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ASSAYING OF ALPHA AMYLASE EXTRACTED FROM MESOPHILIC BACTERIA FROM BISHOFTU LAKES FOR DETERGENT APPLICATIONS

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Declaration

I, Misgana Anbessa, declare that this research paper, prepared for the Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Microbiology at the School of Graduate Studies of Addis Ababa University, is the result of my own efforts. The topic of my research is "Isolation and Characterization of Alpha-Amylase Producing Bacteria Isolated from Bishoftu Lakes, Ethiopia". This research project has completed under the supervision and guidance of my research advisors: Dr. Fitsum Tigu and Dr. Asnake Desalegn.

By Misgana Anbessa_____

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List of abbreviation and acronym

AI- Amylolytic index

ANOVA- Analysis of variance

APS- Ammonium persulfate

DNSA- 3, 5-dinitrosalicyclic acid

DP- Degree of polymerization

EC- Enzyme commission

LSF-Liquid state fermentation

MRVP- Methyl red Voges- Proskauer

OFAT- One factor at a time

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SMF- Submerged state fermentation

SSF- Solid state fermentation

TEMED- Tetramethylethylenediamine

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Abstract

Alpha amylase is a biological catalyst which breakdown large molecules of starch to smaller unit. It has many functions in industries like food, fermentation, detergent, textile, and pharmaceuticals. This study aimed to isolate, characterize, and evaluate alpha-amylase-producing mesophilic bacteria from Bishoftu lakes for potential application in detergent formulation. The samples were isolated using serial dilution and nutrient agar media. Hydrolysis method was employed to screen all isolates for their potential of amylase production by starch plate agar method. Overall, 110 isolates were tested as positive for amylase activity among 169 isolates. Based on amylolytic index (AI), 60 isolates were used for submerged state fermentation and 9 most potent amylase producing isolates were subjected for further analysis. The impact of temperature, pH, incubation time, carbon source, organic and inorganic nitrogen sources, metal ions, inoculum size, and various concentrations of starch, and sodium chloride on the isolates were performed. The maximum alpha-amylase production of all the 9 isolates was observed at 40ºC in 24 h of incubation time, pH 7, glucose, tryptone, beef extract, ammonium chloride, zinc sulphate, potassium phosphate, 1.5% sodium chloride concentration using basal media. The biochemical tests like methyl red, Voges Proskaur, citrate, urease and catalase tests were conducted. A total of 9 isolates were identified. 7 isolates were grouped as *B. cereus* and the rest as, *A. rivipollensis* and *K. pasteurii* based on 16S rRNA, gyrB and elongation factor Tu (*tuf*) gene sequencing. *A. rivipollensis* (G2W1) recorded 4.5 ± 0.1 U/mL after applying all optimum growth conditions, which was the highest one. The second was *K. pasteurii* (G3W1) (4.3 ± 0.4 U/mL) and the third was *B. cereus* (B1W1) (4.1 ± 0.1 U/mL). The optimum conditions for the crude enzyme activity were 45ºC, pH 7, 15 min of reaction time, Ca^{2+}, and in which 5 ± 0.04 U/mL of enzyme activity was recorded by *A. rivipollensis*. Ca^{2+} was the best enhancer among metal ion as cofactors. Partially purified enzyme has a molecular weight of 50 kDa, with specific activity of 10.1 U/mg. The effectiveness of alpha amylase was checked by preparing a detergent and the soap made by this enzyme has successfully cleaned the pieces of cloth that were purposively contaminated by potato waste.

Keywords: Amylase; Bacteria; Bishoftu lakes; Enzyme activity; Optimization

1. Introduction

1.1. Background of the study

More than half of the carbohydrates that humans consume is come from starch, which is the most prevalent source in our diet (Doublier et al., 2007). It occurs in plants as granules, which are most prominent in seeds (especially cereal grains) and tubers, where they serve as a form of storage for carbohydrates (Bertoft, 2007). It is made up of several glucose units connected by glycosidic linkages (Benalaya et al., 2024). The glucose molecules are joined to form a larger molecule known as starch (Bojarczuk et al., 2022).

There are two types molecules in starch: amylopectin, which is branching, and amylose, which is linear (Schirmer et al., 2013). A linear starch polymer, amylose is made up of 1-4 glycosidic linkages and up to 6000 glucose units. It consists of alpha-D-glucose molecules linked by α -1, 4- glycosidic link to form covalent bonds (Doublier et al., 2007). Amylose and amylopectin proportions are influenced by the starch supply, typically, 20–30% amylose is present in most common starches, such as barley, corn, and potatoes (Forssell et al., 2002). Normally, at room temperature, this polymer is insoluble in water. At temperatures higher than 130ºC, it is reported to dissolve in water (Doublier et al., 2007).

Amylopectin is made up of short linear chains with α-1,4 links that contain 10–60 glucose units and side chains with α-1,6 links that have about 1545 glucose units. In average, branch points in amylopectin is 5% ,but varies depending on its origin (Thompson, 2000). The entire amylopectin molecule is one of the biggest molecules in nature, with about 2,000,000 glucose units. It is best explained by the cluster model, which states that the side chains are grouped in orderly clusters on the longer backbone chains (Bertoft, 2007). According to the most widely accepted explanation, amylopectin has a structural element known as a cluster, wherein nearby side chains with varying degrees of polymerization form double helices in the area of their non-branched segments (Nakamura & Kainuma, 2022).

The starch breakdown process contains liquefaction and saccharification. Saccharification is the process by which soluble starch is transformed into glucose units, whereas liquefaction is the process by which starch is transformed into its soluble state and, liquefaction is called hydrolysis

which takes place by alpha-amylase enzyme (De Souza & de Oliveira Magalhães, 2010). Amylase breaks down the starch molecules into glucose unit-based polymers. It can be used in many different industrial activities, including food, fermentation, textiles, and pharmaceuticals (Bajaj, 2019). The most known amylases are classified as alpha, beta, and gamma amylases. Among them, alpha-amylase is the most common enzyme that is used in wide form of industrial application (Souza, 2010).

Even though plants and animals can produce this amylase, the enzyme produced from microorganisms is more advantageous (Burhan et al., 2003). Enzymes from these microorganism are of special interest since they are not usually denatured by high temperature and active at elevated temperatures (Kambourova, 2017). The majority of starch processing industries are function at high temperatures, but when the temperature, pH, and other factors change in an industrial setting, the enzymes derived from microorganisms that can only survive in mild environments are readily denatured (Gebreselema Gebreyohannes, 2015).

Ethiopia has distinct geo-climatic zones that host a rich fauna, flora, and microbial diversity. It is known for having many microbial diversities (Gizaw, 2018). It is expected to get novel bacteria, which have capable of producing amylase with potential application for starch hydrolysis. Although alpha-amylase enzymes are widely studied and utilized globally, there is some research in Ethiopia on isolating and characterizing alpha-amylase-producing microorganisms for industrial applications. Furthermore, it needs to search mesophilic bacteria capable of producing stable and cost-effective amylase suitable for high-temperature industrial processes.

1.2. Statement of problem

Starch is one of the most important substrates that is used in many industrial applications. The efficient breakdown of this starch into its smaller and more usable molecules requires the action of amylase. For industrial applications, it needs a much more stable and cost-effective amylase (Santana & Meireles, 2014). Amylases from microorganisms are the most important even though plants and animals can produce them. Despite the extraction of alpha amylase-producing microbe by many researchers all over the world, it need to study more effectively utilized in practical

applications in Ethiopia (Guta et al., 2024). Therefore, it is important to identify and characterize amylase-producing bacteria to overcome the challenges faced in starch breakdown processes and explore their potential for industrial applications. Bishoftu lakes were selected as a sample collection site to achieve this goal in this study.

1.3. Objectives of Study

1.3.1. General objective

To isolate, characterize, and evaluate alpha-amylase-producing mesophilic bacteria from Bishoftu lakes for potential application in detergent formulation.

1.3.2. Specific objectives

- ❖ To isolate, screen and characterize alpha-amylase-producing bacteria.
- ❖ To optimize alpha amylase production by testing one factor at a time (OFAT) approach.
- ❖ To produce alpha amylase by submerged state fermentation and determine their activity.
- ❖ To partially purify the enzyme and evaluate the specific activity.
- ❖ To check the practical applications of enzyme in detergent industry.

1.4. Scope of the study

To collect and isolate alpha-amylase-producing bacteria from the five selected Bishoftu lakes (out of seven) due to the availability of suitable environmental conditions. Collected samples were conducted to isolation and identification processes to identify potential amylase-producing bacteria. Characterized bacteria was used to assess their enzymatic properties, optimal temperature, temperature stability, pH and substrate specificity. The cell also checked by one factor at a time with different source of nutrients. The enzymatic properties of amylase produced bacteria were determined through various biochemical assays and analysis methods. Partial purification and application of isolated alpha amylase also conducted.

2. Literature reviews

2.1. History of enzyme

Biochemical and biological reactions are catalyzed by biological compound called enzymes. They are doing as accelerating the time of reaction in and out of cell of organisms. Therefore, the term biocatalyst was coined to define an enzyme (Frey & Hegeman, 2007). Life would be difficult to continue without enzymes because of the multitude of biological reactions need for organisms to function fast and effectively. Because of these, enzymes are very important and need for the life of all organisms (Islam, 2016b). Throughout the ages, human civilization has made use of enzymes. For instance, the Egyptians preserved food and beverages by using enzymes. Furthermore, enzymes were used in the production of cheese as early as 400 BC. However, Wilhelm Friedrich Kuhne, He coined the term "enzyme" in 1877 BC while teaching physiology at the University of Heidelberg. The scientific nomenclature of protein molecule was first observed by Kuhne (Welcome & Welcome, 2018). In other way, the Swedish chemist Jon Jacob Berzelius first studied the effect of its catalytic way in 1835 BC. On the other hand, James first isolated an enzyme in pure form in 1926 BC, which earned him the Nobel Prize in 1947, Worthington Biochemical Corporation (Gurung et al., 2013).

2.2. History of amylase

Kirchhoff et al., 1811 discovered the first starch-degrading enzyme in wheat, laying the foundation for the later identification and investigation of amylase. Kuhn et al., 1925 named alpha-amylases due to the hydrolysis products' alpha structure. Ohlsson found another amylase in 1930 that produced β-mannose (monosaccharide sugar that is an isomer of glucose and is involved in glycosylation). He called it β-amylase (S. Kumar & Chakravarty, 2018). Alpha amylases' crystal structure was determined with a 3Å resolution structure, which was then refined to a 1.5 Å resolution. Each form's three-dimensional crystal structure was discovered to be almost identical in the 1990s (Tiwari et al., 2015). In 1894, a fungal-derived enzyme called amylase was the first to be synthesized industrially and utilized as a medication to treat digestive disorder. Biodin and Effront, 1911 were the first to use *B. subtilis and B. mesentericus* for the

production of alpha-amylase on a commercial scale using large fermenters and LSF (Liquid State Fermentation) (Singh et al., 2011).

Amylases (a term used to refer to alpha-amylase, beta-amylase, and glucoamylase) are among the most important enzymes in biotechnology today. Amylases can come from a variety of sources. Many different living things, including bacteria, yeast, and fungi as well as plants and human, produce raw starch-digesting amylases (Shinke et al., 1973). Among the enzymes, alpha amylase plays an important catalytic role in breaking down starch smaller unit to glucose. Nowadays, industries use microorganism-derived enzymes due to their biochemical versatility, increased production rate, stability, and readily available nature (Islam, 2016a). The isolation of microorganisms from water and soil are importance in both biotechnology and microbiology (Abdullah et al., 2014).

2.3. Classification of amylase

2.3.1. Alpha-amylase

Alpha amylases (E.C. 3.2.1.1.) are among the most significant industrial enzymes, with various uses in the reduction of complicated starch to its simpler form. It breaks down starch by hydrolyzing internally α-1,4-O-glycosidic bonds in polysaccharides and produce α-anomeric in smaller unit such as maltose, maltotriose and glucose. However, terminal glucose residues and α -1,6-linkages cannot be cleaved by alpha-amylase (Tiwari et al., 2015). It has several applications across many industries including food, textiles, paper, and detergent, accounting for around 30% of global enzyme output (Mobini-Dehkordi & Javan, 2012). The method of hydrolysis has disadvantages since the operating conditions are highly acidic and at high temperatures. These limitation are removed by hydrolyzing starch using an enzyme to produce high fructose syrup (Souza, 2010).

Kuhn called the α-amylases in 1925 based on the alpha conformation of the hydrolysis products. The majority of alpha-amylases are metalloenzymes with a strong Ca^{2+} affinity. The structure of the majority of alpha-amylases contains at least one calcium ion, which is necessary for both their thermal stability and activity (R. K. Sani & Krishnaraj, 2017). Research suggested that the

optimum pH for alpha amylases enzyme is typically around 7, and the optimum temperature is 37ºC. However, the exact pH and temperature optima can vary depending on the specific source or origin (Janecek et al., 1997).

2.3.2. Beta amylase

Beta amylase (EC 3.2.1.2) is an exoenzyme that breaks down maltose from amylose and amylopectin's non-reducing ends. It hydrolyzes (1, 4)-α-D-glucosidic bonds in large sugar to remove maltose units from non-reducing ends of chains. It is a critical enzyme in the synthesis of maltose, which yeast uses during fermentation (Rejzek et al., 2011). Ohlsson discovered β-amylase in 1930, which produces β-mannose. This enzyme uses an inversion to convert starch, glycogen, and similar polysaccharides and oligosaccharides into beta-maltose. Bacteria, fungi, and plants all have beta-amylase; however, bacteria and cereals are the most heat stable. Two glucose units (maltose) are removed at a time when beta amylase hydrolyzes the second α-1,4 glycosidic bond from the non-reducing end. Starch in fruit during ripening is breaking by beta amylase into maltose which giving its sweet flavor (Horváthová et al., 2000).

Many microorganisms also generate amylase, which degrades extracellular starches. Normally, it is not present in animal tissues, but may be found in digestive system bacteria. The optimal pH range for β-amylase is 4.0-5.5. It found under glyosidic hydrolysis family 14 (Balakrishnan et al., 2019). Beta-amylase can be used for various function in study and industry. It can be applied to structural analyses of variously produced starch and glycogen molecules. It is conducted in the beer and distill industries for fermentation (Quillaguaman et al., 2005).

2.3.3. Gamma amylase

Gamma Amylases (EC 3.2.1.3) break α (1-6) glycosidic linkage, containing the non-reducing end of amylose and amylopectin, which produces glucose in contrast to the other forms of amylase. Its ideal pH is 3, and it works best in acidic environment. It is an exonuclease that individually removes glucose residues from starch molecules by cleaving α (1→4) and α (1→6) glycosidic bonds off the non-reducing end. Like beta amylase, hydrolysis results in a free hemiacetal hydroxyl group that releases beta glucose by translocation. It found in plant and animals ,and some microorganisms (Williams, 2019). They are classified into several GH

families, including glycoside hydrolase family 15 in fungi, glycoside hydrolase family 31 in human, and glycoside hydrolase family 97 in bacterial forms (Janeček & Blesák, 2011).

2.4. Microbial diversity in hot spring environments

Ground water is heated by flow of geothermal force from Earth crust and form hot spring. Geothermal systems are not always connected to volcanoes and can be found in a range of geological environments (Mehta & Satyanarayana, 2013). This hot spring water is heated by geothermal that has no relation volcano environment or by directly contact with lava of volcanic. Environment in non-volcanic area, the ground water that stored deeper into the Earth crust contact with rocks which over heat because geothermal slope whereas volcanic environment that are crustal movement actively area, the significant temperature slope near magma may lead water to become overheated (Zhou et al., 2023). The high pressure may be produced, forcing the water to the surface of Earth via cracks in the crust, where it can emerge as a hot spring or geyser. The heated fluid rises by the Earth's surface; minerals dissolve from the surrounding areas and form high mineralization. Hot spring fluids typically include high quantities of different elements and can be supersaturated with a various of minerals (Pirajno, 2020).

The component of the hot water, which can be extremely acid or alkaline condition, is influenced by the chemical interactions between the rocks and the minerals that create rocks with ascending direction. Its temperature ranges from boiling or superheating at the source to large near the shore (Bucher et al., 2017). Hot springs are an important of physiologically significant compounds because of their diversity in microorganisms. It provides unique natural habitats for thermophilic bacteria. Scholars gathered water and soil samples from a range of hot springs to examine the potential industrial applications of these microorganisms (Soy et al., 2023). Only microbes can grow and survive at both ends of the temperature range that is suitable for living things. A diverse of bacteria survive and thrive at such high temperatures. These bacteria have evolved a variety of structural and chemical adaptations that enable them to survive and grow at high temperatures (Price & Sowers, 2004).

2.5. Ethiopian Rift Valley lakes

Rift Valley of Ethiopian is part of the large East African Rift that is taken as one of the greatest and extended, among rift systems on earth. It is about 6400 Km and 48-64 Km wide and striking geological structure that stretches from Middle East south of Jordan River to Mozambique in southeast Africa (Kaleme, 2011). The Ethiopian Rift Valley offers a singular chance to investigate the shift between new seafloor spreading northward in Afar and continental rifting southward in Kenya and the Gulf of Aden (Mackenzie et al., 2005). The Rift Valley floor is surrounded by magnificent volcanic centers (Ayenew & GebreEgziabher, 2015). Bishoftu crater lakes are found in these Rift Valley lakes. Many researchers studied the diversity of microorganisms in Bishoftu's lakes and areas very well. However, microorganisms that are amylase producing have not been studied well at this study area. Therefore, it needs to isolate, screen, and study amylase-producing microbes in this specific place.

2.6. Industrial application of amylases

Amylase is one of the crucial enzymes in industrial settings. The starch molecules are hydrolyzed by these enzymes into simple sugars like glucose units. Food, detergent, biofuel, agriculture, textile and pharmaceutical industries are some of the industries in which amylases used (Souza, 2010). Enzyme production is done by submerged state fermentation (SMF) or by solid state fermentation (SSF). In Solid-state fermentation, microorganisms are fermented in the absence of free water while SMF is cultivation of microorganisms in free flow water in submerged form. In common substrate like potato peel, corn bran, wheat bran, rice bran, tea waste are used. These substrates are chosen based cost effective and availability for solid-state fermentation (Suganthi et al., 2011). For submerged state fermentation, free oxygen and water is available and it is more moisture when compared to solid-state fermentation. The moisture is impact on enzyme production and microbial growth by changing physicochemical characters of substrate porosity for different diffusion of gas (Vijayabaskar et al., 2012). The process of submerged fermentation is different from solid-state fermentation because producing enzymes within a liquid nutritional medium (has free water) (Suganthi et al., 2011).

2.6.1. Food industry

The employment of microorganisms, including bacteria, yeast, and fungi are common in food making to increase flavor and texture. Additionally, these microorganisms give important economic advantages to various industries. Microbial enzymes are the source of choice more than both plants and animals because of easy to cultivate, economical, and availability and synthesis (Raveendran et al., 2018). To enhance the flavor and quality of bread, alpha-amylase hydrolyzes the starch in flour to produce fermentable sugars that yeast will ferment during bread making. Additionally, when it is used to make bread, it has some anti-staling properties. It can be used as a clarifying agent to enhance clarity while making beer (Okpara, 2022).

Alpha amylase provide break down the starch in wheat flour into small dextrins during dough fermentation, the initial stages of baking, enabling yeast to continue active. Better crumb texture and bread volume are the results of it. Furthermore, these enzymes produce small oligosaccharides and sugars, like glucose and maltose, intensifies the Maillard reactions that give baked goods their distinctive browning and flavor (Lundkvist & Olsen, 2007). In order to improve the digestibility of animal feed, alpha-amylase is used in the pre-treatment phase of processing. To improve juice extraction, clarity, and production, the fruit juice business uses alpha-amylase to hydrolyze the polysaccharides in raw juice (Al-Maqtari et al., 2019).

2.6.2. Textile industry

It is a major industry that take part in the prevention the environmental pollution due to dye, bleaching chemicals, and fabric desizing. Starch paste is used in textile weaving to prevent warping. This strengthens textiles when they are woven. Additionally, because the laid-down wrap gives the surface of the string a softer quality, it reduces the likelihood of string loss from friction, cutting, and make elastic. The cloth is woven, the starch is taken out, and then it is scourged and dyed (Souza, 2010). Enzymes are used in these sectors to facilitate the development of ecologically friendly fiber processing methods. Amylase, cellulase, cutinase, protease, pectinase, and lipase are among the hydrolase group of enzymes in textile industry (Mojsov et al., 2018).

The starch molecules are hydrolyzed by these enzymes into tiny pieces that are easily removed by washing or dissolved in hot water (Kozłowski & Różańska, 2020). Before the grey cloth is

processed further for bleaching and dying, starch is removed using alpha-amylase as a desizing agent. After mashing, many clothes, particularly the ubiquitous jeans, get smaller. Finally, the desired textiles are cleaned and laundered (Saravanan et al., 2012). Alpha-Amylase is used to randomly split starch particles into components that are soluble in water and may be washed off (Gupta et al., 2003). The alpha-amylases do not damage the fibers; instead, they reduce size selectively. Before the discovery of amylases, de-sizing was accomplished by subjecting the fabric to high temperatures and chemical treatments such as acid, alkali, or oxidizing agents. These methods were ineffective at eliminating starch and instead caused the cotton fiber to deteriorate (Gupta et al., 2003).

2.6.3. Biofuel industry

Generation of biofuels in comparison to their advantages, fossil fuels are a major source of pollution. Because fossil fuels are expensive and cause environmental problems, biofuels have lot of attention in recent decades. The principal type of biofuel is ethanol. Biodegradable materials, such crop residue and agricultural byproducts, can be converted into ethanol. The production of fermentable sugars, which in turn produces ethanol, depends on enzymes like alpha amylase and others (Gopalan & Nampoothiri, 2016).

Biomass-based fuels are crucial for reducing our over-reliance on petroleum. Additionally, they reduce greenhouse gas emissions. In the process of producing biofuel, pretreatment is a crucial stage. In addition to being expensive in and of itself, pretreatment significantly affects the price of almost every subsequent processing step (V. Kumar et al., 2016). The crucial substrates in the synthesis of biofuel are starchy and lignocellulosic materials. Globally, alcohol is the most frequently utilized biofuel product. To produce fermentable raw materials for the manufacturing of ethanol, starchy substances such as grains, potatoes, and bagasse are processed with alpha-amylase (Escaramboni et al., 2018). The melting of the starch components creates a viscous suspension solution. Alpha-amylase hydrolyzes and saccharifies the liquid starch molecules into sugar that can be fermented. To produce ethanol, yeast and bacteria uses the fermentable sugar as a substrate (Fabiyi, 2018).

2.6.4. Detergent industry

Alpha-amylase is a crucial ingredient in the laundry and stain remover detergent's formulation (Farooq et al., 2021). The ideal substitute for chemical detergents when removing starchy, fatty, and proteinaceous stains is an enzyme-based detergent. Termamyl, also known as *B. licheniformis*, contains alpha-amylase, which can be used to remove stains from cotton garments and eliminate starchy foods by adding it to detergent. Moreover, the accumulation of waste-water pollution and eutrophication problems in a river may be washed by using enzymatic detergent, which easily decomposes in water (Ahmad et al., 2019). The detergent formulation uses alpha-amylase derived from *Bacillus* or *Aspergillus* species, which exhibits alkaline pH, lower temperature, and oxidative conditions of operation. These species produce alpha-amylase that is mildly acidic and safe for the environment. Utilizing recombinant DNA technology, the drawbacks of alpha amylase's oxidative sensitivity and calcium dependence could be reduced. Researchers worldwide are working to create alpha-amylase that is more resistant to oxidative substances (George & Georrge, 2018).

2.6.5. Agricultural industry

Alpha amylase is speeding up the braking down of the complicated starch that is found in carbohydrates into simpler sugars. In the market of cereals, the alpha amylase is used to hydrolyze starch in crops like corn, wheat, and rice, aiding the extraction of fermentable sugars, which in turn are consumed for the production of alcohol, sweeteners, and other usable items (Sanjaya et al., 2024). Apart from the cereal processing industry, the use of alpha-amylase also exists in the animal feed market. When enzyme products are added to fodder for cattle and poultry, they can make the feed starch more nutritious and more digestible for the livestock. This not only finesses the overall efficiency of animal farms but also is a way of utilizing the agricultural sector effectively because of diminished feed supply and starch-based resource optimization, which altogether boosts the durability of the sector’s sustainability (Bedford, 2018).

2.6.6. Pharmaceutical industry

Alpha amylase hold a significant position in the pharmaceutical industry, especially in digestive health products and the production of valuable sugars. Alpha amylase with the help of high-quality

digestive tonics and medications has been utilized for the hydrolysis of complex carbohydrates in the gastrointestinal tract (Zohra et al., 2016). The biochemical process of starch hydrolysis performed by these enzymes helps to increase the nutritional values by providing a better way to absorb and utilize nutrients, thus these enzymes are indispensable ingredients in the formulation of drugs aiming to cure various digestive disorders (Islam, 2016a). In addition, the enzyme's functionality of hydrolysis starch seems to be a useful application to the pharmaceutical industry. Its ability to break down complex carbohydrates such as starch into simpler sugars to sucrose and maltose is a great asset for pharmaceuticals. These substances are commonly used as sweeteners, fermentation (Souza, 2010).

3. Materials and method

3.1. Description of the Study area

The study area for this research comprises five lakes located in the Bishoftu, Ethiopia, specifically Hora, Babogaya, Kuriftu, Green, and Cheleleka lakes. Bishoftu is located approximately 47 Km from Addis Ababa, Ethiopia. The city has an altitude of 1900 m above sea level. There are seven known lakes in Bishoftu and its surrounding areas. Among the seven known lakes in Bishoftu and its surroundings, the above lakes were selected based on their accessibility for sample collection. The locations of each sampling points were recorded using the GPS Status application. **Hora** (8°45.9870′N, 38°59.6180′E; 8°45.9770′N, 38°59.6870′E; 8°45.9000’N, 38°59.7990’E). **Babogaya** (8°46.9980’N, 38°59.5150’E; 8°46.9580’N, 38°59.5440′E; 8°46.9180′N, 38°59.5730′E). **Kuriftu** (8°44.8980'N, 38°58.9200'E; 8°44.9410′N, 38°58.8910′E; 8°44.9800'N, 38°58.8590′E). **Green** (8°42.0600'N, 38°58.4700'E; 8°42.0890'N, 38°58.4960'E; 8°42.1390'N, 38°58.5250'E) and **Cheleleka (**8°45.7380′N, 38°58.3430′E; 8°45.7790′N, 38°58.3660′E; 8°45.8210′N, 38°58.4130′E).

The physicochemical characteristics including temperature, pH and salinity of each lake were also measured. The temperature was 28 ºC (Hora and Babogaya), 30 ºC (Kuriftu), 31 ºC (Cheleleka) and 35 ºC (Green) lakes. The pH levels 7.3 (Hora) 7.2 (Babogaya), 6.8 (Kuriftu), 7.9 (Green), and 6.9 Cheleleka. Salinity levels also exhibited noticeable differences: 10.8 ppt (Hora), 7.5 ppt (Babogaya), 8.7 ppt (Kuriftu), 14.1 ppt (Green), and 3.9 ppt (Cheleleka).

3.2. Sample collection and processing

Samples were collected in two rounds. During the first round of sample collection on January 13, 2024, a total of 12 samples, including 6 water samples and 6 soil samples, were collected from Babogaya and Hora lakes. In the second round, conducted on January 27, 2024, 18 samples were collected, comprising 9 water samples and 9 soil samples, from Kuriftu, Cheleleka, and Green lakes. Sampling was collected at 4 m to the shore with three water and soil samples taken from each lake at intervals of 150 m to ensure spatial representation and minimize potential bias in the data by purposive sampling method. The water samples were collected to autoclaved bottles, while soil samples were to sterilized plastic bugs. A total of 30 samples (15 soil and 15 water), 6 samples from each lake, were collected for this research study. The decision to collect six samples from

each lake (three water samples and three soil samples) was based on the need for balanced representation of both water and soil environments, allowing for a meaningful comparison of microbial diversity between the two types of samples.

The code was given for all lakes based on their first letter. Hence, Hora (H), Babogaya (B) Kuriftu (K) Cheleleka (C) and Green (G). The water samples were coded by W while the soil samples were coded by S. The first water sample was coded as 1W, the second was coded as 2W, and the third was coded as 3W while the first soil sample was coded as 1S, the second was coded as 2S, and the third was coded as 3S by following the first letter of the lake's name. In addition to these, amylolytic of the positive isolates were also ranked as 1 and 2 which attached to sample code at final. After collection, the samples were carefully transported to laboratory and kept in refrigerator at 4^{o}C for further analysis.

3.3. Isolation of bacteria

Initially, 25 mL water sample was mixed with 225 mL distilled water to make the first 10^{-1} dilution, then serial dilution was done from 10^{-1} to 10^{-4}. 0.1mL aliquots transferred onto nutrient agar plates and incubated at 37ºC for 24 h while 25 g sample soil was blended with 225 mL of distilled water and serial dilution was carried out from 10^{-1} to 10^{-7}. 0.1mL aliquots was transferred to agar plates and incubated at 37ºC for 24 h (Albejo & Hamza, 2017). The study area's temperatures ranged from 28ºC to 35ºC. These temperatures are within the range that supports the growth of mesophilic bacteria, which thrive in moderate temperatures typically between 20ºC and 40ºC (Mishra & Behera, 2008). After 24 h of incubation, the colonies on the agar plates were counted separately for both water and soil samples. The plates with 30 to 300 colonies had selected for counting and selection of microbes for the next experiment. Morphologically distinct colonies were aseptically transferred into broth for further purification. Streak plating was performed to obtain a pure colony. The purified colonies were preserved at -80 ºC with 17% glycerol solution for the next experiment (R. K. Sani & Krishnaraj, 2017).

3.4. Screening for amylase production

Each distinct pure colonies was individually grown on 1% of starch media at 37ºC for 24 h. After incubation, 1% of iodine solution was prepared and flooded on grown colony on starch agar plates. When iodine solution was flooded, the isolate had a clear zone, indicating that it was capable of producing amylase enzymes. The principle is the formation of blueback when iodine reacts with starch. This is iodine test. If no formation of clear zone, it would take as negative result for amylase production. The amylolytic index (AI) (a measurement used to assess the amylase activity of microbial isolates, which indicates the ability of microorganisms to degrade starch) was used to assess the amylolytic microbiological isolates using the following formula:

$$AI = \frac{\text{Clear zone diameter(mm)}}{\text{Colony diameter}}$$

3.5. Submerged state fermentation

To identify the best amylase producers among 110 positive isolates among the total of 169 isolates, a modified submerged state fermentation method was conducted (Al-Johani et al., 2017). The composition of the media was 10 g/L of starch for amylase production test. To provide a suitable environment for microbial growth and enzyme synthesis, 20 g/L of yeast extract and 10 g/L of peptone were added (Abo-Kamer et al., 2023b). The essential minerals and cofactors for the optimal enzyme activity of the organisms were provided by including 0.02 g/L of $MgSO_4$, 0.01 g/L of $FeSO_4$, 0.05 g/L of $CaSO_4$, and 0.05 g/L of K_2HPO_4 and adjusted at pH 7 by phosphate buffer (Samanta et al., 2013). This media was used as basal media for amylase fermentation in this study. Therefore, 60 isolates, which have ≥ 1 mm clear zone, were selected for fermentations, which accounts to 60 isolates. All 60 isolates were fermented in a shaker incubator for 24 h. After that, the isolates were centrifuged for 10 min in 4000 rpm, and the supernatant was collected as crude enzyme.

3.6. Crude enzyme activity

To measure the enzyme activity, alpha amylase standard assay according to Bernfeld spectrophotometric stop reaction method was used. Alpha amylase breaks down starch into maltose, which react with 3,5-dinitrosalicylic acid (DNS) to produce color, which can be seen at

540 nm. The reaction is carried at 37°C and pH 7 by adding 1 mL of 1% starch agar to 1 mL of enzyme sample. After incubated for 15 min, DNS reagent was added, boiled for 10 min, cooled at room temperature, and diluted to measure absorbance (if >1 absorbance). The activity of the enzyme is expressed using a maltose standard curve in which one unit refers to the release of 1 mol of maltose in a time according to Bernfeld assay (Bernfeld, 1955). After getting concentration of maltose, enzyme activity was calculated using the formula:

Amylase activity (U) = (Concentration of maltose x reaction volume x 1000 x dilution factor) / (Molecular weight of maltose x incubation time in min x volume of enzyme). Dilution factor was the result of adding distilled water to solution if the absorbance result is >1 ant 540 nm.

The enzyme activity was measured using a standard maltose calibration curve. Absorbance readings were obtained from a spectrophotometer to calculate the concentration of produced maltose. The preparation of the maltose standard curve is done by taking 0.2, 0.4, 0.6, 0.8, and 1 mL of maltose solutions. Then, each tube was added with 1 mL of DNSA (dinitrosalicylic acid) reagent. The control tube (distilled water) was also made. The tubes were heated in a water bath for 10 min to start the reaction and color formation. Then, all samples were diluted by adding 5mL of distilled water and the absorbance of the solution was measured at 540 nm (Hussein et al., 2020).After doing maltose standard curve, 1 mL of crude enzyme and 1 mL of a 1% starch solution were combined and incubated for 15 min at 37ºC. Following the incubation period, amylase activity was determined by the DNSA method as follows. The DNSA reagent consisted of 1g of 3, 5-dinitrosalicylic acid in 20 mL, 1.6 g of sodium hydroxide, and 35g sodium potassium tartrate tetrahydrate in 100 mL. Each assay was conducted in duplicate and the results were expressed as mean of the two determinations.

After 15 min of incubation at 37ºC, 1 mL of DNSA was added and heated in water bath for 10 min. After cooling at room temperature, the absorbance was measured at 540 nm against a reagent blank (Mamo & Gessesse, 1999). 1 unit (U) of enzyme activity was defined as the amount of amylase that produce 1 micromole of reducing sugar equal to maltose per minute (Senthilkumar et al., 2012). The enzyme activity was calculated by using maltose standard equation and result of spectrophotometer.

3.7. Morphological and cultural characterization

After selecting the top nine isolates based on their enzyme activity, which hade ≥ 1 U, the colony morphology was observed visually and recorded the features of the bacterial colonies incubated for 24 h at 37ºC on the nutrient agar media which adjusted at pH 7 by using phosphate buffer. The colonies on Petri dish were examined and their shape, color, and texture were observed. Whereas cell was studied microscopically (Sousa et al., 2013). For the motility test, a fresh colony that grown on media was prepared. Then, a clean slide was prepared with a drop of water and very small colony was putted on it. It was carefully covered with cover slip and observed under high magnification of convex compound light microscope (V. Kumar, 2012).

The Gram staining procedure was performed by first preparing a clean glass slide and then heat fixing a smear of the bacteria to a slide. The smear was then applied crystal violet stain and it was allowed to sit for 1 min. The slide was then rinsed gently with water to remove any excess of the stain. Then, the iodine solution was applied on it, left for a minute to sit, and rinsed with water. Subsequently, it was decolorized with 96% ethanol for 5 seconds and then rinsed with water. Safranin was then used as a counterstain for 1 min to finish the staining procedure, after which the slide was rinsed with water one last time. The slide was air dry and observed under a light microscope (oil immersion) to classify the bacterial isolate as either Gram-positive or Gram-negative based on the staining characteristics (Coico, 2006). The malachite green spore stain was performed by applying malachite green to a bacterial smear, heating it to allow the dye to penetrate the spore coat, and then counterstaining with safranin to visualize vegetative cells. After staining, the spores appeared green, while the vegetative cells appeared pink (ERGÜL & ÇALIŞKAN, 2018)

3.8. Biochemical characterizations

3.8.1. The methyl red test

It was used to detect whether the bacteria can produce organic acid as product from glucose fermentation or not. This test was done by inoculating a pure culture of the organism into MRVP (Methyl Red and Voges-Proskauer) broth media, which adjusted at pH 7 by using phosphate buffer. Then incubated at 40ºC (optimum temperature of isolates) for 24 h. After incubation, 5

drops of methyl red reagents were added to the culture, and color formation was observed (AL-Joda & Jasim, 2021). A pH of less than 4.4 occurred when there was consistent production of adequate acid to overtake the phosphate buffer by fermenting glucose. The broth became red when the pH was less than 4.4 and the pH indicator (methyl red) is introduced (Kayal et al., 2023).

3.8.2. Voges-Proskauer test

This is to identify bacteria, which can producing butylene as a byproduct. The intermediate product of the reaction is acetoin, which can be found with 40% KOH and 5% alpha-naphthol. The media was the same with methyl red test. Then incubated at 40ºC for 24 h. After incubation, 2 drops of 5% alpha-naphthol and 40% KOH were added to the culture, and color formation was observed. A positive test result was produced when diacetyl reacts with the guanidine component of peptone in the presence of alpha-naphthol, forming a red color (Facklam & Elliott, 1995).

3.8.3. Citrate utilization test

Simmon citrate agar slant was prepared and inoculated with each isolate. The cultures were incubated for 24 h at 40ºC. Any color change was observed after growth. If the media changed from green to blue, it was taken as positive for citrate utilization and if not, taken as negative result for citrate utilization (MacWilliams, 2009). The ability of a bacterial isolate to use citrate as a source of carbon and energy is assessed by the citrate test. A positive diagnostic test requires the generation of alkaline byproducts from citrate metabolism. If the bacteria were positive for citrate test, it would change media from green to blue while negative unchanged media and it would remain green (Kalyan, 2018).

3.8.4. Urease test

The urease test involved inoculating bacterial culture with urea broth and incubating at 40ºC for 24 h. If a pink color formed, it would take as positive for the urease test (Brink, 2010). It is used to identify the bacteria that can hydrolyze urea to form ammonia and carbon dioxide. Urease activity is a crucial trait for species identification to differentiate *Proteus* from other *Enterobacteriaceae* members that do not ferment urea. Two molecules of ammonia and carbon dioxide are produced during the fermentation of urea when the enzyme "urease" is present. The

test result can be determined by looking at how the medium color changes as a result of the pH change (Uotani & Graham, 2015)..

3.8.5. Catalase test

For the catalase test, hydrogen peroxide was dropped onto a clean glass slide, and a small piece of bacterial colonies was added to a drop of hydrogen peroxide. The presence of bubble formation was monitored. The presence of bubbles indicated that the bacteria can break down hydrogen peroxide into hydrogen and oxygen (Reiner, 2010).

3.9. Characterization of amylase production by one factor at a time (OFAT) approach

3.9.1. Temperature

The optimal temperature for higher amylase productivity for bacterial isolates was determined by 10 range at a temperature of 20, 30, 40, and 50ºC by using basal media, which indicated in 3.5 topic. All other supplements to the conditions were the same and the enzymes have collected using the same method. At each temperature, the assay was conducted for each enzyme produced. After that, the amount of reducing sugar produced was calculated and the best temperature was determined by DNSA method (Laiz et al., 2003).

3.9.2. pH

The effect of pH on amylase production was determined by culturing the microbes in the media by adjusting the pH to 5, 6, 7, 8, and 9. The adjustment was carried out using NaOH (1M) and HCl (1M) under phosphate buffer system. The enzyme assay was carried out after 24 h of incubation at 40ºC. This is because the optimum temperature of all isolates is 40ºC temperature (Bakri et al., 2012).

3.9.3. Incubation period

The experiment was carried out individually at various incubation periods of 12, 24, 48, and 72 h to check optimum incubation period (Laiz et al., 2003).

3.9.4. Carbon source

The selection of a suitable carbon source for amylase production was performed by using five different carbon sources; glucose, maltose, sucrose, lactose, and fructose. The importance and selection of the best carbon source was analyzed by adding 1% w/v of each potential carbon source to a fermentation medium (basal media) and activity of enzyme was checked (Vijayabaskar et al., 2012).

3.9.5. Nitrogen Sources

Urea, tryptone and beef extract were used as organic nitrogen sources. The respective nitrogen sources were added as source of nitrogen. Ammonium chloride and ammonia were the inorganic nitrogen sources that were checked in this study. All of the nitrogen (0.5% w/v) came from the addition of the corresponding sources. The enzyme production was observed after 24 h of incubation using standard enzyme assay protocol with 40ºC and pH 7 (Lal et al., 2016).

3.9.6. Inoculation size

Inoculation concentration of bacterial isolates was done by adding bacterium at different inoculum concentrations such as 500, 1000, 1500, and 2000 µL. The potential to produce amylase in the production medium was tested. The enzyme activity was measured after 24 h of incubation using the standard protocol (Vijayabaskar et al., 2012).

3.9.7. Metals ions

The effect of metal ions (0.002% w/v) was checked by using potassium chloride, sodium chloride, zinc sulfate, and manganese sulfate to check their effect on amylase production by isolates. Each metal was added to basal media consecutively to check the effect on the production of amylase within the isolates (Jha et al., 2013).

3.9.8. Starch concentration

The starch concentration (0.5, 1, 1.5 and 2%) also added to basal media for each isolate and checked after 24 h of incubation using the standard protocol (Vijayabaskar et al., 2012).

3.9.9. NaCl concentration

NaCl at different concentrations of (0.5, 1, 1.5 and 2%) were added to basal media to check the production of alpha amylase by the isolates. The enzyme production was observed after 24 h of incubation using standard enzyme assay protocol with 40ºC and pH 7 (Lal et al., 2016).

3.10. Characterization of crude enzyme

3.10.1. Effect of temperature

To determine the optimal temperature of the alpha-amylase activity, the enzymatic activity was measured at different temperatures extending from 35 to 50ºC. This was conducted by adding 1 mL of crude enzyme to 1 mL of 1% of starch and incubating for 15 min, added 1 ml DNSA reagent and boiled at 100ºC for 10 min. The optical density for each test tube was measured at 540 nm using a spectrophotometer as previous technique (Omotoyinbo, 2023).

3.10.2. Thermostability

Regarding thermostability, the alpha-amylase was pre-treated at temperatures ranging from 45 to 65ºC for 30 and 60 min. Residual activity was then measured at optimal conditions described previously. The activity of the non-treated enzyme was considered as 100%, that mean the optimum temperature of enzyme (Omotoyinbo, 2023).

3.10.3. Effect of pH

The medium were prepared by making 1% soluble starch in different pH starting from 5 to 9 using phosphate buffer with the adjustment of 1M NaOH and HCL. In separates test tube, 1 mL of 1% substrate solutions were added with 1 mL of the respective buffers; 1 mL crude enzyme was added to these buffers as well. The prepared solutions were incubated for 15 min at 45ºC. After that, the test tubes were added with DNSA and boiled at 100ºC for 10 min in a water bath and cooled to room temperature. Optical density of each solution was measured at 540 nm using a spectrophotometer according to the above method. The highest pH activity was observed and noted (Simair et al., 2017).

3.10.4. Effect of metal ions

The effect of divalent and monovalent of chloride salts of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Na^{+} and K^{+} at a concentration of 5 mM were used to determine their effects on the activity of amylase. It test was carried out after the enzyme was combined in a 1:1 ratio with the salt solutions and incubated at 45ºC for 15 min. The amylase activity in the presence of salt concentration was compared with activity of amylase without salt concentration (Zohra et al., 2016).

3.10.5. Effect of reaction time

The effect of the reaction time was checked by incubating the enzyme with substrate for 15, 30, 45 and 60 min and the activity of all period was checked with the same method as above (Ahmed et al., 2020).

3.11. Molecular identification

3.11.1. 16S rRNA gene sequencing

For bacterial identification, pair of primer were used: 27 F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492 R: 5′ GGTTACCTTGTTACGACTT-3′, 16S rRNA gene was amplified with the following reaction parameters: 94 °C for 10 min, 32 cycles of 94 °C for 30 sec, 55 °C for 20 sec, 72 °C for 55sec; and final extension at 72 °C for 5 min. A total of 25-µL reaction volume per tube was used. Sanger sequencing method was used with minor modification for amplicon sequencing. In particular, cycle sequencing was carried out using the ABI PRISM® BigDye™ Terminator cycle sequencing kit with the forward primer 27F and the reverse primer 1492R. ABI PRISM Sequencer platform 3730XL was used for the electrophoresis at Beijing Sangon Biotechnology Company. The bacterial isolates sequenced in the current study of 16S rRNA were searched against GenBank database using Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) to find top matches to the sequences. Identification of the species was done using a 97% or higher sequence identity threshold with the type of isolates of related identified bacterial species in the rRNA gene region (Frank et al., 2008).

3.11.2. *Tuf* elongation factor

Even though 16S rRNA gene is widely used for bacterial identification, it had limitation of identify *Bacillus* group due to high sequence similarity in rRNA gene region. To overcome this, the *tuf2* elongation factor was employed as molecular marker with species level discrimination for *Bacillus.* The *tuf2* gene was amplified using the primer tuf2 fw: 5′-AVGGHTCTGCHYTDAAAGC-3′ and tuf2 rev: 5′-GTDAYRTCHGWWGTACGGA-3′ under the following reaction conditions: initial denaturation at 95 °C for 15 min, followed by 30 cycles of 95 °C for 30 sec, 47 °C for 30 sec, and 72 °C for 1 min; with final extension at 72 °C for 15 min. The total volume reaction per tubes was 25 μL which consists of 12.5 TEMPase Hot Start 2 x Master Mix Blue, 0.8 μL of each primer (10 μmol/L), and 0.3 μL DNA template, and 10.6 μL free water nuclease. PCR results were purified and pooled in equimolar rations before sequencing on an Illuminia MiSeq platform using Kit v3 (600-cycle). Then, the result sequence were analyzed and aligned against database to identify *Bacillus* species (Xu et al., 2023).

3.12. Partial purification of enzyme

H3S1 (*B. cereus*) isolates was selected for partial purification. The enzyme purification was completed using ammonium sulphate precipitation and 0.1 M phosphate buffer at 4ºC and pH 7. Ammonium sulphate was added to the crude enzyme in the following saturation ranges: 20-40%, 40-60%, and 60-80%. Magnetic stirrer had stirred the mixture. The precipitated protein was collected from each range of ammonium sulphate concentration (20-40%, 40-60%, and 60-80%). The precipitated protein was collected and centrifuged at 4000 rpm for 10 min. The pellet was then preserved while the supernatant was properly disposed of. To remove the salt and leftover debris, the enzyme solution was then dialyzed against 0.1 M potassium phosphate buffer for 24 h. The protein precipitated was transferred to a membrane filter bag and sealed tightly.

Then, 5 L of buffer was added to the beaker, and the membrane bag containing the protein was placed in the buffer for 24 h. The assay of each concentration of collected enzyme was checked and the best activity was selected for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (I. Sani et al., 2014).

3.13. SDS-PAGE

After obtaining the dialyzed sample, the fraction (60–80% saturation) was selected for SDS-PAGE. First, a running gel was prepared by mixing acrylamide, Tris-HCl buffer (pH 8.8), SDS, APS, and TEMED. This solution was placed in a gel-casting frame and allowed to polymerize for 30 min. After polymerization, a stacking gel was prepared using Tris-HCl buffer (pH 6.8), SDS, APS, and TEMED with reduced concentration of acrylamide. Stacking gel was poured onto the polymerized running gel, which was inserted with a comb well for sample loading. The stacking gel was left to polymerize completely for about 15-20 min. Then, protein sample was mixed with SDS-PAGE loading buffer (SDS, glycerol, β-mercaptoethanol and Tris-HCl) and boiling the mixture at 100°C for 5 min for denaturation. A volume of 20 μL of each sample of crude, 60–80%, and protein marker/ladder, was added to separate well. Electrophoresis was then performed in two steps: an initial voltage of 100V was used to run the proteins through the stacking gel for 15 min and then the voltage was increased to 200V to separate proteins from a running gel for 45 min. After electrophoresis, the gel was removed and stained with Coomassie Brilliant Blue R-250 and destain with the mixture of methanol, acetic acid, and water for 2 h. Finally, a detached gel test was performed to examine protein bands. The molecular weight of the proteins was determined by comparing displacement with a protein ladder (Simpson, 2006 and Manns, 2011).

3.14. Specific activity of enzyme

Enzyme activity measures the total substrate conversion per unit time, while specific activity indicates enzyme efficiency per milligram of protein. In order to determine the specific activity of crude and partially purified enzymes, a series of egg albumin solutions were made using the Bradford method at concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL. An equal volume of Coomassie Brilliant Blue was added to each standard sample, followed by gentle mixing. Incubated at room temperature for 10 min, protected from light then the optical density was measured with a spectrophotometer at 595 nm and standard curve was created using the absorbance measurements to calculate protein concentration. Crude and partially purified enzyme samples were then made and the absorbance was checked at 595nm as standard. After that, specific activity was calculated by the formula: Specific activity = total activity / protein concentration (mg/mL).

3.15. Application of enzyme

To check the effectiveness of the collected enzyme, it was checked by detergent application. Two types of soap were prepared at laboratory condition using sodium hydroxide, coconut oil, sodium chloride and distilled water. One soap was without enzyme while the other was with 3 mL of enzyme in addition to the above chemicals. The enzyme used was taken from G2W1 because the isolate had maximum activity among the nine isolates. Prepared equal pieces of white clothes contaminated with potatoes waste; one was treated with soap without enzyme, another was soap containing enzyme, and compared with clean cloth as a control. Then, 20 g of prepared soap was applied on the pieces of white cloth, which were contaminated by potatoes waste and allowed it to stay for 6 h. After that, rinsed with distilled water and compared the cleanliness of each cloth against control (Roy et al., 2012).

3.16. Statistical analysis

For statistical analysis, a 95% confidence level ($p < 0.05$) was used by Origin Lab 2019b software. To ensure reliability, all analyses were conducted in duplicate. The data are displayed as mean ± standard deviation to clearly illustrate variance.

4. Results

4.1. Microbial isolation for amylase production

The results showed that 110 of 169 isolates tested positive for amylase activity and 59 were negative. Clear zones forming around colonies in starch media indicated starch breakdown due to amylase activity. The size of was measured by calculating the diameter of the clear zone and dividing it by the size of the colony. From 110 amylase positive isolates, 60 colonies exhibiting of ≥ 1 amylolytic index were selected for submerged state fermentation (Table 1). 60 isolates were fermented and assayed for enzyme activities to facilitate further selection (Table 2). After fermentation and enzyme activities was assayed, the nine isolates which had ≥ 1 U/mL were selected (Table 3).

Table 1: Isolates with Amylolytic Index (AI) ≥ 1.

Isolate	AI	Isolate	AI	Isolate	AI
H3W2	1.11 ±0.04	B3W2	1.33 ±0.10	K3W1	1.33 ±0.09
H2S2	1.11±0.01	B3S2	1.27 ±0.3	K2S2	1.01 ±0.06
H2S1	1.2 ±0.04	B2S2	1.01 ±0.01	K2S1	1.34 ±0.05
H1W1	1.23 ±0.09	B1S1	1.25 ±0.2	K2W2	1.11 ±0.11
H2W1	2.56 ±0.12	G2W1	2.65 ±0.13	K1W2	1.11 ±0.04
H1S1	1.2 ±0.01	G2W2	1.32 ±0.22	K2W1	1.56 ±0.07
H3S2	1.3 ±0.12	G1S1	1.44 ±0.08	K3W2	1.03 ±0.11
H2W2	1.23 ±0.22	G1S2	1.33 ±0.09	K3S1	1.42 ±0.05
H3S1	2.21 ±0.08	G1W1	1.31 ±0.08	C2S2	1.02 ±0.07
H1W2	1.21 ±0.03	G1W2	1.12 ±0.08	C2S1	1.22 ±0.01
H3W2	1.1 ±0.1	G3W1	2.23 ±0.09	C1W1	1.31 ±0.04
H1S1	1.29 ±0.12	G3W2	1.32 ±0.3	C1W2	1.01 ±0.12
B3W1	1.49 ±0.09	G3S2	1.12 ± 0.01	C3W1	1.67 ±0.12
B1W1	2.87 ±0.09	G2S1	1.91 ±0.11	C3W2	1.34 ±0.07
B1W2	1.13 ±0.09	G2S2	1.11 ±0	C2W2	1.31 ±0.03
B1S1	1.26 ±0.09	G3S1	1.33 ±0.09	C1S1	1.3 ±0.3
B3S1	1.01 ±0.12	K1W2	1.24 ±0.21	C1S2	1.23 ±0.1
B2S1	1.02 ±0.23	K3S2	1.27 ±0.05	C2W1	1.36 ±0.09
B3W1	1.21 ±0.11	K2S2	1.32 ±0.03	C3S1	1.21 ±0.11
B2W1	1.13 ±0.06	K1S1	1.87±0.13	C3S1	1.09 ±0.8

AI: amylolytic index

Table 2: Enzyme Activity (EA) of 60 Isolates (U/mL).

Isolate	EA (U/mL)	Isolate	EA (U/mL)	Isolate	EA (U/mL)
H3W2	0.22 ±0.04	B3W2	0.84 ±0.01	K2S2	0.53 ±0.03
H2S2	0.15 ±0.01	B3S2	0.74 ±0.01	K2S1	0.98 ±0.01
H2S1	0.58 ±0.04	B2S2	0.13 ±0.01	K1S2	0.62 ±0.05
H1W1	0.7 ±0.04	B1S1	0.48 ±0.03	K2W2	0.57 ±0.03
H2W1	1.37 ± 0.04	G2W1	1.96 ±0.01	K1W2	0.72 ±0.04
H1S1	0.51 ±0.01	G2W2	0.8 ±0.01	K2W1	1.39 ± 0.03
H3S2	0.43 ±0.03	G1S1	1.71 ±0.01	K3W2	0.38 ±0.01
H2W2	0.31 ±0.01	G1S2	0.86 ±0.01	K3S1	1.4 ±0.03
H3S1	1.14 ± 0.03	G1W1	0.89 ±0.03	C2S2	0.15 ±0.01
H1W2	0.44 ±0.03	G1W2	0.53 ±0.03	C2S1	0.51 ±0.01
H3W2	0.33 ±0.01	G3W1	1.40 ± 0.02	C1W1	0.77 ±0.04
H1S1	0.61 ±0.01	G3W2	0.48 ±0.03	C1W2	0.17 ±0.04
B3W1	1.07 ±0.04	G3S2	0.58 ± 0.01	C3W1	0.98 ±0.01
B1W1	1.28 ± 0.05	G2S1	1.73 ±0.01	C3W2	0.92 ±0.03
B1W2	0.44 ±0.03	G2S2	0.53 ±0	C2W2	0.89 ±0.03
B1S1	0.47 ±0.04	G3S1	0.9 ±0.03	C1S1	0.83 ±0.04
B3S1	0.23 ±0.03	K1W2	0.73 ±0.03	C1S2	0.38 ±0 1
B2S1	0.38 ±0.01	K3S2	0.37 ±0.03	C2W1	0.36 ±0.01
B3W1	1.07 ± 0.06	K2S2	0.32 ±0.03	C3S1	0.31 ±0.11
B2W1	0.13 ±0.06	K1S1	1.43 ± 0.09	C3S1	0.39 ±0.8

EA= Enzyme activity

Table 3: The nine best isolates, which hade ≥ 1 U/mL selected for further analysis

Isolate	EA (U/mL)
G2W1	1.96 ± 0.04
G2S1	1.73 ± 0.01
K1S1	1.43 ± 0.09
G3W1	1.40 ± 0.02
K2W1	1.39 ± 0.03
H2W1	1.37 ± 0.04
B3W1	1.07 ± 0.06
H3S1	1.14 ± 0.03
B1W1	1.28 ± 0.05

4.2. Morphological and biochemical characterizations of the nine selected isolates

All the top nine isolates were re-grown on nutrient agar and all the macroscopic and microscopic characterizations were performed. Macroscopic identification like color and texture were checked by observing the colonies on nutrient agar. Isolate B1W1, K2W1, H2W1, G2S1, and B3W1 were transparent colored colonies. Isolate H3S1, K1W1 and G3W1 were white while G2W1 yellowish in color. All the colonies of isolates were dry in texture except G2W1 and G3W1, which were rounded, and mucoid, respectively. Microscopic identification of the cells like shape, gram test, spore and motility tests were performed by microscopic techniques. All the isolates were rod in shape. Except G2W1 and G3W1, all were gram-positive bacteria. In terms of motility, only G3W1is non-motile and all isolates could produce spores with the exception of G3W1 and G2W1 (Table 4).

Table 4: Summary of macroscopic and microscopic characterization of the nine selected amylase-producing isolates

Isolate code	Color	Texture	Shape	Gram test	Motility	Spore
B1W1	Greyish	Dry	Rod	Positive	Motile	Positive

K2W1	Greyish	Dry	Rod	Positive	Motile	Positive
H3S1	White	Dry	Rod	Positive	Motile	Positive
K1S1	White	Dry	Rod	Positive	Motile	Positive
H2W1	Greyish	Dry	Rod	Positive	Motile	Positive
G2S1	White	Dry	Rod	Positive	Motile	Positive
B3W1	White	Dry	Rod	Positive	Motile	Positive
G2W1	Yellowish	Round	Rod	Negative	Motile	Negative
G3W1	White	Mucoid	Rod	Negative	Non-motile	Negative

All the nine isolates were positive test for Methyl red test as shown in Figure 1.

Figure 1: Methyl red result

Seven isolates (B1W1, K2W1, H2W1, G2S1, and B3W1, K1S1, and H3S1) were positive and two isolates (G2W1 and G3W1) were negative for Voges-Proskauer test in the present study as shown in figure 2.

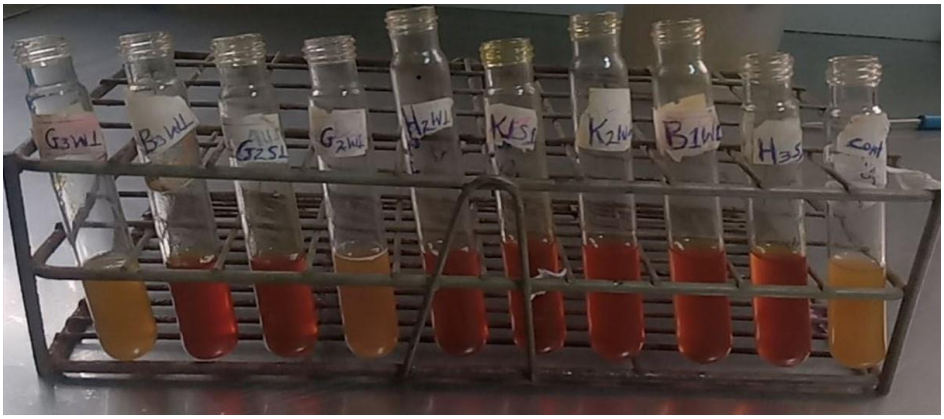


Figure 2: Voges-Proskauer test result

All the isolates of the current study were negative for urease test except G3W1 (Fig.3).

Figure 3: Urease test result.

All the isolates were positive for citrate test except G2W1 (Fig.4).

Figure 4: Citrate test result

All the nine isolates were positive test for catalase test as shown in Figure 5.

Figure 5: Catalase test result

Table 5: Summary of biochemical characterization of best isolates

Code	Methyl Red	Voges-Proskauer	Citrate	Catalase	Urease
1W1	Positive	Positive	Positive	Positive	Negative

K2W1	Positive	Positive	Positive	Positive	Negative
H3S1	Positive	Positive	Positive	Positive	Negative
K1S1	Positive	Positive	Positive	Positive	Negative
H2W1	Positive	Positive	Positive	Positive	Negative
G2S1	Positive	Positive	Positive	Positive	Negative
B3W1	Positive	Positive	Positive	Positive	Negative
G2W1	Positive	Negative	Negative	Positive	Negative
G3W1	Positive	Negative	Positive	Positive	Positive

4.3. Molecular identification

The best 9 isolates were identified by 16S rRNA and *tuf2* elongation factor. Among the 9 best isolates, seven were identified as *Bacillus cereus*, while the reaming were identified as *A. rivipollensis* and *K. pateurii* (Table 6).

Table 6: Summary of biochemical characterization of best isolates

Isolate	**Species**	**Identity (%)**	**Accession Number**
B1W1	*Bacillus cereus*	100	NR_115526.1
B3W1	*Bacillus cereus*	100	NR_115526.1
G2S1	*Bacillus cereus*	100	NR_115526.1
G2W1	*Aeromonas rivipollensis*	99.85	NR_144574.1
G3W1	*Klebsiella pasteurii*	99.71	NR_180640.1
H2W1	*Bacillus cereus*	100	NR_115526.1
H3S1	*Bacillus cereus*	99.86	NR_157736.1
K1S1	*Bacillus cereus*	100	NR_115526.1
K2W1	*Bacillus cereus*	100	NR_157732.1

4.4. Optimization of alpha-amylase producing bacteria by one factor at a time (OFAT) approach

4.4.1. Temperature

The effect of different temperatures on alpha amylase production is displayed in Figure 6. At 40ºC and 24 h incubation time the highest amount of alpha amylase was produced by B1W1 (2.3 ± 0.02 U/mL) followed by K1S1 (2.0 ± 0.02 U/mL). The second-best temperature for amylase production at 50ºC was by B1W1 (2.1 ± 0.1 U/mL). Conversely, the lowest alpha amylase production (0.25 ± 0.03 U/mL) recorded by G2S1 with temperature of 20ºC. An increase of enzyme activities was observed from 20 to 40ºC and decreased at 50ºC. The optimum temperature for all isolates was found at 40ºC. Statistical significant difference of enzyme activity was observed within isolates of different temperature in B1W1, K2W1, H3S1, K1S1, H2W1, G2S1, B3W1 and no significant different within a group of G2W1 and G3W1 except at 20ºC. Between isolates, enzyme activity was significantly difference at 0.05 significant level.

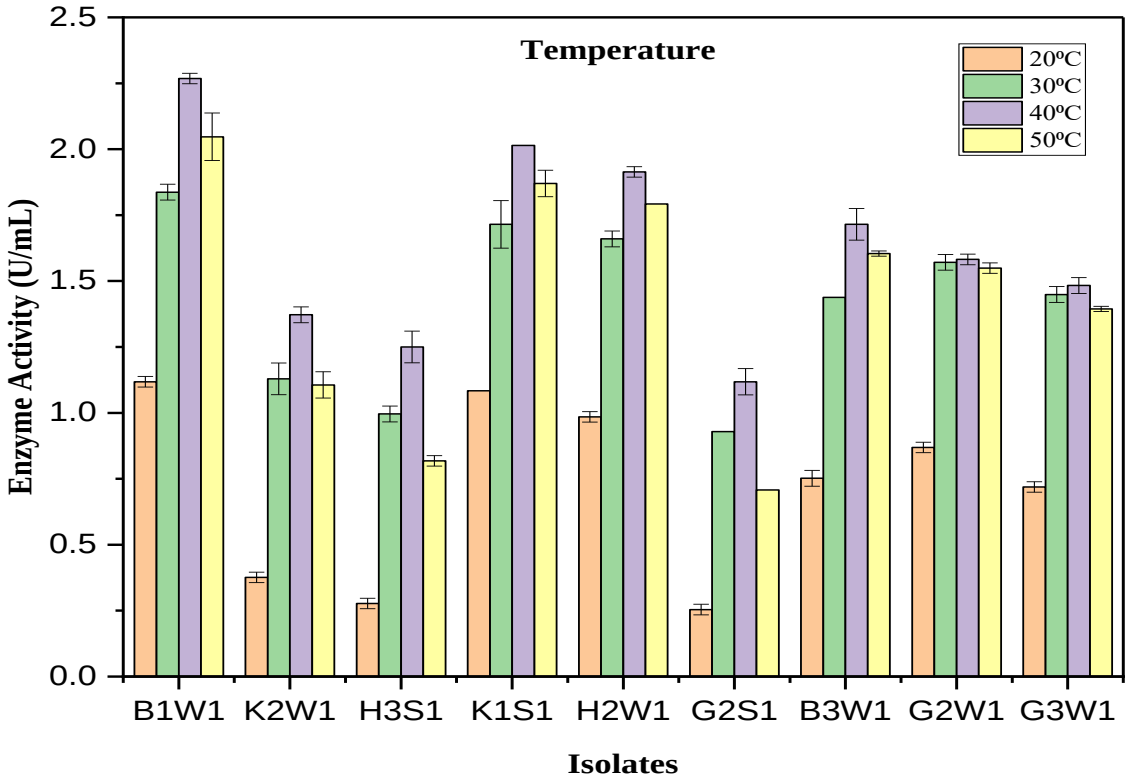


Figure 6: Optimization of temperature

4.4.2. pH

Figure 7 presents the impact of different pH levels on the production of alpha amylase of isolates following 24 h of incubation period at 40ºC. The maximum level of amylase production was measured at pH 7 (2.3 ± 0.03 U/mL) by B1W1, while the lowest level was found at pH 9 (0.87 ± 0.03 U/mL) by G2S1. All isolates' enzyme activities were best at pH 7 while B1W1 recorded the highest activity among the nine isolates. Within isolates, B1W1, K2W1, H3S1, G2S1, were significantly different. K1S1, H2W1, G2W1, G3W1 had no significant difference at 6, 7 and 8 while at 5 and 9 was different. Between isolates, B1W1 was significant difference from all isolates. K2W1, H3S1 and G2S1 had no significant different, but these isolates had significant difference with other isolates. K1S1 and H2W1 had no significant difference. B3W1 was different from the other. G2W1 and G3W1 had no significant difference. All these statistical analysis was done at 0.05 significant level.

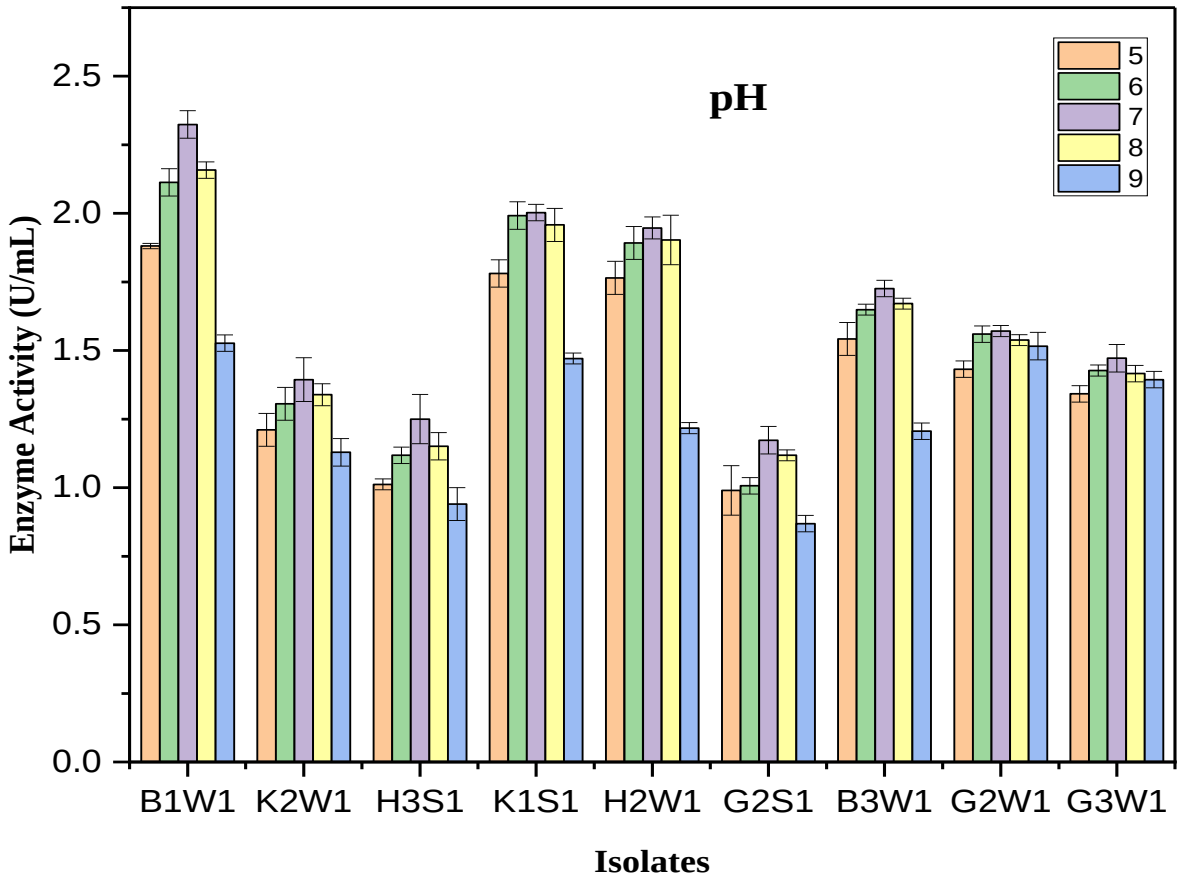


Figure 7: Optimization of pH

4.4.3. Incubation period

Figure 8 shows the optimal incubation time of the nine isolates determined. The highest alpha amylase activity by all isolates was seen after 24 h of incubation at 40ºC and pH 7. With isolate B1W1, it was recorded 2.3 ± 0.02 U/mL, which is the highest enzyme activity among the isolates. All isolates showed a gradual increase in enzyme activity from 12 to 24 h of incubation; however, amylase activity was shown a gradual decrease from 24 to 72 h. This may be due to production of other side products which degrade the alpha amylase enzyme and decrease the activity when time is increasing (Abdullah et al., 2014). The optimum incubation time was identified at 24 h. There was a significant difference within and between all isolates at 0.05 significant level, as *p* values were less than 0.05.

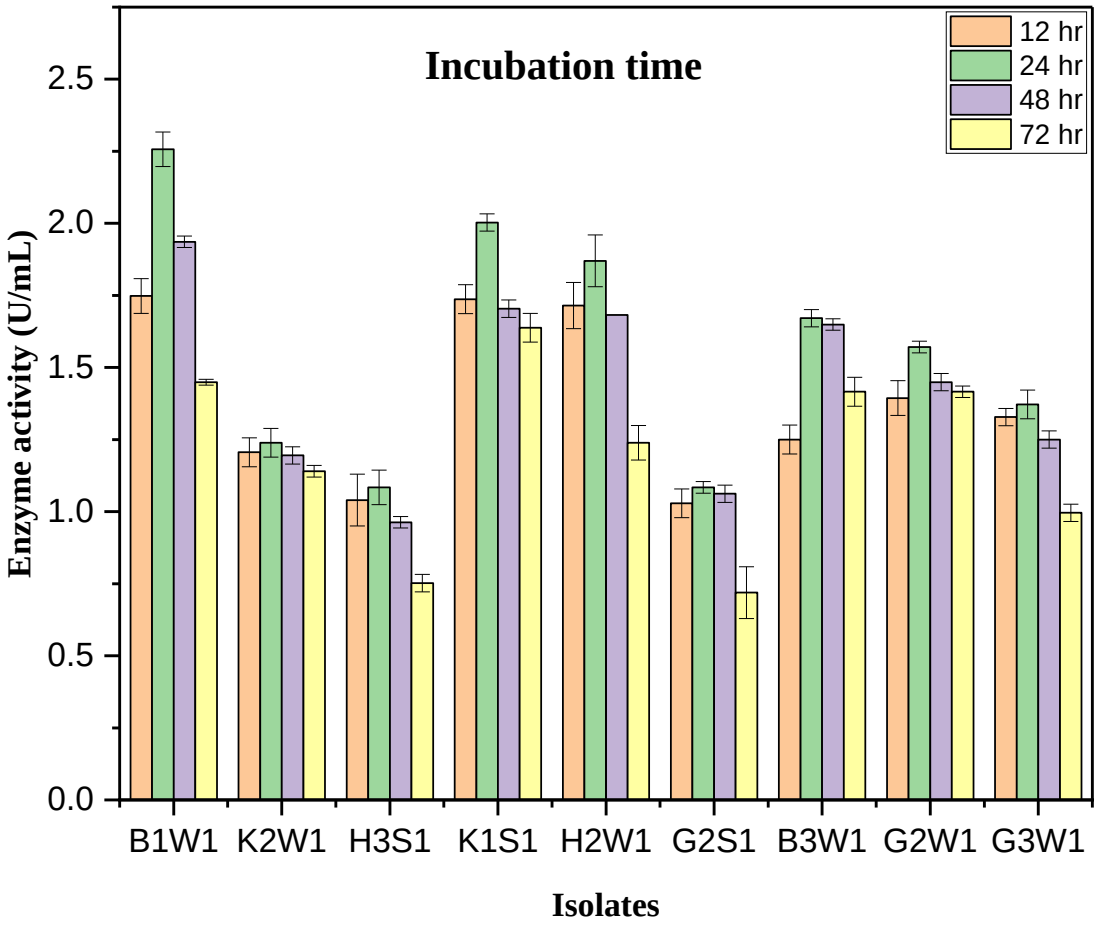


Figure 8: Optimization of incubation time

4.4.4. Carbon source

Each of the following carbon sources was added to the basal medium at a concentration of 1% (w/v): glucose, maltose, sucrose, lactose, and fructose at optimal temperature, incubation time, and pH to see the impact on enzyme production by the selected nine isolates (Figure 9). Among these carbon sources, glucose produced the highest alpha amylase from all isolates and maximum activity was scored by G2W1 (3.4 ± 0.1 U/mL) followed by B1W1 and G3W1 (3.4 ± 0.03 U/mL). The minimum activity was scored by B3W1 in fructose (1.4 ± 0.1 U/mL). Within isolates, all the nine isolates showed significant difference of amylase activity within different carbon source. B1W1, G2W1 and G3W1 had no significant difference. The others had significant difference of enzyme activity at different carbon sources at 0.05 significance level.

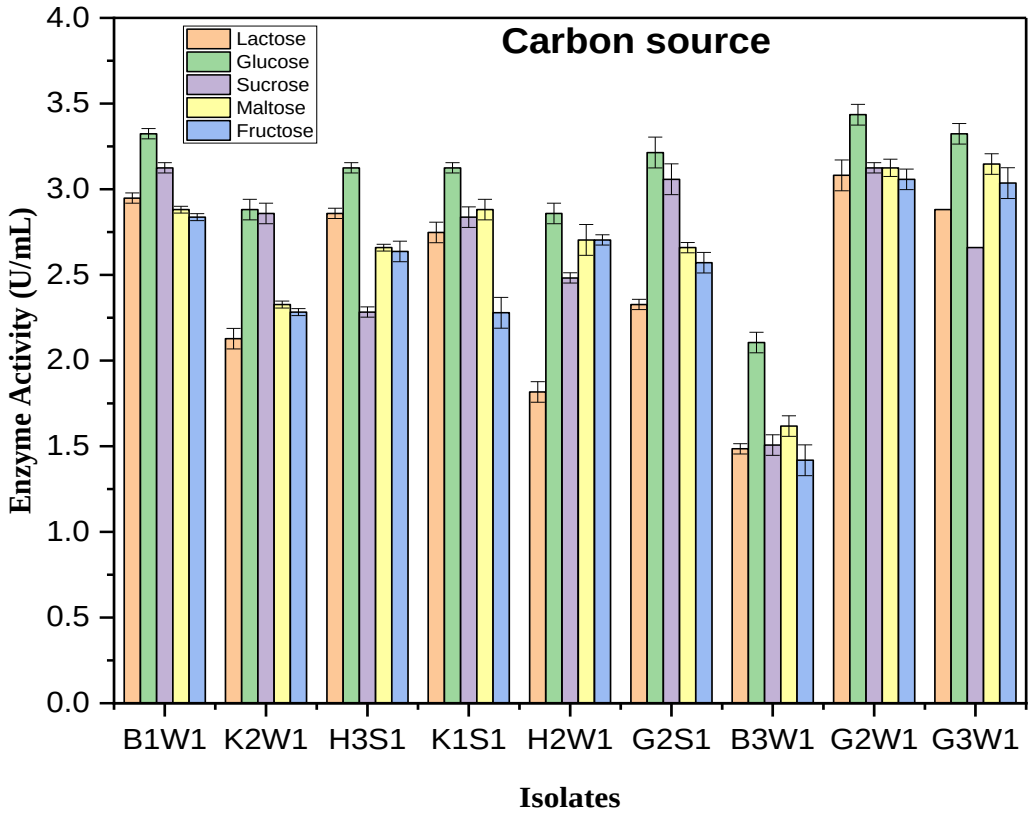


Figure 9: Effect of different carbon source on alpha-amylase activities

4.4.5. Nitrogen Sources

For organic nitrogen source (urea, beef extract and peptone) and inorganic nitrogen source (ammonium chloride and ammonia), B1W1, K2W1, H3S1, K1S1, G2S1, B3W1 were showed high amylase activity in a medium inoculated with beef extract as organic nitrogen source while high activity was showed in a medium inoculated with ammonium chloride as an inorganic source. H2W1, G2W1 and G3W1 were scored high alpha amylase activities in a medium inoculated with tryptone as organic nitrogen source. Different isolates used different nitrogen sources as shown in Figure 10. There was statistically significant difference within isolates of different nitrogen sources. B1W1, H3S1, K1S1, G2S1, G2W1 and G3W1 had no significant difference with each other while different from the other. Overall, the best amylase activity was observed upon utilization of tryptone as a nitrogen source (3.4 ± 0.03 U/mL) with G2W1

followed (3.3 ± 0.1 U/mL) with B1W1 in NH_4Cl. The lowest alpha amylase activity was scored by B3W1 (1.441 ± 0.03 U/mL).

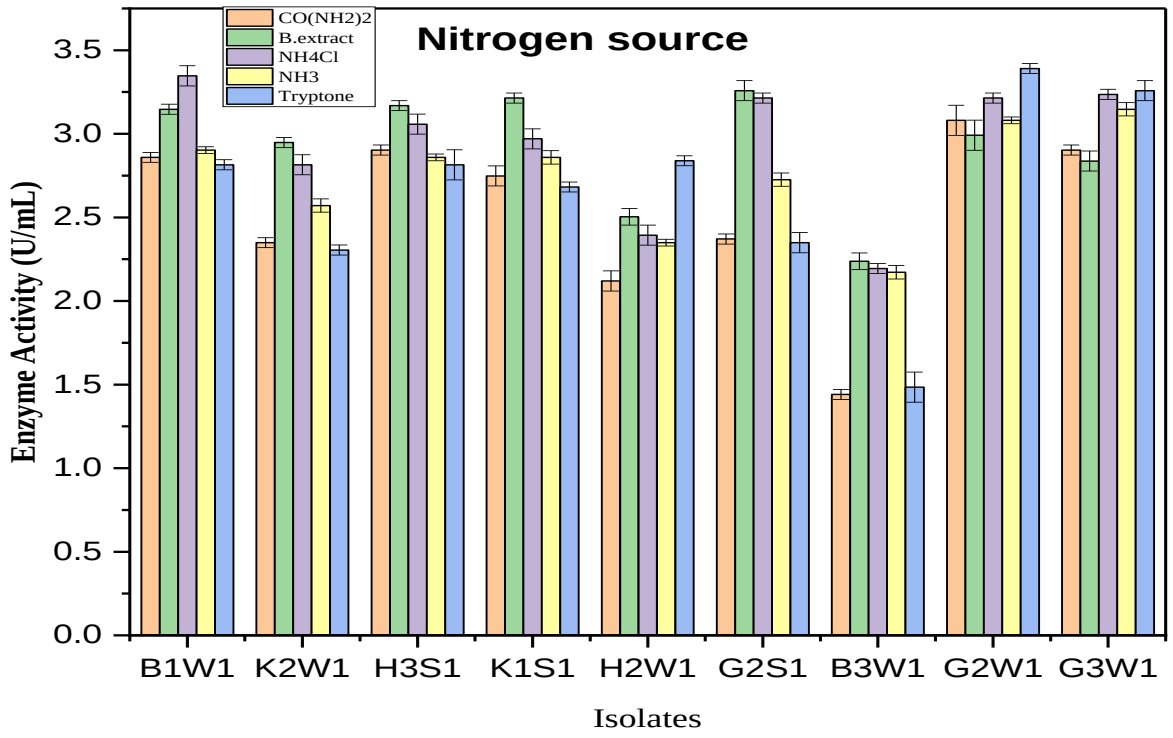


Figure 10: Effect of different nitrogen source on alpha-amylase activities

4.4.6. Metal ions

Different metal ions (K, Na, Zn and Mn) were individually added to basal media to check the production of alpha amylase by the isolates. The six isolates, B1W1, K2W1, H3S1, K1S1, H2W1 and G2S1 were scored high activity with Zn, while B3W1 scored highest activity with Na, and G2W1 scored with K and, G3W1 performed best activity with Mn. It was observed that different isolates used different metal ions for alpha amylase production. Among the metal ions sources, K is the best ion for amylase production for all isolates and maximum activity was scored by G2W1 (3.3 ± 0.1 U/mL) followed by G3W1 (3.2 ± 0.04 U/mL) (Figure 11) and the minimum alpha amylase activity was scored by B3W1 (2.1 ± 0.03 U/mL) with K ion. In most isolates, Na ion source scored lowest production of amylase. Amylase production by different sources of metal

ions in all isolates had significant different between metal ions and different isolates except manganese utilization in G2W1 and G3W1.

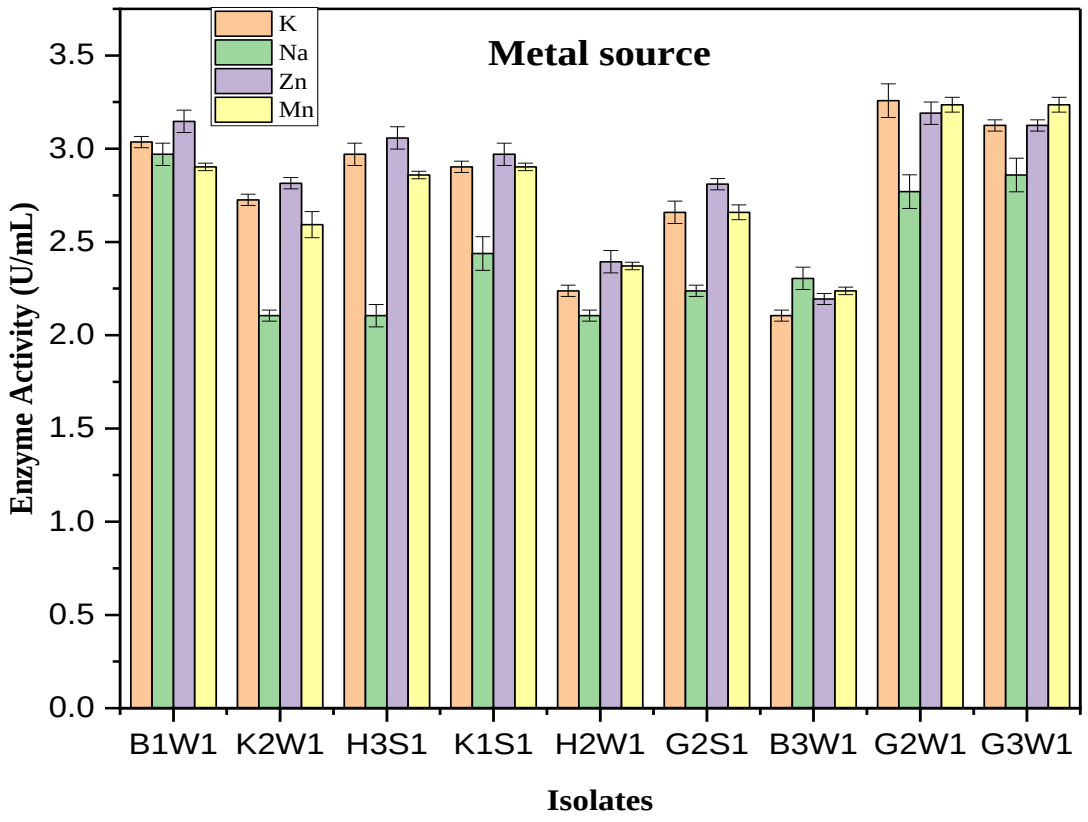


Figure 11: Effect of different metal source on alpha-amylase activities

4.4.7. Inoculation volume

The impact of different inoculation volume on alpha amylase production is displayed in Figure 12. At 1500 µL, the highest (3.3 ± 0.1 U/mL) amount of amylase activity was observed in isolate G2W1, followed by B1W1 (3.3 ± 0.02 U/mL). The activities of all isolates were increasing from 500 to 1500 µL and decreased at 2000 µL. Inoculation of more cell suspension may decrease the activity of enzymes due to nutrient depilation (Abdullah et al., 2014). The optimum inoculum size for all isolates was 1500 µL. Different inoculation sizes showed different enzyme activity in each isolate. Within isolates of different inoculation volume, except G2W1 and G3W1, which only 500 µL different from the other inoculation volume, all isolates were significant difference. Between

isolates, B1W1, G2W1 and G3W1 had no significant difference. H3S1 and K1S1 also had no significant difference with each other but different from other isolates in enzyme production. All the statistical analysis were done at 0.05 significance level.

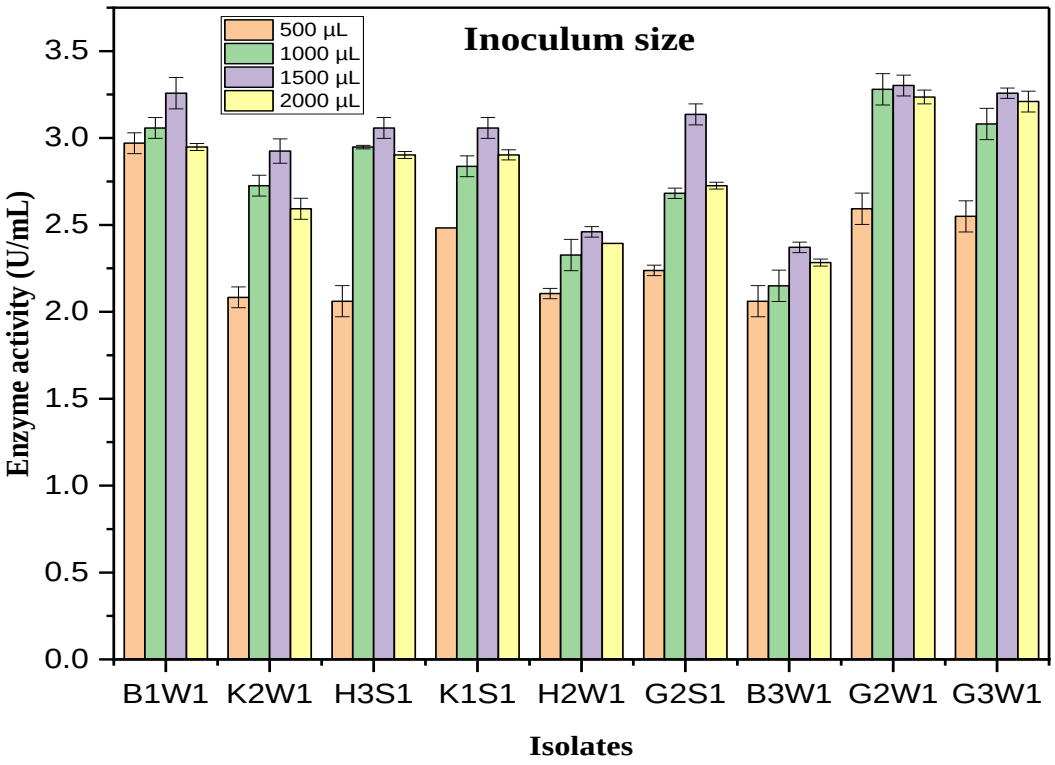


Figure 12: Effect of different inoculation volume on alpha-amylase activities

4.4.8. Starch concentration

The effect of different starch concentrations (0.5%, 1%, 1.5%, and 2%) on alpha-amylase production is displayed in Figure 13. The highest enzyme activity was observed at 1.5% starch concentration for all isolates, indicating that increasing starch concentration enhances amylase production from 0.5 % to 1.5%. Further increase in enzyme activity was not observed when the medium's starch concentration was raised above 1.5%. Among the isolates, B1W1 exhibited the highest enzyme activity (3.3 ± 0.1 U/mL) at 1.5 % starch; conversely, the lowest enzyme activity (2.0 ± 0.1 U/mL) was recorded by H3S1 at 0.5%, indicating that lower starch availability limits enzyme production. Statistical analysis at a 0.05 significance level showed a significant difference in enzyme activity

within isolates for most groups. Between isolates, enzyme activity was significantly different, indicating variability in the ability of different strains to utilize starch for amylase production

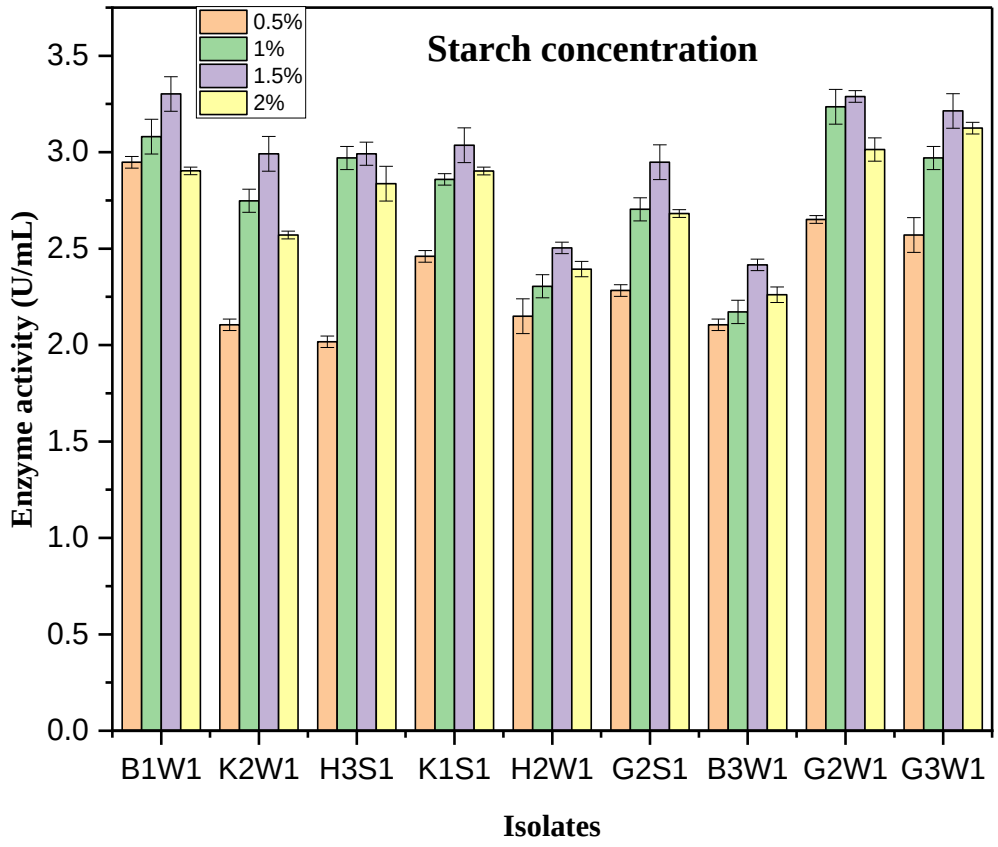


Figure 13: Effect of starch concentration on alpha-amylase activities

4.4.9. NaCl concentration

The effect of different NaCl concentrations (0.5%, 1%, 1.5%, and 2%) on alpha-amylase activity is presented in Figure 14. All isolates scored high activity with 1.5% except G3W1 with 1% of NaCl. The enzyme activity generally increased with NaCl concentration (0.5 to 1.5%) for most isolates. A decrease in enzyme activity was noted at 2% NaCl, indicating potential inhibition at higher salt concentrations. Maximum enzyme activity was scored by G2W1 (3.3 ± 0.03 U/mL) followed by B1W1 (3.2 ± 0.0 U/mL) and the minimum amylase activity was scored by B3W1 (2.0 ± 0.1 U/mL) with 0.5% of NaCl. Statistical analysis at a 0.05 significance level revealed significant differences in enzyme activity between the different NaCl concentrations for most isolates. There

were also notable differences between isolates, highlighting variability in salt tolerance and enzyme production.

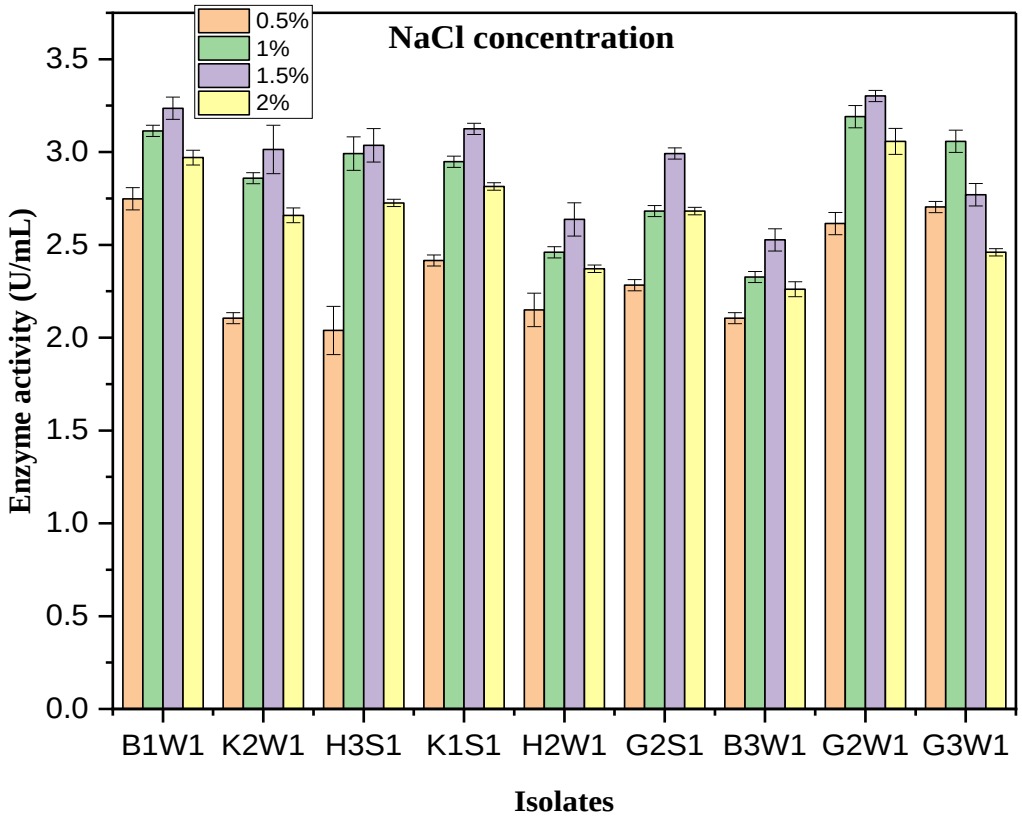


Figure 14: Effect of different NaCl concentration on alpha-amylase activities

4.4.10. Optimal enzyme activity under all optimized conditions

The optimal conditions for alpha-amylase production by the best nine isolates were determined by testing factors such as temperature, incubation time, pH, carbon source, nitrogen source, starch concentration, metal source, and sodium chloride concentration in the basal medium. For most isolates (B1W1, K2W1, H3S1, H2W1, G2S1, and B3W1), the optimal conditions included glucose as the carbon source, beef extract and ammonium chloride as nitrogen sources, zinc as the metal source, 1.5% starch, and 1.5% sodium chloride. However, isolate G2W1 showed the best performance with tryptone as the nitrogen source, potassium as the metal source, while G3W1 performed best with manganese as the metal source, 1.5% NaCl with all other conditions

remaining the same. Among the nine isolates, G2W1 achieved the highest enzyme activity (4.5 ± 0.6 U/mL), followed by G3W1 (4.3 ± 0.4 U/mL), and B1W1 (4.1 ± 0.7 U/mL) (Figure 17). Statistical analysis at a 0.05 significance level showed significant differences in enzyme activity among the isolates, indicates the impact of these optimal conditions on alpha-amylase production.

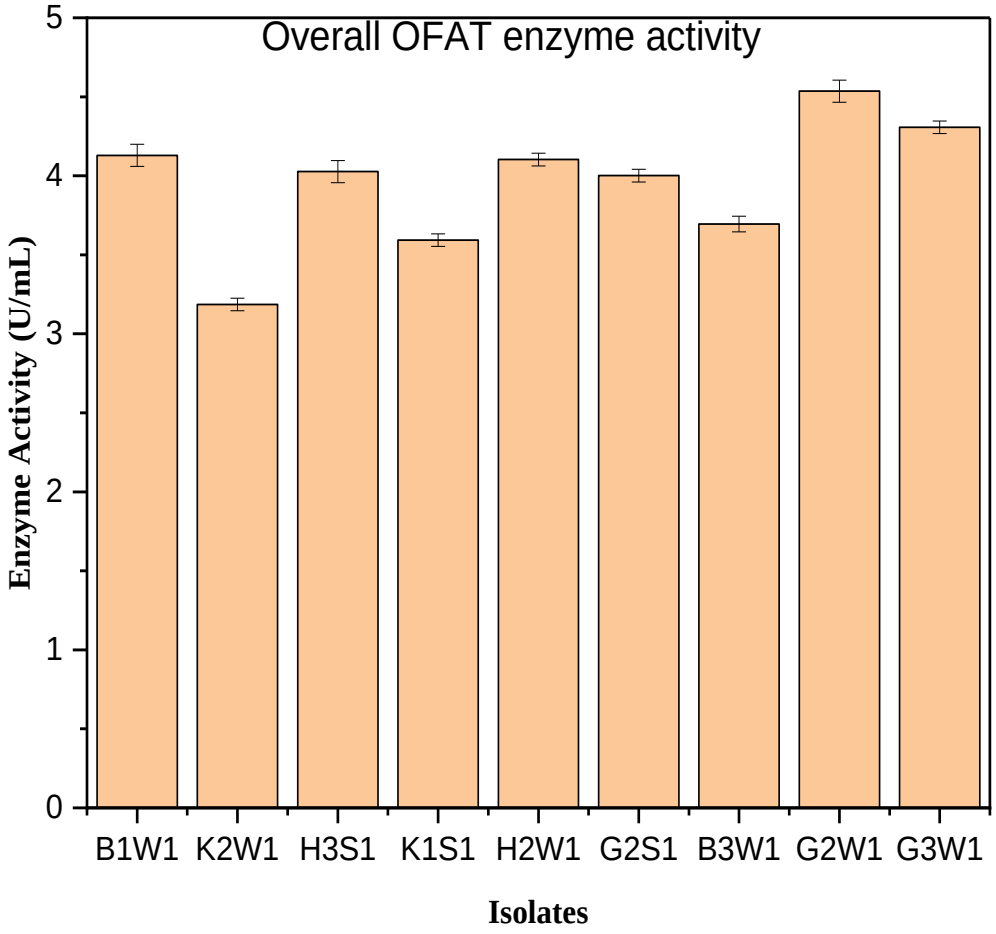


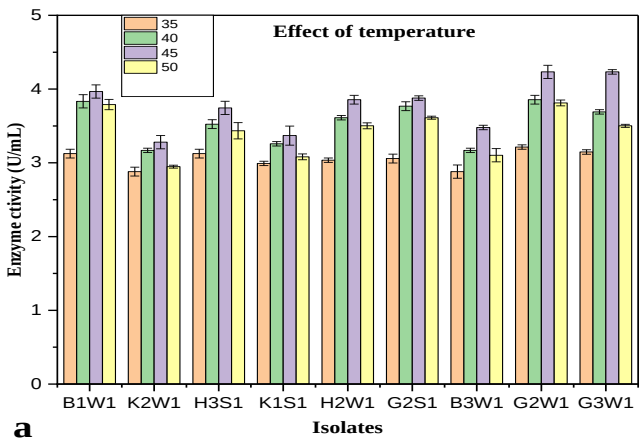
Figure 15: Overall optimal enzyme activity

4.5. Characterization of crude enzyme

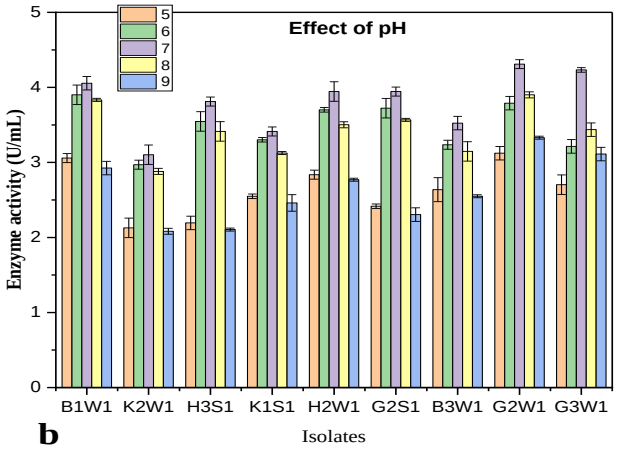
Figure 16 showed enzymological characterization of the crude enzymes extracted from nine isolates. The effect of temperature (a), the effect of pH (b), the effect of metal ions (c), the effect of reaction time (d), thermal stability of treated enzyme for 30 min (e), and thermal stability of treated enzyme for 45ºC for 30 and 60 min, (f) thermal stability of treated enzyme for 55ºC for

30 and 60 min, (g) thermal stability of treated enzyme for 65ºC for 30 and 60 min on amylase activity.

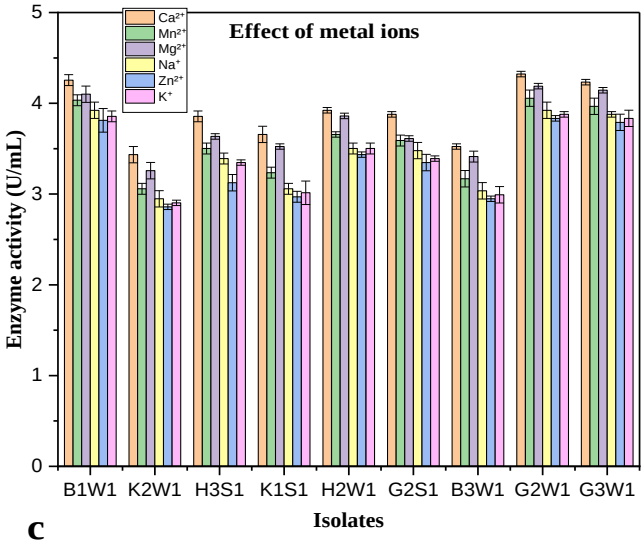
The alpha amylase extracted from the isolates showed high enzymes activity at 45ºC which is slightly greater than the isolates optimum temperature for the enzyme production. The optimum pH of alpha amylase was the same with that of isolates at pH 7. Crude enzymes extracted from isolate G2W1 and G3W1 were the most active in alkaline conditions than acidic conditions when they compared with other isolates' enzyme. The metal ions showed high activity with Ca^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, Zn^{2+}, and K^{+} in respective orders. Among metal ions, Ca^{2+} is the most active ion that enhanced the enzyme activity for all isolates. In the case of reaction time, the amylase activity was decreased with the increasing reaction time and the best reaction time was at 15 min. In thermal stability, when the enzymes pre-treated by incubating at 45, 55, 65ºC for 30 and 60 min, the residual activity of alpha amylase was decreasing as an increase of temperature and time. At 45ºC for 30 min, the residual activity was retained above 90% of activity while for 60 min, all were ≥ 86 %. At 55ºC for 30 min ≥ 80 % and after 60 min, it decreased to below 70%. The residual activity of alpha amylase was still retained greater than 53% of activity at 65ºC when treated for 30 min, but in 60 min, only < 40% was retained compared to the original activity when treated for 60 min. These findings suggest that the enzymes have good thermal stability at moderate temperatures, but is significantly compromised at higher temperatures.



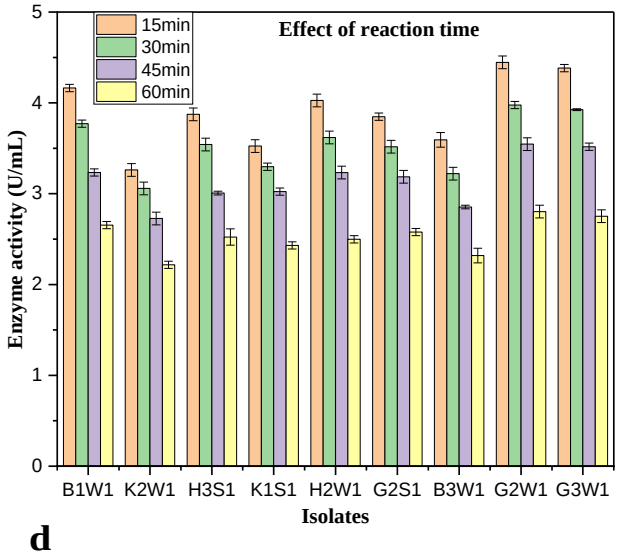
a



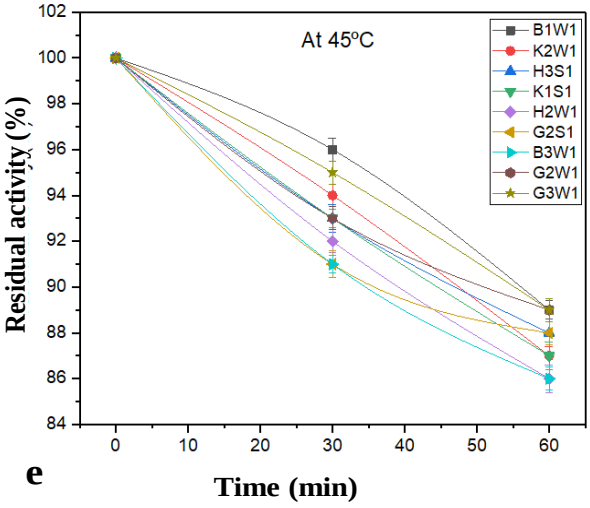
b


Effect of metal ions
Ca²⁺
Mn²⁺
Mg²⁺
Na⁺
Zn²⁺
K⁺
Enzyme activity (U/mL)
B1W1 K2W1 H3S1 K1S1 H2W1 G2S1 B3W1 G2W1 G3W1
Isolates

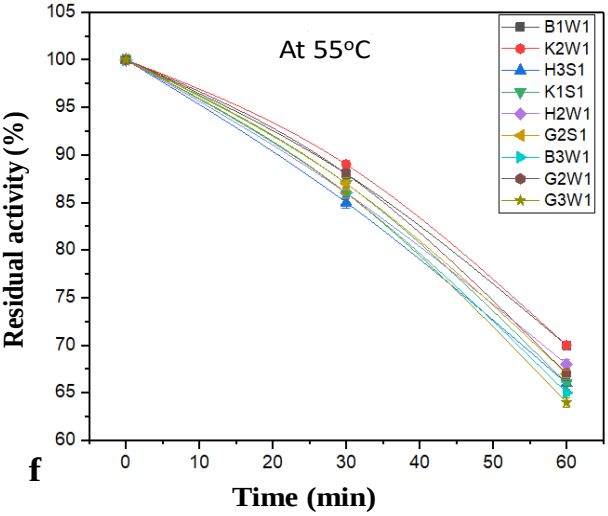
c


Effect of reaction time
15min
30min
45min
60min
Enzyme activity (U/mL)
B1W1 K2W1 H3S1 K1S1 H2W1 G2S1 B3W1 G2W1 G3W1
Isolates

d


At 45°C
B1W1
K2W1
H3S1
K1S1
H2W1
G2S1
B3W1
G2W1
G3W1
Residual activity (%)
Time (min)

e


At 55°C
B1W1
K2W1
H3S1
K1S1
H2W1
G2S1
B3W1
G2W1
G3W1
Residual activity (%)
Time (min)

f

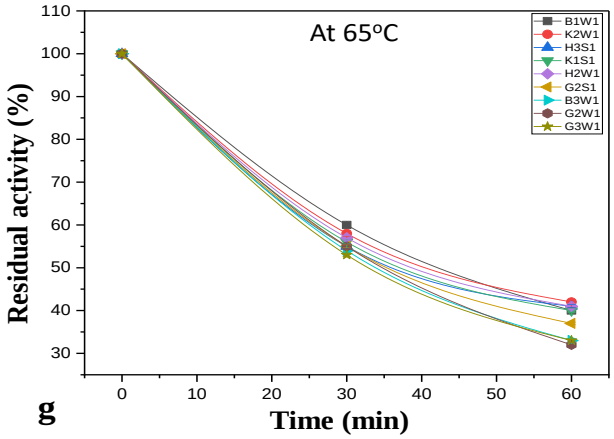


Figure 16: Enzyme characterization

4.6. Partial purification and Molecular weight of alpha-amylase

Bacillus cereus (H3S1) was employed to SDS-PAGE for partial purification using ammonium sulphate precipitation and dialyzed in 0.1 M phosphate buffer for 24 h. The 60-80% fraction and crude extract were loaded to the gel along with protein ladder for estimation of amylase molecular weight. Crude extract had almost no clear bands, but the 60-80% had displayed bands. Based on the molecular weight marker, the amylase band was observed around 50 kDa as shown in the Figure 17.

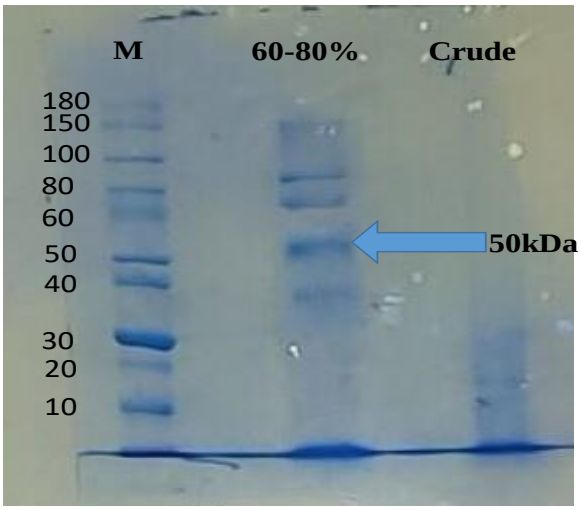


Figure 17: SDA PAGE result

4.7. Specific activity

Specific activity was calculated using Bradford method with standard curve of egg albumin. Initially, the total protein concentration was 46.9 mg/mL with a total activity of 201.5 U/mL for crude enzymes. However, the amount was reduced to 6 mL with a total protein content of 5.9 mg/mL following the precipitation of the crude enzyme using ammonium sulphate at 40–60%. At 60-80%, the volume was further decreased to 3mL with a total protein of 3.2 mg/mL. The maximum specific activity of 4.3 U/mg of protein was obtained from crude enzyme extract. While the partially purified enzyme scored 8.0 and 10.1 U/mg. Specific activity was 2.3-fold compared to the crude enzyme.

Table 7: Crude and partial purified specific activity of alpha amylase

Step	**Volume of fraction (mL)**	**Total protein (mg/mL)**	**Activity (U/mL)**	**Total activity (U/mL)**	**Specific activity (U/mg)**	**Fold**	**Yield (%)**
Crude enzyme	50	46.9	4.03	201.5	4.30	1	100
(40-60 % of $(NH_4)_2SO_4$)	6	5.9	7.9	47.3	8.0	1.9	23.5
(60-80% of $(NH_4)_2SO_4$))	4	3.2	8.2	32.6	10.1	2.3	16.2

4.8. Application of enzyme

Two types of soap were made, one with enzymes from isolates of G2W1 and one without enzymes as mentioned in the method part. This soap was then applied to pieces of clothes contaminated with potato waste, as well as uncontaminated clothing for comparison (control.) The results showed that better than soaps without enzymes. After washing, the piece of cloth treated with enzyme-containing soap had more clarity, indicating that the enzyme successfully broke down the starch in the potato waste. This increase in cleaning efficiency in particular shows the potential use of microbial enzymes in laundry to remove starch stains as the current study.

5. Discussion

B. cereus are gram-positive, rod shape, spore forming, and motile bacteria and known to produce several industrially important enzymes, including alpha amylase (Bottone, 2010). It is widely distributed in nature, and offers a numerous uses across several industries. It is commonly known as a soil-dwelling bacterium, but a study demonstrated its presence in aquatic environments, including lakes and other water bodies (Glasset et al., 2016). During rainfall, *B. cereus* spores can be washed from soil into lakes and rivers, where they persist in water and sediments (Jensen et al., 2003). Additionally, groundwater movement plays a role in the dispersal of *B. cereus* into aquatic systems, as spores can leach into underground water sources and eventually mix with surface water (Vilain et al., 2006). The ability of *B. cereus* to form biofilms on surfaces such as sediments, aquatic plants, and even industrial water pipes further supports its adaptation to water environments (Houry et al., 2010). The presence of *B. cereus* in lakes and other water bodies can also be linked to human activities such as agricultural runoff, wastewater discharge, and contamination from food processing facilities, further supporting its role as both a soil-associated and waterborne microorganism.

Many extracellular enzymes are known to be produced by *Bacillus,* and these enzymes have a broad range of industrial uses (Bakri et al., 2012). The good production of this particular enzyme has been observed in study (Abo-Kamer et al., 2023a). It has been found that several *Bacillus* species, including *Bacillus cereus,* produce alpha amylase (Gebreselema Gebreyohannes, 2015).

Aeromonas species are known as indigenous to aquatic environments and causes many opportunistic infections in humans. *A. rivipollensis* are among these opportunistic pathogens and reported as novel species (Marti & Balcázar, 2015). *A. rivipollensis* are motile, rod shape, gram negative, non-spore forming bacteria (Fono-Tamo et al., 2023). Previously, it was not reported as alpha amylase producing bacteria, but it has highest amylase activity in the present study. *K. pasteurii* are also considered as an opportunistic pathogen to humans. These bacteria are rod-shaped, gram-negative, non-motile, and do not generate spores (Merla et al., 2019). The production of alpha amylase by *K. pasteurii* is also not well studied, but a study showed that *K. oxytoca* has an ability to produce many enzymes including alpha amylase (Yang et al., 2022),

while it was the second most alpha amylase producing bacteria in this study. According to Yang et al., 2022, *K. grimontii, K. huaxiensis, K. michiganensis, K. pasteurii, K. spallanzanii,* and three unidentified novel species make up the nine species that named as *Klebsiella oxytoca*. This group could produce different enzyme including amylase and it may include *K. pasteurii*.

The optimum temperature for most of the isolates was found to be at 40ºC. Increasing the temperature above this level resulted in decreased activity of the enzyme, likely due to denaturation of the enzyme and the isolates entering unfavorable conditions. During sample collection, the environmental temperatures were measured in the range of 28-35ºC. However, the optimum temperature of isolates was above its environmental temperature. A similar finding was reported (Nimisha et al., 2019). The optimum pH of the isolates was at 7. The incubation time for maximum enzyme activity was 24 h, and above this level, the enzyme activity was shown to be declined. This could be because the isolates produce secondary metabolites that aid in the quick depletion of nutrients in the medium and inhibitory compounds that could reduce the catalytic effectiveness of the generated enzyme (Mishra & Behera, 2008).

In case of biochemical test, all the isolates that identified as *B. cereus* were positive for methyl red test (changed glucose phosphate broth to red color) when a few drops of methyl red were added which indicates acid formation. This test indicated that the isolates (*B. cereus*) have the ability to ferment glucose to produce organic acid like lactic acid, formic acid, succinic acid, acetic acid and propionic acid. Many strains of *B. cereus* have positive results in the methyl red test, indicating their ability to perform mixed acid fermentation (Suganthi et al., 2011). While other studies also reported as negative for this test. The variability may be the potential for different strains to exhibit unique fermentation capabilities (Oguntoyinbo & Oni, 2004). All the *B. cereus* in this study were also positive for catalase test which showed breaks down of hydrogen peroxide (H_2O_2) into water and oxygen, helping to protect the cell from oxidative damage. Whereas the isolates were negative for urease test and positive for citrate test similar with other study that reported in *B. cereus* (Al-Dhabaan, 2019).

The isolate identified as *A. rivipollensis* in this study showed characteristics features. This bacterium is catalase positive, capable of producing catalase, which helps to breakdown of H_2O_2 to H_2O and O_2 form bubbles. *A. rivipollensis* ferments glucose, producing acid as a by-product,

and is confirmed with red color change in methyl red glucose broth and negative for Voges Proskauer test (could not produce acetoin during glucose fermentation). On the other hand, *A. rivipollensis* is urease-negative, so it does not hydrolyze urea, resulting in no color change in urea-based media. This species can also utilize citrate as a sole carbon source, evident from a color change to blue on Simmons citrate agar. Each of these tests contributes to a comprehensive biochemical profile that is useful for accurately identifying *A. rivipollensis* and understanding its ecological roles, particularly in aquatic environments as the current study and the other study also found the same result (Marti & Balcázar, 2015). The other related study also identified that biochemical test of *Aeromonas* species are facultative anaerobes with positive catalase and oxidase levels (Abbott et al., 2003 and (Vandijck & Blot, 2007)

The isolate identified *K. pasteurii* was also tested for the above biochemical test. In general, *K. pasteurii* had a negative Voges-Proskauer test, which emphasizes its ability to produce acetoin during glucose reaction, whereas the methyl red test is positive. In the citrate utilization test, *K. pasteurii* showed positive results, indicating that citrate can be used as a sole carbon source. *K. pasteurii* indicated catalase positive test (the ability of the bacterium to break down hydrogen peroxide, indicating its defense against reactive oxygen species). A positive catalase test, as is common in many members of the genus *Klebsiella*, is found indicating whether aerobic or facultative anaerobic characteristics (Yang et al., 2022). The bacterium was the only urease positive from the current isolates which distinguished it from other species. It exhibits urease activity, breaking down urea into ammonia and carbon dioxide, which is characterized by a change in pH and a pink color in urea media. This ability of urea hydrolysis can be of interest especially in the technical and environmental fields, as it can support biocementation processes. This result is the same with the study of (Merla et al., 2019).

The addition of carbon sources, such as mono- or disaccharides, affected the production of amylase. Glucose had a greater (3.4 ± 0.1 U/mL) in this study than the other carbon sources examined for all isolates and high activity was scored by G2W1), which identified as *A. rivipollensis.* The second-best source of carbon was sucrose (3.3 ± 0.03 U/mL) by G3W1 or *K. pasteurii*. Many studies have demonstrated that different carbon sources have variable effects on the production of extracellular enzymes, particularly in strains of *B. cereus* that have been found

to produce high levels of amylase. For example, studies show that *B. cereus* tends to increase amylase production when grown in the presence of starch as a major carbon source. This phenomenon is primarily due to the bacterial adaptation to better utilization of starch, leading to gene expression of amylase metabolism has been revealed (Miller, 2014). In this study, starch is used as basal media for amylase production. Related research study was reported by (Vijayabaskar et al., 2012), who found that glucose increases amylase production. In contrast, sucrose is used best for amylase production. This may be due to the catabolite repression effect, whereby the presence of a weak carbon source down-regulates the enzymes required for the degradation of complex carbohydrates (Soni et al., 2020).

In this study, ammonium chloride was the best nitrogen supplement for the isolates identified as *B. cereus* for alpha amylase production, and beef extract and ammonium chloride were the best as organic and inorganic nitrogen sources, respectively. The highest activity among *B. cereus* recorded as 3.3 ± 0.03 U/mL by B1W1. This report is the same as the study reported by (Sivakumar et al., 2012). In the isolates that were identified as *A. rivipollensis* and *K. pasteurii*, tryptone as organic nitrogen source and ammonium chloride as inorganic nitrogen source. 3.4 ± 0.03 U/mL and 3.3 ± 0.1 were recorded by G2W1 and G3W1, respectively. This is related with the study of (van Bel et al., 2021), but not directly those strains in alpha amylase production as mentioned above. Depending on the source of the enzyme, metal ions and trace elements are frequently required to supplement in the fermentation media for the best production of amylase. According to the current findings, Mn for *K. pasteurii*, K *for A. rivipollensis* and Zn for *B. cereus* raises the production of the alpha amylase production. Compared to other metal ions, calcium chloride increased enzyme activity(Vijayabaskar et al., 2012). The other metal ions checked in this were also needed for the isolates and not as inhibitors.

For isolate *B. cereus*, *A. rivipollensis*, and *K. pasteurii*, which depend on Na^+ for growth and alpha amylase production, sodium chloride is a crucial nutrient component for them. This study found that a concentration of 1.5% NaCl was appropriate for amylase production. The enzyme activity was gradually reduced above this concentration (Mahdavi et al., 2010). This is because the cell gets into hypertonic condition if the environment is a saltier condition leading to plasmolysis (where cells lose water) and inhibiting the growth and enzyme production.

Increasing the concentration of starch was increased the growth of isolates and 1.5% was shown to give the greatest yield of alpha-amylase. The high starch results in the release of toxic metabolic wastes that inhibit bacterial growth and alpha-amylase production. Moreover, elevated starch concentrations made the broth culture more viscous, which hindered O_2 transport and limited the amount of dissolved O_2 needed for microbial development (Mishra & Behera, 2008).

After optimizing the nutrient condition as their need for enzyme production, means at optimum temperature, pH, incubation time and carbon source, organic and inorganic nitrogen source, metal source, starch concentration, the activity of all isolates was assayed. The maximum activity of alpha amylase was recorded as 4.5 ± 0.1 U/mL for *A. rivipollensis* (G2W1), followed by 4.3 ± 0.04 U/mL for *K. pasteurii* (G3W1) and 4.1 ± 0.1 U/mL for *B. cereus* (B1W1). Further studies showed that the optimum conditions for crude alpha-amylase activity was 45°C of temperature, pH 7, reaction time of 15 min. In these conditions, the enzyme showed the highest activity measured at 5 ± 0.04 U /mL, as obtained by *A. rivipolensis* (G2W1). One major finding in this study is that the enzyme activity was much higher in the presence of Ca^{2+} ions when compared to other metal ion cofactors examined. This suggests that calcium ions has significant effect in the enhancement of alpha-amylase activity (Liao et al., 2019). The three species of the alpha amylase produced in this study efficiently work at 45ºC and pH 7 and can be used in industrial application, which match with this temperature, but it needs highest level of purification of this enzyme in order to apply for the food industry. This is mainly because the isolate is considered as an opportunistic pathogen to humans. Although alpha amylase itself is not toxic, the presence of this pathogen may lead to a serious risk if the enzyme is not properly purified. It is therefore important to purify to high level of purification standard to ensure that alpha amylase from the current isolates can be safely used in the food processing industry.

The specific activity was calculated and showed increase from 4.3 U/mg in the crude sample to 10.1 U/mg in the partially purified fraction, increasing the activity by 2.3-fold. SDS-PAGE performed to detect the presence of alpha amylase using protein ladder. A band of approximately 50 kDa was detected after sample loading which showed significant evidence of alpha amylase. This band is the expected molecular weight of alpha amylase, typically 40 to 60 kDa showing the presence of alpha amylase in the protein sample (Marco et al., 1996). This finding confirms

that the purification procedure was performed correctly, indicating that the proteins in the samples are compatible with alpha amylase. This is important for research because a purified enzyme is required for accurate functional analysis. Other small bands near major clusters may represent these isoforms or their potential degradation products. This step is necessary to enable the enzyme to be used in a variety of biochemical applications. Overall, detection of a band of approximately 50 kDa in the SDS-PAGE analysis is a promising indicator of the presence of alpha amylase in the sample.

6. Conclusion

In general, the current research indicates that it is possible to isolate bacteria that produce amylase from the soil and water of Bishoftu lakes. The isolate produced efficient yield of amylase by using submerged state fermentation method. The study confirmed that *Bacillus cereus*, *A. rivipollensis*, and *K. pasteurii* exhibited significant enzyme activity under optimized conditions. The enzyme could effectiveness in breaking down starch and its successful application in detergent formulation highlight its industrial potential. The findings demonstrate the value of locally isolated microbes in enzyme production, reducing reliance on imported enzymes. Further research should focus on enhancing enzyme stability and scalability for commercial applications.

7. Recommendations

- Since the isolates are opportunistic pathogen, further purification of alpha amylase is required to use for food and pharmaceutical industries. Further research is recommended on high purification method like chromatography.
- Culture condition like pH, temperature and nutrient sources should be further optimized to increase the production yield of amylase.
- The current study showed that alpha amylase was successfully applied in detergent. However, future research can investigate the use of this alpha amylase from the stain to biotechnological application like antibiofilm, textile industry, and bioethanol production.

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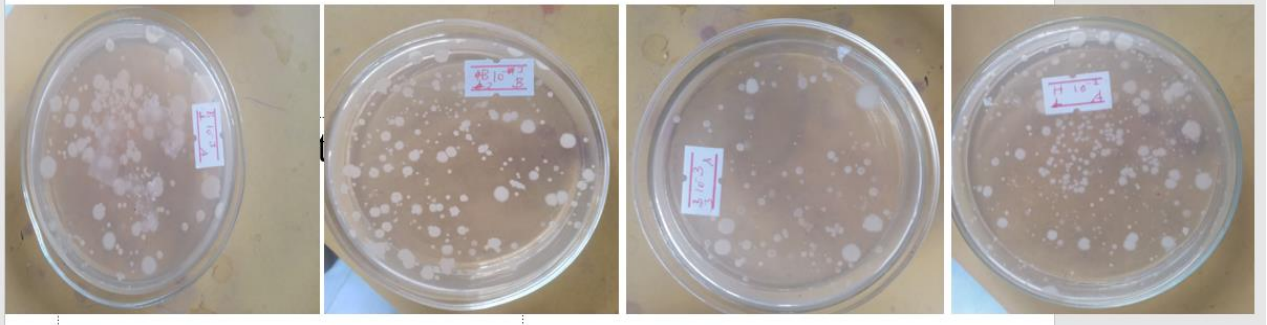
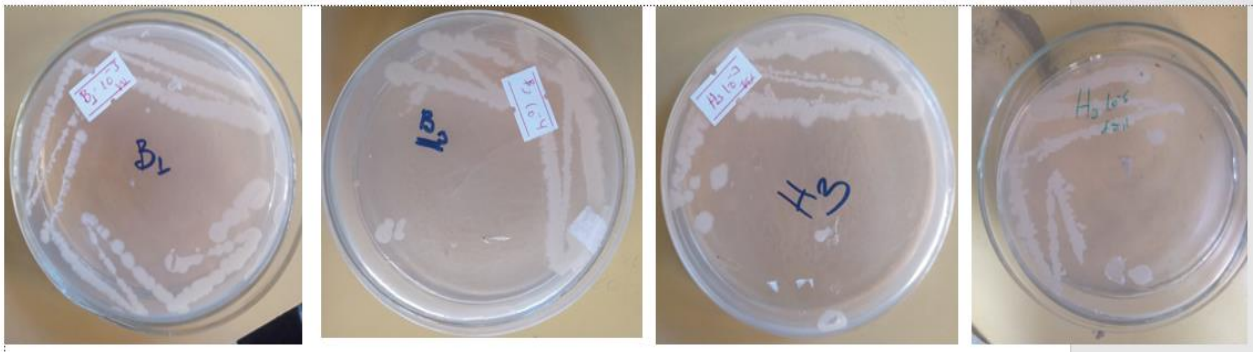
Appendices

Counted colony sample

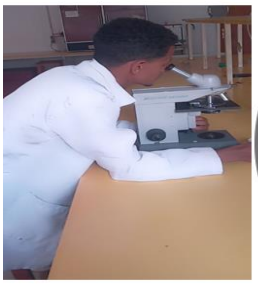


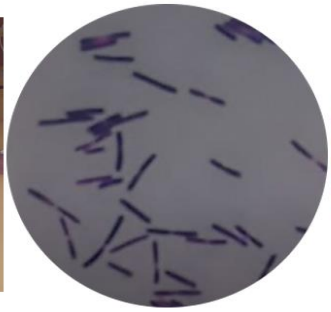
Streak colony samples



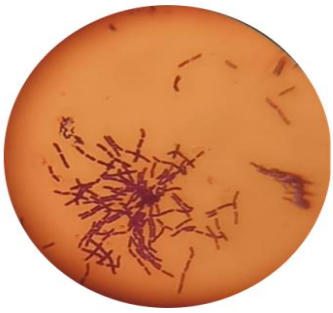
Iodine test sample

Microscopy

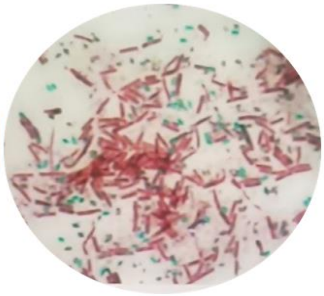




Gram positive

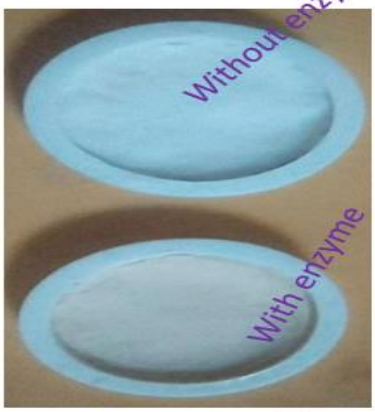


Gram negative sample



Spore positive

Soap formation



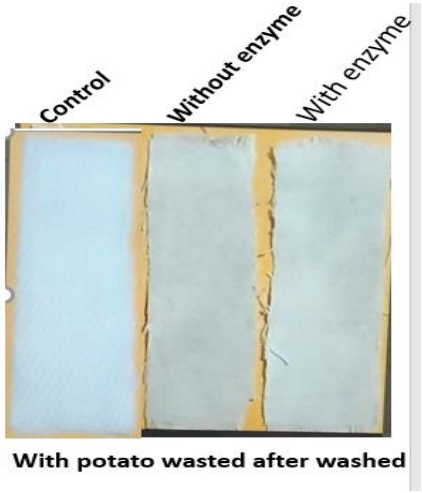
Soap in mold

Formed soap

Enzyme application

Control

With potato waste before wash



With potato wasted after washed