EXTRACTION OF AMYLASE FROM *Bacillus subtilis* ISOLATED FROM SOIL OF DHARAN, SUNSARI

A

Project Work Submitted to

Department of Microbiology

Central Campus of Technology, Tribhuvan University

In Partial Fulfillment for the Award of the Degree of

Bachelor of Science in Microbiology

Submitted by

Chetan Maskey

Roll Number: 80015

Reg. Number: 5-2-0008-0029-2013

August, 2017

RECOMMENDATION

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

……

Mr. Shiv Nandan Sah (Asst. Professor)

Supervisor

Head of Department

Department of Microbiology

Central Campus of Technology

Hattisar, Dharan, Nepal

Date:

CERTIFICATE OF APPROVAL

On the recommendation of Mr. Shiv Nandan Sah, this project of Mr. Chetan maskey entitled "**EXTRACTION OF AMYLASE FROM *Bacillus subtilis* ISOLATED FROM SOIL OF DHARAN, SUNSARI"** has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirements for Bachelor's Degree in Microbiology.

……

Mr. Shiv Nandan Sah (Asst. Professor)

Head of Department

Department of Microbiology

Central Campus of Technology

Hattisar, Dharan, Nepal

Date:

BOARD OF EXAMINERS

Recommended by:

…….

Mr. Shiv Nandan Sah

Supervisor

……

Mr. Prince Subba

Co-supervisor

Approved by:

……

Mr. Shiv Nandan Sah

(Assistant Professor)

Head of Department

Department of Microbiology

Examined by: ……

(Internal Examiner)

Mr. Suman Rai

……

(External Examiner)

Mr. Suman Kumar Lal Das

Date:

ACKNOWLEDGEMENT

I express my heartful sincere gratitude to supervisor Mr. Shiv Nandan Sah, Head of Department of Microbiology, Central Campus of Technology (CCT) T.U. for his valuable guidance, suggestions, support and encouragement for this research work.

Similarly, I am thankful to my co-guide Mr. Prince Subba, lecturer, Department of Microbiology CCT, for his guidance and supervision during the entire period of the research.

Likewise, I am thankful to Mr. Ainra Bahadur Karki and Saru Budhathoki for helping and giving every support in the laboratory of CCT, on time.

I am thankful to Mr. Singhaman Tamang for providing necessary books on time.

Lastly, I would like to express my deepest gratitude to my family members for their continuous support, love and encouragement. I am thankful to my friends especially Mahesh Karki, Himal Kafle, Chunsaha Limbu, Yashodha Rai, Pawan Gurung, Santoshi Ghimire and Rojina Subedi.

.....

Chetan Maskey

Date:

ABSTRACT

Amylases are the enzymes that catalyzes the hydrolysis of starch, glycogen, and related polysaccharides to oligosaccharides, maltose, or glucose. In present study, amylase producing bacteria were isolated from five soil samples of different places of Dharan. A total of 25 bacterial colonies were isolated from collected soil samples. Five bacterial isolates, exhibited zone of clearance in starch hydrolysis test. The isolated displaying maximum amylase activity on quantitation were selected characteristics features of strain indicates that it belongs to the genus *Bacillus* will be later used for further characterization and amylase extraction. Maximum yield of amylase was obtained after 48hours incubation. The optimum pH for enzyme activity was found to be at pH 7.0 and the optimum temperature for the activity was found to be 40^{o}C.

Keywords: Amylase, Characterization, Extraction, Soil, Starch Hydrolysis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ABBREVIATIONS

1. NA- Nutrient Agar
2. DNS- Dinitrosalicyclic Acid
3. hrs- Hours
4. OD- Optical Density
5. rpm- Revolution Per Minute
6. spp.- Species
7. SmF- Submerged State Fermentation

LIST OF PHOTOGRAPHS

PART I

INTRODUCTION

1.1 Background

Amylase is group of important enzyme which is mainly employed in starch processing industries for hydrolysis of polysaccharides like starch into simple sugar (Daniel, Peterson, & Danson, 2010). These enzymes originate from different sources such as plants, animals, and microorganisms. Enzymes from microbial sources generally meets industrial demands, due to their high yield and thermostability. Microbial enzyme presents a wide spectrum of characteristics that make them useful for specific applications. These includes: they are of natural origin and non-toxic, have great specificity of action and hence and can bring reactions, not easily carried out, they work best under mild conditions of moderate temperature and near neutral pH, thus not requiring drastic conditions of high temperature and pH, high acidity and likes which necessitate special expensive equipment. These characteristics and special advantages offered by microbial enzyme made many industries interested in adapting enzymatic methods to the requirement of their processes (Oyeleke & Oduwole, 2009).

Many microorganisms that live in the soil play indispensable role in maintaining life of this planet degrading or chemically modifying molecules. Considerable human interest in soil organism stems from their ability to synthesize a variety of useful chemicals. Industrial microbiology concern itself with the isolation and description of microorganisms from natural environment such as soil and water (Bahadure, Agnihotri, & Akarte, 2010). Amylase is produced by a variety of living organisms, ranging from bacteria to plants and humans. Bacteria and fungi secrete amylases to outside and inside of their cells to carry out extracellular and intracellular enzyme. When they have broken down the insoluble starch, the soluble end products are produce such as glucose or maltose (Anupama & Jayaraman, 2011).

For production of enzymes in industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is continuous

process. Microorganisms have become increasing important as producer of industrial enzymes. Due to their biochemical diversity and ease with which enzyme concentration may be increased by environmental and genetic manipulation, attempts are now being made to replace enzyme, 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 & Elavarasi, 2015).

Due to the wide range of application of amylase enzyme in various sectors such as confectionaries, baking, textile, detergent and many pharmaceuticals; many researches have studies amylase production with variety of substrate and microorganisms like bacteria, yeast and fungi. Due to the ever increasing demand for this enzyme, people are still trying to increase the productivity of amylase by variety of approach like selection of high enzyme producing strains, process optimization, usage of cheap substrate, e.t.c. (Bahadure, Agnihotri, & Akarte, 2010). Therefore, the aim of this study is to extract amylase from bacteria from the soil samples collected from different places of Dharan.

1.2 Statement of problem

The amylolytic enzymes find a wide spectrum of application in food industries, brewing industries, baking, paper, textile and detergent industries. The common sources of amylase production have been animals and plants. They have been extracted from these sources and being used in industries and factories. Since extraction of amylase from plants and animals is expensive, enzyme contents are less predictable and less controllable, plants and animals tissue contain more potentially harmful materials. Therefore, amylase production is more preferred from microorganisms. Amylase from microorganisms are highly stable specifically from bacteria, these bacteria are easily available in soil and screening of amylase producer bacteria are highly recommend.

1.3 Objectives

→ General objective

- To extract the amylase enzyme from soil bacteria

→ Specific objectives

- To isolate and identify amylase producing bacteria from soil sample
- To perform the amylase production from isolated bacteria
- To evaluate the potential of crude amylase extract on the hydrolysis of starch

1.4 Rationale of the study

This study might give the knowledge about amylase similar that human body contains to break down foods into smaller biomolecules. Amylase is an exoenzyme that hydrolyses (cleaves) starch, a polysaccharide into maltose and some monosaccharides such as glucose. Production of amylase commercially and use of this enzyme is helpful in several food fermentation process to convert starch to fermentable sugars. They are also used to partially predigest foods for young children, to clarity fruit juices and in the manufacture of corn and chocolate syrups. Although amylase can be derived from several sources, amylase from bacterial sources are highly stable, less expensive and easy to extract.

1.5 Limitation of the study

a) In this study of extraction of amylase from soil bacteria limited amount of samples had been collected and studied due to the less numbers of petri-plates available.
b) Due to limited time given, amylase was extracted from *Bacillus* spp. only.
c) Due to lack of sufficient amount of budget and equipment's isolated bacteria could not be identified by molecular technique.
d) Extracted crude amylase could not further study in details.

PART II

LITERATURE REVIEW

2.1 Alpha Amylase

Amylase catalyses the breakdown of starch into sugars, alpha amylase can breakdown long chain carbohydrates, ultimately yielding maltose from amylase, or maltose, glucose, and “limit dextrin” from amylopectin. Amylases are produced by a wide spectrum of organisms, although each source produces biochemical phenotypes that significantly differ in parameters like pH and temperature optima as well as metal ion requirement (Sivasudha & Rameshkumar, 2011).

Because of its multifarious application amylase attracts attention of researchers since decades after its first isolation and identification in the year 1894 from a fungal source that was used as additives in pharmaceutical digestive formulations (Crueger & Crueger, 1989). Since the onset of its discovery the research continues till date involving microorganisms as a potential source of amylase. Microorganisms are chosen preferentially for amylase production due to the relative ease of handling, availability, favorable growth conditions, and cheap nutrient requirement compared to the other producers like plant and animals. Amylase occupies a major share (around 25%) of total world enzyme market owing to its high demand eliminating chemical hydrolysis of starch liquefaction process (Rajagopalan & Krishnan, 2008; Reddy & Nimmagada, 2003). It has been utilized also in textile, food, brewing, and pulp industries. Amylase can be subdivided into α,β,γ amylases (Yamamoto, 1988).

Alpha amylases are the calcium containing enzymes, which randomly cleave the α-D-1,4-glycosidic bonds in starch with retention of α-anomeric configuration in the end product (kandra, 2003). They are found in microorganisms where they play a major role in carbohydrate metabolism. For industrial applications bacterial α-amylases are preferred over fungal amylases due to their catalytic efficiency and thermal stability. The thermostable α-amylases in industrial processes operate at higher temperature to minimize the contamination risk and to reduce the reaction time. The history of amylase

began in 1811 when first starch degrading enzyme was discovered by Kirchhoff in wheat and laid down the foundation for the discovery and research on amylase. The α-amylases were named by Kuhn in 1925, because the hydrolysis products are in the alpha-configuration. In 1930, ohlsson discovered another amylase, which yielded a β-mannose. He named it β-amylase. Crystal structure was established using $3A^0$ resolution structure which was further improved to 1.5^0 resolution of α-amylases (Kumari, Singh, & Kayastha, 2012). The three dimensional crystal structures each form were determined in 1990s and found to be effectively identical (Qian, Haser, & Prayan, 1995). As diastase, amylase was first enzyme to be discovered and isolated by Anselme Payen, 1833 (Hill & Needham, 1970). Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as pharmaceutical aid for treatment of digestive disorders (Pandey et al 2000). Biodin & Effron, 1917 were the first to use *Bacillus subtilis* and *Bacillus mesenticus* for the production of α-amylase on commercial scale using large fermentor in sub-merged fermentation (Biodin & Effront, 1917). Employment of bacterial cultures for the production of commercial enzyme was pioneered by them and accepted as an industrial practice throughout the world for the production of bacterial amylases. Prior to the developments, fungal amylases were extensively produced in the United states by SSF techniques as pioneered by Takamine (Takamine, 1914).

2.2 Structural and Functional Characteristics of α-Amylase

The α-amylase (α-1,4glucan-4-glucanohydrolase) can be found in in microorganisms, plants and higher organisms (kandra, 2003). The α-amylase belongs to a family of endo-amylases that catalases the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds (Brayer, Luo, & Withers, 1995; Lulek et al 2000; kandra, 2003). Neither terminal glucose residues nor α-1,6-linkages can be cleaved by α-amylase (Whitcomb & Lowe, 2007). The end products of αamylase 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 and α-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).

2.3 Soil

Soil is the upper layer of earth in which plants grow, a black or dark brown material typically consisting of a mixture of organic remains, clay, and rock particles. Soil is a mixture of minerals, organic matter, gases, liquids, and countless organisms that together support life on earth. Soil is a natural body called the pedosphere which has four important functions: it is a medium for plant growth; it is a means of water storage, supply and purification; it is a modifier of earth's atmosphere; it is a habitat for organisms; all of which, in turn, modify the soil. Soil has been called the Skin of the Earth (Miller A. , 1953) as it interfaces with the lithosphere, the hydrosphere, the atmosphere, and the biosphere (Cheswarth, 2008). The term pedolith, used commonly to refer to the soil, literally translates ground stone. Soil consists of a solid phase (the soil matrix) of minerals and organic matter, as well as a porous phase that holds gases (the soil atmosphere) and water (the soil solution) (Voroney & Heck, 2007; Danoff-Burg, Retrived 2017; Taylor & Ashcroft, 1972) Accordingly, soils are often treated as a three-state system of solids, liquids, and gases (McCarthy, 2006).

2.4 Soil microflora

Soil is the most abundant ecosystem on earth, but the vast majority of organisms in soil are microbes, a great many of which have not been described.

There may be population limit of around one billion cells per gram of soil, but estimates of the number of species vary widely from 50,000 per gram to above million per gram of soil. The total number of organisms and species can vary widely according to soil type, location and depth. One of the chief differences between soil and a heap of inert and powdered rock material is that the soil contains a dynamic population of microorganisms. The cultivated soil has relatively more population of microorganism than sandy eroded soils. Since, plant roots have great influence on soil microflora, crop cultivation in the soil

considerably enhances microbial activity. In a given soil the microbial population is ever changing, but by and large, the microbes are in a dynamic equilibrium. Bacteria are the most dominant group of microorganisms in the soil and probably equal to one half of the microbial biomass present therein. Their population ranges from 100,000 to several hundred million per gram of soil, depending upon the physical, chemical and biological condition of soil. Bacteria live in soil as cocci, bacilli or spirilli. The bacilli are common, whereas spirilli are rare. Some of the bacilli persist in unfavorable conditions by the formation of endospores. These endospores often endure in adverse environments, because of their great resistance to both prolonged deciccation and high temperature (Rangaswami & Bagyaraj, 1996).

2.5 Introduction of *Bacillus* Species

Bacillus species 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 & Thomas, 1984). Ubiquitous in nature, Bacillus includes both free-living (nonparasitic) and parasitic pathogenic species. Under stressful environmental conditions, the bacteria can produce oval endospores that are not true 'spores', but to which the bacteria can reduce themselves and remain in a dormant state for very long periods. These characteristics originally defined the genus, but not all such species are closely related, and many have been moved to other genera of the Firmicutes (Madigan & Martinko, 2005).

2.6 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. Besides these some species are found to be part of the normal flora (Whelch, 1920). Epidemiologic studies on the microbiology of street heroin and injection paraphernalia demonstrated the species as the predominant isolates from both specimens (Tuazon, 2016).

2.7 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 & Rodrigez-Diaz, 2006). *Bacillus subtilis* has proven highly amenable to genetic manipulation, and has become widely adopted as a model organism for laboratory studies, especially of sporulation, which is a simplified example of cellular differentiation. In terms of popularity as a laboratory model organism, *B. subtilis* is often considered as the Gram-positive equivalent of *E. coli*, an extensively studied Gram-negative bacterium. 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 optimum temperature for growth is 25-35^{o}C (Kunst et al 1997). 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 protuberances extending downward from liquid surface pellicles (Errington, 2003). Many strains produce spores with brown pigments.

2.8 Ecology of *Bacillus subtilis*

Soil is the main habitat for endospore forming *Bacillus* organisms. Likewise, *Bacillus subtilis* is most commonly found in soil environment 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 horizon where the oxygen concentration is most abundant and temperature are relatively mild. Scientists have demonstrated that *Bacillus subtilis* concurrently produces antibiotics and spores. Production of antibiotics increases *B. subtilis* 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, Kumthekar, & Ghodake, 2016).

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 & Alsaphar, 2012). Many of the *Bacillus* species 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).

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 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, Kumthekar, & Ghodake, 2016). *Bacillus subtilis* biofilms found in the rhizosphere of plants and 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, Kumthekar, & Ghodake, 2016).

2.9 Application of Amylases these days

a) Starch conversion/processing
b) Detergent additives
c) Bio-fuel alcohol production
d) Food industry (baking, brewing, juice preparation, starch syrups)
e) Removal of starch sizer from textile (desizing)
f) Modification of starch during paper sizing
g) Animal feed processing
h) Elimination of environmental pollutants
i) Digestive enzyme

PART III

MATERIALS AND METHODS

3.1 Materials used

Materials used in this study are listed in the appendix -I.

3.2 Sample Collection

Soil samples were collected from five different places (field, river, jungle, etc) of Dharan. Collected samples were transferred to sterile plastic bags in aseptic conditions.

3.3 Isolation of Amylase Producing Bacteria

Soil sample were pretreated by heating at 80^{o}C for 10 minutes and one gram of the above treated soil samples were weighed and serially diluted was done up to 10^{-10} and spread on nutrient agar (HI-MEDIA fortified with 1% starch). Then the plates were kept in incubation at 37^{o}C for 24 hours.

3.3.1 Media Preparation

For the isolation of amylase producing microorganism, starch agar medium (1% starch) was used. The composition of the media was as given in the appendix I. All the ingredients were weighed and dissolved in distilled water. The ingredients dissolved in water by boiling and shaking. The media was autoclaved at 121^{o}C.

3.3.2 Serial Dilution

Ten tubes were filled with nine ml of sterile distilled water and autoclaved. The tubes were labeled as 10^{-1}, 10^{-2}, 10^{-3},10^{-4}, 10^{-5}, … 10^{-10}. One gram of soil sample was dissolved on 10^{-1} tube and dissolved by shaking and one ml of the solution was taken from 10^{-1} tube and added to 10^{-2} tube. This process was repeated up to 10^{-10} tube. Above same process was done for the different samples.

3.3.3 Inoculation of Sample

0.1 ml of each dilution was added, using the spread plate technique, to starch agar (1% starch). The agar plates were incubated at 37ºC for 24 hours.

3.4 Screening for Amylase Activity (Starch Iodine Test)

Bacterial cultures were screened for amylolytic activity by starch hydrolysis test on starch agar plate. The pure isolated will be streaked on starch agar plates with starch as the only carbon source. After incubation at 37ºC for 24-48 hours, the individual plates were flooded with Gram's iodine to produce a deep blue colored starch-iodide complex. In the zone of degradation, no blue color forms, were a basis of the detection and screening of amylolytic strain. The amylase producers displaying maximum diameter of zone of clearance, was further investigated biochemically after sub-culturing in NA. The pure cultures were sub-culture at regular intervals and starch agar (1% starch) slant were maintained at 4ºC.

3.5 Microscopic and Biochemical Test (Identification of organism)

After the sub-culture of organism was performed, they were tested microscopically and biochemically for their identification. The process of identification by the test involved the following steps and procedure for these biochemical tests are given in appendix iv:

a. Gram staining
b. Catalase test
c. Oxidase test
d. Indole test
e. Citrate utilization test
f. MR-VP test
g. Urease test
h. Gelatin hydrolysis test

3.6 Enzyme Extraction

A loopful bacterial culture was transferred from starch-nutrient agar slants to starch-nutrients broth at pH-7 for activation and incubated in shaker at 40^{o}C at 120 rpm for 24 hours.

The fermentation medium (appendix II) was inoculated with activated culture and incubated in shaker at 37^{o}C for 24 hours. At the end of the fermentation period, the culture medium was centrifuged at 10000 rpm for 15 minutes to obtain the crude extract, which serve as enzyme source.

3.7 Enzyme Production

3.7.1 Enzyme confirmation

Crude enzyme extract obtained after the centrifugation was inoculated in starch agar plates and incubated at 37^{o}C for 24 hours. After incubation period of 24 hours, clear hydrolytic zone was seen in starch agar plates. The result can be seen in photograph number 5.

3.7.2 Measurement of absorbance

Amylase absorbance was assayed as described. Briefly, 1.5ml of 1% starch in 2ml, 0.1M phosphate buffer (pH 6.5) and 0.5ml of dilute enzyme were incubated for 15 minutes at room temperature (37^{o}C). The reaction was arrested by adding 1 ml of DNS reagent and kept in boiling water bath for 10 minutes and diluted with 8 ml of distilled water.

The absorbance was measured at 540 nm against blank prepared as above without incubation. One unit of α-amylase activity was defined as the amount of enzyme that liberates 1 μ mole of reducing sugar (maltose equivalents) per minutes under the assay conditions. The experiment was carried out in triplicate.

3.8 Work Flow Chart

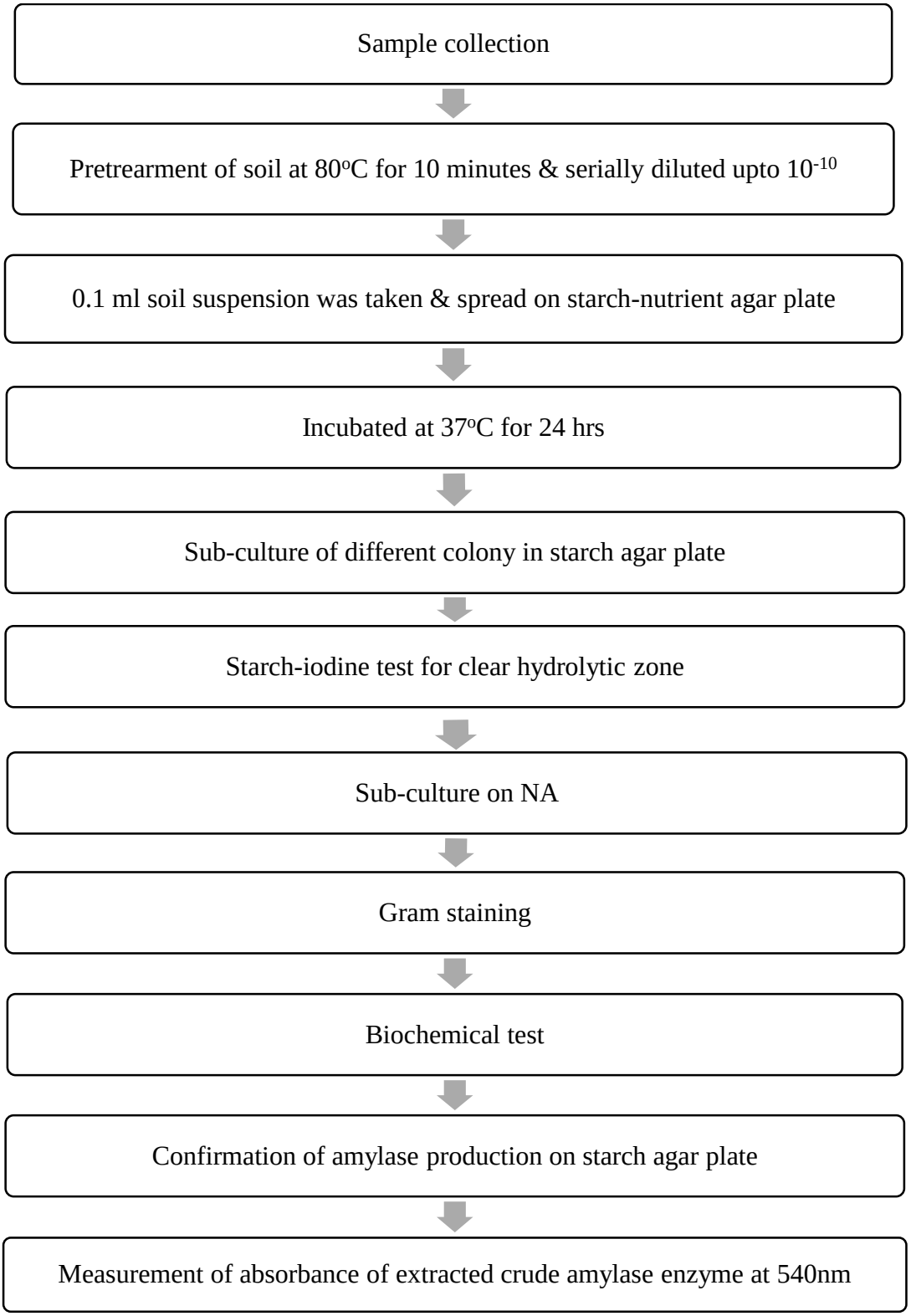


Fig: I Flow chart (Extraction of Amylase from Soil Bacteria)

PART IV

RESULT

4.1 Isolation of Amylase Producing Bacteria

Among the five samples collected for extraction of amylase, five colonies of these samples were positive for the amylase production on starch agar. The amylase production test was done on starch agar plate by the starch-iodine test. The colony with maximum diameter of clear hydrolytic zone were further investigated. The colony characteristics of the isolated bacteria on nutrient agar plate is given below.

Table: Colony characteristics of the isolates of sample on Nutrient agar

SN	Shape	Margin	Elevation	Color	Texture	Opacity	Assumed species
1	Round	Regular	Flat	White	Smooth	Opaque	*B. subtilis*

4.2 Morphological Characterization

Gram staining was performed and viewed microscopically. The isolated bacteria were gram positive, spore forming, rod shaped.

4.3 Biochemical Characterization

Different biochemical tests were performed for the isolated bacteria. According to the results of different biochemical tests, the isolated bacteria were found to be *Bacillus subtilis.* The results of the biochemical tests are given below.

Table: Biochemical test result:

S.N	Tests	Result
1	Starch hydrolysis	Positive
2	Catalase	Positive
3	Oxidase	Positive
4	Indole	Negative
5	MR (Methyl red)	Negative
6	VP (Voges-Proskauer)	Positive
7	Citrate utilization test	Positive
8	Urease	Negative
9	Gelatin liquefaction	Positive
10	Gram staining	Positive

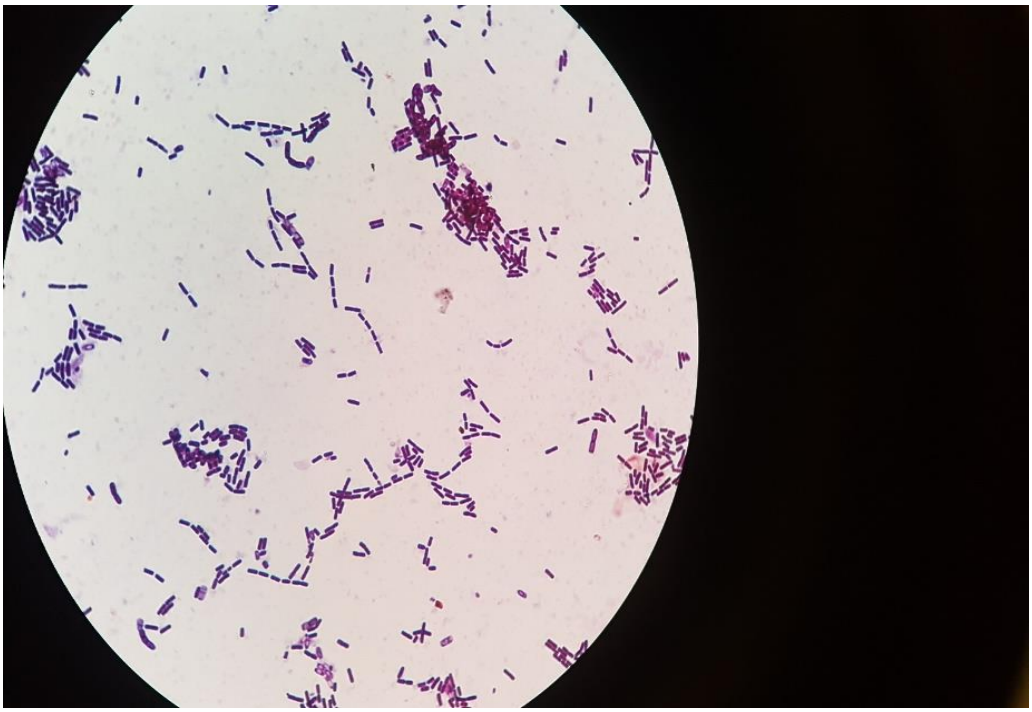
4.4 Analysis of Amylase Production

Crude amylase extract was inoculated in starch agar plate and incubated at 37ºC for 24 hrs, clear hydrolytic zone confirms the production of amylase. Amylase activity was assayed by the measurement of the absorbance at 540nm of crude enzyme extract against the blank solution. One unit of α-amylase activity was defined as the amount of the enzyme that liberates at 1μmole of reducing sugar (maltose equivalent) per minutes under the assay conditions (Miller, 1959). Quantification of the extracted crude amylase in terms of absorbance are given in table below.

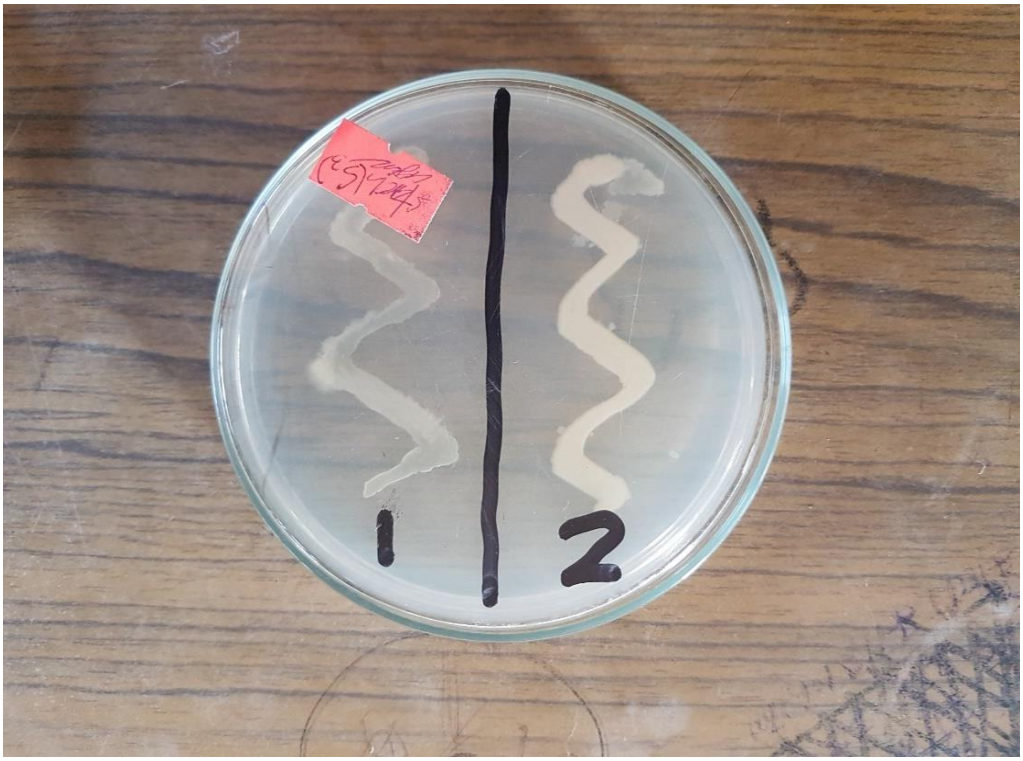
Table: Absorbance of extracted crude amylase enzyme

Isolates	Absorbance(540nm)
S_1	0.04
S_2	0.04
S_3	0.03
S_4	0.01
S_5	0.03

Photograph-1 Bacterial Colony on Starch Nutrient Agar from Soil Sample

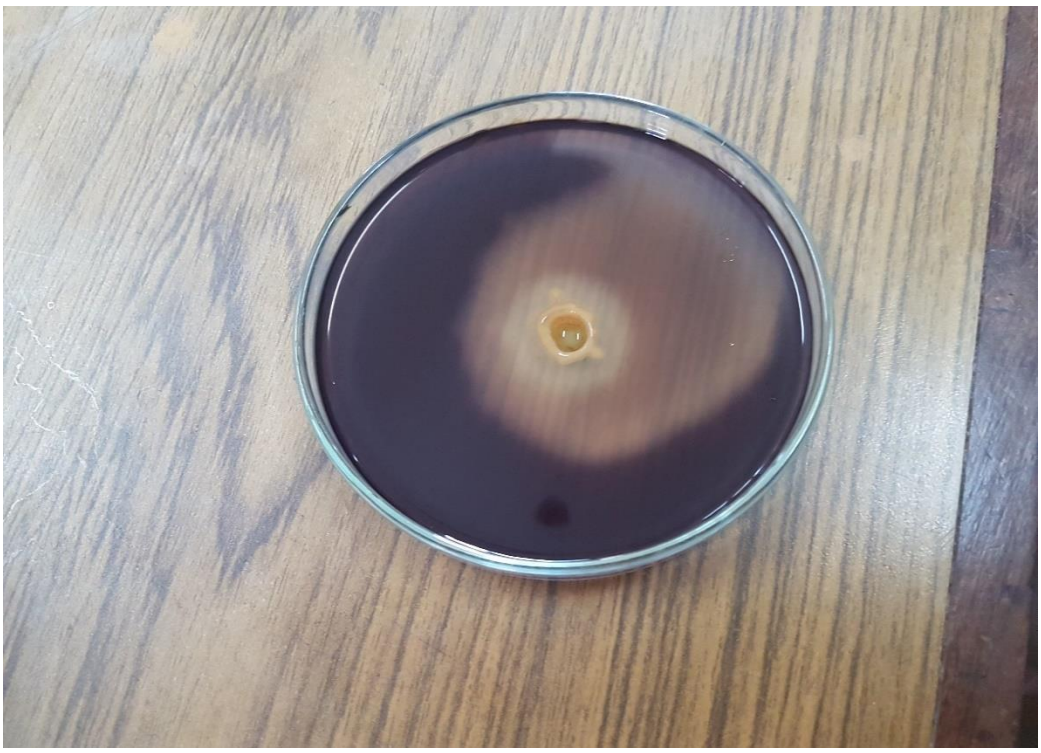


Photograph-2 Gram Stain of Isolated Bacteria



Photograph-3 Sub-Culture on Starch Agar Plates

Photograph-4 Hydrolytic Clear Zone



Photograph-5 Clear Zone on Starch Agar Plate by Crude Enzyme Extract

Photograph-7 Biochemical Test

PART V

DISCUSSION

In this piece of work, extraction of amylase from soil bacteria, amylase was extracted from soil bacteria of Dharan, Sunsari. Amylase is group of important enzyme which is mainly employed in starch processing industries for hydrolysis of polysaccharides like starch into simple sugar (Daniel, Peterson, & Danson, 2010). These enzymes originate from different sources such as plants, animals, and microorganisms. Enzymes from microbial sources generally meets industrial demands, due to their high yield and thermostability. Microbial enzyme presents a wide spectrum of characteristics that make them useful for specific applications.

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 carbohydrates in human diets and is contained in staple foods such as potatoes, wheat, maize, rice, etc. The enzymes that breakdown or hydrolyzes starch into the constituent sugars known as amylases. Amylases can be obtained from several sources such as plant, animal and microbes. Amylases are most important in biotechnology industries with huge application in food, fermentation, textile and paper.

Microorganisms are the most important sources for the enzyme production. Selection of 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 increasing important as producer of industrial enzymes. Due to their biochemical diversity and ease with which enzyme concentration may be increased by environmental and genetic manipulation, attempts are now being made to replace enzyme, which

traditionally have been isolated from complex eukaryotes. An extra-cellular amylase, specifically raw starch digesting amylase has found important application in bioconversion of starches and starch-based substrates. The level of alpha amylase activity in various human body fluids is of clinical importance e.g. in diabetes, pancreatitis and cancer research, while plant and microbial alpha amylase are used as industrial enzymes. The microbial amylases have almost completely replaced chemical hydrolysis of starch in starch processing industry.

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

According to Logan & Rodrigez-Diaz (2006), *Bacillus subtilis* is one of the most common amylase producing bacteria that are mostly found in soil which also matched with the present study.

Fogarty (1983) discussed the application and features of amylases of microbial origin.

According to Lonsane and Ramesh (1990) microorganisms are the most reliable sources of enzymes in large scale productions.

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

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

Liu and Xu (2008) isolate a novel raw starch digesting α-amylase bacteria *Bacillus* spp. YX-1: they worked on purification and characterization of amylase enzyme.

Sani et al (2014) isolates *Bacillus subtilis* for the production, purification and characterization of α-amylase. There results showed that the partially purified enzyme has specific activity of 0.144±0.019 U/mg, these was increase of 33.5 times than the raw enzyme extract.

Bacillus subtilis was the bacteria isolated in this study which was responsible for the production of α-amylase enzyme.

PART VI

CONCLUSION

The isolated bacteria *Bacillus subtilis* from the soil sample of Dharan which has the capability to produce amylase. The present study showed that the isolated amylolytic bacterial *Bacillus subtilis* produced amylase enzyme. Therefore, the study also revealed that the amylase enzyme produced by the isolated bacteria can be used for the industrial application. The ability of produced amylase that could hydrolyze starches in starch agar medium and it showed that these types of enzyme are useful in the sugar industry, food industry (baking and brewing) and fuel alcohol industry. In addition, the potential of these enzymes could also give great importance in animal feed processing because the raw starches especially sorghum and maize are high grain diets used for fattening of farm animals like sheep. The measurement of absorbance of extracted crude amylase concludes that higher the absorbance, higher the enzyme activity on the substrates.

RECOMMENDATION

Following recommendations are suggested

1. Isolated bacteria can be used on the biotechnology industries with huge application in food, fermentation, textile, and paper as amylase producer.
2. Amylases of different biochemical properties are needed when they are used at large scale application. Therefore, characterization of their activity is necessary.
3. Crude amylase enzyme extract can be used to observe the effect and capacity of amylase to hydrolyze the starch.
4. The measurement of hydrolytic clear zone on starch agar plates can be used to identify the bacteria having highest efficiency.
5. Identification of the type of the amylase that the isolate produced would give full information of the amylase for which application could be used. Therefore, identification of extracted amylase and isolated bacteria by molecular technique is necessary.

REFERENCES

Al-faragi J & Alsaphar S (2012). Isolation and identification of Bacillus subtilis as (probiotic) from intestinal microflora of common carp Cyprinus carpio L. proceeding of the Eleventh Scientific Conference: 361.

Angelo R & Rangabhasiyam S (2013). Amylase production from bacteria isolated in the soil receiving kitchen wastes. International journal of pharmaceutical and chemical sciences, **2**(1): 281-287.

Bahadure R, Agnihotri U & Akarte S (2010). International journal of parasitology research: 9-13.

Brayer G, Luo Y & Withers S (1995). The structure of human pancreatic alpha-amylase at 1.8 A resolution and comparisons with related enzymes. In Protein Sci. **4**: (pp. 1730-1742).

Cheswarth W (2008). In Encyclopedia of soil science. Dordrecht, netherlands: Springer.

Collee J (1989). Mackie and McCartney practical medical microbiology.

Crueger W & Crueger A (1989). Industrial microbilogy.

Daniel R, Peterson M & Danson M (2010). Biochemistry journal, **425**(2): 353-360.

Danoff-Burg JA (Retrived 2017). The terrestrial influence: geology and soils. Columbia University Press.

Errington J (2003). Regulation of endospore formation in *Bacillus subtilis.* Nature Reviews Microbiology. **1**: 117-126.

Joshi K, Kumthekar M & Ghodake V (2016). *Bacillus subtilis* Bacteria Impregnation in Concrete for Enhancement in Compressive Strength.

Kandra L (2003). α-amylases of medical and industrial importance. Journal of Molecular Structure (Theochem): 487-498.

Kunst F, Ogasawara N, Moszer I, Albertini A, Alloni G, Azevedo V, . . . Borchert S (1997). The complete genome sequence of gram-positive bacterium *Bacillus subtilis* . Nature. **390**: 249-256.

Logan N & Rodrigez-Diaz M (2006). *Bacillus* spp. and related genera. In Principles and practice of clinical bacteriology. **2**: (pp. 139-158).

Lulek J, Sillva M, Slivinki C, Bloch C, Rigden J & DJ Grossi de sa M (2000). Purification, biochemical characterization and partial primary structure of a new alpha-amylase inhibitor from Secale cereale (rye). Int J Bichem Cell Bio. **32**: 1195-1204.

Maureen B (2000). In Stedman's Medical Dictionary (p. 65). Baltimore, Maryland, USA: Lippincott Williams & Wilkins.

McCarthy DF (2006). In Essentials of soil mechanics and foundations: basic geotechnics (7th edition). Upper Saddle River, United States: Pearson Education (US).

Miller A (1953). In The skin of the earth (1st edition). London, United kingdom: Methuen & Co. Ltd.

Miller G (1959). Use of the dinitrosalicyclic acid reagent for the determination of reducing sugar. In Analytical chemistry **31**(3): (pp. 426-428).

Oyeleke S & Oduwole A (2009). African journal of microbiology research, **3**(4): 143-146.

Panneerselvam T & Elavarasi S (2015). Isolation of α-Amylase Producing *Bacillus subtilis* from Soil. Int. J. Curr. Microbiol. App. Sci. **4**: 543-552.

Poinar JG & Thomas G (1984). Laboratory guide to insect pathogens and parasites. In Bacteria. Springer.

Rajagopalan G & Krishnan C (2008). α-amylase production from catabolite derepressed *Bacillius subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. In Bioresource Technology vol-99: (pp. 3044-3050).

Rangaswami G & Bagyaraj D (1996). Microorganisms in soil. In Agricultural microbiology (2nd edition) (pp. 204-205). Prentice Hall of India Pvt. Ltd, New delhi.

Reddy N & Nimmagada A& (2003). An overview of the microbial alpha amylase family. African journal of biotechnology Vol-2, No-12: 645-648.

Sivasudha A & Rameshkumar T (2011). optimization of nutritionalconstitute for enhanced alpha amylase production by solid state fermentation technology. international journal of microbiological research, vol-2, no-2: 148.

Tangphatsornruang S, Naconsie M, Thammarongtham C & Narangajavana J (2005). Isolation and characterizaton of an alpha-amylase gene in cassava (Manihot esculenta). In Plant Physiol Biochem. **43**: (pp. 821-827).

Taylor SA & Ashcroft GL (1972). In Physical edaphology: the physics of irrigated and nonirrigated soils. San Francisco, California: W.H. Freeman.

Tuazon C (2016). Bacillus species. Last accessed on.

Uhlig H (1998). Industrial enzymes and their applications. John Wiley and Sons.

Van der Maarel M, Van der Veen B, Uitdehaag J & Leemhuis H& (2002). Properties and applications of starch-converting enzymes of the alpha-amylase family. In J Biotechnol. **94**: (pp. 137-155).

Voet D & JG V (2005). In Biochimie (p. 1583). Bruxelles: De Boeck.

Voroney R & Heck RJ (2007). The soil habitat. In Soil microbiology, ecology and biochemistry (3rd edition) (pp. 25-49). Amsterdam, the netherlands: Elsevier.

Whelch W (1920). Papers and Addresses: Bacteriology. Johns Hopkins press.

Whitcomb D & Lowe M (2007). Human pancreatic digestive enzymes. In Dig Dis Sci. **52**: (pp. 1-17).

Yamamoto T (1988). Handbook of amylases and related enzymes: their sources, isolation methods, properties and applications. osaka, japan: pergamon press.

Anupama A & Jayaraman G (2011). Detergent stable, halotolerant a-amylase from *Bacillus aquimaris* VIT4 exhibits reversible unfolding. International Journal of Applied Biology and Pharmaceutical Technology, **2**(2): 366-376.

Bahadure R, Agnihotri U & Akarte S (2010). International journal of parasitology research: 9-13.

Biodin A & Effront J (1917). US Patent **1**, 227, 374.

Daniel R, Peterson M & Danson M (2010). Bichemistry journal, **425**(2): 353-360.

Fogarty W (1983). Microbial amylases in: Microbial enzymes and biotechnology . Applied Science Publishers: 1-92.

Hill R & Needham J (1970). The chemistry of life: eight lectures on the history of biochemistry. Cambridge university press, London, England, 17.

Kumari A, Singh S & Kayastha A (2012). α-amyalse: General properties, mechanism and biotechnologicalapplication- A review. In Current biotechnology. **1**: (pp. 98-197).

Liu X & Xu Y (2008). A novel raw starch digesting α-amylase from newly isolated Bacillus Sp. YX-1, purification and characterization. In Biores Technol., 99: (pp. 4315-4320).

Lonsane B & Ramesh M (1990). Production of Bacterial thermostable α-amylase by solid state fermentation: A potential tool for achieving economy in enzyme production and starch hydrolysis. In Advances in appl. Microbiol. **35**: (pp. 1-56).

Madigan M & Martinko J (2005). Brocks Biology of MIcroorganisms (11 ed). Prentice Hall.

Miller G (1959). Use of the dinitrosalicyclic acid reagent for the determination of reducing sugar. In Analytical chemistry **31**(3): (pp. 426-428).

Oyeleke S & Oduwole A (2009). African journal of microbiology research, **3**(4): 143-146.

Pandey A, Nigam P, Soccol C, Soccol V, Singh D & Mohan R (2000). Advances in microbial amylases. Biotechnol Appl Biochem. **31**(2): 135-152.

Panneerselvam T & Elavarasi S (2015). Isolation of α-Amylase Producing *Bacillus subtilis* from Soil. Int. J. Curr. Microbiol. App. Sci. **4**: 543-552.

Qian M, Haser R & Prayan F (1995). Carbohydrate binding sites in a pancreatic α-amylase subtrate complex, derived from X-ray structure analysis at 2.1 A resolution. In Protein Sci.-**4**: (p. 747).

Sani I, Abdulhamid A, Bello F, Yahaya M & Bagudo A (2014). Isolation, partial purification and characterization of α-amylase from *Bacillus subtilis*. J. Microbiol. Biotech.Res., **4**(1): 49-54.

Takamine J (1914). Enzymes of Asperguillus oryzae and the application of its amyloclastic enzyme to the fermention industry. Ind. Eng. Chem. **6**: 824-828.

APPENDICES

APPENDIX-I: LIST OF MATERIALS

1. **Equipment used:**
    1. Autoclave
    2. Weighting machine
    3. Hot air oven
    4. Incubator
    5. Microscope
    6. Micropipette
    7. Shaker
    8. Photometer
2. **Glassware:**
    1. Test tubes
    2. Pipettes
    3. Beakers
    4. Petri plates
    5. Conical flask
    6. Glass rod and glass tubes
    7. Reagent bottles
    8. Slides
    9. Measuring cylinder
3. **Microbiological and Biochemical media:**
    1. Nutrient-starch media
    2. Nutrient agar
    3. Starch agar media
    4. Fermentation media
    5. Simmons citrate media
    6. MR-VP broth
    7. Urease media
    8. Gelatin media
4. **Chemicals and Reagents:**

1. Catalase reagent (3% H_2O_2)
2. Alpha-napthol (5%)
3. Crystal violet
4. Gram's Iodine
5. Safranin
6. Oxidase reagent
7. Ethanol
8. Lysol
9. Kovacs reagent
10. Methyl red
11. Potassium hydroxide

5. Miscellaneous

1. Aluminium foil
2. Plastic bags
3. Inoculating loop/needles
4. Labelling tape
5. Cotton plugs
6. Blotting paper
7. Cotton swab
8. Test tube holder
9. Bunsen burner
10. Detergent

APPENDIX-II: COMPOSITION OF MEDIA USED

1.Nutrient agar: (pH-7.0)

Peptone	5.0g
Beef extract	3.0g
NaCl	5.0g
Agar	15g
Distilled water	1000ml

2.Starch agar media:

Meat extract	3.0g
Peptone	5.0g
Starch	2.0g
Agar	15g
pH (at 25^{o}C)	7.2±0.1
Distilled water	1000ml

3.Fermentation media:

Soluble starch	10g
Peptone	5.0g
$(NH_4)_2SO_4$	2.0g
KH_2PO_4	1.0g
K_2HPO_4	2.0g
$MgCl_2$	0.01g
Distilled water	1000ml
pH	7.0

4.MR-VP media:

Peptone	7.0g
Dextrose	5.0g
Dipotassium phosphate	5.0g
Distilled water	1000ml
pH (at 25^{o}C)	6.9±0.2

5. Simmons citrate:

Magnesium sulfate	0.2g
Ammonium dihydrogen phosphate	1.0g
Dipotassium phosphate	1.0g
Sodium citrate	2.0g
Sodium chloride	5.0g
Bromothymol blue	0.08g
Agar	15g
Distilled water	1000ml
pH (at 25^{o}C)	6.8±0.2

5.Gelatin media:

Peptone	5.0g
Beef extract	3.0g
Gelatin	120g
Distilled water	1000ml
pH (at 25^{o}C)	6.8±0.2

6.Christensen's urea agar (urease test):

Urea	20g
Sodium chloride	5.0g
Monopotassium phosphate	2.0g
Peptone	1.0g
Dextrose	1.0g
Phenol red	0.012g
Agar	15g
Distilled water	1000ml
pH (at 25^{o}C)	6.7±0.2

7.Tryptone broth media:

Tryptone	1.0g
NaCl	0.5g

Distilled water	100ml
pH	7.5

APPENDIX-III: STAINS AND REAGENTS USED

1. Crystal violet:

Crystal violet	20g
Ethyl alcohol	95ml
Ammonium oxalate	9g
Distilled water	905ml

2. Gram's iodine:

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

3. 95% ethyl alcohol:

Ethyl alcohol	95ml
Distilled water	5ml

4. Safranin:

Safranin	10ml
(2.5% safranin in 95% ethyl alcohol)	
Distilled water	100ml

5. Hydrogen peroxide solution:

Hydrogen peroxide	3ml
Distilled water	97ml

6. Oxidase reagent:

Tetramethyl-p-phynylenediamine Dihydrochloride	0.1g
Distilled water	10ml

7. Kovacs reagent:

Dimethyl amino benzaldehyde	5g
Amyl alcohol	75ml
Conc. Hydrochloric acid	25ml

8. Methyl red solution:

Methyl red	0.05g

Ethyl alcohol	28ml
Distilled water	22ml

9. VP reagent:

VP reagent –I

α- Napthol	5g
ethyl alcohol	100ml

VP reagent –II

Potassium hydroxide	40g
Distilled water	100ml

APPENDIX-IV: PROCEDURE OF BIOCHEMICAL TESTS

1. Starch hydrolysis test:

The bacterial colony was streaked on the starch agar plate and incubated at 37^{o}C for 24 hrs. After incubation gram iodine was poured on the plate. The organism which hydrolyses starch gives clear zone around the colony surrounded by the purple color. In case of starch non hydrolyzing organism the whole media remains purple.

Principle:

The test determines the ability of organisms to hydrolyze starch, by enzymatic action. Iodine is used to detect the disappearances of starch. Iodine combines with the starch to form an intense, deep blue color complex. If the starch is hydrolyzed, the test will fail to maintain the blue color.

2. Indole test:

The bacterial colony was inoculated on tryptone broth and then incubated at 37^{o}C for 24 hours. After 24 hours of incubation, 1ml of Kovac's reagent was added. Appearance of red color (red ring) on the top of media indicates positive indole test.

Principle:

This test is used to determine the ability of bacteria to oxidize the tryptophan by producing tryptophynase enzyme.

3. MR-VP test:

The bacterial colonies were inoculated into MR and VP broth and incubated at 37^{o} C for 24 hours. After incubation, 5 drops of methyl red indicator were added to MR broth and mixed well for MR test. The positive test was indicated by the development of red color, and negative with yellow color. For VP test, 5 drops of Barritt's reagent was added to VP broth and shaken well. Positive test is indicated by the development of pink red color.

Principle of MR test:

The principle of this test is to detect the ability of bacteria to produce and maintain sufficient stable acid from glucose fermentation which is indicated by MR indicator.

Principle of VP test:

This test detects the ability of bacteria to produce a neutral end product, acetyl methyl carbinol (acetoin) from glucose fermentation.

4. Citrate utilization test:

A bacterial colony was stabbed on the butt of the Simmons citrate agar and then streaked on slant by a sterile inoculating needle. Then the inoculated media were incubated at 37ºC for 24 hours. A positive test was indicated by the growth of organism and change of color of media from green to blue. Bromothymol blue is green acidic (pH 6.8 and below) and blue when alkaline (pH 7.6 and higher).

5. Gelatin liquefaction test:

Using sterile inoculating needle, the test organism was stabbed on the nutrient gelatin deep tubes from top to bottom. The inoculated tubes were incubated at 37ºC for 48 hours. After incubation, the tubes were placed into a refrigerator at 4ºC for 15 minutes. The tubes that remain liquefied show positive test for gelatin hydrolysis and those tubes that remain solid demonstrate negative test.

Principle:

Gelatin is protein derived from the animal protein collagen. It dissolves in water at 50º Celsius and exists as liquid above 25º Celsius and solidifies or gels when cooled below 25º Celsius.

6. Catalase test:

3% H_2O_2 was taken in a clean and dry test tube (3ml). A small amount of culture from nutrient agar plate was added and mixed with the help of glass rod. Positive test is indicated by the formation of bubbles of oxygen gas.

Principle:

Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. Bubbles of oxygen are released if the organism is catalase producer.

7. Oxidase test:

The test organism was spread on the filter paper soaked by the oxidase reagent with the help glass rod. The organism producing oxidase enzyme gives purple color on the paper.

Principle:

This test used for determine the presence of cytochrome oxidase in bacteria that catalyse the transport of electrons between electron donor and redox dye. Tetramethyl p-phenylene diamine dihydrochloride in reagent is reduced to deep purple color.