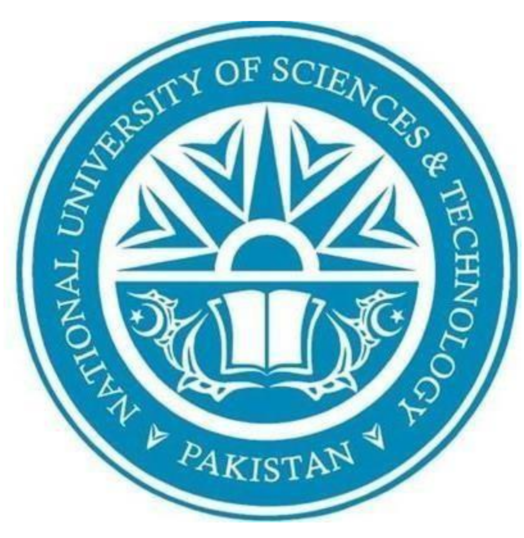
Isolation and Partial Characterization of Phytase from Locally Isolated *Aspergillus* Species.



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

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Dedication

To my family without whom my success would not be possible.

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List of Abbreviations

PDA..........Potato Dextrose Agar

PAP..........Purple Acid Phosphatase

HAP..........Histidine Acid Phosphatase

PTP..........Protein Tyrosine Phosphatase

PSM..........Phytase Screening Media

TCA..........Trichloroacetic Acid

TSCR..........Tausky-Shorr Color Reagent

PCR..........Polymerase Chain Reaction

BSA..........Bovine Serum Albumin

ITS..........Internal Transcribed Spacer

Abstract

Poultry industry is one of the industries which is growing at an exponential rate due to an increased demand and production of broiler chicken by the export markets and local consumers. To balance the amount of phosphorus in animals, the poultry diets are mainly supplemented with seed-based components that store phosphorus in phytic acid form. Phytic acid is indigestible by many animals and it usually makes complexes with other ions like calcium and proteins and as a result hampers their absorption in the animal body. For that matter, phytase enzyme is added in the poultry feed to increase the bioavailability and absorption of phosphorus and other vital ions. This study was conducted to check the potential of local strains of *Aspergillus* species for the production of phytase enzyme and identify high yielding strains for a more environmentally friendly source of the enzyme. In this study, 20 samples were taken from different cities of Pakistan for the screening of phytase producing fungi on phytase specific screening media. The selected strains were subjected to ITS based molecular identification and were identified as *Aspergillus niger* and *Aspergillus flavus*.The fungal cultures were then undertaken submerged fermentation to calculate the total units of enzyme produced. In order to achieve maximum enzyme production, various cultural parameters were optimised, and best units of enzyme were produced with glucose as carbon source, ammonium nitrate as nitrogen source, 120 hours incubation time, 30℃ incubation temperature and 5.5 pH of the fermentation media. Different enzyme units were produced by different strains of *Aspergillus* species. *Aspergillus niger* strain N gave maximum units (25 U/ml) of phytase followed by Aspergillus *niger* strain B4 which gave

19.4 U/ml enzyme. Aspergillus *flavus* strain AF yielded 7.5 U/ml phytase whereas strain G1 produced 16.36 U/ml phytase through submerged fermentation under optimsed conditions.

Chapter 1

Introduction

Isolation and Partial Characterization of Phytase from Locally Isolated *Aspergillus* Species.

1. Introduction to Phytase

Globally, the poultry industry being one of the agro-based industries is growing at an exponential rate due to an increased demand and production of broiler chicken by the export markets and local consumers. It is anticipated that till 2030 the production and consumption of poultry meat in the developing countries will remain at a rate of 3.6% and 3.5% (Global Poultry Feed Market, 2017). In order to balance the amount of phosphorus in animals, the poultry diets are mainly supplemented with seed-based components that store phosphorus in phytic acid form. Since phosphorus is very vital for the well-being of the poultry, therefore it is important to incorporate phosphorus in their feed. Phytic acid is indigestible by many animals and it usually forms complexes with other ions such as calcium and proteins and as a result hampers their absorption in the animal body. For that matter, phytase enzyme is added in the poultry feed to increase the bioavailability and absorption of phosphorus and other vital ions.

Phytases (myo-inositol hexakisphosphate phosphohydrolase EC 3.1.3.8) are enzymes that belong to the group of phosphoric monoester hydrolases that catalyse the release of phosphate from phytate (myo-inositol hexakisphosphate) which is the primary form of phosphorus stored in plants [36]. Phytases have been incorporated in animal feed for improving phosphorus nutrition and also to reduce phosphorus pollution in animal waste [19]. Due to low level of phytase, phytates are not broken down in the monogastric gut and the ingested phytate is released in the environment causing pollution [51]. Therefore, supplementary addition of phosphate is required to avoid phosphorus deficiency in the animals [12].

Phytases are found in animals, plants, and microorganisms. Due to their commercial value, the microbial sources of phytases are preferred and for industrial purposes, different strains of *Aspergillus* species have been used [36]. Phytase has also been produced from various plant sources such as soybean, rice, corn, wheat seeds, rye, maize, dwarf beans and other oil seeds. Phytase has been reported in the blood of calves, birds, reptiles, fish [16]. Today, limited products of phytase are available commercially but more research is being conducted to enhance the production of phytases in order to increase its suitability for use in animal feed [52].

1.1 Production of phytase through fermentation

Fermentation has been extensively used for a long time for the production of useful substances including enzymes and due to its environmental advantages; it has gained significant importance over the years. Since phytase is important in poultry feed industry, therefore various parameters have been studied in order to maximize the enzyme production. Fungi and bacteria yield high amounts of enzymes when fermented on the ideal substrates. Phytase is produced from bacteria, fungi and yeasts using submerged fermentation and solid-state fermentation techniques.

1.2 Major *Aspergillus* species found in environment

Aspergillus is a group of conidial fungi that belongs to the division Ascomycota. This genus was described for the first time in year 1729 by a Florentine mycologist and priest P.A. Micheli [4]. Members of this genus are aerobic and grow well as molds in environments that are high in salt concentration and rich in oxygen. It grows well in soil, potted plants, hay, hospital air- conditioner filters etc. A special characteristic exhibited by species of *Aspergillus* for example *Aspergillus niger*, is oligotrophy where the fungus can grow in nutrient deficient environment. *Aspergillus flavus* is a pathogen of animals, insects and plants and causes rots in several crops and is also a producer of mycotoxin, alfatoxin B_1 which is a naturally produced carcinogen [5].

1.2.1 *Aspergillus* as a source of industrial enzymes

Aspergillus niger is used in biotechnology for the production of extracellular enzymes and citric acid and it is by far one of the most important microorganisms. The first enzymes produced by *Aspergillus niger* were protease, pectinase and amyloglucosidase and they were produced by surface culture technique. Other extracellular enzymes like pectinase, glucose oxidase, α- amylase and recombinant proteins have been reported from *Aspergillus niger* and it is also used for the treatment of waste and biotransformation [38].

1.3 Need of current research

The present study was conducted for the production of phytase enzyme from different *Aspergillus* species using fermentation. Since the potential of indigenous *Aspergillus* strains was unexplored, therefore, this study was conducted to identify the high yielding strains for phytase. The objectives of current project are mentioned as under.

1.4 Objectives of current research:

The main objectives of the study are:

1. Identification of *Aspergillus* species from the environment.
2. Extraction of phytase enzyme from isolated species of *Aspergillus.*
3. Partial characterization of phytase.

Chapter 2

Literature Review

2. Literature review

2.1 Phytase enzyme-An overview

Phytases (myo-inositol hexakisphosphate phosphohydrolase EC 3.1.3.8) are enzymes that belong to the group of phosphoric monoester hydrolases that catalyse the release of phosphate from phytate (myo-inositol hexakisphosphate) which is the primary form of phosphorus stored in plants [36]. Phytases have been used in animal feed for improving phosphorus nutrition and also to reduce phosphorus pollution in animal waste [19]. Due to low level of phytase, phytates are not broken down in the monogastric gut and the ingested phytate is released in the environment causing pollution [51]. Therefore, supplementary addition of phosphate is required to avoid phosphorus deficiency in the animals [12]. To prevent phosphorus deficiency and deteriorating health, animals require phosphorus in their diet. Low phosphorus in the body can result in loss of appetite, weakened immune system, brittle bones, infertility, and weight loss [1].

Phytases are found in animals, plants, and microorganisms. Due to their commercial value, the microbial sources of phytases are preferred and for industrial purposes, different strains of Aspergillus species have been used [36]. Phytase has also been produced from various plant sources such as soybean, rice, corn, wheat seeds, rye, maize, dwarf beans and other oil seeds. Phytase has been reported in the blood of calves, birds, reptiles, fish (16).

Today, limited products of phytase are available commercially but research is being conducted to increase the production of phytases in order to increase its suitability for use in animal feed [52]. Figure 2.1 below shows the structure of phytic acid.

Figure 2.1 Structure of Phytic acid adapted from [20].

In the past few years, there has been a drastic change in consumers' preferences towards buying products of personal and daily use. There has been an exemplary change from cure to prevention and people are opting to buy products that are more organic and environmentally safe. With food safety being the most important issue globally, the role of animal feed with respect to food safety, food security and public health is also considered important worldwide [9]. According to a report by the Freedonia Group (2009), the animal feed enzyme industry will achieve exponential growth in markets, and it would result in increased demand of meat with increasing per capita income. The global demand of enzymes was expected to increase around 7% annually from 2010 to almost $8billion in 2015 [27].

2.2 Classes of Phytases:

Four structurally different classes of enzymes have been defined as phytases namely Purple Acid Phosphatase, Histidine Acid Phosphatase, Protein Tyrosine Phosphatase, β-Propeller Phytase [26] and these classes are explained below:

2.2.1 Purple acid phosphatase (PAP)

Purple acid phosphatases also known as metalloenzymes have been isolated from germinating soybeans. Soybean phytase and purple acid phosphatase (PAP) share a similar active site sequence motif and studies have shown PAP like sequences in bacteria, mammals, plants, and fungi [41]. Soybean phytase (GmPhy) is the only protein acid phosphatase to show significant activity of phytase however, previously a PAP of Aspergillus niger has also been reported but it does not use phytase as a substrate due to its low potential to use it as a substrate [51].

2.2.2 Histidine acid phosphatases (HAPs)

The enzymes belonging to this group catalyse the hydrolysis of phytic acid in 2 steps and all enzymes are active at acidic pH [55]. Phytases of Histidine acid phosphatases family do not need a cofactor for maximum activity and, not all Histidine acid phosphatases are active phytases.

2.2.3 Protein tyrosine phosphatase (PTP)

Protein tyrosine phosphatase is another class of phytase that is known as dual specificity phytase or cysteine phytase since it contains catalytic cysteine and displays maximum activity at acidic pH. Protein tyrosine phytase has been isolated from *Selenomonas ruminantium* which is an anaerobic bacteria and this phytase does not show any sequence similarity with any other class of microbial phytase.

2.2.4 β-propeller phytases (BPP)

β-Propeller phytases belong to a class of microbial phytase are also referred to as alkaline phosphatases since they are active at alkaline pH and require calcium ions for thermostability and optimum activity. These enzymes are not related to histidine acid phosphatases and other classes of phosphatases, but they exhibit 90-98% sequence similarity among themselves [55]. At the cleavage and affinity site, substrate hydrolysis occurs which facilitates and increases the affinity of phytases and other substrates and calcium ions play their role by creating and electrostatic environment that further facilitates the binding [44].

Non-specific acid phosphatases are another subgroup phosphatase that have high rate of hydrolysis for monophosphorylated compounds but show less efficient activity against phytate [32]. Histidine acid phosphatases are extensively studied since they have more specific activity for phytic acid.

2.3 Sources of Phytase

Plants, animals, and microorganisms are sources of phytase. Over the years, it has been found through research that various yeast, fungal and bacterial strains yield high quantity of phytase that can be used on industrial scale and this has urged scientists to check the expression of this enzyme in multiple hosts using different methods. Phytases exhibit different chemical and physical properties depending on the host [37].

2.3.1 Occurrence of Phytases In Microbial Community

Phytase has been reported from various microorganisms including bacteria, yeasts and moulds. Bacteria belonging to genera Enterobacter are extracellular phytase producing bacteria and Escherichia coli phytase is a periplasmic enzyme. Other bacterial species that are a source of phytase include *Pseudomonas, Lactobacillus, Raoultella, Citribacter braakii, Selenomonas ruminantium, Megasphaera elsdenii, Prevetella* species [22]. Many bacterial phytases that are present in the ruminant animals such as cattle and sheep play role in the breakdown of phytic acid present in the grains in order to make phosphorus available for animals. Since non-ruminant animals such as birds, dogs, cats etc. cannot produce phytase therefore to overcome this problem their food is supplemented with phytase so that it may become available to the phytate-bound nutrients such as calcium, phosphorus, proteins, carbohydrates, and minerals. On commercial level, phytases are produced by genetically modified yeast through cellulosic biomass fermentation. Many gut microorganisms possess the ability to produce phytase, but they do not secrete it because of unfavourable pH of the intestine [11].

Extracellular phytase production by Bacillus species T4 was reported by researchers using solid state fermentation [25]. To select the optimal medium for solid state fermentation, combination of individual substrates including wheat bran, corn flour and soybean meal along with nutritive supplements were evaluated. The nutritive supplements included 1% casein hydrolysate, 0.4% $(NH_4)2SO_4$, 0.2% $CaCl_2.2H_2O$, 0.02% $MgSO_4.7H2O$, 0.05% KH_2PO4 and 0.04% K_2HPO_4 respectively. The best results were shown by combination of corn flour with nutritive supplements giving maximum phytase production and this was further used for submerged fermentation study. Maximum phytase production was observed at optimum pH of 7.0 after 84 hours, 4% inoculum density and 55.5% moisture content. The enzyme activity was further enhanced by using galactose and glucose as the carbon source. Phytase activity was reduced by additional nitrogen sources and its synthesis was inhibited by sodium nitrate and corn steep liquor. The T4 phytase produced by solid state fermentation prove as a promising strategy for improving the nutritional content of animal feed and reducing environment pollution in the industry.

2.3.2 Fungal Phytases

In fungi, extracellular phytase has been reported from various species belonging to different genera such as *Penicillium, Mucor* and *Rhizopus* however, it is most abundantly isolated from *Aspergillus* species [37]. *Aspergillus niger* is the most reported and favoured species of the genus*Aspergillus* to produce phytase and other organic acids due to its safe status (GRAS) and tremendous secretory capability [45]. Fungal phytases are preferred over bacterial phytases for use in animal feed due to their greater yield and high tolerance in low pH [48]. It is known that phytases play a vital catalytic role in the degradation of phytic acid and its salts and these enzymes are added in the feed of monogastric animals to reduce the environmental and

nutritional effects of dietary phytate. To improve the phytase production from *Rhizopus oligosporus* 556 strain, submerged fermentation technique was used using 1% wheat bran and optimized culture medium. After 8 days of fermentation, there was an increase in phytase activity with maximum production 39U/ml with 5% glucose, 0.5% peptone and pH 6. Inorganic phosphate content affected phytase production and high levels of inorganic phosphorus suppresses the biosynthesis of phytase [17]. A summary of fungal phytases is given in Figure 2.2 below:

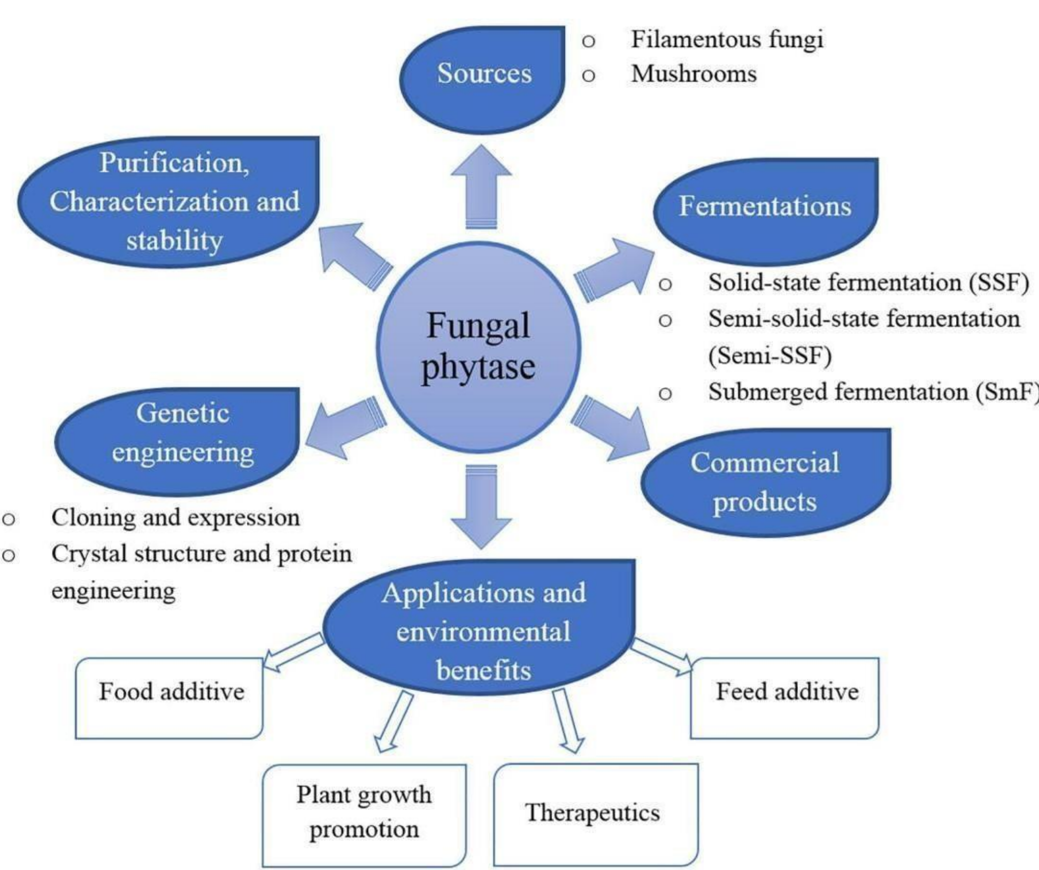

Figure 2.2: Applications of Fungal Phytases. Figure adapted from Jatuwong et al., 2020

2.4 Reported Methods of Fermentation for Phytase Production

Fermentation has been extensively used for a long time for the production of useful substances including enzymes and due to its environmental advantages; it has gained significant importance over the years. Since phytase is important in poultry feed industry, therefore various parameters have been studied in order to maximize the enzyme production. Fungi and bacteria yield high amounts of enzymes when fermented on the ideal substrates. Phytase is produced from bacteria, fungi and yeasts using submerged fermentation and solid-state fermentation techniques.

2.4.1 Solid State Fermentation

Solid state fermentation has gained massive attention in the biotechnological industries because of its proficiency of producing biologically active secondary metabolites and it is now being considered as an alternate to submerged fermentation [33]. In this technique, microorganisms are cultivated on moist solid substrates with the addition of carbon and energy sources. Commonly used substrates are crop residues like wheat bran, rice bran, wheat straw, rice straw, hay; vegetable waste including sweet potato, cassava, sugar cane etc. [35]. Fermentation occurs in the absence of free water in natural environment that is favourable for the microorganisms [34]. Solid state fermentation has benefits over submerged fermentation in the way that it gives higher yield of the products, requires less energy, easy downstream processing energy, less contamination and it is more reliable for production of enzymes. For the fungal enzymes' extraction, solid state fermentation is preferred since they require lesser water potential [49].

2.4.2 Submerged Fermentation

Submerged fermentation is a fermentation technique in which the microorganisms such as bacteria and fungi are cultivated in vessels containing nutrient rich broth (referred to as the fermentation media) and oxygen. This technique is useful for the production of industrial enzymes which are released in the solution by the microorganisms as they breakdown the nutrients (carbon and nitrogen sources) in the broth. Liquid media, molasses, sugars are few examples of the substrates used in the submerged fermentation.

Two common fermentation processes are batch-fed and continuous fermentation. In the batch-fed process the products are harvested in the end starting with the addition of nutrients in the fermenter followed by the growth of biomass whereas in continuous fermentation, the rate of nutrient addition and release of broth is simultaneous. In order to optimise the fermentation process, various parameters such as temperature, pH, carbon dioxide and oxygen are maintained throughout to get maximum output. It is easier to purify the products of submerged fermentation and it is commonly used for the extraction of secondary metabolites used in liquid form. Choosing the appropriate substrate is highly important for this process in order to achieve maximum yield of the fermentation product since every organism responds in a different manner depending on the substrate used. Table 2.1 below shows the differences between Solid State Fermentation and Submerged Fermentation [28].

Table 2.1 Differences Between Solid State Fermentation and Submerged Fermentation

Solid State Fermentation	**Submerged Fermentation**
i. Organisms such as filamentous fungi that require little water for growth are preferred.	i. Water content is greater than the media concentration.
ii. Chances of contamination are low due to less water available.	ii. Greater chances of contamination due to more quantity of water.
iii. No liquid waste produced.	iii. Liquid waste produced in larger quantity.
iv. Time efficient, economical and easy downstream processing.	iv. Expensive and time-consuming downstream processing due to presence of water.
v. Bioreactors of small-normal size can be used.	v. Due to dilute media, large-scale bioreactors are important.
vi. Difficulty in diffusion of nutrients limits the growth.	vi. Diffusion of nutrients in easy due to constant mixing.
vii. Presence of biomass is difficult to quantify.	vii. Online sensors make biomass quantification easy.

2.5 *Aspergillus* as a source of Phytase

Aspergillus is a group of conidial fungi that belongs to the division Ascomycota. Members of this genus are aerobic and grow well as molds in environments that are high in salt concentration and rich in oxygen. It grows well in soil, potted plants, hay, hospital air-conditioner filters etc. They are regarded as opportunistic pathogens as they cause diseases such as aspergillosis in immunocompromised patients. A special characteristic exhibited by species of *Aspergillus* for example *Aspergillus niger*, is oligotrophy where the fungus can grow in nutrient deficient environment.

Aspergillus flavus is a pathogen of animals, insects and plants and causes rots in several crops and is also a producer of mycotoxin, alfatoxin B1 which is a naturally produced carcinogen and is being developed to be used as biological weapon [5]. In humans, species of *Aspergillus* cause mycoses in immunosuppressed patients and among this only Aspergillus fumigatus is important pathogenically as compared to *Aspergillus flavus* [42]. Aspergillus flavus is a saprophytic fungus found in soils and it causes diseases preharvest and postharvest losses to agriculturally important crops such as peanut (yellow mold), cotton seed and maize (ear rot) [21].

Aspergillus niger is used in biotechnology for the production of extracellular enzymes and citric acid and it is by far one of the most important microorganisms. The first enzymes produced by *Aspergillus niger* were protease, pectinase and amyloglucosidase and they were produced by surface culture technique. Other extracellular enzymes like pectinase, glucose oxidase, α- amylase and recombinant proteins have been reported from *Aspergillus niger* and it is also used for the treatment of waste and biotransformation [38].

2.6 Phytase reported from Aspergillus species

According to Edward et al., 2007, phytases have become one of the most important enzymes for environment protection, human well being and animal nutrition as they cleave the orthophosphate group from inositol core of phytate which is the main form of phosphorus found in plants. Phytases have been isolated from various microbes and plants and they are classified based on their catalytic mechanisms and pH (acidic or alkaline). However, the applications of phytases may be directed towards the development of special inositol phosphates and of phytate- bound minerals for human health and nutrition.

Researchers have worked on the commercial production of phytase and concluded that phytases play their role in the breakdown of phytate which the stored form of phosphorus in plants. By replacing and incorporating microbial phytase in animal diet, it resulted in reduced phosphorus excretion by monogastric animals. This reduction in phosphorus excretion contributes towards environment protection and on the other hand, phytase supplementation improves the availability of trace elements and minerals. Apart from its application in animal feed, research is also being carried out for the use of phytase for processing of human food focusing on enhanced mineral absorption and improved food processing. For the commercial production of phytase, fermentation technique has been used over the years and variety of phytases have been produced and characterized. To increase the shelf life of these enzymes, various stabilizing agents are added and to further enhance their activity, different formulation strategies are used [16].

A study reported the production of phytase from *Aspergillus niger* using 10-days fermentation in maize-starch based medium. According to results, the activity of phytase was 1.075 units per minute per millilitre of the crude filtrate at 20 and pH 5.5. To investigate the effect of phytase on availability of potassium and calcium and tibia-toe ash and mineral contents of tibia, a feedingtrial of 4 weeks was conducted on 90 days old broiler chicken where the diets were based on soybean and maize. The treatments that were given involved normal phosphorus level control (4.5g/kg non-phytate phosphorus), normal phosphorus diet with phytase treated soybean and maize and a low phosphorus diet with phytase (1.075 PU/gm of substrate). Compared to the control, in normal phosphorus plus phytase diet the phytase supplementation increased by 2.65 percent and the increase in weight was similar in control diet and low phosphorus plus phytase diet (653g vs 645g). The relative retention of phosphorus and calcium increased by 20.1 and 5 percent in low phosphorus diet treated with phytase in comparison to the control diet. There was an increase in tibial and toe ash contents through normal phosphorus diet added with phytase compared to control diet. In the low phosphorus diet with added phytase, the tibial and toe ash contents were comparable to the control diet (51.2 vs 48.6 and 10.9 vs 10.5). There was no effectof phytase treatment on the minerals in tibia ash however phosphorus and calcium in tibia increased in normal phosphorus plus phytase and low phosphorus plus phytase diets. It can be concluded from the experiment that phytase treatment improves bone mineralization, increases growth and retention of minerals in broiler chicken [2].

A study reported phytase from *Aspergillus niger* NCIM 563 using wheat bran through solid state fermentation. In the study, the statistical approaches used were Placket-Burman design and Box-Behnken design. To study the effects of 11 variables related to phytase production, Placket-Burman design was employed and to further optimise the study, glucose, $NaNO_3$, $MgSO_4.7H_2O$, dextrin and distilled water were used. In order to govern the levels of these variables for maximum enzyme production, Box-Behnken design was used. In dry moldy bran phytase production increased by 3.08 folds from 50 IU/g to 154 IU/g. The culture extract also showed activities of xylanase, amylase and cellulase. The phytase which had been purified partially was active at 55 and pH 6. However, the enzyme showed 75% activity at pH range 2.0-9.5. The approximate weight of phytase on Sephacryl S-200 was 87kDa. It can be concluded that phytase extracted through solid state fermentation from *Aspergillus niger* has wide potential and it can beused in poultry feed since it is stable at wide pH range [6].

In 2014, a group of researchers conducted a research to compare the phytase production from *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 through solid state fermentation and submerged fermentation. They found out that on the 5th day of incubation, both fungi showed maximum phytase production in solid state fermentation media and submerged fermentation media. The enzyme units produced by *Asergillus niger* CFR 335 and *Aspergillus ficuum* in wheat bran solid substrate medium were 60.6 U/gds and 38 U/gds respectively. There was an observed increase in the enzyme level when the solid substrate medium was supplemented with rice bran, wheat bran and groundnut cake in 1:2:1 ratio. In potato dextrose broth, maximum enzyme activity was observed by *Aspergillus ficuum* and *Aspergillus niger* CFR 335 in submerged fermentation [46].

Effect of various media ingredients on the phytase production by *Aspergillus niger* CFR335 in submerged fermentation and solid-state fermentation was studied. The media ingredients whose effects were studied for enzyme production included nitrogen, carbon, inorganic phosphates, metal salts and surfactants. There was a 1.5-fold increase in the enzyme yield after addition of sucrose in both submerged fermentation and solid-state fermentation. Peptone was a good nitrogen source in submerged fermentation. Sodium dihydrogen phosphate yielded 34% more enzyme than the control followed by 19% greater activity in potassium dihydrogen phosphate in solid-state fermentation 0.015% w/v. The growth of the fungus was suppressed by SDS whereas the addition of tween-20 in submerged fermentation exhibited maximum yield of 12.6 U/mL. Calcium chloride stimulated 55% more phytase production in submerged fermentation at 0.01% v/v. However, none of the metal salts played any role in stimulating the enzyme production [13].

In 2017, phytase production by *Aspergillus niger* NCIM 563 using protein rich chickpea flour as substrate through submerged fermentation was studied. Using the Plackett-Burman and Box-Behnken experimental design in shake flasks, there was an increase in phytase activity from 66 IU/mL in 216 hours to 160 IU/mL in 132 hours. There was an increase in productivity from 7.3 IU/mL to 29 IU/mL/day. The enzyme productivity was further increased by using the optimized media and the productivity increased to 164 IU/mL in 96 hours. Using centrifugation, the enzyme in the supernatant was recovered and the stability of phytase for application in animal feed was tested under gastric conditions. In vitro studies of pesticides extensively used in agriculture was also carried out. The pesticides were water insoluble chlorpyrifos and water soluble monocrotophos, methyl parathion. The HPLC analysis showed that phytase was capable of degrading 72% of chlorpyrifos at temperature 35 and pH 7 and at 50 temperature, 91% degradation was possible. Results were similar with monocrotophos and methyl parathion. To

further validate the degradation of chlorpyrifos, phytase was sprayed on harvested green chillies (*Capsicum annuum* L) at normal temperature of 35 and neutral pH 7 and the obtained products were analysed by liquid chromatography-mass spectrometry (LCMS). Therefore, the study confirms the application of phytase for the biodegradation of organophosphorus pesticides [43].

Another experimental study was carried out to evaluate the production of phytase by *Aspergillus niger* through solid state fermentation with triticale waste as the substrate. The process was carried out in the medium with only one nitrogen source and no other supplement. As a result, phytase activity of 7.45 U/g dry substrate was attained. Then in order to optimise the process, various supplements such as lactose, dextrose, potassium chloride and Tween 80 were added in the media. Two experimental designs were used for fermentation: the Plackett-Burman designand the Box-Behnken design. The Plackett-Burman design was used to check the effect of different variables on the phytase production and also to check how significant they are for the process. The Box-Behnken design was used for the determination of the optimal conditions. A maximum enzyme activity of 28.5IU/g dry substrate was obtained. The solid-state fermentation was carried out in trays in order to increase the amount of fermented substrate and the phytase activity was recorded 23.63 IU/g dry substrate. Thus, there is a decrease in the phytase production with the scaling [30].

Chapter 3

Materials and Methods

3. Materials and Methods

3.1 Summary of scheme of study

To isolate phytase from local strains of *Aspergillus* the following methodology was adopted as shown in Figure 3.1 below.

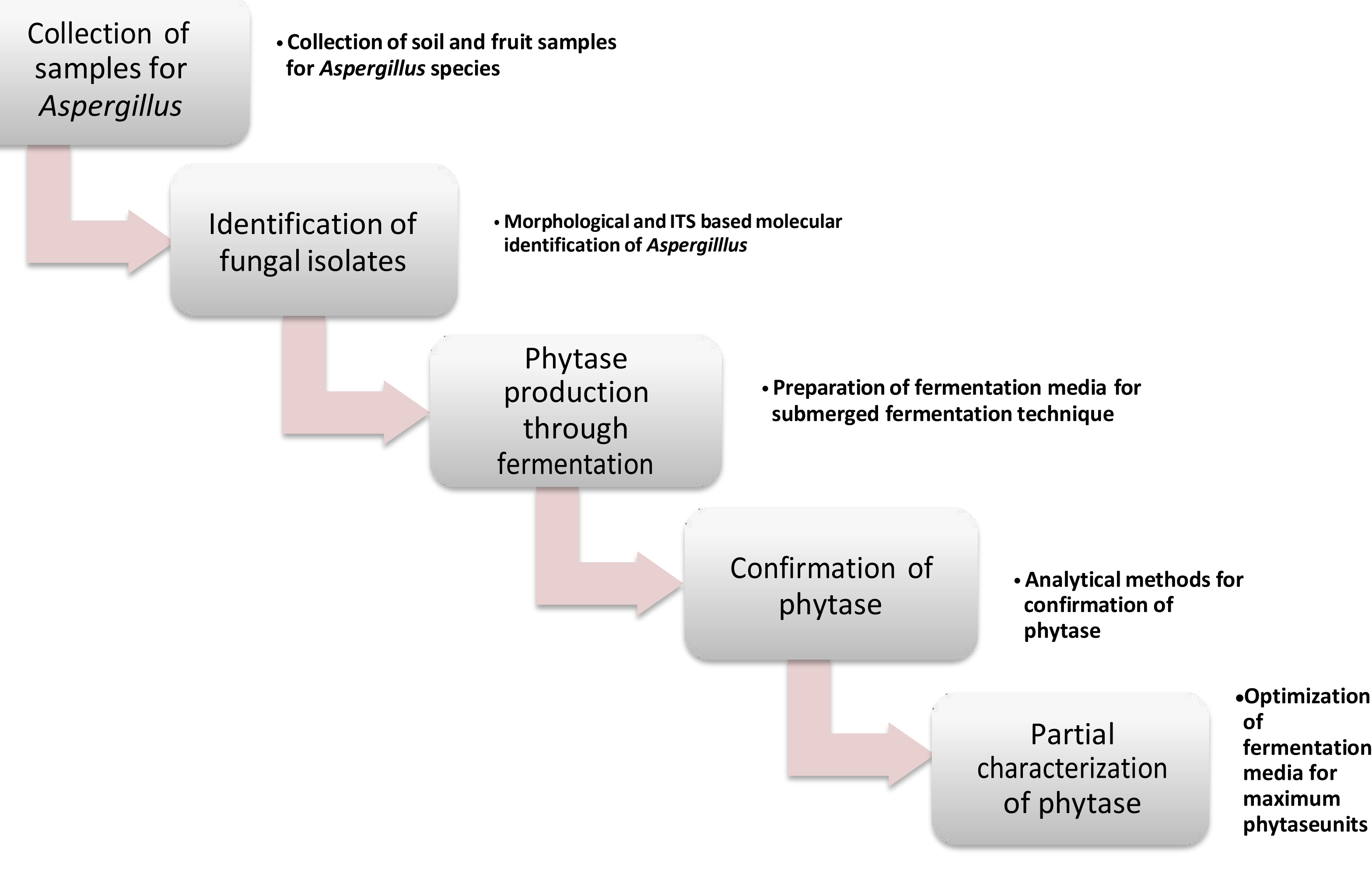

Figure 3.1. Methodology followed for phytase extraction from *Aspergillus* species.

3.2 General Laboratory Practice

The work bench was sterilised with 70% ethanol before starting any experiment and the use of gloves and lab coats was assured while working in the lab. Face masks were worn while dealing with toxic powders and spore bearing fungi. To ensure zero risk of contamination, all the glassware and other materials were autoclaved before using. Preparation of solutions and inoculation of the fungi was done in the biosafety cabinet and all the waste materials were carefully disposed off at the end of each experiment.

3.3 Media preparation

3.3.1 Phytase Screening Media

Phytase specific medium was prepared by dissolving 1.5g glucose, 0.05g Potassium chloride, 0.5g ammonium sulfate, 0.01g magnesium sulphate heptahydrate, 0.001g ferrous sulphate hepta hydrate, 0.02g sodium chloride, 0.02g calcium chloride, 0.001g manganese sulphate, 0.25g sodium phytate and 1.8g agar in 20mL distilled water. All the salts were dissolved by heating and final volume of the media was raised to 100mL. Media was then autoclaved for 15 minutes at 121 degree Celsius and 15 psi as reported by Hosseinkhani and Hosseinkhani, 2009.

3.3.2 Potato Dextrose Agar

Potato dextrose agar (PDA) was prepared by dissolving 39.5g PDA in 1L distilled water and sterilised by autoclaving for 15minutes at 121 degree Celsius and 15 psi.

3.3.3 Fermentation Media for Submerged Fermentation

The fermentation media was prepared by dissolving 7.5g glucose, 2.5g ammonium sulphate, 2.5g potassium chloride, 0.05g magnesium sulphate heptahydrate, 0.1g sodium chloride, 1g calcium chloride, 0.005g iron sulphate heptahydrate, 0.005g manganese sulphate and 1.25g sodium phytate in 500mL distilled water. Media was autoclaved at 121 degree Celsius and 15 psi for 15 minutes [53].

3.4 Reagents preparation

3.4.1 Substrate Solution

To prepare substrate solution, dissolve 0.5g sodium phytate in 25ml of 0.2M sodium acetate buffer.

3.4.2 0.1M $MgSO_4.7H_2O$

0.1 molar magnesium sulphate solution was prepared by dissolving 2.46g salt in 100ml distilled water.

3.4.3 10% Trichloroacetic acid (TCA)

10% TCA solution was prepared by dissolving 5g of TCA in 50ml distilled water.

3.4.4 10% Ammonium Molybdate

Prepare 10% Ammonium molybdate by mixing 10g ammonium molybdate in 10N sulphuric acid. To prepare 10N sulphuric acid, add 27.17ml sulphuric acid in 72.83ml distilled water.

3.4.5 Tausky-Shorr Color Reagent Solution (TSCR)

To prepare the color reagent solution, mix 5g ferrous sulphate in 90ml distilled water and then add 10ml of 10% Ammonium molybdate.

3.4.6 Bradford Reagent

Bradford reagent was prepared for the protein estimation by mixing 0.1g Coomassie brilliant blue G-250, 100mL 85% Phosphoric acid, and 50mL 100% ethanol and raising the final volume to 1L.

3.5 Preparation of buffers

3.5.1 Sodium Acetate Buffer (0.2M)

To prepare 0.2M sodium acetate buffer firstly, 1.64 g sodium acetate was dissolved in 100ml distilled water and 0.2 M acetic acid was prepared by mixing 1.14 mL of absolute acetic acid in 100mL distilled water. Then to prepare the buffer 36.2 mL sodium acetate was mixed with 14.8mL acetic acid.

3.6 Sample Collection from Different Areas of Punjab and Federal Area of Pakistan

For the screening of phytase producing fungi, various soil and fruit samples were collected from different areas of Islamabad, Lahore and Dera Ghazi Khan, Pakistan. Soil was collected by carefully picking up a patch and storing it in ziplock polythene bag. Food samples were collected and stored in the same manner. The map below highlights the sampling areas in Figure 3.2.

Figure 3.2 Map of Pakistan highlighting the sampling areas. The areas are indicated by red balloons.

Table 3.2 List of all the sampling areas.

Sample number	**Area**	**Location**
1	NUST trail	Islamabad
2	Margalla hills trail 5	Islamabad
3	Margalla hills trail 3	Islamabad
4	Margalla hills trail 6	Islamabad
5	Fruit market I-9	Islamabad
6	Fruit market H-9	Islamabad
7	Shakarparian	Islamabad
8	Fruit market F-10	Islamabad
9	Local market	D.G Khan
10	Local market	D.G Khan
11	Field	D.G Khan
12	Field	D.G. Khan
13	Fruit mandi	D.G Khan
14	Jallo park	Lahore
15	Greater Iqbal park	Lahore
16	Safari park	Lahore
17	Nishat fruit market	Lahore
18	Wapda town fruit market	Lahore
19	Hyperstar fruit section	Lahore
20	Lab isolates	Islamabad

3.7 Screening of samples for *Aspergillus* species

Serial dilutions were prepared for the different soil samples which were inoculated on phytase screening media to screen phytase producing fungi. Similarly, inoculation was done from infected tomatoes, grapes, and onions. Preparation of serial dilutions is described below:

i. First of all, 5g of soil was taken in a falcon and dissolved in 50ml distilled water.
ii. The sample was filtered and 1ml of filtrate was poured in 1.5ml Eppendorf tube which was labeled as stock solution.
iii. To prepare 10^{-1} dilution, 100ul of stock was dissolved in 900ul distilled water and to prepare 10^{-2} dilution, 100ul of solution was taken from 10^{-1} dilution and dissolved in 900ul distilled water. Dilutions upto 10^{-8} were prepared in the same manner.
iv. From each dilution, 50ul was picked and spread on PDA and the plates were kept in incubator for 4 days to monitor the fungal growth.
v. Subculturing of the fungus was done after 4 days. The colony of interest was picked up using inoculating loop and transferred to new PDA plate and left in incubator at 30℃ for growth. All these steps were carefully done in biosafety cabinet to ensure aseptic working conditions.

3.8 Morphological Identification of *Aspergillus*

The identification of fungus was done on the basis of morphological characteristics using compound microscope. For microscopy, the slides were prepared in the following steps:

i. To prepare the slides for microscopic examination, the biosafety cabinet was first cleaned with 70% ethanol to minimize any risk of contamination.
ii. 1ul distilled water was put at the center of the slide and spores from pure culture plates were picked up with a loop and inoculated on the water droplet.
iii. After inoculating the fungal spores, the slide was covered with a coverslip and lightly pressed to remove excess water.
iv. The prepared slides were then placed under microscope and analyzed at 40x magnification.

3.9 Molecular Identification of *Aspergillus*

Molecular identification of all the fungal strains was done starting with the total nucleic acid extraction followed by ITS based PCR using different combinations of primers. Total nucleic acid of the fungi was extracted according to the protocol reported in literature by Bhatti et al., 2012.

3.9.1 PCR amplification of ITS region.

PCR amplification of ITS region was done for the selected fungal strains using different combinations of standard primer sets and conditions. The primer sets used were ITS1F & ITS4R, ITSIF & ITS86R and ITS86F & ITS4R as mentioned in Table 3.2 below. PCR amplification was started in the thermal cycler with the first step of pre-denaturation for 3 minutes at 94°C. Then 35 cycles of denaturation were carried out for 45 seconds at 94°C. Primer annealing was

done at 58℃ for 45 seconds and extension at 72℃ for 45 seconds followed by final extension at 72℃ for 7 minutes. The PCR product was analyzed by gel electrophoresis.

Table 3.3 5’-3’ primer sequences used are enlisted in the table.

Sr.no.	**Primer Name**	**5’-3’ primer sequence**
1	ITS1F	TCCGTAGGTGAACCTGCGG
2	ITS4R	TCCTCCGCTTATTGATATGC
3	ITS86F	GTGAATCATCGAATCTTTGAA
4	ITS86R	TTCAAAGATTCGATGATTCAG

40µl reaction mixture was used for PCR and description is given in table 3.3 below:

Table 4.3 PCR reaction mixture

Reagent	**Volume in µl**
Nuclease free water	23
Taq buffer	5
$MgCl_2$	3
2mm dNTPs	2
Primer F	2
Primer R	2
Sample DNA	2
Taq polymerase	1

3.9.2 Agarose Gel Electrophoresis

i. After the total nucleic acid extraction/ PCR products, the samples were analysed through agarose gel electrophoresis as described below:
ii. To prepare 1% agarose gel, 0.5 grams agar was dissolved in 50ml 1x TAE buffer. To ensure a clear solution, the mixture was heated in microwave for few seconds.
iii. After the agarose had completely dissolved in buffer, 5ul ethidium bromide was added and the solution was carefully poured in gel caster and left for solidification.
iv. After the gel had solidified, it was removed from the caster and dipped in gel tank filled with buffer.
v. In the first well of the gel, 5ul 1kb DNA ladder was loaded and in the remaining wells, 2ul 6x loading dye was mixed with 8ul sample and loaded in the respective wells.
vi. The gel was run at 65volts and 120amp current for 120 minutes.
vii. After electrophoresis had completed, gel was visualised in Bio Top UV Transilluminator and then Dolphin Gel documentation software.

3.9.3 PCR Product Purification.

For the product purification, thermo-scientific PCR purification kit was used. The steps of PCR product purification are as follows:

i. Binding buffer and PCR product were mixed in ratio 1:1 and vortexed.
ii. For maintaining the optimum pH ,10ul 3M sodium acetate was added which resulted in color change of solution from orange or violet to yellow.
iii. The solution containing the sample was centrifuged for I minute at 13000rpm and the flow through was discarded.
iv. Then 700ul wash buffer was added centrifuged for another 1 minute.

v. Step 4 was repeated again and the flow through was discarded.

vi. Purified sample was mixed with 30ul autoclaved water and the product was stored at - 20℃.

3.9.4 Sequencing of PCR amplicons

The PCR amplicons were sequenced commercially by Eurofins Scientific Testing company through Sanger sequencing method. The obtained nucleotides were then compared through NCBI Basic Local Alignment Search Tool (BLAST) with the already reported sequences in GenBank.

3.10 Production and Isolation of Phytase *Aspergillus*

For the isolation of phytase from *Aspergillus,* the fungal colonies were first on PDA slants and then the spores from growing colonies were used for inoculation in the fermentation media as discussed below.

3.10.1 Preparation of Fungal Slants

Fungal slants were prepared by pouring PDA in test tubes and leaving the test tubes in tilted position so that the media solidifies in that position. Then fresh spores were inoculated from the edges of growing cultures. The slants were left in incubator for 5 days at 30 for maximum growth.

3.10.2 Inoculum preparation

Spore suspension was prepared by adding 5ml distilled water to the prepared fungal slants and with the help of inoculation loop, the surface of the media was scratched to suspend the spores in water. Then 1ml of the spore suspension was picked and transferred to the flask containing the fermentation media.

3.10.3 Enzyme Production by Submerged Fermentation

25ml of prepared fermentation media was poured in 250ml flask and then 1ml spore suspension was inoculated in each flask under aseptic conditions in biosafety cabinet. The flasks were kept in shaking incubator for 7 days at 130rpm and 30℃.

3.11 Enzyme Extraction

After 7 days incubation, the media was filtered through Whattman filter paper no 1 and the filtrate was further centrifuged at 6000rpm for 10 minutes. The resulting pellet was discarded, and supernatant was stored in falcons for enzyme assays.

3.12 Analytical Method for Quantification of Phytase

To detect the phosphorus released by the hydrolysis of phytic acid, colorimetric method was adopted (Gaind and Singh, 2015). Standard curve of phosphorus standard solution was plotted to evaluate the released inorganic phosphorus.

3.12.1 Standard Curve of Phosphorus

To make the phosphorus standard curve, stock solution of phosphorus was prepared in the ratio 10mg/ml. Then from the stock solution, different dilutions were prepared by taking 2,4,6,8 and 10mL of stock and raising the volume of each solution (except the tube containing 10ml stock) to 10mL by adding distilled water. 0.5mL of 0.1M magnesium sulphate solution was added in each tube. Blank was prepared by mixing 1ml distilled water and 0.5ml magnesium sulphate solution. Tubes were placed in hot water bath for 15 minutes and after 15 minutes the reaction was stopped by adding 1ml Trichloro acetic acid in each tube. After the reaction had completely stopped 1ml TCSR was added in each tube and OD was determined after which the standard curve was plotted as shown in Figure 3.3 below.

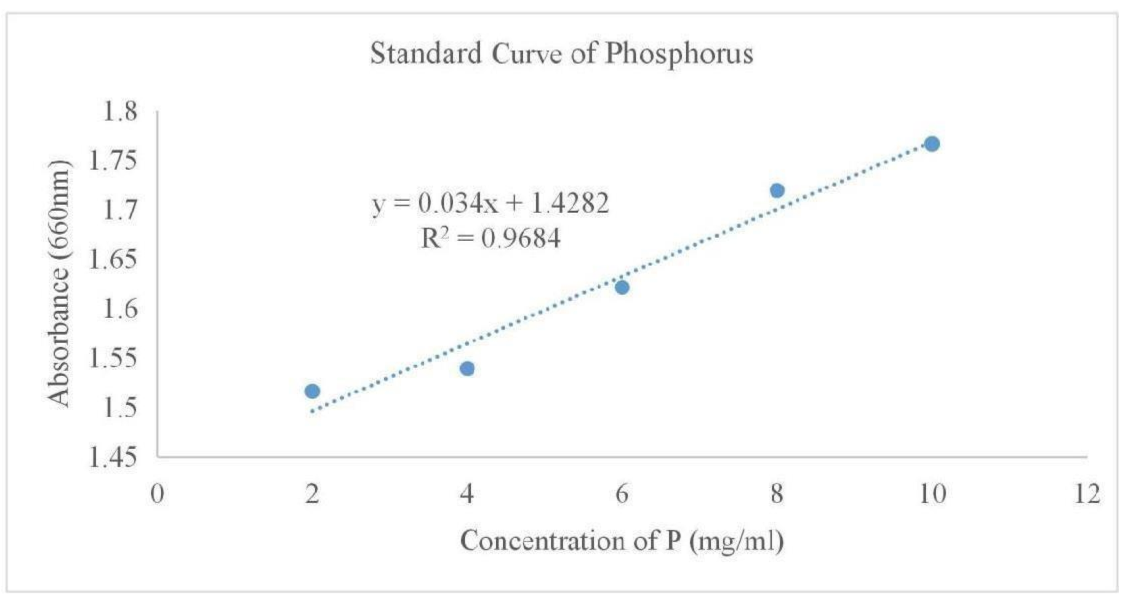

Figure 3.3 Standard curve of Phosphorus with absorbance at 660nm

3.12.2 Enzyme Assay for Quantification of Phytase

For the enzyme assay, 4 test tubes were taken and labelled as blank, control, control enzyme and experimental. Blank was prepared using 2ml distilled water only. Control tube had 0.5ml of 0.1M magnesium sulphate solution and 1ml of 0.2M substrate solution. Control enzyme tube only had 0.5ml enzyme and the experimental tube had 0.5ml magnesium sulphate solution, 1ml substrate solution and 0.5ml enzyme. Tubes were incubated for 15 minutes in water bath at 30℃.To stop the reaction, 1ml TCA was added in each tube. Mix the 2 tubes of control and control enzyme and then add 1ml colouring reagent TSCR in each tube. Measure the absorbance at 660nm through spectrophotometer.

3.12.3 Enzyme Quantification

Under standard assay conditions, the amount of enzyme required to release 1 μmole of phosphorus in one minute of reaction is defined as one unit of phytase. The formula used to calculate enzyme units is given below:

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

3.12.4 Protein Estimation by Bradford Reagent

For the estimation of total protein, assay was performed by Bradford reagent using Bovine Serum Albumin (BSA) as the standard and then the standard curve of BSA was plotted.

3.12.5 BSA Standard Curve

For making the standard curve of BSA, first the BSA stock solution was prepared in the concentration 1 mg/ml and then from the stock solution different dilutions were prepared. For dilutions, tubes were prepared by taking 0.1, 0.2 ,0.3 ,0.4 ,0.5 ,0.6 ,0.7 ,0.8 ,0.9 ,1 ml from stock and raising the volume of each tube to 1 ml (except tube with 1ml stock) by adding distilled water. 5 ml Bradford reagent was added in each tube. Blank was prepared by mixing 1ml distilled water and 5ml Bradford reagent after which all tubes were incubated at room temperature for 5 minutes. OD was taken at 595nm and standard curve was plotted shown in Figure 3.4 below.

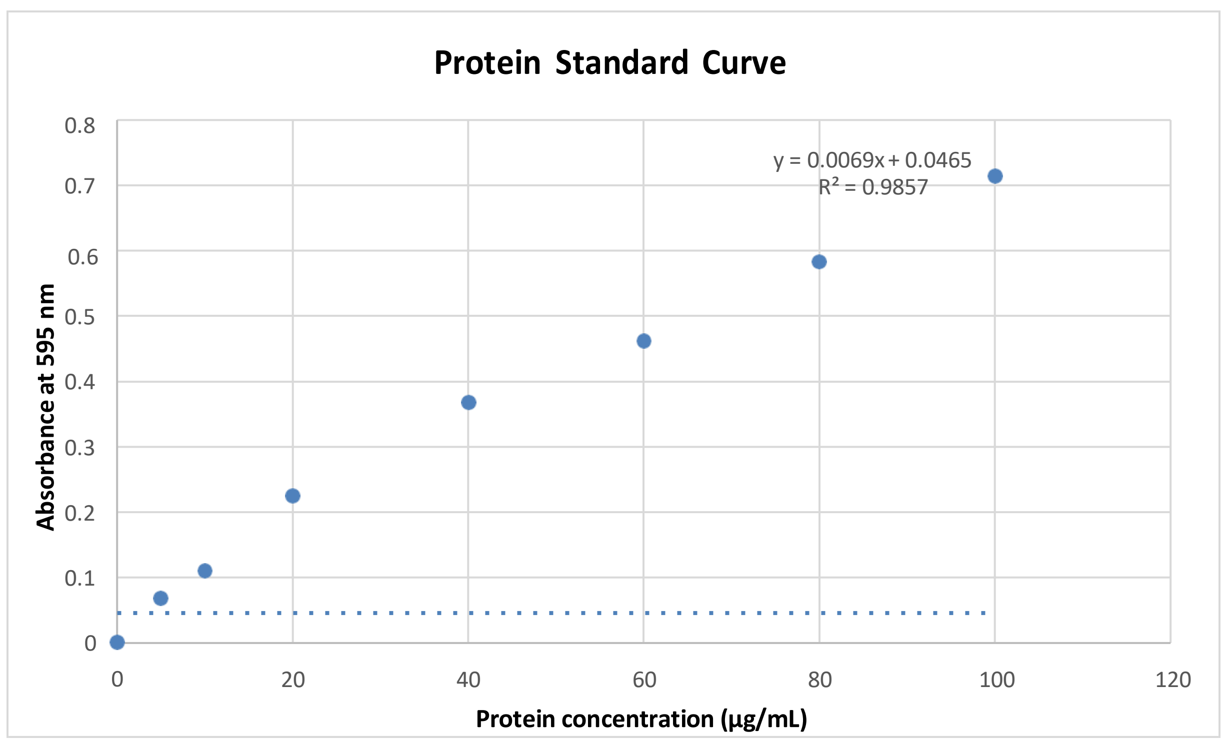

Figure 1.4 Standard curve of BSA with absorbance at 595 nm

3.12.6 Estimation of Sample Protein

The protein present in the sample was estimated using the standard curve. To prepare the tubes, 0.1ml sample was mixed with 5ml Bradford reagent and blank was prepared by mixing 0.1ml sodium phosphate buffer and 5ml Bradford reagent. Incubate tubes at room temperature for 5 minutes and measure OD at 595nm through spectrophotometer.

3.13 Optimization of Culture Conditions

For maximum phytase productions, various culture parameters were optimized which are discussed below. All parameters were optimized according to previous literature reported by Tahir et al., 2010 and Gaind and Singh, 2015.

3.13.1 Optimization of Age of Spores

In order to calculate which cultures gave maximum enzyme units, the age of spores was checked when preparing the inoculum for fermentation medium. 3,4,5,6, and 7 days old cultures were tested for maximum enzyme production.

3.13.2 Optimization of Time of Incubation

The culture flasks were incubated in shaking incubator for maximum 9 days and ODs were checked on days 2,3,5,7 and 9 to deduce that on which day the enzyme activity was maximum.

3.13.3 Optimization of Temperature

The effect of temperature was observed of the enzyme production. The culture flasks were incubated at different temperatures 25℃,30℃,35℃,40℃, respectively.

3.13.4 Optimization of pH

To get the optimum pH, the fermentation media was prepared with varying pH which were 4.5, 5, 5.5 6, 6.5 and its effect was observed on phytase production.

3.13.5 Optimization of Carbon Source

Different carbon sources were used in the fermentation media which were glucose, fructose, sucrose, and lactose. All the carbon sources were tested to check which carbon source gave most yield of the enzyme.

3.13.6 Optimization of Nitrogen Source

Yeast, peptone, ammonium sulphate and ammonium nitrate were used as the nitrogen source in the fermentation media in order to select the best yielding nitrogen source.

3.14 Phytase Production Under Optimized Culture Conditions

The final batch of fermentation media was prepared with the optimised ingredients and conditions in order to get maximum phytase production under optimised conditions.

Chapter 4

Results

4. Results

4.1 Purification of fungal colonies

The fungal colonies that were isolated from soil and plants were grown on phytase screening media to confirm that the desired fungus has the capability to produce phytase enzyme. The pictures of the purified colonies are given below in Figure 4.1.

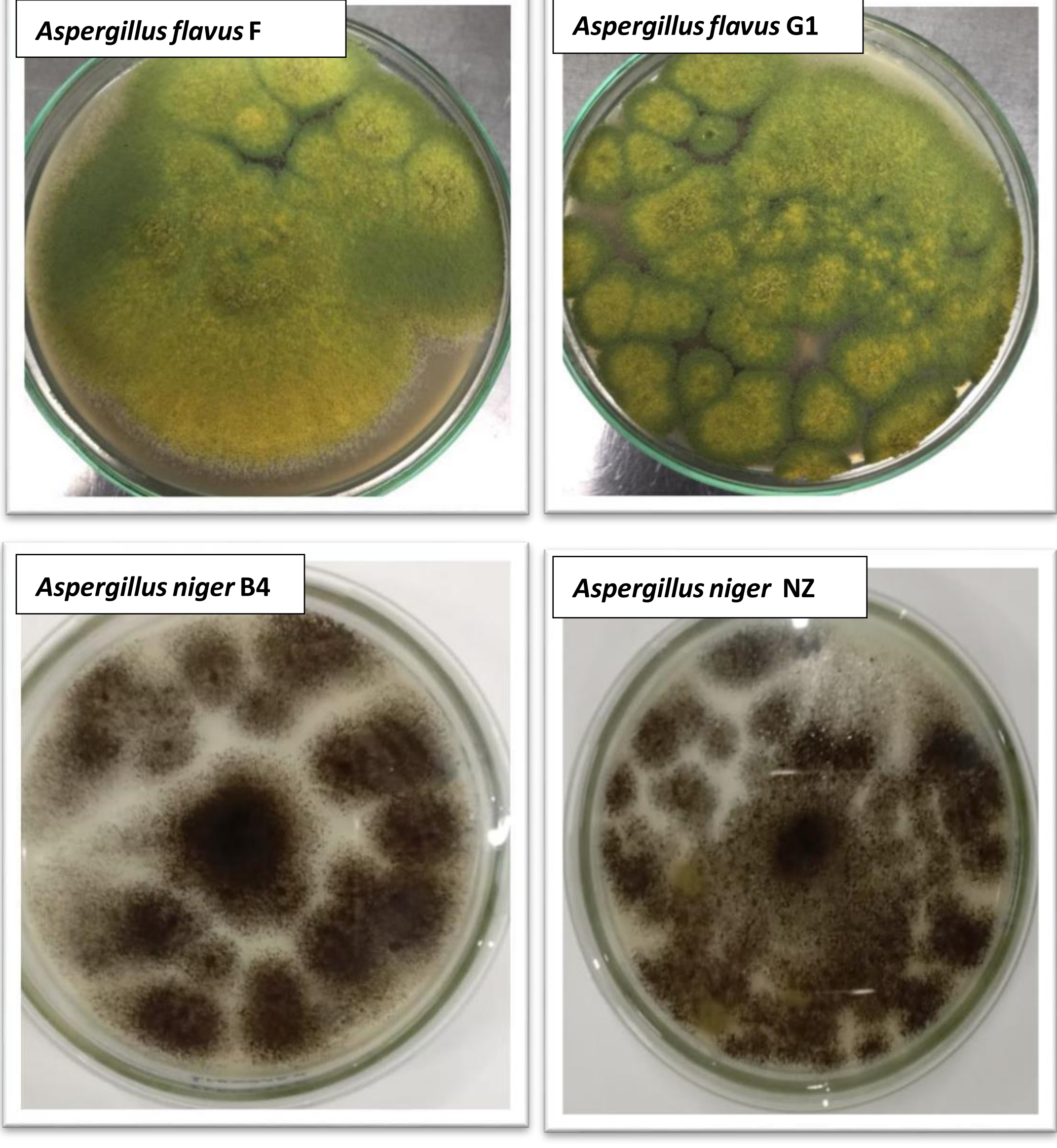

Figure 4.2 Representative Purified Colonies of *Aspergillus* species

4.2 Morphological analysis of fungi

Aspergillus niger has characteristic dark spores which are not present in any other specie of *Aspergillus*. On the other hand, spores of *Aspergillus* flavus are round and globose with smooth surface. The fungal colonies were observed under microscope and the pattern of their spores was studied at different magnification as shown in Figure 4.2 below.

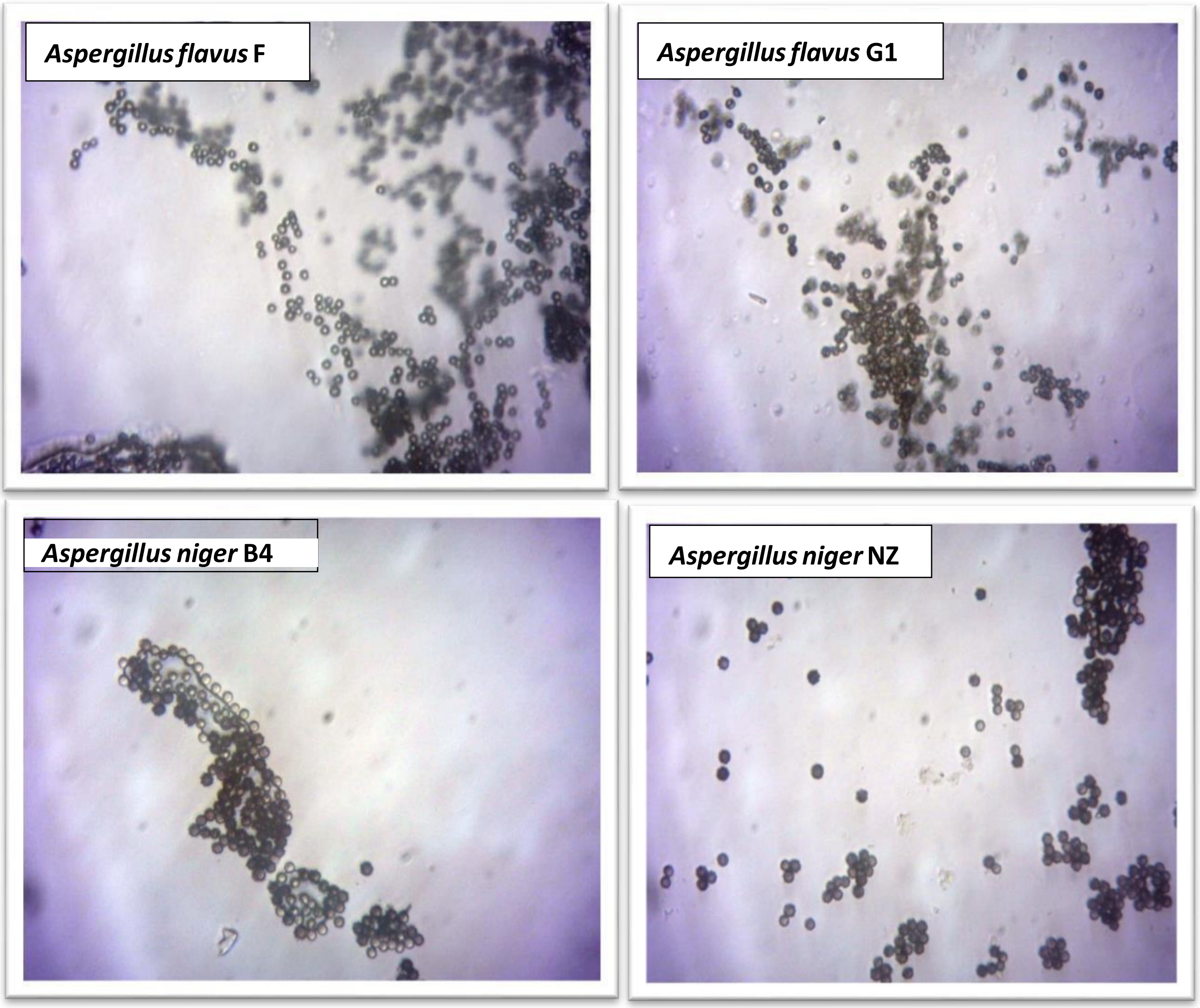

Figure 4.3 Spores of fungal colonies of *Aspergillus flavus* and *Aspergillus niger* under microscope at 40 X magnification.

4.3 Molecular identification of fungal isolates

ITS based PCR was performed for the molecular identification of the fungal isolates. Total 3 primer sets were used for the reaction ITS 1F&4R, ITS 1F&86R, ITS 86F&4R. The gel pictures are below in Figures 4.3 and 4.4.

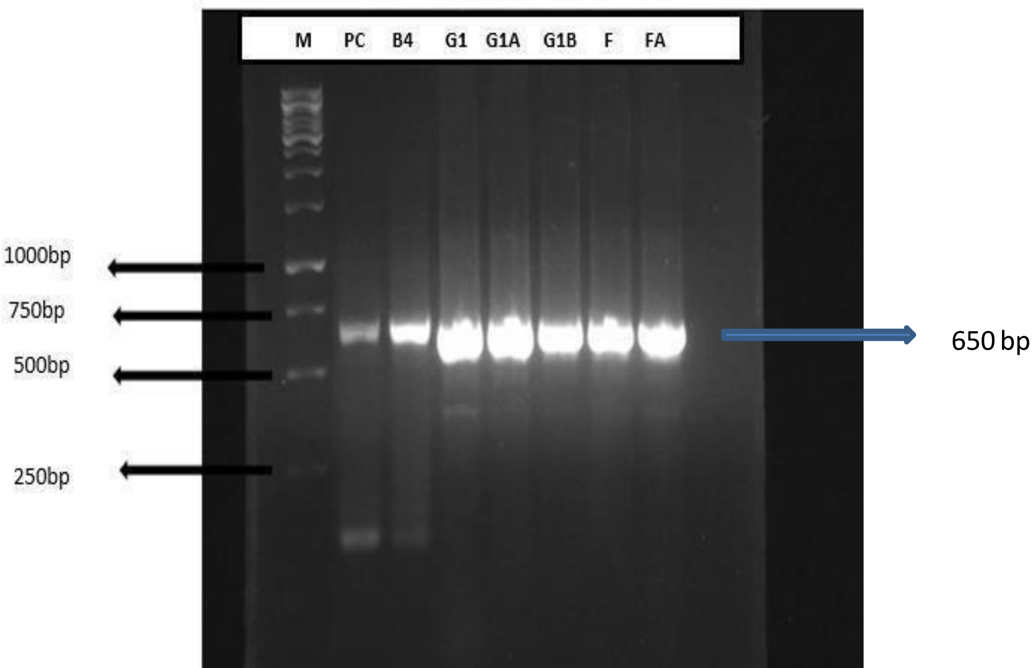

Figure 4.4 Molecular Identification of Fungi. M indicates marker 1 Kb ladder and PC is positive control. Primer set used for lane B4 to FA are ITS1 F & ITS4. All the samples show an amplification of approximately 650 bp.

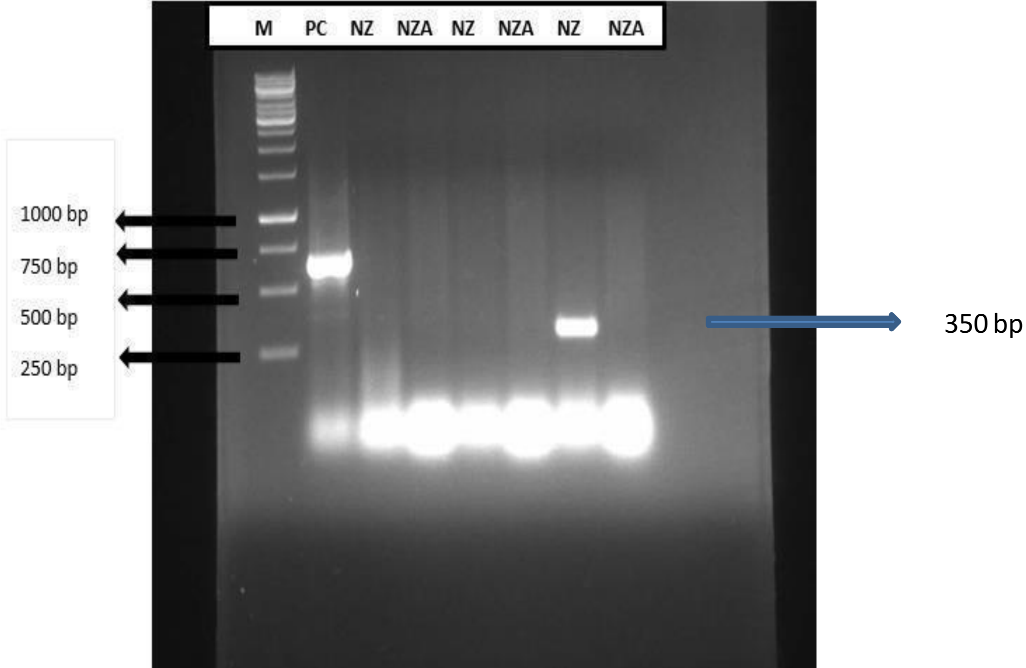

Figure 5.4 Molecular Identification of Fungi. M indicates marker 1kb and PC is positive control. Primer set used for lane 3,4 is ITS 1F&4R. primer set used for lane 5,6 is ITS 1F&86R. Primer set used for lane 7,8 is ITS 86F&4R. The sample shows an amplification of approximately 350bp with primer set ITS 86F&4R.

4.4 NCBI Sequence Submission

The forward and reverse sequences of the 4 isolates NZ, AF, B4, G1 were assembled through Multalin and after assembling the sequence were submitted to NCBI as shown in Table 4.1. The assembled FASTA sequences are listed below.

Table 4.1 Sequences submitted to NCBI

Sr. No.	Fungal Isolate	Genbank Accession ID	Linear DNA Size (bp)
1	NZ	MW426445	251 bp
2	B4	In process	235
3	G1	In process	256
4	F	In process	618

4.4.1 Assembled FASTA sequence of isolate NZ (251 bp)

>NZ

GTACGCCCACAGGNATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAA

GCCCGGCTTGTGTGTTGGGTCGCCGCCCCCTCTCCGGGGGGACGGGCCCGAAAGGC

AGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAG

GATTGGCCGGCGCCTGCCGNACANNTCTTCCNAACNCATTCNTAACCAGGCTGANA

CTCCGATCAGCTAGGGAGACCAGCA

4.4.2 Assembled FASTA sequence of isolate B4 (235 bp)

>B4

ANANNNNNNNNNNANCNCNGGNGGCATGCCTGTCCGAGCGTCNTTGCTGCCCTCAA

GCCCGGCTTGTGTGTTGGNTCGCCGTCACCNTCTCCGGGGGGANNGGCCCGAANNC

CAGNGGNGGCACCGCAAAANATACTNNAGCGAAAGGNNCNTTNNNANATNCTATG

TAANANAAANCANNNCNTNAANANGNNAACAAAACACAAAANAAGATAACNACAA

ACNANNNAAAANA

4.4.3 Assembled FASTA sequence of isolate G1 (256 bp)

>G1

ATTATCACACAAGCATCCCCAACGNNNNNNNNNCGAATTTCCCATTGAGACAAACA

CCAATNNTGTCTTGATTTACCACCCCCGTCGNNGAATGGGCACCAAACCCAGAGGC

GCCAACNGCATNCCAATCCTCGAACCGATGGGAATTTNNNNNNNGTAACCCGCCCT

GTAGACCCGGACAGCCCTCACCAAACGCAAATCAAGAGCTTCAAGGAGACCTCGGA

TCAAGAAAACAAACAAGACTCTATAACAACCC

4.4.4 Assembled FASTA sequence of isolate F (618 bp)

>F

GGCCCAAATCAGCCAAAGTACGGTGAAACTCAAAGAAAGGACCACGACCAAGTGG

AGGGTTCCTACAGAGCCCAACAACACACACGTAATAAATANACCTGAGATCCAACG

GCANNGGCCCAAAAATCATGGCCGACGGGGNNGCCCTAAGACCCGAACCAGCACC

CCAAGGAAACACAACAAACTCGAGGTGACCTAAAGAACTAAANNNGAAAACTAAA

GGGAGCCCAAGATAAAGAGCNNGAAGGGGAAACCCGACGAAAGCGACCAGAAAG

AAAGAGAAAAAAACGAAAGAAGNNGGCGCTAACGCATTNNCAAACGTACCGGTNN

NGATGAATNTAACCACCACACCCGCCNCGCATAAGACTCTACAACNNNNNNNTATC

CATACACNNATTCCGCTNNNGCAGTACGGTAAAAGAAACAACAATAACCAACAAGC

AAGACGTGNTGGAACAAGTCGTAATACCATCACCGCGAGNNNNNCACCCAAAAGA

CAGCGGCGGCACTGNGCCCGATACACNNGAGCGAATGGNGACCTGGTCACACGATC

TGTAGAACCGGCACCCCCAGGCAGAACACAAAACAACATATTCAAGGACACCTCGA

ACCAGCAAG

4.5 Phylogenetic analysis

Phylogenetic tree of the assembled sequence NZ was constructed using MEGA software Neighbor-Joining method and bootstrap value of 1000. Tree is shown in Figure 4.5 below.

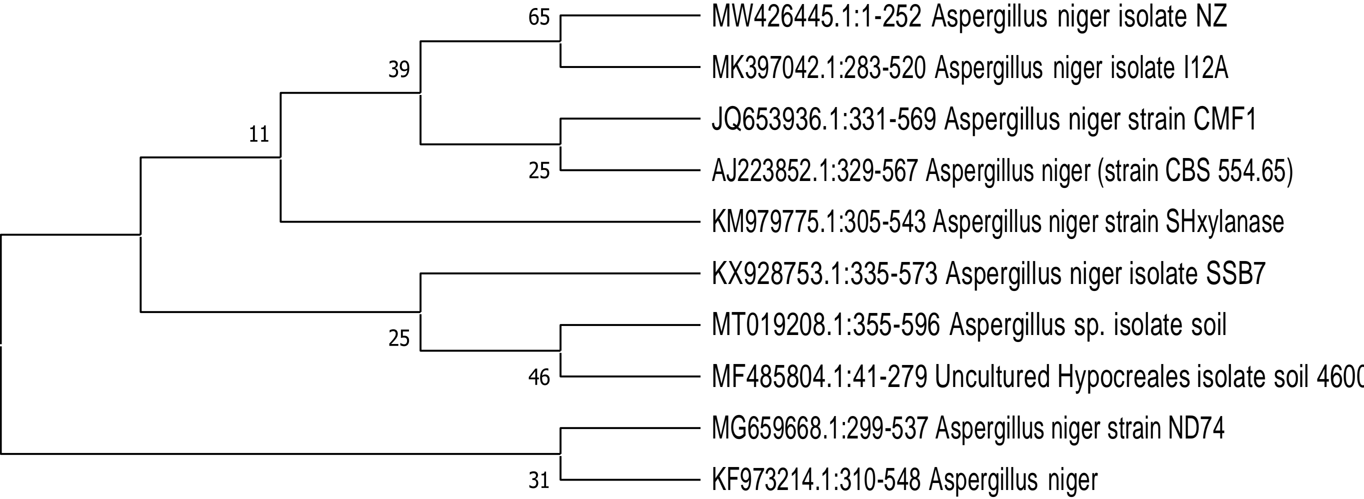

Figure 4.5. Phylogenetic analysis of *Aspergillus* niger isolate NZ**.**

4.6 Phytase Production Through Submerged Fermentation

Enzyme production was done through submerged fermentation technique. Fermentation media was prepared using various salts in different concentrations as mentioned in previous chapter. Fungal slants were prepared on PDA after which the spore suspension was prepared to be inoculated in the media flasks which were then kept in shaking incubator at different temperatures to observe growth and enzyme production. Figure 4.6 below shows the cultures after incubation period.

Figure 4.6 Fungal growth in the fermentation media after 7 days of incubation.

4.7 Optimization of Culture Conditions

For maximum phytase production, the fermentation media was optimized. The optimized parameters are discussed below.

4.7.1 Optimization of Age of Spore

Fungal slants were inoculated and incubated at 30℃ for 7 days to observe that on which day growth was the most. Maximum growth was attained after 3 days of incubation.

4.7.2 Optimization of Incubation Time

The effect of time of incubation on the enzyme production was studied (as shown in Figure 4.1). Maximum enzyme activity was observed at the 5th day of incubation after which it was observed that the enzyme production slightly decreased. The results are depicted in Figure 4.7 below.

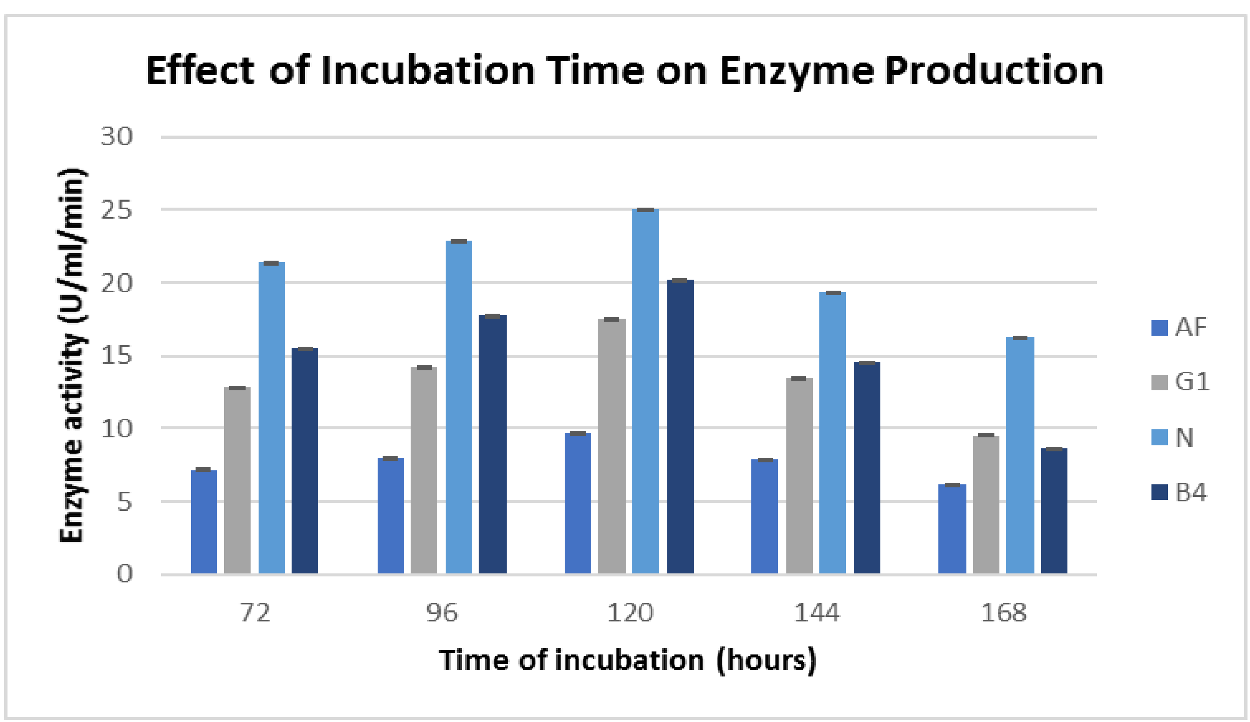

Figure 4.7 Phytase units at different incubation intervals.

4.7.3 Optimization of Temperature

The effect of temperature was studied on phytase production by incubating culture media over temperature range of 25℃-40℃ under submerged fermentation. Results showed that the phytase production was maximum at 30℃ for all fungal species with maximum units produced by *Aspergillus niger* strain N (23.2 units) followed by B4 (21.18 units). Many researchers have reported that the optimum temperature for phytase production is 30 [48][50][51]. A rise in the optimum temperature resulted in decrease in phytase production as shown in Figure 4.8.

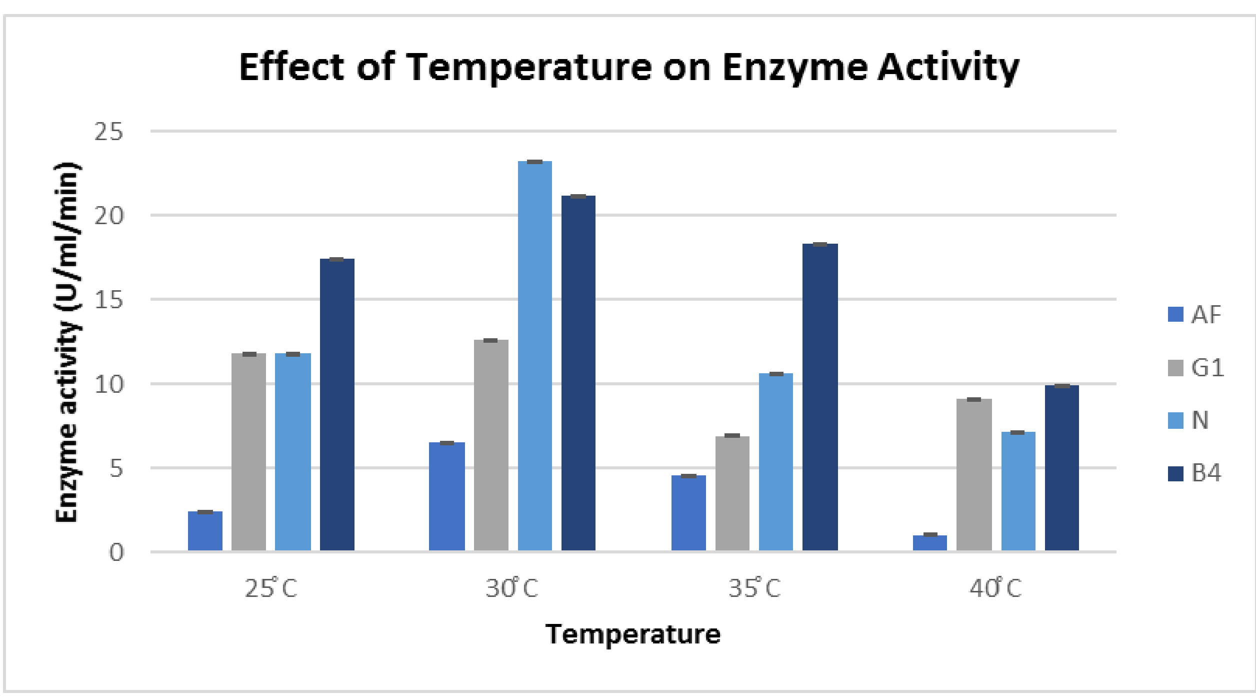

Figure 4.8 Enzyme units at different incubation temperatures.

4.7.4 Optimization of pH

The production of phytase enzyme in all fungal isolates increased from pH 4.5-5.5 and the optimum pH for phytase under submerged fermentation was recorded to be 5.5. Further increase

in pH resulted in decrease in the enzyme production. According to results reported by a study, strain 89 of Aspergillus *niger* gave maximum phytase production at pH 3 whereas Aspergillus *oryzae* gave maximum enzyme units at pH 6.5 [54]. Figure 4.9 below shows the change in enzyme units with change in pH.

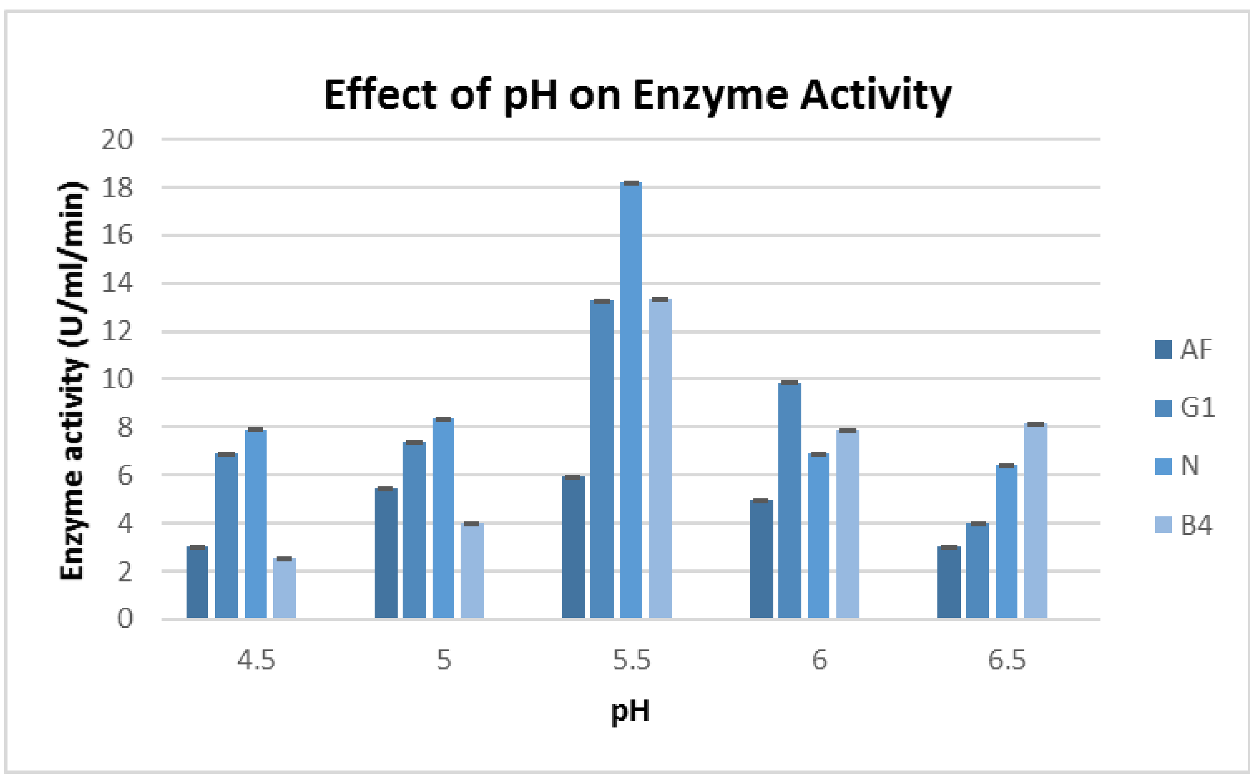

Figure 4.9 Enzyme units at different pH.

4.7.5 Optimization of Carbon source

Different carbon sources such as glucose, fructose, sucrose, and lactose were used in the fermentation media to pick the best. It was observed that glucose was the best carbon source which gave maximum production of phytase. Maximum units of phytase were produced by Aspergillus *niger* strain B4 (21.6 units) followed by another strain of Aspergillus *niger* B4 (19.18 units). The results are shown in Figure 4.10 below.

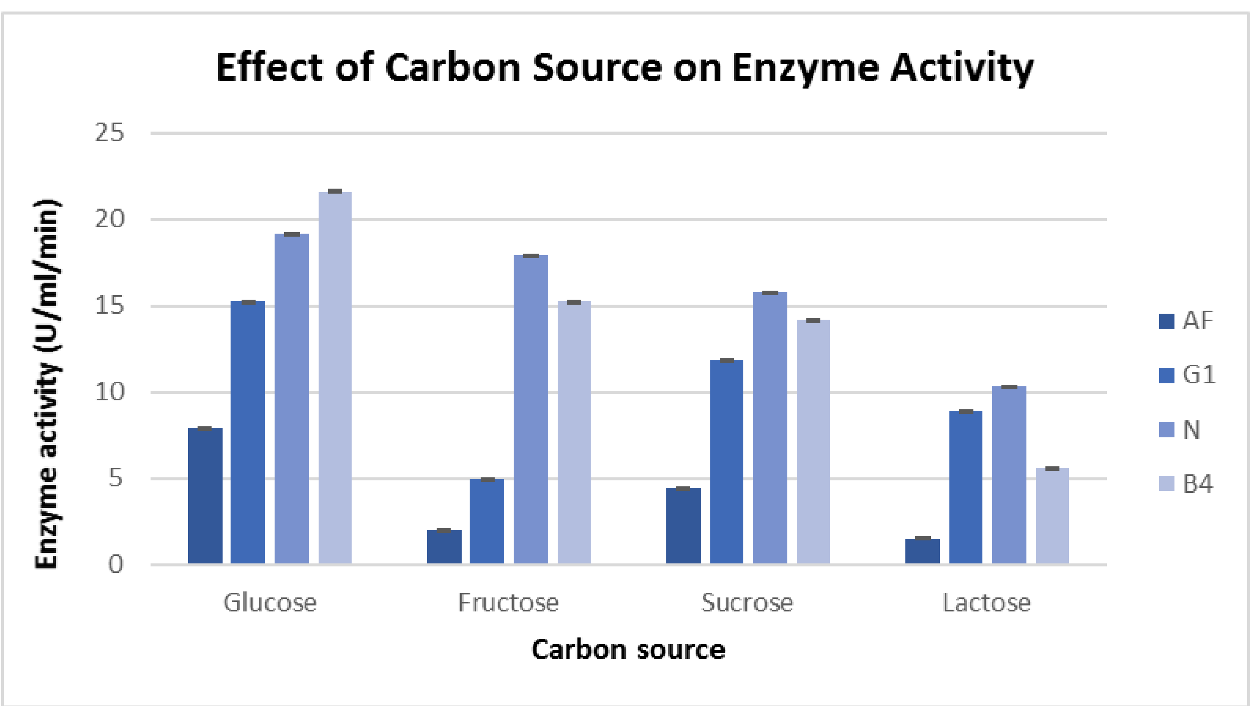

Figure 4.10 Enzyme units at different carbon sources.

4.7.6 Optimization of Nitrogen source

To study the effect of nitrogen source on phytase, the fermentation media was supplemented with different organic sources including yeast, peptone, ammonium sulphate and ammonium nitrate. Among all the sources, the best nitrogen source was ammonium nitrate which gave the maximum titre of phytase in all fungal species. Results shown in Figure 4.11 below.

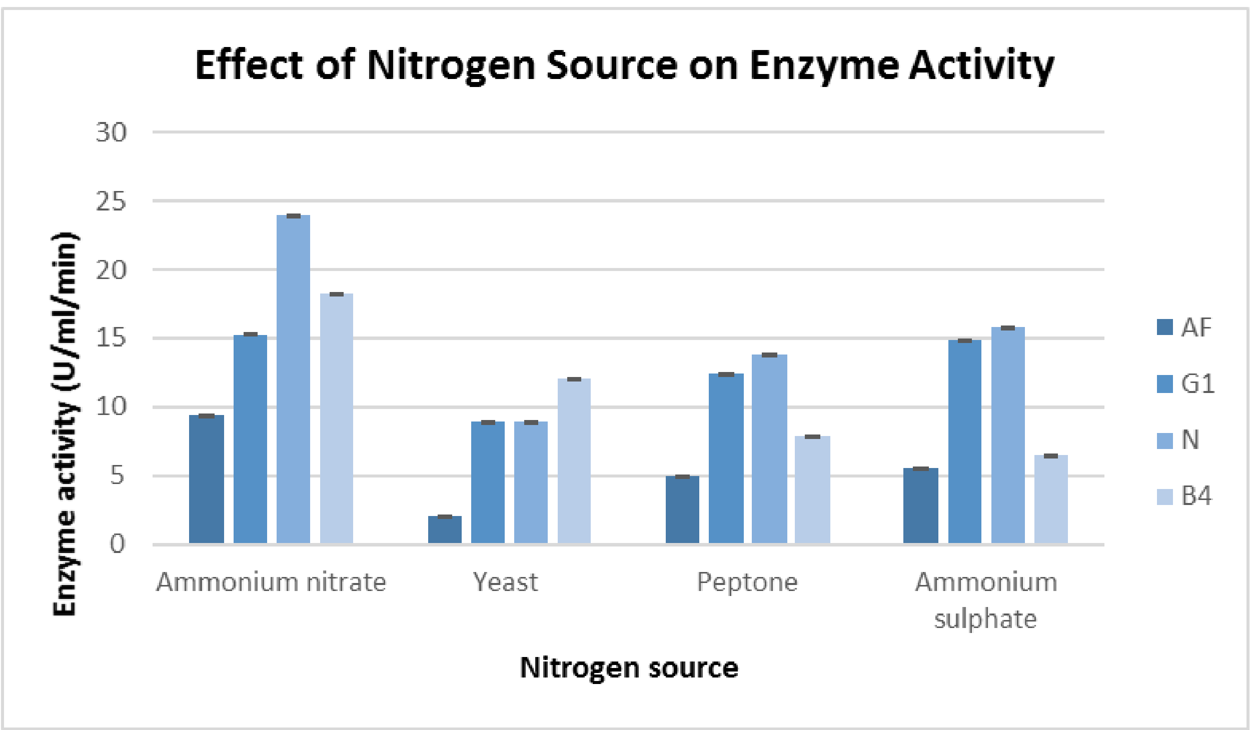

Figure 6.11 Enzyme units with different sources of nitrogen.

4.8 Enzyme production under optimized conditions.

After all the parameters were optimised, a final batch of fermentation was run to observe the units of phytase produced under optimum conditions. The Table 4.2 below enlists the optimum conditions for the enzyme production by the respective fungal species.

Table 5.2 Enzyme production under optimum culture conditions.

Culture conditions	**Fungal sample**			
	F	MG1	NZ	B4
Age of spore (days)	3	3	3	3
Time of incubation (h)	96	96	96	96
Temperature	30℃	30℃	30℃	30℃
Ph	5.5	5.5	5.5	5.5
Carbon source	Glucose	Glucose	Glucose	Glucose
Nitrogen source	Ammonium nitrate	Ammonium nitrate	Ammonium nitrate	Ammonium nitrate

Rest of the conditions of fermentation remained same as mentioned above (Recipe of fermentation media in chapter 3).

4.9 Phytase Units Produced Under Optimized Culture Conditions

The units of enzyme produced by the fungal samples under optimised fermentation media is given in figure 4.12 below.

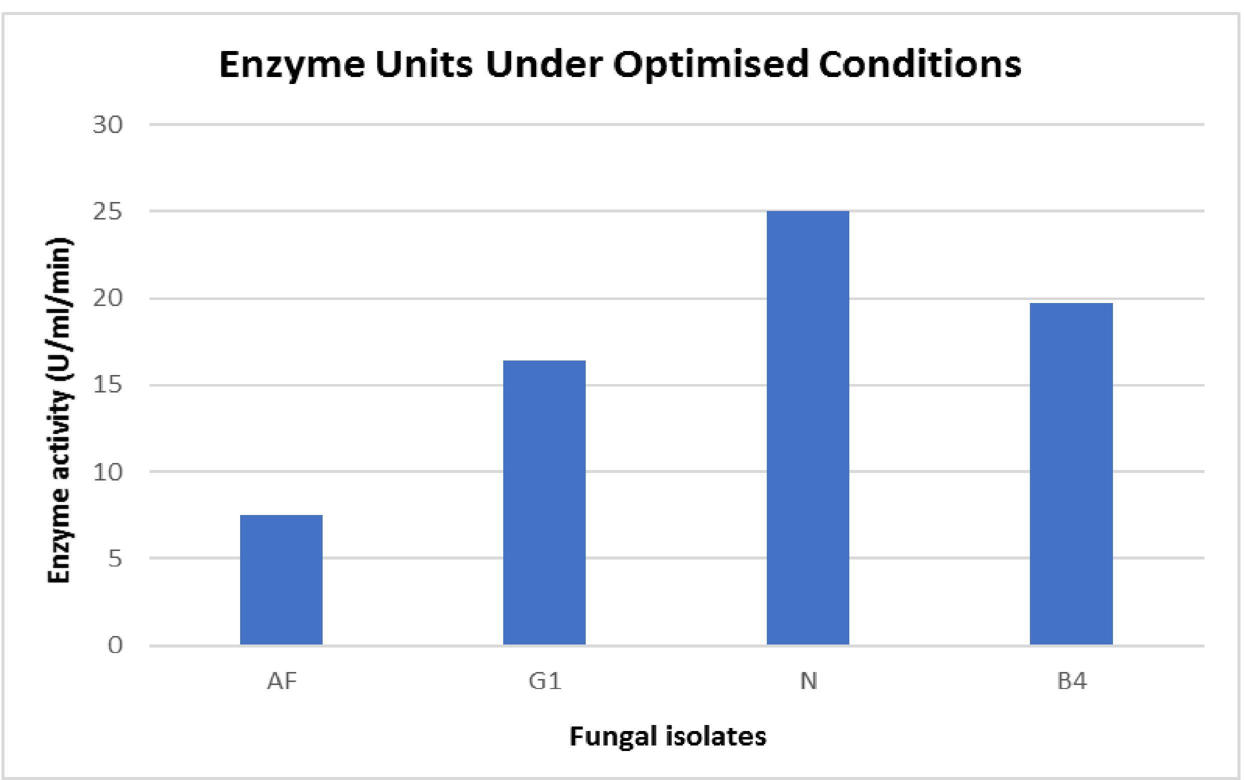

Figure 7.12 Enzyme units under optimized culture conditions.

4.10 Estimation of Phytase Produced through Bradford Assay

The concentration of enzyme is calculated by Bradford reagent. The table 4.3 below enlists the concentration of enzyme extracted from the fungi.

Table 6.3 Enzyme estimation after Bradford assay.

Fungal isolate	**Concentration mg/ml**
F	21.11
NZ	28.3
MG1	26.7
B4	26.7

Chapter 5

Discussion

5. Discussion

Soil and fruit samples were collected from different areas of Punjab and Federal capital for the screening of phytase producing fungi. The primary screening of fungal species was done on PDA by preparing serial dilutions of soil and inoculating on media plates. From the fruit samples, the fungus was directly picked from inoculating loop and point inoculated on PDA plates. After the primary screening, secondary screening of the desired fungal species was done on phytase specific media to check whether the fungus of interest is capable of producing phytase enzyme ornot because PSM only promotes the growth of phytase producing fungi. Similar screening strategy has been previously reported by scientists where the fungus was primarily cultured on PSM since this media specifically contains phytic acid which is the substrate for phytase and it only promoted the growth of phytase producing fungi [39][50]. After the desired colonies were grown on PSM, PDA slants were prepared which were further used for inoculum preparation. The fungal species were initially identified on the basis of morphological characteristics after which they were subjected to ITS based 18s rRNA sequencing. The species were identified as Aspergillus *flavus* and Aspergillus *niger*. For the preparation of fermentation media, various saltswere used, and conditions were optimised for maximum enzyme production through submerged fermentation. 3 days old culture slants were used as active cultures for the preparation of spore suspension for inoculation in fermentation media. Researchers have reported maximum phytase production form *Aspergillus flavus* when 72 hours (3 days) old cultures were used [11].

The optimum incubation time for maximum enzyme units was recorded 5 days. All the fungal species produced maximum enzyme on the 5th day of incubation. Maximum units after the incubation time were produced by *Aspergillus niger* isolate N (22 U/ml). 38.5 (U/ml) of phytase were recorded at the 4th day of incubation by *Aspergillus niger* after submerged fermentation [39] and according to other reported results, 68 U/ml phytase activity was recorded by *Aspergillus niger* NCIM 563 after 11 days of incubation period [7]. Aspergillus *flavus* strain G1 produced 17.5 units of phytase through submerged fermentation on the 5th day of incubation and strain AF produced 9.7 units of phytase under same conditions. Maximum phytase 1.916 units have been reported by *Aspergillus flavus* after 6 days of incubation through solid state fermentation [11].

Carbon source for the fermentation media was also optimised and the best yielding carbon source was glucose as suggested by many other researchers. Maximum enzyme units were produced by Aspergillus *niger* strain B4 21.6 units followed by another strain (of same species) N which gave 19.18 units of phytase. Aspergillus *flavus* strain G1 yielded 15.26 enzyme units with glucose. This outcome is supported by studies carried out by other researcher for instance, it was observed that glucose is the best carbon source for phytase units from *Aspergillus niger* isolate NCIM 612 [10].

Ammonium nitrate was found as the best nitrogen source among other nitrogen sources that were tested. It gave maximum yield with 24 units of phytase from *Aspergillus niger* N and *Aspergillus flavus* G1 gave 15.26 phytase units with the same nitrogen source. Similar results have been reported by scientists [50]. Malt extract has been concluded as the best nitrogen source for phytase production through solid state fermentation by *Aspergillus flavus* yielding 93.10 enzyme units per gram of substrate [11].

Another important factor which was optimised for maximum enzyme production was temperature. The optimum temperature at which all the strains gave good yield of enzyme was recorded 30 . This observation is supported by results reported by studies which state that *Aspergillus niger* gave maximum enzyme production at 30 under submerged fermentation after which the enzyme units decreased [39]. Similar findings are reported by Gull et al., 2013 that suggest that *Aspergillus niger* and *Aspergillus flavus* gave maximum activity at 30 under solid state fermentation.

pH 5.5 was recorded to be the optimum pH for all fungal strains for maximum production of phytase enzyme. *Aspergillus niger* strain N produced 18.2 phytase units followed by strain B4 which produced 13.35 phytase units at pH 5.5. This finding is supported by results reported by other researchers. Research suggested that phytase production of *Aspergillus niger* increased between pH 3-5 after which a decrease in production was observed both in solid state fermentation and submerged fermentation [39]. Other researchers have reported their findings which support the fact that phytase production is maximum when the pH of fermentation media is acidic to neutral. Since each organism has its own specific pH at which it yields maximum enzyme therefore, any change in the optimum condition results in change in the units of product produced [47].

To upscale the production of phytase for industrial application, the locally isolated *Aspergillus* species may be subjected to various cloning and expression based genetic modifications. For the characterization and purification of isolated enzyme, Next Generation Chromatography (NGC) may be performed in order to get fractions of the purified protein sample. 2D gel electrophoresis and SDS may be done to determine the size of the isolated protein.

Chapter 6

Conclusion

6. Conclusion

From the study it can be concluded that *Aspergillus niger* is a potent source of phytase enzyme with isolate NZ yielding maximum enzyme units i.e. 25 U/ml under optimized fermentation conditions. Moreover, the phytase production is greatly influenced when the fermentation conditions are optimised resulting in enhanced production of enzyme units. Various parameters significantly affect the enzyme activity by either increasing or decreasing the production. Current study and further research in this domain will help in upscaling the biosynthesis of phytases on industrial level which will further assist in utilizing the enzyme in different industrial sectors. For example, in the poultry feed, the phytase enzyme can be broadly used as a feed supplement for the breakdown of phytic acid and absorption of phosphorus in animals and this in turn can prove to be very beneficial and significant for nutritionists and environmentalists.

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