pi (1723.04 ± 45.32 µg/ml) significantly after 24 h. ICP-MS analysis showed that dephytinization of maize bran leads to minerals release in ascending order of Ca < Zn < Mn < Mg < P < Fe.
- Ammonium sulphate precipitation (0-85 %), followed by ion exchange with DEAE chromatography, were used to purify *A. niger* NT7 phytase to homogeneity.
- An 11.49 purification fold was achieved with 34.93 U/mg.
- The purified phytase has a monomer with a molecular mass of ~ 70 kDa.
- Purified phytase is most active at pH 5.0 and 50 °C with a half-life of 288.75 min.
- The observed values of V_{max} and K_M for purified phytase are 99 U/mg and 1.98 mM, respectively.
- Using a UV-Vis spectrometer a maximum absorption peak at 352 nm confirms the synthesis of ZnO nanoparticles (ZnP) by the chemical precipitation method.
- FE-SEM imaging showed that particles are spherical in shape and the average size varies from 40-45 nm.
- XRD analysis showed that ZnP are crystalline, and its average grain size is 14.28 nm.
- FTIR analysis confirms the immobilization of phytase on ZnO nanoparticles (Phy-ZnP).

- The immobilized phytase showed enhanced operational stability (optimal pH 4.0; temperature 70 ºC) and thermotolerance (optimally active at 70 ºC; $t_{1/2}$ 144.37 min) compared to the free enzyme ($t_{1/2}$ 52.90 min).

- The observed values of V_{max} and K_M for Phy-ZnP are 96.15 U/mg and 4.87 mM, respectively. The catalytic efficiencies (K_{cat}/K_M) for Phy and Phy-ZnP were 1.31 x 10^4 and 3.9 x 10^4 M^{-1} $sec^{-1,}$ respectively, which showed that Phy-ZnP has an improved catalytic efficiency than the Phy.

- Immobilized phytase can be reused up to the ten catalytic cycles (retaining 50% activity).

- Purified phytase hydrolyzes metal-phytate and protein-phytate complexes optimally in a time-dependent manner more efficiently at 50 ºC than at 37 ºC.

- Purified phytase effectively releases nutrients (Pi, proteins, sugar and amino acids) under simulated gastric and intestinal phase conditions.

- The observed Pi, reducing sugar, proteins, and free amino acid release after test gastric phase treatment were 10463.2 ± 13.694 µg/ml, 7926.707 ± 27.71 µg/ml, 1121.4 ± 38.33 µg/ml and 678.58 ± 4.53 µg/ml, respectively. After Test intestinal phase, the observed Pi, reducing sugar, proteins and free amino acid residues released were 13406.80 ± 105.84 µg/ml, 13213 ± 37.21 µg/ml, 1677.15 ± 37.21 µg/ml and 1557.50 ± 17.97 µg/ml, respectively.

- The digestibility of Zn and Fe metal ions is more in the gastric phase, whereas the Mn and Ca have more digestibility in the intestinal phase**.**

- Immobilized phytase dephytinizes the maize flour more efficiently than the free soluble enzyme and improves the release of bound nutrients. The phytic acid content was reduced to 0.23 ± 0.03 µg/ml and 0.13 ± 0.02 µg/ml by free and immobilized phytase, respectively.

In a nutshell, our phytase enzyme from *A. niger* NT7 shows almost all desired characteristics such as thermotolerant, active at acidic pH range and catalytically efficient in *in vitro* conditions; therefore, the current study proposes that this enzyme can be a promising candidate for the food and feed industries.

FUTURE SCOPE

- Because the enzyme is thermostable, acid stable, and protease-resistant, it can be used to reduce phytates in the food and feed sectors. However, the current study demonstrated improved phytase production through optimization studies. Further increase in enzyme titers can be achieved by cloning and over-expression of the phytase gene in an appropriate host.
- Production of phytase can be performed at a scaled-up level.
- Development of different feed and food formulations using *A. niger* NT7 phytase.
- *In vitro* stability, assessment and efficacy of phytase can be studied more elaborately in gut conditions to establish it as a promising feed/food additive. An improved version of the simulated digestive model could be opted in future (having an oral compartment and dialysis membrane to decipher the bioavailability of released nutrients).
- For clinical evaluation, studies can be validated in animal models (Chickens, pigs).
- Moreover, protein engineering can be used for further enhancement of the desirable properties of fungal phytase.

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CHAPTER 5

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CHAPTER 6

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ANNEXURES

ANNEXURE-A (CHAPTER 3)

A.1 *Aspergillus niger* isolate NT-7 identification by 18S rRNA sequencing (Genbank ID: MN337269)

aacaaaaaatgggtttgggaaaaaccttggggcccaacctcccatccgtgtctattgtaccctgttgcttcggcgggcccgccgcttgtc
ggccgccgggggggcgcctctgccccccgggcccgtgcccgccggagaccccaacacgaacactgtctgaaagcgtgcagtctg
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A.2 Standard curve and calculation for phytase assay

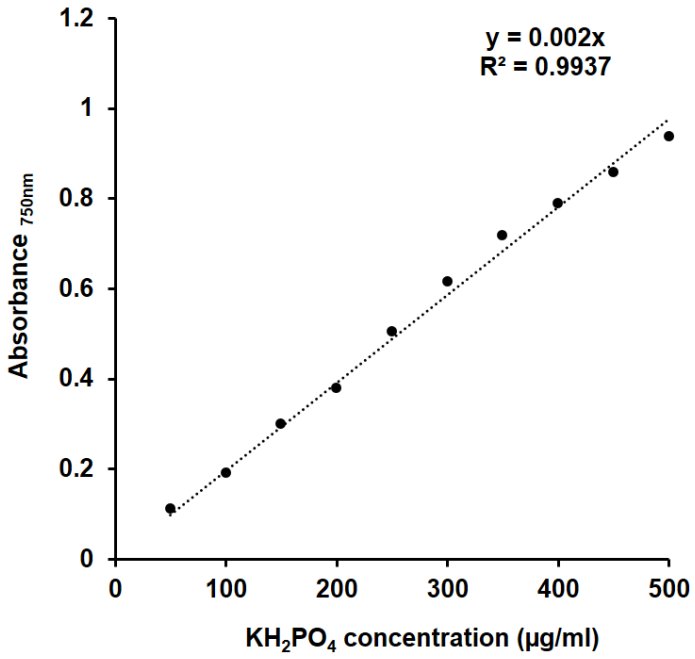

Figure 1. Standard curve of KH_2PO_4 (50-500 ug/ml).

The phytase activity was calculated from equation as follows:

$$\textbf{\textit{Enzyme activity}}(\boldsymbol{U/ml}) = \frac{(O.D_T\_O.D_c) * Value\ from\ graph * Reaction\ volume}{Molecular\ weight\ of\ product * Time\ (min) * Volume\ of\ enzyme(ml)} * 1000$$

A.3 Characterization of wheat bran

METHODOLOGY

WB quantification

Total Kjeldahl nitrogen (TKN), total carbon and Total solids (TS), and volatile solids (VS) of WB were measured using APHA (standard procedures for the assessment of water and wastewater American Public Health Association, Washington). The pH of the substrate was tested by mixing 1g of WB with 10 ml of distilled water and stirring it for one hour. The pH of the clear supernatant was measured after one hour.

Determination of total solids and volatile solids

1 g WB was placed in a pre-weighed crucible and oven-dried at 103°C for 1-2 hours to remove any remaining water. The dish was chilled and placed in a desiccator until a steady weight could be achieved for TS calculations. To compute VS, the TS were cooled, weighed, and dried in a furnace at 550 °C for 3 hours.

Determination of total carbon content

WB was digested in an acidic solution containing potassium dichromate, H_2SO_4 and phosphoric acid. Following digestion, sodium fluoride was added, followed by adding diphenyl indicator to calculate total carbon content.

Determination of total Kjeldahl nitrogen (TKN)

In the Kjeldahl flask, materials were digested with copper sulphate, potassium sulphate (1:5), as well with H_2SO_4 acid. Digestion took place in a fume hood at 100 °C for 2 hours, till it turns solution to colourless or light greenish. The titration of digested WB against 40 % sodium hydroxide was carried out using the Kjeldahl distillation equipment until the solution turned light brown. Distillate samples were titrated against 0.1 N HCl with 4% boric acid. Samples were titrated after adding methyl red until the solution turned from green to pale pink. The TKN was calculated using titrated values.

RESULTS

The WB has optimal level of C/N ratio which supports the phytase production as shown in table below.

Table 1. Characteristics of wheat bran.

Characteristics	Values
pH	6.24 ± 0.41
Total solids (mg/g)	243 ± 4.5
Total carbon (%)	43.78 ± 1.23
TKN (%)	3.276 ± 0.22
C/N ratio	13.36 ± 0.28
Volatile solids (mg/g)	191.33 ± 3.68

ANNEXURE-B (CHAPTER 4)

B.1 Bradford reagent composition and BSA standard plot

The working Bradford reagent was filtered through Whatman No. 1 filter paper and stored in a brown glass bottle at room temperature until further use.

Table 1. Reagents of Bradford for protein quantification.

S. No.	Ingredient	Composition
1	**Bradford stock solution**	
	Ethanol (95%)	100 ml
	Phosphoric acid (88%)	200 ml
	Coomassie blue G-250	350 mg
2	**Bradford working solution**	
	Distilled water	425 ml
	Ethanol (95%)	15 ml
	Phosphoric acid (88%)	30 ml
	Bradford stock solution	30 ml

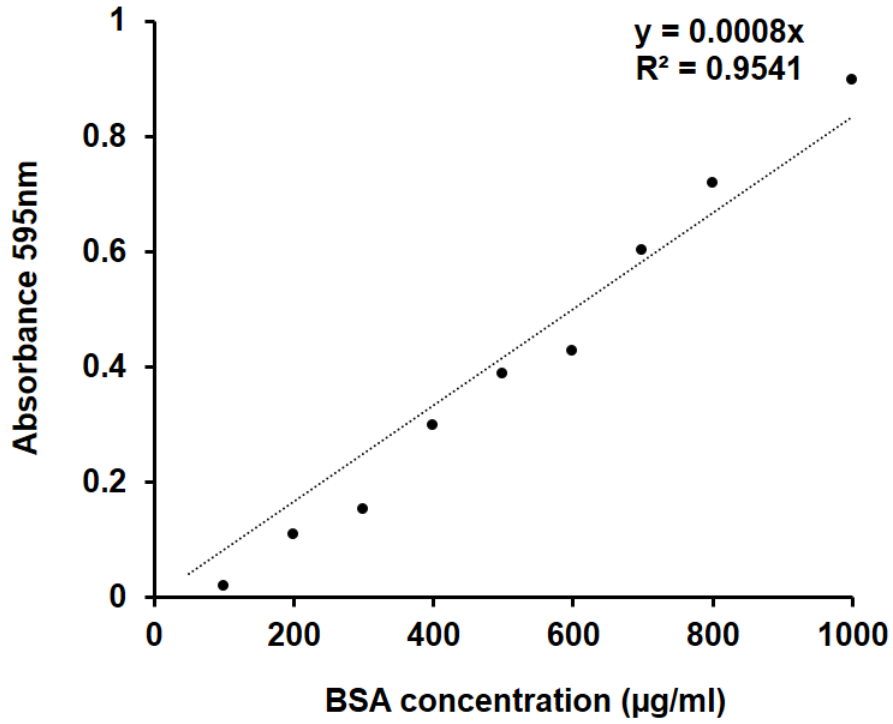

Figure 1. Standard of BSA (100-1000 µg/ml).

B.2 DNSA reagent composition and glucose standard plot

The prepared DNSA reagent stored in a brown glass bottle at room temperature until further use.

Table 2. Composition of DNSA reagent.

S. No.	Ingredient	Composition (g/100ml)
1	Dinitrosalicylic acid (DNSA)	1
2	NaOH	1
4	Sodium sulphite	0.05
5	Potassium-sodium tartarate	20

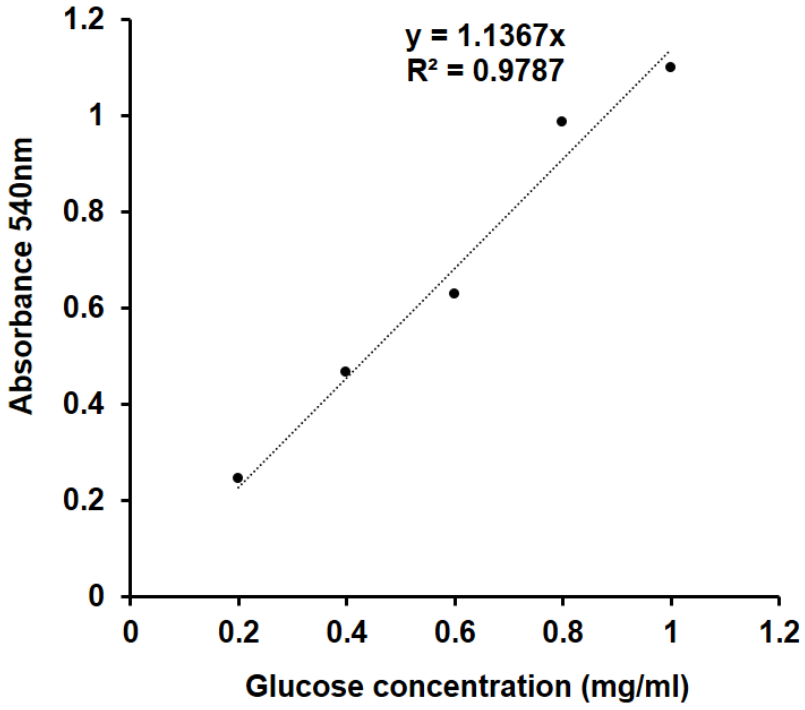

Figure 2. Standard of Glucose for DNSA assay (0.2-1 mg/ml).

ANNEXURE -C (CHAPTER 5)

C.1 Polyacrylamide Gel Electrophoresis (PAGE) analysis

Reagents of PAGE

Stock solutions for native-PAGE and SDS-PAGE

(i) Bis-acrylamide (0.8 g) and acrylamide (29.2 g) dissolved in 100 ml distilled water
(ii) 2 M (pH 8.8)Tris-HCl
(iii) 1 M (pH 6.8)Tris-HCl
(iv) 10% Sodium Dodecyl Sulphate (SDS)
(v) 10% Ammonium Persulphate (APS)
(vi) 50% Glycerol
(vii) 1% Bromophenol blue
(viii) β-Mercaptoethanol (BME)

Preparation of electrophoresis buffer

14.4 g glycine, 3.0 g Tris base, and 1.0 g SDS were dissolved in distilled water. The pH was adjusted to 8.3 before making the final volume 1 l for SDS-PAGE. However, SDS was omitted in the electrophoresis buffer for native-PAGE.

Preparation of Sample buffer (5x) for SDS and Native PAGE

10 ml of sample loading buffer for SDS-PAGE was prepared by mixing 0.6 ml of 1M Tris-HCl (pH 6.8), 5 ml of glycerol (50%, v/v), 2 ml of SDS (10%, w/v), 0.5 ml of BME, 1 ml of bromophenol blue and 0.9 ml of H_2O. However, SDS and BME were replaced by H_2O for the sample buffer of native-PAGE.

Preparation of SDS-PAGE and Native-PAGE gel

The electrophoresis was carried out using a Bio-Rad electrophoresis instrument. The separating gel mixture was placed between two glassware plates layered along with a 1.0 mm spacer to form one gel of 1.0 mm thickness for native and SDS-PAGE. After 30 min, the gel solution was overlaid with a top layer of distilled water and left to the polymerization reaction. The filter paper was used to absorb the aqueous layer on top, and then a stacking (5%) gel solution was poured over it. The comb was deliberately put to exclude air pockets and left for 1 h to polymerize. The formulation of the separating gel and stacking gel for native and SDS-PAGE analysis is shown in Table 1.

Table 1 The ingredient and composition of Native and SDS-PAGE.

	Native PAGE		SDS PAGE	
Ingredients	**Separating Gel**	**Stacking Gel**	**Separating Gel**	**Stacking Gel**
Percentage	8%	5%	12%	5%
Acrylamide/Bis-acrylamide solution	2.6 ml	0.67 ml	4 ml	0.67 ml
2M Tris-HCl	2.5 ml	-	2.5	-
1M Tris-HCl	-	1 ml	-	1 ml
10% APS	50 µl	30 µl	100 µl	50 µl
10% SDS	-	-	100 µl	50 µl
Distilled Water	4.8 ml	2.3 ml	3.3 ml	2.3 µl
TEMED	5 µl	5 µl	5 µl	5 µl

Electrophoresis for molecular weight determination

The samples for SDS-PAGE were prepared by adding the protein solution to a microcentrifuge tube with 5x sample loading dye in a 4:1 (v/v) ratio and heated at 100°C in a boiling water bath. The prepared samples were then loaded on an SDS-PAGE gel, and electrophoresis was performed at a constant voltage of 120 V till the dye front touched 0.5 cm above the bottom of the gel. In contrast, for native-PAGE, the samples were prepared by adding 5x sample loading buffer without SDS and BME without heat treatment. The samples were then loaded on a native-PAGE gel. The electrophoresis was run at a constant current of 40 mA while maintaining a low temperature to avoid any denaturing and hatting effect of protein.

Gel staining for visualization of bands

After electrophoresis, the gel was carefully transferred to a clean gel staining box containing a fixative (100 ml) and kept on a rocker at room temperature for 10-20 min. After removing the fixative, the gel was washed three times with a washing solution. The gel was then treated with a reducing agent for 1 min and washed thrice with deionized water. Then the gel was stained with silver stain for 20 min and then washed thrice with deionized water. The gel was then incubated with a developer until the bands became clearly visible. Finally, a stop solution was added to stop the further development of protein bands. The composition of solutions of silver staining is tabulated in Table 2.

Table 2 Reagents for silver staining of the gel.

S. No.	Ingredient	Composition
1	**Fixative solution**	50% water 40% Methanol 10% acetic acid
2	**Wash solution**	30% ethanol
3	**Reductant**	0.02% Sodium thiosulfate
4	**Silver stain**	0.2% Silver nitrate 20 µl Formaldehyde
5	**Developer**	3% Sodium carbonate 5 mg Sodium thiosulfate 50 µl Formaldehyde
6	**Stop solution**	5% glacial acetic acid

The protein molecular weight markers (Bio-Rad; cat number: 161-0374) used for molecular mass determination.

C.2 Elution of phytase

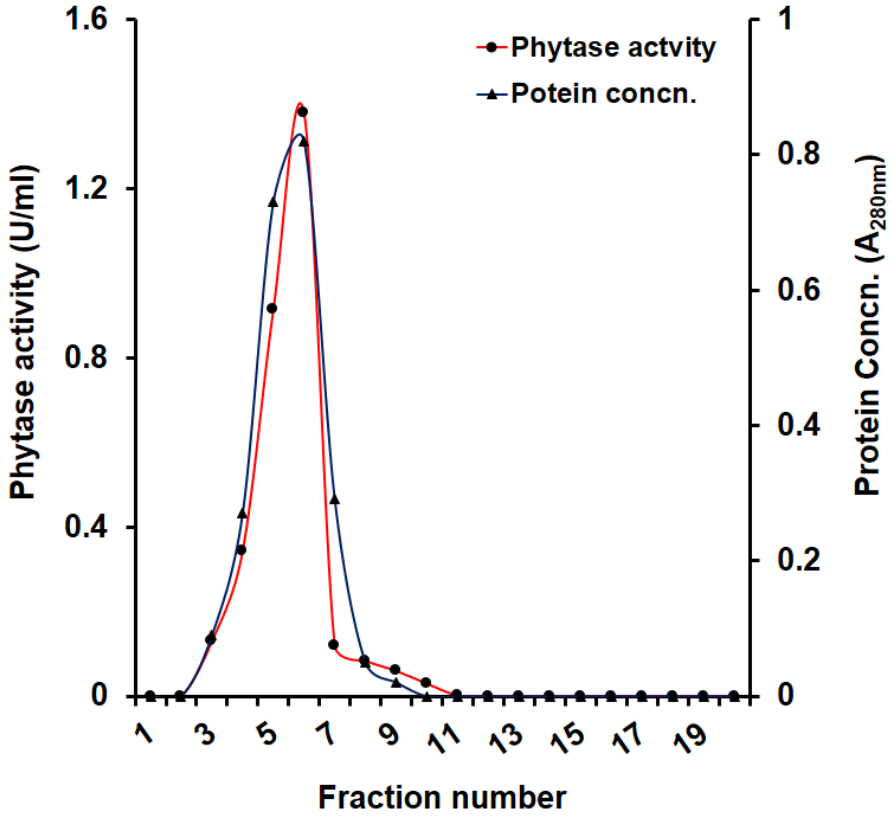

Figure 1. Elution of phytase of *A. niger* NT7 on ion exchange chromatography (DEAE-sepharose).

C.3 Lineweaver-Burk plot

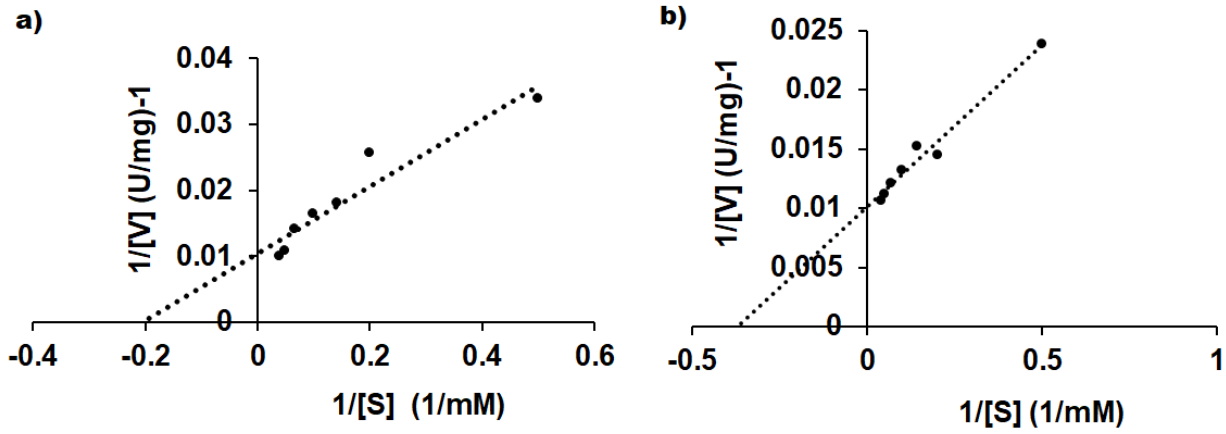

Figure 2. Kinetic parameters of **a)** Phy and**, b)** Phy-ZnP; Experimental conditions of Phy: Sodium phytate (2, 5, 7, 10, 15, 20 and 25 mM, at 50 ºC, pH 5.0 (0.1 M sodium acetate buffer) for 30 min); Experimental conditions Phy-ZnP: Sodium phytate (2, 5, 7, 10, 15, 20 and 25 mM, at 70 ºC, pH 4.0 (0.1 M sodium acetate buffer) for 30 min).

PUBLICATIONS AND CONFERENCES

JOURNAL PAPERS

1) **Neha Kumari** and Saurabh Bansal, "Production and characterization of a novel, thermotolerant fungal phytase from agro-industrial byproducts for cattle feed," *Biotechnology Letters*, vol. 43, no. 4, pp. 865–879, Apr. 2021, DOI: 10.1007/s10529-020-03069-8. (**IF: 2.46, Indexing: Scopus, SCI, SCIe)**
2) **Neha Kumari** and Saurabh Bansal, "Statistical modeling and optimization of microbial phytase production towards utilization as a feed supplement," *Biomass Conversion and Biorefinery*, Jun. 2021, DOI: 10.1007/s13399-021-01672-x. (**IF: 4.98, Indexing: Scopus, SCIe**)
3) **Neha Kumari** and Saurabh Bansal, "Purification of *Aspergillus niger* NT7 phytase and evaluating its dephytinization potential for sorghum flour in simulated gastric and intestinal phase digestion" (Ready to communicate)
4) **Neha Kumari** and Saurabh Bansal, "Immobilization of phytase from *A. niger* NT7 on ZnO nanoparticles and evaluation of its dephytase activity" (Under preparation)

CONFERENCE (PAPER PRESENTED)

- **Neha Kumari** and Saurabh Bansal, Optimization of Physio-chemical Factors Affecting Extraction Process for Full Recovery of Phytase from *A. niger* NT7 in Solid State Fermentation. Presented a **technical paper** in an International E-conference on frontiers in industrial biotechnology (AICTE Sponsored) 27-29 July 2020, St. Joseph College of Engineering.

- **Neha Kumari** and Saurabh Bansal, Optimization of Cultural Parameters and Extraction Process for Recovery of Phytase Enzyme from *A. niger* NT7 in Solid State Fermentation. Oral presentation at 2nd International Conference on "Application of Biotechnology in Industry and Society (ABIS-2019)" (TEQIP-III Sponsored) 14-16 November 2019, NIT Jalandhar.

- **Neha Kumari** and Saurabh Bansal, Optimization of Cultural Parameters by X6 Fungal isolate in Solid-State Fermentation by OVAT (One Variable at A Time) approach. Oral presentation at International Conference on recent trends in Biotechnology and Bioinformatics (ICBAB-2019), 01-03 August 2019, Jaypee University of Information Technology, Waknaghat, Solan, H.P.

- **Neha Kumari** and Saurabh Bansal, One Variable at A Time Approach to Enhance *Lactobacillus* L-Arginase Production. Oral presentation at Science Congress IIT, Mandi, 2018, H.P.

CONFERENCE ATTENDED

- **Neha Kumari** attended a virtual international conference on "Technologies for an Environmental Sustainability and Smart Agriculture" Organized by the Department of Biotechnology and Bioinformatics, JUIT, September 18-19, 2020.
- **Neha Kumari** attended a two-day online international conference on Covid-19: Challenges in Testing Prophylaxis & Management organized by ISF College of Pharmacy, Moga (SPER & ISPOR Sponsored) 22-23 May 2020.

REVIEW ARTICLES (PUBLISHED)

- **Neha Kumari** and Saurabh Bansal, "Arginine depriving enzymes: applications as emerging therapeutics in cancer treatment," *Cancer Chemotherapy and Pharmacology*, vol. 88, no. 4, pp. 565–594, Oct. 2021, DOI: 10.1007/s00280-021-04335-w. **(IF: 3.333, Indexing: Scopus, SCI, SCIe)**

BOOK CHAPTERS (SUBMITTED)

1) **Neha Kumari** and Saurabh Bansal, Book chapter entitled "Valorization of sugar industry waste for value-added products" in Waste Valorization for Value-added Products (Submitted to Bentham Science)
2) **Neha Kumari** and Saurabh Bansal, Book chapter entitled "Reduction of process induced Toxin by Green Technology" in Green Chemistry in Agriculture and Food Production (Submitted to Taylor and Francis, Boca Raton, FL, USA)

OTHER RESEARCH PUBLICATIONS

- Himanshi Gautam, **Neha Kumari** and Saurabh Bansal, "Enhancing the production of therapeutic enzyme arginase from *Lactobacillus acidophilus* using response surface methodology," *Brazilian Archives of Biology and Technology*, vol 16, pp. 65, oct 2022. **(IF: 0.7, Indexing: Scopus, SCI)**

- **Neha Kumari** and Saurabh Bansal, "Computational based analysis of phylogenetic, structural and functional aspects of *Aspergillus niger* phytase" (under preperation)
- **Neha Kumari** and Saurabh Bansal, "Phytase from novel *Bacillus spp*: Production optimization by using economic agri-waste wheat bran and partial purification for its utilization as a feed supplement" (Under preparation)