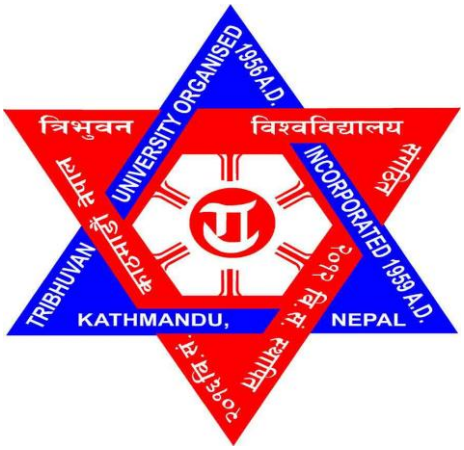
EXTRACTION OF AMYLASE FROM FUNGI ISOLATED FROM SOIL OF DHARAN, NEPAL



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Project Work Submitted to

Department of Microbiology

Central Campus of Technology, Tribhuvan University

In Partial Fulfillment for the Award of Degree of

Bachelor of Science in Microbiology

Submitted by

Mahesh karki

Roll No.: 80017

Registration No.: 5-2-0008-0033-2013

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RECOMMENDATION

This is to certify that **Mr. Mahesh Karki** has completed this project work entitled **"EXTRACTION OF AMYLASE FROM FUNGI ISOLATED FROM SOIL OF DHARAN, NEPAL"** as a part of partial fulfillment of the requirements of Bachelor's degree in Microbiology under my supervision. To my knowledge, this work has not been submitted for any other degree.

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Date:

CERTIFICATION OF APPROVAL

On the recommendation of **Mr. Shiv Nandan Shah**, this project of **Mr. Mahesh Karki** entitled "**EXTRACTION OF AMYLASE FROM FUNGI ISOLATED FROM SOIL OF DHARAN, NEPAL**" has been approved for the examination and is submitted to the Tribhuvan University on partial fulfillment of the requirements for B.Sc. degree in Microbiology.

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Date:

ABSTRACT

Enzymes are the most important substances which are used today in so many areas like research, in industries and in medical field. There are different types of enzymes prevailing in the nature. Among them, amylases are one of the most significant and are widely used in the industries. Amylases are starch hydrolyzing enzymes that have wide spectrum application in industrial and non-industrial sectors. The aim of the current study was to isolate and identify amylase producing fungi from soil collected from different parts of Dharan, and use in the extraction of enzyme. Amylase is extracted from different sources such as plant, animal and microbes. Amylases are widely extracted from the microorganisms, more specifically from the fungi. Extraction of amylase was done by isolating and identifying fungi from the different soil sample. Soil sample was primarily inoculated in Potato Dextrose Agar containing 1% of the starch. The 3 well developed colonies were further sub cultured into the starch agar medium. The production of amylase was tested by using Gram's iodine solution, and the one fungal colony was selected for the production of amylase on the basis of maximum zone of clearance after the application of iodine. The selected fungal colony was stained by lacto-phenol cotton blue staining technique and was identified as *Aspergillus niger*. It was subjected in the fermentation medium and was worked accordingly for the production of enzyme (amylase). The enzyme extracted was amylase, and was verified by pouring on the starch agar medium in which zone of clearance was obtained around the holes containing the enzyme. The amylase enzyme was assayed by using Dinitrosalicylic acid (DNS) method. At the end of DNS method, the absorbance of the crude enzyme was estimated and was found to be 0.03 at 540nm

Keywords: Amylase, *Aspergillus niger*, DNS, Fermentation,.

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LIST OF ABBREVIATIONS

1. PDA- Potato Dextrose agar
2. SAM- Starch Agar Medium
3. FAO- Food and Agricultural Organization
4. WHO- World Health Organization
5. GRAS- Generally Regarded As Safe
6. FDA- The Food and Drug Administration
7. rpm- Rotation Per Minute
8. spp.- Species

CHAPTER I

INTRODUCTION

1.1 Background of the study

Many chemical transformation process used in various industries have inherent drawbacks from a commercial and environmental point of view. High temperatures/pressures needed to drive reactions lead to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving high temperatures, pressures, acidity, or alkalinity need high capital investment, and specially designed equipment and control systems. Unwanted by-products may prove difficult or costly to dispose off. High chemicals and energy consumption as well as harmful by-products have a negative impact on the environment. These drawbacks cab be virtually eliminated by using enzymes (Pandey et al 2013).

Soil is the habitat of diverse array of organisms which include both micro flora and micro fauna. Soil microorganisms play a very important role in soil fertility not only because of their ability to carry out the biochemical transformation but also due to their importance as a source and sink of mineral nutrients. Fungi are one of the important group of microorganisms in soil after bacteria and have cryptic lifestyles in soil or on dead matters. Fungi are the member of the group of microorganisms that includes microorganisms such as yeast and mold. Yeasts are unicellular whereas molds are multi-cellular. Generally, molds are found in abundance in soil while the yeasts are found in body tissues of animals (Biswas and Murkherjee, 2001).

The use of microorganisms as the sources of industrially relevant enzymes has led to an increased interest in the application of microbial enzymes in various industrial processes. Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from the complex eukaryotes. Amylases are

widely distributed in plants, animals and microorganisms which show varying action patterns depending on the source (Pandey et al 2000, Saboury, 2002). However, amylases from microbial sources, especially fungi have gained much attention because of the availability and high productivity of fungi, which are also amenable to genetic manipulation. Fungi are involved on variety of industries ranging from food, chemical, detergent, textile and paper industries (Moreira et al 1999, Moreira et al 2001, Kathiresan and Manivannan, 2006).

Amylases are capable of digesting glycosidic linkages found in starch to give diverse products including dextrin and progressively smaller polymers composed of glucose units. They are among the most important enzymes and are of great significance in present day biotechnology. Amylases are obtained from diverse sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. In spite of the wide distribution of amylases, microbial sources are mainly used for industrial production. Amylases from filamentous fungal and bacterial sources are the most commonly used in industrial sectors (Pandey et al 2000; Senthikumar et al 2012). This is due to their advantages such as cost effectiveness, consistency, less time and space required for the production as well as ease of process modification and optimization. (El-Fallal et al 2012).

Many enzymes are known to the present world, only few are industrially exploited. There are about 3000 enzymes known but, a very less are used in different industries and factories. These enzymes are mainly extracellular hydrolytic enzymes, which degrade naturally occurring polymers such as starch, proteins, pectins and cellulose (Panneerselvam and Elavarasi, 2015). The alpha amylase is used in the production of glucose syrup in the first step of enzymatic degradation yielding a mixture of glucose and fructose with high fructose content. The amylase can be extracted from different sources such as plants, animals and microbes. The microbial extraction of amylase meets industrial demand because it is economical when produced in large quantities (Gurung et al 2013). The amylase has been extracted from several fungi. Bacteria, yeasts and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in the industrial sectors.

Starch is a polysaccharide composed of two units; amylose and amylopectin. Amylose is linear in structure while the amylopectin is the branched form. It is the most common carbohydrates in human diets and is contained in large amount of foods such as potatoes, wheat, maize, rice, etc. Amylase degrades the starch into the mixtures of glucose and fructose with high fructose content. Amylase production from fungi is economical because the amylase production rate is higher in fungi as compared to the other microorganisms. The isolation and manipulation of microorganisms have a great importance on biotechnology as well as in microbiology.

The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. Microbial amylase has almost surpassed the synthetic sources in different industries. (Singh et al 2011) Amylolytic enzymes are widely distributed in bacteria and fungi. Generally fungi like *Aspergilllus* species, *penicillium* species, are widely used for the extraction of amylase.

There are various reports on fungi from different sources and respective amylase activity. In the present study, the extraction of amylase from fungi isolated from the soil sample collected from different places of Dharan was reported.

1.2 Statement of the problem:

Amylases are very important enzymes and are highly applicable in different industries and factories. They are used in textile industries, paper industries, and different other industries where there is need for starch break down. The common sources of amylase production have been animals and plants. They have been extracted from these sources and are being used in the industries and factories. However, the methods of production/extraction of amylases from animals and plants are quite expensive and tiresome. Since extracting amylase enzyme from expensive samples is ineffective, the use of microbes can be done for this purpose. Since, soil is generally the best habit for most of the microbes, it can be used for the production of amylase. Microorganisms like fungi and bacteria can be isolated from simple and inexpensive sample like soil and can be used for the production of amylase. Fungi are present in the soil in large number and can be used for the production of amylase enzyme extensively. Amylase

extracted from the fungi can be used in different industries and factories. This can be useful to reduce the expenses in the production of amylase from the animals and plants. Amylase from fungal source is cheap and effective, and it can be produced in large amount since fungi are available freely in the soil.

1.3 Rationale of the study:

Enzymes are vital components in the life of living organisms. They are found inside the organisms and perform various functions which in turn run the life of those organisms. In the present world, the use of enzyme has outburst in many different ways. They are used in different types of industries and factories like in textile industry, paper industry, etc. Amylase is one of those many enzymes which have found applications in different types of industries. They can be extracted from plants, animals as well from microorganisms. So, it can be said that these enzymes are the basis of those industries. This research is totally based on the production of amylase from the soil fungi which is very much cheaper. The findings of this research can be helpful for the people and they can produce amylase from the cheap source like soil instead of extracting from plants and animals which is considerably expensive and tiresome process.

1.4 Objectives of the study

1.4.1 General objective:

- To extract the amylase enzyme from fungi isolated from the soil of Dharan of Sunsari.

1.4.2 Specific objective:

- To isolate and identify the fungi having the capacity to produce amylase enzyme.
- To extract amylase enzyme produced by the isolated fungi from the fermented broth.
- To perform DNS test for enzyme activity.

1.5 Limitations of the study:

In this study, extraction of amylase was done from the fungi isolated from soil samples of some parts of Dharan. Thus, this study does not support the direct use of fungi in the production of amylase in industrial level. Due to limited time period, soil samples from more areas of Dharan could not be collected. In this study, only one fungus was taken for the production of amylase. So, the result of this study can't be taken into generalization.

CHAPTER II

REVIEW OF LITERATURE

Introduction:

Soil is the outermost covering of the earth which consists of loosely arranged layers of materials composed of inorganic and organic constituents in different stages of organization. Soil provides the physical support needed for the anchorage of the root system and also serve as reservoir of air, water and nutrients which are essential for plant growth (Rao, 1999). Soil is the habitat of diverse array of microorganisms which include both micro-flora and micro-fauna. Soil microorganisms play a very important role in soil fertility not only because of their ability to carry out biochemical transformation but also due to their importance as a source and sink of mineral nutrients. This is done in soil general because of exo-enzymes that those microbes release in the environment and degrades the soil components (Biswas and Mukherjee, 2001). Soil receiving the garden is one of the rich sources of micro organisms. A very wide range of sources are used for the commercial enzyme production from Actinoplanes to Zymomonas, from spinach to the snake venom. Of the hundred or so enzymes being used industrially, over a half is from fungi and yeast and over a third are from bacteria with the reminder divided between animal (8%) and plant (4%) sources (Patel, 2015). Although many microorganisms produce amylase enzyme, the ones most commonly used for their industrial production are *Bacillus subtilis, Bacillus licheniformis*, *Aspergillus niger* and *Bacillus amyloliquifaciens* (Sivaramakrishnan et al 2006). Amylase is an enzyme that catalyses the breakdown of the starch into sugars, different organisms has been studied to produce this enzyme, a widely used is *Aspergillus niger*. Sometimes other strains of fungi like *Rhizopus*, *Penicillin*, other species of *Aspergillus* are also significantly used for the production of amylase in industries.

2.1 Soil microorganisms:

Soil microbiology is the study of the organism that are mostly found in soil, their functions, and how they affect soil properties. It is believed that between

two and four billion years ago, the first ancient bacteria and microorganisms came in the Earth's oceans. They were able to fix nitrogen, in time multiplied and as a result released oxygen into the atmosphere. Microorganisms in the soil are important because they affect soil structure and fertility. Soil microorganisms can be classified as bacteria, actinomycetes, fungi, algae and protozoa. Each of these groups has characteristics that define them and their functions in soil (Paul, 2006)

Amylases can be obtained from several sources such as plants, animals and microbes (Mishra and Behera, 2008). Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge applications in food, fermentation, textile and paper (Pannerselvam and Elavarasi, 2015).

Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized into exo-acting, endo-acting and de-branching enzymes. Among the amylases, beta amylase is exo-acting whereas alpha amylase is endo-acting enzyme. Extraction of amylase is possible from various sources, but the must well known and convenient one is from the fungi.

2.2 Amylase:

An amylase is an enzyme that catalyses the hydrolysis of starch into sugars. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Foods that contain large amounts of starch but little sugar, such as rice and potatoes, may acquire a slightly sweet taste as they are chewed because amylase degrades some of their starch into sugar. The pancreas and salivary gland make amylase (alpha amylase) to hydrolyze dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose supply the body with energy. Plants and some microorganisms also produce amylase.

a. Alpha Amylase

α-Amylases are the enzymes that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch in low molecular weight products, such as glucose, maltose and maltoriose units (Gupta et al 2003; Kandra 2003; Rajagopalan and

Krishnan 2008). Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market (Rajagopalan and Krishnan 2008; Reddy et al 2003). They can be obtained from several sources, such as plants, animals and microorganisms. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch processing industry.

b. Beta Amylase:

Another form of amylase is beta-amylase (alternative names: 1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase) is also synthesized by bacteria, fungi; and plants. Working from non-reducing end, β-amylase catalyses the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time. During the repining of fruit, beta-amylase, results in the sweet flavor of ripe fruit. Both alpha and beta amylases are present in seeds; beta-amylase is present in inactive form prior to germination, whereas alpha amylase and proteases appear once germination has begun. Many microbes also produce amylase to degrade extracellular starches. Animal tissues do not contain beta amylase, although it may be present in microorganisms contained within the digestive tract. The optimum pH for beta amylase is 4.0-5.0.

2.3 Structural and Functional Characteristics of α- Amylase:

The alpha amylase can be found in microorganisms, plants and higher organisms (Kandra 2003). The α-amylase belongs to a family of endo-amylases that catalyse the initial hydrolysis of starch into shorter oligosaccharides through the cleavages of α-D-(1-4) glycosidic bonds (Brayer et al 1995; Ilulek et al 2000; Kandra 2003; Tangphatsornruang et al 2005). Neither terminal glucose residues nor α-1,6-linkages can be cleaved by α-amylase (Whitecomb and Lowe 2007). The end products of α-amylase action are oligosaccharides with varying length with an α-configuration and α-limit dextrins (Van der maarel et al 2002), which constitute a mixture of maltose, maltoroise, and branched oligosaccharides of 6-8 glucose units that contain both α-1,4 and α-1,6 linkages

(Whitecomb and Lowe 2007). Others amylolytic enzymes participate in the process of starch breakdown, but the contribution of α-amylase is the most important for the initiation of this process (Tangphatsornruang et al 2005).

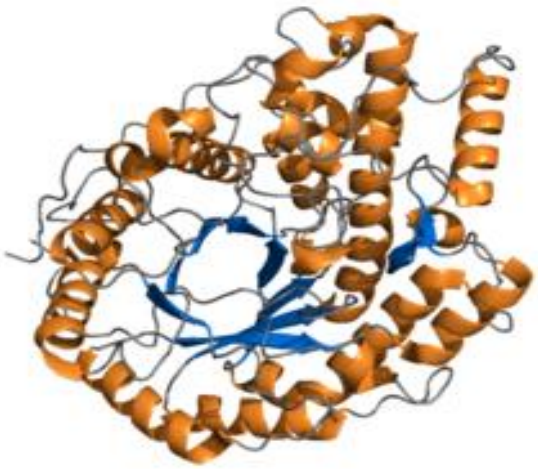

Fig. Alpha amylase

2.4. Applications of Amylases:

a. Starch conversion/processing:

The most widespread applications of α-amylases are in the starch industry, where they are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups (Nielsen and Borchert, 2000). The enzymatic conversion of all starch includes: gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further hydrolysis (Gupta et al 2003).

b. Detergent additives:

Detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe (Hmidet et al 2008). Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid

detergents contain these enzymes (Mitidieri et al 2006). These enzymes catalyze the hydrolysis of glucosidic linkages in starch polymers, commonly found in foods such as potatoes, custard, pasta, fruit, chocolate, baby food, barbecue sauce and gravy. As colored stains, their removal is of interest in both detergent and dishwashing contexts. Removal of starch from surfaces is also important in providing a whiteness benefit, since it is known that starch can be an attractant for many types of particulate soils (Mukherjee et al 2009; Mobini-Dehkordi and Javan, 2012).

c. Bio-fuel alcohol production:

Ethanol is the most utilized liquid bio-fuel. For the ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world (Chi et al 2009). In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars (Sanchez et al 2008). The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using an amylolytic microorganisms or enzymes such as alpha-amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganisms such as yeast *Saccharomyces cerevisiae* (Oner, 2006).

d. Food industry (baking, brewing, juice preparation, starch syrups):

Amylases are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (Couto and Sanromán, 2006). These enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. The addition of α-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product. Moreover, it generates additional sugar in the dough, which improves the taste, crust color and toasting qualities of the bread. Besides generating fermentable compounds, α-amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods, increasing the shelf life of these products (Gupta et al 2003).

In beer industries microbial amylases are used to aid cereal amylase in the production of fermentable sugar (Kirk et al 2002). Amylases are also used for the clarification of haze formed in beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility of fiber (van der Maarel et al 2002; Ghorai et al 2009).

e. Removal of starch sizer from textile (desizing):

Amylase from *Bacillus* strain was employed in textile industries for quite a long time (Haq et al 2010). In textile industry, strength of the textile is improved by warping the starch paste to textile weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of string due to laid down warp. After weaving the cloth, the starch is removed and the cloth goes to scouring and dyeing. The starch on cloth is usually removed by application of α-amylase. The α-amylases remove selectively the size (Feitkenhauer, 2003).

2.5 Starch:

Starch is a polymer of glucose linked to another one through the glycosidic bond. Two of types of glucose polymers are present in starch are: amylose and amylopectin. Amylose and amylopectin have different structures and properties. Amylose is a linear polymer consisting of upto 6000 glucose units with α-1,4 glycosidic bonds. Amylopectin consists of short α-1,4 linked to linear chains of 10-60 glucose units and α-1,6 linked to side chains with 15-45 glucose units. Granule bound starch synthase can elongate malto-oligosaccharides to form amylose and is considered to be responsible for the synthesis of this polymer. Soluble starch synthase is considered to be responsible fot the synthesis of unit chains of amylopectin. α- Amylase is able to cleave α-1,4 glycosidic bonds present in the inner part of the amylose or amylopectin chain (Muralikrishna and Nirmala 2005; Sorensel et al 2004; Tester et al 2004; Van der maarel et al 2002).

2.6 Starch conversion:

The most widespread applications of α-amylase are in the starch industry, which are used for the starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups (Nielson and Borchert, 2000). The enzymatic conversion of all starch includes: gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further hydrolysis (Gupta et al; Prakash and Jaiswal 2009).

2.7 Amylase producing fungi:

Different fungal species have capacity to produce amylase enzyme. Some well-known fungal species famous for amylase production are *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium* spp, *Rhizopus* spp, etc. The exclusive production of amylase is achieved by *Aspergillus niger* (Selvakumar et al 1998; Wang et al 2006), *Aspergillus oryzae* (Biesebeke et al 2005), and *Aspergillus terreus* (Berka et al 1992) in enzyme industry. These strains are already reported to produce substantial amount of glucoamylase in submerged (Berka et al 1992)

Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* species and to only one species of *Penicillium*, *P. brunneum* (Haska and Ohta, 1994) *Aspergillus oryzae* is a filamentous fungus, which has an ability to secrete large amounts of hydrolytic enzymes. It is the most prominent fungus producing amylase. It is also widely used in manufacture of traditional fermented soy sauce in Asia. The extracellular proteins in soybean koji inoculated with *A. oryzae* S. contain different protein profiles including neutral and alkaline protease, amylase, glutaminase, and metallopeptidase (Liang Y, 2009).

Similarly, another promising strains of fungi capable of amylase production is *Rhizopus* spp. The species *Rhizopus oryzae* and *Rhizopus microspores* (also known as *Rhizopus oligosporus*) are denoted as GRAS (Generally Regarded as Safe) by FAO (Food and Agricultural Organization) (Nityavardhana & Khanal, 2011); Rani & Ghosh, 2011). This is especially important when the enzyme produced is used in food processes (Sarouth et al 2012)

2.8 *Aspergillus* Species:

Aspergillus is a genus consisting of a few hundred mold species found in various climates worldwide. *Aspergillus* was first catalogued in 1729 by the Italian priest and biologist Pier Antonio Micheli. Viewing the fungi under a microscope, Micheli was reminded of the shape of an *Aspergillum* (holy water sprinkler), from Latin spargere (to sprinkle), and named the genus accordingly (Bennet, 2010). *Aspergillus* is an asexual spore forming structure common to all *Aspergillus* species; around one- third of species are also known to have a sexual stage. Some of them, however, are known to have a telemorph (sexual state) in the Ascomycota, so with DNA evidence forthcoming, members of the genus *Aspergillus* can tentatively be considered members of the Ascomycota (Geiser, 2009)

Members of the genus possess the ability to grow where a high osmotic concentration (high sugar, salt, etc.) exists. *Aspergillus* species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on the surface of a substrate, as a result of the high oxygen tension. A*spergillus* species are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees.

Species of *Aspergillus* are important medically and commercially. Some species can cause infection in humans and other animals. Some infections found in animals have been studied for many years, while other species found in animals have been described as new and specific to the investigated disease, and others have been known as names already in use for organisms such as saprophytes. More than 60 species of *Aspergillus* are medically relevant pathogens (Thom & Church, 1926).

Other species are important in commercial microbial fermentations. For example, alcoholic beverages such as Japanese sake are often made from rice or other starchy ingredients (like manioc), rather than from grapes or malted barley. Typical microorganisms used to make alcohol, such as yeasts of the genus Saccharomyces, cannot ferment these starches.

Aspergillus species are also commonly used in production of native and foreign enzymes, including glucose oxidase, lysozyme, and lactase. *Apergillus niger* is the major source of citric acid; this organism accounts for over 99% of global citric acid production, or more than 1.4 million tonnes per year. One widely used application of some *Aspergillus* is the production of amylase enzyme in the industries.

2.9 Description of *Aspergillus niger:*

Aspergillus niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mould on certain fruits and vegetables such as grapes, apricots, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported room indoor environments, where its black colonies can be confused with those of Stachybotrys (species of which have also been called "black mould").

Some strains of *Aspergillus niger* have been reported to produce potent disagree, claiming this report is based upon misidentification of the fungal species. Recent evidences suggests some true *A. niger* strains do produce ocratoxins A (V Morya, 2008).

Aspergillus niger is included in *Aspergillus* subgenus Circumdati section Nigri. The section Nigri includes 15 related black spored species that may be confused with *A. niger*, including *A.tubigensis, A. foetidus*, etc.

Aspergillus niger is relatively harmless compared to other filamentous fungi. Despite this fact, there have been some medical cases that have been accounted for, such as lung infections or ear infections in patients that have an weakened immune system, or an immune system that have been impaired by a disease or medical treatment. (V Morya, 2008).

The species of filamentous fungi produces several secondary metabolites, one of the most important being ochratoxin A. Ochratoxin A is an abundant food contaminating mycotoxin. Human contact with this toxin usually occurs through consumption of food which has not been stored and taken care of appropriately. Nevertheless, studies have shown that less than 10% of the *A. niger* strains were

tested positive for ochratoxin A under conditions that were favourable. The production of ochratoxin A from *A. niger*, is liable to cause immunotoxicity in animals. The effects on animals include a decrease in antibody responses, a size reduction in immune organs, and an alteration in the production of cytokine which are proteins and peptides specifically used in signalling. Food that has been contaminated by *A. niger* metabolites has a major effect on the poultry industry. Different animals, such as chicken, turkey, and ducks, are very prone to ochratoxin.

2.10 Ecology

Many black *Aspergilli* have been isolated from all over the world. *A. niger* is a filamentous fungus growing aerobically on organic matter. In nature, it is found in soil and litter, in compost and on decaying plant material. Reiss (1986) collected data on the influence of tempertaure, water activity and pH on the growth of various *Aspergilli* (Reiss, 1986). *Aspergillus niger* is able to grow in wide temperature range of 6-47 ^{o}C with relatively high temperature optimum at 35-37 ^{o}C. the water activity limit for growth is 0.88, which is relatively high compared with other *Aspergillus* species. *A. niger* able to grow over an extremely wide pH range: 1.4-9.8. These abilities and the profuse production of conidiospores, which are distribution via the air, secure the ubiquitous occurrence of the species, with a higher frequency in warm and humid places (Rippel-Baldes, 1955).

2.11 Industrial use of *Aspergillus niger*:

A. niger became an industrially used organism when citric acid was first produced by fermentation in 1919. Citric acid is widely used in a variety of industries and, by sales volume, greatly exceeds other metabolites such as gluconic acid (Roukas, 2000). Citric acid is the primary acidulant in the food and beverage industries. It is used in foods such as soft drinks, fruit juices, desserts, jams, jellies, candy and wine. In the pharmaceutical industries, iron acetate is used as a source of iron and citric acid as a preservative for stored blood; in the cosmetics and toiletries industries it is used as buffer, pH adjustment and as an anti-oxidant. The Food and Drug Administration (FDA)

has listed *A. niger* as source of citric acid (21 Code of Federal Regulations ξ 173.280).

In addition to citric acid, *A. niger* is rich source of enzymes. Pectinase, protease and amyloglucosidase were the first to be exploited, and were originally produced in surface culture (Frost and Moss, 1987). Several additional enzymes like cellulase and hemicellulase were manufactured using black *Aspergillus* strains in stirred tank processes.

For the manufacture of many products, starch- one of the most abundant carbohydrates- must be hydrolyzed to syrups, which contain glucose, maltose and low molecular weight dextrins. Amyloglucosidase, also referred to as glucoamylase, is an exo-amylase catalysing the release of successive glucose units from the non- reducing ends of starch by hydrolysing α-1,4-D-glucosidic linkages. The glucose syrup and the alcohol industries are the principal users of amyloglucosidase produced by *A. niger*.

Pectin, a heteropolysaccharide, is a principal component in commercially important fruits and vegetables. Several enzymes, including pectin esterases, endo- and exo- polygalacturonidases and pectin lyases, produced from *A. niger* degrade pectin; they are used in wine and fruit juice production to reduce juice viscosity before pressing and improve clarification (Grassin and Fauguenbergue, 1999).

It is established practice to improve the baking process by adding hemicellulases from *A. niger* when mixing the dough. The enzymes modify the rheological properties of the dough and give higher loaf volume and better crumb structure of the bread and pastry.

A. niger glucose oxidase and catalase are used for determination of glucose mainly in diagnostic enzyme kits, for the removal of either glucose or oxygen from foods and beverages and for the production of gluconic acid from glucose (Berka et al 1992).

FAO/WHO experts have repeatedly reviewed and accepted enzyme preparations from *Aspergillus niger* including the organism itself (FAO/WHO 1972, 1978, 1981, 1987, 1990), listing them with an Acceptable Daily Intake of “not

specified". The FDA in the United States has accepted numerous enzymes for the food use: in the early 1960s. The FDA issued opinion letters recognizing that alpha amylase, cellulase, amyloglucosidase, catalase, glucose oxidase, lipase and pectinase from *A. niger* can be 'generally regarded as safe' (GRAS) under the condition that non pathogenic and non-toxigenic strains and current good manufacturing practices be used in production. In addition to these enzymes, Godfrey and Reichelt (1983) claimed GRAS status for β-galactosidase and protease from *A. niger*. Carbohydrates and cellulase from *A. niger* are also approved as a secondary direct food additive by the FDA as an aid in clam and shrimp processing (21 Code of Federal Regulation, 173.120)

CHAPTER III

MATERIALS AND METHODS

3.1 Site of the study:

The study was carried out in the lab of Department of Microbiology, Central Campus of Technology, Dharan, Sunsari.

3.2 Research method:

The method for this study was qualitative as well as quantitative. This study was based on the culture method.

3.3 Type of study:

The study was of descriptive type

3.4 Population and Sample:

a. Sample: Soil

b. Description of the research site:

The soil sample for this study was asceptically collected from different places: Sami Chowk, Bijaypur, Bhanu Chowk, Panmara and Zero Point.

c. Work Flow chart:

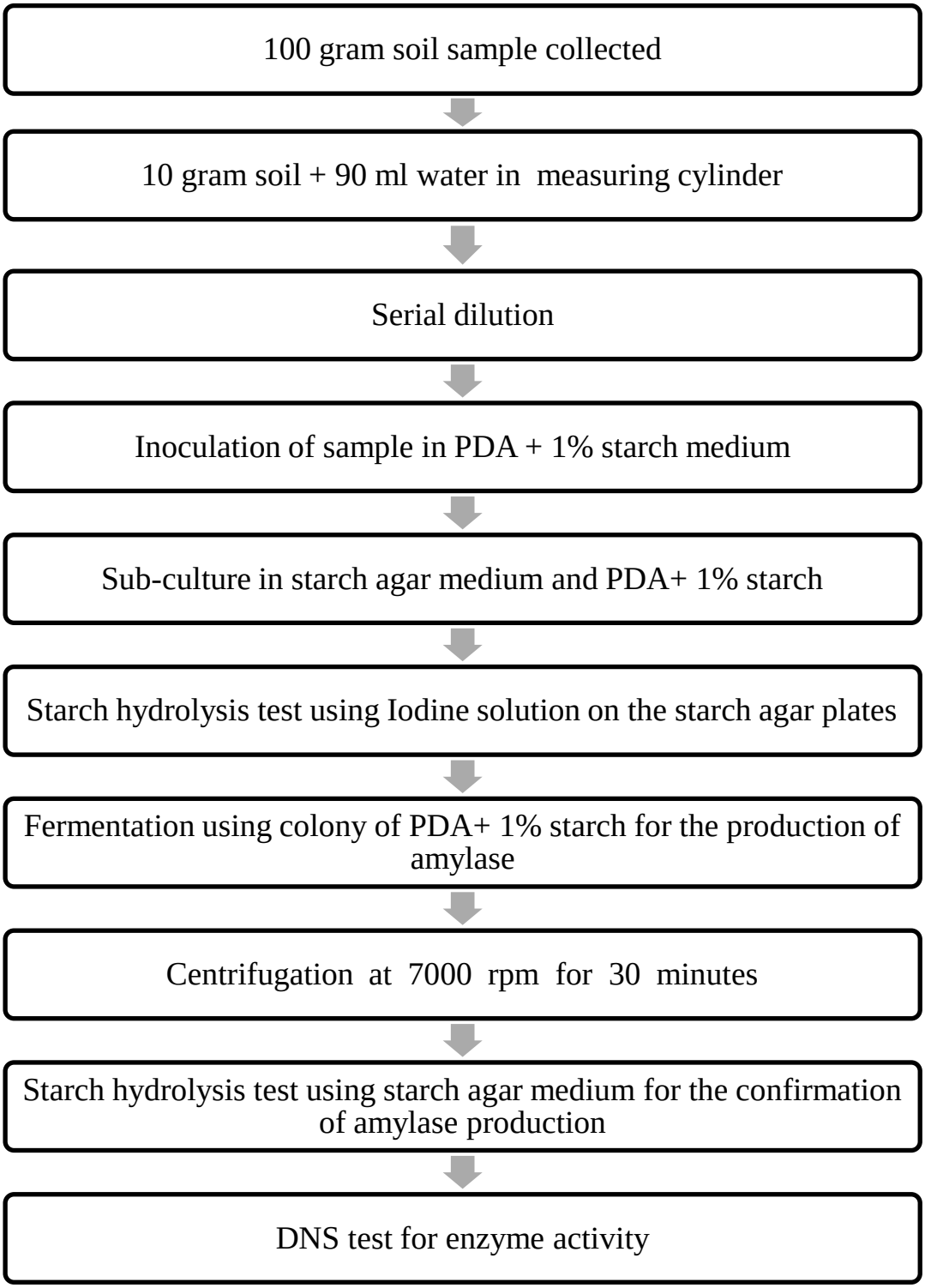

Fig 1: Flow chart of the study

d. Sample Collection:

Soil samples were asceptically collected from different sites of Dharan. For this study, agricultural fields where organic matters are deposited were selected as a sample site. Soil was collected from about 10 cm deep from the surface of the field into sterile plastic bags. Since number of fungi in soil is lesser, more amount of soil sample was collected for the research activity.

e. Transportation of sample:

The samples were then transported from the site of collection to the laboratory for further processing.

3.5 Screening and selection of fungi for amylase production:

a. Media preparation:

For the isolation of amylase producing fungi, PDA (Potato Dextrose Agar) and starch agar media were used. PDA was prepared with the addition of 1% starch for the growth of starch degrading fungi. Starch agar medium was composed of 2 gm of starch. The media were autoclaved at 121 ^{o}C for 15 mins.

b. Culture:

The collected soil sample was coded by B for Bijaypur, BC for Bhanu Chowk, P for Panmara, Z for Zero Point and S for Shami Chowk. Ten grams of soil sample was added to the measuring cylinder containing 90ml of sterile dilution blank. Then the serial dilution was done upto 10^{-6} using the test tubes containing 9ml of sterile dilution blank. 0.1ml of sample was spread plated onto (PDA + 1% starch) media plate. pipetted out to the petri-plate containing PDA+ 1% starch by spread plate technique. The plates were incubated for 3-5 days at 28^{o}C. The process was repeated for the different soil samples separately.

c. Sub-Culture:

After 72 hours, the growth of fungi was observed and the colonies were further sub-cultured in starch agar medium and also in potato dextrose agar containing 1% starch. The pH of starch agar media was adjusted to 6.5 with the help of conc. HCl. The plates containing starch agar media were inoculated with single

fungi from previous PDA plates while the plates containing PDA+ 1% starch were inoculated with two fungi colony by a single streak, separated with margin in between them. The fungi of starch agar media were for starch hydrolysis test while the fungi developed in PDA + 1% starch were for fermentation process. The colony which was sub-cultured in the starch agar media, was also correspondingly sub cultured in PDA + 1% starch. In this way, 3 well-grown fungal colonies were sub-cultured. The sub-cultured plates were incubated at 28^{o}C for 3-5 days.

d. Starch Hydrolysis Test:

The fungal colonies grown on starch agar plates were tested for the production of amylase. For that, Gram's Iodine (Gram's iodine- 250 mg iodine crystals added to 2.5gm potassium iodide solution and 125ml of water, stored at room temperature) was poured in the plates containing fungi. The production of amylase is indicated by clear visible zone of hydrolysis (violet color) around the fungal colonies. The free starch do not show violet color The 3 starch agar plates were tested for amylase production using iodine solution, and the fungal colony showing the maximum zone of hydrolysis was selected correspondingly from the potato dextrose agar plates for the production of amylase.

e. Identification of Fungi:

The fungi which showed the maximum zone of clearance was selected for the production of amylase. This selected fungus was identified by lacto-phenol cotton blue staining technique. In this technique, few drops of lacto-phenol cotton blue stain were poured on a clean and dry slide. After that, the selected fungi was dropped into the stain with the help of inoculating loop and mixed properly. At the end, the test sample was covered by cover-slip and was observed under the microscope.

3.6 Enzyme Production (Fermentation):

The fungi showing greater zone of hydrolysis was selected for the production of amylase. For this, fermentation media was prepared and the pH of the media was maintained 6.5 for the production of amylase from fungi. Then, about one

loopful fungal isolate was inoculated in the beaker containing fermentation media and was incubated in a shaking incubator for 48 hours at 200 rpm and 28^{o}C (Sethi & Gupta, 2015)

3.7 Centrifugation:

The fermented broth was initially filtered to remove the solid debris floating in the test sample. Then, it was centrifuged at 7000 rpm for 30 minutes. After centrifugation, the supernatant was poured into clean separate beaker. This supernatant was further used for the confirmation and activity of amylase.

3.8 Confirmation of Amylase Production:

The supernatant obtained was used for the confirmation of production of amylase. Starch agar media was prepared and the supernatant was added into the holes made in the media. The media was incubated for 24 hours. After incubation, the plates were flooded with Gram's iodine and observed for the zone of hydrolysis.

3.9 Measurement of absorbance of crude amylase:

Amylase activity was assayed for by the Dinitrosalicyclic acid (DNS) method. In a test tube, the reaction mixture (containing 1ml of soluble starch solution mixed with 1 ml of potassium phosphate buffer, pH 6.9) was mixed with 0.1 ml of the crude enzyme source from one of the labeled conical flasks and incubated for 15 mins at room temperature. After the incubation, 2 ml of the DNS reagent was added and the reaction was terminated by immersing the tube in boiling water (100 ^{o}C) for 10 mins. The same technique was applied to the rest of the crude enzyme sources. The absorbance was measured at 540nm against blank prepared as above without incubation. One unit of α-amylase activity will be defined as the amount of enzyme that liberates 1 µ mole of reducing sugar (maltose equivalents) per minutes under the assay conditions (Miller, 1959).

CHAPTER IV

RESULTS

The samples were collected from different places of Dharan like Bijaypur, Dharan-16, Bhanu Chowk, Panmara and Zero Point. Out of different ranges of temperatures, maximum colonies were recorded at 28^{o}C. Since different fungal colonies developed from a single soil sample, it was difficult to gather information regarding the colonial characteristics of fungi. So, the colonial characteristics of fungi (especially amylase producing fungi) were observed by sub-culturing in starch agar media.

4.1 Colonial characteristics of the isolates of sample:

Different types of fungal colonies were developed in Potato dextrose agar plates initially. In order to get proper information on fungal colonies and to isolate the fungi capable of producing amylase, the well- grown fungal colonies were sub-cultured on starch agar plates. The three fungi sub-cultured on starch agar media have significant colonial characteristics which are mentioned in the table below.

Table I: Colonial characteristics of the isolates of sample on starch agar and PDA.

Serial number	Colony morphology	Probable identity
1	Velvety, black, creamy	*Aspergillus niger*
2	Green color colonies on PDA plates	*Penicillum* spp.
3	Colonies grow rapidly, resemble cotton candy.	*Rhizopus* spp.

4.2 Isolation of Amylase producing Fungi:

Five samples were collected for the screening of amylase producing fungi, among which some of them gave positive result for the production of amylase. Initially, the sample were inoculated in PDA+ 1% starch media, but the test for amylase production was done in starch agar media with the aid of starch-iodine test. Three fungal colonies were tested for amylase production, among which the fungi producing maximum zone of hydrolysis was selected for enzyme production. The amylase production test and its result is mentioned in table below.

Table II: Screening for Alpha Amylase Production (starch hydrolysis test).

Serial number	Probable fungi isolated	Result(mm)
1	*Aspergillus niger*	8.2
2	*Penicillium* spp.	5.2
3	*Rhizopus* spp.	5.0

4.3 Morphological characterization:

Only one fungal colony which produced maximum zone of clearance was observed under the microscope by lacto-phenol cotton blue staining. The fungus was found out to be filamentous mold with septate hyphae, brush-like conidiophores chain conidia. Hence, the fungi selected for the amylase production was found out to be *Aspergillus niger*.

4.4 Analysis of crude enzyme production:

Crude amylase extract which was inoculated in the agar-well of starch agar medium gave zone of clearance. This proved the production of amylase. This crude amylase was used for the measurement of absorbance at 540nm against the blank solution.

Table III: The absorbance at 540nm by amylase produced by *A. niger*

Serial number	Crude enzyme source	Absorbance (540nm)
1	Blank solution	0.00
2	Sample (S)	0.03

CHAPTER V

DISCUSSIONS

In this piece of work, amylase producing organisms (fungi) were isolated and identified from the soil. Then the isolated fungus was used for the production of amylase. The fungi were isolated from the soil by plating method. The inoculation of the sample was done in Potato Dextrose Agar. For the sake of identification of fungi and production of amylase, the fungal colonies were sub-cultured in starch agar media. The sub-cultured fungal colonies were studied on the basis of their colonial characteristics and were examined for the starch hydrolysis test. The fungal colony giving larger zone of clearance was selected for the fermentation process, i.e., production of amylase. The selected fungus was viewed under microscope and was found to be *Aspergillus niger*. The broth produced was centrifuged for the separation of pellets and supernatant liquid. The supernatant liquid obtained was crude amylase enzyme and was used for the confirmation. For the confirmation of production of amylase, starch hydrolysis test was again performed on starch agar media. Small holes were made on the media and crude enzyme was added to those holes. It was then incubated at 28^{o}C for 24 hours. Following the incubation, gram's iodine was flooded on the media and zone of clearance was noticed which indicated the production of amylase.

This produced amylase was then use for amylase activity. Amylase activity was assayed for by the Dinitrosalicyclic acid (DNS) method. In a test tube, the reaction mixture (containing 1ml of soluble starch solution mixed with 1 ml of potassium phosphate buffer, pH 6.9) was mixed with 0.1 ml of the crude enzyme source from one of the labeled conical flasks and incubated for 15 mins at room temperature. After the incubation, 2 ml of the DNS reagent was added and the reaction was terminated by immersing the tube in boiling water (100^{o}C) for 10 minutes (Monga et al 2011).

The absorbance was measured at 540nm with the help of photometer, and was found to be 0.03. The crude enzyme was tested again and again for the absorbance, and each time the absorbance produced was obtained as 0.03.

In similar type of work by Intesar Ali Mezeal and Noor Muafak Alwaan (2013), *Aspergillus niger* was isolated and identified for the production of amylase enzyme. In this work, eight *A. niger* isolates were differentiated on the basis of colony morphology, and all the isolates were tested for the starch hydrolysis. The zone of clearance was measured and was recorded as 5.2mm, 3.7mm, 6.1mm, 5mm, 6.2mm, 8.2mm, 3.6mm and 8mm. Then the fungal colonies were used for the production of amylase enzyme under different enzyme conditions like variable nitrogen source, carbohydrate source, different pH, and different temperatures.

Sonia Seth and Saksham Gupta (2015) isolated four fungal isolates and were identified as *Aspergillus, Penicillium, Fusarium* and *Microsporium.* These isolates were used for the production of amylase and effects of different conditions on the production were examined. In this research, *Aspergillus* was found to produce more amount of amylase in optimum conditions.

Rinku. Sundar, Lila. T, Rajila. C, and P. Suganyadevi (2012) performed similar type of research using *Aspergillus niger* and concluded that higher yield of alpha-amylase production from *A. niger* is possible by submerged fermentation. The maximum production with 450 U/mg of alpha-amylase was observed at room temperature (28^{o}C) with a pH of 7.0.

Amylase is one of the most widely used and demanded enzyme in different industries and factories. The most widespread applications of α-amylases are in the starch industry, where they are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups (Nielsen and Borchert, 2000). The enzymatic conversion of all starch includes: gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further hydrolysis (Gupta et al 2003)

Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes (Mitidieri et al 2006). These enzymes catalyze the hydrolysis of glucosidic linkages in starch polymers, commonly found in foods such as potatoes, custard, pasta, fruit,

chocolate, baby food, barbecue sauce and gravy. As colored stains, their removal is of interest in both detergent and dishwashing contexts. Removal of starch from surfaces is also important in providing a whiteness benefit, since it is known that starch can be an attractant for many types of particulate soils (Mukherjee et al 2009; Mobini-Dehkordi and Javan, 2012).

Amylases are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (Couto and Sanromán, 2006). These enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. The addition of α-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product. Moreover, it generates additional sugar in the dough, which improves the taste, crust color and toasting qualities of the bread. Besides generating fermentable compounds, α-amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods, increasing the shelf life of these products (Gupta et al 2003).

This study has showed us the path for the production of expensive amylase enzyme from the cheap sources (sample) like *Aspergillus niger*. This can be revolutionary in the field of enzyme production since low cost sample can yield expensive enzymes.

CHAPTER VI

CONCLUSION

It is concluded that the fungi isolated from the soil is capable of producing amylase enzyme. Therefore, soil can be used for the production of very sophisticated products (like amylase enzyme) by using very simple methods and equipments. Fungi like *Aspergillus niger*, *Penicillium* spp and *Rhizopus* spp are capable of producing amylase enzyme. They can be used extensively for the production of various biotechnological products. The results obtained from our study can prove the importance of different types of fungi in the industrial field. In addition to these, this study forms a strong basis for the use of different microorganisms (especially fungi) in the production of different enzymes, organic acids, fermented foods, etc. The results obtained from this study support the isolation and identification of different types of fungi for the production of different useful substances.

RECOMMENDATIONS

1. Further research can be carried out to determine the amylase activity in different parameters such as temperature, humidity, nutritional condition, pH, etc.

2. Further study can be done for the purification of amylase.

3. Due to time limitation, only few samples were mobilized in this study. For more conclusive result, the number of samples can be increased.

4. Further study can be done for the production of other enzymes by taking rhe concept of this techniques and procedures.

5. Study of isolated fungi can be done on molecular level for further investigation.

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