**SCREENING OF STARCH DEGRADING *Bacillus sp.* FROM SOIL IN DHARAN**

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A Project Work Submitted to the Department of Microbiology,

Central Campus of Technology, Tribhuvan University,

Dharan, Nepal,

In Partial fulfilment of the Requirements for the Award of

 Degree of Bachelor of Science in Microbiology

**Submitted by**

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September, 2016

 **RECOMMENDATION**

This is to certify that **Ms.** **Sneha Shrestha** has completed this project work entitled **“Screening of starch degrading *Bacillus Sp.* from soil”** 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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**CERTIFICATE OF APPROVAL**

On the recommendation of Mr. Suman Rai, this project of Ms. Sneha Shrestha entitled **“SCREENING OF STARCH DEGRADING *Bacillus* *Sp.* FROM SOIL”** has been approved for the examination and is submitted to the Tribhuwan University in Partial fulfillment of the requirements for B.Sc. degree in Microbiology

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 …….……

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**ABSTRACT**

Enzymes are the most important substances which are used today in so many areas either in research, medicine or commonly in industries. Among the most important enzymes, amylases are of great significance in present day industries. Amylases could be extracted from different sources such as plant, animal and microbes. These bacteria are screened from natural resources including soil, biogas plant, kitchen waste and domestic waste water for its ability to grow on cheap substrates, producing enzymes at high stable rate and no toxic substances. Starch is the reserve store of plants and is one of the most abundant biopolymers on earth. Starch degrading bacteria are mostly important in food, texture, fermentation and paper industries. The isolation and identification of starch degrading microorganisms from soil through the conventional culture method have a great importance in biotechnology. In this study, cultural, morphological and biochemical characteristics of the bacterial isolates were studied. Out of seven samples, only five samples showed positive result. The pure culture of the isolated organisms was obtained by subculture from starch agar media and on the basis of gram’s staining and a series of biochemical test. *Bacillus subtilis* and *Bacillus cereus* were identified.

**Keywords**: Soil, Starch, Amylase, Bacilli, Hydrolysis

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**CHAPTER I**

**INTRODUCTION**

**1.1. Background of the study**

 Soil is the outer covering of the earth which consist of loosely arranged layers of materials composed of inorganic and organic constituents in different stages of organization. It is the natural medium in which plants live, multiply and die and thus providing a perennial source of organic matter which could be recycled for plant nutrition. Soil provides the physical support needed for the anchorage of the root system and also serve as the reservoir of air, water and nutrients which are so essential for plant growth ([Rao 1999](#_ENREF_23)).

 Soil is the habitat of a diverse array of organisms which include both microflora 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 ([Biswas and Mukherjee 2001](#_ENREF_3)).Bacteria are the most dominant group of microorganism in soil and probably equal one half of the microbial biomass in soil. They are present in all types of soil but their population decreases as the depth of soil increases. Bacteria live in soil as cocci, bacilli or spirilli. The bacilli are common in soil whereas spirilli are very rare in natural environment ([Dar 2009](#_ENREF_5)).

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. 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 complex eukaryotes. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper ([Panneerselvam and Elavarasi 2015](#_ENREF_19)) . Amylase are obtained from various origins like plant, animal, bacterial and fungal. Several researchersproduce amylase enzyme using Bacillus sp ([Souza 2010](#_ENREF_29)).

There are about 3000 enzymes known today only few are industrially exploited. These are mainly extracellular hydrolytic enzymes, which degrade naturally occurring polymers such as starch, proteins, pectin’s and cellulose ([Panneerselvam and Elavarasi 2015](#_ENREF_19)).In the production of glucose syrup the α-amylase is used in the first step of enzymatic degradation yielding a mixture of glucose and fructose with high fructose content. The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands because it is economical when produced in large quantities ([Gurung et al 2013](#_ENREF_10)). Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors.

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. They are categorized in to exo-acting, endo-acting and debranching enzymes. Unusual bacterial amylases are found in acidophilic, alkalophilic and thermos acidophilic bacteria (Patel 2015).

 Starch is a heterogenous polysaccharide composed of two high molecular- weight components, called amylase and amylopectin ([Aiyer 2005](#_ENREF_1)). It is the most common carbohydrates in human diets and is contained in large amount of staple foods such as potatoes, wheat, maize (corn), rice etc. Starch degrading bacteria are common inhabitants of soil. They are most important for industries such as food, fermentation, textile and paper. Enzymes are important substances used in so many areas either in research, medicine or commonly in industries ([Sharma 2014](#_ENREF_26)). Amylase production from bacteria is economical because the enzyme production rate is higher in bacteria as compared to other microorganisms. The isolation and manipulation of pure culture of starch degrading microorganism from soil have a great importance on biotechnology as well as microbiology field ([Patel 2015](#_ENREF_20)).

There are various reports on starch degrading microorganisms from different sources and respective amylase activity (Mishra and Behera 2008). In the present study, we report the isolation and identification of starch degrading bacteria from the soil samples collected from different places of Dharan.

**1.2. Statement of the problem**

The use of enzymes in industrial processes is beginning to deliver its promise. Enzymes have high catalytic rates and work in aqueous solution. By industrial standards, only moderate temperatures and pressures are required. Thus, industrial exploitation of enzymes is making the development of cleaner, environmentally friendly processes possible. Furthermore, their exquisite specificity keeps unwanted side reactions to a minimum, maximizing yield. In industrial processes, however, we are still asking enzymes to perform under conditions for which they have not evolved. Because of this, a major problem in the industrial exploitation of enzymes is lack of stability. An example that illustrates both the possibilities, and also the limitations of the industrial use of enzymes, is starch processing, which is considered an unqualified success of modern industrial biotechnology. Amylase has a great deal of application in starch saccharification. The amylolytic enzymes find a wide spectrum of applications in food industry for production of glucose syrups, high fructose corn syrups, maltose syrups, reduction of viscosity of sugar syrups, reduction of haze formation in juices, solubilisation and saccharification of starch for alcohol fermentation in brewing industries, and also find a wide range of application in baking, paper, textile and detergent industry ([Guzmán‐Maldonado et al 1995](#_ENREF_11)).

**1.3 Objectives of the study**

 **1.3.1. General objective:**

* To isolate and identify starch degrading bacteria from different soil sample.

 **1.3.2. Specific objective:**

* To compare starch degrading bacteria from different place in Dharan Sub- Metropolitan City.
* To perform biochemical test for isolation and identification of starch degrading bacteria.
	1. **Rationale of the study**

These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries. Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry ([Gupta et al 2003](#_ENREF_9)).

Sujata (2010) has produced the thermostable α-amylase using the bacteria isolated from soil in a liquid media. Screening of important nutrient parameters from selected seven medium components ([FORM](#_ENREF_8)). Oueleke and Oduwole (2009) obtained ten grams (10 g) of soil sample obtained from a cassava waste and analysed bacteriologically. One gram (1.0 g) of the sample inoculated into a liquid soluble starch medium generated reducing sugar with a concentration of 1.65 mg/ml after 72 hours. Characterization of the soluble starch amylases revealed an optimum temperature of activity of 70°C. Optimum pH for activity was between 6.5 and 7.5. The most frequently occurring amylolytic bacteria were *Bacillus subtilis* (37.5%), followed by *Bacillus subtilis. Bacillus megaterium* and *Bacillus coagulans* (18.75% each) ([Oyeleke and Oduwole, 2009](#_ENREF_18)). Senthilkumar et al (2012) isolated the bacteria from the soil samples and the bacteria isolated was identified as *Bacillus* sp. based on Staining techniques, motility test, plating on selective media and biochemical tests. Amylase production by *Bacillus* sp. was detected by the disappearance of blue colour in the starch agar medium around the microbial colonies after incubation. Cassava was used as the substrates for the amylase production. Solid state fermentation was carried out for the production of amylase using *Bacillus* spp ([Senthilkumar 2012](#_ENREF_25)).

**1.5. Limitations of the study**

1. Due to limited time given, soil samples couldn’t be collected from all the parts of Dharan.

2. Due to lack of sufficient amount of budget and equipment’s genes of different isolated organisms could not be identified by molecular study.

 **CHAPTER II**

 **REVIEW OF LITERATURE**

**2.1. Introduction**

 Soil is the habitat of a diverse array of organisms which include both microflora 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 microbe release in the environment and degrades the soil components ([Biswas and Mukherjee 2001](#_ENREF_2)). Soil receiving the garden is one of the rich sources of microorganisms. In this starch degrading microorganisms, isolated sources plant. Biologically active enzymes may be extracted from any living organisms ([Paul 2006](#_ENREF_21)). A very wide range of sources are used for commercial enzyme production from Actinoplanes to Zymomonas, from spinach to 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](#_ENREF_20)). Although many microorganisms produce amylase enzyme, the ones most commonly used for their industrial production are *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquifaciens* and *Aspergillus Niger* ([Sivaramakrishnan et al 2006](#_ENREF_28)*)*. Amylase is an enzyme that catalyses the breakdown of starch into sugars, different organisms has been studied to produce this enzyme, a wide studied is *Bacillus subtilis* was studied to develop different forms of amylase such as alpha-amylase, β-glucanase (gummase) and hemicellulose ([Souza 2010](#_ENREF_29)).

**Soil microorganisms:** soil microbiology is the study of organisms 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](https://en.wikipedia.org/wiki/Bacteria) and microorganisms came about in Earth's oceans. These bacteria could fix nitrogen, in time multiplied and as a result released oxygen into the atmosphere. This led to more advanced microorganisms. Microorganisms in 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](#_ENREF_21)). Soil microorganisms have had another direct importance for humans—they are the source of most of the antibiotic medicines we use to fight diseases. Some of the starch degrading bacterial genera found in soil are: *Pseudomonas, Arthrobacter, Clostridium, Bacillus, Micrococcus, Sarcina, Mycobacterium, Corynebacterium* etc.

 Amylases can be obtained from several sources such as plant, animal and microbes ([Mishra and Behera 2008](#_ENREF_17)). Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper ([Panneerselvam and Elavarasi 2015](#_ENREF_10)).

 Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized into exo-acting, endo-acting and de-branching enzymes. Among the amylases, β**-**amylase is exo-acting whereas α-amylase is endo-acting enzyme. Unusual bacterial amylases are found in acidophilic, alkalophilic, and thermo- acidophilic bacteria ([Boyer et al 1979](#_ENREF_4)). There are various reports on starch degrading microorganisms from different sources and respective amylase activity ([Mishra and Behera 2008](#_ENREF_17)).

Amylases are a family of enzymes that degrade starch (polymers of glucose) into smaller disaccharides (maltose). A molecule of water is also split during this reaction and the OH- and H+ ions bind to the exposed ends of the broken starch polymer. This type of reaction is called a hydrolysis (water splitting). Hydrolysis is a common mechanism used by enzymes to break chemical bonds.

**2.2 Alpha Amylase**

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

**2.3 Structural and Functional Characteristics of α-Amylase**

The α-amylase (α-1,4-glucan-4-glucanohydrolase) can be found in microorganisms, plants and higher organisms (Kandra 2003). The α-amylase belongs to a family of endo-amylases that catalyses the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds (Brayer et al 1995; Iulek et al 2000; Kandra 2003; Tangphatsornruang et al 2005). Neither terminal glucose residues nor α-1,6-linkages can be cleaved by α-amylase (Whitcomb & 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, maltotriose, and branched oligosaccharides of 6-8 glucose units that contain both α-1,4 andα-1,6 linkages (Whitcomb & 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).

**2.4 Starch**

Starch is a polymer of glucose linked to another one through the glycosidic bond. Two types of glucose polymers are present in starch: amylose and amylopectin. Amylose and amylopectin have different structures and properties. Amylose is a linear polymer consisting of up to 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 for 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 & Nirmala 2005; Sorensel et al 2004; Tester et al 2004; Van der maarel et al 2002).

**2.5 Starch conversion**

The most widespread applications of α-amylases are in the starch industry, which are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups (Nielsen & 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; Prakash & Jaiswal 2009).

**2.6 Introduction of *Bacillus Spp:***

Bacillus *spp* are spore forming, aerobic rod shaped bacteria that gives gram stain positive or gram variable. But few species have no pathogenic potential. They have never been associated with disease in human or animals. Members of the genus have significant microbiological uses ([Poinar Jr and Thomas 1984](#_ENREF_22)). Numerous enzymes, antibiotics and other metabolites have agricultural, pharmaceutical, medical and other industrial applications. Some of the examples of antibiotics formed by Bacillus spp include bacitracin which is formed by B. licheniformis or B. subtilis, similarly polymyxin by B. polymyxa and gramicidin by B. brevis. Certain strains of Bacillus have been utilized as biological controls in antibiotics and other assays.

**2.7 Epidemiology**

Bacillus organisms are widely distributed in the environment although the primary habitat is the soil. These organisms are usually found in decaying organic matter, dust, vegetables and water. Beside these some species are found to be part of the normal flora. ([Welch 1920](#_ENREF_33)). Epidemiologic studies on the microbiology of street heroin and injection paraphernalia demonstrated that Bacillus spp as the predominant isolates from both specimens ([Tuazon 2016](#_ENREF_30)).

**2.8** **Description of *Bacillus Subtilis***

 *Bacillus subtilis* is one of the most common starch degrading bacteria that are mostly found in soil. *Bacillus subtilis* was originally named as *Vibrio subtilis* in 1835. Later, in 1872, this organism was renamed as *Bacillus subtilis*. Similarly, other names for this bacterium also include *Bacillus uniflagellatus*, *Bacillus globigii*, and *Bacillus natto*. *Bacillus subtilis* bacteria were one of the first bacteria to be studied. These bacteria are a good model for cellular development and differentiation ([Logan and Rodrigez-Diaz 2006](#_ENREF_15)).

Cells of *bacillus subtilis* are rod-shaped, Gram-positive bacteria that are naturally found in soil and vegetation. *Bacillus subtilis* grows best in the mesophilic temperature range. The optimal temperature for growth is 25-35oC ([Kunst et al 1997](#_ENREF_14)). In this environment stress and starvation are common. Therefore, *Bacillus subtilis* has evolved a set of strategies that allow survival under these harsh conditions. One strategy, for example, is the formation of stress-resistant endospores.

*Bacillus subtilis* is also a model organism for studying endospore formation in bacteria. Endospores in *Bacillus subtilis* bacteria are mostly formed in the tips of protuberances extending downward from liquid surface pellicles ([Errington 2003](#_ENREF_7)). Many strains produce spores with brown pigments.

**2.9 Ecology**

Soil is the main habitat for endospore forming *Bacillus* organisms. Likewise, *Bacillus subtilis* is most commonly found in soil environments and on plant undergrowth. Historically, these mesophilic organisms have been considered strict aerobes. Thus, they are most likely to be found in O and A surface soil horizons where the oxygen concentration is most abundant and temperatures are relatively mild. Scientists have demonstrated that *Bacillus subtilis* concurrently produces antibiotics and spores. Production of antibiotics increases *B. Subtilis's* chance at survival as these organisms are capable of producing spores as well as toxin that might kill surrounding gram positive microbes that compete for the same nutrients ([Joshi et al 2016](#_ENREF_12)).

These microbes usually form spores in times of nutrient exhaustion. When the nutrients required for the growth of bacteria are abundant, they exhibit metabolic activity. During sporulation, these organisms can produce antibiotics. Some of the examples of the antibiotics that *Bacillus subtilis* can produce are polymyxin, difficidin, subtilin, and mycobacillin ([Al-Faragi and Alsaphar 2012](#_ENREF_2)). Many of the *Bacillus spp* can degrade polymers such as protein, starch, and pectin, therefore, they are thought to be one of the important contributor to the carbon and nitrogen cycles. When they cause contamination, they may result in decomposition ([Uhlig 1998](#_ENREF_31)).

*Bacillus subtilis* supports plant Growth. As a member of *Bacillus*, this bacterium often plays a role in replenishing soil nutrients by supplying the terrestrial carbon cycle and the nitrogen cycle. *Bacillus subtilis* bacteria form rough biofilms, which are dense organism communities, at the air and water interface. *Bacillus subtilis* biofilms are beneficial ([Joshi et al 2016](#_ENREF_12)). *Bacillus subtilis* biofilms found in the rhizosphere of plants promote growth and serve as a bio controller. B. subtilis benefits by deriving nutrients and surface area for biofilm formation from the plant's root structure. *Bacillus subtilis* strains can act as bio fungicides for benefiting agricultural crops and antibacterial agents. *Bacillus subtilis* also reduces mild steel corrosion ([Joshi et al 2016](#_ENREF_12)).

**2.10** **Description and significance of Bacillus cereus**

*Bacillus cereus* is a large, 1 x 3-4 µm, Gram-positive, rod-shaped, endospore forming, facultative aerobic bacterium ([Vinnerås et al 2006](#_ENREF_32)). It was first successfully isolated in 1969 from a case of fatal pneumonia in a male patient and was cultured from the blood and pleural fluid ([Dudding et al 1972](#_ENREF_6)). *B. cereus* is mesophilic, growing optimally at temperatures between 20°C and 40°C, and is capable of adapting to a wide range of environmental conditions. *B. cereus* is also a contributor to the microflora of insects, deriving nutrients from its host, and is found in the rhizosphere of some plants ([Sabzevar](#_ENREF_24)).

**2.11 Ecology**

*B. cereus* interacts with other microorganisms in the rhizosphere, the region surrounding plant roots. Plants benefit from the presence of *B. cereus* since it is capable of inhibiting plant disease caused by protest pathogens and also enhances plant growth. *B. cereus* is a also found in the gut microflora of invertebrates, and is an intestinal symbiont of arthropods where it exhibits filamentous growth in sow bugs, roaches, and termites ([MIGAP 2014](#_ENREF_16)).

As a ubiquitous bacterium, small amounts are consumed by humans from foods. Therefore, it is a contributor to the human intestinal microflora .In addition, *B cereus* is widely known to affect humans by causing food poisoning and infections as an opportunistic pathogen ([Kotiranta et al 2000](#_ENREF_13)).

**CHAPTER III**

**METHODOLOGY**

**3.1. Site of the study**

 The study was carried out in Dharan, Sunsari.

**3.2. Research method:**

The method for this study was qualitative study. 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 sample for this study was ascepticaly collected from seven different places of Dharan viz Bishnupaduka, Phusre, Panmara, Bargachi, Railway and dharan-16. Laboratory works for the isolation and identification of starch degrading bacteria was carried out in the laboratory of Department of Microbiology, Central Campus of Technology, Dharan. The laboratory was provided with all the necessary materials and equipments that were required to carry out this study.

**c. Research Design**

 100 gm of the soil sample collected

 1gm sample + 10 ml water

 Heated upto 800c for 5 min

cooled

 Culture by spread plate technique on Starch agar media

 Subculture on NA

 Addition of starch to the colony for clear zone formation

Positive colonies subjected to identification

Gram Staining

 Biochemical tests

 Identification of the organism

**d. Sample Collection:** For this study, agricultural fields where organic matters are deposited were selected as sample site. Soil samples were collected from different sites of Dharan. Soil was collected from about 10 cm deep from the surface of the field. Sterile plastic bags were used for collecting soil sample.

 **e. Transportation of the sample:** The samples were then transported from the site of collection to the laboratory aseptically without any damage.

**3.5. Isolation and identification**

1. **Media preparation:**

 For the isolation of starch degrading bacteria, starch agar medium was used as selective media. Starch agar media was composed of 2% starch in nutrient agar. Similarly, biochemical test media were also prepared for the identification of starch degrading bacteria. The media was autoclaved at 121ᵒC for 15 min.

Following is the list of the biochemical test media prepared for the identification process of the study.

1. Citrate Agar
2. MRVP Broth
3. Tryptone Broth
4. TSIA Agar
5. Urease Agar
6. Gelatin liquefaction
7. **Sample processing**

After the samples were collected, 1 g of the soil sample was mixed with 10 ml of water and was heated at 80o C for 5 minutes. The solution was allowed to cool for few minutes and it was subjected to culture.

1. **Culture:** 0.1 ml of the heated solution was cultured on the pre- prepared Starch agar plates with the help of the sterile pipette by spread plate technique. The plates were then incubated at 37o C for 24 hours.
2. **Observation:** After the 24 hours of incubation**,** the culture plates were observed for the growth of the colonies. The production of amylase was indicated by a clear visible zone of starch hydrolysis surrounding the colony of isolated samples. Further confirmation was achieved by flooding the plate with iodine solution, as the starch free areas do not show violet colour**.** The colonies were then observed for their colour, shape, size, configuration, elevation and the number of colonies was counted on the individual culture media plates.
3. **Sub culture:** After observation, the individual type of bacterial colonies that showed the clear zone on the addition of starch from each of the media plates were further sub cultured on the nutrient agar plates by Streak plate method and were again incubated at 37o C for 24 hours.
4. **Biochemical test:** After the sub culture of organism was performed, they were tested biochemically for their identification. The process of identification by the biochemical test involved the following steps:
5. **Gram staining**: It is the first step of the biochemical test which is performed in order to differentiate the bacteria i.e. whether the bacteria is gram positive or gram negative.
6. **Catalase test**: This test is primarily used in order to demonstrate the presence of catalase enzyme in the bacteria.
7. **Oxidase test**: The basic principle of this test is to determine the ability of bacteria to produce oxidase enzyme. cytochrome c oxidase, an enzyme of the bacterial electron transport chain.
8. **TSIA** **test**: TSI stands for the Triple sugar iron test. TSI test is used for the determination of carbohydrate fermentation and hydrogen sulphide production. This test is carried out in the slant of TSIA media which consist of three sugars: lactose, sucrose and a very small amount of glucose.
9. **MRVP:** This test consists of four different tests based on the biochemical activities of the organisms, they are: indole production, methyl red, Voges-Proskauer and citrate utilization. Indole test was performed on the trypton broth by using kovac’s reagent. Methyl red and Voges-Proskauer test was performed on the MRVP broth by using methyl red as the reagent in the MR test and VP reagent (KOH and alpha naphthol). Citrate test was performed by streaking the organisms in the slant of Simmon’s citrate agar media.
10. **Urease test**: This test is performed for the identification of bacteria based on their ability to hydrolyse the urea.
11. **Gelatin Hydrolysis test**: Gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefy gelatin.

 **CHAPTER IV**

**RESULTS**

As the samples were collected from seven different places of Dharan like Bijaypur, Panmara, Phusre, Bagarkot, Bargachi, Railway and Dharan-16. Out of different ranges of temperature, maximum colonies were recorded at 37o C. Also, we observed that the colonies were cream whitish in color. All colonies were with irregular and round margin (Table 1). The strain closely resembled to *Bacillus* species. Likewise, out of seven petri plates prepared five of them showed positive results than others after incubation period of 24 hours at room temperature. These were selected due to clear zone showed in the centre of the petri plates. This showed the ability of starch degrading bacteria and those five samples were used for microorganism identification, biochemical analysis and microscopic observation. The two samples from Dharan 16 and Bargachhi did not reveal the amylolytic organism.

**4.1 Morphological characteristics of the isolates of sample-**

All isolates isolated from the different sites were found to be Gram positive rods but isolates from Panmara and Phusre sites were motile while that of Bijaypur, Bagarkot and Railway were non-motile. According to Table no 1, sample 1 (Panmara) and sample 3(Phusre)gave similar morphological characteristics i.e. irregular, curled, raised, white, smooth and opaque. Similarly, sample 2, 4 and 5 also gave similar morphological characteristics i.e. round, lobate, flat, white, smooth and opaque.

**4.2 Biochemical and cultural characteristics of the isolates of sample**

According to table 2, sample 1 and 2 gave similar biochemical test i.e. Indole (-), MR (-), VP (+), Urease (-), Catalase (-), Citrate (-), Oxidase (+), Gelatin (+) and Starch hydrolysis (+). Likewise, sample no 2, 4 and 5 gave similar biochemical test i.e. Indole (-), MR (+), VP (+), Urease (-), Catalase (+), Citrate (+), Oxidase (+), Gelatin (+) and Starch hydrolysis (+). TSIA test showed that the isolates from Panmara and Phusre were able to ferment the three sugars in the medium under anaerobic condition but cannot ferment under aerobic condition while the isolates from Bijaypur, Bagarkot and Railway were able to ferment the three sugars under both conditions. Similarly, the isolates from Panmara and Phusre were unable to produce H2S while the isolates from Bijaypur, Bagarkot and Railway were able to produce H2S. Lastly, all the isolates from five sites were found to be gas producers.

Hence, the isolated bacteria from the above five samples were identified. The isolated bacteria from the samples 1and 3 were identified as *Bacillus cereus* with white colored colony on starch agarplates*.* Similarly, the isolated bacteria from the remaining samples 2, 4 and 5 were identified as *Bacillus subtilis* with white colored colony on starch agar plates.

**CHAPTER V**

**DISCUSSION**

In this piece of work, starch degrading organisms were isolated and identified from the soil. Soil is a mixture of minerals, organic matter, gases, liquids, and countless organisms that together support life on earth. It is the natural medium in which plants live, multiply and die and thus providing a perennial source of organic matter which could be recycled for plant nutrition. Likewise, starch is the reserve energy store of plants and is one of the most abundant biopolymers on earth. It consists of large number of glucose units joined by glycosidic bonds. It is the most common carbohydrate in human diets and is contained in staple foods such as potatoes, wheat, maize, rice etc. The enzymes that break down or hydrolyze starch into the constituent sugars are known as amylases. Amylases can be obtained from several sources such as plant, animal and microbes. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper.

*Bacillus sp* are capable of degrading the starch due to their ability to produce alpha amylase. Alpha amylase is a protein enzyme that hydrolyses alpha bonds of large, alpha linked polysaccharides, such as starch and glycogen, yielding glucose and maltose (Maureen BP 2000). It is the major form of amylase found in humans and other mammals (Voet D et al 2005). It is also present in seeds containing starch as a food reserve and is secreted by many fungi.

According to [Logan and Rodrigez-Diaz (2006](#_ENREF_15)), *Bacillus subtilis* is one of the most common starch degrading bacteria that are mostly found in soil which also matched with the present study.

Similarly, according to Collee (1989), *Bacillus subtilis* gives VP reaction positive and are acid producers which matched with the present study.

According to Angelo & Rangabhashiyam (2013), pure strain of *Bacillus* species was isolated from the soil in which the *Bacillus* gave positive result for starch hydrolysis, which is similar in case of the present study as well.

According to Madhav, Verma & Tanta (2011), *Bacillus subtilis*, *Bacillus cereus* and *Bacillus megaterium* were isolated which were capable of hydrolyzing the starch. *Bacillus subtilis* and *Bacillus cereus* were the bacteria isolated in the present study which were responsible for the hydrolysis of the starch. But, *Bacillus megaterium* was not isolated in this study.

**CHAPTER VI**

**CONCLUSION**

From this study, it is concluded that *Bacillus subtilis* and *Bacillus cereus* present in the soil sample are capable of hydrolysing the starch which was present in the soil. Bacillus species are capable of hydrolysing the starch due to their ability to produce alpha amylase.

**RECOMMENDATIONS**

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

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**APPENDICES**

**APPENDIX I: MATERIALS USED**

**Glasswares:**

 Pipettes

 petri plates

 conical flask

 beakers

 glass rods

 glass tubes

 slides

**Equipments:**

 Autoclave

 Hot air oven

 Microscope

 Refrigerator

**Chemicals:**

 Ethanol

 Chloroform

 Ethyl acetate

**Materials:**

 Test tube rack

 Wash bottle

 Burner

**Others:**

 Cotton swab

**Sample:**

 Soil

 **APPENDIX II: COMPOSITION OF MEDIA USED**

1. **Nutrient Agar:** (pH-7.0)

Peptone: 5.0 g

Beef extract: 3.0 g

NaCl: 5.0 g

Agar: 15 g

Distilled Water: 1000 ml

1. **Starch agar media**

2% starch 10 g

Na 14 g

 Agar Agar 5 g

**APPENDIX III: TABLES**

**Table I:** Morphological characteristics of the isolates of sample on starch agar

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **S.N.** | **sample** | **shape** | **margin** | **elevation** | **colour** | **texture** | **Opacity** | **Assumed species** |
| 1 | Panmara | irregular | curled | raised | white | smooth | Opaque | *B. cereus* |
| 2 | Bijaypur | round | lobate | flat | white | smooth | Opaque | *B. subtilis* |
| 3 | Phusre | irregular | curled | raised | white | smooth | Opaque | *B. cereus* |
| 4 | Bagarkot | round | lobate | flat | white | smooth | Opaque | *B. subtilis* |
| 5 | Railway | round | lobate | flat | white | smooth | Opaque | *B. subtilis* |

**Table II:** Biochemical and cultural characteristics of the isolates of sample

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **S.N.** | **Gram staining** | **Shape** | **Motility** | **TSI** | **H2S** | **Gas** |
| 1. | +ve | Rod | motile | K/A | - | + |
| 2. | +ve | Rod | Non-motile | A/A | + | + |
| 3. | +ve | Rod | motile | K/A | - | + |
| 4. | +ve | Rod | Non-motile | A/A | + | + |
| 5. | +ve | Rod | Non-motile | A/A | + | + |

|  |  |  |
| --- | --- | --- |
| **SN** | **Biochemical test** | **Assumed species** |
| *B. cereus* | *B. subtilis* | *B. cereus* | *B. subtilis* | *B. subtilis* |
| 1 | Indole | **-** | **-** | **-** | **-** | **-** |
| 2 | MR | **-** | + | **-** | + | + |
| 3 | VP | + | + | + | + | + |
| 4 | Urease | **-** | **-** | **-** | **-** | **-** |
| 5 | Catalase | **-** | + | **-** | + | + |
| 6 | Citrate | **-** | + | **-** | + | + |
| 7 | Oxidase | + | + | + | + | + |
| 8 | Gelatin hydrolysis | + | + | + | + | + |
| 9 | Starch hydrolysis | + | + | + | + | + |