Biosynthesis and Partial Characterization of Phytase from Rhizopus, Penicillium and Trichoderma spp. for Industrial Applications

by Zahra Ali

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2020

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Thesis submitted to the National University of Sciences and Technology Islamabad in partial fulfillment of the partial requirement for degree of Master of Science in

Plant Biotechnology

Supervisor:
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2020


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Dedications

Dedicated To

My family, husband and my friends without them

my success would not be possible.

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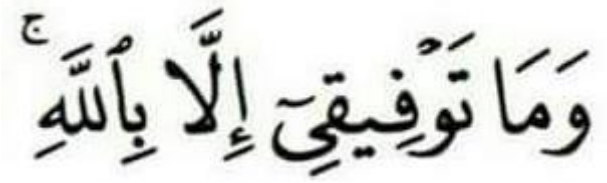
All praises & glory to **Almighty Allah (S.W.T)** the most beneficent and merciful. Because of His help and blessings that I was able to complete my research work.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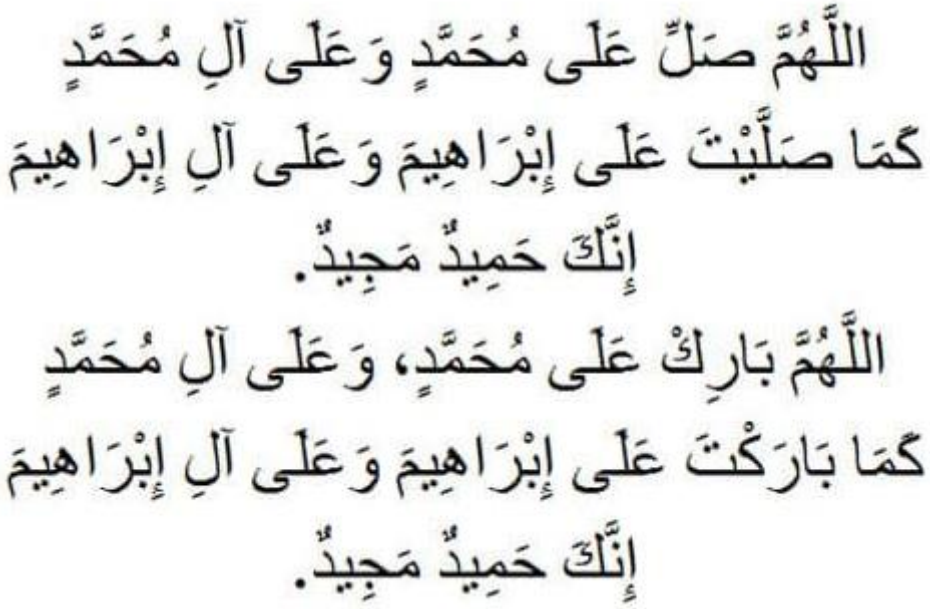
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List of Abbreviations

ITS	Internal Transcribed Spacer
BSA	Bovine serum Albumin
SSF	Solid State Fermentation
SmF	Submerged Fermentation
PCR	Polymerase Chain Reaction
TSCR	Tausky-Shorr Color Reagent
PDA	Potato Dextrose Agar Media
UV	Ulra Voilet
PSM	Phytase Screening Media
TCA	Trichloroacetic Acid
OD	Optical Density

Abstract

Phytases belongs to the family of phosphatase enzymes that are important from industrial perspective. Phytases are produced from a number of sources among which most important are the fungal sources. The present study includes the production of phytase from three fungal strains. The study was initiated by the sampling from various fields and fruit markets from different sites of Punjab and Islamabad and used for the isolation of phytase enzyme. From all samples three phytase producing strains were selected and identified morphologically as well as molecularly. There strains were identified as *Rhizopus oligosporous, Penicillium oxalicum* and *Trichoderma longibrachiatum*. All these strains were then subjected to submerged fermentation to analyze their phytase producing ability and different fermentation parameters such as age of spore, pH, temperature, incubation time, and carbon and nitrogen sources were optimized for each fungal isolate to obtain maximum enzyme production. Maximum enzyme production was observed at 3 days spore stage after 120 hours of incubation time, at 25^{o}C temperature, pH 6.0 with carbon source being glucose and peptone as nitrogen source when it was inoculated with *P. oxalicum. R. oligosporous* gives its high phytase yield by using 3 days spore after 96 hours of incubation time, at 30^{o}C temperature, pH 5.5 with fructose and ammonium sulphate as carbon and nitrogen sources respectively. *T. longibrachiatum* yields maximum phytase production using 4 days spore after 168 hours of incubation time, at 30^{o}C temperature, pH 5.5 with sucrose as carbon source and ammonium sulphate as nitrogen source. The yield of phytase from *Penicillium oxalicum* appears to be the important source for enhancing the sustainable and cost effective processes through which phytase can be used in poultry industry.

1. Introduction

Phytase or myoinositol hexakisphosphate hydrolase (EC 3.1.3.8) belongs to the group of phosphoric monoester hydrolases, and catalyzes the hydrolysis of myoinositol hexakisphosphate to inorganic monophosphate and lower phosphoric esters of myoinositol or, in some cases, to free inositol

Many researches on phytase have been performed for almost around 87 yrs. till the time of its discovery [29]. The first phytase was commercialized in 1993-1994 by Gist-Brocades. The first animal source of phytase was from blood and liver of veal in 1908 and first plant source was discovered from rice bran in 1907. Phytase as animal feed additive was first used in 1962 for enhancing the nutrition. The first commercial animal feed enhancer was commercialized by the name of Natuphos extracted from *A. Niger* in 1991 by BASF (Abelson, 1999).

1.1 Sources of phytase

Phytase has been reported from various sources such as plant sources, animal sources and microbial sources including fungal and bacterial sources both. Various plant sources include maize, rice, wheat, beans, rye, lettuce and many oil seeds. It has also been observed that phytase is responsible for phytin degradation in the germinating pollens. Phytase was detected in the blood of lower vertebrates such as birds, reptiles, fishes, and sea turtle. Because phytate acts as an antinutritional factor for animals, the presence of phytase in the gastrointestinal tract of various animals was investigated. The presence of phytase was also reported in the intestine of pig, sheep, and cow. (Morgan et al., 2017)

There are various bacterial sources of phytase as well. Most of the phytases produced by the bacteria are associated with the cell with the exception of a few such as *Lactobacillus amylovorus, Enterobacter* sp. And *Bacillus subtilis* (Goyal et al., 2012).

An important application of phytase is their use in animal feed as an additive. Microbial phytases when added in to animal feed enhances the availability of proteins and other nutrients to be absorbed into the animals and hence increasing the animal growth. As phytases helps to degrade the indigestible phosphorous into digestible phosphorous hence it helps to reduce the phosphorous pollution from the environment (Danial et al., 2011).

1.2 Fermentation techniques

There are many ways to extract phytase from various sources. The most common methods of the production of phytase are submerged, solid state and surface culture fermentation techniques. In submerged fermentation the essential nutrients are mixed into fermentation media and enzyme is produced submerged in the liquid medium whereas in solid state a solid substrate is used to extract the enzyme. Among all three the surface culture technique is the oldest one in which enzymes are released and collected from upper layer of the liquid. Various fermentation factors like temperature, pH and incubation period were controlled in the fermenter (Correa & Villena, 2010).

1.3 *Rhizopus* – a common bread mould

Rhizopus belongs to phylum zygomycota. They are also known as saprophytic fungi. The species of this genus are mostly found on mature fruits and vegetables and jellies, syrups, peanuts and tobacco. There are 8 known species of this genus. Until now many enzymes have been extracted from *Rhizopus* species so far. Most of the species of this genus are a potential source for lipase including *R.oryzae, R.arrhizus* and *R.oligosporous.* (Nahas E, 1988) Other enzymes extracted from genus *Rhizopus* include protease, xylanase and galacturonase and most importantly phytase. (Sabu AH et al., 2002)

1.4 *Penicillium* as an enzyme source

Penicillium belongs to the phylum Ascomycota. The members of this genus usually feeds on biodegradable substances and rotten citrus fruits. The members of this genus grows best in temperature ranging from 25-30°C. Around 300 species of genus *Penicillium* has been discovered and identified so far. *Penicillium* has been reported to be the source of various enzymes such as phytase, pectinase (Kohli P & Gupta R, 2015), lipase [10], amylase [15], xylanase [3] and β-Galactosidase [20].

1.5 *Trichoderma*- a biological control agent

Trichoderma belongs to the phylum Ascomycota and into fungal family of hyprocreaceae. The members of this genus are mostly and widely present in the soils (forest as well as agricultural soil) and on the bark of deteriorated wood. These are the plant symbionts which are a virulent. They are fast growers and usually grow in all temperatures. They are a potential source of lipase, cellulase and phytase [24].

1.6 Aims and outline of the research

This research is conducted for the production of phytase from local strains of *Rhizopus, Trichoderma* and *Penicillium* by submerged fermentation. The fermentation factors will be optimized like incubation time, temperature, pH and carbon and nitrogen sources for the maximum production of phytase and determination of enzyme activity by spectrophotometer.

1.7 Objectives of the research

The study involves the following main objectives:

1. Identification of *Rhizopus, Penicillium* and *Trichoderma* spp. from environment.
2. Production of phytase enzyme from *Rhizopus*, *Penicillium* and *Trichoderma* species.
3. Improving enzyme production by optimizing various conditions.

2. Literature Review

2.1 Overview of phytase enzyme

Applications of phytase have been reported which decrease feed cost, protect environment and making phytic acid more available to animal in feed. It also hydrolysis phytate into phosphoric acid and inositol. The *Trichoderma viride* strin was isolated through mutation. The optimized conditions were 96 hours at 30℃ with pH 6. for production of enzyme. The carbon sources were also optimized, and maximum activity was obtained with straw rice bran and moisture content 60%.

In 2000, [8], studied the benefits of phytase in various industries and most importantly the poultry industry. Recently the market of phytase is the topmost market of worth millions. He suggested that other sources of phytase proved to be very expensive sources of phytase. Usually the cost of the proteins is high so this study was conducted to produce phytase from plant and fungal sources to be used in animal feed. The plant and fungal phytases could lead to very low-cost effective sources of phytase for industrial use.

Researchers worked on the maize starch-based medium by 10-day fermentation by using *Aspergillus niger*. As a result, he found that at the temperature of 40°C and the pH 5.5, the action of the enzyme was calculated to be 1.075 phytase units per minute per ml of the crude culture filtrate. Furthermore, the old broiler chicken of 90 days which was fed with soybean and maize was taken for a feeding trial of 4 weeks. The result of this experiment gives a usual phosphorous diet added with phytase enzyme along with a low phosphorous diet plus phytase (1.075 PU/gm of substrate)[1].

It was found that phytases can be achieved from various sources such as plants, micro-organisms and animals. By his tests he clarified that although a few strains of microscopic organisms, yeasts and parasites have been utilized for creation under various conditions, yet

two strains of *Aspergillus* sp., *A. niger* and *A. ficuum* have most generally been utilized for its commercial creation. His examination assessed 19 strains of lactic acid creating microorganisms of the genera *Lactobacillus* and *Streptococcus* benifically for the generation of phytase. He created a few bacterial phytases by utilizing distinctive bacterial strains, for example, *E. coli, Lactobacillus amylovorus, B. subtilis, Klebsiella* sp. *and B. amyloliquefaciens* and so forth. Besides various they showed the compound movement in the fermentation medium. As the conclusion of his examination and test works, he presumed that, *Lactobacillus amylovorus* B4552 delivered the greatest measures of phytase, running from 125±146 units/ml in submerged fermentation utilizing glucose and inorganic phosphate. He also studied various yeast phytases and used several strains of yeast which includes *Rhodotorula gracilis, Schwanniomyces castellii, Hansenula polymorph, S. occidentalis and Arxula adeninivorans,* etc. Intelligently, he found that phytase production using yeast cultures has effectively been carried out in submerged fermentation systems. [22]

A researcher proposed his work on a thermophilic fungus, *Rhizomucor pusillus*. By using solid-state fermentation, he isolated it from composting soil and studied it for phytase production. The optimization of production of phytase enzyme by methodology of surface response based on a one-variable-at-a-time approach (OFAT), independent variables (pH of medium, age of culture and incubation period) was selected for optimization by (RSM) using a Box– Behnken design of experiments. Each variable was studied at three different levels (1, 0, +1). The levels for pH were 4.0, 6.0 and 8.0, ages of inocula were 0, 12 and 24 h and periods of incubation were 1, 2 and 3 days., He carried out phytase production using Solid state fermentation (SSF) in a 250 ml flask containing wheat bran and basal medium of the following composition: asparagine, corn steep liquor as nitrogen sources, KCl, $MgSO_4$, $FeSO_4$, $MnSO_4$, pH (variable) and inoculated with a 1 ml culture, age of culture (variable), and incubated for 1-3 days (variable) at 50°C. Besides, the incompletely cleansed phytase

was ideally dynamic at 70°C and pH 5.4, however the protein demonstrated 80% action over a wide pH run, 3.0-8.0. The phytase was found to have wide substrate specificity. The substance of the flask was gathered with acetate buffer (50 mM, pH 5.0) and centrifuged to expel the waste. The rough supernatant was utilized for testing the phytase activity. [6]

2.2 Industrial applications of phytases

A study explained how phytase cause the degradation of indigestible phosphorous by cleaving their orthophosphate groups from the core enzyme, which is most commonly found in the plants. He gave the classification of phytases on the basis of various factors such as pH and their catalytic activity i.e. β-propeller, purple acid or histidine acid phosphatases. Phytases can also be acid phytase and alkaline phytases. However, potential applications of phytases may extend to release dietary phytate-bound minerals for human nutrition and to develop special inositol phosphates for human health [8]

A group of researchers worked on the production of phytase through solid state fermentation by using various different substrates such as sesame oil cake, cottonseed oil cake, palm kernel cake and groundnut oil cake. He worked on three strains of fungus named *Rhizopus* spp which are *R. oligosporus* NRRL 5905, *R. oryzae* NRRL 1891 and *R. oryzae* NRRL 3562. The results showed that coconut oil cake was the substrate that gives the maximum phytase yield with all the three strains. Palm kernel cake and groundnut oil cake was found to give maximum phytase yield with *R. oryzae* NRRL 1891 whereas olive oil cake and cottonseed oil cake gives poor results with this strain [25].

2.3 Sources of phytase

Study on one of the most thermo stable and salt tolerant phytase was conducted in which the researcher produced phytase using *Bacillus amyloliquefaciens* US573. The soil from this bacteria was isolated belong to Southern Tunisia and this bacteria has capability to produce

extracellular phytase. The phytase produced has various unique features such as it was bile stable and calcium dependent. The phytase produced from *B. amyloliquefaciens* US573 was named as PHY US573. The optimum conditions for the stability was found at pH 7.5 and temperature at 70°C. The thermo stability of enzymes showed that even after heating it to 100 and 90°C for around 10 mins the enzyme can resume its activity to up to 50-62%. Also this phytase can withstand a wide range of pH from 4-10 [14]

Some worked on the production of phytase by a mold called as *Sporotrichum thermophile* through submerged fermentation technique. The factors affecting the enzyme production were optimized to enhance the yield of enzyme. Peptone, sodium phytate, starch and Tween-80 were found to be the vital factors that affect the production of phytase enzyme by Plackett–Burman design. The phytase production was increased by 3.73-folds when starch (0.4%), Tween-80 (1.0%), peptone (0.3%) and sodium phytate (0.3%) was added in to the media [28].

2.4 Fungal phytases

A group of researchers performed their study on the phytase production in solid-state fermentation of *Rhizopus oligosporus*. He clarified the advantages and the use of phytase in the industry of animal feed to enhance performance of animals as there is a considerable and developing enthusiasm among swine and poultry makers in the use of phytase to enhance the nourishing content in the feed of animals. He showed benefits which included enhanced feed yield proportions with the decrease in the environmental expenses related with the disposal of waste of animals, He, subsequent to working with strong state aging utilizing coconut oil cake as substrate detailed the creation of extracellular phytase by *R. oligosporus.* Maximum production of phytase enzyme (14.29 U/g of dry substrate) happened at pH 5.3, 30°C, and 54.5% dampness content after incubation of 96 h. Also, the factor that stops the production of

enzyme was the consequence of expansion of additional supplements to the substrate. He additionally took a shot at optimizing of different physicochemical parameters required for greater production of phytase by *R. oligosporus* under strong state fermentation. The various process parameters optimized for increased production of phytase includes time of incubation (24–168 h), temperature of incubation (25-60°C), the mineral salt solution pH (3.0–8.0), initial level of moisture (37.5-70.5%), size of inoculum (0.5 × 106 to 5 × 106 spores), extra sources of carbon (sucrose, mannitol, lactose, glucose, sorbitol and maltose at 1% [w/v]), extra organic and inorganic sources of nitrogen (ammonium sulfate, peptone, malt extract, potassium nitrate, yeast extract, ammonium chloride, beef extract and sodium nitrate at 1% [w/v]), and impact of the addition of phytic acid (0.1–0.5% [w/v]) [7]

Production of phytase enzyme by *Aspergillus niger* NCIM 563. This phytase was produced extracellular by submerged fermentation technique. The fermentation conditions were kept at 30 °C with two carbon sources that is glucose and dextrin along with one nitrogen source. The nitrogen source used was sodium nitrate. The results showed that by using dextrin as the carbon source maximum activity of phytase was observed (41.47 IU/mL at pH 2.5 and 10.71 IU/mL at pH 4.0). Soni found that it was not only dextrin that gives the maximum production of phytase but the combination of dextrin along with glucose and sodium nitrate gives the best phytase yield. Almost an increase of 13 folds in the phytase activity was seen when KH_2PO_4 (0.004 g/100 mL) form of phosphorous was added in to the fermentation media [29]

Production of phytase from *Klebsiella* sp. that were collected from soils of the poultry fields. Extracellular phytase was produced using submerged fermentation technique by bacteria *Klebsiella* sp. The distinctive factor of the research is that he used orange peel as the substrate. Orange peel mixed with wheat bran was found to show the maximum yield of the phytase. This media of fermentation was also supplemented with other nutrients such as

sucrose, cell suspension and age old spores was calculated. It was found that 24hrs spore age with incubation temperature of 50°C and incubation time of 72hrs give maximum yield of phytase [18].

Chandra and Tewari (2013) studied that the phytases are myo-inositol 1,2,3,4,5,6-hexakisphosphate phosphohydrolases which belong to the family of histidine acid phosphatase. In the light of their examination they proposed answer for the issue of phosphorus insufficiency in animals and plants feed together with its contamination in regions of serious domesticated animal's generation. They found that phytase appears to be bound to end up progressively critical. Henceforth, for both ecological and monetary concerns, phytases and phytase-creating organisms are drawing in noteworthy mechanical intrigue. Moreover, they depicted different uses of phytases in industries.

Awad *et al.* (2014) worked on the production of phytase by fungus named *Penicillium purpurogenum* GE1 that was obtained from the root nodules of beans. He produced phytase using solid state fermentation and a mixture of substrate including corn bran and cob of corn. The conditions of the solid state fermentation media was optimized using OFAT technique. The optimization revealed that highest phytase yield from *P. purpurogenum* was obtained at pH 8, at 27 °C temperature and moisture cintent of 66%. Peptone and glucose were found to be optimized and yielding highest phytase production as (92 ± 5.6 U/g ds, 125 ± 4.9 U/g ds). He also used surfactant in the fermentation media which was optimized to be Tween 80. Basically his research yields that corn bran and cob can be used as a cheap medium for the enhanced production of phytase.

Harcharan *et al.* (2017) chipped away at the characterization and production of an impartial phytase of *Penicillium oxalicum* EUFR-3 confined from Himalayan area. Micronutrient bioavailability to monogastric animals from oats is poor because of the chelating effect of

phytase present in the seeds and its exogenous utilization of phytases is required to defeat anti nutritional impact of phytate. In this research, 40 isolates of fungus including *Talaromyces, Aspergillus, Trichoderma, Toliposporium, Penicillium and Cladosporium* and so on from various environments were screened positive for phytase action on particular media. It was found from the study that while producing phytase from solid state fermentation *Penicillium oxalicum* strain EUFR-3 was found to be the highest yielding strain having phytase activity of 12.8 U/g. The *P. oxalicum* EUFR-3 phytase (PhyP-EUFR3) had most extreme activity at pH 7.0 and temperature 40 °C. It was steady in pH scope of 3-8 with more than 60 % action all through examination.

3. Material & Methodology

3.1 Media preparation

3.1.1 Potato Dextrose Agar Medium (500 ml)

To prepare 500ml PDA, 19.5g PDA was dissolved in 500ml distilled water and then for 15 minutes it was autoclaves at 121psi. The media was then poured in sterilized petri plates, incubated overnight at 37^0C and thus ready for fungal colony growth.

3.1.2 Potato Dextrose Infusion Agar

It was prepared by dissolving potato infusion (20g), dextrose (2g) and agar (1.5g) in 20 milliliter of d.H_2O, homogenized it by heating and then the volume was upraised to 100 milliliter. Sterilized the media in autoclave (model: WAC-60, Wisd, WiseStri, Germany) at 121°C, 15 psi for 15-20 minutes.

3.1.3 Phytase Screening Medium

Phytate specific medium was prepared by adding glucose (1.5g), ammonium sulfate (0.5g), KCl (0.05g), Magnesium sulphate hepta hydrate (0.01g), NaCl (0.02g), $CaCl_2$(0.02g), Ferrous sulphate hepta hydrate (0.001g), manganese sulfate (0.001g), sodium phytate (0.25g) and agar (1.8g) in 20 mL distilled water. Then media was dissolved by heating and d.H_2O was used to increase the volume to 100 milliliters. It was sterilized at 15 psi, 121 degree Celsius for 15-20 min and then medium was poured into sterilized petri plates aseptically. [13]

3.1.4 Fermentation Medium

To prepare fermentation media, the following chemicals were used: glucose (10g), ammonium sulphate (3g), KCl (0.5g), MgSO4 (0.5g), CaCl2 (0.1g), KH2PO4 (3g) was formulated by dissolving components in 250 milliliter d.H2O, then volume was levelled with

distilled water to one liter. Media was then autoclaved at 121 degree Celsius for 15-20 minutes

3.2 Reagents Preparation

3.2.1 Sevag Solution

The Sevag solution used in the extraction of DNA to remove the proteins was prepared by adding Chloroform and Isoamyl alcohol in the ratio 24:1.

3.2.2 3M Sodium Acetate Solution (1 Litre)

Sodium acetate solution was prepared by adding sodium acetate (408.3g) and sterile water (800ml). Both the components are dissolved, and the pH is then adjusted to 5.2 with glacial acetic acid or to 7.0 with dilute acetic acid. The final volume of the solution is raised to 1 liter with sterile water and dispensed into aliquots and sterilized by autoclaving.

3.2.3 TAE Buffer Solution (50x)

The TAE buffer solution was prepared by adding Tris base (60.5g), Glacial acetic acid (14.3ml) and 0.5 M EDTA (25ml). All of the above ingredients are mixed, and the final volume is raised up to 250ml.

3.2.4 TAE Solution (1x)

The TAE buffer (1x) for running the gel electrophoresis was prepared by adding 50x TAE buffer (10ml) and sterile water (490ml). Both the ingredients are mixed well and kept at room temperature.

3.2.5 0.1M $MgSO_4.7H_2O$

The magnesium sulphate (0.1 Molar) solution was formed by mixing 2.46g of salt in 100 milliliters of $d.H_2O$.

3.2.6 Substrate Solution

Sodium phytate substrate solution was formed by mixing 0.5g of sodium phytate in 25 milliliter of sodium acetate (0.2 M) buffer.

3.2.7 10% Trichloroacetic acid (TCA)

Trichloroacetic acid which is the stopping reagent was formulated by dissolving 5g of TCA in 50 milliliters of d.H_2O.

3.2.8 10% Ammonium molybedate

Prepare 10% ammonium molybedate by adding 10 g of ammonium molybedate in 10 N sulfuric acid (27.17 mL of sulfuric acid in 72.83 mL distilled water).

3.2.9 Tausky-Shorr Color Reagent Solution (TSCR)

The color reagent was formulated by mixing 5g of ferrous sulphate ($FeSO_4.7H_2O$) in 90 milliliters d.H_2O and after that add 10mL of 10% ammonium molybedate.

3.2.10 Bradford reagent

Protein estimation was done through bradford reagent. It was formulated by mixing 0.1g comassie brilliant blue G-250, 100mL phosphoric acid (85%) and 50 milliliter of absolute ethanol and the volume was raised to 1000ml with d.H_2O. The reagent was kept safe from light to prevent photo-oxidation.

3.3 Buffers

3.3.1 Extraction Buffer Solution (200 ml)

Extraction buffer solution (200ml) was prepared by adding 0.5M EDTA having pH 8, 8ml 1M Tris-HCl having pH 7.5, 4 ml 10% SDS, 20ml 2M sodium chloride and 100ml sterile water. From 100 ml water 68ml was mixed above well to prepare final buffer solution (200ml; 20mM EDTA, 20mM Tris-HCl (pH 7.5) 1% NaCl and 1% SDS) in a bottle of volume 250ml.

3.3.2 Sodium Acetate Buffer (0.2 M)

Sodium acetate with 0.2 molarity was formed by dissolving 1.64g sodium acetate in low volume of d.H_2O which was then raised up to 100 milliliters. Acetic acid (0.2M) was formulated by mixing 1.14 milliliter of 100% acetic acid (17.4 M) in 100 milliliters of d.H_2O. Sodium Acetate (36.2ml) was mixed with acetic acid (14.8ml) to make 100 ml buffer having pH 5.5. The buffers of pH 4.5-6 used for characterization of enzyme was then prepared by maintaining the pH of sodium acetate with acetic acid.

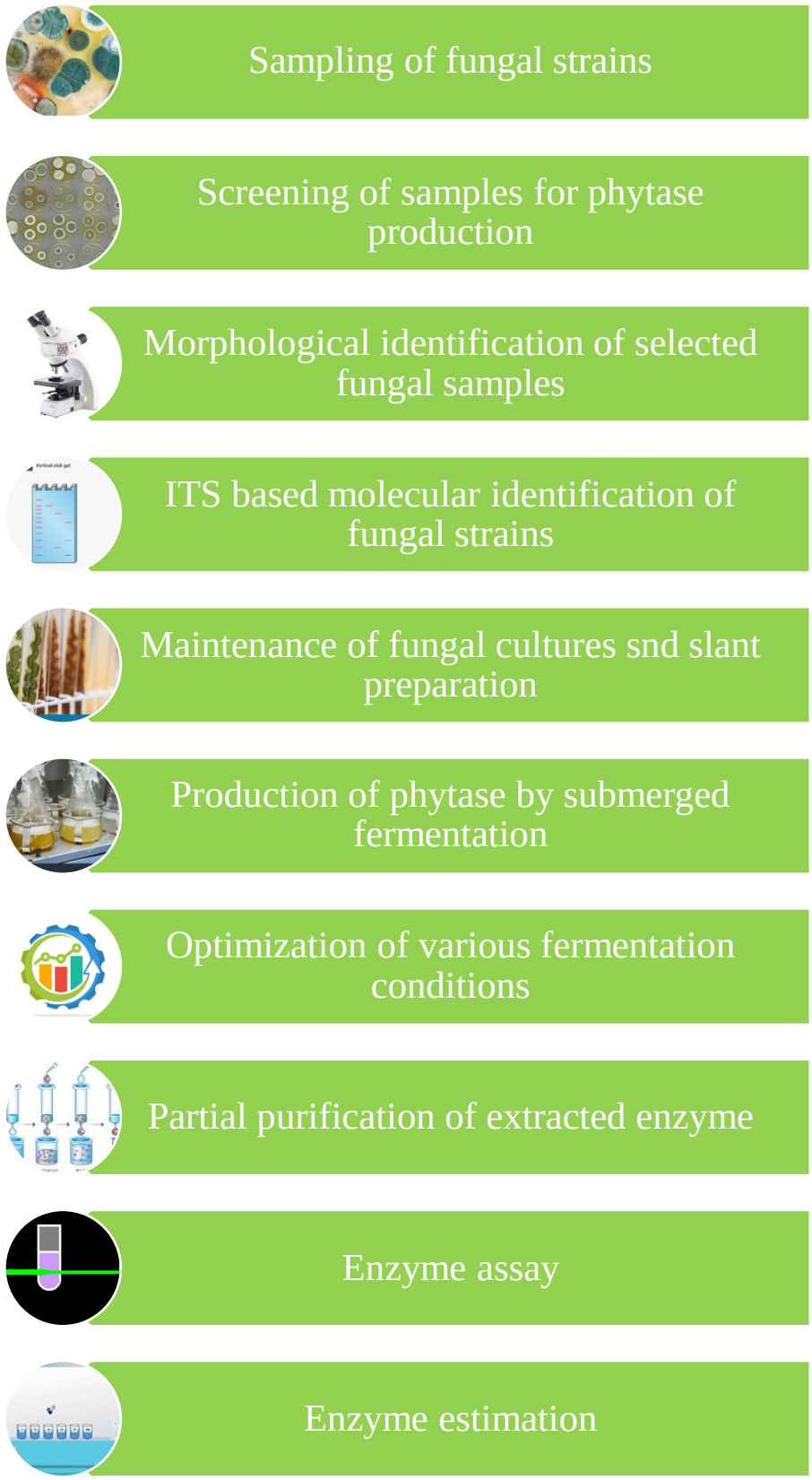


Figure 3.1: A flow sheet diagram representing the step wise scheme of research to produce phytase enzyme

3.4.1 Basic Lab Practices

Before starting any work in the lab basic lab practices should be followed to prevent any loss or damage and for personal safety. First of all, the protective lab coat and the gloves are put on before handling any chemical. The workstation is always sterilized with ethanol for the clean and safe environment. Solutions are always prepared in the safety cabinets and fume hoods to avoid contamination and the hazardous fumes. At the end of each experiment the proper disposal of waste material is ensured to avoid any harm. The lab equipment should not be taken out of the lab to avoid any unfavorable contact of harmful chemicals with the environment. Any sort of food and drink should not be taken into the lab. The workspace should always be labelled to avoid any mishap.

3.4.2 Sample collection

The phytase producing fungus were selected and for their isolation different samples of fruits and soil were collected from different areas by digging the soil up to 2-3cm with the help of spatula and added in the sterilized polythene bag and collecting the rotten fruits from different fruit markets. The areas are selected on the basis of their climate and soil differences. At each area the samples were collected from various places to ensure maximum sampling. The samples collected from different habitats as given in Table 3.1.

Sample no.	Area	Location
1	Margalla trail 5	Islamabad
2	Margalla trail 3	
3	Margalla trail 6	

4	Nust hiking trail	
5	Sunday fruit market h-9	
6	Fruit mandi I-9	
7	Fruit market F-10	
8	Shakarparian	
9	Jallo park	Lahore
10	Greater Iqbal park	
11	Safari park	
12	Nishaat fruit market	
13	Hyperstar fruit section	
14	Wapda town fruit mandi	
15	Local market	D.G Khan
16	Fields	
17	Fields	
18	Local market	
19	Fruit mandi	
20	Lab isolates	NUST, Islamabad

Table 3.1 Sampling areas for fungal screening

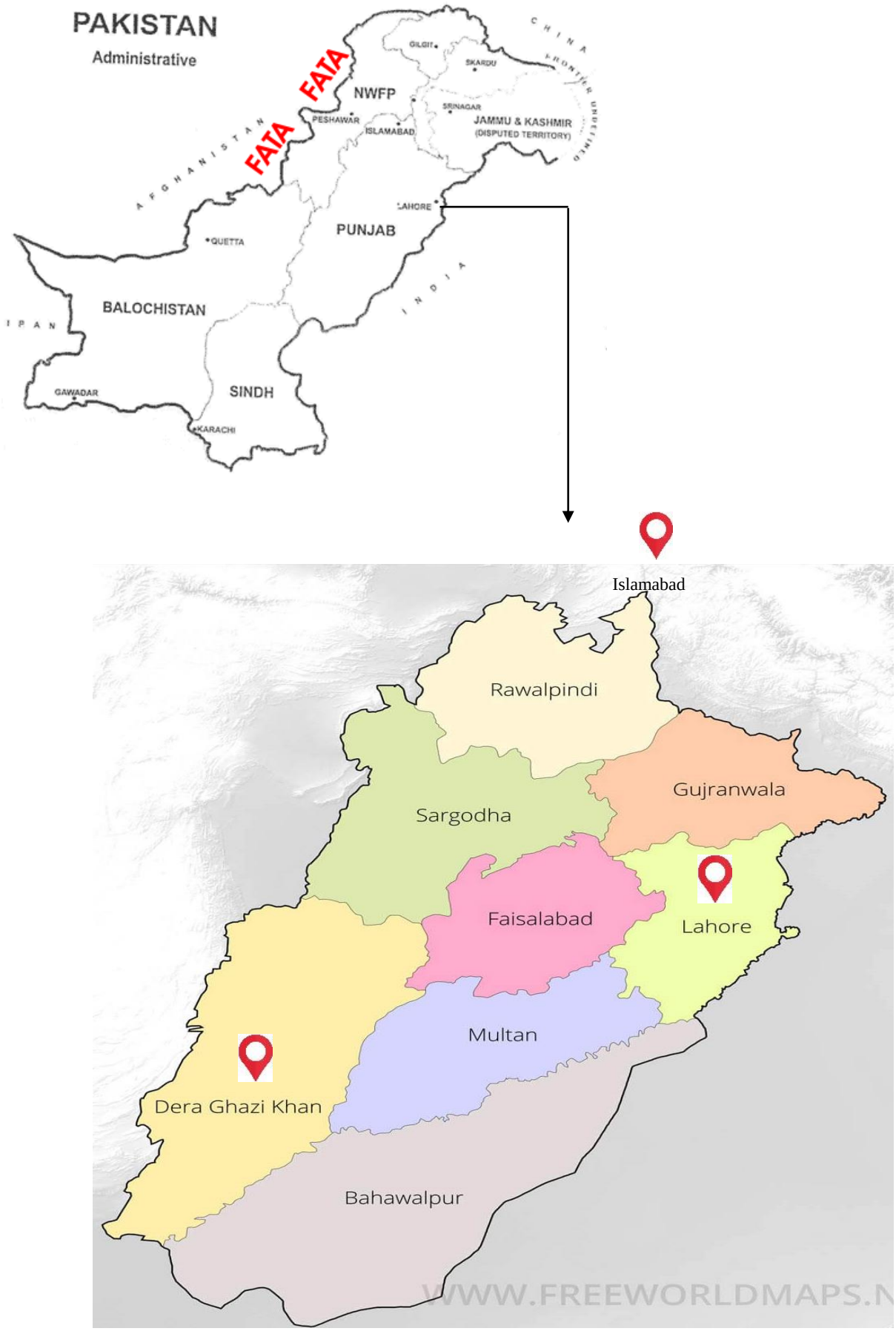


Figure 3.2: Pictures showing map of Pakistan with highlighted areas of sampling.

3.4.3 Isolation and Purification of Fungi

The isolation and purification of the fungus includes the following steps:

1. First, 5g of soil was taken from each sample and dissolved in 50ml of distilled water in a falcon tube.
2. The solution was then filtered, and filtrate was collected as the stock solution.
3. To prepare various dilutions stock solution is used. 100ul stock solution is taken in an Eppendorf and 900ul distilled water was added to make 10^{-1} dilution. Similarly, in order to make 10^{-2} dilution 200ul stock and 800ul distilled water was added. Dilutions up to 10^{-8} were prepared in the same manner.
4. From each dilution 50ul was taken and spread on plated containing phytase screening media and left in incubator for 4 days for the fungal growth. The phytase screening media only allows the phytase producing fungus to grow.
5. Sub culturing of the desired fungus was then done on PDA plates with the help of inoculating loops and pure colonies were maintained. [9]

3.4.4 Pure cultures

After growth of fungal colonies on plates they were inoculated on the slants of PDA and kept at 30 degree Celsius for 3 to 4 days and were preserved at 4 degree Celsius (model: MPR-1410, SANYO, Japan)[23].

3.4.5 Morphological Analysis of Fungi

The morphological identification of fungal isolates was performed with the help of compound microscope following fungal compendium. For the slide preparation the slides were first cleaned properly with 70% ethanol. The spores of the purified fungal colonies were then placed in the center of the slide on the water droplet in the Bio Safety Cabinet. The spores on the slide are then covered with a clean cover slip and pressed slightly to remove all the air

and water bubbles. These prepared slides were then observed under microscope at 40x resolution.

3.4.6 Total nucleic acid extraction

For screening DNA in fungal isolates, total nucleic acid extraction of fungi was performed as adopted from literature reported by [4].

3.4.7 Agarose gel electrophoresis

Fungal genome and extracted from fungal mycelia was analyzed performing Agarose gel electrophoresis described as follows.

1. 1 X TAE buffer was made from 50X TAE by taking 490 ml sterile water and 10 ml of 50X TAE buffer from stock.
2. After making 1X TAE buffer, 1% Agarose was measured and added in beaker containing 1X TAE. For making 1% Agarose gel , 50 ml 1X TAE was taken in a beaker and 0.5 grams Agarose was added and to make 1.5% Agarose gel, 0.75 Agarose was added to 50 ml 1X TAE.
3. Heated the mixture for 1 and half minute in a microwave oven and then added 5 ul ethidium bromide before pouring to side-sealed caster.
4. After gel solidification, side seals and comb were removed from caster and just the Agarose gel was placed in gel tank filled up to a recommended limit mentioned on tank.
5. In first well 3.6 ul 1 kb Thermo Scientific DNA ladder was loaded as a marker and 10 ul of total nucleic acid extracted sample was mixed with 2 ul Thermo Scientific 6x loading dye and complete mixture was loaded into well.
6. Finally, at 55V and 120A, the Agarose gel was run for 2-3 hours.

7. After complete gel electrophoresis, gel was visualized using Bio Top UV Trans-illuminator and gel analysis was performed using Dolphin Gel Documentation software.

3.4.8 PCR amplification of ITS region

ITS based PCR amplification of fungus was performed using standard primers set and conditions (Church and Buckler., 1999; Kendall and Paul., 2005). The two primers sets used are ITS1 F, ITS2 R, ITS5 F, ITS4 R, ITS86 F and ITS86 R.

3.4.9 Inoculum preparation

Spore suspension was made by adding 10 milliliter autoclaved water in the fungal slant. It was gently scratched by using red hot inoculating loop to form spore suspension.

3.4.10 Enzyme production by submerged fermentation

Media was prepared by adding 25 mL medium in the 250 milliliter flask. Media was autoclaved at 121 degree Celsius and 15 psi for fifteen minutes. Media was prepared according to recipe as shown in section 3.1.4. Then 1 milliliter of spore suspension was inoculated in the flask aseptically containing medium and it was thoroughly mixed and placed at 30 degree Celsius in a shaking incubator (model: Innova^{R} 43 Incubator shaker series) for 7 days. The production was ran in triplicate.

3.4.11 Enzyme extraction from different fungal species

After incubation and enzyme production (Section 3.4.10), enzyme containing medium was filtered with the help of Whattman filter paper no 1. Centrifugation of enzyme was performed at 6000 rpm for 10 min using (SIGMA laboratory centrifuges 3K30) and pellet was discarded. The obtained supernatant was then used for estimation of enzyme activity.

3.4.12 Media Optimization for Enhanced Phytase Production

Different factors and conditions of fermentation media were changed and their effect on the production of phytase enzyme was observed. The optimization is usually performed by OFAT technique also known as one factor at a time technique. This technique allows us to change single parameter of the fermentation media while keeping other factors constant. Thus multiple batches of fermentation were carried out as shown in section 3.4.10. using optimized conditions to enhance the production of phytase enzyme. Mentioned below are some of the parameters that effect the growth of fungal isolates:

1. Optimization of various age of spores for maximum production of phytase enzyme
2. Optimization of various incubation time for maximum production of phytase enzyme.
3. Optimization of various temperatures for maximum production of phytase enzyme.
4. Optimization of various pH for maximum production of phytase enzyme.
5. Optimization of various carbon sources for maximum production of phytase enzyme
6. Optimization of various nitrogen sources for maximum production of phytase enzyme

3.4.12.1 Optimization of various age of spores for maximum production of phytase enzyme

Spores of various ages were inoculated in the fermentation medium to check their effect on production of phytase enzyme. Various ages of spores were ranging from 2days, 3 days, 4 days, and 5 days up to 7 days.

3.4.12.2 Optimization of various incubation time for maximum production of phytase enzyme

Various times of incubation were used for the fermentation medium to check their effect on production of phytase enzyme. The different incubation periods range from 72 hours, 96 hours, 120 hours, 144 hours and 168 hours.

3.4.12.3 Optimization of various temperatures for maximum production of phytase enzyme

Various temperatures were used in the fermentation medium to check their effect on production of phytase enzyme. Various temperatures used in the fermentation medium includes 25^{o}C, 30^{o}C, 35^{o}C and 40^{o}C.

3.4.12.4 Optimization of various pH for maximum production of phytase enzyme

Various pH was used in the fermentation medium to check their effect on production of phytase enzyme. Various pH used in the fermentation medium ranges from 4.5-6.5.

3.4.12.5 Optimization of various carbon sources for maximum production of phytase enzyme

The effect of different sources of carbon on enzyme production was checked and monitored to identify maximum yielding source. Glucose, fructose, sucrose and lactose were the different carbon sources used in the media to find the optimized substrate giving maximum units of phytase.

3.4.12.6 Optimization of various nitrogen sources for maximum production of phytase enzyme

Different sources of nitrogen that are peptone, yeast extract, potassium nitrate and ammonium sulphate were used as nitrogen source to study their effect on the production of phytase. The source giving the best phytase yield was found and identified.

The volume of all the fermentation media was raised to 100ml in each flask and rest of the conditions remains same as that of phytase screening media. NaOH (1N) and HCl (1N) was used to maintain the pH of the medium. After the pH has been maintained the media was autoclaved for 15 minutes at 15psi at 121°C. The flasks were inoculated with 5ml spore

suspension under aseptic conditions and the inoculated flasks were incubated in shaking incubator at 140rpm.

3.4.13 Phytase production using optimized conditions

The optimized fermentation media for the production of phytase was prepared by using all the ingredients with their optimized quantities in distilled water. The rest of the composition of fermentation medium remains same as that of phytase screening media.

3.5 Analytical methods

3.5.1 Estimation of inorganic phosphorus by Colorimetric method

Colorimetric method is used detect the indigestible or inorganic form of phosphorous that is released by the breakdown of phytic acid (Gaind and Singh, 2015). A graph was plotted for the evaluation of the inorganic phosphorus by preparing standards of phosphorous solutions and constructing a standard graph of it.

3.5.2 Procedure of Standard curve

Phosphorous standard curve was prepared by preparing the stock solution of phosphorus i.e. 10 mg/ml. Then different dilutions were prepared by adding 2, 4, 6, 8 and 10 milliliter from the stock solution. Then volume of each tube except tube containing 10 mL stock solution was increased to 10 milliliters by adding d.H_2O. Then 0.5 ml MgSO4 sol (0.1 M) was added in each tube. The black was prepared by adding 1 milliliter d.H_2O and 0.5 milliliter magnesium sulphate solution. The tubes were placed in boiling water for 15 minutes. Reaction was stopped by rapidly adding 1 mL TCA (Trichloro acetic acid) in all tubes. After that add 1 mL of coloring reagent (TSCR) in all tubes and then by using the O.D standard curve was plotted.

3.5.3 Enzyme assay

Enzyme assay was performed by taking 4 test tubes and labeled them as blank, control, control enzyme and experimental. Blank was prepared by adding 2 mL water. Control was prepared by adding solution of 0.5 mL $MgSO_4$ and 1 mL Na_2CO_3 substrate solution (0.2 M). Control enzyme tube contained 0.5 mL of enzyme. In experimental tube 0.5 mL magnesium sulphate solution, 1 mL sodium acetate substrate solution and 0.5 mL extracted enzyme was added. Blank, control, control enzyme and experimental tubes were incubated in water bath for 15 minutes at 30 °C (Model: WSB-30, Wisd, WiseBath, Germany). Tricholoracetic acid 1(ml) was then in each tube. Reaction was stopped. Add control enzyme in control tube and then add 1 mL of TSCR in each tube. Absorbance was taken at 660 nm by using spectrophotometer (model: Model: UV – 1700, SHIMADZU Corporation, Japan)

3.5.4 Enzyme units

“The quantitiy of enzyme required to release per minute of 1 µmole of inorganic phosphorus at standard conditions of assay is called as one unit of phytase.” (Rachmawati *et al.*, 2017)

$$\text{Units/ml enzyme} = \frac{(\text{micromoles of phosphorus released})\,(df)\,(5)}{(T)\,(0.5)\,(2)}$$

3.5.6 Protein estimation by Bradford reagent

The total protein estimation was performed by using standard of BSA (Bovine Serum Albumin) and Bradford reagent. Standard curve of BSA was plotted to evaluate protein.

3.5.7 Procedure of standard curve

Standard curve of BSA was prepared by preparing different dilutions from the stock solution having concentration 1 mg/ml. Then different dilutions were prepared by adding 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL from the stock solution. Then volume of each tube except

tube containing 1 mL stock solution was raised up to 1 mL by adding distilled water. Then 5 mL Bradford reagent was added in each tube. In blank 1 mL distilled water and 5 mL Bradford reagent was added. Incubate the tubes for 5 minutes at room temperature. Absorbance was taken at 595 nm to plot the standard curve.

3.5.8 Estimation of enzyme

To estimate protein in sample standard curve was used. For estimation 0.1 mL sample and 5 ml Bradford reagent was taken. In blank add 0.1 mL sodium phosphate buffer instead of sample along with 5 mL Bradford reagent. Incubate the tubes for 5 minutes at room temperature before taking absorbance. O.D was taken at 595 nm using spectrophotometer. Then compare the absorbance taken with the standard curve of BSA.

4. Results

4.1 Screening of fungal samples

The fungal samples collected from various areas of D.G Khan, Lahore and Islamabad were screened for the production of phytase by growing these cultures on phytase screening media. The fungal strains showing best growth were selected, named and subjected to morphological identification. Based on the morphology following isolates were selected for enzyme production.

Sample name	**Fungus**	**Area**	**Location**
MH4	*Rhizopus oryzae*	Fields	D.G Khan
PT	*Penicillium oxalicum*	Fruit market	Lahore
A8	*Trichoderma longibrachiatum*	Lab isolates	Nust Islamabad

Table 4.1: Selected fungal samples to produce phytase

4.2 Pure Colonies

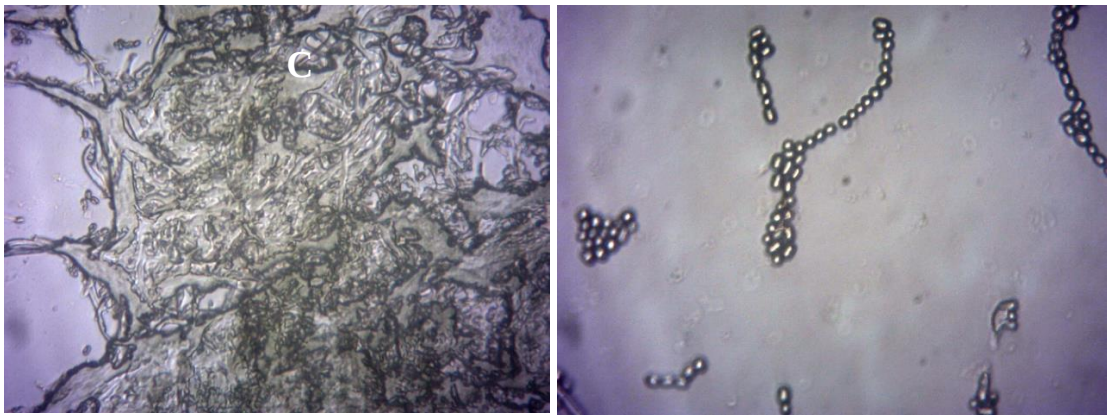
The pure cultures of the selected samples as mentioned in table 4.1 were maintained on the PDA plates. The pure colonies are shown in figure 4.1.

Rhizopus oryzae ***Penicillium oxalicum*** ***Trichoderma longibrachiatum***

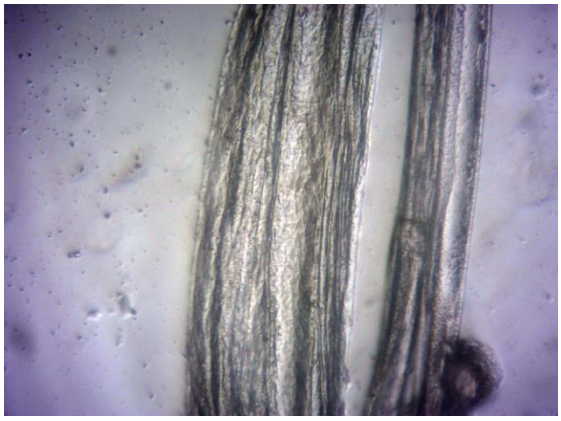
Figure 4.1: Picture showing pure colonies of *Rhizopus oryzae, Penicillium oxalicum* and *Trichoderma longibrachiatum* on PDA plates

4.3 Morphological Analysis of Fungi

The pure fungal isolates are then morphologically identified as each fungal strain has its own specific morphology and patterns upon which they are identified and classified. The morphological identification was carried out by preparing the slides as explained in the section 3.4.5. The slides of fungal strains are then observed under microscope at 40X resolution.



Trichoderma longibrachiatum ***Penicillium oxalicum***



Rhizopus oryzae

Figure 4.2: Images showing spores of *Trichoderma longibrachiatum, Penicillium oxalicum* and *Rhizopus oryzae* at 40x resolution.

4.4 Molecular Identification of the fungal isolates

The morphological identification enables us to find and predict the genus of the fungal strain and sometimes the specie as well. The morphological identification is confirmed by molecular identification. The molecular identification of fungal isolates was done on the basis of ITS based PCR by total nucleic acid extraction followed by the agarose gel electrophoresis using three sets of primers. The primer sets used are

Sr.no.	Primer Name	5'-3' primer sequence
1	ITS1F	TCCGTAGGTGAACCTGCGG
2	ITS4R	TCCTCCGCTTATTGATATGC

Table 4.2: The above table shows 3-5' primer sequences

Figure 4.3 shows the ITS gel of sample MH4 which was morphologically identified as *Rhizopus oryzae*. This sample was amplified using the primer set 1F and 4R and the size of the amplicon is about 700bp. The ITS based amplification of sample PT was also performed using the primer set 1F and 4R and the amplicon shows the size of about 650bp as shown in figure 4.4 below.

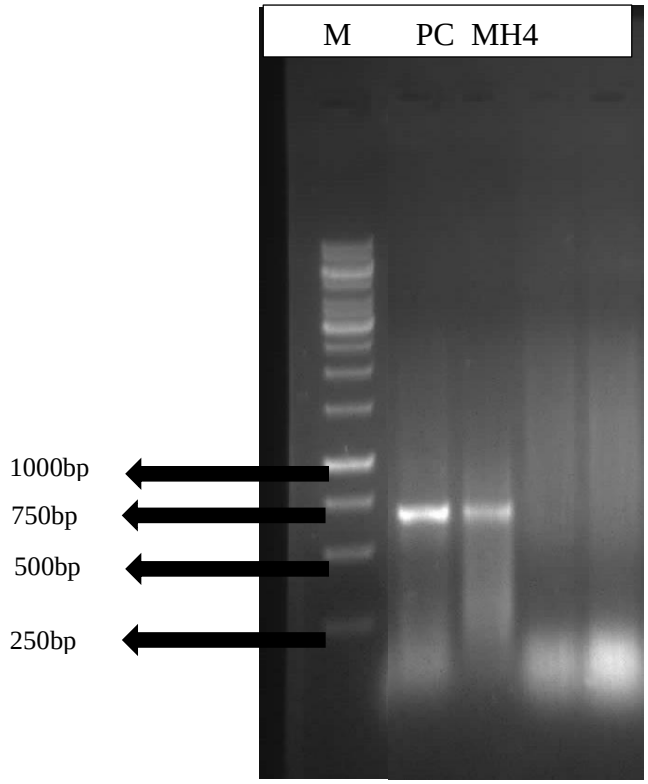


Figure 4.3: Picture showing ITS gel of fungal isolate where M indicates 1Kb ladder, PC denotes for positive control and MH4 shows the sample amplified using primer set ITS1F and ITS4R. The sample shows an amplification of approximately 700bp.

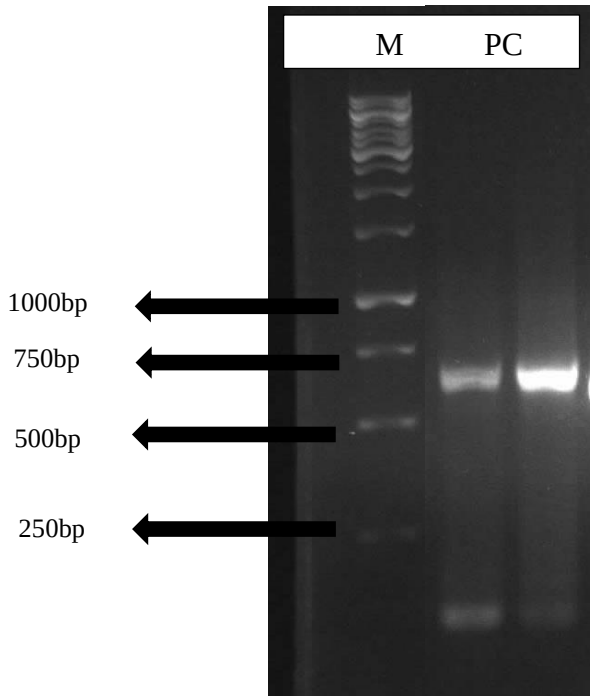


Figure 4.4: Picture showing ITS gel where M indicates ladder of 1Kb, PC denotes for positive control and PT is the sample amplified using the primer set ITS1F and ITS4R. The sample shows an amplification of approximately 650bp.

As the third sample was collected from lab so it was pre-identified. Based on ITS the selected fungal isolates are identified as shown in table 4.3 below.

Sample name	**Scientific name**	**Identification**	**Accession No.**
A8	*Trichoderma longibrachiatum*	Pre-identified lab isolate	KY967258
MH4	*Rhizopus oryzae*	ITS based identification	MW434070
PT	*Penicillium oxalicum*	Pre-identified lab isolate	KY967256

Table 4.3: Fungal isolates along with their ITS based identified names.

4.5 Submerged Fermentation

The identified fungal isolates are then subjected to submerged fermentation to produce phytase. The slants of fungal isolates were prepared on PDA and spore suspension was inoculated in fermentation media in 250ml flasks and flasks are then placed at 30°C for 7 days initially. After 7 days the fermentation media was filtered and centrifuged to get partially purified enzyme. The enzyme assays are then performed to find the quantity of phytase obtained. The results showed that while using un-optimized media *P.oxalicum* yields maximum phytase production and *T.longibrachiatum* yields the lowest. Table 4.3 shows the ODs of extracted enzyme recorded by spectrophotometer at 660nm.

Fungal isolate	**OD at 660nm (mg/ml/min)**
Rhizopus oryzae	1.902±0.01
Penicillium oxalicum	2.017±0.03
Trichoderma longibrachiatum	1.724±0.002

Table 4.4: Phytase obtained by using phytase media for the submerged fermentation.

4.6 Optimization of fermentation conditions

The fermentation media was then optimized for maximizing the production of phytase using OFAT (One Factor at a Time) technique.

4.6.1 Optimization of age of inoculum

The fungal slants were prepared and kept at 30°C for 2 to 7 days and observed. *R.oryzae* and *P.oxalicum* shows maximum growth in 3 days whereas *T. longibrachiatum* shows maximum growth in 4 days.

4.6.2 Optimization of incubation time

The effect of various incubation periods on the enzyme production was studied and observed. Figure 4.5 shows graph plotted based on ODs recorded at 660nm by spectrophotometer at different incubation times.

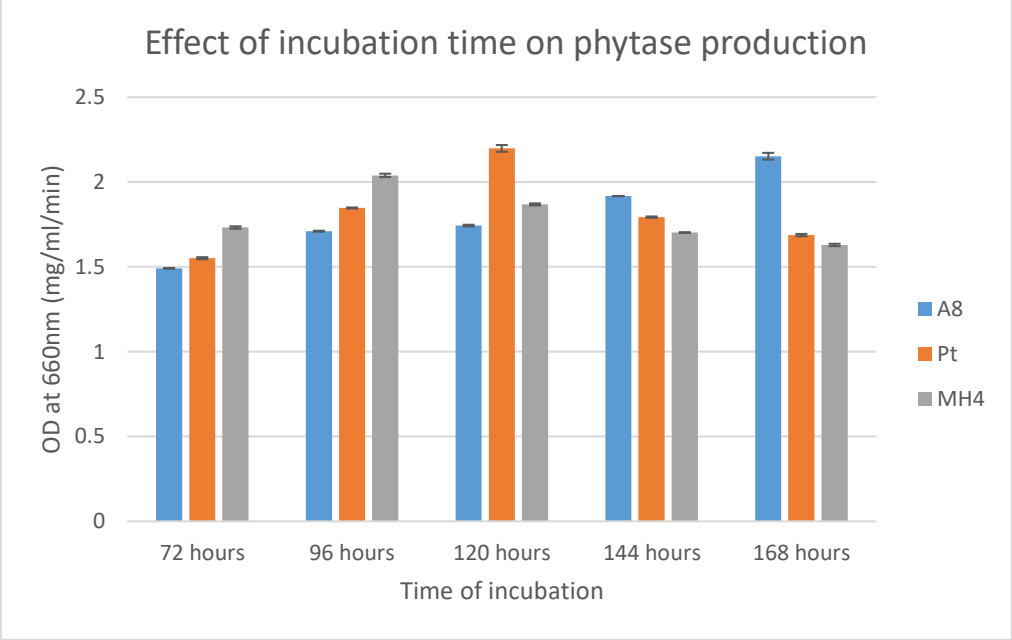


Figure 4.5: Effect of various incubation time on phytase production

The results showed that *T. longibrachiatum* gives maximum phytase production (2.152±0.02 mg/ml/min) in 168 hours, *P.oxalicum* (2.198±0.02 mg/ml/min) in 120 hours and *R.oryzae* (2.039±0.01 mg/ml/min) in 96 hours.

4.6.3 Optimization of temperature

The effect of various temperatures on the enzyme production was studied and observed and graph was plotted based on ODs.

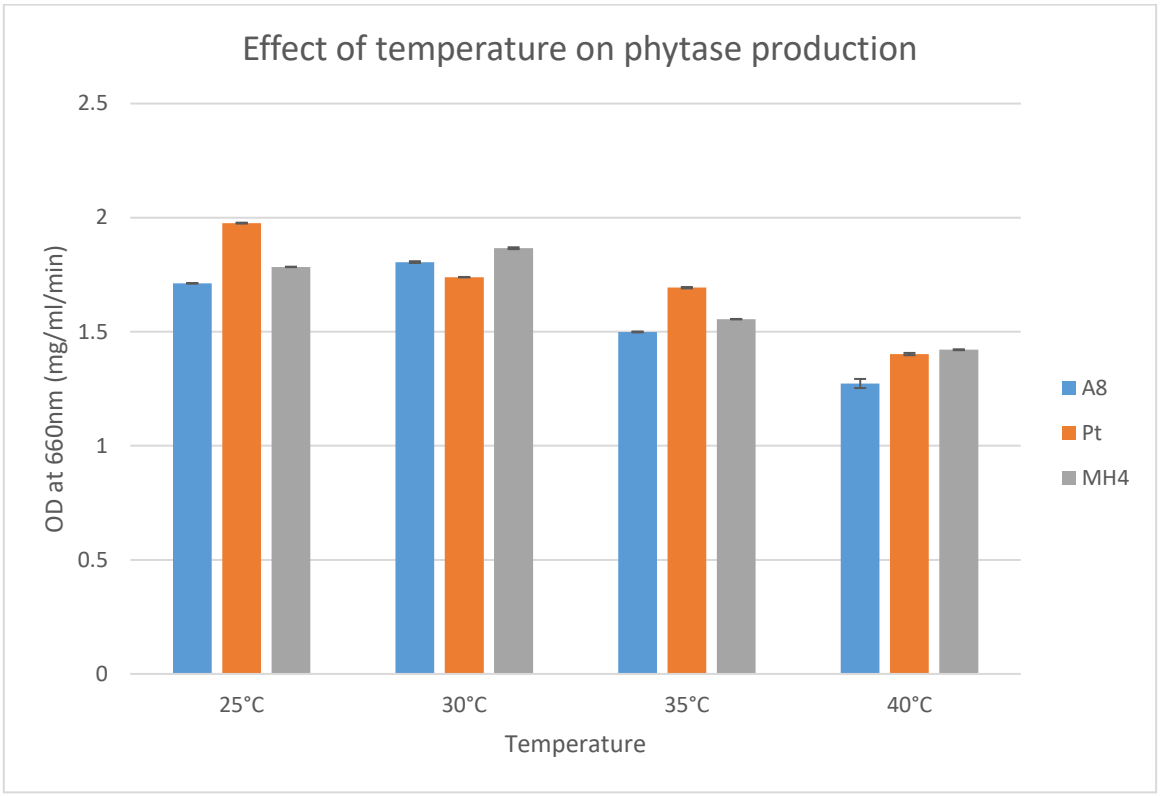


Figure 4.6: Effect of various temperatures on phytase production

The results showed that *T.longibrachiatum* and *R.oryzae* showed maximum OD at 660nm (1.805±0.004 mg/ml/min) (1.866±0.004 mg/ml/min) respectively and hence yields maximum phytase production at 30ºC and *P.oxalicum* (1.976±0.002 mg/ml/min) at 25ºC.

4.6.4 Optimization of pH

The effect of various pH on phytase production was studied and observed. The ODs at various pH were noted at 660nm from spectrophotometer and a graph was plotted as shown below.

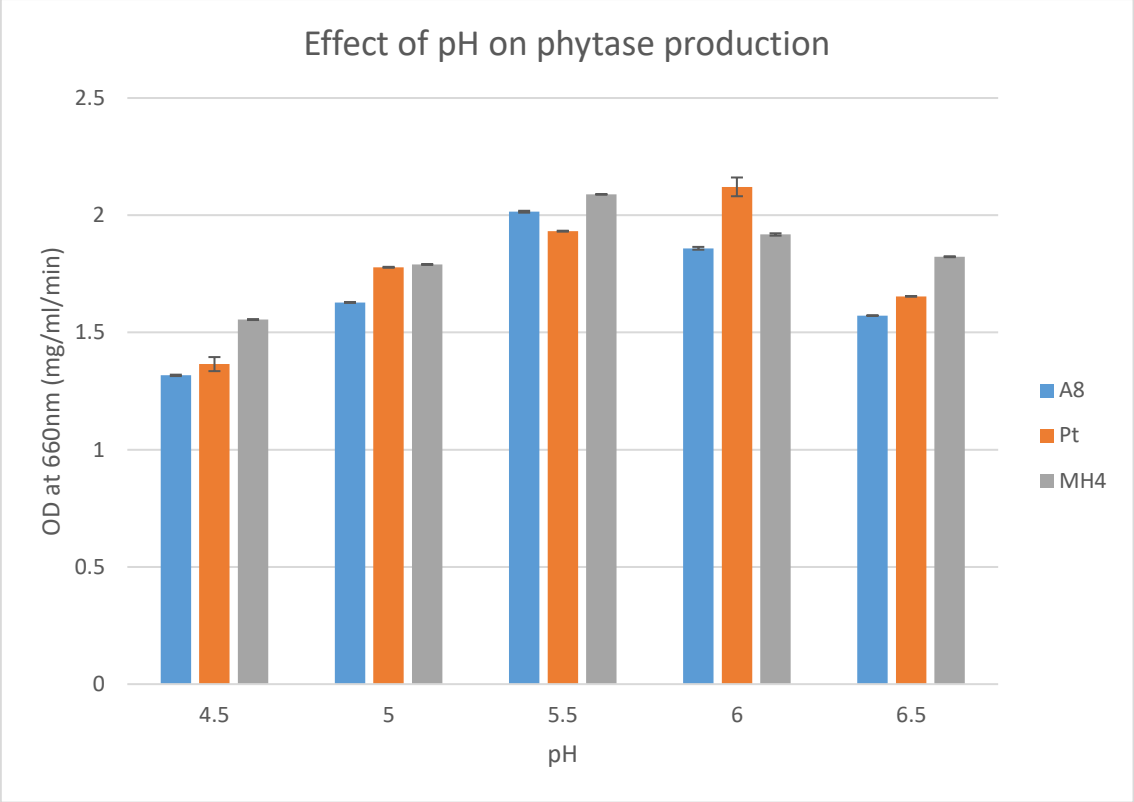


Figure 4.7: Effect of various pH on phytase production

The results showed that *T.longibrachiatum* (2.0152±0.004 mg/ml/min) and *R.oryzae* (2.089±0.001 mg/ml/min) gives maximum phytase production at pH 5.5 and *P.oxalicum* (2.121±0.004 mg/ml/min) at pH 6.

4.6.5 Optimization of carbon sources

The effect of various carbon sources on the enzyme production was studied and observed and the optimized carbon source for each fungal strain was identified. A graph was plotted on the basis of the ODs recorded at 660nm by spectrophotometer as shown below.

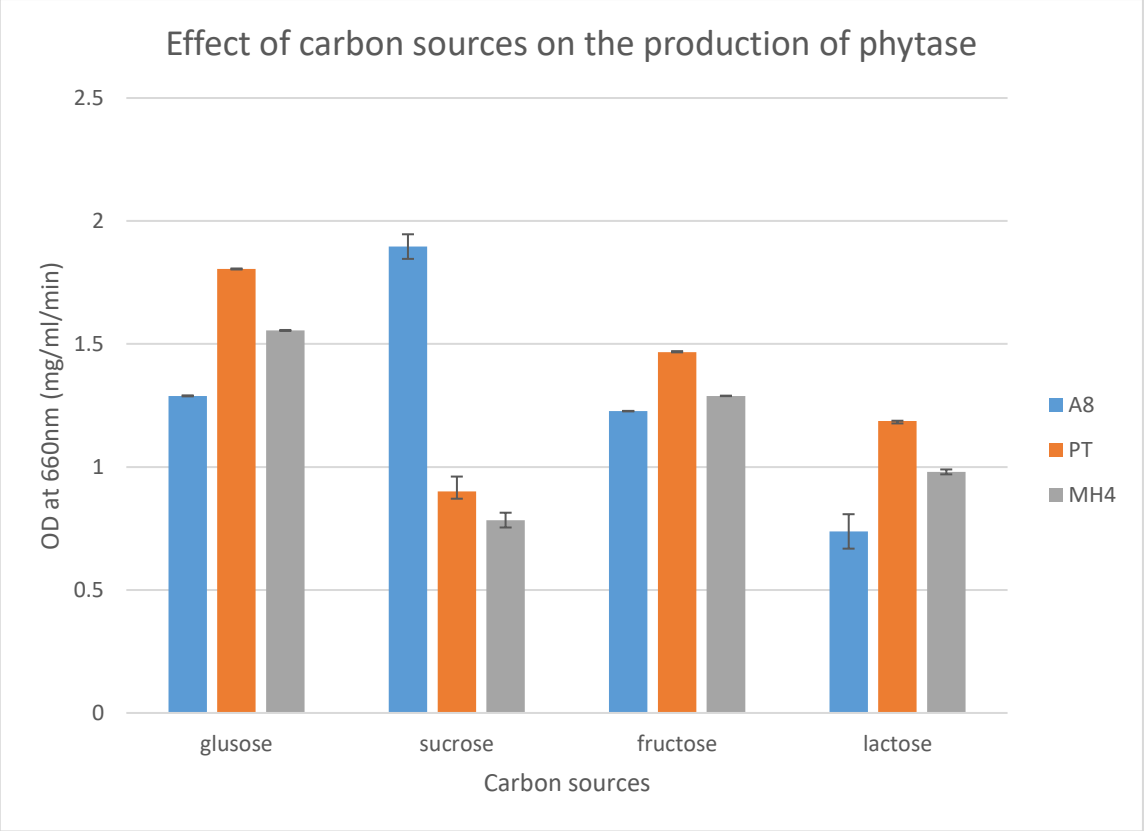


Figure 4.8: Effect of various carbon sources on phytase production

The results showed that *T. longibrachiatum* (1.896±0.05 mg/ml/min) gives maximum phytase production by using sucrose and *P.oxalicum* (1.805±0.002 mg/ml/min) and *R.oryzae* (1.555±0.002 mg/ml/min) using glucose as carbon source in fermentation media.

4.6.6 Optimization of nitrogen sources

The effect of various nitrogen sources on the enzyme production was studied and observed. The ODs recorded at 660nm by using different nitrogen sources are plotted in a graph below.

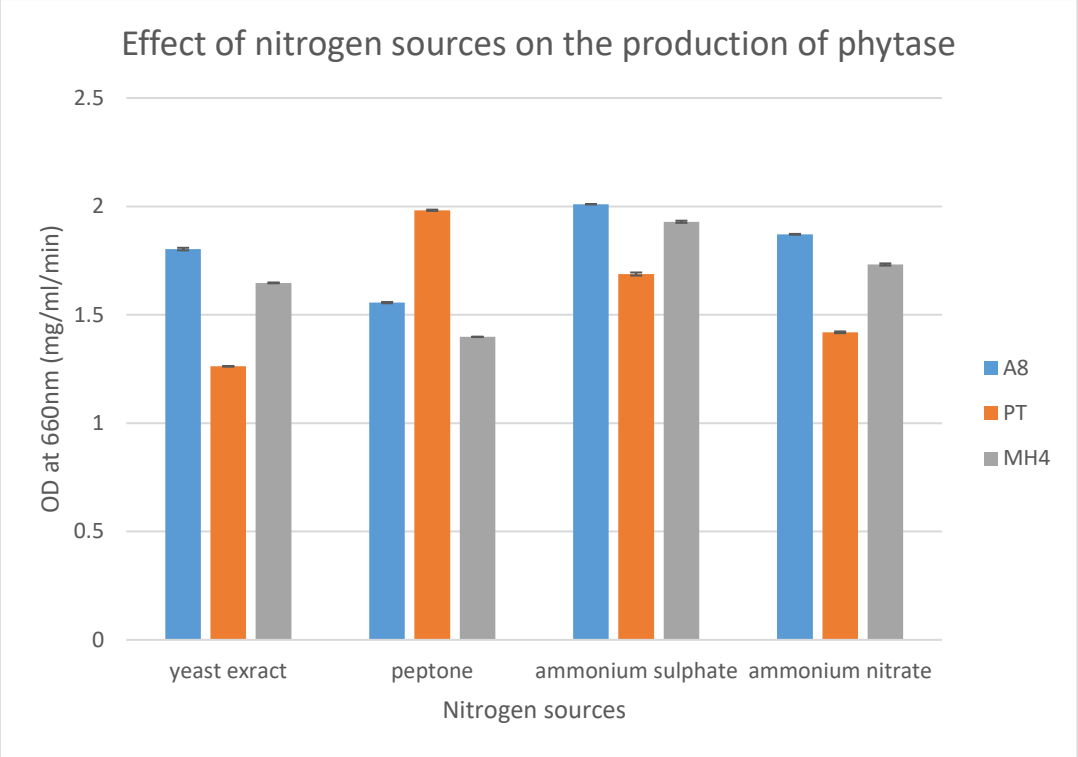


Figure 4.9: Effect of various nitrogen sources on phytase production

The results showed that *T. longibrachiatum* (2.010±0.001 mg/ml/min) gives maximum phytase production by using ammonium sulphate, *R.oryzae* (1.929±0.005 mg/ml/min) using ammonium nitrate and *P.oxalicum* (1.982±0.003 mg/ml/min) using peptone as nitrogen source.

4.6.7 Optimized fermentation conditions

The fermentation of all the isolates were carried out with the optimized condition for the maximum production of phytase. The optimized conditions for all three fungal isolates are listed below. The rest of the fermentations ingredients for each fungus remains same as enlisted in phytase screening media.

Cultural conditions	**Fungal isolate**		
	A8	**PT**	**MH4**
Age of spore	4	3	3
Incubation period	168hrs	120hrs	96hrs
Temperature	30^{o}C	25^{o}C	30^{o}C
pH	5.5	6.0	5.5
Carbon source	Sucrose	Glucose	Glucose
Nitrogen source	Ammonium sulphate	Peptone	Ammonium nitrate

Table 4.5: Optimized fermentation conditions for maximum phytase production.

These fermentation media was then partially purified and the enzyme assays are performed. The ODs of the samples with optimized fermentation conditions are as follows.

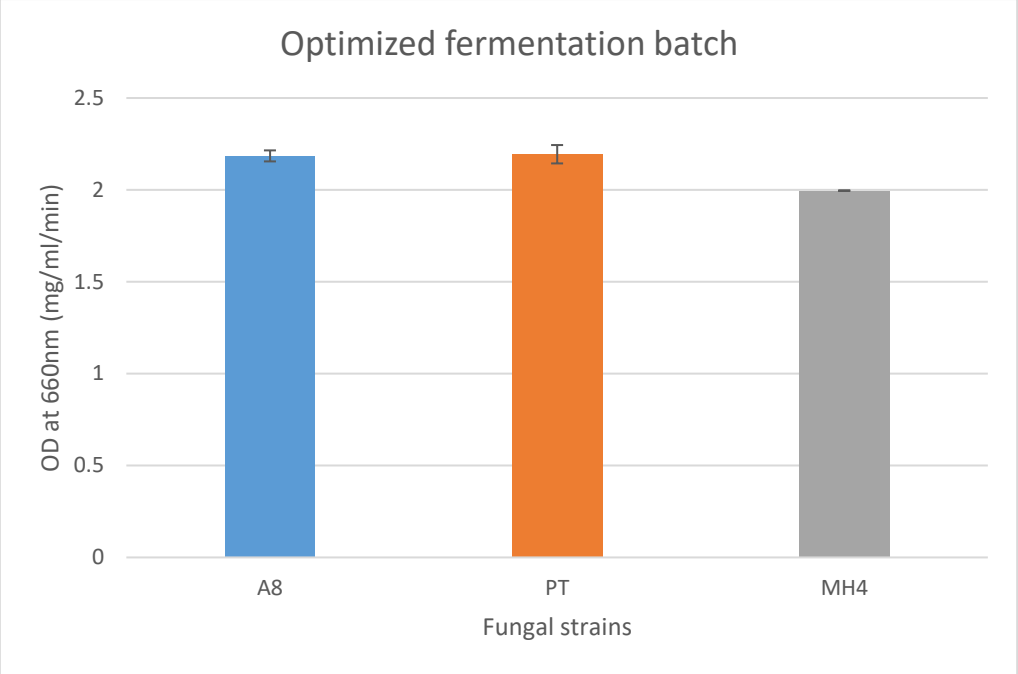


Figure 4.10: ODs recorded at optimized fermentation conditions for maximum phytase production

4.7 Enzyme Quantification

The enzyme units indicate the phytase activity or the amount of phytase required to release 1umol of inorganic phosphorous per minute. These are calculated by plotting a graph according to section 3.5.2 for the standard phosphate which act as a standard and along with the rest of the ODs are compared. The standard of phosphorous is shown below.

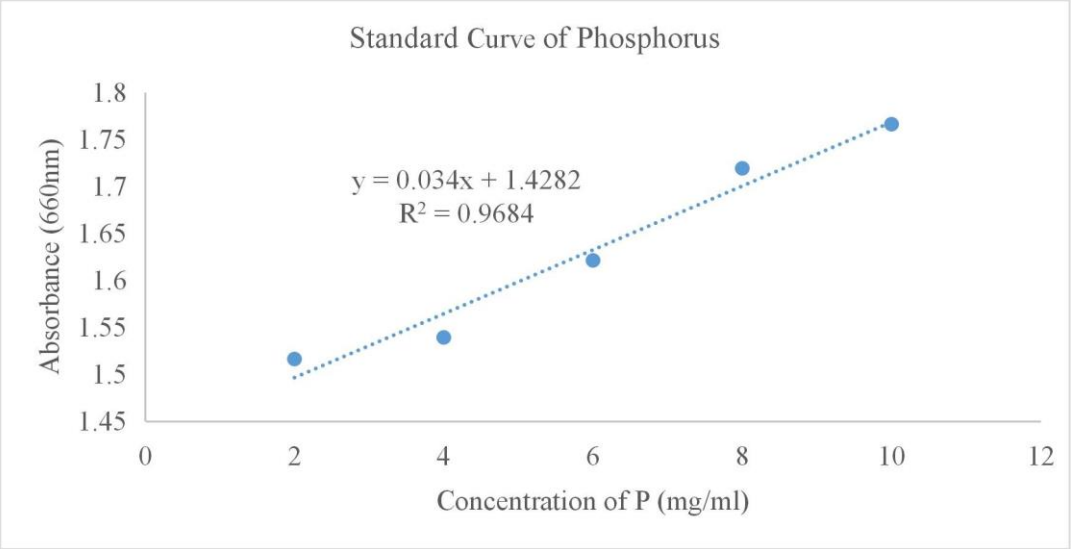


Figure 4.11: Standard curve of phosphorus showing absorbance at various concentrations of phosphorous.

The ODs of the optimized batch are then compared with the standard and the units of the enzyme are calculated by a formulae that is stated in section 3.5.4. The enzyme units are calculated as follows:

Sample name	Enzyme units (ug/mol/min)
A8	21.10
PT	25.50
MH4	20.7

Table 4.6: Units of phytase enzyme (ug/mol/min)

Table 4.6 shows the maximum units of phytase obtained after the optimization of various fermentation conditions or factors by all three fungal isolates. Among all three *P.oxalicum*was the highest yielding local strain for phytase.

4.8 Enzyme Estimation

The enzyme estimation is done to find the concentration of the protein obtained. The enzyme estimation is done by Bradford reagent which also act as a standard for this process. Before

finding the concentration of samples, a graph of standard BSA (Bovine Serum Albumin) is plotted to compare the readings according to the methodology mentioned in section 3.5.7.

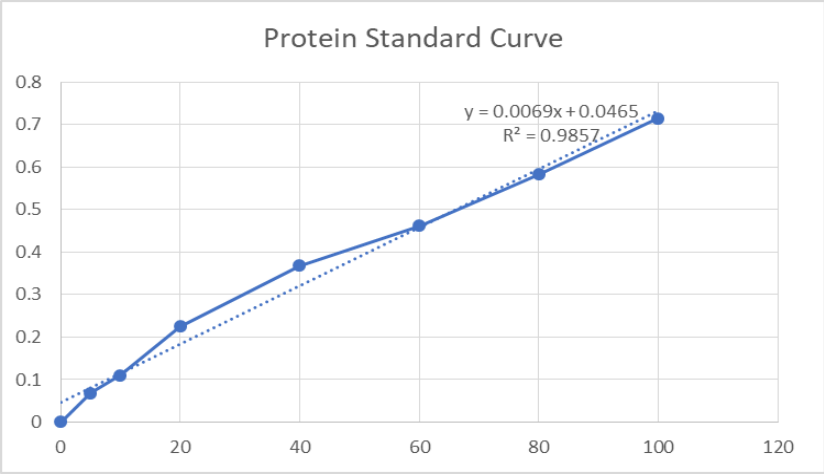


Figure 4.12: Standard Curve of BSA

The concentration of fungal protein obtained after fermentation is calculated by Bradford Reagent. The concentration of enzyme extracted from the fungus is as follows:

Sample name	**Concentration mg/ml**
A8 *Trichoderma longibrachiatum*	0.241
PT *Penicillium oxalicum*	0.252
MH4 *Rhizopus oryzae*	0.249

Table 4.7 Concentration of phytase enzyme mg/ml

5. Discussion

Phytases are the enzymes that belongs to the group of phosphoric monoester hydrolases. In the recent times fungal phytases are emerging as a potential source to be used in various industries such as poultry industry to reduce the phosphate pollution from environment. In the present study the fungal sampling was done from various regions of Punjab as well as Islamabad. These samples were screened for the production of phytase enzyme using the phytase screening media and three potential fungal sources of phytase belonging to different genera were selected. These selected fungal strains were subjected to morphological as well as molecular identification and identified as *R. oryzae, P.oxalicum* and *T. longibrachiatum.* These fungal strains were then used for the production of phytase enzyme using submerged fermentation. The various ingredients of fermentation media were then optimized for maximum production of phytase and the units and concentration of phytase enzyme produced by optimized fermentation media was then calculated.

There are many fungal sources of phytase. Previously the phytase have been produced by *R. oryzae* (Rani R & Ghosh S., 2011) using solid state fermentation and by *R. oligosporous* using solid state fermentation (Sabu AH et al., 2002) and submerged fermentation as well (Haritha K & Sambasivarao KRS., 2009). Phytases from *P.oxalicum*are also reported from solid state (Awad G et al., 2011) as well as submerged fermentation (Tseng YH et al., 1999). The production of phytase from *T. longibrachiatum* is yet to be done from submerged fermentation and only a few are reported from solid state fermentation (Prado DZ et al., 2019).

The incubation time for fermentation was optimized for all the three fungal strains. It was found that *T. longibrachiatum* shows maximum phytase activity (24.96 units) after 168hrs in submerged fermentation however highest phytase activity (23.2 units) after 120hrs using

solid state fermentation has been reported by (Prado DZ et al., 2019). *P.oxalicum* shows maximum phytase activity (20.2 units) after 120hrs which is in accordance with the study of (Awad G et al., 2014). *R. oryzae* yields (17 units) after 96hrs of incubation similar to the study of (Rani R & Ghosh S., 2011)

The incubation temperature for fermentation was optimized and results showed that *T. longibrachiatum* yields highest phytase activity(12.8 units) at 30°C, *P.oxalicum* gives maximum units of phytase (15.2 units) at 25°C and *R. oryzae* gives the highest production of phytase (18.8 units) at 30°C. All these results are in accordance with the previous studies performed on phytase production from *T. longibrachiatum* (Prado DZ et al., 2019), *P.oxalicum* (Awad G et al., 2014) and *R. oryzae* (Rani R & Ghosh S., 2011). However some species of *penicllium* are also reported to show maximum phytase production at 27°C (Tseng YH et al., 2000).

The optimization of pH of the fermentation media was done and it was found that *T. longibrachiatum* gives maximum phytase production (16 units) at pH 5.5 in contrary to (Prado DZ et al., 2019) where the fermentation was carried out at 5.0 pH by solid state fermentation. The pH at which *P.oxalicum* produce high phytase yield (18.6 units) is pH 6.0 but 5.5 pH was optimized by (Tseng YH et al., 2000) using solid state fermentation. High phytase production was achieved at pH 5.5.

The carbon sources greatly affects the enzyme yield and optimizations of carbon source yields glucose to be the best carbon source for phytase production by *P.oxalicum* (12 units) as reported in (Awad G et al., 2014) and in *R. oryzae* resembling to the study of (Ramachandran S et al., 2005). *T. longibrachiatum* shows maximum phytase production (19 units) by using sucrose as the carbon source.

The nitrogen source used in the fermentation media was also optimized for maximum phytase yeild. Ammonium sulphate was optimized for *T. longibrachiatum* (15.45 units), peptone for *penicllium oxalicum* (12.8 units) and ammonium nitrate for *R. oryzae* (17.71 units). The results of *penicllium* are in accordance with the study of (Awad G et al., 2014) in which peptone was the optimized nitrogen source for maximum phytase production by solid state fermentation.

6. Conclusion

The local fungal strains *Trichoderma longibrachiatum, Rhizopus oryzae* and *Penicillium oxalicum* were identified as the potential source of phytase production among which *Penicillium oxalicum* is the highest yielding fungal strain (25.50±0.05 U/ml/min). Moreover, optimization of the fermentation conditions greatly enhanced the phytase production. Moreover enzyme activities and concentrations of the extracted phytases were also calculated. This study will help in devising a process for the future which is economical and environment friendly for mass production of phytase to be used in various industries for example phytase can be widely used in animal feed as supplement and are of great importance to nutritionists and ecologists.

7. Future Recommendations

- Phytase produced through local fungal strains can be purified completely using ammonium sulphate precipitation method for further commercial uses.
- Phytase production by local fungal strains can be enhanced through physical or chemical mutations for industrial level production of enzyme.
- Complete characterization of phytase including Next Generation Chromatography (NGC) can be performed.
- Fungal phytases could be used as a feed additive in poultry industry for reducing the phosphate pollution from environment.

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