Isolation and Partial Characterization of Phytases Obtained from *Botrytis cinerea* and *Fusarium oxysporum* Species for Their Potential Use in Industrial Application

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A thesis submitted in partial fulfillment of the requirement for the degree of MS Plant Biotechnology

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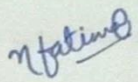

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Dedication

Dedicated to my Dearest Mama, Papa, my beloved Husband, my grandparents, and my lovely siblings who have never left my side and for being there for me throughout this course to achieve my target.

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Nitasha Fatima

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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 are enzymes utilized as feed additive substance that improve the phosphorus and mineral take-up in monogastric animals and lessen the degree of phosphate discharge in their manure. Fungal extracellular enzymes are easy to cultivate and due to their high production fungi are consider one of best wellsprings of phytase that can be used in the feed business. The present study deals with morphological and ITS based molecular identification of phytase producing plant pathogenic fungal strains. Samples were taken from lab and some samples were collected from area of Gujranwala to get phytases producing fungi on phytase screening media.18S rRNA based sequencing of selected strains was performed and identified as *Botrytis cinerea* and *Fusarium oxysporum.* Fungal cultures were subjected to submerged fermentation to find out total units of enzyme produced. Various culture conditions including incubation time, temperature, pH, source of carbon and nitrogen were optimized to get maximum enzyme yield. Maximum enzyme units were achieved with glucose as carbon source, ammonium sulphate and ammonium nitrate as nitrogen source ,6 days of incubation, 25℃ incubation temperature with acidic pH 5 of fermentation media. All strains produce different units *Botrytis cinerea* strains KST6 and KST51 gave maximum units (31.73U/ml) and (27.36 U/ml) of phytase while *Fusarium oxysporum* strain NF1 yielded 24.81 U/ml through submerged fermentation under optimized conditions.

CHAPTER 1

Introduction

Introduction

1.1 Enzymes and their Role n Living World

Concept of life without enzyme is back breaking. Enzymes are known as biocatalyst with magical powers of enhancing the rate and speed of biochemical reactions in living bodies. Speed of number of important biological and commercial processes can be increased using enzymes (Robinson, 2015). Technology enables easy access and use of enzymes in food and non-food industries by extracting and purifying it from biological materials. Enzymes are biological substances, so they belong to biological origin such as plants, animals, and microorganisms, among which microbial enzymes account for most of the industrial enzymes.

One can extract enzymes from multiple sources such as microbes, plants, and animals but microbes are considered the most systemic and stable to get enzymes. Microbial enzyme can culture in short time with high yield by fermentation due to their sensitivity to gene manipulation and biochemical diversity. To fulfill the current enzyme requirement Industries are looking for new microbial sources (Anbu, Gopinath, Cihan and ChaμLagain,2013). Enzymes found their utilization in number of important products production, such as bakery products, detergents, fermented products, pharmaceuticals, clothing products, leather processing and beer production (Singh, Kumar, Mittal and Mehta, 2016).

1.2 Enzyme Production through Fermentation.

Enzymes has been used in number of food and other industries for thousands of years. Fermentation has been used for long time to produce useful substances including enzymes and fermentation has gained importance over years. Phytase is enzyme used in feed industry for animals such as poultry,

swine, and fish. With ideal condition and substrate bacteria and fungi give high yield of enzyme using fermentation. Bacteria fungi and yeast are good producer for phytase enzyme.

Solid state fermentation along with submerged fermentation are main types of fermentation used commercially to produce enzymes. In submerged fermentation microbes are cultivated in liquid medium or broth whereas in solid-state fermentation growth of microbes is supported by solid substrate that include bran of grains such as rice and wheat.

1.3 Major Environmental species of *Botrytis*

Species of *Botrytis* have their effects on broad range of plants ranging from general to specialized plant pathogen. ***Botrytis*** is an anamorphic fungus and belong to Ascomycota. The other name for botrytis is grey mold due to its grey color hyphae appearance. This fungus has almost 30 species. Maximum species of botrytis infect fruits such as grapes and strawberries other affect dicots such as beans and lentils some of them also infect onions,tulips and lilies), (Batt and Robinson, 2014).

Important candidates of botrytis include *Botrytis allii, Botrytis carnea , Botrytis cinerea ,Botrytis fabae, Botrytis artocarpi, Botrytis anacardia, Botrytis ampelophila* (MicroscopeMaster, 2020).From above mentioned species *Botrytis cinerea* has ability to affect wide variety of fruit plants along with range of ornamental plants and vegetables.

1.4 Poultry Industry in Pakistan

In Pakistan poultry was established in 1962 at commercial level. This industry is playing great role in minimizing the gap between demand and supply of meat protein. Poultry industry is one of the major agricultural based industry of Pakistan with investment of 1,168 Billion rupees. With the constant reduction in supply of red meat, poultry provide cheapest replacement of protein source.

Directly or indirectly poultry industry provides source of income to 15 lac people of Pakistan (An Overview of Poultry Industry, 2020).

One of the most important sectors of poultry industry is feed. Ingredients that are mostly used in feed are plant based rich in phosphorous content such as cereal grains, protein meals, oil seeds and some minerals. Phosphorus is foremost nutrients required for chicken growth. Feed additives from plant source have fifty to eighty percent of their total phosphorus in form of phytate (Harland and Morris, 1995). Naturally occurring compound phytate in plant sources restrict the availability of Phosphorus (P.) Minerals and phosphorus when bound to the phytate become poorly available to monogastric animals (Broz et al., 1994).

1.5 Role of Phytase in Feed Industry

Phytate is also known as myo-inositol hexakisdihydrogen phosphate. Phytic acid contain 6 phosphorus atoms bound to a carbon ring. Phytate is an anionic acid which have anti digestible properties (Yoon, Thompson and Jenkins, 1983). Animals need phosphorus for their optimal growth which is unavailable to them because cereal grains and oilseed meals contain phosphorus in phytate form. Absence of ample amount of phytase enzyme in gastrointestinal tracts of monogastric animals cause unavailability phytate phosphorus (Gontia-Mishra et al., 2013)

Globally feed enzyme production level is increasing due to increase in rate of rearing of poultry, swine and other monogastric. The feed enzymes have vital role in suppressing the antinutritive activity of phytic acid and other phenolic compounds. Phytases (myo-inositol hexakisphosphate phosphohydrolases) belongs to distinct class of phosphatases which helps in the hydrolysis of phytates to digestible inorganic phosphates (Vats and Banerjee, 2004).

Poultry needs to digest feed phytate to reduce its anti-nutritional effects by enhancing inorganic phosphorus content of feed. Phytase release phosphate to elevate nutritional quality of feed components for simple stomach animals (Konietzny and Greiner, 2004). Microorganisms produce phytase that is used as feed supplement to control ecosystem related and nutritional distress due to phytate (Gontia-Mishra et al., 2013)

Phosphorus present in phytate is necessary for the development of bones in poultry and to protect ecological system from pollution from excessive manure phosphorus runoff. Our local industries are importing most of the enzymes from other countries. These enzymes can be produced locally & this can reduce the import cost. With expanding consumption of phytase for poultry feed industry and keeping in analysis the commercial importance of phytase.

1.6 Need of Current Research

Present study was conducted for production of phytase enzyme from different strains of *Botrytis cinerea* and *Fusarium oxysporum.* Potential of strains of Botrytis cinerea for phytase production was unexplored. This study was conducted to identify the phytase yielding capacity of *Botrytis cinerea* and *Fusarium oxysporum* and to produce economically and ecologically efficient phytase on industrial scale in Pakistan, using solid state fermentation to achieve following objectives:

1.7 Objectives:

- Identification & isolation of environmental strains of ***Botrytis*** and ***Fusarium species***.
- Extraction of enzyme from ***Botrytis*** and ***Fusarium species***
- Improving enzyme production by optimizing various factors including pH incubation time, C and N source etc.

CHAPTER 2

Review Of Literature

Review of Literature

Enzymes are natural living substance that without being used give boost to biochemical reactions in living organism. Enzymes are highly specific in their action. Nearly all of the life related chemical reactions going on in all kinds of living entities including human beings depend on enzymes. These molecules are responsible for millions of metabolic reactions that sustain life. Maximum enzymes are biodegradable. Regardless all enzymes are produced within living cells, but they can show their performance outside of cell as well making them useful in industrial processes and enzymes preoccupancy in food processing (Kuddus, 2019). Enzymes have good record of use in food and feed industries along with other important industries. In past few decades, enzymes have found their applications in various commercial processes such as baking, brewing, detergents, paper and medicine. They are also used frequently in processing pharmaceuticals, textiles, leather artificial sweeteners, cheese, meat etc. They also find their application in medicine and animal feed additives. Animal feed industries are using feed enzymes that play important role in world agro-industrial activities. Globally feed enzyme production is growing vigorously with increase in rearing of monogastric animal such as swine poultry and pigs. Enzymes used by animal feed industry help in breakdown of crude fiber, starch, proteins, and phytates to enhance nutritive quality of feed and suppress antinutritive activity of phytic acid and other phenolic compounds.

2.2 Phytic acid: Overview

Primary storage form of phytic acid in number of oilseeds and grains is phosphorous and named myo-inositol hexaphosphoric acid, IP6. Fifty to eighty percent of phosphorous contents in cereals and grains is in the form of phytate. Phytic acid is fiber-linked component of wheat, rice bran and legumes. Cereal, legumes, and oil seeds contain salts of phytic acid that act as chelating agents

(Tomschy et al., 2002). Phytic acid affect the nourishing and healthful effect of seeds by forming bond and complex with minerals and proteins that are necessary for nourishment of seeds (Bohn, Meyer, and Rasmussen, 2008).

2.2.1 Sources Occurrence and Distribution of Phytic acid.

Series of phosphorylation steps are involved in synthesis of phytic acid. It is synthesized from myo-inositol limited information is present in literature about the presence and functions of the intermediates in phytic acid biosynthesis within cell (Figure 2.1). Phytic acid is present in seeds and nuts especially in the bran or outer hull. Tubers contain phytic acid too. (SAMOTUS, 1965). Trace amounts of phytic acid also show its availability in some fruits and vegetables. Salts of phytic acid that are phytate mostly show their occurrence in plant seeds and phytate are present in animal and soil. Main source of acid from where they originate are natural mineral sources.

Phytic acid is present in multiple parts of grain as a cation such as K^+, Mg^{2+} and Ca^{2+}. Aggregation of phytic acid into the seed occur during the ripening period. Phytate is formed during the process of maturation of plant seeds and grains (Coulibaly, Kouakou and Chen, 2010). Phytate also subsist at variable sites in protein depositing vacuoles in some of plant seeds. It exists in the aleuronic layer of cells and embryo of grains in wheat, rice, and barley, (Bohn, Meyer and Rasmussen, 2008). Germ of corn kernel contain maximum phytate about 80-90 percent. Cereal contain phytic acid in outermost layer of. Studies showed that leafy vegetables and fruits lack phytic acid content. Most important and highest phytase producers are seeds and bran that is more than 5 times of some of highly indigestible varieties of soybeans (Coulibaly, Kouakou and Chen, 2011).

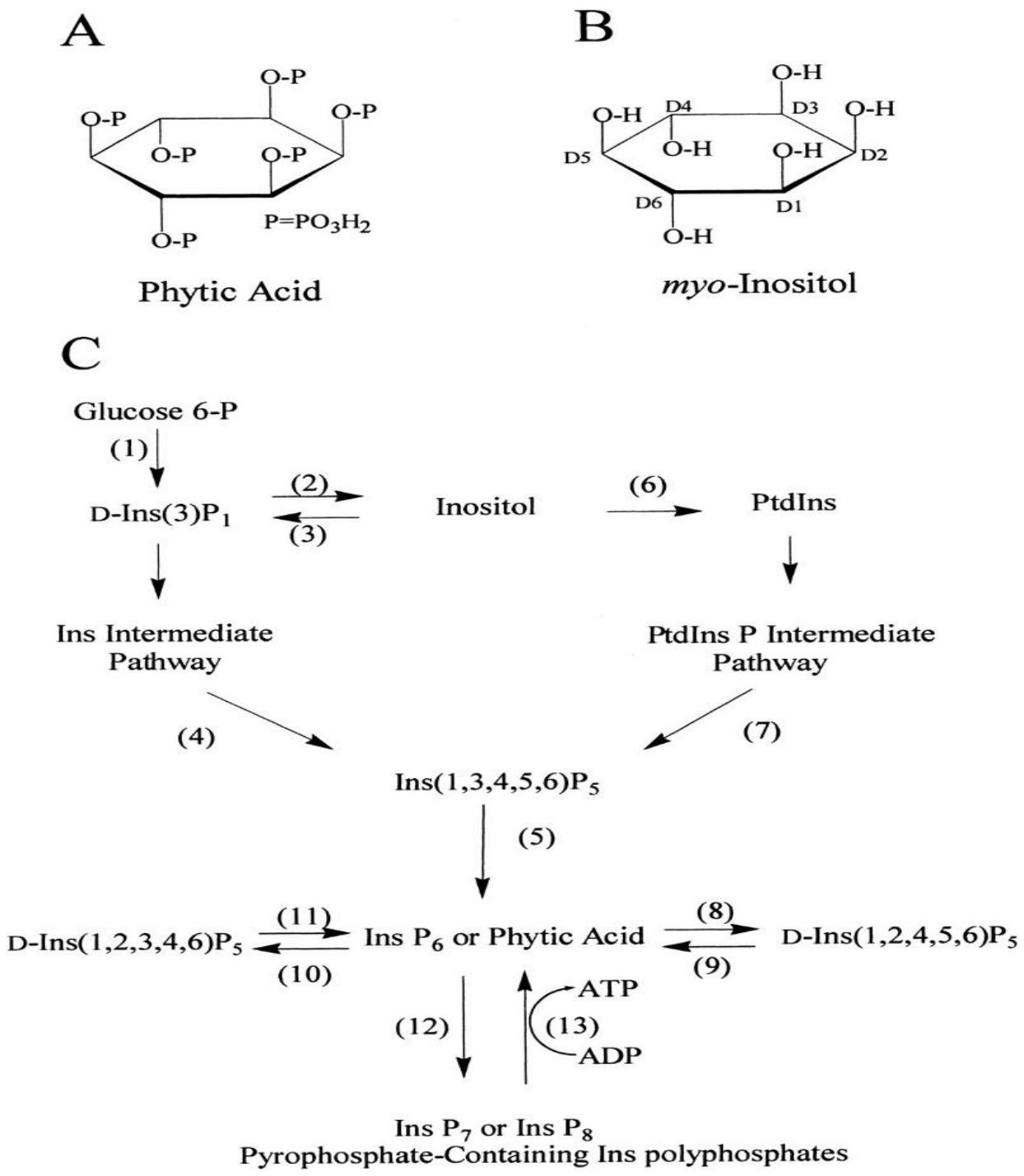

Figure 2.1 Phytic acid synthesis pathway (myo-inositol-1, 2, 3, 4, 5, 6- hexakisphosphate or IP6) in cell of eukaryote: (A) phytic acid structure. (B) Inositol structure. (C) Biochemical pathways (Loewus and Murthy, 2000).

2.2.2 Physiological Function of Phytic acid

Phytic acid also known for storing energy in the form of cations (Phillippy, Lin and Rasco, 2004). Phytic acid occur as salt that rapidly accumulate in grains and seeds when they are near to mature.Phytic acid is a unique molecule with variety of functionalities that included its role in DNA repair, transport of RNA and vascular trafficking and cell signaling (Bohn, Meyer and Rasmussen, 2008). Dormancy of seeds is due to chelation of some important minerals and phosphorus that play their role in seed germination. After chelating to phytate germination process is ceased. (Doria et al., 2009). Phytic acid also play some physiological functions in plants such as phosphorous, energy and cations storage, cause dormancy of seeds etc. Along with these it also performs some other critical functions in seed. (Bohn, Meyer and Rasmussen, 2008).

2.1.3 Molecular structure of phytic acid

Ring structure of phytate contain carbon molecules and each ring has phosphate group in it as shown in (figure 2.2). This ring structure contain 6 phosphate molecules (Yao et al., 2011). The exact structures for phytic acid have been derived from X-ray analysis (Blank, Pletcher and Sax, 1971) and 31P-NMR (Johnson and Tate, 1970). Inositol is present in phytate which is a hexa-hydroxy cyclohexane in a chair configuration with six phosphate ester bonds. Negative charge on this molecule is due to presence of phosphate group due to which it act as chelating agent and amino acids and minerals bioavailability in diet reduced (Haros, Bielecka and Sanz, 2005).

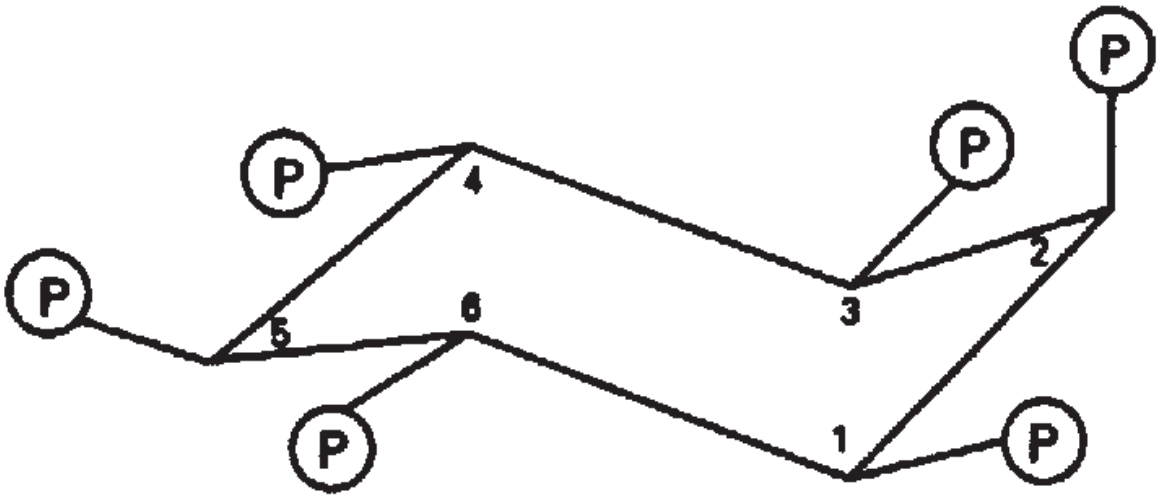

Figure 2.2 Molecular structure of phytic acid

2.1.4 Plants based phytases.

Mature seed contain organic phosphorous in the form of phytic acid. Phytic acid is phosphate storage house with about 70 percent of total phosphorous. In most of cereals and legumes phytic acid stockpile in the layer of endosperm and globoids, respectively (Reddy and Sathe, 2002). The endosperm of some plant lacks phytate and it reside in the germ portion such as wheat and rice grains. Different plants have variable amount of phytate content in them like cereals has ability to store 2.2 % maize crop is the one with highest content 2.2% and among legumes and beans (5.92 - 9.15%) (Reddy and Sathe, 2002).

Table 2.1 Phytic acid content in seed fruits and vegetable consumed by human. (Lott1 et al., 2000)

Plant	% Phytic Acid
Peas	1.00
Wheat	1.02

Oat	1.02
Barley	1.02
Cocoa bean	1.04
Sorghum	1.06
Cucumber	1.07
Watermelon	1.36
Pistachio	1.38
Eggplant	1.42
Almond	1.42
Soybean	1.55
Tomato	1.66
Peanut	1.70
Hemp	1.74
Cashew	1.97
Mustard	2.00
Sunflower	2.10
Canola	2.50
Linseed	3.69
Pumpkin	4.08
Sesame	4.71

2.1.5 Potential Problem of Phytic Acid

In monogastric animals phytic acid and its salt phytate both had strong antinutritive effect depending upon surprising molecular structure of it (Vohra and Satyanarayana, 2003). Various beneficial minerals bind with phytic acid in gut of monogastric and other animals before their absorption in digestive tract and influence digestive enzymes. Digestibility of starches, proteins, and fats are reduced by phytates (Andrews, 2013) Bioavailability and utilization of nutrients is decreased by phytic acid. (Bohn, Meyer and Rasmussen, 2008). Phosphate groups have negative charge on them which make its reaction easy with cations to form insoluble complexes (Urbano et al., 2000). These insoluble complexes of phytate cause low ingestion and absorption of essential minerals in gastrointestinal tract of monogastric (Shobirin et al. 2009). Phytate-mineral complexes that are insoluble in nature their formation in the intestinal tract halt mineral absorption. Essential minerals become unavailabe (Bohn, Meyer and Rasmussen, 2008). Zinc is a trace element, and its bioavailability is most affected by phytic acid. Phytic acid also show its influence on some other enzymes such as trypsin, pepsin, α-amylase, and β-galactosidase, by reducing their functional activities (Singh and Satyanarayana, 2008).

2.2 Phytases and Its Types

Enzyme phytases be allied to phosphatases that remove phosphate from phytic acid (Shin et al., 2001). Phytic acid and its salts(phytates) are hydrolyzed by catalysis activity of phytase that yield inositol, inositol monophosphate, and inorganic phosphate (Shivanna and Venkateswaran, 2014). Phytase enzyme have 2 different types. 3-phytase is first type and the second one is 6-phytase (Bohn et al. 2000). Enzyme phytases find their application in feed additives and gained good share in

market. Fungal and bacterial species produce different types of phytases fungi produce 3-phytase and bacterial species produce 6-phytase (Dvořáková et al., 2000b)

2.3 Microbial Source of Phytase

Phytase is widespread in nature and occurs in a diversity of organisms including miniatures, plants and animal tissues. Few studies revealed that phytases from microbes are best for usage in biological applications. Several microorganisms like bacteria, moulds and yeasts have showed their capacity of producing phytase enzyme. However, fungal sources are more auspicious to produce phytases on industrial level (Pandey et al., 2001). Generally, fungi produce extracellular enzyme whereas the bacteria produce phytase that is mostly inside the cell.

2.3.1 Bacteria as Phytase Source

Number of bacterial species were reported as phytase producers. In general enzymes produced from bacteria are cell associated but few bacterial species produce extracellular enzymes (Table 2.2). Optimum pH of some bacterial phytases fall in range of 6.0 to 8.0 (Shimizu, 1992). That why bacteria phytases are considered serviceable to use in animals feed due to similarity in there optimum pH with animal feed pH.

Table 2.2 Different Phytase producing bacterial strain.

Bacteria	**Location of the enzyme**	**References**
Bacillus pantothenticus	Extracellular	Anis Shobirin et al., 2009
Bacillus licheniformis	Extracellular	Joseph and Raj, 2007
Bacillus subtilis	Extracellular	Kerovuo et al., 1998

Citrobacter braakii	Intracellular	Kim et al., 2003
Enterobacter sp.4	Extracellular	Yoon et al., 1996
Lactobacillus plantarum	Extracellular	Saribuga et al., 2014
Lactobacillus	Extracellular	sanfranciscensis De Angelis et al., 2003
Pseudomonas putida	Extracellular	Richardson et al., 1997
Serratia marcescens	Extracellular	Mukesh Kumar et al., 2011

2.3.2Fungal source of phytase

Most frequent phytase activity among microbes is noticed in fungi , and most notable species belongs to Aspergillus. In different moulid and yeast species phytase has also been detected. In number of fungal species both intracellular and extracellular phytases have been produced. (Table 2.3) There was great variance between extra- and intracellular phytase activities among species. Among microbial source of phytase fungi is one of most important and high yielding organisms used on industrial scale for enzyme production.

Table2.3: Phytase producing fungal strains.

Fungi	**Location of the enzyme**	**Reference**
Aspergillus candidus	Extracellular	Howson and Davis, 1983
Aspergillus carneus	Extracellular	Ghareib, 1990

Aspergillus ficuum	Extracellular	µLlah & Dischinger 1993a; Ebune et al., 1995; Kim et al., 1999a; Kim et al., 1999b
Aspergillus flavus	Extracellular	Shieh and Ware,1968
Aspergillus fumigatus	Extracellular	Rodriguez et al., 2000
Aspergillus niger	Extracellular	Phillipy and Mullaney, 1997
Aspergillus niger 11T25A5	Extracellular	Da Silva et al., 2005
Aspergillus oryzae AK 9	Extracellular	Chantasartrasamee et al., 2005
Aspergillus terreus	Extracellular	Mitchell et al., 1997
Botrytis cinerea	Extracellular	Howson and Davis, 1983
Fusarium verticillioides	Extracellular	Marlida et al., 2010
Mucor piriformis	Extracellular	Howson and Davis, 1983
Myceliophthora thermophila	Extracellular	Pasamontes et al., 1997
Pencillium sp.	Extracellular	Shieh and Ware, 1968
Rhizoctonia sp.	Extracellular	Marlida et al., 2010
Rhizopus oligosporus	Extracellular	Casey and Walsh, 2004
Rhizopus oryzae	Extracellular	Rani and Ghosh, 2011
Rhizopus stolonifer	Extracellular	Howson and Davis, 1983
Sporotrichum thermophile	Extracellular	Javed et al., 2010
Thermomyces lanuginosus	Extracellular	Berka et al., 1998

2.3.3 Plants and Animal as Source of Phytases

Phytase enzymes is mostly present in microbes but it also occurs widely in the plant kingdom as well as in monogastric animals. Plants have been considered as good source for phytase isolation and has mostly been characterized from maize, wheat, rice, and barley. Wide variety of beans also gave good yield of phytase (Kumar et al., 2010). Phytase functioning is also observed in leafy vegetables, grass potato and radish (Li et al., 1997). Phytase is produced in the mucosal membrane of intestines, complex stomach, blood cells and liver of the animals (Konietzny and Greiner, 2002). Humans along with monogastric do not have phytase in their gastrointestinal tract for digestion (Elkhalil et al., 2011) and minerals attached with phytate, due to which proteins and phosphorus cannot be released (Jin et al., 2007).

2.4 Impact of physical parameters on phytase activity.

Physical parameters are important for the development of every living entity and have profound effect on production of different metabolites in organisms. Temperature, pH, agitation, inoculum size and quality, etc are physical parameters effecting the growth of living entities.

2.4.1 Influence of Temperature on Phytase Production

Temperature is one of decisive factors effecting the growth of microbes along with the production of enzymes and metabolites by microbes. Phytase produced from the microbial source is mostly heat sensitive. Slight changes in temperature affects activity of the phytase produced by microbes. Best working temperature of phytase ranges from 45–77°C. The optimum fermentation temperature reported for maximum production of phytase from fungi using submerged fermentation is 30°C (Vats and Banerjee, 2002; Gargova et al., 2003). Both fungal and yeast cultures were known for

highest phytase production at 28°C (Lambrechts et al., 1992; Segueilha et al., 1992; Marlida et al., 2010). Phytase production from different microbial sources has also been reported at temperature ranges from 35° to 37°C in SmF and SSF (Yoon et al., 1996; Dahiya et al., 2009; Ries and Macedo, 2011; Rani and Ghosh; 2011). After considering multiple studies its summarized that optimum temperature to produce phytases in maximum microbe fall in the range of 25 to 36°C.

2.4.2 Effect of pH on enzyme yield

Depending on pH enzyme phytase can be divided into two groups that are alkaline phytase and acidic phytase (Oh et al., 2004). Most preferable phytases for food or feed additives are acidic phytases because stomach of animals has acidic ph. That is the reason acidic phytases have been examined substantially for their systematic and well-structured performance in acidic medium and environment (Mullaney and ullah, 2003).

The pH got its ample role in production of microbial enzymes and pH of medium also has a profound effect on the production of the enzyme. The optimum pH to produce phytase from most of microbial sources such as bacteria and fungi range between 5.0 and 7.0.

2.4.3 Influence of Nutrients on Phytase Activity.

Composition of media components, have great impact on the production of phytase. Major chemical components of media that can affect phytase production includes, carbon and nitrogen sources, in addition to minerals and metal ions, particularly phosphorus. Optimal concentration of carbon and source of carbon are key factors to produce phytase. Glucose has been considered the most suitable substrate for phytase production (Papagianni, Nokes and Filer, 1999). Glucose was also found as

the best carbon source for most of the bacteria and yeast for their growth and phytase production (Vohra and Satyanarayana, 2001)

Nitrogen source in the culture medium is another important nutritional parameter that affect and control the growth and production of enzymes. Phytase production from Bacillus sp. DS11 (Kim et al., 1998), Aspergillus niger van Teighem (Vats and Banerjee, 2002) and Aspergillus niger St-6 (Tahir et al., 2010) was found maximum with ammonium nitrate as nitrogen source.

2.5 Applications of phytase

Hydrolysis of phytic acid yield phosphorous some minerals and salts that gained nutritional importance for animals and human. Impressively, supplementation of enzyme phytase in animal feed has been found to play their role in the reduction of total phosphorus concentrations over the past few years (Lei et al.,2013b). So phytase has therefore, potential applications in feed and food industries (Vohra and Satyanarayana, 2003).

2.5.1 Application of Phytases in Food

Phytase has an appreciable value, and it is utilized in fabrication of foods for human use (Kumar et al. 2010) Diet of huge section of world population is rich in cereal fibers, legumes and soy protein which results in more intake of phytate. Vegetarians mostly eat vegies and plant-based food and unbalanced food that mostly contain cereals. (Famularo et al. 2005) Phytase can play its role as anti-cancerous agent(Afnah et al. 2010). Stomach and intestines of monogastric animals have low capability of degrading phytate into useful products. (Gilani et al, 2005), for that purpose supplementation of phytase in feed preparation and in the GIT (gastrointestinal tract) is considered

beneficial. Because phytate has very severe effects regarding to mineral deficiency (Gilani et al. 2005).

Proper functioning of phytase is only achieved when it is able to bear heat enough to tolerate the low pH of the esophagus and to tolerate the enzymes in the digestive tract and high cooking temperature it is not heat stable it will degrade before usage or during processing (Haefner et al. 2005). Phytate content can be reduced in food by playing with their genetics and making their mutants with low phytate in barley, soybean, rice and maize or using genetic engineering technique to increase seed phytase (augspurger et al. 2007).

2.5.2 Phytase as Feed Additives

Feed supplements are core need of diet for many animals. Phytase is used as additive in the diet of fishes and poultry. Phytate is non digestible component of feed (Kumar et al. 2010) and phytase is an enzyme that break down the phytate to enhance nutritional factors such as minerals and salts in feed (Brinch-Pedersen et al. 2002). The use of phytate phosphorus increases when phytase is used as additive in the birds feed (Yao and Fan 2000). Phytase also have negative effect it can effect immune system (Baur et al. 2002)

2.5.3 Role of phytase in Poultry

Generally, phytase is used as diet supplement in poultry and in feed of monogastric animals. Phytase hydrolyze the phytate that is acting as a anti-nutritional factor in feed. Once phytate is hydrolyzed it enhance the availability of number of bounded nutrients in return lessen the price of phosphate and added in feed as phosphate source and reduce the excess phosphorus from livestock waste to water stream and cause water eutrophication. Availability of phosphorus present in phytate is

increased by adding microbial phytase in feed and it also enhance the absorption of minerals (Tran, 2010)

CHAPTER 3

Materials & Methods

Materials & Methods

3.1 Laboratory Precautionary Measures

Basic precautionary and safety measures were adopted while working in laboratory before starting any experiment. Protective laboratory coat/overall and gloves were used while working with hazardous, carcinogenic, and toxic chemicals such as ethidium bromide, chloroform, phenol and many other acids. Protective face masks were worn while working with carcinogenic fumes and toxic powdery texture chemicals. While working in presence of UV proper care was taken to avoid contact with UV. Safety cabinet were used in fungal mycelia extractions. All working area were cleaned with 70% ethanol before starting any experiment. All waste material including gel after electrophoresis were put in disposable autoclave bags, sealed properly and placed in a container for disposal.

3.2 Washing and Sterilization.

All Laboratory glassware was properly dipped in solution of bleach overnight and then washed. All glassware, plastics and solutions autoclaved at temperature 121^0 C and pressure at 15 psi for 15 minutes and then dried in drying oven at temperature 55-75^0C for 2-3 hours except Sodium dodecyl Sulfate because it is a detergent and it can burst during autoclaving.

3.3 Media Preparations

3.16.4.1.1 3.3.1 Potato Dextrose Agar Media (PDA 500ml)

PDA media was prepared by adding potato dextrose agar (19.5g) in clean media bottle and distilled water up to 500 ml. Mixed them well and autoclaved. Autoclaved media was poured in to sterilized glass/plastic petri plates, incubated overnight at 36-37°C. PDA media plates ready for fungal colony growth.

3.3.1 Preparation of Phytase Screening Media

Fungal isolates were screened on phytase screening medium (PSM) to know whether our strains of fungi has ability to produce phytase . Phytase screening media was prepared by following procedure reported in literature (Hosseinkhani, Emtiazi and Nahvi, 2009).

First of all chemicals were measured using weighing balance. Glucose 1.5 g, ammonium sulphate 0.5 gram, KCl 0.05 g, Magnesium sulphate hepta hydrate 0.01g, sodium chloride 0.02 g, $CaCl_2$ 0.02 g, Ferrous sulphate heptahydrate 0.001g, manganese sulphate 0.001 g, sodium phytate 0.25 g, and agar 1.8 g were measured and added in 20 ml distilled water. Media was mixed and heated to dissolve it and the volume was raised by adding distilled water upto 100ml.PSM was sterilized at 15 psi.121 degree Celsius for 15-20 minutes using autoclave. After sterilization media was cooled down and poured into sterilized petri plates.

3.16.4.1.2 3.3.2 Fermentation Media for Submerged Fermentation

Fermentation media for submerged fermentation was prepared using glucose phosphate broth having composition glucose 10g, ammonium sulphate 3g, KCl 0.5g, Magnesium sulphate hepta hydrate 0.5g, CaCl2 0.1 g, KH2PO4 3g were measured and dissolved in 250 ml distilled water. Fermentation media was sterilized using autoclave at temperature 121 C℃ and 15 psi for 15 to 20 minutes.250ml glass flask were used and each flask was filled with 25ml media.

3.4 Reagents Preparations

3.16.4.1.3 3.4.1 Sevag Solution

For sevag solution Chloroform 24 ml and Isoamyl alcohol 1 ml was mixed well and sevag solution is ready. Always use both in ratio 24:1(chloroform to Isoamyl alcohol). This solution contains chloroform which was used to remove protein during total DNA extraction.

3.16.4.1.4 3.4.2 TAE Solution (1x)

50 x TAE buffer 10 ml was taken and added in distilled water 490 ml. Mixed well and kept at room temperature. TAE buffer solution is used for gel electrophoresis of fungal DNA and Fungal virus.

3.16.4.1.5 3.4.3 0.1M $MgSO_4.7H_2O$

The magnesium sulphate (0.1 M) solution was formed by mixing 2.46g of salt in 100ml of distilled water.

3.16.4.1.6 3.4.4 Substrate Solution

Sodium phytate substrate solution was prepared by adding 0.5g of sodium phytate in 25ml of sodium acetate 0.2 M buffer.

3.16.4.1.7 3.4.5 10% Trichloroacetic acid

TCA is s was formulated by dissolving 5g TCA in 50 ml of dH_2O. TCA is a reagent used to stop chemical reaction

3.16.4.1.8 3.4.6 10% Ammonium Molybedate

Prepare 10% ammonium molybedate by adding 10 g of ammonium molybedate in 10 ml diluted sulfuric acid (27.17 ml sulphuric acid I n 72.83ml of distilled water).

3.16.4.1.9 3.4.7 (TSCR) Tausky- Shor Color Reagent Solution

This solution was formulated by adding 5 grams of ferrous sulphate ($FeSO_4.7H_2O$) in 90 ml distilled water along with 10 ml of 10% ammonium molybedate.

3.16.4.1.10 3.4.8 Bradford Reagent

Bradford reagent was formulated by mixing 0.1g Coomassie brilliant blue, 100ml (85%) phosphoric acid and 50 ml 100% ethanol. Then volume was raised to 1 L and covered with aluminum foil to avoid light

3.5 Sample collection.

Fungal samples of botrytis were collected from Kamoke city in Gujranwala district Punjab, Pakistan. Fruit samples were collected. Strawberries with expected fungal contamination were collected from field.

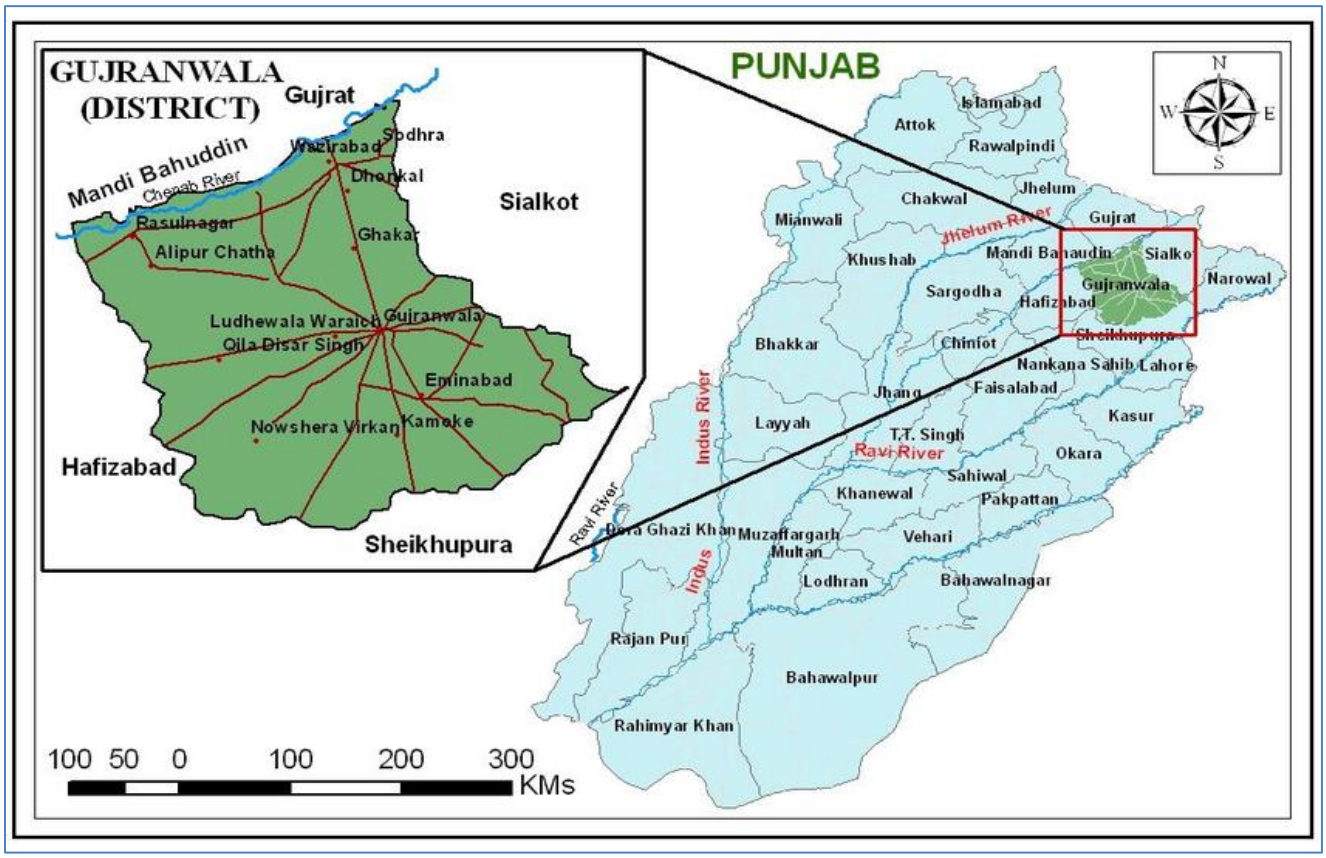

Fig 3.1 Map showing area of sample collection

3.6 Pure Cultures

Fungal colonies that seems to be botrytis having morphological similarities were picked and plugs were inoculated on PDA media plates and incubated for 3 to 4 days at 26 °C to get pure cultures.

3.7 Morphological Analysis of Fungi

Identification of fungal morphology was performed with the help of compound microscope following fungal compendium (Barnet, 1960 & Subrahmanian,1971).

Steps performed were as given below:

1. Slide which had to be used for microscopic examination was properly cleaned with 70 % ethanol.
2. Secondly, in Bio Safety Cabinet, PDA plates consisting of purified fungal colonies were opened spores of pure fungal colony were placed onto water droplet present in the center of the slide.
3. Fungal spores placed on the slide were covered with over slip and pressed with finger to avoid water bubbles.
4. Prepared slide was placed under microscope at different resolutions, first at resolution of 40X and then at resolution of 100X for each purified fungal colony.
5. Morphological analysis of fungal hyphae and spores was performed for the purpose of identification.

3.8 Total Nucleic Acid Extraction

To identify fungal isolates, total nucleic acid extraction of fungi was performed as adopted from literature reported by (Bhatti et al., 2012).

3.9 Agarose Gel Electrophoresis

Fungal genome 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 gel was made for that 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 about two minutes in a microwave oven and then added 5 µL ethidium bromide before pouring to caster.
4. After gel solidification comb was removed from caster and then the agarose gel was placed in gel tank and filled up gel tank to a recommended limit mentioned on tank.
5. In first well 4-5 µL 1 kb Thermo Scientific DNA ladder was loaded as a marker.
6. In other wells 10 µL of total nucleic acid extracted sample was assorted with 2 µL Thermo Scientific 6X loading dye and complete mixture was loaded into well.
7. At last at 55V and 120A, the agarose gel was run for 1.5 to 2 hours.
8. After completion of gel electrophoresis, gel was visualized using Bio Top UV Trans-illuminator and gel analysis was performed using Dolphin Gel Documentation software.

3.10 PCR Amplification of ITS Region

PCR amplification of ITS region (Conrad et al., 2012) of viral infected fungus was performed using standard primers set and conditions (Church and Buckler., 1999; Kendall and PaµL., 2005). The two primers sets used are ITS1 F, ITS4 R, ITS86 F and ITS86 R (table: 3.1)

Table 3.1: 5'-3' primer sequences used are listed as follows

Sr.no.	Primer Name	5'-3' primer sequence
1	ITS1 F	TCCGTAGGTGAACCTGCGG
2	ITS4 R	TCCTCCGCTTATTGATATGC
3	ITS86 F	GTGAATCATCGAATCTTTGAA
4	ITS86 R	TTCAAAGATTCGATGATTCAG

3.11 PCR Thermal Profile

PCR thermal profiling used is given below:

1. PCR amplification started with pre-denaturation at 94^0C for 3 minutes.

2. 35 rounds of denaturation at 94^0C for 45 seconds, primer annealing at 58^0C for 45 seconds and extension at 72^0C for 45 seconds were processed and final extension at 72^0C for 7 min was done in a PCR Thermal Cycler.
3. Finally, the amplified product was analyzed by gel electrophoresis.

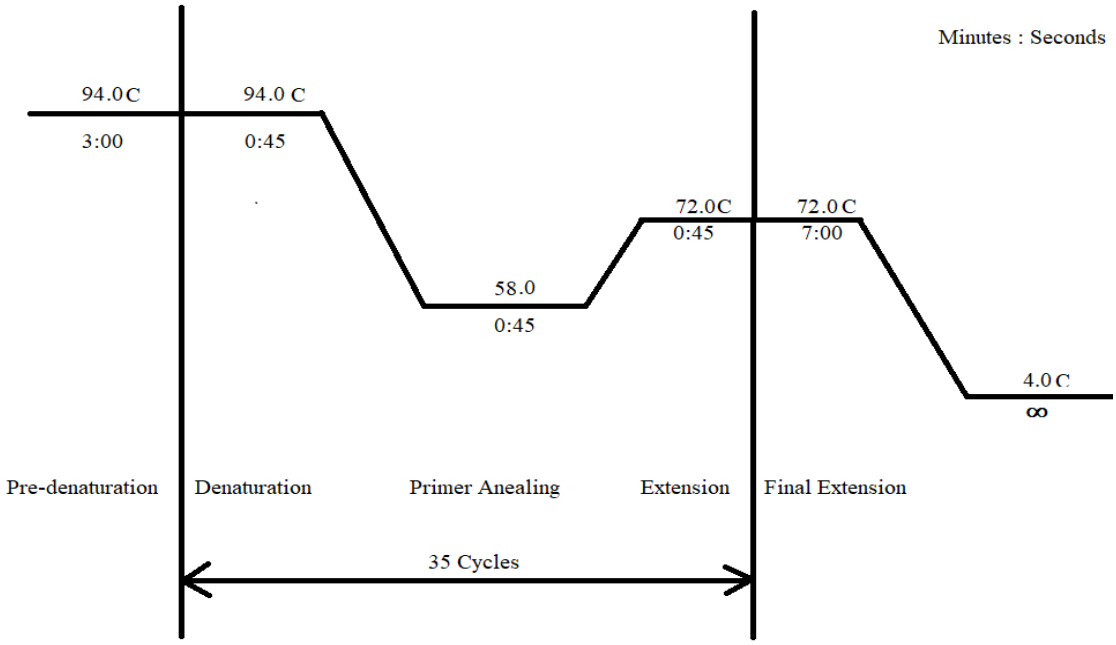

Figure 3.2: PCR thermal profile used for amplification of ITS region.

The total 25 µL PCR reaction mix was used as described by the table below.

Table 3.2: PCR reaction mixture with individual component quantity.

Reagent used	Volume in µL
Nuclease free water	11
Pfusion buffer 10X	2.5
25mM $MgCl_2$	2.5
DMSO 100%	1
2 mM dNTPs	2
Template DNA	2
Primer F	1.5
Primer R	1.5

3.12 Purifying PCR Product.

PCR purification kit (thermo-scientific) was used for the purification of primer dimers attached to PCR product obtained.

1. At first 1:1 proportion of binding buffer was added into PCR product and both were mixed by vortex.
2. 10 µL 3M sodium acetate was added to maintain optimal pH for binding of DNA which was indicated by change in color of solution from orange or violet to yellow color.
3. Centrifugation at 13000 rpm was performed for 1 minute and flow through was discarded.
4. Wash buffer 700 µL was added to the same Eppendorf and centrifugation was repeated for 1 minute.
5. Step 4 was repeated for sample to be sequenced.
6. After discarding flow through, eluted the DNA into 30 µL of sterile water and either gel electrophoresis was performed and the PCR purified product was stored at -20 ^{0} C for future use.

3.13 Amplicon Quantification

Before sequencing of PCR product, it was quantified using Nanodrop (Thermo scientific) 2000c by following procedure:

1. For measuring accurate concentration, blank was performed prior loading the sample by adding 1 µL of sterile water onto the lens and blank was performed using respective software.
2. Lens was cleaned with lint-free lab wipe and 1 µL nucleic acid was loaded onto lens.
3. Concentration of nucleic acid was measured by observing its UV absorption peak of 260 λ.

3.14 PCR Amplicon Sequencing

Sequencing of PCR amplicons was performed following Sanger method (F. Sanger et al., 1977) commercially by Eurofins Scientific (Testing Laboratories Company). After sequencing the nucleotide sequences obtained were compared though Nucleotide using basicTool (BLAST) in

National Center for Biotechnology Information (NCBI) with the sequences already reported in Gene Bank.

3.15 Optimizing Fermentation Media for Phytase Production.

Different physical and chemical factors that are media components were optimized using one factor at a time technique to enhance enzyme yield. Variables affecting the growth of fungal isolates were optimized as discussed below:

- Optimization of age of spores for maximum production of phytase.
- Optimization of incubation time for enhanced production of phytase.
- Temperature for maximum production of phytase.
- Managing pH for enhanced yield of phytase.
- Optimization of carbon sources for maximum production of phytase.
- Optimization of N sources for maximum yield of phytase.

3.15.1 Optimization of age of spores/mycelia for maximum production of phytase.

Spores/mycelia of various age were inoculated in the fermentation media to check their effect on production of phytase enzyme. Various aged spore/mycelia ranging from 2 ,3,4 upto 7 days were used for that purpose.

3.15.2 Optimization of incubation time for enhanced production of phytase.

Variable incubation times were used for fermentation media to evaluate their effect on enzyme production. Incubation time ranges from 72 hours, 96 hours 120 hours, 144 hours and 168 hours.

3.15.3 Optimization of temperature for maximum production of phytase

Various temperature was used for fermentation media to find their effect on production of phytase enzyme. Various temperatures used in fermentation medium includes 20, 25 ,30 ,35 and 40 Celsius.

3.15.4 Optimization of pH for enhanced production of phytase

Various acidic pH ranges were maintained for fermentation media to check increased yield capacity of phytase. Various pH ranges were used including 3,4,5,6.

3.15.5 Optimization of carbon sources for maximum production of phytase.

Different carbon sources were used in fermentation medium to check maximum production of phytase enzyme. Glucose, fructose, and sucrose were assessed to check which help producing maximum enzyme.

3.15.6 Optimization of N sources to get increased phytase growth.

Different nitrogen sources were used in fermentation medium to check maximum production of phytase enzyme. Yeast extract, peptone, ammonium sulphate, potassium nitrate were used to check which help producing maximum enzyme.

3.15.7 Phytase enzyme yield using optimum conditions.

All above optimized factors were taken and their optimum quantities were added in 100mL distilled water. Rest of media remain same as that of phytase screening media.

3.16 Analytical Methods.

3.16.1 Estimation of Inorganic Phosphorus by Colorimetric method.

The inorganic phosphorous that is produced by hydrolysis of phytic acid was detected by the colorimetric method. To evaluate the inorganic phosphorous standard curve of phosphorus solution was plotted.

3.16.2 Standard Curve

Phosphorous standard curve was formed by preparing the stock solution 10mg/ml. Then different dilutions were made by adding 2,4,6,8, and 10 ml from stock solution. Volume of each tube was raised to 10 milliliters by adding distilled water. Then 0.5ml magnesium sulphate solution (0.1M) was added in each tube. Blank was prepared by adding 1ml distilled water and 0.5 ml magnesium sulphate solution. Tubes were placed in boiling water for 15 minutes. Reaction was stopped by adding 1ml TCA in all tubes. After that add 1ml color reagent TSCR in all tubes and then by using the OD taken by spectrophotometer standard curve was plotted as shown in fig.

3.16.3 Enzyme Unit

Phytase activity is described as quantity of enzyme that is required to free 1.0 µmole of inorganic phosphorous per minute under the standard assay conditions (Rachmawati et al., 2017).

$$\text{Units/ml Enzyme} = \frac{\mu\text{mole of phosphorous releases) (dilution factor) (5)}}{\text{(Time) (0.5) (2)}}$$

3.16.4 Protein Estimation by Bradford reagent

Estimation of total protein was assayed by Bradford reagent using Bovine Serum Albumin (BSA) as standard curve of BSA was plotted to evaluate protein.

3.16.5 Standard curve Procedure

In Bradford assay BSA was used as standard and BSA solution was made by dissolving 10mg BSA in 10ml distilled water. For standard curve 10 dilutions were made 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1ml. For dilution 0.1ml stock solution and raised to 1ml by adding distilled water. Then 5ml Bradford reagent was added in each tube. For blank 1ml water was added in 5ml Bradford reagent. Incubate for 5 minutes at and OD was taken at 595nm wavelength for plotting standard curve.

3.16.6 Enzyme estimation by Bradford reagent

For enzyme sample 0.1ml sample was added in test tube along with 5ml Bradford reagent. In blank 0.1ml sodium phosphate buffer was added in 5ml Bradford reagent and OD was taken at 595nm wavelength using spectrophotometer.

CHAPTER 4

Results

Results

4.1Fungal Colonies Purification

Fungal colonies of *Botrytis cinerea* and *Fusarium oxysporum* that were collected from fruit sample and lab stocks were purified by growing on phytase screening media to find out fungus of our choice has capacity of producing phytase enzyme or not. Fungal isolates that grew on phytase screening media are shown in Figure 4.1.

Figure 4.1 Representative colonies of Botrytis cinerea and Fusarium oxysporum

2 Morphological Identification of Fungi

Microscopy was used to identify my fungal samples on basis of their morphology. Out of three phytase yielding isolates two are *Botrytis cinerea* and one isolate belongs to *Fusarium oxysporum*. Microscopic character of identified fungal isolates are shown in Figure 4.2 and Table 4.1

Table 4.1 Morphological identification of phytase producing fungal isolates.

Isolates	**Macroscopic characters**		**Microscopic characters**	**Identification**
	Obverse side	**Reverse Side**		
KST 6 **KST 51** **KST 54**	Compact white fluffy cottony textured appressed with concentric rings	White appressed with concentric rings seen on reverse side of plate.	Conidia are non-septate, ovoid and one celled. Hyaline branched & septate mycelia conidiospore produce directly from hyphae	*Botrytis cinerea*
NF1	Cottony mycelium smooth and fluffy and colony first appear white and changes color to pink to purple	Grow radially and seems dark in color in middle and whitish on edges	Mycelia are delicate, conidiospores are short single lateral and Sickle shaped conidia,	*Fusarium oxysporum*

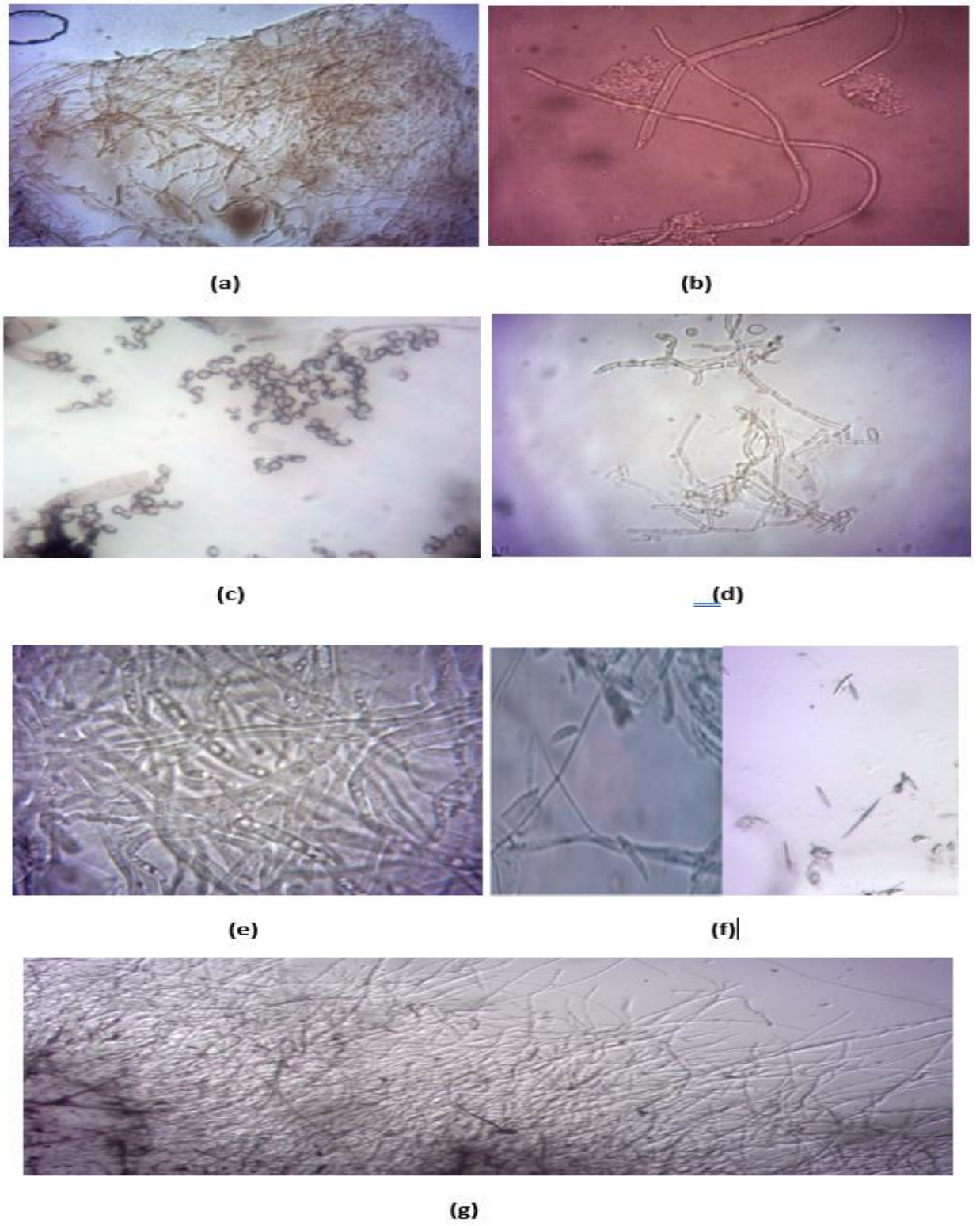

Figure 4.2 (a),(b),(c),(d),(e) show microscopic view of hyphae and spores of *Botrytis cinerea* and *Fusarium oxysporum* (f),(g) at 40X magnification

4.3 Molecular Recognition of Phytase Producing Fungal strains

Nanodrop method was performed after DNA extraction of fungal isolates to come across the concentration of DNA (Table 4.2). Amplification of ITS (Internal transcribed spacer region) gene of my fungal isolates was performed using PCR with universal primers. Then these amplified amplicons were resolved by agarose gel (1%) electrophoresis (Fig 4.3). Band of nucleic acid observed was in range of 500-550 base pairs. Sequencing of all amplicons was done using same universal primers. These sequenced amplicons were submitted in Gen Bank (NCBI). Gen Bank accession numbers of fungal isolates revealed that 3 out of 4 isolates were Botrytis cinerea while remaining 1 isolate was Fusarium oxysporum. These results verify the above-mentioned results of morphology of fungal strains (Table 4.1).

Table 4.2: DNA concentration values using Nanodrop.

Fungal isolates	DNA (ng/µL)	260/280
KST 6	42.3	1.92
KST 51	15.8	1.82
KST 54	26.6	1.98
NF 1	19.1	1.78

Table 4.3: Molecular identification of phytase Producing fungi.

Isolate	Accession Number	Species	Length (Bases)
KST6	MW320529	*Botrytis cinerea*	527
KST51	MW320533	*Botrytis cinerea*	431
KST54	MW320535	*Botrytis cinerea*	370
NF1	Not assigned	*Fusarium oxysporum*	

Figure 4.3: Agarose gel electrophoresis of fungal isolates (KST6, KST51, KST54, NF1) on the basis of ITS region gene (500-550bp**)**

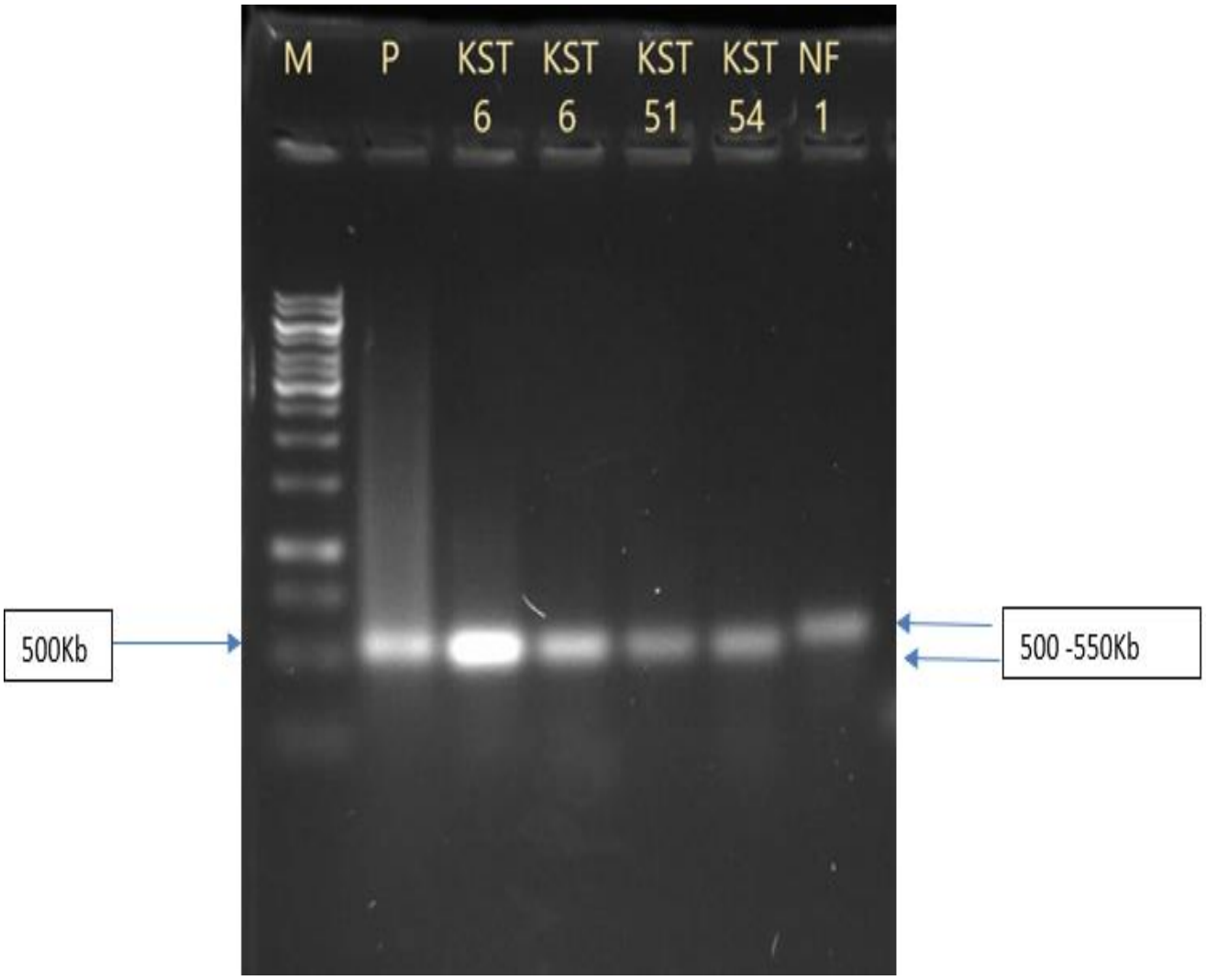

Figure 4.3: Agarose gel electrophoresis of fungal isolates (KST6, KST51, KST54, NF1) on the basis of ITS region gene (500-550bp**)**

4.4 Optimization of Physical and Chemical conditions of fermentation media for phytase production

4.4.1 Optimization of age of spores/mycelia

Fungal slants were prepared and then incubated at 30℃ for about 7 days and fungal slants were observed on which day fungus growth was maximum. Ranging from day 2 to day 7 maximum growth was attained on day 6 for Botrytis cinerea and day 5 for Fusarium oxysporum

4.4.2 Optimization of incubation time

Effects of incubation time on enzyme production was studied fermentation media was incubated for 7 days and maximum enzyme was produced by botrytis cinerea on day 7 in contrast fusarium oxysporum show its maximum phytase production on 6^{th} day as shown in (Table 4.4) and (Fig4.4)

Table 4.4: Effect of incubation time on phytase production

Specie	Isolate	Incubation time				
		3 days	4 days	5 days	6 days	7 days
Botrytis cinerea	KST 6	1.90	3.61	8.16	15.05	19.06
	KST 51	1.24	3.08	7.47	14.44	20.91
Fusarium oxysporum	NF 1	2.01	4.98	12.25	19.10	15.21

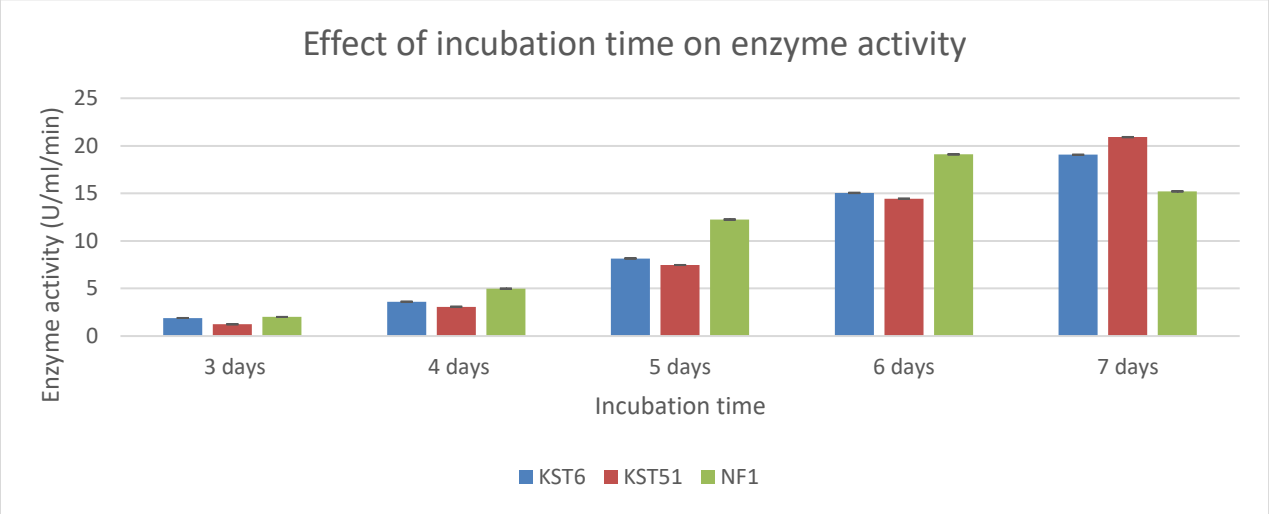

Figure 4.4: Effect of incubation time on phytase production

4.4.3 Optimization of temperature

Five temperature values that were falling in range of selected fungal strains were selected and studied for their ability to produce enzyme and fungal growth by incubating them for 7 days. Optimum temperature was measured by observing maximum phytase activity. The results of our study clarify the fact phytase yield is directly proportional to the growth of fungal isolate as well as temperature. The effect of temperature ranging from 20℃ and 45℃ on growth for each isolate was investigated. (Fig 4.5) Optimum temperatures found to be 25℃, All of 4 selected isolates produced highest phytase at 25℃. (Table 4.5)

Table 4.5: Effect of temperature on phytase production

Specie	Isolate	Temperature				
		20℃	25℃	30℃	35℃	40℃
Botrytis cinerea	KST 6	18.16	20.61	14.16	7.37	3.06
	KST 51	16.24	19.08	12.47	7.44	2.91
Fusarium oxysporum	NF 1	16.01	18.98	13.25	6.10	2.21

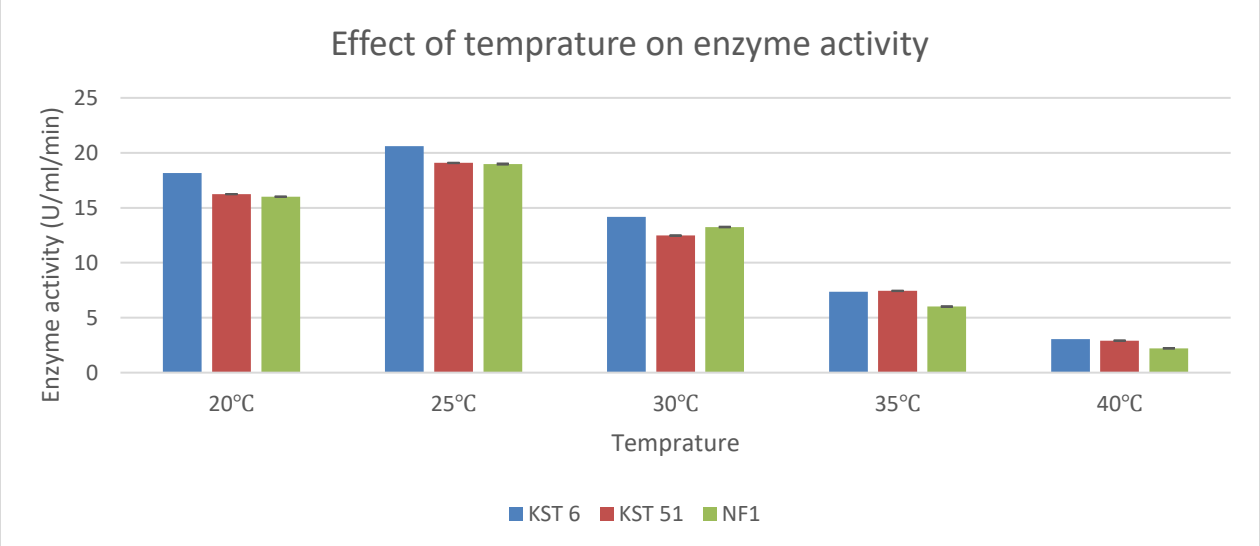

Figure 4.5: Effect of temperature on phytase production

4.4.4 pH Optimization

Likewise, temperature pH plays its role in effecting growth and yield of enzyme phytase. pH of fermentation media with ranging from 3-6 was used to measure yield of enzyme phytase under submerged fermentation is shown in (Table 4.6) and (Figure 4.6). Current results revealed that all Botrytis cinerea produced maximum phytase (12.16, 10.47 units µg/mol) at optimum pH of 5. Fusarium oxysporum produced maximum phytase (13.25 units µg/mol) at optimum pH of 5.

Table 4.6: Effect of pH on phytase production

Specie	Isolate	pH			
		3	4	5	6
Botrytis cinerea	KST 6	7.12	9.24	12.16	7.37
	KST 51	4.24	7.08	10.47	6.74
Fusarium oxysporum	NF 1	5.01	6.98	13.25	5.16

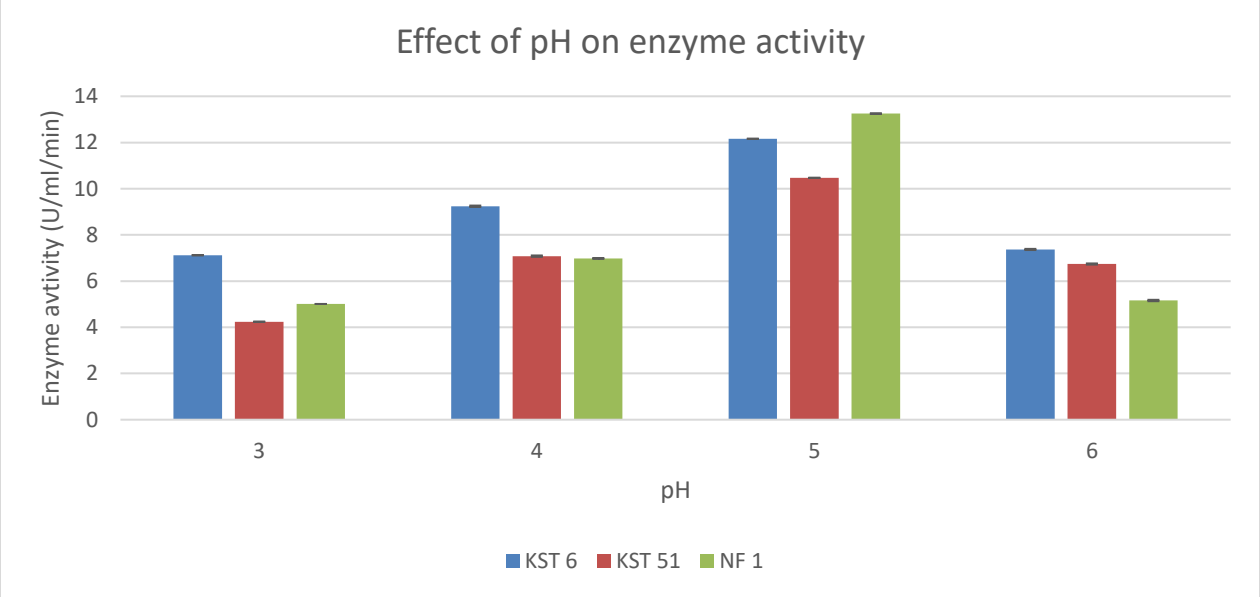

Figure 4.6: Effect of pH on phytase productio

4.4.5 Optimization of Carbon sources

carbon sources have their vital role in affecting enzyme production with fermentation and enzyme production was studied by incubation with four different carbon sources including glucose, starch, maltose, and sucrose for 7 days. Results revealed that fungal isolates showed maximum phytase production with glucose source (Table 4.7). The outcome of varying carbon sources on phytase production for each isolate was determined. All of 4 selected isolates produced highest phytase with glucose source (Fig 4.6).

Table 4.7: Effect of carbon source on phytase production

Specie	Isolate	Carbon Source			
		Glucose	Starch	Sucrose	Maltose
Botrytis cinerea	KST 6	21.48	17.24	5.16	3.37
	KST 51	18.72	16.21	5.47	2.74
Fusarium oxysporum	NF 1	18.01	15.06	4.25	2.16

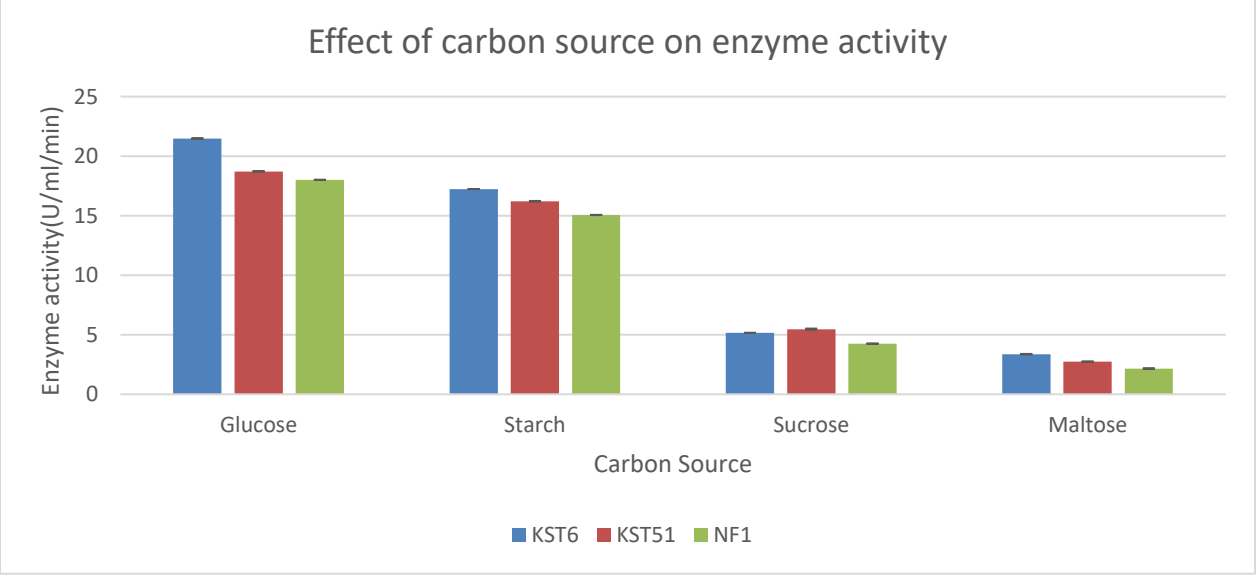

Figure 4.7: Effect of carbon source on phytase production

4.4.6 Optimization of Nitrogen sources

Effect of nitrogen source on enzyme production and under submerged fermentation was studied by incubation with four different sources including Yeast extract, peptone, ammonium sµLphate, ammonium nitrate for 7 days. It was observed that fungal isolates showed maximum phytase production with ammonium and nitrate source. The effect of varying nitrogen sources on phytase production for each isolate was determined (Table 4.8). All of 4 selected isolates produced highest phytase with ammonium and nitrate source (Fig 4.8).

Table 4.8: Effect of nitrogen source on phytase production

Specie	Isolate	Nitrogen source			
		Yeast extract	Peptone	Ammonium sµLphate	Ammonium nitrate
Botrytis cinerea	KST 6	5.54	6.24	18.42	20.01
	KST 51	5.01	5.22	16.90	17.43
Fusarium oxysporum	NF 1	5.89	4.98	15.22	15.19

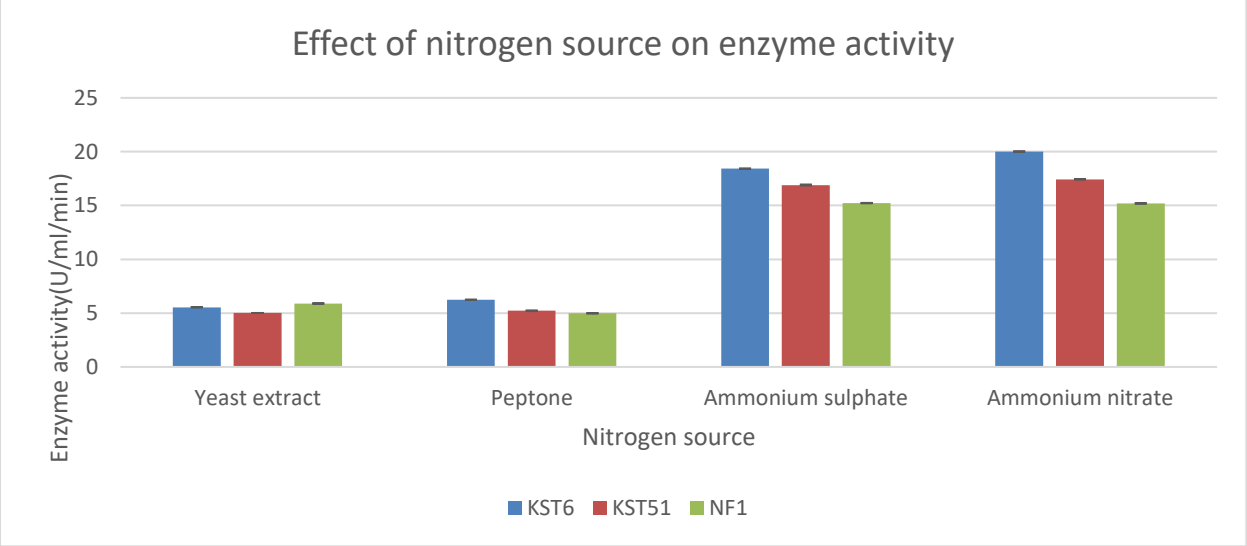

Figure 4.8: Effect of nitrogen source on phytase production

4.4.7 Optimized Fermentation Condition

Fermentation of all isolated were carried out with optimized condition to produce maximum quantity of phytase enzyme. All the factors mentioned above with high yield were used together (table 4.9). Other factors of fermentation media remain same as mentioned in recipe of fermentation media.

Table (4.9): Optimized condition of all isolates for phytase production**.**

Culture Conditions	**Fungal Isolates**		
	KST 6	**KST 51**	**NF1**
Age of spores	6 days	6 days	5 days
Incubation time	168 hrs	168 hrs	144 hrs
Temperature	25℃	25℃	25℃
pH	5	5	5
Carbon source	Glucose	Glucose	Glucose
Nitrogen source	Ammonium nitrate	Ammonium nitrate	Ammonium sulphate

4.5 Enzyme Units

Enzyme units indicate the phytase activity or the amount of phytase required to release 1 µmol of inorganic phosphorous per minute. Enzyme unit are calculated for botrytis cinerea and fusarium oxysporum are mentioned (Table 4.10)

Table 4.10: Enzyme production at optimum conditions.

Isolates	Enzyme units µg/mol/min
KST 6	31.73
KST 51	27.36
NF 1	24.81

4.6 Protein Concentration Estimation by Bradford Assay

Bradford assay is used to fin the concentration of enzyme. Bradford protein curve obtained by using 10 dilutions 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1ml. Absorbance was measured at 595nm.Protein standard curve was obtained by using MS Excel that automatically calculate concentration of interest using its OD value at 595nm. Equation containing X value that determine concentration of protein of interest. whereas R tells that Bradford reagent has been prepared correctly. Values of absorbance of BSA (Bovine Serum Albumin) diluted with distilled water (Table 4.11) .5ml Bradford reagent is added to all dilutions. For sample protein 0.1ml sample was added in test tube along with 5ml Bradford reagent.

Table 4.11: Bovine serum albumin absorbance value at 595nm in different dillutions.

BSA concentration [ug/ml]	Absorbance at 595nm
0	0.001
5	0.069

10	0.111
20	0.225
40	0.368
60	0.462
80	0.583
100	0.714

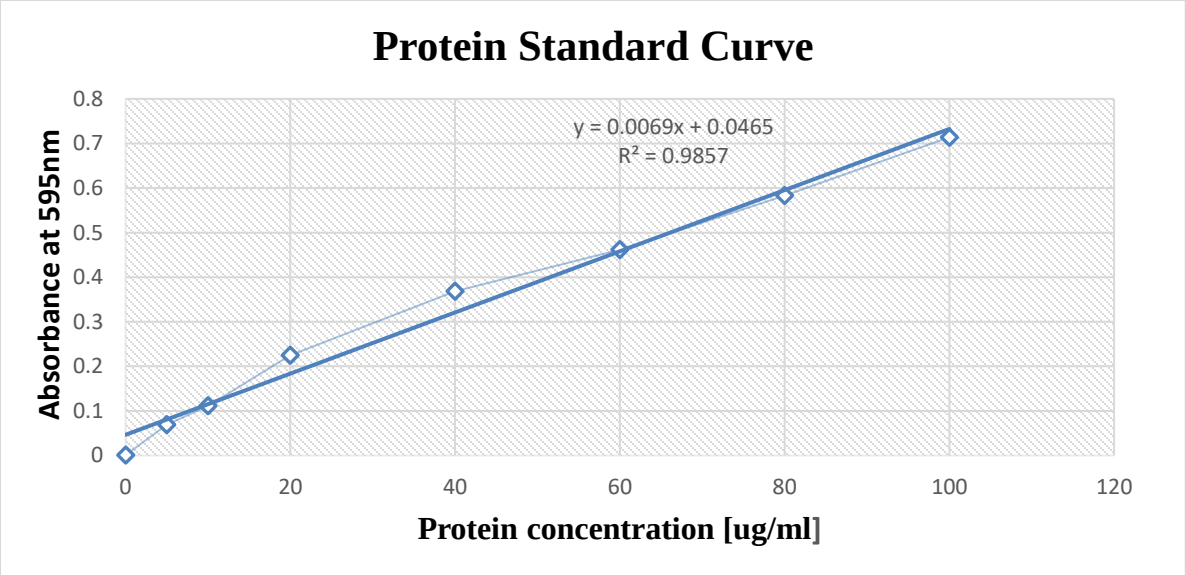

Fig 4.9 Bradford protein curve

Absorbance of crude protein extract is measured at 595 nm. Concentrations are measured using formula y=0.0069x + 0.0465. Where y is value of absorbance and x is value of concentration of protein.

Table 4.12: Absorbance and concentration of Crude protein extract

Sample	Absorbance	Conc in µg/ml
KST6	0.105	15.17
KST51	0.110	15.89
NF1	0.237	34.30

CHAPTER 5

Discussion, Conclusion & Future Recommendations

Discussion

With increased studies and research on enzymes fungi gained considerable value commercially due to their refined byproducts (Kunamneni A et al., 2014). Most of the commercially produced enzyme approximately 50% belongs to fungal origin and attain great success in industrial process and applications. Enzymes used I most of industries are hydrolytic depolymerases. Some renowned fields where fungal enzymes are used include and paper, textiles, detergents, food, feeds, nutraceuticals, and therapeutics. In a commercial sense, the main enzymes include protease, cellulase, xylanase, lipase, amylase, phytase, pectinase, β-fructo furanosidase and L-asparaginase and fungal genera utilized widely and repeatedly in the production of industrial enzymes are *Aspergillus*, *Rhizopus*, and *Penicillium.*

Botrytis cinerea and *Fusarium oxysporum* both are well known plant pathogen. *F. oxysporum* causes vascular wilt agriculture and horticulture plant species whereas *Botrytis cinerea* has been considered as one of the most important post-harvest necrotrophic fungal pathogens in fresh fruits and vegetables especially grape, strawberry, and tomato. *B. cinerea* secretes a battery of enzymes utilized for the degradation and consumption of the host plant.

The present study aimed at identification of plant pathogenic fungal strains having capability of producing phytase enzymes and partial characterization of enzyme from fungal infected fruits or vegetables. Fruit samples and some local sample of fungal strains from lab were studied. Fruit (strawberry) samples were collected for *Botrytis cinerea* from Kamoke city of Division Gujranwala Punjab, Pakistan. After surface sterilization, the fungal infected sample were cultured on PDA plates to get pure *Botrytis cinerea* strains. Isolates of *Fusarium oxysporum* taken from lab and Botrytis cinerea pure cultures were grown on phytase screening media to get those strains that have capacity to produce phytase enzyme.10 isolates of botrytis was grown on PSM and 2 isolates of

Fusarium oxysporum were grown on PSM out of which 2 Botrytis cinerea and 1 *Fusarium oxysporum* show their growth on phytase screening media.

For their confirmation morphological and molecular identification were performed. Microscopy was used to identify morphological features spores and hyphae of fungal isolates which showed resemblance with Botrytis cinerea and Fusarium oxysporum. Total nucleic acid extraction of fungal colonies was performed for screening of fungal isolates and after total nucleic acid extraction as reported by (Bhatti et al., 2012). ITS region amplification of fungal isolate KST6, KST 51 and NF1 was performed using Total one primer set including ITS1 F & ITS4 R and and sample were found to be *Botrytis cinerea* and Fusarium oxysporum.

Phytase producing fungal isolates were then shifted to fermentation media using submerged fermentation technique to get phytase enzyme. Multiple physical and chemical factors used in fermentation process were optimized to get enhanced yield of enzyme and far beyond my expectation Botrytis and Fusarium both show good results. The results obtained showed higher enzyme yield in both species in the presence of glucose, while ammonium sulphate and ammonium nitrate were found to be a favorable nitrogen source for SmF at optimum temperature 25℃ with acidic pH of 5 with 6 days incubation for Fusarium oxysporum and 7 days incubation for Botrytis cinerea. As there is no previous work done on Botrytis cinerea for phytase enzyme production. KST6 and KST51 produced 31.73 and 27.36 U/ml and Fusarium oxysporum isolate NF1 produce 24.81 U/ml. For protein concentration Bradford assay was used KST6, KST51 showed 15.17 and 15.89 µg/ml and Fusarium oxysporum showed 34.30 µg/ml

For further enzyme characterization SDS PAGE of crude protein will be performed to get exact size of protein.

In future this study will help to know about new source of fungi that can also produce phytase enzyme one of the emerging and highly utilized enzyme in feed industry.

Conclusion& Future Recommendations

In this study very careful optimization steps were carried out and it is concluded Botrytis cinerea KST 6 and KST 51 and *Fusarium oxysporum* NF 1 could produce phytase in submerged fermentation media. Literature on phytase production from botrytis cinerea is very limited and no proper work has done before on production of phytase from botrytis species. In this work local strain of both *Botrytis cinerea* and *Fusarium oxysporum* were identified morphologically and molecularly and submerged fermentation was performed to get phytase. KST6 and KST51 strains of *Botrytis cinerea* produced 31.73 and 27.36 U/ml and *Fusarium oxysporum* isolate NF1 produce 24.81 U/ml. Further protein purification using next Generation Chromatography (NGC) for these above-mentioned strains will be performed to find the purity of protein. This result conveys the very economized production of phytase locally and by further purification of phytase it will be capable of using in poultry industry and can reduce the import cost of enzyme. Thus, it will be beneficial for the economy of Pakistan.

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