WOLKITE UNIVERSITY
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE
DEPARTMENT OF BIOTECHNOLOGY

A SENIOR RESEARCH ON: ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF AMYLASE-PRODUCING BACTERIA FROM THE SOIL SAMPLE AND WASTE WATER EFFLUENT IN ZEBIDAR BEER FACTORY, GUBRYE TOWN, WOLKITE, ETHIOPIA

PREPARED BY

NAME	ID NO
ADDISE ABEBE	NSR/ 0146/13
ABAYNEH AMARE	NSR/ 0002/13
CHERNET ATALAY	NSR/ 0552/13
DESALEG N TESFAYE	NSR/0663/13
MIHRET DEBAS	NSR/ 2470/13
HABTU KEBEDE	NSR/ 1025/13
MEKUANINT ANTENEH	NSR/1437/13
YITAYAL ANRSAW	NSR/2230/13
YOUSUF HUSENA	NSR/ 2295/13

Submitted to the Department of Biotechnology for the Partial Fulfillment of the Requirement of Degree of Bachelor of Science in Biotechnology

ADVISORS: HENOK T. (MSc)

MAY, 2024
WOLKITE, ETHIOPIA

ACKNOWLEDGMENT

We would like to thank Wolkite University for providing the necessary material and laboratory consumables. We would also like to express our special thanks of gratitude to biotechnology and biology laboratory technicians for their support during sample collection, availing equipment and media preparations material and technical support We would like to express our deepest and strongest gratitude to our advisor Mr. Henok Tadesse (MSc) and also laboratory cassistance Mr. Ayansa Kebenessa and Mr. Debebe Landina for contribution for the overall quality of the paper from the beginning of the title selection up to the end of this paper development.

LIST OF ABBREVIATIONS AND ACRONYMS

MR……..……. Methyl Red

VR…….……...Voges Proskauer

TSI ……..…… Triple Sugar Iron Test

K. ……Alkaline=no fermentation

A. ……Acidic sugar fermentation

SS……..……Soil Sample

SW……..…..Sample of Wastewater

SWF……..Sample of Wastewater Final Effluent

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ABSTRACT

Amylase has been one of the most widely used enzymes in industries for decades. Its main function is to hydrolyze the starch molecule into glucose unit. Among different types of amylases, α-amylase is in high demand to due its wide spread use in food, textile, baking and detergent industries. It can be acquired from microorganisms, plants and animals. The present study was conducted with the aim to isolate, identify and characterize amylase-producing bacteria from soil and waste water. The soil and waste water effluent samples were collected from the different places in the Zebidar beer factory, Gubrye town. Serial dilution of the samples from 10^{-3} to 10^{-6} were prepared and amylase producing bacteria were isolated from soil and waste water samples. Samples were cultured on a nutrient agar and Sub-culturing of clear colonies was made on Starch Plate Agar. The bacterias producing highest clear zone were identified and further used for amylase production and biochemical characterization. The screening of amylase producing bacteria was carried out by starch hydrolysis test on starch agar plates. Total of forty isolates were obtained by primary screening technique from which thirty isolates showed amylolytic activity. All isolates were found to be positive for gram reaction, catalase test, TSI test, VP test. However, they were found to be positive and negative for Simmon's cit rate test and MR-test.

Key words: Amylase producing Bacteria,Biochemical test, Starch hydrolysis, Gram staining

1. INTRODUCTION

1.1. Background

Amylase is any of several enzymes that hydrolyz starch and glycogen to simple sugars, such as glucose. Amylase is an enzyme that hydrolyses starch into its monomer compounds, the smallest being glucose. The glycosidic bonds that hold the monomers together are broken down by this enzyme. This is a very common and essential reaction that takes place within various living organisms in order to generate or store energy. Hence, amylase is a very prevalent enzyme produced biologically by various kinds of living things. This includes plants, animals, humans and micro-organisms.

Among the various types of amylase, microbial amylase meets the industrial demands (Akcan *et al*., 2011). Quite large varieties of microorganisms have been identified and chosen as the source of amylase production because of the availability and simplicity of the ways in which they yield amylase. Soil is a primary source of these bacteria which can be isolated and commercially grown in large numbers to produce a vast amount of amylase. In order to provide this, industries use fermentation shake flasks to grow bacteria (Dash *et al*., 2015).There have been great advances in the use of amylase in industrial sectors as well. A large portion of the enzyme market share is owned by amylase (Gupta *et al*., 2003). A wide range of industries such as food industries, garments, textiles and beverage industries along with medicinal and clinical chemistry use amylase to manufacture their products. This requires a constant production of amylase enzyme. Extraction of this huge quantity of amylase directly from nature is not feasible and hence various methods are being constantly established to develop the mass production of commercial amylase (Dash *et al*., 2015).

In addition, the amylases that are extracted require optimum conditions to show the greatest activity. This includes parameters such as temperature and pH (Sivarama krishnan *et al*., 2006). For this reason, it is important to find out the optimum conditions for amylase activity through research before it can be used for industrial purposes. The enzyme amylase breaks down carbohydrate molecules into smaller products. Their main substrate is starch which is broken down into smaller components such as dextrin, maltose, maltotriose, and glucose.

The enzyme hydrolyses the α-1, 4 - glycosidic bonds that hold the glucose units together (Gurung *et al*., 2013). Hence, amylase is also addressed as 'glycoside hydrolases' (Kaur *et al*., 2012). Apart from starch hydrolysis, other forms of amylase known as transglycosylating enzymes, cause starch modification (Sivaramakrishnan *et al*., 2006). Amylase is a very common enzyme found in nature. They are known to be produced most widely by microorganisms such as bacteria and fungi as compared to plants and animals (Dash *et al*., 2015). The enzymes are produced outside the cell, to carry out extracellular digestion (El-Fallal *et al*., 2012).Bacillus is a common choice of source for industrial amylase production. Different strains of Bacillus have different optimal growth conditions and enzymatic production profiles. Additionally, the genus produces amylase that is thermo stable, and has retention to extreme pH, osmolarity, and high pressure. Other advantages of this bacterial enzyme production include the use of short fermentation cycles, capacity to produce an extracellular enzyme, simple cost-effective production, eco-friendly behavior, less handling hazard for workers, mass production and easy manipulation of bacterial genes (Gurung *et al*., 2013). All these characteristics are ideal for industrial amylase production.

The Production and characterization of amylase is a hot topic of research. This is due to the vastness of applications of amylase in various sectors, especially in industries. Studies and experiments are being carried out on a large scale to detect and determine the easiest ways and optimal conditions to acquire amylase to meet the demands of product manufacture in the industrial world.

1.2. Statement of the problem

Bacteria have potential to produce industrial enzymes is found in different waste disposal areas. Bacteria that produce amylase from soil and waste water will be isolated and characterized in Wolkite university gubrye campus. Enzymes are not more used in industry due to cost, availability, limited/no production, and a limited search for microorganisms producing industrially important enzymes. The greatest challenge to the environmentalists is the eco-friendly management of waste. Different organic and inorganic wastes are released into the environment, industry uses different chemicals during the processing of industrial products however the release of industrial chemicals into the environment leads to human health hazards and harms biological diversity on the earth. Therefore, this study would be

intended to isolate and characterize the potential amylase producing bacteria strain from the soil and waste water for different purposes.

1.3 Objective of the Study

1.3.1 General Objective

- ❖ To isolated and characterized of Amylase producing Bacteria from soil and waste water.

1.3.2 Specific Objectives

- ✓ To isolated amylase producing bacteria from soil and waste water.
- ✓ To characterized amylase producing bacteria from soil and waste water.

1.4 Research Question

How much of the isolated bacteria will be positive for starch hydrolysis test?

How much of the isolated bacteria will be positive for gram reaction, Catalase test, MR test, TSI test, VP test and Simmon's citrate test?

1.5 Significance of Study

This study will be important to search for different amylase producing bacteria in Gubrye town. In this study bacterium having the potential to produce Amylase enzyme from soil and waste water will isolate and characterize. Amylase reduces the effect of environmentally hard chemicals used in industrial processes and used for environmental remediation. Thus the study will be provided information about the source of bacteria for Amylase production. Microbial enzymes are often more useful than enzymes derived from plants or animals because of more stable than their corresponding plant and animal enzymes and their production is more convenient and safer.

1.6 Scope of the Study

This study wase conducted at Wolkite University, particularly in the Biotechnology and Biology laboratory of the Gubrye campus. The Study focus on the isolation and characterization of amylase-producing bacteria from waste soil and wastewater effluent in Zebidar beer factory samples were collected from Gubrye town.

2. LITERATURE REVIEW

2.1 Historical Background Enzyme Discovery

In 1833, diastase (a mixture of amylases) was the first enzyme to be discovered by a French chemist named Anselme Payen a few decades later. The term enzyme was first used in 1878 by Wilhelm Kuhne, a German physiologist. Thus, after a lot of hard work, the scientists were able to find the missing connection. They were able to demonstrate that it is not life but a large biomolecule made of amino acids, which behaves as a catalyst in the functioning of the cell. These large molecules not only speed up the reactions but also make them happen at a moderate temperature which can support life. This biomolecule takes part in all the formations and breakdown of compounds that follow specific pathways.

The use of enzymes has been evident among the human civilization for centuries. For instance, Egyptians used enzymes to preserve food and beverages. Moreover, the process of cheese making included the use of enzymes from as early as 400 BC. However, the term 'enzyme' was first introduced in 1877 by Wilhelm Friedrich Kuhne who was a professor of physiology at the University of Heidelberg. He was the first person to observe the scientific terminology of the protein molecule (Gurung *et al*., 2013). On the other hand, their catalytic action was first studied by the Swedish chemist Jon Jakob Berzelius in 1835.A good industrial catalyst should be stable under the toughest operating conditions and for long durations (A. Pal and F. Khanum., 2010).

2.2 Structure of an Enzyme

Enzymes are a linear chain of amino acids, which give rise to a three-dimensional structure. The sequence of amino acids specifies the structure, which in turn identifies the catalytic activity of the enzyme. Upon heating, the enzyme's structure denatures, resulting in a loss of enzyme activity, which typically is associated with temperature. Compared to its substrates, enzymes are typically large with varying sizes, ranging from 62 amino acid residues to an average of 2500 residues found in fatty acid synthase. Only a small section of the structure is involved in catalysis and is situated next to the binding sites. The catalytic site and binding site together constitute the enzyme's active site. A small number of ribozymes exist which serve as an RNA-based biological catalyst. It reacts in complex with proteins.

Enzymes are globular folded protein arranged in a three dimensional structure (Mojsov, 2012). Furthermore, enzymes are specific molecules. The specificity arises due to the selective binding site each type of enzyme possesses for its respective substrate. The substrate attaches to the binding site of the enzyme, which is known as the 'active site'. Here, various chemical bonds are broken and formed in the substrate, converting it into products. So, an enzyme is made up of a protein portion called the apo-enzyme attached to the co-factor called the co-enzyme which makes an entire molecule called the holo enzyme. Besides, different enzymes have different optimal conditions of temperature and pH for its maximum level of activity. Holoenzyme attached to apo-enzymes and different types of cofactor (Fruk, L., Kuo, C. H., Torres, E., & Niemeyer, C. M. (2009).

2.2.1 Amylase

Amylase is a digestive enzyme predominantly secreted by the pancreas and salivary glands and is present in other tissues at minimal levels. Amylase was initially described in the early 1800s and is one of the pioneering enzymes to undergo scientific investigation. Although this enzyme was originally termed diastaste, it was later renamed "amylase" in the early 20th century. The enzyme amylase breaks down carbohydrate molecules into smaller products. Their main substrate is starch which is broken down into their smaller components such as dextrin, maltose, maltro triose, and glucose. The enzyme basically hydrolyses the α-1, 4 - glycosidic bonds that hold the glucose units together (Gurung et al., 2013).

2.2.2 Types of Amylase

Amylases are crucial enzymes that have been massively used in industries. Amylases are divided into three types Depending upon found in different organisms, those are alpha amylase,beta-Amylase,gamma amylase, they hydrolyze starch into smaller units such as glucose and dextrins (Dash BK, Rahman MM, 2015:) .

2.2.2.1 Alpha Amylase

α-amylases are found in humans, animals, plants, as well as in microbes. They are calcium metallo enzymes that cleave random α-1,4 glycosidic bonds to yield either maltose and maltotriose from amylose chains or glucose, maltose and dextrin from amylopectin chains.In humans, amylase secreted from the pancreas and salivary gland is α-amylases. (Sharma, H., Singh, J., 2019). Because they can break random bonds in the starch chain, they tend to work

more quickly than the beta-amylases. Their optimum pH is 6.7 – 7.0. They are a member of the glycosidic hydrolase family α- Amylase are enzymes that helps in the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such glucose, maltose, and maltotriose units(R. Gupta,and P. Gigras, 2003).

2.2.2.2 Beta Amylase

The beta-amylases are found in microbes and plants. They hydrolyse the second α-1,4 glycosidic bond in the starch molecule and yield two maltose molecules at a time.. At the time of ripening of fruits, the starch is hydrolysed into maltose which gives a sweet taste to the fruits. Their optimum pH is 4.0 – 5.0. beta-amylases are found in seeds in an inactive form prior to germination.

2.2.2.3 Gamma Amylase

Gamma-amylases are found in plants and animals. They cleave the last α-1,4 glycosidic bond and the α-1,6 glycosidic bond in the starch molecule to yield glucose molecules. Their optimum pH is 3. They are a member of the glycosidic hydrolase family 15.In addation Amylase comes in two different types, endo acting or endo hydrolases and exo-amylase or exohyrolases (Parmer and Panday, 2012)

2.2.3 Structure of Amylase

Amylase, like all human enzymes is a tertiary protein. It has some special structural traits that help it carry out its role effectively. It has a globular (roughly spherical) shape. Tightly folded polypeptide chains cause these globular shapes. This shape allows amylase to form an active site where the substrate molecule can bond.The outside of the amylase enzyme contains hydrophilic (water-loving) groups that make it soluble. This allows amylase to be easily transported around the body.generaly The protein is made up of 496 amino acid arranged in alpha helices and beta sheets. The structure also contains 170 water molecules, one calcium ion and one chloride ion. Three water molecules and one of each amino acids asparagine, aspartate, arginine and histamine are bound to the calcium ion. Calcium is needed for the enzyme to function. Also, two arginines, one asparagine and one water molecule is attached to the chloride ion (Roy *et al*., n,d, para.5).

The main substrate that amylase works on is starch. It is a complex polysaccharide containing α 1, 4-glycosidic bonds between its glucose monomers. This glycosidic bond is stable at high

pH but breaks down at low pH. Starch is a carbohydrate and is a very common and useful source of energy for plants, animals, micro organisms and humans (El-Fallal et al., 2012). Starch is naturally produced mostly by higher plants as a result of photosynthesis, hence it is considered as a renewable raw material (Leszczynski, 2004). During dark periods, starch is stored in the plastids as a source of respiration. Some algae also produce starch called phyto glycogen (El-Fallal *et al*., 2012). Starch has been widely used in industries to provide textural properties to many foods. It has been used as thickeners, colloidal stabilizers, gelling and bulking agents and water retention agents in food and many industrial processes (Mojsov, 2012).

2.2.4 Microbial Amylase

Amylases are extracellular enzymes that hydrolyze starch molecules to give such diverse products as dextrins and progressively smaller polymers composed of glucose units. Amylases are divided into two classes, endo amylases and exo amylases. Endo amylases act randomly on the α linkages. Amylase is a very common enzyme found in nature. They are known to be produced most widely by micro organisms such as bacteria and fungi as compared to plants and animals (Dash *et al*., 2015). The enzymes are produced outside the cell, to carry out extracellular digestion. Among bacteria, the Bacillus spp is well recognized as amylase producers. Some of these include Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus stearothermophilus, Bacillus cereus, Bacillus polymyxa, Bacillus coagulans and Lactobacillus splantarum (Dash *et al*., 2015; Parmer and Pandey, 2012; El-Fallal *et al*., 2012). Other bacteria such as Clostridium thermo sulphuro genes, Proteus and Pseudomonas spp are also acknowledged as amylase producers (El-Fallal *et al*., 2012; Parmer and Pandey, 2012). Bacillus is a common choice of source for industrial amylase production. Different strains of Bacillus have different optimal growth conditions and enzymatic production profile. Additionally, the genus produces amylase that is thermo stable, has retention to extreme pH, osmolality and high pressure (Gurung *et al*., 2013). The α bonds, which are the branching points of amylopectin and glycogen, remain un attacked. This enzymatic action can be characteristically demonstrated by the ability of amylases to reduce rapidly the viscosity of starch solutions, to liberate some reducing groups, and to form dextrins of varying chain length. The α-amylases are one type of endoamylase. The chapter discusses the industrial applications of microbial amylases, the biosynthesis of amylase, and the physical and chemical characteristics.

2.2.5 Application of Microbial Amylase in Industries

Amylase has a wide range of applications in food industries, textile mills and paper industries. In addition, it is used in the production of alcohol, detergents, bread, glucose and fructose syrup, fruit juices including fuel ethanol. Clinical, medical and analytical chemistry also uses bacterial α-amylases (Gurung *et al*., 2013). Starch is an important constituent of the human diet and is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. Starch-converting enzymes are used in the production of maltodextrin, modified starches, or glucose and fructose syrups. A large number of microbial α-amylases has applications in different industrial sectors such as food, textile, paper and detergent industries. The production of α-amylases has generally been carried out using submerged fermentation, but solid state fermentation systems appear as a promising technology. The properties of each α-amylase such as thermostability, pH profile, pH stability, and Ca-independency are important in the development of fermentation process. This review focuses on the production of bacterial and fungal α-amylases, their distribution, structural-functional aspects, physical and chemical parameters, and the use of these enzymes in industrial applications.

Amylases have a wide range of application in various industries such as in the food, bread making, paper industries, textiles, sweeteners, glucose and fructose syrups, fruit juices, detergents, fuel ethanol from starches, alcoholic beverages, digestive aid, and spot remover in dry cleaning. Bacterial α-amylases are also being used in clinical, medicinal, and analytical chemistry (A. Pandey, P. Nigam., 2000).Liquefaction:In this process, insoluble starch granules present in the aqueous solution are dispersed by partial hydrolysis using thermostable amylases. Thermostable α-amylase is employed as a thinning agent to reduce the viscosity and partial hydrolysis of starch . Textile industry: Alpha-amylases are used in the textile industry to hydrolyze and solubilize starch, which is then washed out of the fabric to improve the stiffness of the finished product. Alpha amylase is used as desizing agent to remove the starch from the grey cloth before further its processing like bleaching and dyeing .Bread industry:Enzymes like malt and fungal alphaamylase have been employed in breadmaking.Using the enzymes in the flour improves the bread's flavour, fragrance.Indrakshi Bhattachearjee, (Deepika Mazumdar *et al.*,2019).

3. MATERIALS AND METHODS

3.1 Description of Experimental Site

The present study was conducted at Wolkite University, College of Natural and Computational Science,Biotechnology laboratory, which was found in Gurage zone wolkite town in south-western Ethiopia. It was the administrative center of the Central Ethiopia Regional State and Gurage zone. The town's latitude and longitude of 8°17′N 37°47′E and an elevation is between 1910 and 1935 meters above sea level. Gubrye town was located 164km southwest of Addis Ababa.

3.2. Study Design

The study design was a completely randomized design (CRD) based experimental procedure for isolation, screening for amylase-producing bacteria, and identification and characterization of amylase-producing bacteria. Biochemical characterization techniques were used to determine with a clear zone hydrolysis test amylase.

3.3. Sampling Method

3.3.1 Collection of Samples from Soil and Waste Water

The Soil samples were collected from two different places in the beer by-product from sludge incised at10 cm below in the amount of 15g from Zebidar beer factory around Gubrye, Ethiopia, and the soil samples were transferred in two different sterile plastic and labeled with SS1 for the sample dehydration machine and SS2 for the sample sludge and then transported to biotechnology laboratory and kept at 4^0 C for preservation.

A domestic wastewater sample was collected from two different places in the amount of 200ml from the Zebidar beer factory in Gubrye. The Sample was transferred to two different sterile Falcol test tubes and labeled with SW for the sample pond wastewater and SWF for the sample final effluent wastewater and then transported to the biotechnology laboratory and kept at 4^0C for preservation.

A  B  C  D 

Figure 1: The sample collection process; A= SS1 (dehydration machine), B= SS2 (Sludge), C=SW (Pond wastewater), and D=SWF (Final effluent wastewater).

3.3.2 Preparation of Nutrient Agar

Nutrient agar was prepared in the amount of 6.72 grams of the dehydrated powder or lab-prepared nutrient agar (see the manufacturer's instruction) was added to 240ml of distilled or deionized water in the erlenmeyer flask. The suspensions were heated to boiling to dissolve the medium completely. The dissolved mediums were autoclaved at 15 lbs pressure, 121°C temperature for 15 minutes. Once the autoclaving process was completed the Erlenmeyer flask was taken out and left to cool to a temperature of about 40-45°C. The media was poured into sterile Petri plates under sterile conditions. Bacterial cultures were incubated at 37^{o}C for 24-48hrs and it was preserved in sterile conditions.

3.4 Isolation of Bacterial Cultures

One gram of the soil was dissolved in 10 ml of sterilized distilled water in each of two different test tubes. a serial dilution was made taking 1ml from the dissolved sample and adding 9ml distilled water. 1 ml of the sample was taken and added to the neighboring test tube sequentially in a series of 10^{-1} up to 10^{-6}. After the sequential dilution of bacterial suspended, isolated bacteria from a small bacterial population from 10^{-4} up to 10^{-6} acontained test tubes were pipetted into a sterile petri dish overlaid with 20ml of nutrient agar by spread plate method and then incubated at 37^{o}C for 24-48 hours and then after 24-48 hours bacterial pure colonies weresubcultured. Pure colonies were kept at 4 C for future tests.

1.0ml of waste water sample was mixed with 9.0 ml of sterilized distilled water in the sterile test tube. Serial dilutions were prepared by adding 1 ml of the sample to the neighboring test tube sequentially in a series of 10^{-1} up to 10^{-6}. After the sequential dilution of bacterial suspended, then the bacterial cultures were inoculated in the sterile Petri dish plates under sterile conditions. (Vasekaran s *et al,* 2010). It isolated bacteria from a small bacterial

population from 10-4 up to 10-6 contained test tubes and were pipetted into a sterile petri dish overlaid with 20ml of nutrient agar. Isolation of bacteria was performed by serial dilution and spread plate method and then incubated at 37^{o}C for 24-48 hours. then after 24-48 hours bacterial pure colonies were cultured. Pure colonies were kept at 40 C for future tests.

3.4.1 Screening of Amylase Producing Bacteria

After the incubation time sub-culturing process on nutrient agar by Streak Plate: a sterile loop or inoculating needle was used to transfer a small or individual pure colony of the bacteria from the original culture to the surface of a fresh nutrient agar plate. The bacteria were streaked across the plate in a zig-zag pattern to isolate pure colony bacteria. Then cultures were incubated at 37^{o}C for 24-48hrs (Soumya Vaidya and Pragya Rathore., 2015).

After the pure culture plates were incubated check for amylase production by adding gram's iodine solution with a dropper for 30 seconds on the starch agar plate. The production of amylase was indicated by the clear zone of starch hydrolysis surrounding the colony. the isolated bacteria was screened for amylolytic activity by starch hydrolysis test on a starch nutrient agar plate (M. Al-awsy et al., 2017). The blue color was present around the growth indicating a negative result and a clear zone of hydrolysis around the growth indicating positive result (Dipali Parmar, A. P. 2012).

3.5. Identification of Amylase Producing Bacteria

3.5.1. Morphological Identification

Clean free slides were taken and a loop of the sample was transferred to the microscope slide. Spread the sample to an even-thin film. Air dry the sample, and for staining for 1 minute with crystal violet staining reagent, then the slide was washed in distilled water for 2 seconds, Flood the slide with the mordant (Gram's iodine) waited for 1 minute and the slide was washed in distilled water 2 seconds, Flood slide with decolorizing agent(alcohol) and waited 15 seconds then the slide was washed in distilled water for 2 seconds, finally safranin was flooded and the slide left for 30 to 60 seconds, then the slide was rinsed in distilled water for 2 seconds until no color appears in the effluent and air dried. The slide was then observed under a compound light microscope (Gephardt et al, 1981).

3.5.2. Biochemical Characterization

3.5.2.1. Reaction of the organism in the triple sugar iron (TSI) agar slant

The appropriate amount of TSI Agar powder media was measured at 13 grams and dissolved in 200 ml of distilled water of the required volume in a conical flask. The media was boiled and dissolved entirely in distilled water. Dispensed about 5 to 7 mL of the medium in each test tube and loosely put on the cap or cotton plug the opening. Then the tubes were autoclaved at 121°C and 15 lbs pressure for 15 minutes. They were left to cool and solidify at a slanting position (around 30°) to form an agar slant with a deep butt of about 3 to 5 cm. An 18 to 24 hours old well- isolated colony from a fresh culture of the test bacterium was taken using a sterile inoculating wire. The tube was incubated aerobically (with a loose cap) at 37°C for about 24 hours. It was examined for color change of the slant and butt, recorded, and reported within 24-48 hours of incubation (Wind R. D. et al., 1994).

3.5.2.2. Catalase Test

A small amount of bacterial colony was transferred to the surface of a clean, dry glass slide using a sterile wire. A drop of 30% H2O2 was placed onto the slide and was mixed. A positive result were the rapid evolution of oxygen (within 5-10 seconds), as evidence by bubbles. A negative result were no bubbles or only a few scattered bubble. Dispose of your slide in the biohazard glass disposal container(Jahan et al., 2015).

3.5.2.3 Methyl red and Voges Proskauer Test

The MR and VP agar were formulated in the amount of 3.4 g dissolved in 200ml distilled water, boiled and 7 ml were added to each test tube. The test tubes were autoclaved to sterilize the broth. After that, for each test tube a loop of 24 hours, fresh cultures were inoculated. The test tubes were incubated at 37°C for 24-48 hours (Koneman, E. W.1997).

3.5.2.3.1. voges proskauer (vp) test

After incubating at 37°C for 24-48 hours 6 drops of 5% alpha-naphthol were added and mixed well to aerate. 2 drops of 40% potassium hydroxide were added and mixed well to aerate (Koneman, E. W.2016).

3.5.2.3.2. Methyl Red Test

After 24-48 hours hours of incubation five drops of the methyl red indicator solution were added to the MR-VP medium.Methyl red mediumwas incubated at 37°C for 24-48 hours (Koneman, E. W.2016).

3.5.2.4. Simmon's Citrate Test

The media were prepared using 4.9g of Simmon's Citrate agar by dissolving in 200 ml distilled water and 7 ml medium was added to each test tube. Then each test tubes were autoclaved and then left to cool at a slant position to create a butt and slant. An inoculated needle was used to take a single colony from a 24-hour fresh bacterial culture. The slant of the media was streaked from bottom to top using a zigzag motion with the needle. The test tube was incubated at 37°C for 24-48 hours.

4. RESULTS

4.1. Isolation of Bacterial

A total of 40 bacterial isolates were obtained from four different samples (two different soil samples and two different west water) used for the test as listed in (Table 1). Among the soil

sample one dehydration machine (SS1) isolates 9 colonies and soil sample two of sludge (SS2) were isolated 12 colonies ,8 colonies were isolated from pond west water sample one (SW), 11 colonies from the final effluent wast water sample (SWF) are separately counted. Those isolates were given a name individually for identification in the further characterization tests.

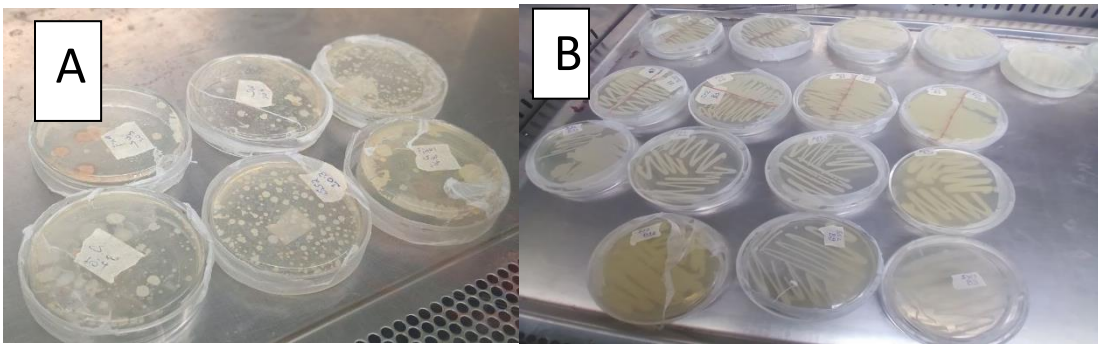


Figure 2: Bacterial growths on the nutrient media; A) primary inoculation B) subculture

Table 1; Different sample sites selected for sample collection to isolate amylase-producing bacteria

NO	Sample site	Number of samples	Colony number	Labeled as
1	dehydration machine for soil	SS1	9	SS1A-SSA9
2	sludge	SS2	12	SS2B-SSB12
3	pond wastewater	SW	8	SWA1-SWA8
4	Final effluent wastewater	SWF	11	SWFB1-SWFB11

4.2 Screening of Amylase Producing Bacteria

Potential amylase isolates were screened by gram's iodine solution on colonies grown on starch nutrient agar plate as shown (below Figure 2). Then added gram's iodine solution clear zone observed around the colonies. Among 40 bacterial isolates were tested.

30 displayed clear zones around their colonies, indicating amylase activity in the hydrolyzed.

while,10 isolates bacteria weren't exhibit clear zones around their colonies, suggesting no amylase activity in those regions



Figure 3: Amylase-producing bacteria

Table 2: Number of bacterial Colony from Starch agar plate

NO	Types of samples	Label	Number of bacterial Colony from Starch plate agar	Amylase-producing bacteria (clear zone)
1	SS1	SS1A1-SS1A9	9	6
2	SS2	SS2B1-SS2B12	12	10
3	SW	SWA1-SWA8	8	5
4	SWF	SWFB1-SWFB11	11	9

4.3 Identification of Amylase-Producing Bacteria

4.3.1 Morphological Identification

The morphological character of the isolates was determined by observing at physical features of the colonies. The bacterial isolates SS1, SS2, and SWF were large colonies and SW were medium-sized colonies. Two bacteria colonies appeared circular in shape (SS2B5) and (SWF1B9). Others were white colored; (SS1A1) and (SW1A6). Both of these colonies were rod-shaped and -whitish-yellow colored.

Table 3: Morphological characterization of amylase-producing bacteria

Isolates	**Colony pigment**	**Shape**	**Size**	**Gram Staing**
SS1A1	White	Rod	Large	Gram-positive
SS2B5	White	Circular	Large	Gram-positive
SW1A6	White	Rod	medium	Gram-positive
SWF1B9	White	Circular	Large	Gram-positive

4.3.2 Biochemical Characterization

4.3.2.1 Starch hydrolysis

As As indicated in Figure 3 below, two of the isolates from the soil sample (SS1A2 and SS2B9) and two of the isolates from the wastewater sample (SWA3 and SWFB6) have shown positive results by clear zone formed for starch hydrolysis test. The other two isolates from the wastewater sample; (SWA1 and SWA3) showed negative tests indicating non-hydrolyzed regions exhibited by blue-black zone around the colonies for starch hydrolysis negative isolates.

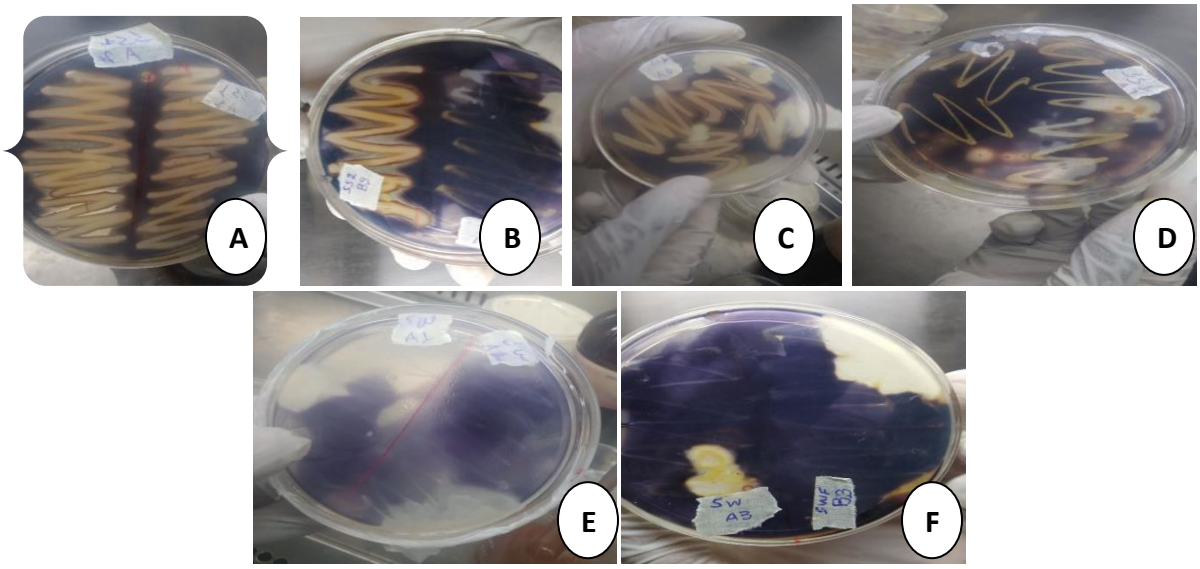
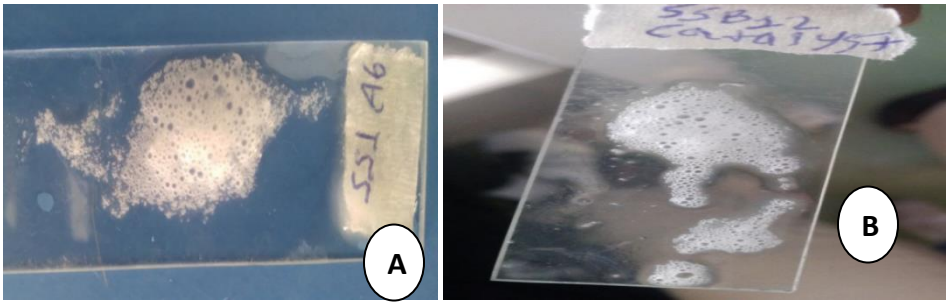


Figure 4: Starch hydrolysis test; indicating that A (SS1A2), B (SS2B9), C (SWA3), D (SWFB6), E (SWA1) and F (SWA3)

4.3.2.2 Catalase Test

Catalase test isolates were produced bubbles. All isolates were confirmed to be catalase-positive results.




C 
D

Figure 5: Catalase test; indicating A (SS1A6), B (SS2B12), C (SWA5) and D (SWFB6)

4.3.2.3. Triple sugar iron (TSI) agar test

TSI agar was used for the presumptive identification based on their fermentation of glucose patterns. This specific result (yellow butt/red slant) on TSI agar suggested that the bacteria fermented only glucose. It served as a crucial indicator for further identification tests to pinpoint the specific bacterial species within the family and Positive ResultAcid production from fermentation turns the medium yellow (glucose fermentation). Gas production may result in cracks or lifting of the agar.Negative Result: No color change or gas production.All test isolates showed results for the triple sugar iron (TSI) test as there were bacteria fermented only glucose there was formation.



Figure 6: TSI agar test: SS2A2, SS1A6, SS2B12, SS2B14, SWA6, SWFB5 and SSWFB7

4.3.2.4 Simmon's Citrate Test

Growth on the slant and change in color to the blue of the medium indicates positive results and color to green of the medium indicates negative results.

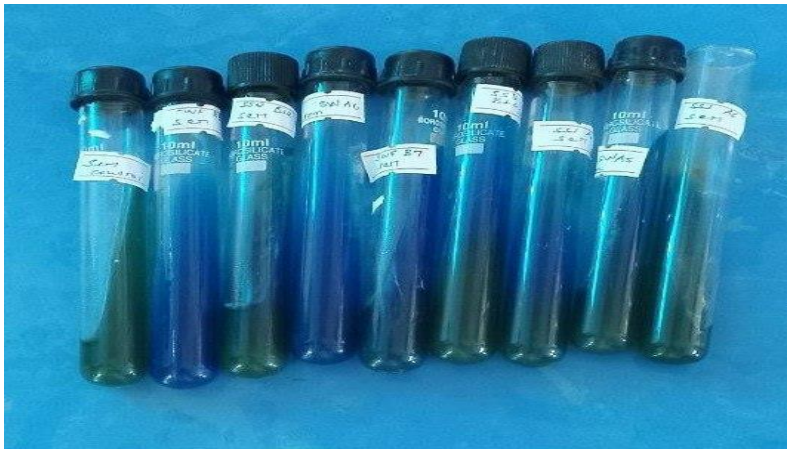


Figure 7; Result of SimmonSimon's citrat test

4.3.2.5 Methyl red (MR) test

The test isolates (SS2A2, SS1A6, SS2B12, SS2B14, SWA6, SWFB5, and SSWFB7) confirmed a positive result, with the color of the culture medium changing from bright yellow to red. while the negative result no color change.



Figure 8; MR test (SS2A2, SS1A6, SS2B12, SS2B14, SWA6, SWFB5 and SSWFB7).

4.3.2.5.2 Vogues proskauer (VP) Test:

The Voges-Proskauer (VP) test was performed on bacterial isolates SS2A2, SS1A6, SS2B12, SS2B14, SWA6, SWFB5, and SSWFB7. A reaction between alpha-naphthol and potassium

hydroxide produced a red color for isolates, confirming a positive VP test.

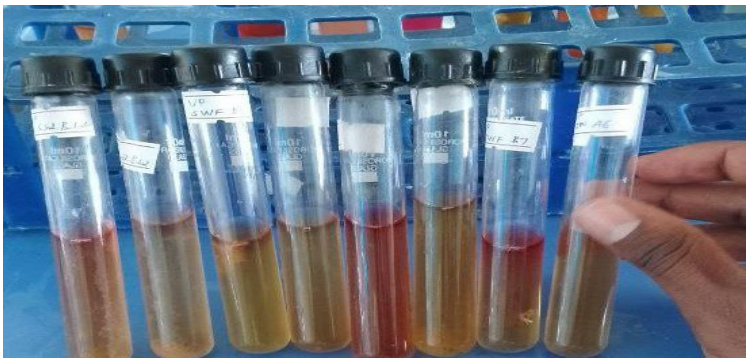


Figure 9; VP test (SS2A2, SS1A6, SS2B12, SS2B14, SWA6, SWFB5 and SSWFB7).

5. DISCUSSIONS

Starch hydrolysis test detects amylase activity,Gram's iodine forms a blue-brown complex with starch. After hydrolysis, a clear zone around bacterial growth indicates starch breakdown by amylase. After the addition of iodine, the clear surrounding the bacterial growth indicates starch hydrolysis and no large clearing around the streaked culture was not indicating starch hydrolysis, based upon the differences in fermentation product starch hydrolysis test detects amylase activity,Gram's iodine forms a blue-brown complex with starch. After hydrolysis, a clear zone around bacterial growth indicates starch breakdown by amylase. After the addition of iodine, the clearing surrounding the bacterial growth indicates starch hydrolysis and no large clearing around the streaked culture was not indicating starch hydrolysis, based upon the differences in fermentation products according to (Talaiekhozani,A.2013).

To identify and classify amylase-producing bacteria based on their morphology, several characteristics are considered.Microscopic examination revealed that the isolated bacteria were Gram-positive. Interestingly, their morphology was varied, with some isolates appearing spherical and rod-shaped . Gram staining was a widely used technique to differentiate bacteria based on the structure of their cell walls. Bacteria can be classified as Gram-positive (retain the crystal violet dye) or Gram-negative (do not retain the dye). This staining helps in the initial characterization of amylase-producing bacteria according to (Talaiekhozani,A.2013

The Triple Sugar Iron (TSI) agar slant was a medium used to differentiate and identify bacteria based on their ability to ferment sugars. The TSI agar contains three sugars: glucose, lactose, and sucrose, as well as the pH indicator phenol red.Amylase was an enzyme that breaks down starch into simpler sugars. While TSI agar does not directly test for amylase production the Voges-Proskauer (VP) test identifies the production of neutral end products like acetoin and butanediol associated with the butanediol fermentation pathway according to(Wind R. D. *et al*., 1994).

Our study found that all isolates exhibited positive catalase activity. When hydrogen peroxide (H_2O_2) was applied to the cultures, gas bubbles were observed. This suggests that the amylase-producing isolates also produced catalase. Catalase breaks down H_2O_2 into oxygen and water, leading to the observed foam formation according to (Jahan et al., 2015).

The Voges Proskauer test was used to detect the presence of acetoin, a metabolic byproduct of glucose fermentation, in a bacterial culture.In the context of amylase-producing bacteria.Voges Proskauer test was used to indirectly assess the activity of amylase-producing bacteria the Voges Proskauer (VP) test identifies the production of neutral end products like acetoin and butanediol associated with the butanediol fermentation pathway,while the Methyl Red (MR) test detects the production of a variety of acidic end products during mixed-acid fermentation according to (Koneman, E. W.2016).

This study was performed that isolated amylase-producing bacteria exhibited a positive simmon citrate test. These positive isolates displayed a blue color change. This color change indicates the bacteria's ability to utilize simmon’s citrate as an energy source.These bacteria likely convert citrate into oxaloacetate, which was further broken down into CO2 and pyruvate according to(S. Sahoo, S. Roy, and S. Maiti,2016).

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

From this study difference biochemical test which are important for the isolation of amylase producing bacteria were performed. Biochemical methods like: Triple Sugar Iron (Tsi) Agar, Simmon's Citrate. The bacterias were identified as amylase producing and identified based on color and shape. Gram-positive bacteria was preformed purple or blue in color, while gram-negative bacteria was either pink or red in color was spherical. methyl red, Vogues proskauer (VP) Test and catalase were to characterize the isolates. The study revealed that the amylase produced from microorganism was capable for the industrial use. Hence, the present study showed that organisms with potential to produce amylase producing bacteria can be obtained from soil and waste water effluent can be considered as the worth source for amylase producing bacteria.

6.2. Recommendations

Depend Depending on the results obtained from this study the following recommendations should be considered for future studies:

- This study showed some morphological and biochemical characterizations could be helpful and identifying amylase-producing bacteria. Therefore, further optimization biochemical tests would be beneficiary.
- DNA-based molecular characterization should be performed for detail analysis of the bacteria isolates.

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