ISOLATION, IDENTIFICATION AND CHARACTERISATION OF AMYLASE ENZYME PRODUCED BY DIFFERENT MICROORGANISMS ISOLATED FROM SOIL.

A Dissertation Submitted to **Central Campus of Technology**, **Department of Microbiology**, Tribhuvan University in Partial Fulfillment of the Requirements for the Award of Degree of

Master of Science in Microbiology **(Public Health)**.

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ABSTRACT

Amylase is one of the most widely used enzyme in industries from decades. Its main function is to hydrolyze the starch molecule into glucose unit and oligosaccharides. Among different types of amylases, α-amylase is in high demand due its wide spread use in food, textile, baking and detergent industries. It can be acquired from microorganisms, plants and animals. This study was conducted with the aim to isolate, identify and to characterize the amylase enzyme produced by microorganisms from soil. The soil sample were collected from the different places of Dharan. The screening of amylase producing microorganisms were carried out by starch hydrolysis test on starch agar plates. The organisms producing highest zone of clearance were identified and further used for amylase production and characterization. Based on colonial characteristics, cultural characteristics, staining and biochemical test the organisms were identified as *Bacillus subtilis* whereas the isolated fungi was identified as *Aspergillus niger* on the basis of colonial characteristics and LPCB staining. The identified organisms were further used for the production of amylase on submerged fermentation. The amylase activity was assayed by DNS method. The produced enzyme was centrifuged and partially purified using ammonium sulphate. The amylase production was found to be maximum at 40ºC and 6.5 pH for *Bacillus* amylase and 30 ºC and pH 7.5 for *Aspergillus niger*. Further, Characterization of the enzyme was done for the industrial application of the enzyme. Crude amylase showed its maximal activity at pH 7 and 45 ºC For *Bacillus subtilis* and 30 ºC and pH 6.5 for *Aspergillus niger.* Crude amylase showed great potential for its application in detergent industry.

Keywords –amylase, enzyme, submerged fermentation, DNS method, ammonium sulphate, characterization.

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ABBREVIATIONS

DNSA = Dinitrosalicyclic acid

EDTA = Ethylenediaminetetraacetic acid

EGTA = Ectazic acid

GRAS = Generally Recognized As Safe

KDa = kilo Dalton

LPCB = Lacto phenol cotton blue

MR = Methyl red

NA = Nutrient agar

SA = Starch agar

SBD = Starch Binding Domain

SIM = Sulphide Indole motility

SmF = Submerged fermentation

SSF = Solid state fermentation

VP = Voges-proskauer

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CHAPTER I

INTRODUCTION

1.1 Background

Enzymes are biological catalyst, which initiates and accelerates the biochemical reaction in living cells. Major sources of enzymes are the biological organisms, plants, animals and microorganisms. Enzymes produced by microorganisms are either inducible or constitutive. Enzymes may either be extra cellular or end enzyme (Bansode 2010). The production of microbial enzymes has advantages over the chemical catalyst. The disadvantages of using the chemical catalyst include the need of high temperature and pressure for catalysis and the moderate specificity. However, enzymes are highly specific and catalyze reaction faster than chemical catalyst (Krishna 2011). Enzymes are now being used in various sectors of industries such as detergents, paper, textiles, food, pharmaceuticals and many more. They have been used in saccharification of starch, production of beverages like beer, treatment of digestive disorders, production of cheese from milk etc (Gupta et al 2003) . Among many enzymes that are widely used, amylases are in high demand due to its role in starch hydrolysis and its application in hydrolytic action (Drauz et al 2012).

Amylases are enzymes that breaks the starch into glucose, maltose, maltotriose and dextrin by hydrolysis of glycosidic bonds. It constitute one of the most important group of industrial enzyme and accounts for nearly 25% of the total sale of enzymes (Crueger and Crueger 1990). It is also the first enzyme to be industrially produced from fungal source in 1894, which was used as the pharmaceutical aid for the treatment of digestive disorders. Amylases can be derived from different sources such as plants, animals and microorganisms. Today large numbers of microbial sources are being used for amylase production commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing. Amylases can be divided into three different types depending upon the bond they break in starch molecules. They are alpha-amylase, beta-amylase and gamma-amylase. Alpha amylase is an end hydrolase enzyme that catalyzes the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield glucose and maltose. Beta amylase is an exohydrolase enzyme

that acts from the non-reducing end of a polysaccharide chain by hydrolysis of α-1, 4-glucan linkages to yield maltose units (Sivaramakrishnan et al 2006). Gamma-amylase cleaves α (1-6) glycosidic linkages, in addition to cleaving the last α (1-4) glycosidic linkages at the non-reducing end of amylose and amylopectin to yield glucose (Konsoula and Liakopoulou-Kyriakides 2007) .

Most amylases used in industries are derived from the microbial sources due to great microbial diversity present in the environment, high enzymatic activity in a wide range of conditions such as pH, temperature, osmolarity, pressure etc. It is simple and cost effective in production as well as it is easy to manipulate the desired characters of enzyme. α-amylase is produced by several bacteria, fungi and genetically modified species of microbes. *Bacillus* spp are the most widely used source for α-amylase production among the bacteria and *Aspergillus* spp are mainly used as the fungal source of amylases. *Bacillus amyloliquefaciens* and *Bacillus licheniformis* are widely used for the commercial production of the enzymes. Some of the bacterial species used for the production of amylases are *B. subtilis, B. cereus, B. stearothermophilus, B. megaterium, B. polymyxa* etc (Erdal and Taskin 2010) *Aspergillus niger, A. oryzae, A. awamori, A. kawachhi, Streptomyces rimosus* etc are some of the fungal sources of the enzymes (Couto and Sanroman 2006).

Solid state fermentation and submerged fermentation are used in the production of α-amylase on a commercial scale. Low cost medium is formulates for the production of α-amylase in industries (Kunamneni et al 2005). Submerged fermentation uses free flowing liquid substrates such as molasses and broths whereas solid state fermentation uses solid residues as substrates such as bran, bagasse and paper pulp. It is used for those which require less moisture for growth. SSF is favorable for fungal source and SmF is favorable for bacterial source. Though SmF is the traditional way for the production of industrially important enzymes. SSF has replaced it because it provides the natural habitat for microorganisms as well as it is simple, low-cost investment, requires low energy, less water and lack of foam built up. There are various physical and chemical parameters such as pH, temperature, duration of fermentation, agitation, carbon sources, nitrogen sources, phosphates and different metal ions that affect the production of amylase in industries for the production of enzymes

in large quantities. These parameters vary depending on the microbial source, desired end product, method of fermentation employed and many other such factors.

The enzyme activity is determined by measuring the reducing sugars released as a result of the action of alpha amylase on starch and by measuring the extent of hydrolysis by reading the absorbance of starch-iodine complex. Some of the commonly used measures are Dinitrosalicyclic acid method (DNS), Nelson-somogyi (NS) method and Indian pharmacopoeia method. Enzymes used for industries are usually crude preparations whereas the enzymes need to be highly purified to be used in clinical and pharmaceutical industries. Depending upon the properties of the enzymes desired, various types of purification method are employed such as precipitation, chromatography, liquid-liquid extraction etc. Chromatography techniques applied in purification are ion exchange chromatography, gel chromatography, column chromatography, affinity chromatography etc. Traditionally, the purification of amylases from fermentation has been done in several steps which include centrifugation of the culture, selective concentration and selective precipitation of the enzyme by ammonium sulphate precipitation or solvents like ethanol on the cold. Then finally the enzyme is subjected to the chromatography and gel filtration.

Nowadays, the demand of α-amylase has been increased due to its starch hydrolyzing properties and the activity carried out by its own property. It has replaced the use of chemical method of hydrolysis in various industries. It has wide spread application in food, brewing, textile, detergent and pharmaceutical industries (Bano et al 2011). They are mainly used for starch liquefaction to reduce the viscosity, production of maltose, oligosaccharides, high fructose syrup. It is also employed in desizing of starch in textile industries and to improve the cleaning effect of detergents. As α-amylases are produced by using microbes by SSF which uses byproducts and waste products of other processes, it helps to keep the environment clean. The commercial production of enzyme is limited only to the few selected strains of bacteria and fungi so more researches should be done focusing on other strains of microbes for the production of the enzyme. Hence this study was conducted with the aim to isolate the amylase

producing microorganisms from soil, to partially purify the amylase enzyme and to characterize the amylase enzyme produced by isolated microorganisms.

1.2 Objectives

1.2.1 General objectives

To characterize the amylase enzyme produced by different bacteria and fungi.

1.2.2 Specific objectives

- To isolate amylase producing microorganisms from soil.
- To optimize the production parameters for amylase production.
- To partially purify the amylase enzyme produced by microorganisms.
- To characterize the enzyme produced by microorganisms.

CHAPTER II

LITERATURE REVIEW

2.1 Starch

Starch is an important constituent of the human diet. It is used chemically and enzymatically in different products such as starch hydrolysates, glucose syrup, fructose, maltodextrin derivatives and in food industry. It is a major reserved carbohydrate of all plants. Starches are produced commercially from the seeds of plants such as corn, wheat, sargum or rice; from the tubers and roots of plants such as cassava, potato, arrowroot etc. starch contributes greatly to the textural properties of many foods and is widely used in food and industrial applications as a thickener, colloidal stabilizer, gelling agent, bulking agent and water retention agent (Singh et al 2007) .

Starch is a glucose linked to one another through glycosidic bond. The two types of glucose polymer of starch are amylose and amylopectin. Depending upon the plant, starch generally contain 20-55% amylose and 75-80% amylopectin. Starch may be separated into two components by addition of a polar solvent such as n-butanol. The insoluble amylose complex can then be separated from the soluble amylopectin fraction. Amylose is a linear polymer consisting of upto 6000 glucose units with α-1,4-glycosidic bonds. Amylopectin consists of short α-1,4 glycosidic bond linked to linear chains of 10-60 glucose units and α-1,6 linked to side chains of 10-60 glucose units. The hydrolysis of starch may be carried out using either acid or enzymes as catalyst. It is hydrolysed into smaller oligosaccharides by α-amylase enzyme, which is one of the most widely used commercial enzyme.

2.2 Amylase

Amylase is an enzyme that hydrolyzes starch molecules to give small polymers composed of glucose units (Windish and Mhatre 1965) . It is present in human saliva, where it begins the chemical process of digestion. It is produced by two parts of the human body; the pancreas and the salivary gland. As food is chewed and mixed with saliva, amylase break food into smaller molecules and is neutralized by gastric acid and the starch, partially breaks down and goes onto

smaller intestine and further breaks down by pancreatic amylase resulting into glucose. They are also produced by plants, animals and microorganisms.

Amylase belongs to amylolytic enzymes which act on starch and related polysachharides. All amylases are glycosidic hydrolases and act on α-1,4-glycosidic bonds. They were one of the 1st enzymes to be produced commercially from microorganisms. It was discovered by krichoff in 1811 from wheat. The α-amylase was named by Kuhn in 1925, because the hydrolysis products are in the alpha configuration. It was first produced in 1894 and was used for the treatment of digestive disorders. Boidin and Effront (1917) were the first to use *Bacillus subtilis* and *Bacillus mesentricus* for the production of α-amylase on commercial scale using large fermentors in submerged fermentation (Boidin and Effront 1917). Amylase approximately covers 25 % of the enzyme market (Asghar et al 2005), Burhan et al (2003) and Rao et al (1998). Amylases can be divided into two categories, exoamylases and endoamylases. Endoamylases catalyse the hydrolysis in a random manner in the interior of the starch molecule. Exoamylases hydrolyze from the non-reducing end, successively resulting in short chain end products. Depending upon the place of hydrolysis in polysaccharide, amylases are divided into three types, they are α-amylase, β-amylase and γ-amylase.

2.2.1 Alpha-amylase

Alpha-amylase (α-1,4-α-D-glucan-4-glucanohydrolase; EC 3.2.1.1) is a endohydrolase enzyme that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch to yield glucose and maltose. It belongs to family 13 (GH-13) of glycoside hydrolase of enzyme (Kandra 2003) . It is a calcium-metallo enzyme i.e.it depends on the presence of a Ca^{2+} for its activity, structural integrity and stability. In animals it is a major digestive enzyme and its optimum pH is 6.7-7.0. It is found in wide variety of microorganisms, belonging to the archaea and bacteria. Alpha amylase is divided into two catagories on the basis of degree of hydrolysis of the substrate. They are saccharifying and liquefying α-amylase. Saccharifying α- amylase hydrolyses 50-60% and liquefying α-amylase cleave about 30-40% of the glycosidic linkages of the starch. α-amylases tend to act faster than β-amylases because it can act randomly on the substrate (Liu et al 2010) . In human, both salivary and pancreatic amylases are

α-amylases. α-amylase has become an enzyme of crucial importance due to its starch hydrolyzing activities and the activities that can be carried out due to hydrolysis.

2.2.2 Beta -amylases

Beta amylase (1,4-α-D-glucanmaltohydrolase,glycogenase;EC 3.2.1.2) is another form of amylase which works on the non-reducing end, catalysing the hydrolysis of α-1,4 glycosidic bond cleaving glucose into maltose. β-amylases are unable to cleave the branched linkages in branched polysaccharides such as glycogen and amylopectin, the hydrolysis is incomplete and dextrin units remain. It belongs to family 14(GH-14) of glycoside hydrolase of enzyme. β-amylases are of plant origin. Some of the microorganisms producing β-amylases are *Bacillus polymyxa, B. cereus, Sreptomyces sp, Pseudomonas sp* and *Rhizopus japonicum*. The seeds of higher plants and sweet potatoes are the primary sources of β-amylases. During the ripening of fruits, β- amylases breaks starch into maltose, giving sweet flavor to ripen fruit. Its optimum pH range is 4.0-5.5. It can be used for structural studies of starch and glycogen. Animal's tissue does not contain β-amylase. In industries it is used for fermentation in brewing and distillery industry. It is also used to produce high maltose syrups.

2.2.3 Gamma amylase

Gamma amylase (glucan 1,4-α-glucosidase,glucoamylase, EC 3.2.1.3) cleaves α-1,4 glycosidic linkages at the non-reducing end of amylase and amylopectin, giving glucose. It also cleaves α-1,6 glycosidic linkages. It belongs to GH family 15. It is most abundant in acidic pH and has optimum pH 3 (Drauz et al 2012).As other amylase, it is also used in brewing and fermentation industries, textile industries, paper industries etc. It is used for production of sweet syrups, for modification of food for infants and for removal starch in jelly production.

2.3 Molecular weight of α-amylase

The molecular weight of α-amylases are usually in the same range of 40-70 KDa (Gupta et al 2003) . The highest molecular weight of α-amylase is 210 KDa produced by *Chloroflexus auranticus* (Omar 2011) and the lowest molecular weight is 10 KDa which is produced by *Bacillus caldolyticus* (Gupta et al 2003).

2.4 Molecular structure of α-amylase

The three-dimensional structure of α-amylase have revealed monomeric, calcium containing enzymes with a single polypeptide chain folded into three domains (A-C). The A domain consists of a highly symmetrical fold of eight parallel β- strands arranged in barrel encircled by 8 α-helices. The highly conserved amino acid residues of α-amylase family involved in catalysis and substrate binding are located at the C- terminal of β- strands in this domain. B-domain of α-amylase protrudes between β-sheet no 3 and α-helix no.3, ranges from 44 to 133 amino acid residues and plays a role in substrate or Ca^{2+} binding(Van Der Maarel et al 2002) . All known α-amylases, with a few exceptions, contain a conserved Ca^{2+} binding site which is located at the interface between domain A and B (Linden et al 2003). C domain of α-amylase is relatively conserved and folds into anti-parallel β-barrel. Depending on the type and source of α-amylase, the orientation of domain C varies to domain A. C domain has an additional starch binding domain (SBD) positioned at C-terminal of amylase which helps amylase to bind and hydrolyse native granular starch. This domain is also responsible for thermostability of the amylase.

2.5 Biochemical properties of α-amylase

The substrate specificity of α-amylase varies from microorganisms to microorganisms. α-amylase has highest specificity towards starch followed by amylose, amylopectin, cyclodextrin, glycogen and malto-triose. The pH optimum of the α-amylase vary from 2 to 12 (Vinihen and Mantsiila 1989) . Most of the bacterial and fungal amylases have pH optimum in the acidic to neutral range. In some cases, the pH optimum was found to be dependent on the temperature and calcium. The temperature optimum for the activity of amylase is related to the growth of the microorganisms. The lowest temperature optima is reported to be 25-30 ˚C for *F. oxysporum* (Chary and Reddy 1985) and highest 100-130 °C for *Pyrococcus furiocus* (Rose 1980) and P. *woesei* (Ray et al, 1995)respectively**.** In some cases, temperature optima are dependent on calcium and sodiumchloride. There are several metal cations, especially heavy metal ions, EDTA, EGTA, BSA, iodoacetate, Sulphdryl group reagent that inhibit the α-amylase.

2.6 Sources of α- amylases

Alpha amylases can be derived from several sources such as plants, animals and microbes. It can also be derived from barley and rice plants (Oboh 2005) . The microbial source of amylase enzyme meets the industrial demand and a large number of them are available commercially (Pandey et al 2000) . Although α-amylases have been derived from yeast, fungi, bacteria and actinomycetes, amylases produced by bacteria and fungi has dominated the industry. There are 2 major reasons for increasing demand of microbial enzymes;

- Microorganisms can grow rapidly, increasing the speed of production of enzyme. They are easy to handle, requires less space and are cost effective too.
- ii) They can be easily manipulated using genetic engineering and can be subjected to strain improvement, mutation and other changes by which production of enzyme can be optimized. (Pandey et al 2000 and Fossi et al 2009).

Among the various bacterial species, *Bacillus* spp are widely used for thermostable α-amylase production to meet industrial needs. *Bacillus subtilis, B. licheniformis, B. amyloliquefaciens a*nd *B. stearothermophilus* are known to be good producers of the alpha amylase (Kim et al 1995). *B. amyloliquefaciens and B. licheniformis* are widely used for the commercial production of enzyme. *B. subtilis, B amyloliquefaciens and B. stearothermophilus* are known as the good producer of thermostable α-amylase. Thermostability is an important characteristic as enzymatic and liquefaction and saccharification of starch are performed at high temperature. Thermostable enzymes isolated from thermostable microorganisms have found a number of commercial applications because of its stability. Some of the bacteria other than Bacillus producing amylases are *Halobacillus spp, Haloarcula hispanica, Halomonas meridian, Chromohalobacter sp* etc.

Most of the α-amylase producing fungi are mesophilic fungi. Fungal sources are limited to terrestrial isolates, mostly to *Aspergillus* and *Penicillium* (Kathiresan and Manivannan 2006). *Aspergillus* species are the mostly used fungal source for the production of α-amylase. They produce a large variety of extracellular

enzymes and amylases are the ones with most significant industrial importance (Hernandez et al, 2006). Apart from *Aspergillus* few species of *Penicillium* has also been used for the production of alpha amylase by submerged fermentation (Kaur and Sharma 2014). *P. chrysogenum* and *P. exoansum* are also used for producing enzyme by solid state fermentation. *A. awamori, A. niger and A. oryzae* are the most commonly used species of *Aspergillus* for the commercial production of α-amylase. The thermophilic fungi *Thermomyces laguginosa* and *Thermoascus auranticus* are also an excellent producer of amylase (Kunamneni et.al, 2005 and Arnesen et.al 1998).

2.7 Production of α-amylase

Commercial production of amylases is carried out in various steps because of the environmental factors requires for the growth of microorganisms being employed for production may differ from those requires for the production of amylases. The parameters include nutrients supplementation, pH of the medium, temperature, osmotic relationship and the control of the contamination during fermentation. The α-amylase has been produced by two methods for commercial use. They are by Submerged fermentation (SmF) and Solid-State Fermentation (SGBJMNSF). Although submerged fermentation has been used for longer period of time, SSF has gained renowed interest in the production of enzymes.

SmF is suitable for microorganism that requires high moisture contents for their growth. It employs free flowing liquid substrates such as molasses and broths for the enzyme production. The substrates are utilized rapidly in this process, so the substrates need to be constantly replenished. The products are secreted in the fermentation broth. It has several advantages such as it allows the utilization of genetically modified organism to greater extent than SSF. The sterilization of the medium and purification process of the end product can be done easily. The process parameters such as pH, temperature, aeration and moisture can be controlled conveniently (Kunamneni et al 2005).

SSF is suitable for those microorganisms that require low moisture contents for their growth. It uses solid substrates such as bran, bagasse and paper pulp for the production of enzymes. The substrates are utilized very slowly and steadily so the same substrate can be used for longer duration. The main advantage of SSF

is that the nutrient rich material is easily recycled and used as substrate. It needs simpler equipment, have higher volumetric productivity, have higher concentration of products and lesser effluent generation.

Though both fermentation methods are employed for the production of amylase from fungal microbes, SSF is found to be the appropriate process for the production of enzymes. It is cost effective and it provides the suitable medium that resembles the natural habitat of fungal species for growth (Laderman et al 1993).

2.8 Production parameters of α-amylase

The production parameter of alpha amylase varies depending upon the microbial source, desired end product, method of fermentation employed and many other such factors. The various parameters of enzyme production include composition of medium, temperature, pH, agitation, carbon and nitrogen source etc (Fogarty and Kelly 1979).

2.8.1 Temperature

The influence of temperature on amylase production is related to the growth of enzyme takes place. α-amylase has been produced at wide range of temperature among the bacteria. The optimum temperature for growth and amylase production were found to be 45-50^{o}C for *Bacillus subtilis.* Temperature as high as 80^{o} C has been found for amylase production from hyper thermophilic *Thermococcus profundus*. Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25- 37 ^{o}C. Optimum yield of α- amylase were achieved at 30-37 ^{o}C for *Aspergillus oryzae* (Giri et al 2000).

The enzyme production increases with increase in temperature till it reaches the optimum. With the further increase in temperature the enzyme production decreases due to the loss of moisture in the substrate which adversely affect the metabolic activities of the microbes leading to the reduced growth and decline in enzyme production (Prakasham et al 2007) .

2.8.2 pH

Enzymes are pH sensitive. It plays an important role in enzyme production by inducing morphological change in the organism and in enzyme secretion. The pH serves as a valuable indicator in the initiation and end of the enzyme's synthesis. Most of the *Bacillus* sps commercially used for the production of bacterial amylases have optimum pH of 6.0-7.0 for growth and enzyme production. The optimum pH for the enzyme production *by Bacillus* isolated from the dhal industry was found to be 6.5 whereas *Aspergillus* sps such as *A. oryzae* and *A. niger* showed maximum production at pH of 5.0-6.0 in SmF. The thermophilic microorganism *Thermococcus profundus* has optimum production of amylase at pH 5.0 (Swamy and Seenayya 1996) .

2.7.3 Fermentation Duration

Duration of fermentation directly affect the production of enzyme. The enzyme activity increases with increase in incubation time till it reaches the optimum duration. In most of the cases, the production of enzyme decreases with increasing incubation period due to depletion of nutrients in the medium or due to release of toxic substances(Raul et al 2014) .*Bacillus amyloliquefaciens* showed an optimum amylase production activity after 72 hours of fermentation whereas *Bacillus subtilis* showed high yield of α -amylase after 48 hours of incubation.

2.7.4 Substrate source; induction of amylase

Alpha amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose (Tomomura et al 1961) . The carbon sources such as glucose and maltose has been used for the α- amylase production. However, the use of starch remains promising and ubiquitous. Lactose (Kelly et al,1997) , casitone (Cheng et al,1989) , fructose (Welker and Campbell 1963) and starch producing waste water(Jin et al,1999) are also used for the production of α- amylase. Some agro-byproducts such as wheat bran(Lonsane and Ramesh 1990) are also used. Wheat bran has been used as substrate *by B. licheniformis* and *A. niger* for amylase production. Banana waste was also used for the production by *B. subtilis.*

2.7.5 Nitrogen source

Both organic and inorganic sources of nitrogen are used for the production of α-amylase. Most commonly used organic source of nitrogen include peptone, yeast extract and soyabean meal (Paqeut et al, 1991). Ammonium chloride, Ammonium sulphate and Ammonium hydrogen phosphate are some inorganic sources of nitrogen. Yeast extract increased the production of α-amylase by 110-150 % by *A. oryzae* when used as an additional nitrogen source than ammonia was used as a sole source of nitrogen(Pedersen and Nielsen 2000) . Peptone is the highest yielding source of nitrogen when employed in SSF. Peptone showed maximum production of enzyme for α-amylase by *Penicillium expansum*. For *A. fumigates* and *A. niger* (Moller et al, 2004), peptone, sodium nitrate and casein hydrolysate are found to be good source for enzyme production. Various other organic nitrogen sources are also reported to support maximum amylase production by bacterial strains.

2.7.6 Moisture

Moisture content may vary depending upon the microbial source. Fungal source requires the less moisture content whereas bacterial source requires higher moisture content (Leveque et al, 1999). The enzyme production usually increases with the high initial moisture content reaching an optimal level followed by decrease in enzyme yield with the further increase in moisture content. At high moisture content, the swelling of substrates take place which ensures the better uptake of nutrients by microbes. But at low moisture, there is high water tension and low solubility of nutrients leading to low yield of enzyme.

2.7.7 Agitation

Agitation influences the mixing and oxygen transfer rates in many fungal fermentations and thus influences the mycelia morphology and product formation. Higher the agitation speed, higher the damage of mycelia growth and decrease in enzyme production. However, at constant specific growth rate, it was found that there is not any effect on enzyme production due to change in agitation rate. Normally 300 rpm has been employed for the production of amylase from various microorganisms.

2.8 Determination of enzyme activity

Alpha amylases are generally assayed by using soluble starch on modified starch as the substrate. The reaction is monitored by an increase in the reducing sugar level or decrease in the iodine color of the treated substrates. There are various methods for the determination of enzyme activity based on decrease in starch-iodine color intensity, increase in reducing sugar, degradation of colour complexed substrates and decrease in viscosity of starch suspension.

2.8.1 Decrease in starch-iodine intensity

Starch forms a deep blue complex with iodine and by hydrolysis of the starch changes into reddish brown. The absorbance can be read after the enzyme substrate reaction has been terminated. It gives the measure of the extent of hydrolysis of starch by α-amylase.

2.8.1.1 Determination of dextrinizing activity

The dextrinizing activity of α-amylase employs soluble starch as substrates and dilute HCl as terminator of reaction, iodine solution (KI) is added. The decrease in absorbance at 620 nm is then measured against a substrate control. One percent decline in absorbance is considered as one unit of enzyme(Fuwa 1954) . The major disadvantage of this method is the interference of media components of luria broth, tryptone, peptone, corn steep liquor etc and thiol compounds with starch iodine complex. In interference with these media components, copper sulphate and hydrogen peroxide protect the starch-iodine colour.

2.8.1.2 Indian pharmacopoeia method

This method is used to calculate α-amylase activity in terms of grams of starch digested by a given volume of enzyme. It is usually employed for estimating α-amylase activity in cereals. In this method various dilution of enzyme solution is incubated with starch at 40 ^{o}C for 1 hour after forming the coloured complex by addition of iodine, the tube that shows no blue colour is used to calculate the enzyme activity.

2.8.2 Dinitrosalicyclic acid method (DNSA)

This method determines the increase in reducing sugars as a result of amylase action on starch (Bernfeld P 1955). In this method, aliquots of the substrate stock solution are mixed with the enzyme solution and incubated at 50 ^{o}C for 10 min. Then DNS reagent is added to the test tube and mixture is incubated in boiling water bath for 5 min. After cooling to room temperature, the absorbance of supernatant is measured at 540 nm. The major defect in this method is a loss in colour produced and destruction of glucose by constituent of DNSA reagent.

2.8.3 Decrease in viscosity of starch suspension

It is generally used in the bakery industry to assess the quality of the flour. There are two methods used to measure the activity in terms of decreasing viscocity of the starch suspension. They are Falling number (FN) method and Amylograph /Farinograph test. The FN method is accepted internationally for assessing cereal α-amylase activity in flour enzyme preparation at 100^{o}C (Perten 1984) . It is modified and standardize for measuring both cereal and fungal α-amylase at 300°C, by replacing a part of the flour with pre-gelatinised starch. Amylograph test is used in milling and baking industry to assess the diastatic activity of flours. It is based on the relationship of peak viscocity of starch slurry and the enzyme activity level(American Association of Cereal 1989) .

2.9 Purification of α-amylase

Industrial enzyme produced in bulk generally require little down streaming process hence are usually crude preparation whereas the enzymes used in clinical and pharmaceutical industry requires to be highly purified. The purified enzyme is also a prerequisite for studying the structure-function relationship and biochemical properties of the enzyme(Gupta et al, 2003) .

Depending upon the enzymes desired, a series of precipitation, chromatography and liquid-liquid extraction is carried out to achieve high purity of amylase. The crude extracellular mass obtained from the fermented mass by filtration and centrifugation can be precipitated and concentrated using ammonium sulphate precipitation or organic solvents. The precipitated sample is then dialysed against water or buffer for further concentration (Shih and Labbe 1995) and then subjected to chromatographic techniques like ion exchange chromatography, gel

chromatography and affinity chromatography for further purification. These multi-step methods require expensive equipments at each step, making them labourious, time consuming and may result in the increasing loss of the desired product. Liquid-liquid extraction is the transfer of certain components from one phase to another when immiscibe or partially soluble liquid phase is brought into contact with each other. It is widely used in the industry due to its simplicity, low cost and ease of scale up**.**

2.10 Application of α-amylase

Amylases are the most important hydrolytic enzymes for all starch-based industries and it has found wide spread application in food, detergents, textiles and in paper industries for hydrolysis of starch. They are also used in pharmaceutical and fine chemical industries. Some of the applications of amylases are as follows;

- Amylases are used in the clarification of fruit juices for jelly production.
- Fungal amylases are used in the preparation of dried baby foods and cereal products.
- Amylases are used in the production of chocolate syrups.
- Amylases are also used in the production of high conversion syrup.
- Amylases are used in the brewing industry in the initial breakdown of starchy substances.
- Amylases are used in the textile industry as a desizing agent.
- Amylases are used in the laundry to remove the spots from the clothes.
- Amylases are used as a digestive enzyme to digest carbohydrates.

CHAPTER III

MATERIALS AND METHOD

3.1 Materials

Different laboratory materials, equipments, media and reagent are used during the study are used which are listed in Appendix A.

3.2 Methods

3.2.1 Study duration

The study was conducted from May 2017 to October 2017.

3.2.2 laboratory set up

All the laboratory works were performed in Microbiology laboratory, Central Campus of Technology, Dharan.

3.2.3 Sampling method and sample size

Simple random sampling was done for the collection of the sample. Total of 20 soil samples were collected from different places of Dharan sub-metropolitan city.

3.2.4 Sample collection and Transport

Soil samples were collected from the different places of Dharan sub-Metropolitan city on sterile Petriplate using sterile spatula and was transported as soon as possible to the laboratory of Central Campus of Technology,Dharan.

3.3 Processing of samples

3.3.1 Laboratory analysis of sample

3.3.1.1 Isolation of Bacteria

1 gm of soil sample was weighed and added to 9 ml of sterile distilled water. Sterile dilutions were prepared up to 10^{-9} dilutions and 0.1 ml of each dilution was added using spread plate method to starch agar plates. The starch agar plates were incubated at 37^{o}C for 24 hours.

3.3.1.2 Selection of the microorganisms

Microorganisms that survive on starch agar medium producing a clear zone around the colonies when flooded with grams iodine was considered as amylase

producing microorganisms. The bacteria forming the largest halo zone were further used for studies.

3.3.1.3 Identification of the microorganisms

Various morphological characteristics, cultural properties were observed as well as grams staining and biochemical test were performed for the identification of bacteria .

3.3.2 Isolation and Identification of fungi

1 gm of soil sample was taken and added to 9 ml of distilled water. Sterile dilutions were prepared up to 10^{-4} dilutions and 0.1 ml of each dilution were added by spread plate method to the Potato dextrose agar. It was incubated at 25 ^{o}C for 7 days.

Morphological characteristics were observed and Lactophenol cotton blue staining was performed for the identification of the fungi (Aneja 2007).

3.3.2.2 Selection of amylase producing fungi

Streak inoculation of *A. niger* was performed on the starch agar plate and 1% PDA plates and were incubated at 25^{o}C for 3 days. Then the surface was flooded with grams iodine. The *A. niger* forming largest halo zone was used for the further studies.

3.3.3 Culture conditions and amylase production

The bacteria were grown aerobically at 37^{o}C in basal medium (BMA) (containing 0.2% beef extract, 0.2% peptone and 0.1%NaCl). Amylase production was carried out in a volume of 20 ml BMA taken in a 100 ml conical flask inoculated with loopful of cells from 24 hrs old slant culture and was kept in 37^{o}C in a rotatory water shaker bath at 120 rpm. After 24 hrs of incubation, the content was centrifuged at 10,000 rpm for 5 min to remove the bacterial cell. The supernatant was used for amylase activity.

The *A. niger* was grown in liquid medium containing 0.3 % yeast extract,0.1 %KH_2PO_4,0.05% $MgSO_4.7H_2O$ and 1% $(NH_4)_2HPO_4$. The pH was adjusted to 7.5 and sterilized by autoclaving at 121^{o}C for 15 min. starch (15%) was added. Then the flask was inoculated with *A. niger* and then incubated at 28^{o}C for 7 days in rotatory water shaker bath. After incubation the contents were

centrifuged at 5000 rpm for 5 min to remove the mycelia. The supernatant was used for the amylase activity.

3.4 Confirmation of amylase production

The supernatant obtained was used for the confirmation of production of amylase enzyme. Starch agar media was prepared and the supernatant was added into the Wells made in the media. The media was incubated for 48 hours. After the incubation, the plates were flooded with gram's iodine and observed for the zone of hydrolysis.

3.5 Determination of α-amylase activity

Amylase activity was determined according to DNSA method. In a test tube, the reaction mixture (containing 1 ml 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 1 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 mixture was terminated by immersing the tube in boiling water (100 ^{o}C) for 10 min. the absorbance was measured at 540 nm against blank prepared as above without incubation. One unit of α- amylase activity is defined as the amount of enzyme that liberates 1μ mole of reducing sugar (maltose equivalents) per minutes under the assay conditions.

3.6 Optimization of production parameters

3.6.1 Effect of temperature on amylase production

The optimum temperature for the amylase production was determined by incubating fermentation media at different temperature (20, 25,30,35, 40 and 45ºC) for 24 hours for *Bacillus* amylase and 72 hours for *Aspergillus* amylase.The enzyme assay was carried out by DNS method.

3.6.2 Effect of pH on amylase production

The optimum pH for the amylase production was carried out by incubating fermentation media i.e., basal media at different pH (5.5,6.6.5,7,7.5 and 8). The enzyme assay was carried out after 24 hours for *Bacillus* amylase and 72 hours for *Aspergillus* amylase.

3.7 Partial purification of α- amylase enzyme

Bacteria grown in BMA was centrifuged at 10,000 rpm for 5 min to remove cells. The supernatant was subjected to fractionated ammonium sulphate precipitation for enzyme purification. Ammonium sulphate crystals were added to the supernatant to bring the saturation to 65% in an ice bath. After 2 hours, the precipitate was collected by centrifugation at 10,000 rpm for 20 min. The enzyme was recovered by suspending the precipitate in 0.1mM citrate buffer at pH 6.

Fungi grown in media for 6 days was centrifuged at 5,000 rpm for 10 min and was subjected to ammonium sulphate precipitation for enzyme purification. Ammonium sulphate crystals were added to the supernatant to bring the saturation to 65% in an ice bath. After 2 hours, the precipitate was collected by centrifugation at 5,000 rpm for 5 min. The enzyme was recovered by suspending the precipitate in 0.1 mM citrate buffer at pH 6.

3.7 Characterization of α-amylase enzyme

The characterization of partially purified amylase produced by both *Bacillus subtilis* and *Aspergillus niger* were done on the basis of temperature and pH .The starch hydrolysis was observed on pH (3,4,5,6,6.5,7,8,9) and temperature (20,25,30,35,40 and 45 ºC). The reaction mixture was prepared by adding 1.5 ml of starch and 0.5 ml of partially purified amylase enzyme in 2 ml of phosphate buffer pH 8.

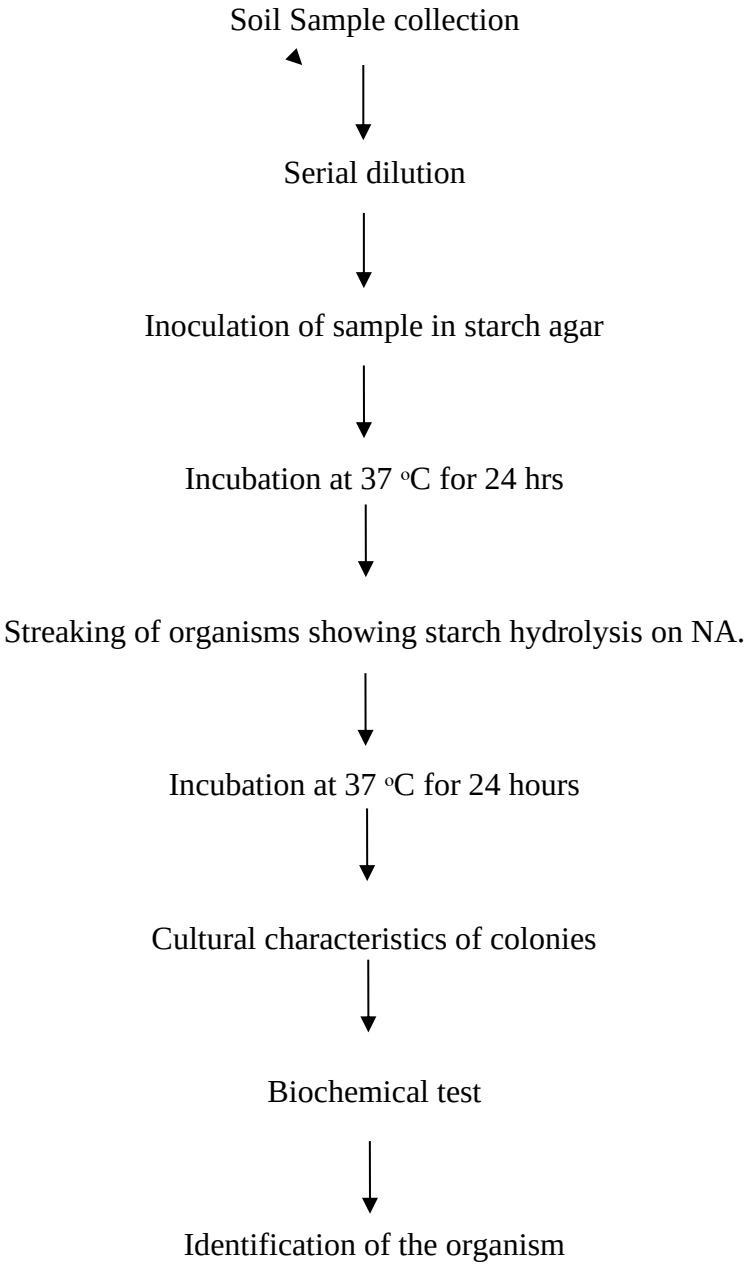
3.7.1 Determination of optimum temperature

The optimum temperature for the amylase activity was determined by incubating the reaction mixture at different temperature ranging from 20 ºC-45ºC for 15 min. The reaction mixture was prepared by adding 1.5 ml of starch and 0.5 ml of partially purified amylase enzyme in 2 ml of phosphate buffer having pH 8.0. The reaction was terminated by adding 1 ml DNS reagent and the mixture was incubated in boiling water for 10 min. After cooling to room temperature, the enzyme activity was determined by taking the absorbance at 540 nm.

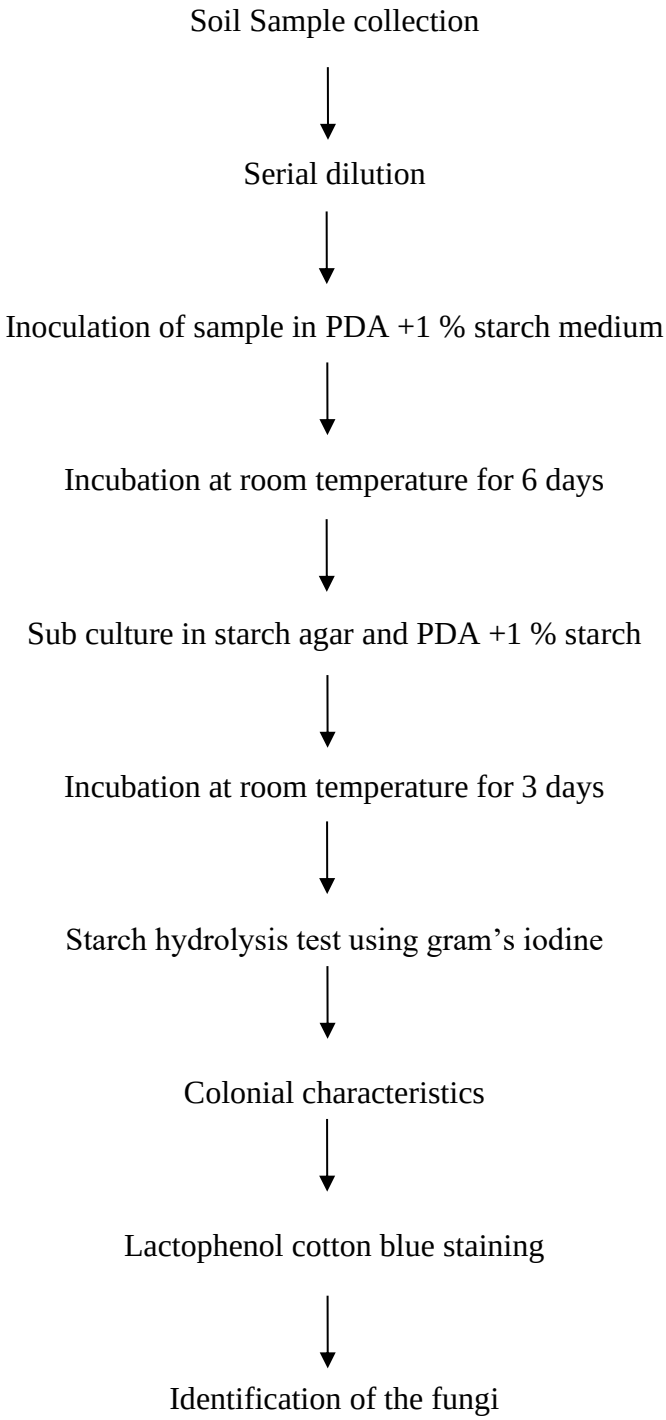
3.7.2 Determination of optimum pH

The optimum pH for the amylase activity was determined by incubating the reaction mixture containing 1.5 ml of starch and 0.5 ml of partially purified amylase in the phosphate buffer at pH 3,4,5,6,6.5,7,8 and 9. Then the mixture was incubated for 15 minutes in room temperature and the reaction was terminated by adding 1 ml DNS reagent and the mixture was incubated in boiling water for 10 min. After cooling to room temperature, the enzyme activity was determined by taking the absorbance at 540 nm.

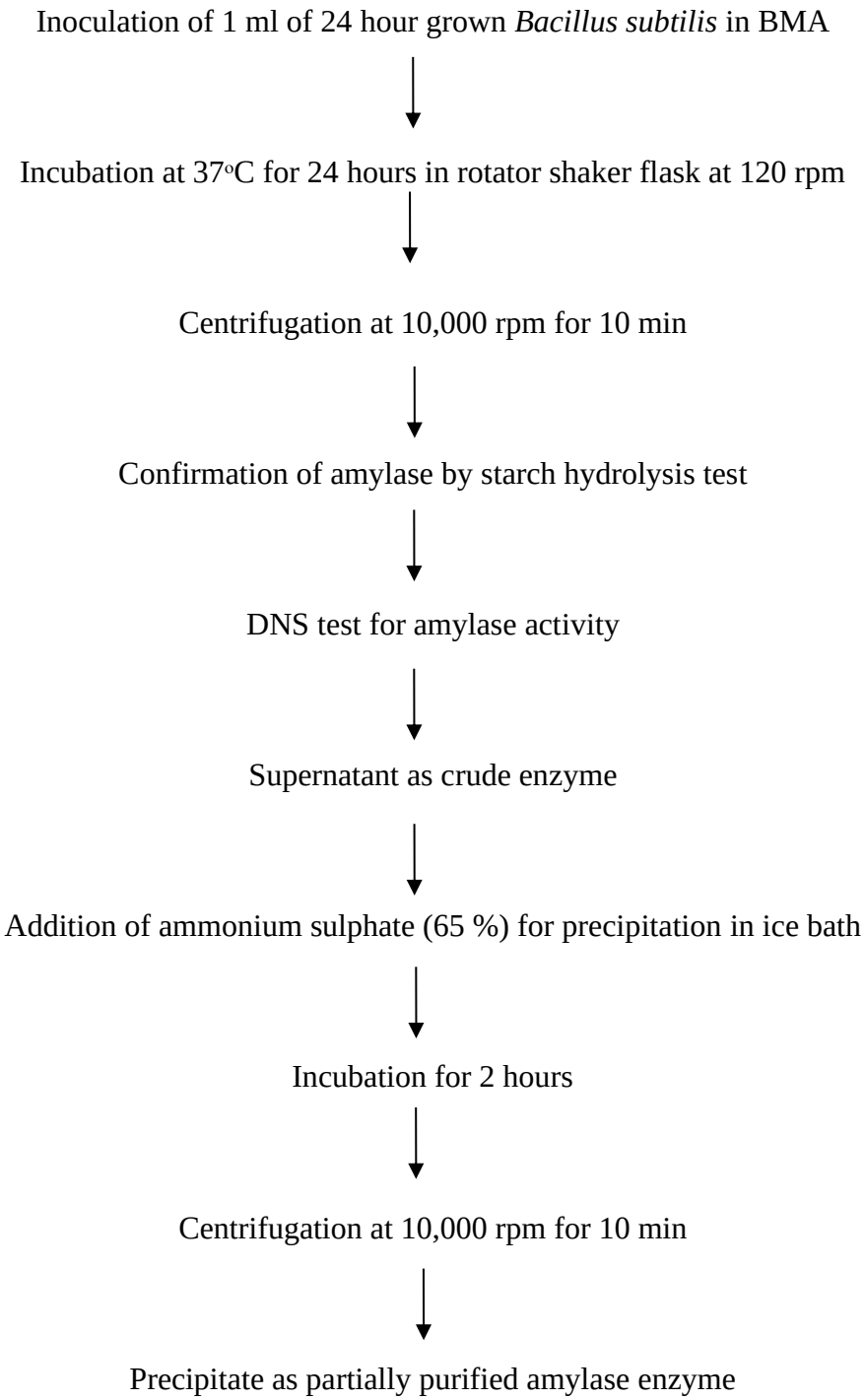
Flow chart for isolation and identification of amylolytic bacteria



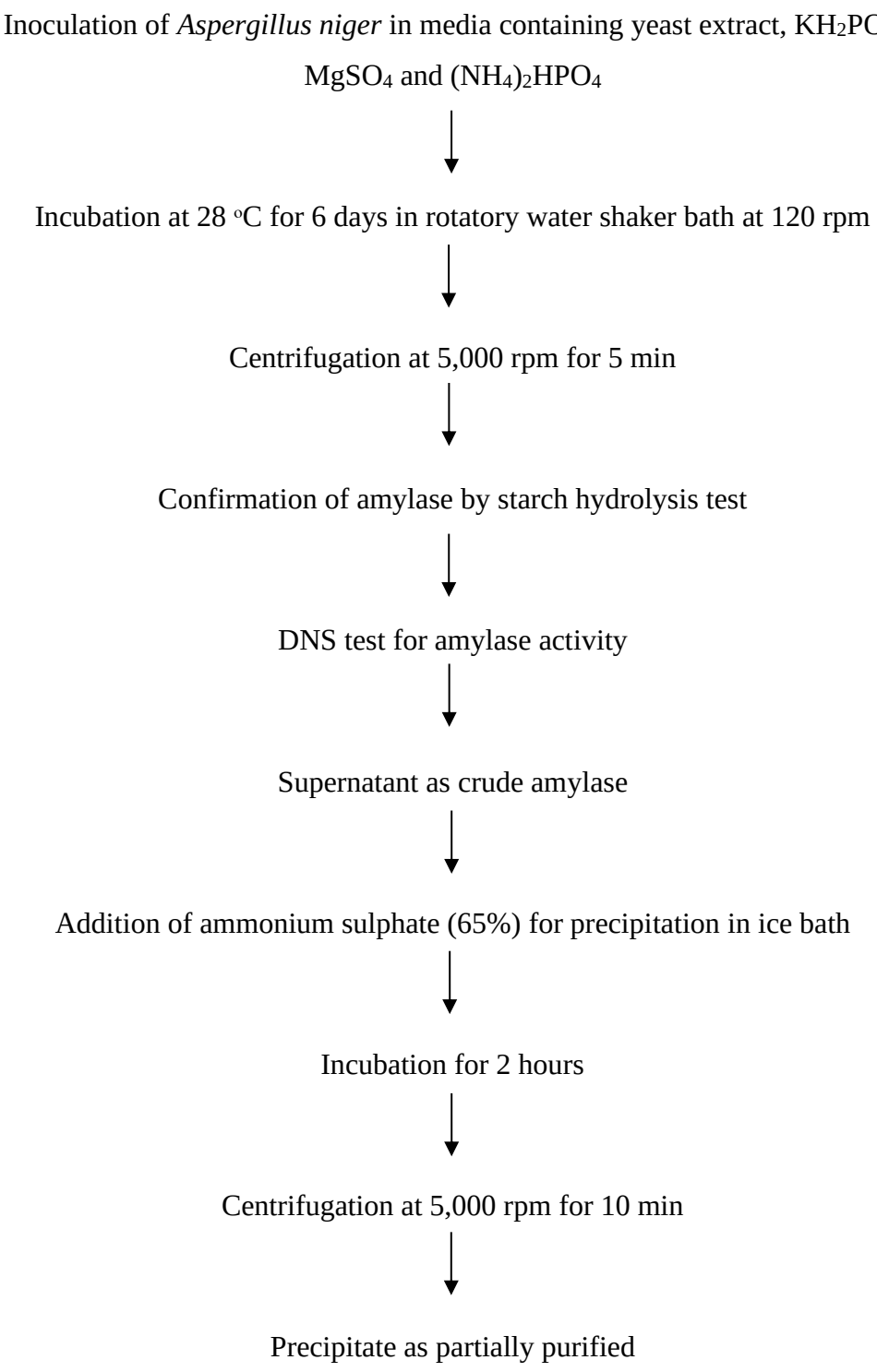
Flow chart for the isolation and identification of amylolytic fungi



Flow chart for the production and partial purification of *Bacillus* enzyme



Flow chart for the production and partial purification of *Aspergillus* enzyme



CHAPTER IV

RESULTS

4.1 Isolation and Identification of amylase producing Fungi

Out of 20 samples, 10 *Aspergillus niger* were isolated. Among them, only 4 *Aspergillus niger* showed the amylase activity. The *Aspergillus niger* were identified on the basis of colonial characteristics and Lactophenol cotton blue staining. The results are illustrated in the Appendix C.

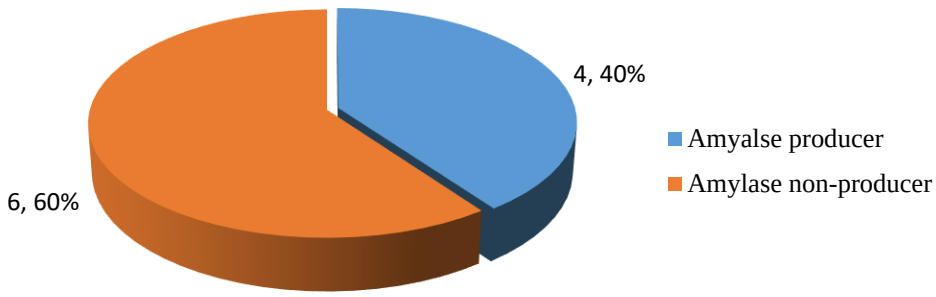


Figure 1: Chart showing the amylase producing and non-producing *Aspergillus niger*

4.2 Isolation and identification of amylase producing Bacteria

Out of 20 soil samples collected from different places of Dharan, 13 isolates were identified as *Bacillus subtilis*. Among 13 isolates, 5 isolates showed the positive result for the amylase production. The bacteria were identified on the basis of their colonial characteristics, staining and various biochemical test. The results are illustrated in the Appendix.

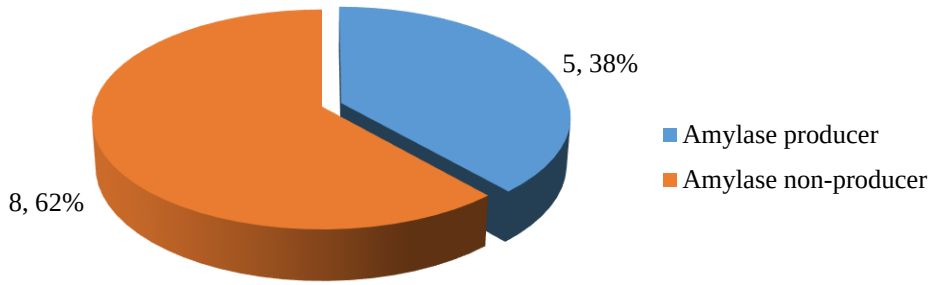


Figure 2: Chart showing the amylase producer and non-producing *Bacillus subtilis*

4.3 Analysis of amylase activity

Amylase activity was assayed by the measurement of the absorbance at 540 nm of the crude enzyme against the blank solution. One unit of α-amylase activity is defined as the amount of the enzyme that liberates 1 μmole of reducing sugar (maltose equivalents) per minutes under the assay conditions.

Table 1: Analysis of *Bacillus amylase*

Crude enzyme source(bacteria)	**Absorbance (540 nm)**
S2	0.21
S5	0.11
S6	0.09
S9	0.25
S10	0.12

Table 2: Analysis of *Aspergillus amylase*

Crude enzyme source (fungi)	Absorbance (540 nm)
S12	0.54
S15	0.51
S18	0.14
S20	0.65

4.4 Optimization of production parameters

4.4.1 Effect of temperature on amylase production

The effect of temperature on amylase production was studied in the Basal medium for amylase production at different temperature (20-45ºC) for 24 hours for Bacillus subtilis. It was found that the amylase production was low at 20ºC which increased with raising temperature giving optimum production at 40ºC. whereas the Optimum temperature for the *Aspergillus* amylase was recorded at 30 ºC after 72 hrs of incubation in basal medium. The production of amylase was found to decrease with the rise in temperature.

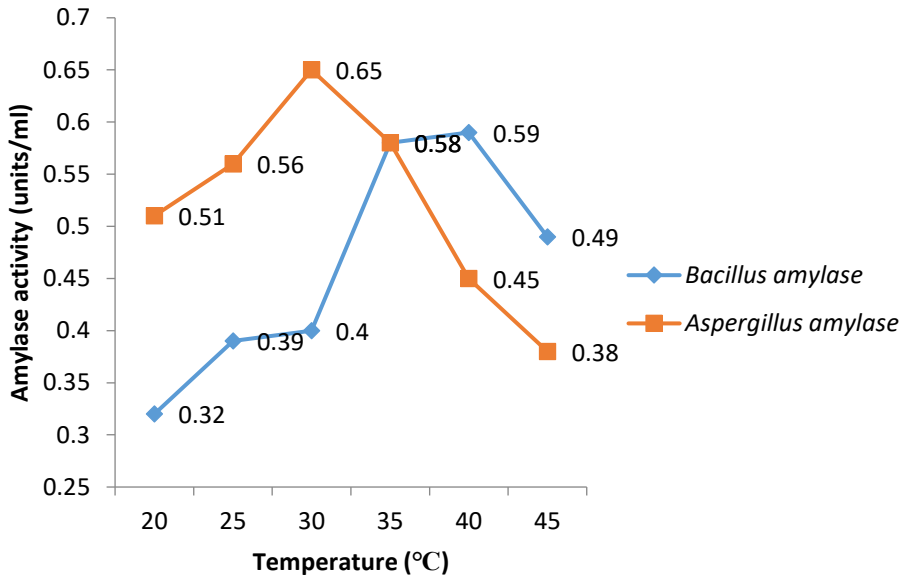


Figure 3: Effect of temperature on production of enzyme

4.4.2 Effect of pH on amylase production

The amylase production in relation to the pH of the medium was studied for both *Bacillus subtilis* and *Aspergillus niger* at various pH range.The study reported for the maximum production of amylase by *Bacillus subtilis* was at pH 6.5.Whereas the Optimum pH for *Aspergillus* amylase was recorded at pH 7.5.

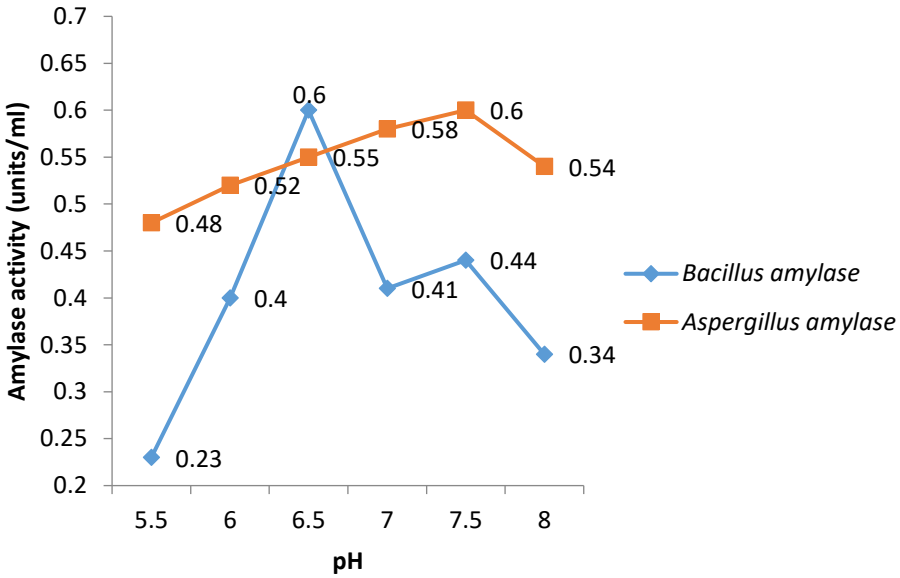


Figure 4: Effect of pH on the production of enzyme.

4.4 Partial purification of amylase enzyme

The fermented broth of *Bacillus subtilis* was centrifuged at 10,000 rpm for 20 min to remove the cells as well as the fermented broth of *Aspergillus niger* was centrifuged at 5,000 rpm for 10 min to remove the mycelia. The cell free broth i.e., crude enzyme was then used for the partial purification of the enzyme.

Enzymes were precipitated from cell free broth using ammonium sulphate. The ammonium sulphate was added to the supernatant to bring 65% saturation in an ice bath and the precipitate was collected by centrifugation. The enzyme was recovered by suspending the precipitate in 0.1 mM citrate buffer and was dried. The amount of dried enzyme was weighed respectively.

Table 3: Dry weight of amylase from *Bacillus subtilis*

S. N	Sample no.	Weight of dry enzyme(gm)
1	S2	0.5
2	S5	0.5
3	S6	0.6
4	S9	0.7
5	S10	0.5

Table 4: Dry weight of amylase from Aspergillus niger

S. N	Sample no.	Weight of dry enzyme(gm)
1	S12	0.6
2	S15	0.6
3	S18	0.7
4	S20	0.7

4.5 Characterization of α- amylase enzyme

4.5.1 Effect of temperature on α-amylase activity

The optimum temperature for the partially purified amylase was determined by measuring the activity at different temperatures. The optimum temperature for enzyme activity of amylase produced by *Bacillus subtilis* was found at 45°C. The enzyme activity was low at initial temperature which increases with increasing temperature giving highest activity at 45°C. whereas, the optimum temperature for the amylase produced by *Aspergillus niger* was recorded at 30 °C. The activity of amylase in this case was found to be lowest at 20 °C resulting highest at 30 °C which decreased with rise in temperature. (Fig 3)

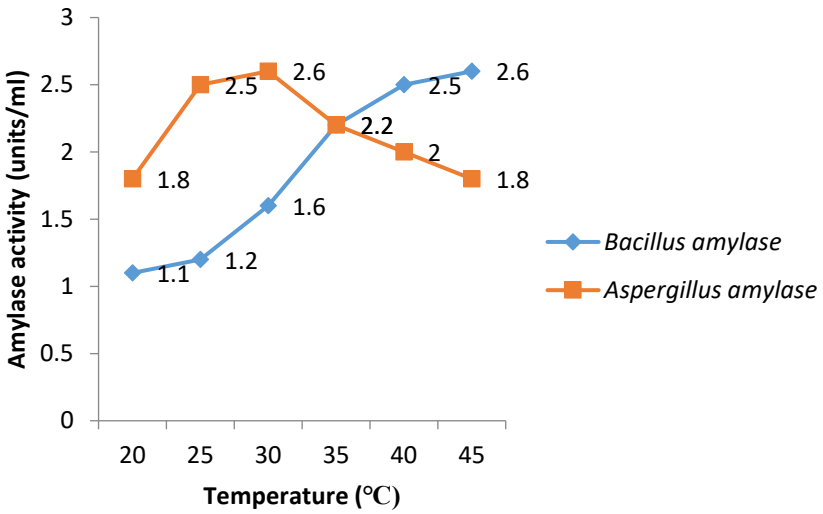


Figure 5: Effect of temperature on α-amylase activity

4.5.2 Effect of pH on α-amylase activity

The activity of *Bacillus* enzyme was low at pH 3 and 4 which rapidly increase with pH giving optimum activity at pH 7. However, the activity decreases from pH 8 to pH 9. But in case of *Aspergillus* enzyme the activity of the enzyme was moderate at pH 3 and 4 which increased at pH 5 and 6 resulting highest activity at pH 6.5. However, the enzyme activity decreased from pH 8 and 9. (Fig 6)

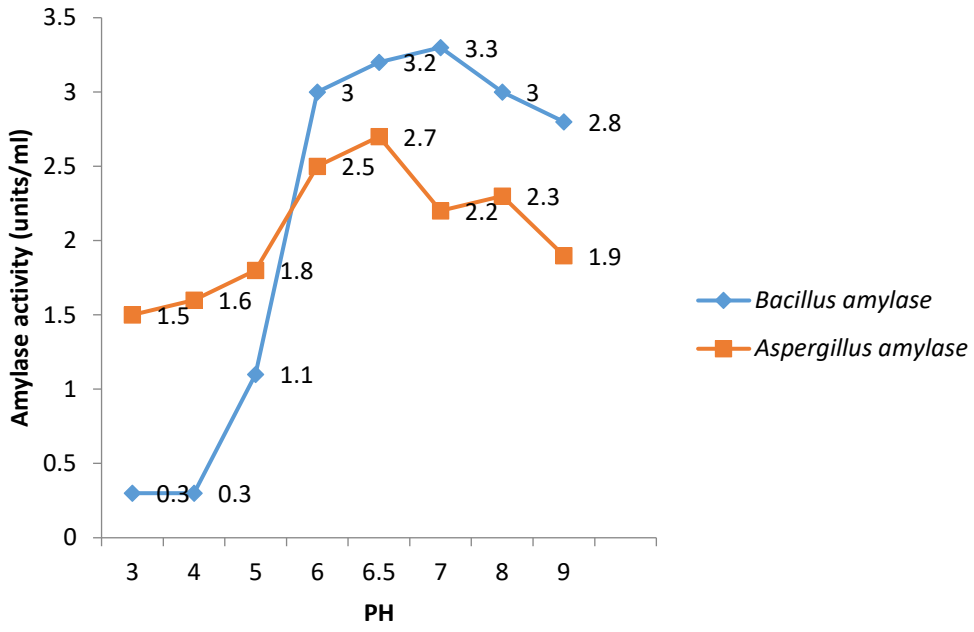
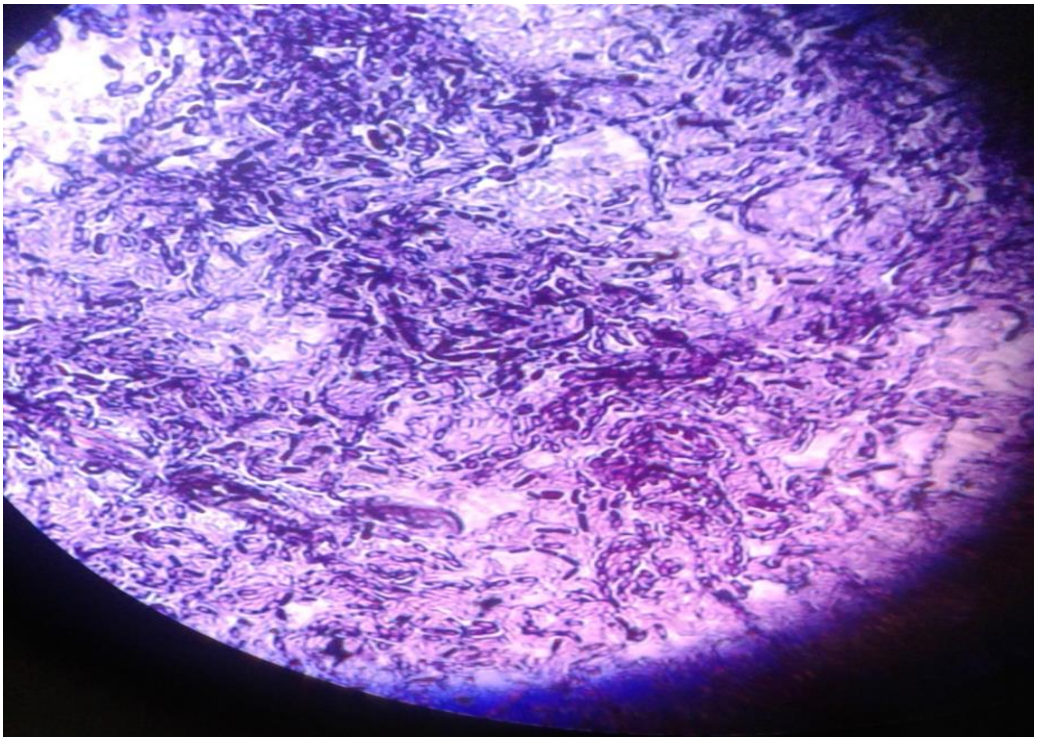


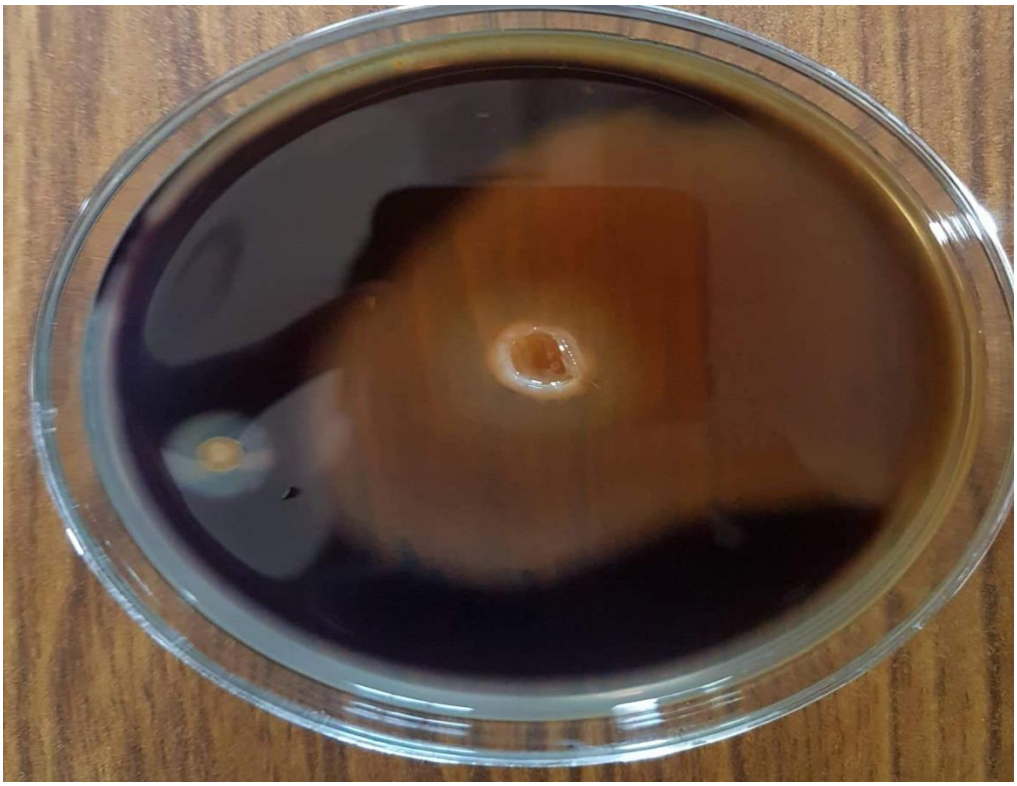
Figure 6: Effect of pH on α-amylase activity

PHOTOGRAPHS

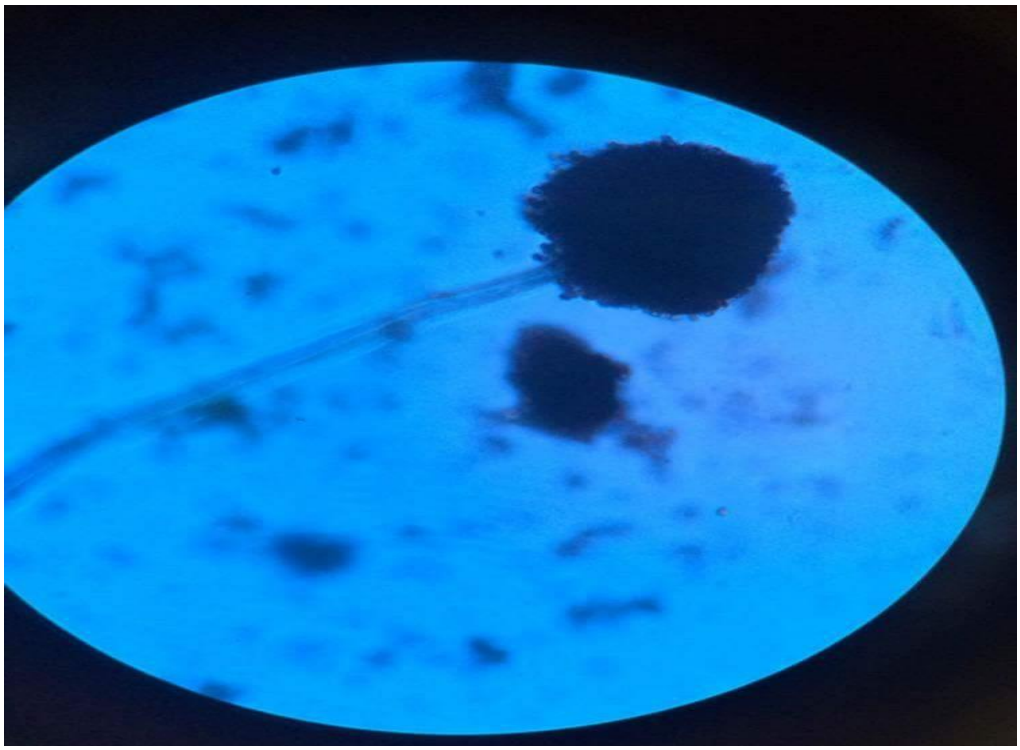


Photograph 1: Gram staining of *Bacillus subtilis*

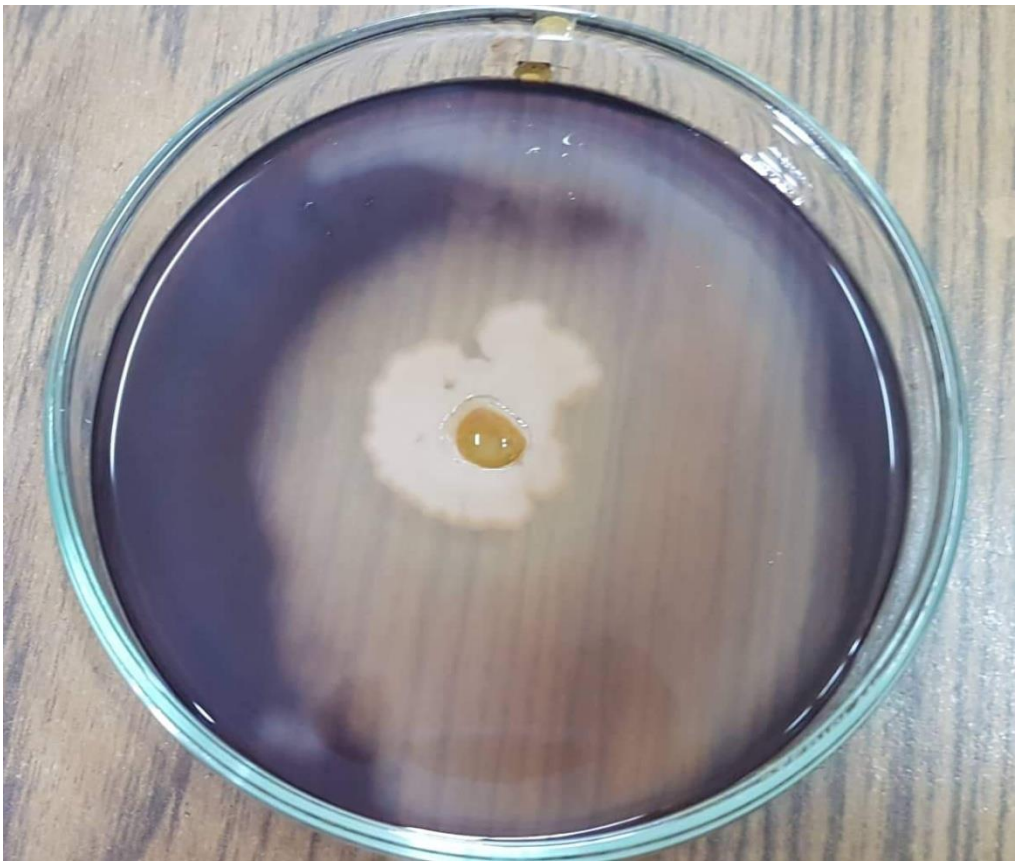
Photograph 2: Starch hydrolysis test for isolated *Bacillus subtilis*



Photograph 3: Confirmation of amylase enzyme from *Bacillus subtilis*



Photograph 4: Lactophenol cotton blue staining of *Aspergillus niger*



Photograph 5: Confirmation of amylase enzyme from *Aspergillus niger*

CHAPTER V

DISCUSSION

Enzymes are biological catalyst, proteinaceous in nature, produced by living cells to bring about specific biochemical reactions, generally forming parts of the metabolic process of the cell . They are highly specific in their action on substrates. Among different types of enzymes, amylases are the group of enzymes that has high demand in industries for its hydrolyzing ability. They are produced by varieties of living organisms ranging from microbes to plants to humans . Although there are many microbial sources of amylase, only few such as *Bacillus subtilis*, *Bacillus licheniformi*s, *Bacillus amyloliquefaciens* and *Aspergillus niger* are widely used for the commercial production of amylase (Sivaramakrishnan et al, 2006) .

As amylases for industries production are usually extracted from microorganisms,it is mandatory to isolate high amylase producing strain. In this study, isolation, identification and characterization of the amylase enzyme produced by various microorganisms were done. Due to the availability of various types of microorganisms in soil, it was chosen as the source of microorganism.The soil sample was collected from different places of Dharan sub-metorpolitan city. A total of 20 soil samples were studied on the basis of its ability to produce amylase enzyme. The production of amylase was confirmed by flooding of the starch agar plates with gram's iodine. Iodine forms the blue coloration with starch but the amylase producing microorganisms degrade the starch by the action of amylase surrounding them and utilize as sole source of carbon giving transparent zone. The primary selection of amylase producing microorganisms were done on the basis of clear zone formation on iodine flooded starch agar plates. The clear zones produced were due to the absence of starch which was hydrolysed by the enzyme secreted by microorganisms.The colonies forming the largest clear zone were further identified and used for the production of the amylase enzyme on submerged fermentation. Out of 20 samples, 9 samples showed the positive results for the production of amylase. Among 9 positive samples, 5 positive results were shown by bacteria and 4 positive results were shown by fungi. All the positive results showing bacteria were identified on the basis of their colonial characteristics, gram staining and biochemical test and

were identified as *Bacillus subtilis*. The fungal colonies were identified as *Aspergillus niger* on the basis of colonial characteristics and Lactophenol cotton blue staining.

In the earlier study of Singh and Kaur (2014), 14 different soil samples were studied for the amylase producing bacteria in which 2 sps of *Bacillus* were isolated and identified as *Bacillus subtilis* and *Bacillus brevis* where Bacillus *subtilis* showed the highest amylolytic activity. Similarly, Lawal et al (2014) isolated and identified three strains of *Aspergillus niger* as the amylase producing fungi from the cassava processing site and reported 2 of the strains as the highest amylase producer. Though the number and types of amylases producing organisms on soil depends on the types of soil, it was reported that Bacillus *subtilis* was the most frequently occurring amylolytic bacteria followed by *Bacilus cereus* and *Bacillus brevis*. In this study also *Bacillus subtilis* were isolated and identified for the production and characterization of amylase enzyme. Though different types of soil harbor large amount of amylolytic organisms, soils from potato fields and kitchen waste dump sites are considered as the better source of amylolytic bacteria (Peltier and Beckard 1945).

In this study, the crude amylase enzyme was produced by submerged fermentation carried out a in rotatory water shaker bath. For the production of bacterial amylase, the BMA was incubated with 1 ml of the 24 hours old culture of *Bacillus subtilis* and was incubated at 37^{o}C for 24 hours. Similarly, the *Aspergillus* amylase was also produced by submerged fermentation in a medium for 6 days at 25^{o}C. The fermented broths were centrifuged and the supernatant was used for the confirmation of amylase production. The confirmation was carried out by starch hydrolysis test on starch agar plates. Submerged fermentation is generally preferred for bacteria as it requires high moisture content. It is also used because the product recovery is very simple (Subramaniyan and Vimala 2012). Then the supernatant i.e., crude enzyme was further assayed by DNS method and the absorbance was measured at 540 nm. DNS method is one of the simplest and most widely used method to determine the amount of reducing sugar produced and hence an indication of the enzyme activity (EL-Fallal et al 2012). Amylase produces reducing sugar by breaking down starch into glucose. So, a greater concentration of glucose indicates a greater level of starch break down and highest activity. In this study, the lowest activity recorded was 0.11 U/ml by sample S5 and highest activity recorded was 0.25 U/ml by sample S9 in case of

Bacillus amylase. While in case of *Aspergillus* amylase highest activity of 0.65 U/ml was recorded by sample S20 and lowest activity of 0.14 U/ml by sample S18. The isolate having the greater level of activity were selected for study.

To enhance the production of amylase enzyme various parameters associated with the production of amylase were studied in the medium used for the amylase production. Among the various physical parameters for the optimum production of amylase enzyme, temperature and pH of the growth medium plays an important role by inducing the morphological change in the organism and in enzyme secretion. Thus, the optimization of production parameters on the basis of pH and temperature were carried out in this study. The optimum temperature of 40 °C was recorded for the production of *Bacillus subtilis* amylase. The amylase production was recorded low at the initial temperature which increases with increasing temperature giving maximum production at 40°C. Similar findings has been recorded in the study of Vijayalakshmi et al (2012) and Shruti and Banik (2018). Ashraf et al (2005) also reported maximum yield at 40ºC for Bacillus licheniformis. Whereas the optimum temperature of 45°C was recorded on the study of S. Shanmugasundaram et al (2015) where strain of *B. subtilis* SUS3 was used for enzyme production. In contrast to the present study,the optimum temperature of 35°C was recorded in the study of Singh and Kumari (2016) and pokharel et al (2011) where alpha amylase producing bacteria was isolated from sewage enriched soil. In the present study, the optimum temperature of 30°C was recorded for the enzyme produced by *Aspergillus niger*. The study conducted by Alva et al (2007) also reported the maximum temperature of 30°C for *Aspergillus* sp. JGI 12 isolated from different seeds obtained from various location. Similar finding has been recorded in the study of Kathiresan and Mannivan (2006) for amylase produced by *Penicillum fellutanum*. As well the same result was found in the study of Alva et al (2007) and Suganthi et al (2011). In contrast to this study, the optimum temperature of 45°C was reported in the study conducted by Asrat and Girma et al (2018) and the optimum temperature of 22+2°C was recorded by Varalakshmi et al (2008).

Different variation in temperature was found in various studies which was probably due to the preference of various strains to their optimal growth. As the temperature exceeds the maximum, the moisture content becomes lower than the optimal growth

thereby influencing the enzyme production. The influence of temperature on amylase production is related to the growth of the organism. From this study both the organisms i.e., *Bacillus subtilis* and *Aspergillus niger* are found to be mesophilic in nature. In the present study, the optimum temperature for enzyme production was comparatively low which can be due to the mutation of the strain or the environment where the organism was grown.

pH of the growth medium plays an important role in enzyme production by inducing morphological change in the organism and in enzyme secretion.In most of the cases, the pH used is not specified. Most of the strains of *Bacillus* used in commercial production of amylase enzyme by smF have an optimum pH between 6.0 and 7.0 for growth and enzyme production. In the present study also, the optimum pH for the production of alpha amylase was recorded at pH 6.5 for *Bacillus subtilis.* The amylase production was found to be low at the initial pH resulting highest at the pH 6.5. In a study conducted by Pokharel et al (2011), *Bacillus* spp was isolated from sewage which showed its maximum production at pH 7. Similarly, optimum pH of 7 was also recorded in the study of Vijayalakshmi et al (2012) and Singh and kumari (2016) and Kushwaha et al (2011) Whereas the optimum pH of 6 was recorded in the study of S. Shanmugasundaram et al (2015) for the *Bacillus subtilis* SUS3.Whereas the maximum production of 0.6 U/ml amylase was recorded at pH 7.5 for *Aspergillus* enzyme which is similar to the finding of Varalakshmi et al (2008) where *A. niger* JGI 24 was used for study. Similarly, the optimum pH of 6 was recorded in the study of Asrat and Girma et al (2018) and Shinde et al (2014) for the amylase enzyme produced by *Aspergillus*. Suganyadevi et al (2012) isolated *Aspergillus niger* from sweet potato and reported its optimum pH at 7. Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth (Lehninger 1982).

The partial purification of the crude enzyme was carried out by ammonium sulphate precipitation. Ammonium sulphate is the most widely used salt for the enzyme precipitation. It is carried out in various percentage of saturation for the partial purification of the enzyme. In this study, the ammonium sulphate was used at 65 %. It is useful as precipitant due to its high solubitlity, low density, readily available, cheap in cost and it stabilizes the protein structure.

In this study, the characterization of partially purified amylase was done to find the optimum temperature and pH at which the amylase has optimum activity. Characterization of an enzyme leads to the optimum fermentation conditions for that enzyme. The properties of amylase should meet its application and hence it is very important to check optimum condition which can be done by characterization. (Sivaramakrishan et al.2006). There are various methods to characterize an enzyme. In this study, the DNS method was used to determine the amount of reducing sugar produced at different temperature and pH. Amylases are known to be active in wide range of temperature (40-90^{o} C) and pH (4-11).

In present study, it was observed that the amylase showed the highest activity at 45°C for Bacillus amylase. The enzyme activity was found to be low at 20°C which increased with increasing temperature from 25 to 45^{o}C showing highest at 45 °C. Similar finding was found in the study conducted by E. Demirkan (2011) in which *Bacillus subtilis* and its mutant derivative EBUE5-3 was studied. Similarly, the temperature of 40 °C was recorded in Raul et al (2014) where *Bacillus subtilis* (MTCC 121) was used for enzyme production. In the study of Vaidya and Rathore (2015) the optimum temperature of 40^{o}C was recorded for the amylase activity where Bacillus strain AP1B2 was isolated from potato dump site. However, temperature higher than 45°C were also found to be recorded in the various studies for thermophilic *Bacillus*. In the study conducted by Fattah et al (2013) the amylase produced by *Bacillus licheniformis* isolate A120 showed the highest activity between 60-80 °C. While in case of *Aspergillus* amylase, the optimum activity was recorded 30°C which is found to be lower than the one studied by Patel and Nampoothiri (2005) in which optimum activity was recorded at 50^{o}C for *Aspergillus Oryzae*. Similarly in the study conducted by the Varalakshmi et al (2008), the optimum temperature for amylase enzyme produced by *Aspergillus niger* JGI24 was recorded at 30^{o}C. The activity later decreased with increasing temperature. In another study of Alva et al (2007), the maximum activity was recorded at 30 °C for *Aspergillus* sp.JGI 12 which is similar to the present study.

When characterizing the enzyme to identify the optimum pH ,it was observed that *Bacillus* amylase showed the highest activity at pH 7.From pH 3 to 5,the activity was moderate and gradually increasing but at pH 6 it increases drastically upto pH 7 and

started showing lower activity as pH moved from 8-9.From this results it can be concluded that enzyme works best at neutral pH .similarly, the optimum pH of 7 was also recorded in the study of Vaidya and Rathore(2015) where *Bacillus* strain APIB2 was isolated from potato sump site in Madhya Pradesh,India. Similar findings have been reported in the study of Vipul et al (2011) and E., Demirkan (2011). Whereas in the study conducted by Shilpi et al (2018) the optimum pH of 8.0 has been recorded for the amylase activity of *Bacillus subtilis* JS-2004. In the determination of optimum pH for *Aspergillus* amylase the optimum activity was recorded at pH 6. The activity slowly increases from pH 3 to 4 and rapidly increases at 6 giving highest activity but then the activity decreases as the pH moved from 8 to 9. Thus, it can be said that the amylase enzyme from *Aspergillus niger* worked best at acidic pH. Different study showed different variation in pH for the optimum activity of fungal amylase. In the study of Alva et al (2007) two pH i.e., one acidic pH 5.8 and one basic pH 9 were recorded for the *Aspergillus* sp JGI 12. In the study of Patel et al (2005) the optimum pH of 5 was recorded for the amylase produced by *A. oryzae* whereas the optimum pH of 9.5 was reported in the study of (Varalakshmi et al, 2008).

Alpha amylases for industrial application have to be selected appropriately as per the demand which can be done by characterization. In the present study, *Bacillus* amylase was found to be active at 40°C and pH 7 indicating its application in various industries. Similarly,the amylase produced by *Aspergillus niger* also had potential to be used in the industries for large scale production.As reported the optimum temperature for Aspergillus amylase at 30°C and pH 6.5. The enzymes produced by both the microbes in this study have high potential to use in the detergent industry as both are found to be active in low temperature. Moreover, they can be applied in the industries which required neutral or acidic pH.They can also be employed in food and textile industries.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Amylase is an enzyme that has wide spread application in industries due to its starch hydrolysis activity and the action carried out due to its starch hydrolyzing capacity. The search of new microorganisms for the production of amylase is a continuous process due to its wide spread use in various industries. The present study shows that it is possible to isolate the amylase producing microorganisms from the soil of Dharan municipality.

Determination of the optimum temperature and pH for the amylase production are important to optimize the production of amylase enzyme. The results obtained from this study indicated that the amylase produced by both *B. subtilis* and *Aspergillus niger* were appreciable. As well as the characteristics of amylase showed that they can be applied in the industries which required low temperature and neutral or acidic pH like in detergent or textile industries. The study revealed that the amylase produced from microorganisms is capable for the industrial use. Hence, the present study showed that organisms with potential to produce amylase can be obtained from soil and can be considered as the worthy source for amylase production as the enzymes was produced at low cost by inexpensive media. Moreover, further research based on genetic modification can be done to acquire desired properties of enzymes from *Bacillus subtilis* and *Aspergillus niger* which can be applied in the industries for the commercial purpose.

6.2 Recommendations

1. Optimization of the production parameters can be done further with respect to other parameters to scale up the amylase production.
2. Genetic modification can be done for the improvement of the strain.
3. The crude amylase can be purified.
4. Further study can be done for the production if other enzymes by using the concept of this technique and procedures.
5. Characterization can be done further with respect to surfactants, chelators and other additives in order to determine factors required to maintain enzyme activity and stability.

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APPENDIX A

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

1. Equipment

Hot air oven	Inoculating loop	Autoclave
Incubator	Weighing balance	Refrigerator
Microscope	Water shaker bath	Centrifuge
Micropipette		

2. Microbial and biochemical media

Nutrient agar	Starch agar
Potato dextrose agar	Nutrient broth
MR-VP broth	Simmon's citrate agar
Gelatin agar	

3. Chemicals and reagents

Ethanol	Crystal violet
Gram's iodine	Safranin
Lysol	Catalase reagent (3%H_2O_2)
Methyl red	Alpha- naphthol
Oxidase reagent	Malachite green
Kovac's reagent	Lactophenol cotton blue

4. Glasswares

Test tubes	Slides
Petri plates	Measuring cylinder
Conical flask	Pipettes
Glass rods	Glass tubes

APPENDIX B

COMPOSITION AND PREPARATION OF CULTURE MEDIA AND REGENTS

I. Composition and preparation of culture media

1. Nutrient Agar (NA)

Ingredients	Grams/Litre
Beef extract	1g
Yeast extract	2g
Peptone	5g
Sodium Chloride	5g
Agar	15g
Distilled water	1 Litre

All ingredients were weighted properly and were dissolved in 1000 ml of distilled water by heating the medium and was sterilized by autoclaving at 121ºC for 15minutes. It was then cooled and poured in sterile petri plates.

2. Potato Dextrose Agar (PDA)

Ingredients	Grams/Litre
Potatoes, infusion form	200.00
Dextrose	20.00
Agar	15.00
Final pH	5.6 ± 0.2

39gm of the medium was suspended in 1000ml distilled water and heated to boiling to dissolve the medium completely. Then, the medium was sterilized by autoclaving for 15lbs pressure at 121ºC for 15 minutes.

3. Starch Agar

Ingredients	Grams/Litre
Peptone	5.000
Sodium chloride	5.000
Yeast extract	1.500
HM peptone	1.500
Starch, Soluble	2.000
Agar	15.000
Final pH (at 25 ºC)	7.4 ± 0.2

30gm of the medium was dissolved in 1000 ml of distilled water heating. The medium was then sterilized by autoclaving at 15 lbs pressure (121 ºC) For 15minutes. It was then cooled to 45-50 ºC and poured in sterile petriplates.

II. Biochemical test media

1. Sulphide Indole Motility (SIM) medium

Ingredients	Grams/Litre
Beef extract	3.00
Peptic digest of animal tissue	30.000
Peptonized iron	0.200
Sodium thiosulphate	0.025
Agar	3.000
Final pH (at 25 ºC)	7.3 ± 0.2

36.23 grams of medium was suspended in 1000ml distilled water and boiled to dissolve the medium completely. Then the medium was dispensed in tubes and sterilized by autoclaving at 15lbs pressure (121 ºC) for 15 minutes. The tubes were allowed to cool in an upright position.

2. Simmons Citrate Agar

Ingredients	Grams/Litre
Magnesium Agar	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000
Final pH (at 25 ºC)	6.8 ± 0.2

24.28 grams of the medium was suspended in 1000ml distilled water and boiled to dissolve the medium completely. The medium was mixed well, distributed in tubes and sterilized by autoclaving at 15lbs pressure (121ºC) for 15 minutes. After autoclaving, the tubes containing medium were tilted to form slant.

III. Staining test and reagents

1. For Gram's stain
   a) Crystal Violet solution

Crystal Violet	20.0g
Ammonium oxalate	9.0g
Ethanol or methanol	95ml
Distilled water to make	1 litre

Preparation: In a clean piece of paper, 20g of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and 64.32g

of the medium was suspended in 1000ml distilled water and boiled to dissolve the medium completely. The medium was mixed well, distributed in tubes and sterilized by autoclaving at 15lbs (121 ºC) pressure for 15 minutes. The tubes containing sterilized medium was allowed to set in sloped form with a butt about 1 inch of thickness.

b) Lugol's Iodine

Potassium iodide	20.0g
Iodine	10.0g
Distilled water	1000ml

Preparation: To 250ml of distilled water, 20g of potassium iodide was dissolved. Then 10 gram of iodine was mixed to it until it was dissolved completely. Finally, the volume was made 1 litre by adding distilled water.

c) Acetone-alcohol decoloriser

Acetone	500ml
Ethanol (absolute)	475ml
Distilled water	25ml

Preparation: To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then, immediately, 500ml acetone was added to the bottle and mixed well.

d) Safranin (counter stain)

Safranin	10.0g
Distilled water	1000ml

Preparation: In a clean piece of paper, 10g of Safranin was weighted and transferred to a clean bottle. Then, 1 litre water was poured into it.

2.) For lactophenol cotton blue staining

a) Lactophenol cotton blue

Aniline blue	0.05g
Phenol Crystals ($C_6H_50_4$)	40ml
Lactic acid	20ml
Distilled water	20ml

Preparation: The reagent solution was prepared by dissolving them in 20ml of distilled water. The mixture was heated and transferred to dissolve the stain. The solution was mixed well and kept at room temperature.

4) Test reagents

a) For catalase test

Hydrogen peroxide	3ml
Distilled water	97ml

Preparation: To 97ml of distilled water, 3ml of hydrogen peroxide was added and mixed well.

b) For oxidase test

Oxidase reagent (impregnated in what man's No.1 filter paper)

Tetramethyl p-phenyle ne chamine

Dihydrochloride	1g
Distilled water	100ml

Preparation: The reagent solution was made by dissolving 1gm of TDP in 100ml distilled water. To that solution, strips of whatman's No.1 filter paper were soaked and drained for about 30 seconds.

Then, these strips were freeze dried and stored in a dark bottle tightly sealed with screw cap.

c) For indole test

Havoc's indole reagent

Isoamyl alcohol	30ml
P-dimethyl aminobenzaldehyde	2.0g
Hydrochloric acid	10ml

Preparation: In 30ml of isomylalcohol, 2g of P-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that 10ml of concentrated HCL was added and mixed well.

d) Dinitrosalicyclic (DNS reagent)

Ingredients	Amount
Sodium sulphite	0.05g
Potassium sodium tartrate	40.0g
3,5 Dinitrosalicyclic	1.0g
Sodium hydroxide	1.0g
Phenol	0.3ml
Distilled water	100ml

IV. Fermentation media

a) for fungal isolate

Ingredients	Amount
Starch	2gm
Yeast extract	0.05gm
KH_2PO_4	0.14gm
$MgSO_4.H_2O$	0.01gm
$(NH_4)_2.HPO_4$	1gm
D/w	100ml
pH	6.5

Preparation: All the ingredients were mixed in a clean conical flask and was sterilized at 121ºC for 15 minutes.

b) For bacterial isolate

Ingredients	Amount
Beef extract	0.1g
Peptone	0.1g
NaCl	0.5g
D/w	100ml

Preparation: All the ingredients were mixed in a clean conical flask and was

APPENDIX C

METHODOLOGY OF BIOCHEMICAL TEST FOR THE IDENTIFICATION OF BACTERIA

A. Methyl red test

This test is used to determine which fermentation pathway is used to utilize glucose by organism. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic and formic acid). The stable production of enough acid to overcome the phosphate buffer will result in a pH below 4.4. If the pH indicator (methyl red) is added to an aliquot of the culture broth and the pH is below 4.4, a red colour will appear. If the MR turns yellow, the pH is above 6.0 and the mixed acid fermentation pathway has not been utilized.

Procedure

A pure colony of organism was inoculated into 2 ml of MRVP broth and was incubated at 37°C for 24 hours. After incubation 5 drops of methyl red reagent was added and mixed well. Positive test was indicated by the development of bright red colour that indicates acidity.

B. Voges- proskauer (VP) test

The voges-proskauer test detects the presence of acetoin, a precursor of 2,3 butanediol during fermentation of carbohydrates. If the culture is positive for acetoin, it will turn “brownish-red to pink”. If the culture is negative for acetoin, it will turn “brownish-green to yellow”.

Procedure

Pure colony of test organisms was inoculated into of broth and was incubated at 37°C For 24 hours. After incubation, about 5 drops of barritts reagent was added and shaken well for maximum acetoin and kept for 15 min. positive test was indicated by the development of pink red color.

C. Indole production test

Bacteria has the ability to produce enzyme tryptophanase, which can convert the amino acid, tryptophan to indole. Indole reacts with added Kovacs

reagent to form red colour. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving ring intact in the form of indole.

Procedure

A pure bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and incubated at 37°C for 24 hours. After 24 hours of incubation, 2-3 drops of Kovacs reagent were added. Appeareance of red colour on the top of media indicates indole positive test.

D. Citrate Utilization test

This test is done to determine if an organism can use citrate as its sole carbon source. If an organism is capable of utilizing citrate as a carbon source, the enzyme citrase hydrolyzes citrate into oxaloacetic acid and acetic acid. The oxaloacetic acid is then hydrolyzed into pyruvic acid and CO_2. If CO_2 is produced, it reacts with the components of the medium to produce an alkaline compound (e.g., Na_2CO_3). The alkaline pH turns the pH indicator (bromthymol blue) from green to blue.

Procedure

A loopful of test organism was streaked on the slant area of citrate agar and incubated at 37°C for 24 hours. A positive result was indicated by the growth of organisms and change of media color from green to blue due to alkaline reaction.

E. Catalase test

This test is done to identify organisms that produce the enzyme, catalase which detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The positive result is indicated by the production of bubbles resulting from the liberated oxygen gas.

Procedure

A small amount of 24 hours old culture sample from NA was taken in a clean grease free slide with the help of inoculating loop. About 2-3 drops of 3% H_2O_2 was poured on its surface. Positive results were indicated by production of active bubbling of oxygen gas.

F. Oxidase test

This test is used to identify microorganisms containing the enzyme cytochrome oxidase which is important in the electron transport chain. Cytochrome oxidase transfers electrons from the electron transport chain to oxygen and reduces it to water. In the oxidase test, the artificial electron donors and acceptors are provided. When the electron donor is oxidized by cytochrome oxidase it turns a dark purple.

Procedure

A piece of Whatman s no. 1 filter paper was soaked with few drops of oxidase reagent i.e., 1 % tetra methyl-p -phenylene-diamine dihydrochloride. Small amount of test organisms was smeared on the filter paper. The appearance of blue-purple color immediately indicates the positive test.

G. Motility test

This test is done to determine whether the test organism was motile or non-motile. SIM tubes are inoculated with a single stab of culture to the bottom of the tube. If an organism is motile than the growth will radiate from the stab mark and make the entire tube appear turbid. Motile organisms migrate from stab line and diffuse into medium causing turbidity whereas non-motile bacteria show growth along the stab line.

H. Carbohydrate fermentation test

This test is done to determine an organism's ability to ferment sugar glucose as well as its ability to convert the end product of glycolysis, pyruvic acid into gaseous byproducts. This test is commonly used to identify bacteria which are glucose fermenters and produce gas.

The medium also contains the pH indicator, phenol red. So, when an organism is capable of fermenting the sugar glucose, then acidic byproducts are formed and the pH indicator turns yellow.

The end product of glycolysis is pyruvate. Organisms that are capable of converting pyruvate to formic acid and formic acid to $H_2(g)$ and $CO_2(g)$, via the action of the enzyme formic hydrogen lyase, emit gas. This gas is trapped in the durham tube and appears as a bubble at the top of the tubes.

APPENDIX D

Table I: Isolation of amylase producing organisms

Soil sample	Amylase producing organism
S1	-
S2	+
S3	-
S4	-
S5	+
S6	+
S7	-
S8	-
S9	+
S10	+
S11	-
S12	+
S13	-
S14	-
S15	+
S16	-
S17	-
S18	+
S19	+
S20	-

Where (+) = positive for amylase production and (-) = negative for amylase production

Table II: cultural characteristics of suspected bacterial colonies

Culture (Sample no)	color	elevation	surface	edge	shape
S2	white	raised	smooth	filamentous	irregular
S5	creamy	flat	smooth	Undulate	Round
S6	White, dull	Flat	granular	undulate	round
S9	white	raised	smooth	Filamentous	irregular
S10	creamy	convex	mucoid	undulate	punctiform

Table III: Morphological characteristics of suspected organisms

Sample no	Cell shape	Cell arrangement	endospore	motility
S2	rod	Short chain	+	+
S5	rod	Short chain	+	+
S6	rod	Short chain	+	+
S9	rod	Short chain	+	+
S10	rod	Short chain	+	+

Table IV: Staining reaction of suspected organisms

Sample no	Gram staining	Spore staining
S2	+	+
S5	+	+
S6	+	+
S9	+	+
S10	+	+

Table V: Biochemical tests of suspected organisms

Biochemical test performed	**S2**	**S5**	**S6**	**S9**	**S10**
Indole test	-	-	-	-	-
Methyl red test	+	+	+	+	+
Voges proskeur test	+	+	+	+	+
Citrate utilization test	-	-	-	-	-
Catalase test	+	+	+	+	+
Oxidase test	-	-	+	+	+
Starch hydrolysis test	+	+	+	+	+
H2S production	-	-	-	-	-
Motility test	+	+	+	+	+
Nitrate reduction test	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+
Sucrose fermentation	+	+	+	+	+
Lactose fermentation	-	-	-	-	-
Dextrose fermentation	+	+	+	+	+

Table VI: colonial characteristics of suspected Fungi

Sample no	**Colonial characteristics**
S12	Velvety, black
S15	Creamy, black
S18	Black creamy and white
S19	Velvety, black

Table VII: Microscopic examination of fungi

Sample no	Microscopic characteristics
S12	Septate hyphae, brush like conidiophores
S15	Septate hyphae, brush like conidiophores chain conidia
S18	Septate hyphae, brush like conidiophores chain conidia
S19	Septate hyphae, brush like conidiophores chain conidia