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SCHOOL OF GRADUATE STUDIES



College of Natural and Computational Science

**SCREENING AND CHARACTERIZATION OF THERMOSTABLE ALPHA- AMYLASE FROM
BACTERIA ISOLATED FROM HOT ENVIRONMENTS**

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DECLARATION

I, declare that this research paper entitled, “Screening and Characterization of Thermostable Alpha-Amylase from Bacteria isolated from selected hot Environments found in Addis Ababa” is the result of my own efforts and which conducted for the Partial Fulfillment of the Requirement for the Degree of Master of Science in Applied Microbiology at the College of Natural and Computational Science Addis Ababa University. This research project has completed under close advice and guidance of my research advisors Dr Fitsum Tigu and Dr Asnake Desalegn.

Abbreviation and Acronym

APS-Ammonium persulfate

ANOVA- Analysis of variance

DNSA-Dinitrocyclicalic acid

EC-Enzyme Commission

ICT- Information Communication Technology

M-marker

MR- Methyl red

OFAT- One factor at a time

RNA-Ribonucleic Acid

rpm- Revolution per minute

SDS-PAGE- Sodium dodecyl sulfate polyacrylamide Gel Electrophoresis

SmF- Submerged state fermentation

TEMED-Tetramethylethylenediamine

TIM- Triose phosphate isomerase

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Abstract

Alpha-amylase is one of the most important enzymes for undergoing in starch saccharification and liquefaction to results simple sugar. Despite there were numerous resources to isolate thermostable alpha-amylase producing bacteria however little research was conducted in our country. The objective of this study was to screen, and characterize thermostable alpha-amylase producing bacteria. A totally of 35 samples were gathered from selected hot environments around Addis Ababa. A total of 188 bacterial isolates were screened for alpha- amylase production through starch hydrolysis test. Of which, 161 isolates were obtain to be positive for starch hydrolysis test. Sixty-six isolates with amyolytic index ($AI \geq 1.5+0.3$ mm) were selected for submerged state fermentation and the enzyme assay was conducted through DNSA method. Based on their fermentation performances, 6 isolates, FBW2-3A, FHW5-1A, KS1-1A, SJS1-4A, KHW2-2A and SAS1-2A were selected for further amylase production optimization process. All the six bacterial isolates were phenotypically and molecularly characterized into species level. They belonged to *Bacillus cabrialesii* (FBW2-3A), *Acinetobacter haemolyticus* (FHW5-1A), *Enterobacter quasiharmachei* (SJS1-4A), *Bacillus cereus* (KHW2-2A) and *Pseudomonas plecoglossicida* (SAS1-2A) while KS1-1A isolates was not identified. The effect of temperature, pH, incubation period, carbon and nitrogen sources, starch and salt concentration, metal ions and inoculum size were optimized based on one factor at a time approach. For all isolates the optimal growth temperature, pH and incubation time was obtained at 50°C, pH 7 and 24 h, respectively. Crude alpha-amylase was optimally active at 55°C with pH 7. The crude enzyme was thermally stable above 90% at 55°C for 30 min incubation. Partially purified amylase from *Bacillus cabrialesii* (FBW2-3A) has a specific activity of 10.12U/mg, which is 1.8-fold than the crude enzyme with molecular weight lies approximately 20kDa. The food industrial efficiency of the alpha amylase was investigation by applying of crude alpha-amylase into wheat flour fermentation and it showed a good dough raising capacity.

Key terms: Alpha-amylase, starch, characterization, optimization, application.

1. Introduction

1.1. Background

Enzymes are biological catalysts that help to initiate and speed up chemical reactions. An enzyme is an organic substance that is a protein by origin, colloidal in nature, and synthesis by living things that catalyze various chemical (Bhatia, 2018). They are essential for nearly every biological process; they regulate the energy required for genetic information routes and speed up range of metabolic events (Wei et al., 2021). Enzymes have unique characteristics such as the ability to synthesize compounds under benign reaction circumstances and the conversion of substrates into the desired products (Elmarzugi et al., 2014). Enzymes are sharply specific in their action on substrates to change into product (Fang et al., 2021). Enzymes have different application and have been used since ancient times in an empirical manner and specialized knowledge has been passed down from generation to generation housewives, cooks, and craftsmen (James & Simpson, 1996 and McGlynn, 2020). Currently, there are many enzymes which are applied in many industries, including food, detergent, textile, pharmaceutical, paper and saccharification starch, (Chapman et al., 2018). There are many enzymes which are applied in many industries. It breaks down starch molecules into small fragments like dextrin, maltose and glucose, through the hydrolysis of glycoside bonds. It is among the most significant classes of industrially utilized enzymes, accounting for around 30% of all enzyme products worldwide (Sivaramakrishnan et al., 2006 & Mondal et al., 2022). Amylase is derived from a variety of sources, including microorganisms, fungi, plants, and animals (Ahmad et al., 2020). Among several sources now a day, microbes are a more intriguing source of amylase and extensively employed in industrial processes due to their extensive availability, low cost, high productivity, chemical stability, preservation of the environment, and versatility (Chovatiya et al., 2014). Microorganisms live everywhere and occupying a variety of ecological niches, including as compost, deep organic dumps, volcanic areas, hot springs, and deep oceanic environments (Salano et al., 2017). However, they have been widely investigated in hot springs around the world and are abundant in nature because it is most important habitats for thermophilic microorganisms (Aanniz et al., 2015b). Thermophilic microorganism can live and grow 40-90°C but they grow an optimal between 55 and 80°C whereas growing best hyper thermophiles develop over 80°C (Medová et al., 2013). These microorganisms thrive and adapt such as high temperatures through unique molecular processes. These adaptations modify the thermal stability of their proteins, lipids, nucleic

acids, and cell membranes, allowing them to function effectively even in such hostile conditions (Yassin et al., 2021). The thermal adaption of thermophilic microorganisms best source of thermostable amylases which can fit the need of several industrial applications (Lévêque et al., 2000 & Sharif et al., 2023). A desirable feature of the majority of commercial enzymes is thermostability(Ullah et al., 2021). According to Vaseekaran et al., 2011 *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α -amylase, and these have been widely used for commercial production of the enzyme for various applications. Due to high thermal stability, decrease risk of contamination, increase high diffusion rate, resistance against chemical agents: and decreased viscosity the interest of thermostable amylase for industrial application is increasing at internationally level including Ethiopia(Liu & Kokare, 2023). Contamination and instability of enzymes at high temperature are among the big problem associated with industries that are working on mildly condition, in which we must undertake the fermentation activity under extremely circumstances where contamination is virtually free (Atsbha et al., 2015). Because of this, the majority of the starch-processing industries are built to run at elevated temperatures but when temperatures are high, pH, and other parameters are low or high, the mesozyme can only survive in mild environments readily become denatured(Pandey et al., 2000).Therefore finding of new bacterial strain which produce thermostable amylase for starch degrading in at elevated temperate and other parameter can solve this problem. One of the best alternative to study thermostable amylase producer microbes population are that can live in hostile environment such as hot environments. Therefore, this study aimed to screened and characterized thermostable α -amylase-producing bacteria and optimized and as well as characterization of α -amylase with different parameters and tested food industrial application of crude α -amylase.

1.2. Statement of the problem

Thermostable amylases are mainly obtained from thermophilic microorganisms which commonly found in a number of different hostile and extreme geographical habitats all over the world including Africa (Zahoor et al., 2016). Ethiopia is part of East Africa country and the fortunate to have a variety of geographical feature like low land (Rift valley), highland and different climatic zones, and hot springs as well as hostile habitats are home to a wide variety of microorganisms and found in different parts of the country (Husen et al., 2012). Addis Ababa is the capital city in which variety of hot environments is found and which can be best source for thermostable alpha-amylase producing bacteria. Despite this enormous resource little's research has been conducted in some hot springs and other area to identify thermostable alpha-amylase producer bacteria. This leave a gap in understanding the full potential of Addis Ababa's hot environments as source of thermostable alpha-amylase producing bacteria and also being identified alpha-amylase producer bacteria did not scale up for production of alpha-amylase to deliver its sufficient amount for industrial application in our country. Because of this, Ethiopia is used imported microbial enzymes for a different of industrial applications in order to meet their growing demands. For example in Ethiopia more than 40,000 ton of malt with a cost of about US\$ 40 million is imported every year and about 25 -30% of the malt is replaced by imported amylase (Gessesse et al., 2011). Therefore, this research was aimed to screen and characterize thermostable alpha-amylase producing bacteria from selected hot environment around Addis Ababa for testing towards the biotechnological application of this enzyme to different industries. Therefore this research was aimed to screened and characterized thermostable alpha-amylase producing bacteria from selected hot environment found in Addis Ababa and also characterization of thermostable alpha-amylase and tested its industrial application.

1.3. Objective of the research

1.3.1. General objective

This study was aimed to isolate, characterize and optimize the thermostable alpha-amylase producing bacteria from selected hot environments found in Addis Ababa.

1.3.2. Specific objective

- To screen and characterize bacterial isolates for alpha-amylase production
- To phenotypic and molecular identification of alpha-amylase producing bacteria
- To optimize alpha-amylase producing bacteria by one factor at a time approach
- To characterize alpha-amylases with different enzymological parameters
- To purify and evaluate the biotechnological potential of alpha-amylases in food industry application

1.4. Scope of the study

Alpha-Amylase producing bacteria were found almost in all types' of environments but this research focused on selected hot environments (Qoshe) which were found in Addis Ababa.,

1.5. Significance

Being this study has been conducted, to screened and isolated of thermostable alpha-amylase producing bacteria, in which used a source of alpha-amylase that may be applied in numerous industrial fields, including the food, textile, paper, and detergent industries by scale upping its production. Because finding of new amylase producing bacteria may be a source of beset alpha-amylase for industrial processes, which in turn to minimize the need of importing amylase and encourage its production in our country.

2. Literature Review

2.1. Starch

Starch is a polymeric carbohydrate made up of several glucose units and the most prevalent type of stored sugar in plants and the main source of meals for humans (Putri & Nakagawa, 2020). It is made up of two components; amylose, a linear polymer of α -D-glucose units linked by α -1,4 glycosidic connections, and amylopectin, a branching polymer of α -D-glucose units linked by α -1,4 and α -1,6 glycosidic linkages (J. Singh et al., 2010). Amylose is basically a linear molecule with a molecular weight ranging from 5×10^5 to 10^6 , consisting of anhydroglucose units joined by α (1,4) bond (Takagi & Hizukuri, 1984). The low degrees of branching and structure with 3–11 chains of roughly 200–700 glucose residues per molecule are caused by the less than 0.5% of glucoses in amylose that have α [1/6] links. Depending on where the branches are placed within the structure, the low degree of branching in dissolved amylose can lead to the formation of insoluble semi-crystalline aggregates (Huang et al., 2015). Amylopectin has larger size, a molecular weight of roughly 10^8 and a potential double bond of more than one million (Mezeal et al., 2019). Amylopectin has a highly branched, tree-like structure with a complicated molecular architecture that can vary significantly between different starches in terms of branch length and location (Ortega-Ojeda et al., 2004). Depending up on the plant, starch generally contains 20-55% amylose and 75-80% amylopectin. Plant roots like those of cassava, potatoes, arrowroot, and other plants can be used to obtain starch. It is broadly utilized in food and industrial applications as a thickener, colloidal stabilizer, gelling agent, bulking agent, and water retention agent. It significantly enhances the textural qualities of many foods (Batchelor et al., 1996) (Farooq et al., 2021). Therefore the starch needed hydrolysis agent in industrial application to use it as raw material in order to hydrolyze into smaller oligosaccharides. So for this purpose amylase is well known and one of the most widely used commercial enzymes. Especially α -amylases, one of the common enzymes that hydrolyze starch, in which essential for starch processing sector. Since they break down α -1,4-glycosidic bonds in starch, they are also known as glycoside hydrolases (Van Der Maarel, Van der Veen, et al., 2002 & Kaur et al., 2021).

2.2. Amylase

Amylase is one of the earliest identified enzymes. It was discovered by Kirchoff(1815)and the first starch-degrading enzyme the enzyme(Gojiya et al., 2021). Amylase is utilized to break down starch molecules into smaller polymers made of glucose units. It is an extracellular enzymes that hydrolyze starch molecules to produce a variety of products, including dextrin's and ever smaller polymers made of glucose units(Windish & Mhatre, 1965&Sobhy et al., 2023).It produce from different living sources such as human body and as food is chewed, it mixed with salivary amylase breaks down starch containing food into smaller molecules and is neutralized by gastric acid and the starch is partially breakdown and goes on to smaller intestine for further break down by pancreatic amylase into glucoses. Amylase belongs to amylolytic enzymes which act on starch and related polysaccharides and applied in different starch industries(Xia et al., 2021) . All amylases are glycoside hydrolases and act on α -1, 4-glycosidic bond. Amylase can be classified as exo-amylase and endo-amylase(Ahmad et al., 2019).

Endo-amylase: randomly breaks the a-1,4-D-glycosidic bond between adjacent glucose units in the product chain while keeping the product's anomeric carbon structure and also referred to as a-amylases, are well known for cleaving internal a-1,4 bonds to produce a-anomeric compounds(Li et al., 1992&Srishti et al., 2022).

Exo-amylases: acting on the non-reducing ends of polysaccharides, to generate low molecular weight products like glucose and maltose. Some enzymes only cleave a-1,4-glycosidic bonds (like b-amylase) or both a-1,4 and a-1,6-glycosidic bonds (like glucoamylase and glycosidase)(Rana et al., 2013).

2.3. Classification of amylase

The conversion of polysaccharides like starch and glycogen into short-chain sugars is catalyzed by amylases. These enzymes have a crucial function in metabolism and the most common enzyme in starch conversion process both in the body of living things and in industrial process. Three varieties of amylases have been identified based on their catalytic function and enzyme structure: α -amylase (EC 3.2.1.1), β -amylase (EC 3.2.1.2), and γ -amylase (EC 3.2.1.3)(R. S. Singh et al., 2019)

2.3.1. Alpha-amylase

Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α -1,4-O-glycosidic bonds in polysaccharides with the retention of α -anomeric configuration in low molecular weight products, such as glucose, maltose, and maltotriose units (Patil et al., 2022). The final byproducts of the amylase activity are variable-length oligosaccharides with α -configuration and limit dextrins, which comprise a blend of branched oligosaccharides with 6–8 glucose units that contain both -1,4 and -1,6 links, as well as maltose and maltotriose (Whitcomb & Lowe, 2007 & Fincan et al., 2021). Alpha-amylases are among the most significant industrial enzymes with a wide range of applications, including the conversion of polysaccharide compound (starch) into simple sugar (Fernandes et al., 2020). It plays a significant role in the starch hydrolysis process by decreasing viscosity, increasing the number of starch chain molecules, and decreasing the starch chain molecular weight (Putri & Nakagawa, 2020). Because they are metalloenzymes, the majority of α -amylases depend on calcium ions (Ca^{2+}) for stability, structural integrity, and activity. These enzymes have a TIM barrel structure with an 8 (α/β) structure that includes the catalytic site residues and has four highly conserved areas where primary sugar syrups are produced for the pharmaceutical industry's cyclodextrins (Ranjani et al., 2014). The majority of microbial alpha-amylases have an ideal pH of 6 to 7 (Higashihara & Okada, 1974).

2.3.2. Beta amylase

Beta-amylase is another type of amylase that functions on the non-reducing end. It catalyzes the hydrolysis of α -1,4-glycosidic bonds, which cleaves glucose into maltose. In branched polysaccharides like glycogen and amylopectin, beta-amylase cannot cleave the branched connections; as a result, the hydrolysis is not fully completed, leaving dextrin units behind (Parés Viader et al., 2021). Beta-Amylase, is a true saccharifying enzyme, forming maltose directly from starch by cleaving disaccharide units from the open ends of chains (De Schepper et al., 2021). It is not dependent on calcium, is responsible for facilitating the breakdown of α -1,4-glycosidic bonds at the non-reducing end of starch molecules, resulting in the production of a maltose unit. It is an industrially important glycosidase that is used in the production of high-maltose syrup, food products and glycosylation of pharmaceutical drugs (Genghof, 1969). Currently, most of the β -amylases used in the industry are derived from cereals such as barley and soybean, but the demand

for cereals is increasing every year, and there are concerns about the stability of supply due to competition with their use in the food industry(Murakami & Osanai, 2022). The molecular weight of microbial Beta-amylases was found to vary. Beta-Amylases of low molecular weight were reported from *Thermoactinomyces* sp. (3.16×10^4) by (Ray & Nanda, 1996).

2.3.3. Gamma amylase

A type of amylases that can cleave alpha 1,4-glycosidic bonds and alpha 1,6-glycosidic connections in amylose and amylopectin are known as gamma amylases(Vaidya et al., 2015). Gamma amylase (EC3.2.1.3) not only cleaves the final (1-4) glucose bond, but it also cleaves the (1-6)-glucose bond.-glucose link between the non-reducing end of amylose and amylopectin, as opposed to the other amylase form that produces glucose. Rapid hydrolysis of 1, 6- α -D-glucosidic bonds can be achieved by most kinds of glucoamylase when 1,4- α -D-glucosidic bonds follow in the saccharide sequence(Norouzian et al., 2006).Furthermore, a subset of glucoamylases is capable of hydrolyzing 1,6- and 1,3- α -D-glucosidic linkages seen in other polymers. Comparatively faster on polysaccharides than oligosaccharides (Stuart, 1978). Gamma amylase has an ideal pH of 3 and is most effective in an acidic environment.(Saini et al., 2017).The majority glycosidic hydrolase families to which these gamma amylase enzymes may belong are the glycosidic hydrolase family of bacteria species.(Van Der Maarel, Van Der Veen, et al., 2002). Most commonly, glucoamylase is used to saccharify partially processed starch or dextran, converting it to glucose, a necessary substrate for a variety of fermentation processes as well as the food and beverage industries(P. Kumar & Satyanarayana,2009).

2.4. Biochemical properties of amylase

The substrate specificity amylase varies from microorganism to microorganism alpha-amylase has highest specificity towards starch(Yu et al., 2018). The optimal pH range for apha-amylase varies between 2 and 12. The optimal pH range for the majority of bacterial amylases is acidic to neutral. It was discovered that the ideal pH occasionally depended on calcium and temperature(Ceci & Lozano, 2002). The ideal temperature for amylase activation is correlated with microbial growth. The lowest temperature optima are reported be 25-30°C for *Fusarium oxysporium* (Crtary & Eddy, 1985) and highest 100-130°C for *Pyrococcus furiosus* respectively. There are numerous metal ions,

particularly have metal ions EDTA, EGTA, BST, iodoacetate, group reagent that inhibits the alpha-amylase, and in some cases temperature optima are reliant on calcium and sodium chloride (Y. chuan Wang et al., 2020).

2.5. Source of amylase

There are several sources of amylases, including plants, animals, and microbes. However microbial enzymes are extensively utilized in industrial processes due to their wide availability, low cost, high productivity, chemical stability, protection of the environment, and plasticity ((Elmarzugi et al., 2014). Among different sources currently microorganisms, especially extreme environment living and thermophilic microorganisms, such as bacteria archaea and fungi are more interesting source of amylase and best used for production of amylase and are known to serve high degree of industrial needs ((Lonsane & Ramesh, 1990)). Because of their heat stability, thermostable amylases that were separated from thermophilic organisms have found several industrial uses(Arbab et al., 2022). Thermo-stable amylolytic enzymes have been researched recently to enhance industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose, and maltodextrins(Zamost et al., 1991). This is because enzymatic liquefaction and saccharification of starch are carried out at high temperatures (100–110°C)(Vidya et al., 2011). Alpha-amylase can obtained from many fungi, yeasts, bacteria, and actinomycetes have produced alpha-amylase; yet, the majority of enzyme uses in industrial sectors have come from bacterial and fungal sources(Tanyildizi et al., 2005). The primary advantages of using microorganisms to create amylases are their capacity for inexpensive bulk production and their simplicity in manipulation to produce enzymes with the necessary characteristics(Kumari et al., 2019). Microorganisms are inexpensive, easy to manage, and require little space. They may multiply quickly, which speeds up the manufacturing of enzymes(R. Singh et al., 2022). In the help of genetic engineering, they are easily manipulated and susceptible to mutation, strain enhancement, and other changes that allow for optimum enzyme production (Sachdev et al., 2016). Microorganisms especially thermophiles are the best source of thermostable amylase. Although the various bacterial species *Bacillus spp.* are widely used for thermo-stable alpha amylase production to meet industrial needs(Rasooli et al., 2008). *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α -amylase, and these have been widely used for commercial production

of the enzyme for various applications (T. P. Souza et al., 2014). The capacity to withstand high temperatures is crucial since starch is liquefied, saccharified, and enzymatic processes all require it (Mohammad et al., 2017). The stability of thermo-stable enzymes derived from thermophiles microorganisms has led to a multitude of commercial uses etc. (Narang & Satyanarayana, 2001).

2.6. Diversity of microorganisms in hot spring and other extreme environment

Thermophilic microorganisms are occupying several biological niches, including hot springs, deep marine, volcanic sites, compost and deep organic landfills. However, they have been widely investigated in hot springs around the world and are abundant in nature. Hot springs have been identified as natural habitats that are ideal for thermophile colonization. Hot springs are some of the most important habitats for thermophilic microorganisms ((Aanniz et al., 2015a). Hot springs are springs that are geothermal heated and are located all over the earth, on all continents and even beneath the seas and oceans (Zahoor et al., 2016). Hot springs in Ethiopia are found in different region and climatic zone of the country like east, south and central parts Ethiopian including Addis Ababa (Asefa et al., 2020).

2.7. Hot environments in Addis Ababa

Addis Ababa is capital city of Ethiopia' and situated at an elevation of roughly 2000 meters above mean sea level entirely covered with volcanic rocks, including basalt, trachyte, ignimbrite, and rhyolite and surrounding by Entoto mountains to the North, the Wechacha Mountains to the West, Yerer Mountain and other volcanic cones to the East and South, and other high rising mountains encircle. Because of it is a part of Rift Valley in which frequent and varied volcanic eruptions the city Geology is primarily composed of volcanic rocks with acidic and basic compositions such ignimbrite, trachyte, rhyolite, and basalt.. There's not a big river that flows near Addis Ababa, though several smaller rivers cross from north to south (Engidasew & Abay, 2016). The presence volcanic area indicates the city is connecting with Great Rift Valley which leads to formation of hot spring in different parts of the city. In which there are many hot springs in Addis Ababa but there is no concert written information rather than oral. Nevertheless Filwoha is one the well knows natural hot spring found in Arada sub-city around Arat Kilo bête mengist at central part of Addis Ababa. Filwoha is Amharic word which means boiling water with 55-60⁰C also known as "Finfinnee". The name was assigned by Empress Taitou she was the wife of Emperor Menelik II, in 1986 (Grönlund, 2010). It has around four main sources and by combining those four source which gives bath room

service. Other hot spring also found in Kirkos sub-city around in Leghar at the back the former train station office now it is the part of Ethiopian Orthodox Saint Jon Holy Tsebel (Korme, 1999) and also other many hot springs are found in Lemikura sub-city in Hayat and Koye Fech around ICT park and in Akaki Kality sub city. In addition to hot spring there is large landfill which is known as Qoshe which means dirt in Amharic which loaded huge organic and inorganic waste materials. The site is located in the South part of Addis Ababa in border of Nifas-Silk lafto and Kolife Keranio and cover around 36 Hector(Yirgalem, 2005).Because of microbial action the accumulated wastes were created high unpleasant odor. The physiochemical of properties of the dump is characterized by highly moisturized balk soil(contain various nutrients, minerals, organic matter, and microorganisms) with average temperature 48⁰C(Parés Viader et al., 2021).

2.8. Production of amylase

Due to environmental conditions that must be met for the growth of the microorganisms used in commercial amylase production may differ from those needed for amylase production, several steps were involved in the process, supplementing nutrients, the medium's pH, temperature, the osmotic connection, and the management of contamination during fermentation are the common parameters during amylase production(Kammoun et al., 2008).Alpha amylase has been produced by submerged and solid state fermentation(Far et al., 2020).

Submerged state fermentation (SmF) method is requires an unusually high level of moisture, which is necessary for the growth of microorganisms in the medium mostly bacteria that produce alpha-amylase. Owing to its high moisture content, sterilizing, producing, purifying, and maintaining a regulated pH, temperature, and nutrition content are all easily accomplished(Mezeal et al., 2019). It has been used for long period of time. For microbes that need a high moisture content to flourish, submerged fermentation is appropriate. Enzyme synthesis occurs via the use of freely flowing liquid substrates like broth and molasses. Within the fermentation broth, the products are secreted. Convenient control over the process's parameters(Kunamneni et al., 2005).

2.9. Production parameters of alpha amylase

The production parameter of α -amylase varies depending upon the microbe's the type of fermentation used, and numerous other variables all affect the alpha-amylase production. The

different conditions that affect the production of enzymes include the medium's composition, temperature, pH, and sources of carbon and nitrogen, incubation time, inoculum concentration. (Rani, 2012) (*New York Science Journal*, 2012; 5(1); [Http://Www.Sciencepub.Net/Newyork](http://www.sciencepub.net/newyork) *Amylase Production By*, 2012),(R. Singh et al., 2017)

2.9.1. Temperature

Temperature has an impact on the production of alpha-amylase because it causes the enzyme to increase. Bacteria have been shown to produce alpha-amylase across a broad temperature range. For *Bacillus subtilis*, the ideal temperature range for growth and amylase production was determined to be 45–50°C *Thermococcus profundus* (Bedan et al., 2014). Hyperthermophilic bacteria, has been shown to produce amylase at temperatures as high as 80°C. As the temperature rises, the production of enzymes increases until it reaches its optimal level. But as the temperature rises higher, the production of enzymes decreases because of the loss of moisture and microbes, which inhibits their growth (Kaliappan & Ramasamy, 2013)

2.9.2. pH

Enzymes are sensitive to pH. It plays a significant role in the synthesis of α -amylase by initiation of the morphological change of the organisms. The ideal pH range for the majority of *Bacillus* species used commercially to produce bacterial amylase is 6-7, which is also the ideal range for enzyme synthesis (Prakasham et al., 2007). The thermophilic microbe such as *Thermococcus profundus* produces amylase at its maximum level at pH 5 (Gre et al., 2006)).

2.9.3. Incubation time

Enzyme production was directly impacted by the length of fermentation. Over the course of the incubation, the α -amylase activity increases until it reaches the ideal duration. Most of the time, when the incubation period increases, the amount of enzyme produced decreases because either the medium's nutrients are depleted or harmful substances are released (Ojha et al., 2020) *Bacillus amyloliquefaciens* demonstrated peak amylase production activities during 72h fermentation period, while *Bacillus subtilis* demonstrated a high alpha amylase yield following a 48h incubation period (Perego et al., 2003).

2.9.4. Carbon sources

According to Tomomura et al., (1961), α -amylase starch's hydrolysis enzymes, and which has been produced using carbon sources including glucose and maltose. Nonetheless, starch is still widely and potentially useful, make alpha amylase(WELKER & CAMPBELL, 1963).

2.9.5. Nitrogen sources

Alpha amylase is produced using both organic and inorganic sources of nitrogen. Such as Yeast extract, soybean meal, and peptone are the most widely used organic sources of nitrogen ((Paquet et al., 1991). Among the inorganic sources of nitrogen are ammonium hydrogen phosphate, ammonium sulfate, and ammonium chloride that used to enrich the nutrient supply for production of alpha- amylase(Van Der Maarel, Van Der Veen, et al., 2002).

2.9.6. Ions of metals

Since most α -amylases are known to be metallo-enzymes, adding salts of specific metal ions to the fermentation media promoted better microbial growth and, consequently, better enzyme synthesis. The bulk of these enzymes are said to contain Ca^{2+} ions. The production of enzymes was enhanced by the addition of CaCl_2 to the fermentation media (Francis et al., 2003).

2.10. Determination of enzyme activity

Amylase is generally assayed by using soluble starch as substrate. The amylase enzyme binds to the starch substrate and catalyzes the hydrolysis reaction, breaking the starch molecules into their constituent sugars. The reaction is monitored by an increase in the reducing sugar level or decrease in the iodine color of the treated substrate. There are various methods of the determination of enzymes activity based on decrease in starch iodine color intensity, increase in reducing sugar, degradation of color complex substrate and decrease in viscosity of starch suspension.

2.11. Dinitrosalicylic acid method (DNSA)

According to Bernfield, (1955) this approach measures the rise in reducing sugars caused by amylase's action on starch. This approach involves mixing an aliquot of the substrate stock solution with the enzyme solution and incubating it for 10 min at 37°C . After adding the dinitrosalicylic acid

(DNSA) reagent, the mixture is heated to a boiling water bath and left for five minutes. At 540 nm, the absorbance of the supernatant is measured after it has cooled to room temperature. The primary flaw in this technique is the loss of color caused by the DNSA reagent's component that destroys glucose

2.12. Purification of alpha- amylase

Industrial enzymes are typically prepared crudely since they are generated in large quantities and don't require much downsizing, while the enzymes utilized in the pharmaceutical and healthcare industries must be quite pure. The purified enzyme is also necessary to investigate the biochemical characteristics and structure-function relationship of the enzyme ((Windish & Mhatre, 1965). To obtain purity, amylase a range of procedures including precipitation, dialysis and SDS electrophoresis. Ammonium sulfate with multiple saturation ratios (20, 40, 60, and 80%) were added, on the given crude extracellular mass that was extracted from the fermented mass by filtration and centrifugation. The mixture was then slowly stirred with a magnetic stirrer in an ice bath, and the solution was centrifuged for 25 min at 6000 rpm. Protein content and enzyme activity were measured for each distinct fraction after the supernatant was dropped, the precipitate was taken, and it was dissolved in 5 mL of distilled water(Scopes, 1995). After the precipitated sample is dialyzed against water or a buffer for additional concentration. In order to undergo further purification, the prepared amylase enzyme was refrigerated at 4°C. The enzyme solution obtained from dialysis was subjected to gel filtration using a Sephadex G-200 column (2 x 40 cm) that was conditioned with 0.2 M phosphate buffer at a pH of 6.5. The fractions were retrieved from the column and their absorbency measured at 280 nm. (Petersen & Daniel, 2006).

2.13. Application of amylase

The most significant hydrolytic enzyme for all starch-based businesses is amylase, which is widely used in the food, detergent, textile, and paper sectors to hydrolyze starch. It is also employed in the fine chemical and pharmaceutical industries(Rajagopalan & Krishnan, 2008). Following are a few examples of applications.

2.13.1. Food industries

Amylases are widely employed in many different industrial processes in the processed food business, such as baking, starch syrups, fruit juices, cakes, and numerous digestive aids. Alpha-amylases are extensively employed in the baking industry, when added to bread dough; they break down the starch in the flour into smaller dextrin, which are then fermented by the yeast. Increasing the rate of fermentation and reducing the viscosity of the dough improves the volume and texture of the final product(Nandakumar & Wakayama, 2015).

2.13.2. Brewing Industry

Amylases are essential to the brewing sector. Amylases convert complex starches found in grains like barley into fermentable sugars in the early stages of brewing. After that, during fermentation, yeast uses these sugars to create alcohol(Jujjavarapu & Dhagat, 2019))

2.13.3. Textile Industry

Amylases are used as a desizing agent in the textile sector. The process of desizing involves taking out the sizing chemicals that are used on textiles when they are being manufactured. Amylases aid in the breakdown of starch-based sizing agents, making it easier to remove them and enhancing the fabric's quality(Ahlawat et al., 2009).

2.13.4. Detergent and laundry

Detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe. Alpha-Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes ((Hmidet et al., 2010).

2.13.5. Production of fuel alcohol

Ethanol is mostly used as a biofuel of liquid nature. Starch is the most widely utilized substrate for the synthesis of ethanol since it is a cheap and readily available raw material in most of the globe(Chi et al., 2009).Liquefaction and saccharification are the first steps in the bioconversion of starch into ethanol. Using an amylolytic microbe or enzymes like amylase, starch is converted into

sugar. Next, sugar is fermented to produce ethanol using a microorganism that ferments ethanol, like yeast, *Bacillus cerevisiae*(Article, 2008).

2.13.6. Paper industry

Alpha-amylases are used in the pulp and paper industry to produce low-viscosity, high molecular weight starch by modifying the starch of coated paper. The purpose of the coating process is to increase the paper's writing quality by making the surface suitably robust and smooth. The natural starch's viscosity in this application is too high for paper sizing, but it can be reduced by using amylases to partially break down the polymer in batch or continuous operations. In addition to being an excellent coating for paper, starch is a good sizing agent for paper finishing, enhancing the quality. The size increases the strength and stiffness of paper.(Van Der Maarel, Van Der Veen, et al., 2002)

3. Material and methods

3.1. Description of the study area

Addis Ababa is the capital city of Ethiopia and divide in to 11 sub city administration (Kalle Hirvonen, Elia Machado, 2024) which lies at 9° 1'48"N latitude and 38°44'24"E longitude. According to Tashebo, (2021) the city has a sub-tropical highland climate with maximum temperatures of 24.4 and lowest temperatures of 6°C . The city's morphologic structure provides a clear illustration of the various geologic processes, including tectonic activity, erosion, and volcanic stratigraphic successions and also only a few of the notable volcanic feature that around the city. The main tectonic structure lava force resulted formation of faults such as Filwoha fault. Due to these features Addis Ababa is parallel fault in Great Rift valley in Ethiopia(Tamiru Alemayehu, 2001) and also a number of hot spring are found in deferent sub-city. Acidic and intermediate lava flows make up the majority of these distinctive volcanic topographies(Worku, 2017).The physiochemical properties of the soil in the city is polluted with different heave metals , salts, fuel oil, plastic materials and other chemicals which generated from industries, garage and households(kitchen and bathroom), because of approximately 65% of industries and huge number of population were in the city(Dessie et al., 2021).Therefor in the current study different hot environment(hot spring) were sited specially founded in Yeka, Kolfe Keraniyo, Arada, Lemi Kura, Kirkos and Akaki Kality sub-cities that include ten sample sites (picture in appendix: A).These ten sample sites were described as follows below.

Sample site one was Saint Jon Holy Tseble which was found in Kirkos sub-city around Mexico at the back of the former Ethiopia Train Station. It has 46°C, pH7.6 and its surrounding soil was accommodated by decayed leaves, grass, and straw.

Samples site two Koye Fech hot springs which is found in Lemikura sub-city in Eastern part of Addis Ababa. In this area there were three hot springs found at the alongside of medium river of on the right and left side and have a temperature between (45-50°C) and pH (6.6- 7.5) respectively. It surrounded by grass and filled with variety of dried leaves and other suspended materials.

Samples site three was Filwoha bathroom service outlets which is found in Arada sub-city around Arat Kilo ete mengist. It has many outlets which connected which with different bath room in which generate waste water. The physiochemical characteristics of the outlets water were contained

different derbies, soap bubble and particles that discharged from bathroom service and vary with temperature and pH and also most the outlet water temperature was between (44- 56°C) and pH (6.2-7.8).

Samples site four was Biru Banti Filwoha (hot spring) bath room service outlets which is found Koye Fech Lemikura sub-city in Eastern part of Addis Ababa around ICT Park. It has two main outlets which discharged waste water from the two separated bath room and has 47°C, pH6.2 and 48° pH6.8

Sample site five was Filwoha hot springs which is found in Arda sub-sity around Arat kilo bete mengist closed to Sheraten Addis Hotel. There are five hot springs sources in the compound of Filwoha with different temperature (54-60°C) and pH (5.9-7.2).The compound is accommodated with verity grass, shrubs, animal dungs and decayed plant materials.

Sample site six was Qoshe landfill (damp) which is located in the southeastern part of Addis Ababa and situated within the boundaries of Nifas Silk-Lafto and Kolfe Keranio sub-city districts in the southwestern area of the city. The damp was accommodated with a huge organic and inorganic matter. The soil was highly moisturized and hot. Due the decomposition of the accumulated matter by microbes which create unpleasant odor and has a temperature 46°CpH6.5 and 48°C, pH5.7

Sample site seven was Yeha Science and Technology Bio-gas found in Yeka sub-city around Ferensay Legasion at the Northern part of Addis Ababa. The biogas was used animal dungs and plant residue as source of energy which stored in fermentation pool. The fermentation of the animal dung and plant residue creates hot condition with a temperature 46°C pH7.3.

Sample site eight was Hyat Medihaniyale Hot Holy Tsebel which found in the Eastern part of Addis Ababa in Lemikura sub-city. It is found alongside of the medium river and surrounding by shrubs, grass, herbs and its waste water flow toward river and it has 44°C pH 6.6.

Sample site nine was Sallo George Hot Holy Tsebel and Sallo hot spring which is found at the southern part of Addis Ababa in the boundaries of Shager city and Akaki Kality sub-city. The physiochemical properties of the Holy Tsebel and the hot spring were marsh and covered by grasses and herbs 45°C, pH6.8 and (46°C, pH6.3).

Sample site ten was Saint Angle Gebreal Hot Holy Tsebel found in the southern part of Addis Ababa within the boundaries of Shager city and Akaki Kaliti sub-city. The physiochemical properties of the Holy Tsebel were a temperature 45°C, pH 6.8 and surrounded by grass

3.2. Sample collection

From ten sample sites water and soil samples were collected using purposive sampling method. Because this sampling method used to select and collect specific samples. In which water samples were carefully collected from each hot springs and bathroom outlets by using separated autoclaved bottles first by measuring the temperature and pH of the water. At the same time soil samples specifically the (hot soil) or mud soil was collected from each hot springs (hot area) surrounding by digging with sterilized spatula and taken in sterilized plastic bag. Collected samples were symbolized by a short codes which was given by combination of the first and or the second letter of the local name of the sample sites and the sample type (water/ soil) followed by number (1, 2, 3...) which indicated number of samples per site and then transported in Addis Ababa University Microbiology laboratory and put in refrigerator at 4°C. From each (ten) sample sites samples were collected with different date between January and February and as follows.

The first sample was collected from Saint Jon Holy Tsebel sample site in January 2/1/2024. From this site one soil sample was collected from its surrounding and coded as SJS1 (46°C, pH 7.6) which means S (Saint), J (Jon), S (soil) and 1 (sample one).

The second samples were collected from Koye Fech hot springs sample site in January 7/1/2024. From this site three water and three soil samples were collected and samples were coded as KHW1/KHS1 (48°C, pH 7.2) KHW2/KHS2 (45°C, pH 6.6), and KHW3/KHS3 (50°C, pH 7.5). The code indicates K (Koye Fech), H (hot spring), W (water sample), S (soil sample) and the number 1, 2 and 3 indicates (sample number 1, 2, and 3)

The third samples were collected from Filwoha hot bathroom service outlets sample site in January 13/1/2024. From this site five water samples were collected and coded as FBW1 (54°C, pH 7.2), FBW2 (45°C, pH 6.2) FBW3 (49°C, pH 6.8) and FBW4 (52°C, pH 7.8) and FBW5 (56°C, pH 6.7). The code FBW means FB (Filwoha hot spring bath room) and W (water sample).

The fourth samples were collected from Biru Banti hot spring bath room service outlets in sample site January 19/1/2024. From this sample site two water and two soil samples were collected and

coded as BHW1/BHS1 (47°C, pH6.2) and BHW2/BHS2 (48°CpH6.8). The codes, B, (Biru Banti) H (hot spring), w (water sample) and S (soil sample).Four samples were collected from this sample site.

The fifth samples were collected from Finfinnee hot springs sample site in January 23/1/2024.From this site five water and five soil samples were collected and coded as FHW1/FHS1(54°C,pH6.1),FHW2/FHS2(57°C,pH7.4),FHW3/FHS3(60°C,pH5.9),FHW4/FHS4(59°C,pH7.2)and FHW5/FHS5(55°C,pH6.5). The code (FHW1 and FHS1) indicates (Finfinnee hot springs water and its surrounding soil sample one).

The sixth samples were collected from Qoshe (Koshe) landfill (damp) sample sites in January 30/1/2024. From this site two soil samples were collected and coded as KS1 (46°C pH6.5) and KS2 (48°CpH5.7). The code KS indicates, K(Koshe),S1/S2(soil sample one and two).

The seventh samples were collected from Yeha Science and Technology Biogas in February 4/2/2024.From this site one soil sample were collected and coded YBS146°C pH7.YB(Yeha Science Biogas) and S(soil sample)

The eighth samples were collected from Hyat Medihaniyalem hot Holy Tsebel in February 10/2/2024. From this site one water samples from Tsebel service swage and one soil sample from its surrounding and coded as HTW1(45°C, pH 6.6) and HTS1. HTW/HTS (Hayat Medihaniyalem hot Holy Tsebel water and soil sample) respectively.

The ninth samples were collected from Sallo Saint George hot Holy Tsebel and its surrounding other hot springs in February 16/2/2024.The compound was surrounding by farm land in which different plant residues generated towards the Holy Tsebel compound especially from consequent stream or steep land as it is sunken place. From this site one soil sample from hot Holy Tsebel surrounding and coded as SGHS1 (45°C, pH7) and also one water and one soil sample from the other hot spring found closed the hot holy Tseble and coded as SW1 (46°C, pH6.5) and SHS1 The code SGS indicate (Sallo Geroge hot Holy Tseble soil sample), SHW1 (Sallo hot spring water sample) and SS1 (Sallo hot spring surrounding soil sample).

The tenth samples were collected from Saint Angle Gebreal hot Holy Tsebel in February 20/2/2024. The physiochemical properties of the springs were a temperature 45°C, pH6. From this site one soil

sample was collected and coded as SAS1. SAS (Sallo Angle Gebreal Tesbel surrounding soil sample). In general from ten sample sites a total of 35 water (17) and soil (18) samples were gathered.

Table 1. Summary of number of samples per sample site, sample type and date of collection

No	Sample site	Sample types per sample site				Total sample per site	Date of collection
		Water	Code	soil	code		
1	Saint Jon Holy Tsebel	-		1	SJS	1	2,1/2024
2	Koye Fech hot springs	3	KHW	3	KHS	6	7,1/2024
3	Filwoha hot bath room	5	FBW	-	-	5	13,1/2024
4	Biru Banti hot bath room	2	BHW	2	BHS	4	19,1/2024
5	Finfinnee hot springs	5	FHW	5	FHS	10	23,1/2024
6	Qshe Damp	-	-	2	KS	3	30,1/2024
7	Yeha Sience Biogas	-	-	1	YBS	2	4,2/2024
8	Hayat M/lem hot Holy Tsebel	1	HTW	1	HTS	2	10,2/2024
9	Sallo St. Geroge hot Holy Tsebel	-		1	SGS/	1	16,2/2024
	And Sallo hot spring	1	SHW	1	SHS	2	16,2/2024
10	Sallo Angle G/real hot Holy Tsebel	-	SAW	1	SAS	1	20,2/2024
	Total sample	17		18		35	

3.3. Isolation of bacteria

Ten grams of soil or 10 ml of water sample was measured in separated way and added in 90 mL of sterile distilled water in a 250 mL flask and well shaken to obtain a homogenized sample (P. E. et al., 2022a). A serial dilution of 10⁻¹ to 10⁻⁷ (for soil sample) or 10⁻¹ to 10⁻⁴ (for water sample) with duplicated was performed and 0.1 mL of aliquots was transferred into nutrient agar plates (P. E. et al., 2022a). A serial dilution of 10⁻¹ to 10⁻⁷ or 10⁻¹ to 10⁻⁴ with duplicated was performed for soil and water sample respectively, and 0.1 mL of aliquots transferred into nutrient agar plates for incubation with spread method (Wise, 2016). The plates were then incubated for 24h that relate with the temperature of each sample source. After 24 h of incubation, the plates were monitored and countable colonies for each plate were identified. Only the countable colonies (30-300 colony forming unit) plates were selected and morphologically distinct colonies were aseptically picked with inoculated loop and transferred in to 5 mL newly prepared nutrient agar slants and incubated for 24 h. After repeated streak plating method, pure colonies were obtained ((Katz, 2008). After pure colonies were obtained they were keep in slant media.

3.4. Screening and selecting of alpha-amylase producer bacteria

Amylase producing bacteria were checked by growing a purified isolate on freshly prepared starch agar plate by dot method, which used to mix a single colony by picking the colony with sterilize loop and then stab in the starch media and incubated for 24 h with related temperature of the isolates source (Al-awsy et al., 2017). Following 24 h incubation, the plates were flooded with 1% iodine solution (Lal & Cheeptham, 2012). The mixture was then left undisturbed for 5 to 10 min, and the iodine solution was removed by decanting from each plate. The formation of any clear zone around each colony's monitored and the diameter circumference of clear zone was measured using ruler and recorded. Isolates with the bigger (above 1.5mm) clear zone surrounding the colony was chosen and proposed for submerged state fermentation (Aynadis et al., 2013).

3.5. Determination of clear zone ratio

To calculate the clear zone ratio, a loop of 24h culture of every isolate was put in the middle 50 mL volume fresh starch plate that prepared for screening of amylase producing bacteria and incubated for 24 h with related the temperature of the each sample source. After 24 h incubation the plates were took out and flood with 1% iodine solution and kept for few min, and recorded the clearance diameter using ruler at the end of decolonization. Then, using the method developed by Latorre and associates, the diameters of the colony and the zone of clearance were measured to determine the clear zone ratio (Latorre et al., 2016)

$$\text{Clear zone ratio (mm)} = \frac{\text{Diameter of zone of clearance}}{\text{Diameter of the bacterial colony}}$$

3.6. Submerged state fermentation media for alpha-amylase production

In this study to identify the best alpha-amylase producer's bacterial isolates, SmF was conducted using the following composition of media (peptone 10 g/L, yeast extract 20 g/L), starch 10 g/L, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.015 g/L KH_2PO_4 0.05 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.05 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 g/L, and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L pH was placed at pH 7.0. 1mL of fresh starch positive bacterial isolates higher (above 1.5mm) clear zone forming isolate) was added to 50 mL of fermentation broth. The mixture was then incubated for 24 with related the temperature of the each sample source on a rotary shaker at 120 rpm. After 24 h incubation the media was centrifuged with 4,000 rpm for 10 min and the supernatant (crude alpha-amylase) was collected for enzyme assay (Ashwini et al., 2011)

3.7. Enzyme assay and determination of crude alpha-amylase activity

The activity alpha-amylase was measured using DNSA reagent which consisted of 1g of 3, 5-dinitrosalicylic acid in 20 mL, 1.6 g of sodium hydroxide, and 35g potassium sodium potassium tartrate tetrahydrate in 100 mL(Sokač et al., 2023) and Fisher and Stein(Ashwini et al., 2011).In accordance with protocol, 1 mL of crude amylase was mixed with 1 mL of substrate (1% starch solution) in test tube and incubated for 15 min with related the temperature of the each sample source. Then the test tube was taken out from incubator and 1 mL of DNSA reagent was immediately added to each tube, and boiled for 10 min in water bath. The absorbance was measured using a spectrophotometer (540 nm). Maltose standard curve was developed(in appendix 3) from different maltose concentration (0-1 mg/mL) and the amount of total reducing sugar present as a percentage of starch equivalents was calculated using a linear regression analysis. One unit (U) of enzyme activity was defined as the amount of amylase that liberates 1 mole of reducing sugar equivalent to maltose per minute under the test conditions (Bernfeld, 1955).

$$\text{Amylase Activity} = \frac{\text{release of maltose} * \text{reaction volume} * \text{dilution factor}}{\text{molecular mass of maltose}(342\text{g}) * \text{incubation time}} \times 1000$$

If the activity of amylase greater than 1nm during measuring of its activity in Spectrophotometer (540nm) it should be dilute and finally the activity of that enzyme result obtained by multiplying with the dilution factor.

3.8. Identification and characterization of selected bacterial isolates

Selected isolates were identified and characterized based on morphological and biochemical techniques.

3.8.1.1. Morphological identification and characterization of selected isolates

The morphology of six selected isolates was identified with macroscopic and microscopic technique(Nisa et al., 2021).

3.8.1.2. Macroscopic characterization of Isolates

The six selected isolates were identified and characterized their morphological feature through observing the colony size, shape, color, elevation, texture on the agar plate(Sousa et al., 2013)

3.8.1.3. Microscopic characterization of isolates.

According to Bergey, 1994 selected isolates were also further described by their morphology characteristics. Following several staining techniques, the bacterial isolate was examined under a compound light microscope. Some microscopic identification was here.

3.8.1.4. Gram staining

Every isolate underwent gram staining to determine its morphological characteristics. On a glass slide, a tiny smear of isolated bacterium was prepared using sterile water. The smear was allowed to air dry and heated before being flooded with crystal violet for a min. After a minute crystal violet was rinsed by running tap water. Then gram iodine was added to the slide and left for an additional minute. After decolorization, the slide was washed with gentle running water. Finally, safranin was added to the slide as a counter stain. After being allowed to air dry, the slide was examined under a compound light microscope with an oil immersion objective (1000X). Bacterial isolates were classified as gram-positive or gram-negative based on the color of their cells, which were purple or pink, respectively (MITTWER, 1952).

3.8.1.5. Spore test

Under aseptic circumstances, a selected bacterial smear was prepared. The smear slide was covered with malachite green after air drying, and put on the top of boiling water containing beaker and heated for 5 min. After boiling the smear was washed and stained with safranin for 30 second and cleaned again. Then the smear was examined under a compound light microscope of oil immersion objective (Kane et al., 2016).

3.8.1.6. Motility test

A loopful of fresh colony was put on the clean glass slide and water was dropped and covered with clean cover slide carefully. The motility was investigated under a compound light microscope(Sharma et al., 2022) in which motile bacterium was moving here and there, while non-motile species remain stationary.

3.8.2.1. Biochemical Characterization of isolates

The six selected isolates were characterized through several biochemical tests. The experiment was conducted by preparing fresh bacterial culture medium from selected culture media (Talaiekhosani, 2022). Some biochemical tests were here.

3.8.2.2.Catalase test

To determine catalase test a loopful of isolate was placed in to on a glass slide and 2-3 drops of 3% H₂O₂ was added. For a positive catalase test result bubbles was observed. Based on the formation of bubble isolates were identified as positive catalase and while non-bubble formation negative catalase(Headon & Walsh, 1994).

3.8.2.3.Methyl Red Test

MR- test was conducted after being inoculated the bacterial isolates, in 5 mL of prepared MR broth and incubated for 24 h at 50°C. Following incubation, the tubes were filled with a few drops of methyl red indicator solution. MR broth changed to a cherry red color indicated positive methyl red (MR) test and while remain unchanged negative methyl red (MR) test (Azhar et al., 2014).

3.8.2.4.Citrate utilization test

To conducted citrate utilization test, a loopful of isolates was inoculated in freshly prepared Simmons citrate agar slant and incubated at 50°C for 24 h. Following incubation the color change was observed in which change in the slant color from green to blue was indicated a positive result, while non-color change negative citrate utilization(Azhar et al., 2014).

3.8.2.4.1. Urease Test

The urease test was performed; by inoculating 1 mL of fresh isolated bacterial culture in to prepared urea broth, which were then incubated for 24 h at 50°C. According to (Brink, 2010) the urease test would be considered positive if a pink tint appeared while remained negative test

3.8.3. Determination of optimum growth condition for amylase production by one factor at a time approach

3.8.3.1.Temperature

The optimal temperature for effective alpha-amylase synthesis for each isolate was determined by incubating in 50 mL of production media individually at various temperatures 30(for comparison),40, 50, and 60oC for 24 h with pH 7 used as control and amylase assay was measured under standard assay condition (Perego et al., 2003).

3.8.3.2.pH

The effect of pH on alpha-amylase synthesis was determined by incubating the selected isolates in 50 mL of synthesis media at various pH values 5, 6, 7, 8, and 9 by adjusting the pH with 1M of KOH and HCl base and acid, respectively and incubated for 24 h at 50°C being identified as optimal temperature. After 24 h of incubation the enzyme assay was conducted (Soni et al., 1992).

3.8.3.3.Incubation period

The optimal incubation time on alpha-amylase synthesis was determined by incubating the selected bacterial isolates in 50 mL of production media and incubated in different hours, 12, 24, 48, and 72 h at 50°C with pH 7 earlier identified as an optimal temperature and pH. The enzyme assay was performed after each of incubation period (Y. Kumar et al., 2014).

3.8.3.4.Effect of carbon source

Various carbon sources have been employed in the process of alpha-amylase production(Raj & Hemashenpagam, 2012) The effect of carbon source for alpha-amylase synthesis by selected bacterial isolate, was determine by supplemented the growth medium with sucrose, maltose, glucose, lactose and fructose (1% (w/v) level individually and the isolates were inoculated in 50 mL (pH 7.0)following incubating at 50°C with 24 h. After 24 h of incubation, supernatant from each test tube was collected following centrifugation of the cultures and enzyme assay was performed for each carbon source (Vijayabaskar et al., 2012).

3.8.3.5.Effect of nitrogen source

To determine the role of various nitrogen sources on alpha-amylase synthesis, the basal medium was enriched with various organic and inorganic nitrogen sources. Organic nitrogen sources include meat extract, urea, tryptone and soybean , while inorganic nitrogen sources include, sodium nitrate, ammonium chloride, ammonium sulphate and potassium nitrate 1% (w/v) level and the isolates were incubated at 50°C for 24 h (pH 7.0). After 24 h of incubation, the standard (Aqeel & Umar, 2008).

3.8.3.6.Effect of inoculums size

The best culture inoculum concentration for alpha amylase synthesis was determined by incubating isolates with different inoculum size 0.5, 1 ,1.5, and 2 mL in 50 mL fermentation media (pH 7.0) at

50°C for 24 h and after 24 h of incubation the activity of amylase was measured (Simair, Qureshi, et al., 2017).

3.8.3.7. Effect of starch concentration

Starch is the major substrate for alpha-amylase (Padhiar & Kommu, 2016). In this study the concentration of starch on alpha-amylase synthesis was determined by addition of different starch concentrations (1, 2, 3 and 4% w/v) in the basal media (pH 7). Then isolates were inoculated in 50 mL media that contain different starch concentrations and incubated for 24 h and after incubation the culture was centrifuged and the supernatant was collected to conduct enzyme assay.

3.8.3.8. Effect of metal ions

The effects of several metal ions on the synthesis of alpha-amylase were investigated by supplementing the basal media with different metal ions (0.002M) of level (Arekemase et al., 2020). Metals like K^+ , Na^+ , Zn^+ and Mn^+ were used in order to examine the impact of metal ions on the production of alpha-amylase. These metals were added individually, in basal media for enrichment and isolates were inoculated in 50 mL of enriched basal media and incubated for 24 h in shaker at 50°C. Following incubation the culture was centrifuged at 4000 rpm for 10 min and the supernatant was collected and enzyme assay conducted.

3.8.3.9. Effect of salt concentration

The effect of NaCl concentrations on alpha-amylase activity was measured by incubating isolates with various salt concentrations of NaCl (Aida & Hanan, 2016). In this study to assess the effect of salt concentration on alpha-amylase production was measured by incubating selected bacterial isolates in various (0.5, 1, 1.5 and 2% w/v) NaCl concentrations with pH 7.0 at 50°C for 24 h. After 24 h following centrifugation the absorbance of alpha-amylase was measured and the enzyme assay conducted.

3.9. Characterization of crude alpha-amylase enzymes

3.10.1. The effect of temperature for alpha-amylase activity

The effects of temperature on alpha-amylase activity were studied by incubating 1 mL of crude alpha-amylase in 1% of 1 mL starch solution (pH 7) at different temperatures (45, 50, 55, and 65°C) in water bath for 15 min. Following 15 min incubation the absorbance of enzyme was measured with Spectrophotometer (540 nm) and enzyme assay was performed

3.10.1.1. Effect pH

The effect of pH on alpha-amylase was measured by preparing several starch solution as substrate (Islam, 2016). The impact of pH on alpha-amylase activity was measured by preparing several starch solution as substrate (Islam, 2016). In this study 1% of starch solution was adjusted at different phosphate buffer ranges from pH 5 to 9. Then 1 mL of crude alpha-amylase was added in each starch solution with different pH. Then the test tube was incubated for 15 min at 50°C and after 15 min incubation period, 1 mL of DNSA solution was added and the reaction was stopped by boiling at 100°C to a 10 min in boiler. Following boiling the absorbance of alpha-amylase was measured

3.10.1.2. Effect of starch concentration

The concentration of starch on alpha-amylase activity was determined by incubating 1mL of crude alpha-amylase in 1 mL of in different starch concentration (1, 2,3 and 4% w/v) (pH7) for 15 min at 50°C. After 15 min then the reaction mixture boiled in water bath for 10 min for 100°C and stopped by adding 1mL of DNSA solution and enzyme assayed was conducted.

3.10.1.3. Effect of metal ions

Many metals have play important role on amylase activity (Rehman, 2016). In this study the effects of different metal ions on the alpha-amylase activity was investigated by incubating the 1mL crude alpha-amylase in 1mL of different metal ions solution (0.002M) level (pH7) at 50°C (Arekemase et al., 2020) for 15min. Metal ions such as K^+ , Na^+ , Zn^+ , Mn^+ , Fe^{+3} , Ca^{+2} , Mg^{+2} and Na^+ were used in order to determine their effects on the alpha-amylase activity and enzyme assay was conducted.

3.10.1.4. Effect of salt concentration

The effect NaCl concentrations on alpha-amylase activity was measured by incubating the crude alpha-amylase in various salt concentration (Shafiei et al., 2012). In this study to the salt stability of alpha-amylase was assessed by incubating 1mL of alpha-amylase in to various (0.5, 1, 1.5 and 2% w/v) of NaCl concentration at pH 7.0 from 15 min at 50°C. After 15 min the absorbance of alpha-amylase was measured and enzyme assay was conducted.

3.10.2. Thermal stability of crude alpha-amylase enzymes

The thermal stability of crude alpha-amylase was assessed by incubating 1 mL of crude extracted alpha-amylase at different temperatures (55, 60, 65 and 70°C and without substrate for 30, and 60 min (Arekemase et al., 2020). Because the optimal temperature was obtained at 55°C. Then after pre-incubation 1 mL of alpha-amylase was mixed in 1% of 1 mL starch solution and incubated for 15 min at in the same temperature and following incubation the reaction mixture was boiled at 100°C for 10 min and stopped the reaction with DNSA solution and its residual activity was measured.

$$\text{Residual Activity} = \frac{\text{Individual enzyme at each concentration activity}}{\text{The highest enzyme activity in the block}} * 100$$

3.10.3. Molecular identification of best isolates

The six best selected isolates were molecularly characterized by using the primer pair 27 F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492 R: 5' GGTTACCTTGTTACGACTT-3, the full-length of 16 S rRNA gene was amplified with the following reaction parameters: 94 °C for 10 min, followed by 32 cycles at 94 °C for 30 sec, 55°C for 20 sec, and 72 °C for 55 sec; and a final extension at 72 °C for 5 min. Each tube had a 25 µL total reaction volume. With a few minor adjustments, the Sanger sequencing approach as outlined in (Frank et al., 2008) was used to sequence the amplicons. Specifically, the ABI PRISM® BigDye™ Terminator cycle sequencing kit was used to perform cycle sequencing utilizing the forward primer 27F and the reverse primer 1492R. Beijing Sangon Biotechnology Company's ABI PRISM Sequencer platform 3730XL was used for the electrophoresis. The Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) /BLAST/ was used to search the Gene Bank database for the 16S rRNA sequences of the bacteria isolates sequenced in this investigation in order to identify the best matches. 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

3.10.4. Partial purification of alpha-amylase

Bacillus cabrialesii strain was selected for partial purification of alpha-amylase which was performed by following three main steps was followed

Precipitation: Partial purification of alpha amylase was conducted by gradually adding 40-80% ammonium sulfate (NH₄)₂SO₄ in approximately 50 mL of crude culture filtered alpha-amylase

and precipitated using stirring constantly for up to 24 h at 4°C. Every precipitate was separated from the supernatant using centrifugation at 4,000 rpm for 10 min at 4°C. After centrifugation, the solid precipitate was dissolved in phosphate buffer (pH 6.5) to generate a more concentrated enzyme solution.

Dialysis: The ammonium sulfate-precipitating alpha-amylase solution was entered in a semipermeable membrane bag and tied with tiny strand and then it inserted in large volume (5 L capacity) of phosphate buffer (pH 6.5) for 24 h at 4°C, with continuously swirling using magnetic stirrer it against a large volume (appendix: D). A magnet stirrer was used to mix the enzyme solution in a bag made of cellophane that was selectively permeable. Whereas proteins with large molecules cannot pass through the holes in the semipermeable membrane, tiny molecules like ammonium and sulfate ions can pass through until there is a balance of volume outside and within the bag. The enzyme solution was having a larger volume after dialysis and that ensured all ammonium sulfate and lower molecular weight metabolites were removed from enzyme solution (Sethi et al., 2016). After dialysis in each salt precipitate crude alpha-amylase activity was measured with spectrophotometers (540 nm) and the best precipitated result was used to determine the molecular weight of the protein in SDS (PAGE) running process.

SDS(PAGE): The dialyzed alpha-amylase enzyme was loaded in SDS polyacrylamide gel electrophoresis to determine the protein profile and molecular weight according to analysis methods of Cannon-Carlson & Tang, (1997)¹. First the resolving and stacking gel was prepared from different salts and buffers (Tris, acrylamide SDS, APS, TEMED) and distilled water respectively. Then 20 mg of protein samples were loaded in small prepared hole of the running gel and run by gel electrophoresis in SDS running buffer (25 mM TrisHCl, 192 mM Glycine, 0.1% SDS), the gel was electrophoresed at 100 V for 15 min. After 15 min the protein was passed down the stacking gel in to resolving gel and again electrophoresed at 200 V for 45 min. After 45 min the protein sample take out and the gel was stained by using Coomassie blue R250 for 30' min and finally de-stained the gel an overnight in shaker 25 rpm by using de-staining agent with the combination of 25 mL methanol and 10 mL acetic acid 65 mL distilled water (100 mL total volume). The protein profile and molecular weight was determine by using conventional protein marker (BioRad)(Abdel-Fattah et al., 2013).

3.10.5. Determination of specific activity of partially purified enzyme

In order to determine the specific activity of alpha-amylase of selected isolate the Bradford method of protein estimated was used (Ernst & Zor, 2010). In which the specific activity of partially purified amylase was conducted by mixing of 1mL crude amylase with Coomassie reagent and incubated at room temperature for 10 min and its absorbance was measured (595 nm). Finally, the specific activity of the unknown purified protein sample was calculated first by plotting the protein standard curve from egg albumin protein solution which was prepared in five different test tubes with different volume (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL).

Specific activity (U/mg) = (Enzyme activity (U/mL) / protein concentration (mg/mL))

3.10.6. Application of crude alpha-amylase in food industry

The starch hydrolysis performance of alpha-amylase was tested through application of alpha-amylase in wheat flour fermentation (Albejo & Hamza, 2017). To conduct the application of alpha-amylase on fermentation process, four flasks were taken and 20g of wheat flour was blended with water to make wheat flour dough and autoclaved. Autoclaving used to kill the natural yeast that occurred in the wheat flour but didn't affect the formation activity by market yeast. After autoclaving, the four wheat flour containing flasks were arranged by coding with number and added 4 mL of crude alpha-amylase, plus yeast (*saccharomyces cerevisiae*) (1st) flask, only crude alpha-amylase (2nd) flask, only yeast in other (3rd) flask and neither yeast nor crude alpha-amylase (4th) flask for comparison and control (negative) respectively. Following 2 h of incubation at room temperature, the raising height of the wheat flour dough was measured for all the yeast, yeast plus enzyme and the crude enzyme containing wheat dough and compared its fermentation capacity of alpha-amylase.

3.10.7. Culture preservation

Culture preservation was performed by using 20% glycerol broth in which one loopfull of bacterial inoculums was transferred into 5ml nutrient medium and incubated at 4°C for overnight. Then 830µL bacterial suspension was mixed with 170µL sterilized glycerol in 1.5 mL centrifuge tube and sealed with parafilm tapes and stored in freezer. (Aman et al., 2019)

3.10.8. Analysis of data

The data was analysis using Origin lab 2019b software at $p < 0.05$ statistical tests, and figures were represented by graph. The statistical data results conducted by analysis of variance (ANOVA) at a 95% significance level. Every experiment was carried out in duplicated, and the mean \pm standard error (standard deviation) was used to display the results.

4. Result

4.1. Isolation and screening of thermostable producing bacteria from hot environment

From ten sample sites a total of 35 samples (17, water & 18, soil) samples were collected (Table 2)

Table 2: The number of isolates per each sample.

	Sample site	Number of isolates per sample							
		water	code	Isolates		Soil	code	Isolates	
				+Ve	-Ve			+Ve	-Ve
1	Saint JonHoly Tsebel	-				1	SJS1	5	-
2	KoyeFech hot springs	3	KHW1	4	2	3	KHS1	5	1
			KHW2	6	-		KHS2	4	1
			KHW3	5			KHS3	5	1
3	Filwoha hot bath room	5	FBW1	5	-				
			FBW2	6	2				
			FBW3	5	-				
			FBW4	4	2				
			FBW5	5	1				
4	Biru Banti hot bath room	2	BHW	3	1	2	BHS1	4	1
			BHW	4	1		BHS2	5	2
5	Finfinnee hot springs	5	FHW1	5	1	5	FHS1	4	-
			FHW2	4	1		FHS2	6	-
			FHW3	4	-		FHS3	4	2
			FHW4	4	-		FHS4	5	-
			FHW5	5	1		FHS5	4	1
6	Qshe Damp		-			2	KS1	5	-
							KS2	5	1
7	Yeha Science and Technology , Biogas		-			1	YBS1	6	
8	Hayat M/lem hot Holy Tsebel	1	HTW	3		1	HTS1	5	1
9	Sallo St. Geroge hot Holy Tsebel					1	SGS1	4	2
	And Sallo hot spring	1	SHW1	4		1	SHS1	5	-
10	Sallo Angle G/real hot Holy Tsebel					1	SAS1	4	2
11	Total sample from water & soil sample	17				18			
	Total samples from ten sample sites	17+18=35							
12	Total isolates from 35 samples			76	12			85	15

13	Total isolates from ten sample sites	76+12+85+15=188
14	Total starch test(+Ve) and (-Ve)	(+Ve) isolates (76+85=161) and (-Ve) isolates=(27)

#In Table (2) the (+Ve) means positive starch hydrolysis test while (-Ve) negative starch hydrolysis test result.

4.1.1. Hydrolysis test of the screened pure bacterial isolates

As the result indicated in the above Table (2) from 35 total water and soil samples based on morphological observation (colony shape, size and color) 188 isolates were isolated and screened. Out Of which, 161 isolates were positive for starch hydrolysis test (Appendix:2). The positive hydrolysis test of the bacteria indicated that they produce amylase, that hydrolysis the starch which detected after incubation of starch agar and with the addition of iodine solution, in which the hydrolysis part of the starch by the amylase form clear zone around the growing bacterial colony due to the absence of starch in the surrounding, while the negative hydrolysis tests form a blue back around the growing bacteria colony surrounding because the iodine solution react with starch and form blue black color. From ten samples site high number of positive isolates were obtained from Finfinnee hot springs (44) and Filwoha hot bath room(25 isolates) respectively. The minimum and maximum rang of clear zone in all isolates was obtained between (1.2-3.46mm) respectively and from each sample isolates at least one isolates that form 1.5mm and above clear zone was obtained. From ten samples site high number of positive isolates were obtained from Finfinnee hot springs (44) and Filwoha hot bath room(25 isolates) respectively. The minimum and maximum rang of clear zone in all isolates was obtained between (1.2-3.46mm) respectively and from each sample isolates at least one isolates that form 1.5mm and above clear zone was obtained. After comparing their clear zones formation, from all 35 samples isolates (188), 66 isolates were found to have above 1.5 mm diameter clear zone around their colony (Table 3) and selected for submerged state fermentation process. There was significant difference isolates (66) clear zone formation ($p>0.05$).The maximum diameter clear zone was recorded by isolate KHW2-1A 3.46 ± 0.05 .The code (KHW2-1A) indicates as KH (Koye Feche hot spring), W (water sample),2,(sample two from that sample site), 1(isolate one from this sample two) and letter A, (the first(highest) clear zone formed isolate from the total isolates of the sample two) respectively.

$$\text{Clear zone ratio (mm)} = \frac{\text{Daimeter of zone of clearance}}{\text{Daimetre of the bacterial colony}}$$

Table 3: Starch hydrolysis performance of bacterial isolates in terms of clear zone formation

No	Isolates	Clear Zone	No	Isolates	Clear zone	No	Isolates	Clear zone
1	SJS1-4A	2.71±0.06	23	FBW5-1A	2.13±0.1	45	BHW1-2A	2.01±0.1
2	SJS1-3B	1.95±0.07	24	FBW5-2B	1.9±0.01	46	BHW2-3A	1.8±0.13
3	KHW1-1A	1.95±0.07	25	FHS1-3A	2.62±0.04	47	BHS1-3A	1.7±0.18
4	KHW1-2A	1.82±0.09	26	FHS1-4B	2.33±0.07	48	BHS2-1A	1.56±0
5	KHW2-2A	3.46±0.05	27	FHW1-1A	2.01±0.02	49	KS1-1A	2.7±0.12
6	KHW2-3B	2.23±0.04	28	FHW1-2B	1.78±0.03	50	KS2-2A	2.08±0.06
7	KHW3-4A	2.2±0.13	29	FHS2-2A	1.83±0.07	51	KS2-1A	1.93±0.1
8	KHW3-1B	2.13±0	30	FHS2-5B	1.69±0.09	52	KS2-3B	1.82±0.04
9	KHS1-3A	1.95±0.07	31	FHS3-2A	2.11±0.06	53	YB1-2A	2.74±0.06
10	KHS1-4B	1.81±0.08	32	FHS3-1B	1.85±0.1	54	YB2-3B	2.16±0.09
11	KHS2-3A	2.29±0.02	33	FHW2-3A	2.34±0.06	55	HTW1-1A	1.94±0.14
12	KHS2-2B	2.27±0.03	34	FHW2-2B	1.67±0.03	56	HTW1-3B	1.7±0.08
13	KHS3-3A	2.27±0.09	35	FHW3-4A	1.84±0.02	57	HTS1-1A	2.72±0.02
14	KHS3-5B	1.95±0.08	36	FHW3-2B	1.64±0.05	58	HTS1-2B	2.32±0.08
15	FBW1-4A	2.38±0.07		FHW4-3A	2.23±0	59	SHS1-2A	2.51±0.09
16	FBW1-2B	2.65±0.03	38	FHW4-4B	2±0.04	60	SHS1-1B	2.38±0.04
17	FBW2-3A	3.09±0.12	39	FHW5-1A	2.640±0.1	61	SHW1-4A	2.07±0.1
18	FBW2-2B	2.17±0.07	40	FHW5-2B	1.95±0.08	62	SHW1-3B	2.01±0.19
19	FBW3 -1A	2.1±0.01	41	FHS4-1A	1.78±0.1	63	SGHS1-3A	2.47±0.12
20	FBW3 -2B	2.07±0.09	42	FHS4-5B	1.63±0.05	64	SGHS1-2B	1.95±0.08
21	FBW4 -2A	2.7±0.1	43	FHS5-4A	1.65±0.09	65	SAS1-2A	3.13±0.01
22	FBW4-1B	2.07±0.09	44	FHS5-1B	1.52±0.03	66	SAS1-4A	2.79±0.1

4.1.2. Submerged state fermentation test of the 66 selected isolates from alpha-amylase production.

To identify best alpha-amylase producing bacterial isolates, all the 66-starch positive bacterial isolates were subjected to submerged state fermentation. The result showed in (Table 4), among 66 isolates, 11 isolates were performed better amylase production in which the enzyme activity rang was obtained between the minimum and the maximum result (1.67 ± 0.07 to 2.19 U/mL) which achieved by FHS5-4A and SJS1-4A respectively under submerged state fermentation conditions (showed in Table 4)

Table 4: Alpha-amylase production performance of 66 selected isolates in SmF.

No	Isolates	Clear zone	No	Isolates	Clear zone	No	Isolates	Clear zone
1	SJS1-4A	2.19 ± 0.08	23	FBW5-1A	1.23 ± 0.03	45	BHW1-2A	1.27 ± 0.01
2	SJS1-3B	0.85 ± 0.09	24	FBW5-2B	0.71 ± 0.01	46	BHW2-3A	1.54 ± 0.02
3	KHW1-1A	1.74 ± 0.02	25	FHS1-3A	0.92 ± 0.03	47	BHS1-3A	1.41 ± 0.06
4	KHW1-2A	0.91 ± 0.02	26	FHS1-4B	1.19 ± 0.05	48	BHS2-1A	1.07 ± 0.04
5	KHW2-2A	1.81 ± 0.03	27	FHW1-1A	1.35 ± 0.06	49	KS1-1A	1.68 ± 0.06
6	KHW2-3B	0.96 ± 0.02	28	FHW1-2B	1.19 ± 0.02	50	KS2-2A	1.01 ± 0.04
7	KHW3-4A	1.26 ± 0.01	29	FHS2-2A	1.88 ± 0.07	51	KS2-1A	0.58 ± 0.01
8	KHW3-1B	1.18 ± 0.07	30	FHS2-5B	0.96 ± 0.03	52	KS2-3B	1.34 ± 0.01
9	KHS1-3A	1.52 ± 0.01	31	FHS3-2A	0.96 ± 0.03	53	YB1-2A	1.89 ± 0.06
10	KHS1-4B	0.52 ± 0.01	32	FHS3-1B	0.05 ± 0.01	54	YB2-3B	1.36 ± 0.01
11	KHS2-3A	1.19 ± 0.01	33	FHW2-3A	1.12 ± 0.01	55	HTW1-1A	1.36 ± 0.02
12	KHS2-2B	1.19 ± 0.01	34	FHW2-2B	1.15 ± 0.01	56	HTW1-3B	0.89 ± 0.01
13	KHS3-3A	1.38 ± 0.01	35	FHW3-4A	2.01 ± 0.03	57	HTS1-1A	0.28 ± 0.04
14	KHS3-5B	0.85 ± 0.09	36	FHW3-2B	1.19 ± 0.05	58	HTS1-2B	0.94 ± 0.03
15	FBW1-4A	0.52 ± 0.06		FHW4-3A	1.11 ± 0.02	59	SHS1-2A	1.34 ± 0.02
16	FBW1-2B	1.47 ± 0.02	38	FHW4-4B	1.50 ± 0.01	60	SHS1-1B	1.00 ± 0.01
17	FBW2-3A	2.18 ± 0.03	39	FHW5-1A	2.13 ± 0.02	61	SHW1-4A	0.96 ± 0.03
18	FBW2-2B	1.30 ± 0.02	40	FHW5-2B	1.53 ± 0.01	62	SHW1-3B	0.59 ± 0.07
19	FBW3 -1A	0.60 ± 0.01	41	FHS4-1A	1.43 ± 0.06	63	SGHS1-3A	1.77 ± 0.03
20	FBW3 -2B	1.45 ± 0.06	42	FHS4-5B	1.11 ± 0.01	64	SGHS1-2B	1.3 ± 0.02
21	FBW4 -2A	1.30 ± 0.08	43	FHS5-4A	1.62 ± 0.08	65	SAS1-2A	1.72 ± 0.09
22	FBW4-1B	1.15 ± 0.04	44	FHS5-1B	1.55 ± 0.05	66	SAS1-4A	0.9 ± 0.03

4.1.3. Better alpha-amylase producing bacterial isolates

As the results indicated in (Table,4), 66 isolates that were checked for their fermentation activity 11 isolates were showed better fermentation performances. These 11 isolates were selected and their alpha-amylase production capacity was re-checked before their optimization and characterization process was conducted. Hence as the result indicated in (Table 5) from 11 isolates the alpha-amylase production capacity and enzyme activity results of the 5 isolates did not consistently produced or there were showed fluctuation of enzyme active one and two ,while the six isolates enzyme production showed consistency or showed relatively similar to compared that of the 5 isolates.

Table 5:11 better Alpha-amylase producing bacterial isolates enzyme activity results

No	Isolates	EA(U/mL)	EA(U/mL)
1	SJS1-4A	2.19±0.08	2.17±0.08
2	KHW1-1A	1.74±0.02	1.12±0.02
3	FHS2-2A	1.88±0.07	1.41±0.07
4	KHW2-2A	1.813±0.03	1.89±0.03
5	KS1-1A	1.68±0.06	1.71±0.06
6	YB1-2A	1.89±0.06	1.01±0.06
7	SAS1-2A	1.72±0.01	1.81±0.01
8	FHW5-1A	2.13±0.06	2.08±0.06
9	FHW3-4A	2.01±0.03	1.54±0.03
10	FBW2-3A	2.18±0.01	2.2±0.01
11	SGHS1-3A	1.77±0.03	1.14±0.03

4.1.4. Best 6 alpha-amylase producing bacterial isolates

As results showed in above (Table,5) 6 isolates alpha-amylase production and enzyme activity was showed consistency. So based on the fermentation enzyme activity and consistency alpha-amylase production, 6 more potent and best isolates were selected for further analysis and optimization process (Table,6).

Table 6:11 better Alpha-amylase producing bacterial isolates enzyme activity result

No	Isolates	EA(U/mL)
1	SJS1-4A	2.19±0.08
2	KHW2-2A	1.813±0.03
3	KS1-1A	1.68±0.06
4	SAS1-2A	1.72±0.01
5	FHW5-1A	2.13±0.06
6	FBW2-3A	2.18±0.01

4.1.5. Morphological and biochemical characterizations of best six selected isolates

4.1.5.1. Morphological characterization

The morphological feature of the six selected isolates identified macroscopically and microscopically. Macroscopic identification like color and size of the colonies were identified by observing the colonies on nutrient agar in which all the six isolates have white color and medium colony size respectively. And also in terms of margin isolate KS1-1A and SASA1-2A have entire and wavy margin respectively but SJS1-4A, KHW2-2A, FHW5-1A and FBW2-3A have smooth margin. Microscopic identification of the cells like shape, gram reaction, spores and motility tests were performed by microscopic techniques. As shown in Table7, all the isolates were rod in shaped, except KHW2-2A, and FBW2-3A, all were gram negative bacteria. In terms of motility, only FHW5-1A is non-motile and all isolates could produce spores with the exception of KHW2-2A, and FBW2-3A (Table7)

Table 7: Morphological characteristics of the six best selected isolates

Isolates feature	FBW1-3A	FHW5-1A	KS1-1A	SJS1-4A	KHW2-2A	SAS1-2A
colony color	white	White	White	white	White	White
colony shape	rod	Road	Rod	rod	Rod	Rod
Margin	smooth	smooth	Entire	smooth	Smooth	Wavy
Texture	Moist	Slimy	Dry	smooth	Slimy	Moist
Elevation	flat	Raised	Convex	flat	Flat	Raised
Colony size	medium	Medium	Medium	medium	Medium	Medium
Gram staining	Positive	Negative	Negative	Negative	Positive	Negative
Spore staining	Positive	Negative	Negative	Negative	Positive	Negative
Motility	motile	Non motile	Motile	motile	Motile	Motile

4.1.5.2. Biochemical characterization of isolates

Six selected isolates were characterized through several biochemical tests such as,

4.1.5.2.1. Urease test

As the result showed in Table 8 the three isolates FBW1-3A, KS1-1A and SAS1-2A showed ureases positive test which indicated that these isolates can hydrolyze urea to form ammonia and carbon dioxide in which change to purple. Two molecules of ammonia and carbon dioxide are produced during the fermentation of urea when the enzyme "urease" is present. However other three isolates were FHW5-1A SJS1-4A and KHW2-2A urease test negative.

4.1.5.2.2. Methyl red test

As the result showed in Table 8 FBW1-3A, KHW2-2A and SAS1-2A showed methyl red positive which the isolates indicate the mixed acid fermentation bacteria they produce acids for fermentation of different substrate (glucose) as their energy sources in which media was changed in a cherry red color

4.1.5.2.3. Catalase test

As the result showed in Table 8 except KHW2-2A all isolates showed catalase positive. This indicate that isolates produced enzyme catalase, which is responsible for hydrogen peroxide's (H₂O₂) catalytic release of oxygen and consequently, bubbles was formed from the quick synthesis of oxygen gas (O₂)

4.1.5.2.4. Citrate test

As the result showed in Table 8 the three isolates FBW1-3A SJS1-4A and KHW2-2A showed citrate positive. This indicated that the bacteria use citrate as a source of carbon and energy and change the media (citrate) from green to blue while negative unchanged media while other three isolates FHW5-1A KS1-1A and SAS1-2A showed citrate negative

Table 8: Biochemical characterization of the selected isolate

Biochemical test	FBW1-3A	FHW5-1A	KS1-1A	SJS1-4A	KHW2-2A	SAS1-2A
Urease test	Positive	Negative	Positive	Negative	Negative	Positive
catalase test	Positive	Positive	Positive	Positive	Negative	positive
MR test	Negative	Positive	Positive	Positive	Negative	Positive
Citrate test	Positive	Negative	Negative	Positive	Positive	Negative

4.1.6. Molecular identification of best isolates

In the current study all the six best alpha-amylase producing isolates were characterized by using 16s rRNA sequencing technique in which the results showed that (Table 9) five of them FBW2-3A, FHW5-1A, SJS1-4A KHW2-2A, and SAS1-2A have 99% of similarity with *Bacillus cabrialesii*, *Acinetobacter haemolyticus*, *Enterobacter quasihormaechei*, *Bacillus cereus*, *pseudomonas plecoglossicidia*, respectively, while KS1-1A strain remain unidentified with this method.

Table 9: Molecular identification of six selected isolates

No	New code	Species	Identity (%)	Accession No
1	FBW2-3A	<i>Bacillus cabrialesii</i>	99.72	NR_180419.1
2	FHW5-1A	<i>Acinetobacter haemolyticus</i>	99.58	NR_117622.1
3	KS1-1A	Unknown	96.4	NR_117622.1
4	SJS1-4A	<i>Enterobacter quasihormaechei</i>	99.65	NR_126208.
5	KHW2-2A	<i>Bacillus cereus</i>	99.66	NR_115526.1
6	SAS1-2A	<i>Pseudomonas plecoglossicida</i>	99.79	NR_114226.1

4.2. Optimization of amylase production by one factor at a time (OFAT) approach

4.2.1. Effect of Temperature

Temperature is a significant physical component that affects the synthesis of enzymes. In this study effect of temperature on amylase synthesis was studied in basal medium by incubating selected isolates at different temperatures (30, 40, 50 and 60°C) for 24 h with (pH 7) and the optimal temperature for alpha-amylase production by all isolates (FHW5-1A, SJS1-4A, KHW2-2A, SAS1-2A and KS1-1A) was observed at 50°C which resulted in amylase activity ranged from SJS1-4A (3.3±0.09) to FHW5-1A (4.1±0.06)U/mL (Figure 1). There was a significant

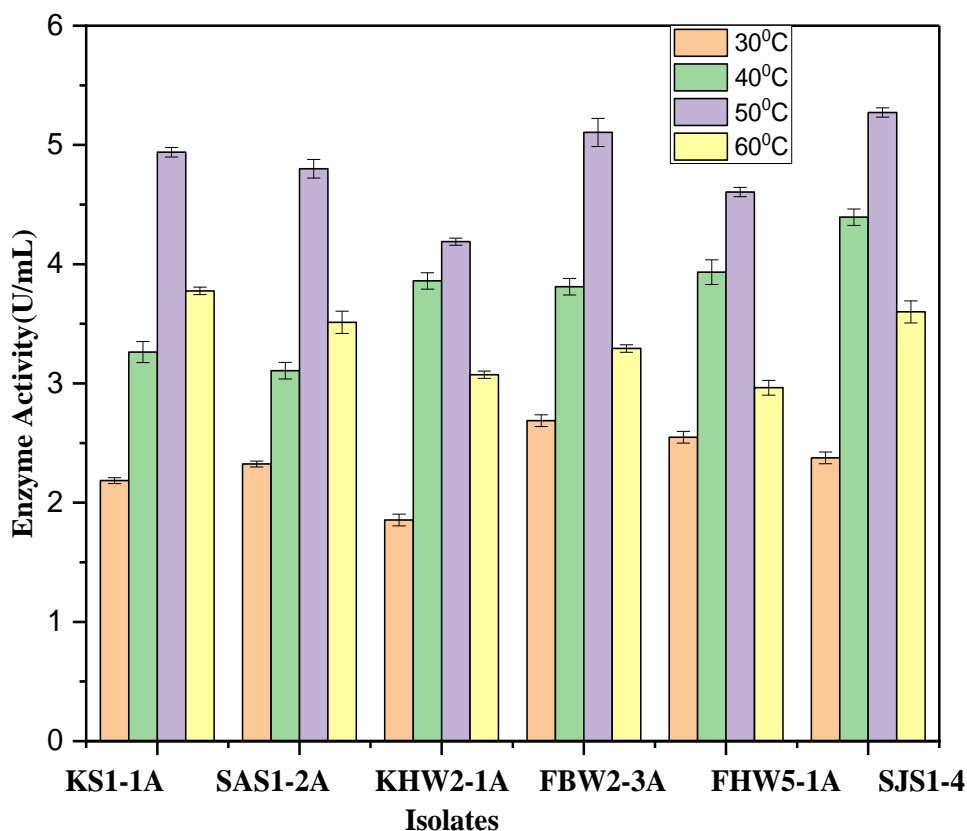


Figure 1: Effect of Temperature on amylase production

4.2.2. Effect of incubation period

The effects incubation period on amylase synthesis were determining by incubating the selected isolates in different incubation length of time (12, 24, 48, and 72 h) at 50°C with (pH 7). Among the four different incubation times that was tested for alpha-amylase production, the optimum incubation period for all isolates was occurred at 24 h of incubation period and the maximum and minimum alpha-amylase activity FBW2-3A(4.2±0.03) and SJS1-4A (3.6±0.03 U/mL), respectively (Figure 2). At 24h there was significant difference in the activity of alpha-amylase synthesized by isolate at various incubation time and also different isolates at the same incubation time (P<0.05).

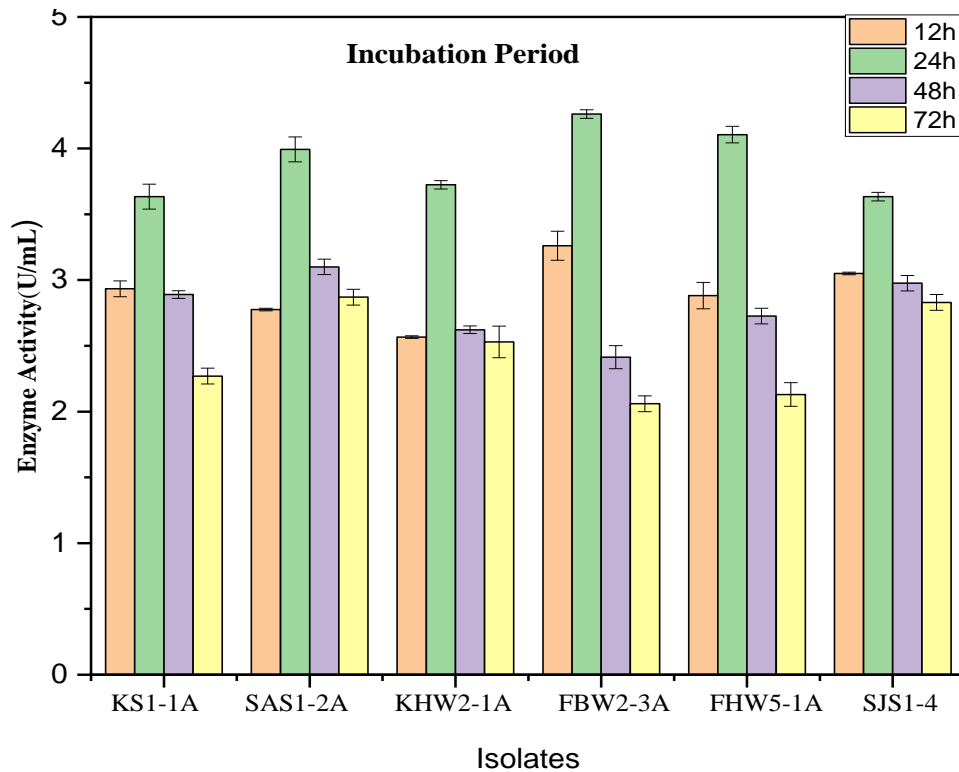


Figure 2: Effect of incubation period on alpha-amylase production

4.2.3. Effect of pH

In this study, the effect of pH on alpha-amylase synthesis was determined by preparing the basal media with different pH and incubating each isolate in pH 5 to 9 at 50°C for 24h. The optimal pH for all isolates was obtained at pH 7 and the maximal and lowest amylase activity was obtained by FBW2-3A (4.3 ± 0.05) and SJS1-4A (3.8 ± 0.01 U/ml/), respectively (Figure 3). At pH7, all isolates there was significant difference ($p < 0.05$) in the production of alpha-amylase among isolates at various pH and different isolate with the same pH (SAS1-2A, FBW2-3A and FHW5-1A and but KS1-1A, KHW2-A and SJS1-4A was not showed significant difference at the same pH ($p > 0.05$)).

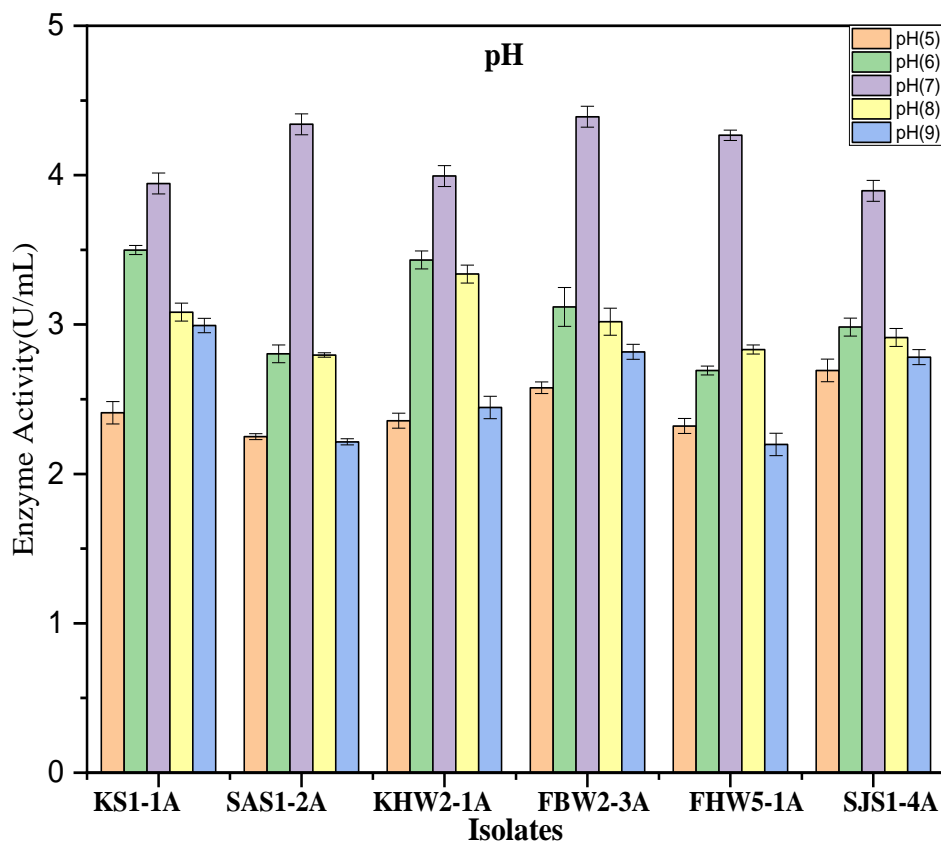


Figure 3: Effect of pH on alpha-amylase production

4.2.4. The effect of carbon source

In this study, the effect of different carbon source on alpha-amylase synthesis was detected by enriching the basal media with various carbon sources (1% w/v) such as lactose, sucrose, fructose maltose and glucose by incubated at 50°C with pH7 for 24h. As the result indicated in Figure 4, three isolates high alpha-amylase when lactose used as carbon source (KHW2-1A (5.9 ± 0.09), FBW2-3A (6.2 ± 0.04) and SJS1-4 (5.5 ± 0.04 U/mL), while in other three isolates KS1-1A (5.7 ± 0.09), SAS1-2A (6.0 ± 0.1), and FHW5-1A (6.1 ± 0.1 U/mL), maltose, glucose and fructose, respectively. The highest amylase production was observed by lactose FBW2-3A (6.2 ± 0.09), but the production of alpha-amylase was showed highly suppression for the two isolates, SAS1-2A (1.6 ± 0.03) and FHW5-1A (1.7 ± 0.09) in the presence of sucrose. There was a significant difference in the alpha-amylase synthesis among isolates at different carbon sources and also different isolates at the same carbon source ($P < 0.05$).

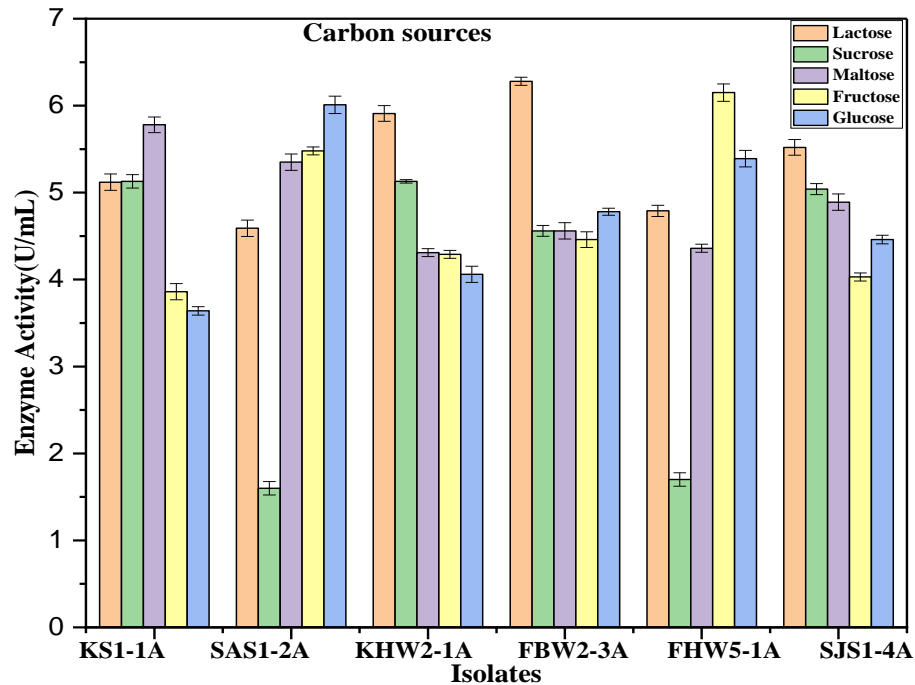


Figure 4: The effect of carbon source for alpha-amylase production

4.2.5. Effect of organic nitrogen sources

In the current study, the effect of organic nitrogen sources on alpha-amylase production was determined by supplementing the basal media with different organic nitrogen source (at 1% level) like urea, tryptone, beef extract, and soybean with (pH 7) and isolates were incubated individually at 50°C for 24 h. As the results indicated in Figure 6, the maximum amylase production for the three isolates, SAS1-2A (5.0±0.03), KHW2-1A(5.2±0.08) and FBW2-3A (5.6±0.1) was achieved by the urea supplement while the other two isolates, KS1-1A (4.9±0.1) SJS1-4A (5.2±0.03) and isolate FHW5-1A (5.1±0.07U/mL) was exhibited by the presences of tryptone and soybean, respectively(Figure 5). The highest result was observed in urea by FBW2-3A (5.6±0.01 U/mL) while the least one was in beef extract KS1-1A (4.9±0.03U/mL). There was significant different in alpha-amylase production between isolates at different organic nitrogen sources and also different isolates at the same organic nitrogen source (p<0.05).

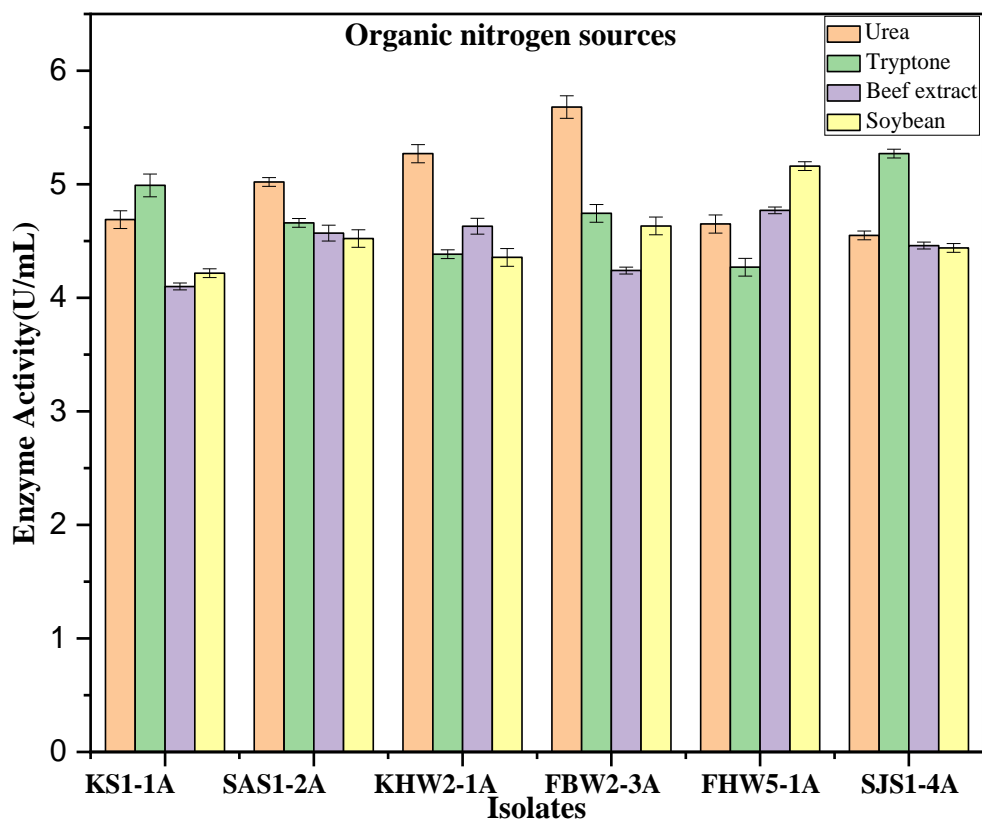


Figure 5: Effect of organic nitrogen sources on alpha-amylase production

4.2.6. Effect of inorganic nitrogen source

In this study, the effect of inorganic nitrogen sources on alpha-amylase synthesis was examined by enriched the basal media with (1%) level of various inorganic nitrogen sources like potassium nitrate, sodium nitrate ammonium chloride, and ammonium sulphate. As the results showed in Figure 6, the three isolates, KS1-1A (4.5 ± 0.1), KHW2-1A (4.9 ± 0.07) and SJS1-4A (5.1 ± 0.03 U/mL) the optimum amylase synthesis was obtained in the of presence of $(\text{NH}_4)_2(\text{SO}_4)$, while other two isolates, SAS1-2A (4.9 ± 0.1 and FHW5-1A (5.2 ± 0.03 U/mL) and isolate FBW2-3A (5.3 ± 0.04 U/mL) in the of presences NaNO_3 and KNO_3 , respectively. There was a significant difference in alpha-amylase synthesis between different inorganic nitrogen source between isolates and the same inorganic nitrogen source with different isolates ($p < 0.05$).

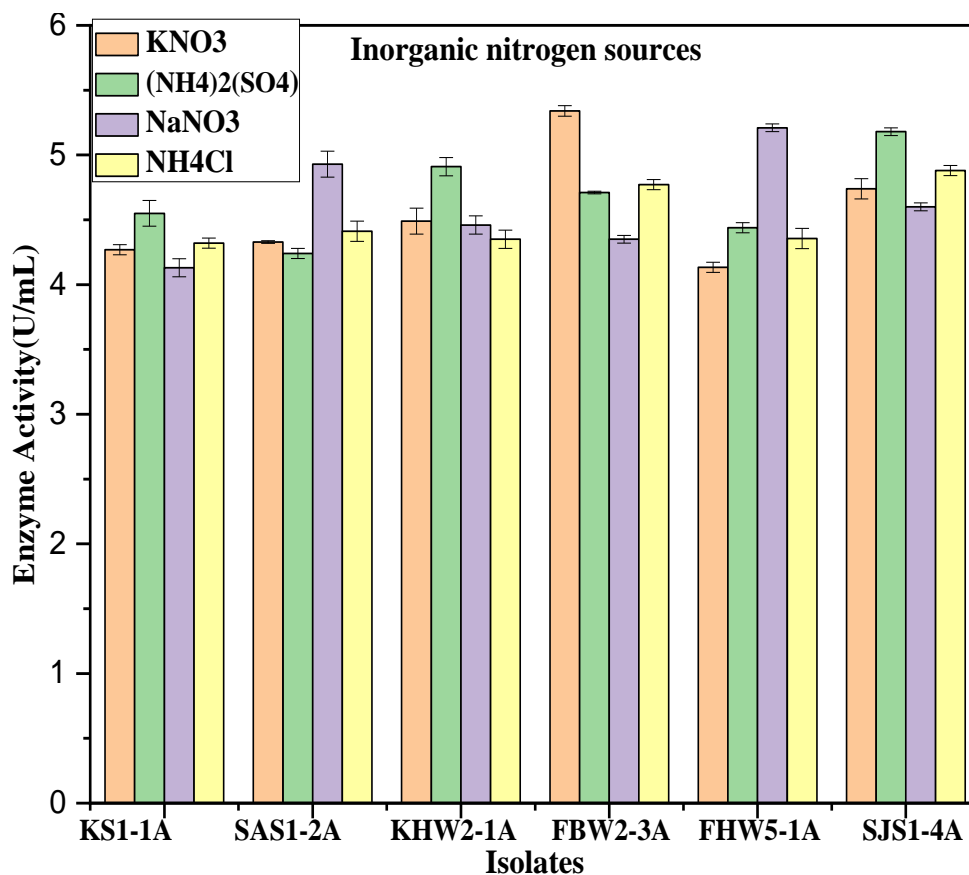


Figure 6: The effect of inorganic nitrogen source on alpha-amylase production

4.2.7. Effect of starch concentration

In this study, the effect of starch concentration on alpha-amylase synthesis the basal medium was enriched with various starch concentrations ranging from 1, 2, 3, and 4% w/v and the optimal starch concentration for the production of alpha-amylases for all isolates was at 2% level which resulted the maximum and minimum activity achieved by isolates FBW2-3A (6.5 ± 0.1) and SAS1-2A (5.1 ± 0.05 U/mL), respectively (Figure 7). There was significant difference in amylase production between isolates at different starch concentrations and also different isolates with the same (2%) starch concentration ($p < 0.05$).

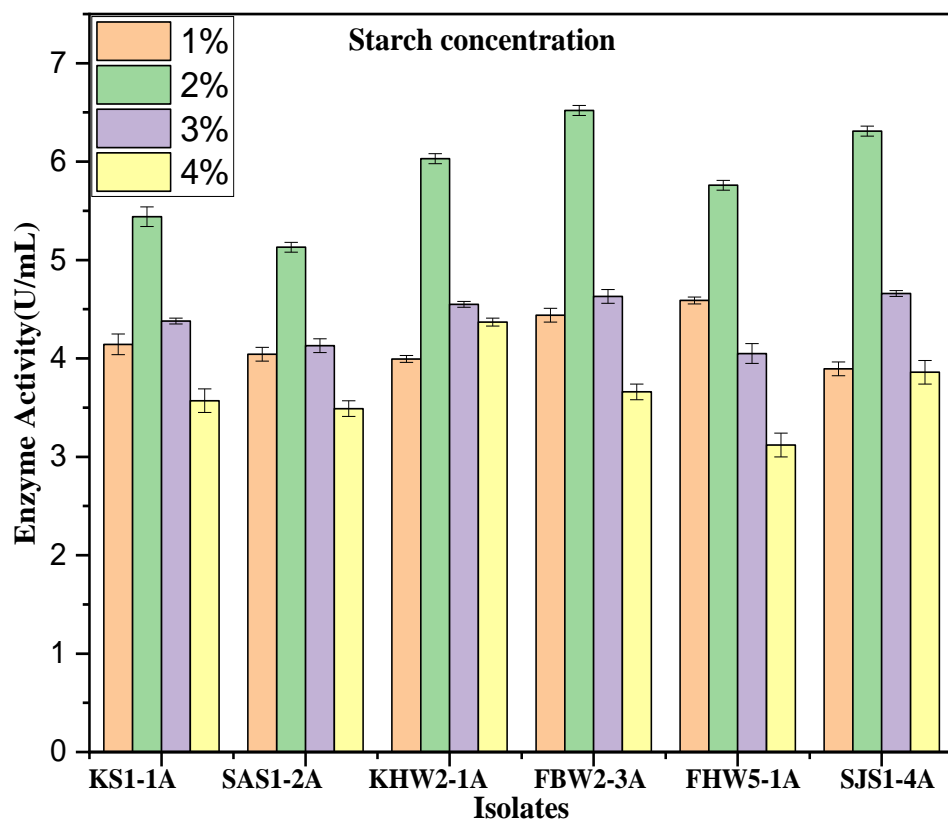


Figure 7: The effect of starch concentration on alpha-amylase production

4.2.8. Effect of metal ions

In this study, the effect of metal ions on alpha-amylase production was determined by addition of different metal ions on basal media as enrichment such as K, Mn, Zn and Na. As results indicated in Figure 9, the three isolates, FHW5-1A (5.3 ± 0.1), KS1-1A (5.2 ± 0.08) and KHW2-1A (5.2 ± 0.1 U/mL) the highest amylase synthesis was achieved in presence of Mn while the other two isolates, FBW2-3A (5.33 ± 0.1) and SJS1-4 (5.2 ± 0.1) in K and the rest one SAS1-2A (5.2 ± 0.03 U/mL) isolate in Na, respectively (Figure 8). When (K, Na and Mn) used metal ion sources in the optimization of the selected isolates help to synthesis of high alpha-amylase for all isolates and the maximum result was observed by isolate FBW2-3A (5.33 ± 0.1 U/mL) but when Zn ion, used as metal ion source the production of alpha-amylase in all isolates showed high suppression and leads to lowest alpha-amylase activity was obtained by isolate KS1-1A (2.7 ± 0.08 U/mL). There was significant difference ($p < 0.05$) in the production of alpha-amylase by individual isolate between different metal ions and also between different isolates with same metal ions ($p > 0.05$).

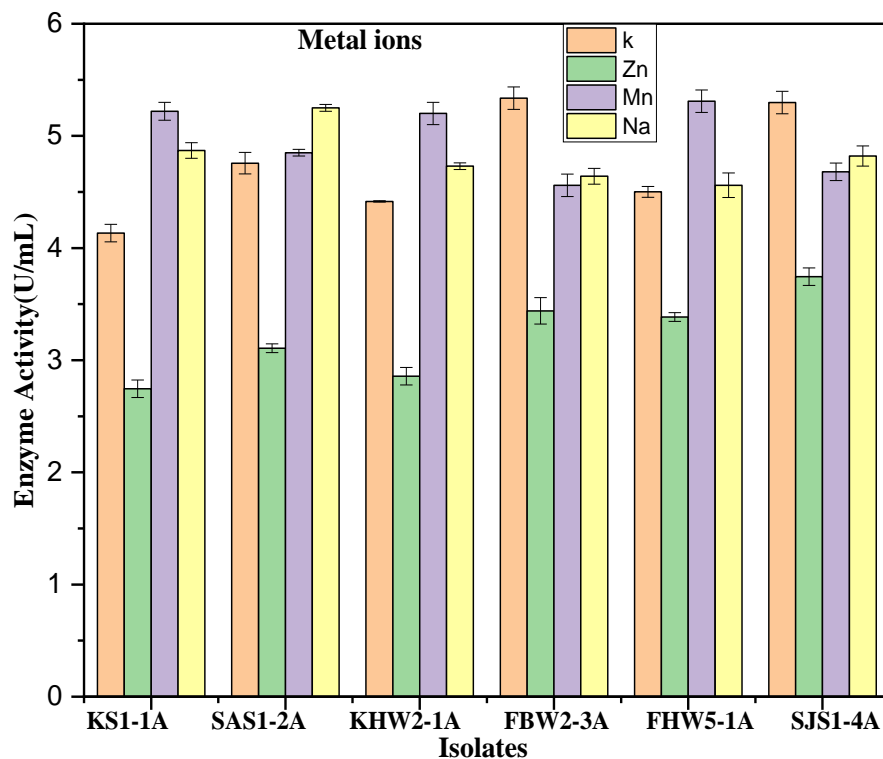


Figure 8: The effect of metal ions on production of alpha-amylase

4.2.9. Effect of inoculum concentration

In this study, the effect of inoculum concentration in alpha-amylase synthesis was determined by incubating the cell culture with various volumes (0.5 l, 1.5 and 2% v/v) of broth. As results showed in Figure 9, all isolates the optimal cell concentration on alpha-amylase production was achieved at 1mL and the maximum and the minimum activity was noticed by isolate FBW2-3A ($4.9 \pm 0.07 \text{ U/mL}$) and SJS1-4A ($3.8 \pm 0.08 \text{ U/mL}$), respectively. There was a significant difference on the synthesis of alpha-amylase among isolates between different inoculum concentration and different isolates with the same inoculum concentration ($p < 0.05$) except KS1-1A and SAS1-2A did not show significant difference with the same inoculum volume ($p > 0.05$).

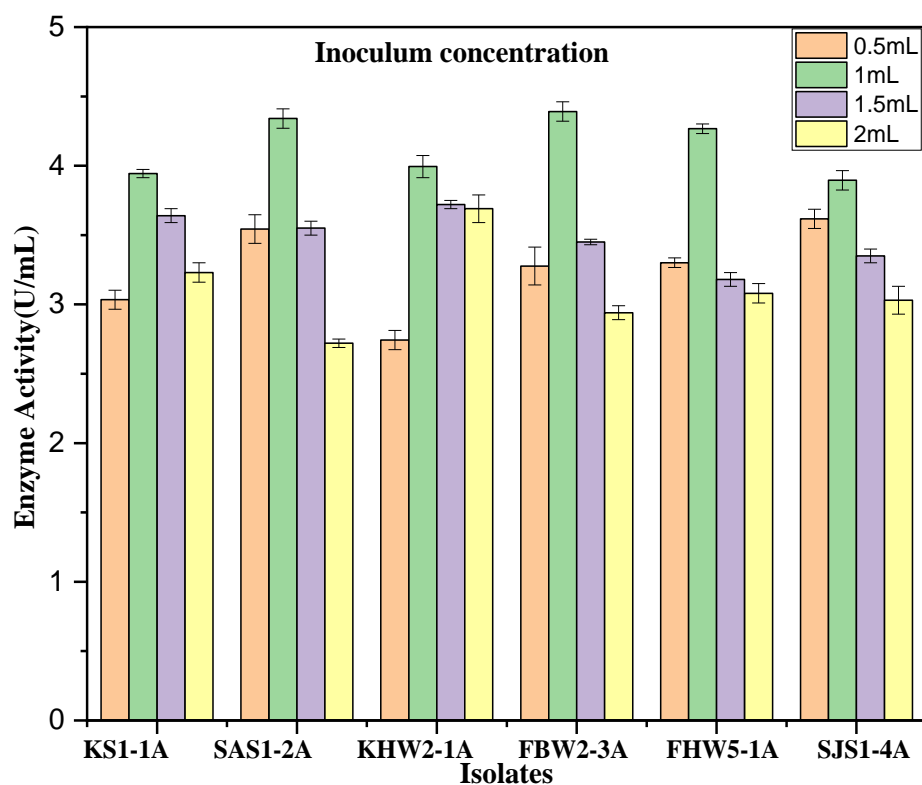


Figure 9: The effect of inoculum concentration

4.2.10. Effect salt (NaCl) concentration

In the current study, the effect of NaCl concentration on alpha-amylase synthesis was optimized by adding various NaCl concentrations in basal media, including 0.5, 1, 1.5 And 2.0% w/v. The optimum salt concentrations on alpha-amylase production for all isolates were obtained at a concentration of 1.5% w/v. As the results indicated in Figure 10, the highest and lowest amylase activity was observed by isolate FBW2-3A ($5.8 \pm 0.1 \text{ U/mL}$) and SAS1-2A ($4.6 \pm 0.04 \text{ U/mL}$), receptivity. All isolates showed a significant different on the alpha-amylase production among isolates and at different salt concentration ($p < 0.05$) and also between different isolates with the same salt concentration except FBW2-3A and SJS1-4A did not show significant difference with salt concentration of 1.5% w/v ($p > 0.05$).

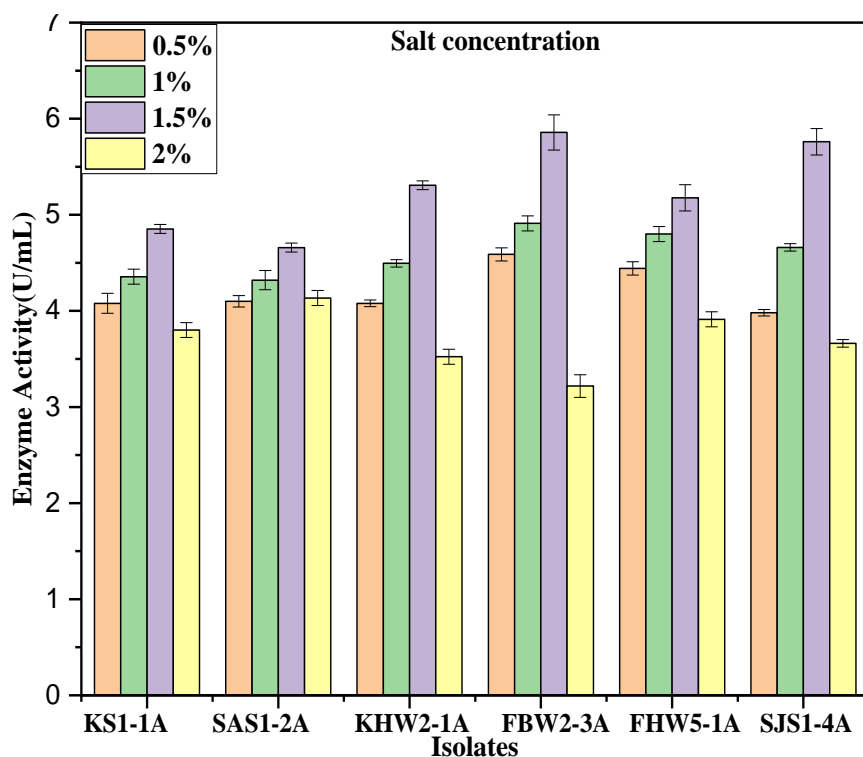


Figure 10: The effect salt (NaCl) concentration on production of alpha-amylase

4.3. Optimal enzyme activity under all optimized conditions

In the current study the optimal condition of temperature, pH, incubation time, nitrogen and carbon source, enzyme, salt, starch and inoculum concentration and metal ions were added in fermentation media and their amylase production capacity was tested. The optimal condition of all selected isolates temperature 50°C, pH,7 salt and starch concentration (1.5 and 2%) and inoculum site (1mL) was the same preference. However, with other parameters their preference was vary in each isolate in which the optimal condition for KS1-1A was maltose, tryptone (NH₄)₂(SO₄) and Mn SAS1-2A, glucose, urea, NaNO₃, and Na, KHW2-A lactose, urea, (NH₄)₂(SO₄), and Mn, FBW2-3A lactose, urea, KNO₃ and K for FHW5-1A fructose, soybean, KNO₃ Mn and SJS1-4A Lactose, tryptone (NH₄)₂(SO₄), and K. From all the six selected isolates the highest amylase result was achieve by FBW2-3A(Figure 11)There was a significant different on the alpha-amylase production between KS1-A SAS1-2A and FBW2-3A and other three isolates but the three isolates (SJS1-4A ,KHW2-1A and FHW5-1A) did not show significant difference with different parameters (p>0.05).

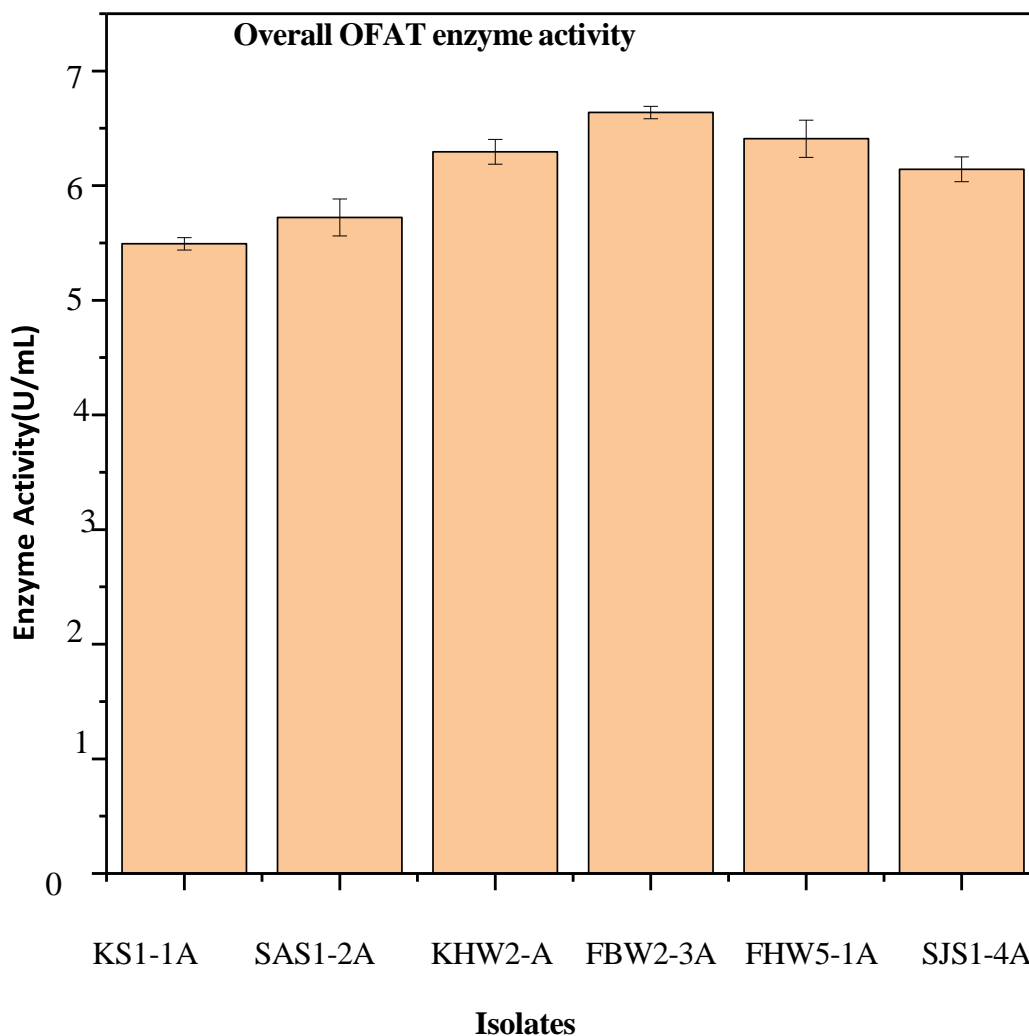


Figure 11: Optimal enzyme activity under all optimized condition

4.4. Characterization of crude alpha amylase enzyme..

4.4.1. The effect of temperature

In this study, the effects of temperature on alpha-amylase activity was determined by incubating the 1mL of crude alpha-amylase in starch solution (1%) with phosphate buffer (pH 7) at different temperatures (45, 50, 55, 60 and 65°C) in water bath for 15 min and the optimal temperature for all isolates for alpha-amylase activity was achieved at 55°C. As the result showed in Figure 12a, the highest and the lowest crude alpha-amylase activity was obtained by FBW2-3A (5.1±0.1U/mL) and

KS1-1A (4.3 ± 0.04 U/mL), respectively. There was a significant difference on the activity of alpha-amylase under different incubation temperature ($p < 0.05$) and between different isolates crude alpha-amylase activity at same temperature except KS1-A, KHW2-A and FHW5-1A isolates ($p > 0.05$).

4.4.2. Effect of pH

In the current study, the effect of pH on amylase activity was measured by preparing 1% of starch solution as substrate with different phosphate buffer, pH ranges from 5 to 9 and incubated with 1mL crude alpha-amylase at 55°C for 15 min and the optimal pH for all isolates extracted alpha-amylase activity was at pH 7. In Figure 12b the results showed that the maximum and minimum activity obtained by isolate FBW2-3A (4.9 ± 0.1) and SJS1-4A (4.18 ± 0.07 U/mL), respectively. There was a significant difference on the crude alpha-amylase activity at various pH ($p < 0.05$) and between different isolates of at same pH 7.

4.4.3. Effect of metal ions

In the current study, the effect of pH on amylase activity was measured by preparing 1% of starch solution as substrate with different phosphate buffer, pH ranges from 5 to 9 and incubated with 1mL crude alpha-amylase at 55°C for 15 min and the optimal pH for all isolates extracted alpha-amylase activity was at pH 7. In Figure 12b the results showed that the maximum and minimum activity obtained by isolate FBW2-3A (4.9 ± 0.1) and SJS1-4A (4.18 ± 0.07 U/mL), respectively. There was a significant difference on the crude alpha-amylase activity at various pH ($p < 0.05$) and between different isolates of at same pH 7.

4.4.4. Effect of starch concentration

In the current study, the effect of starch concentration on alpha-amylase activity was determined by incubating 1mL of crude alpha-amylase in 1mL of different starch concentrations (1, 2, 3 and 4% w/v) (pH 7) for 15 min at 55°C . As the result showed in Figure 12d the optimal starch concentration was at 2% for all isolates crude alpha-amylase and the maximum and the minimum crude alpha-amylase activity recorded by isolate FBW2-3A (6.6 ± 0.1) and KS1-1A (5.5 ± 0.05 U/mL), respectively. There was a significant difference in each isolate crude alpha-amylase activity at different starch concentration and between different isolates' crud alpha-amylase activity at the same starch concentration (2%) ($p < 0.05$) except isolates, FBW2-3A and SJS1-4A did not showed significant difference at same starch concentration (2%) ($p > 0.05$).

4.4.5. Effect of salt concentration

The effect NaCl concentrations on alpha-amylase activity was measured by incubating 1mL of crude alpha-amylase in various (0.5, 1, 1.5 and 2% w/v) of NaCl concentration at pH 7.0 for 15 min at 55°C. In this study, the optimal alpha-amylase activity was obtained at 1.5% NaCl concentration and the highest and the lowest crude alpha-amylase activity revealed by isolate SJS1-4A(5.81±0.1) and SAS1-2A (4.78±0.1U/mL), respectively (Figure 12e). There was a significant difference in the activity of crude alpha-amylase at different NaCl salt concentration ($p < 0.05$). However, significant difference was not showed in amylase activity of different crud alpha- amylase at the same NaCl salt concentration ($p > 0.05$).

4.4.6. Thermal stability

In this study, the thermal stability of alpha-amylase was assessed by pre-incubating 1mL of crude alpha-amylase at various temperatures (55, 60, 65 and 70°C without substrate for 30 and 60 min. which mean first incubated for 30min with different temperature (55-70°C) and also at the same way incubated for 1h and then its stability was measured. As indicted in (Figure 13 f, g,h&i) at 55°C for 30' the crude alpha-amylase was retained its thermal stability above 90%. However when the temperature increase above 55°C its thermal stability decreased and lost its original activity a round at 70 and 65°C about 70% at 30 min and 1h, incubation time respectively(Simair, Khushk, et al., 2017)

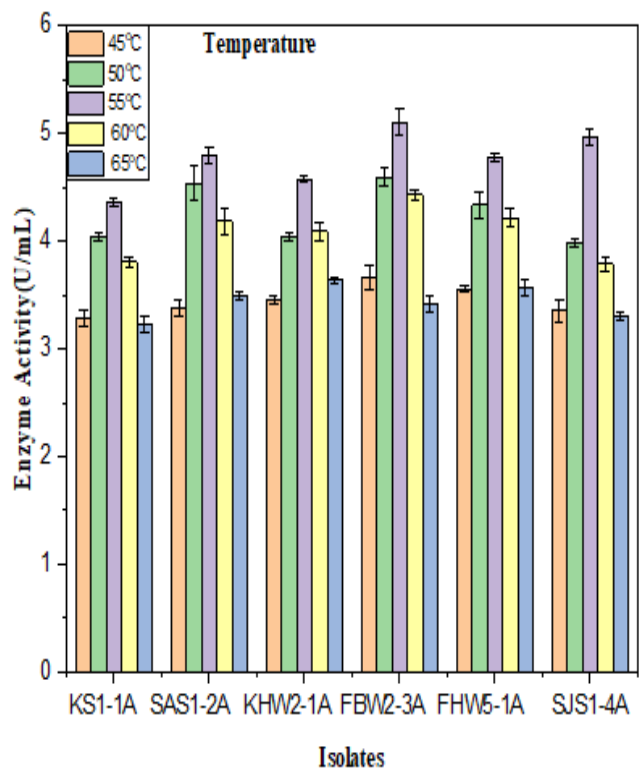


Figure (12a)

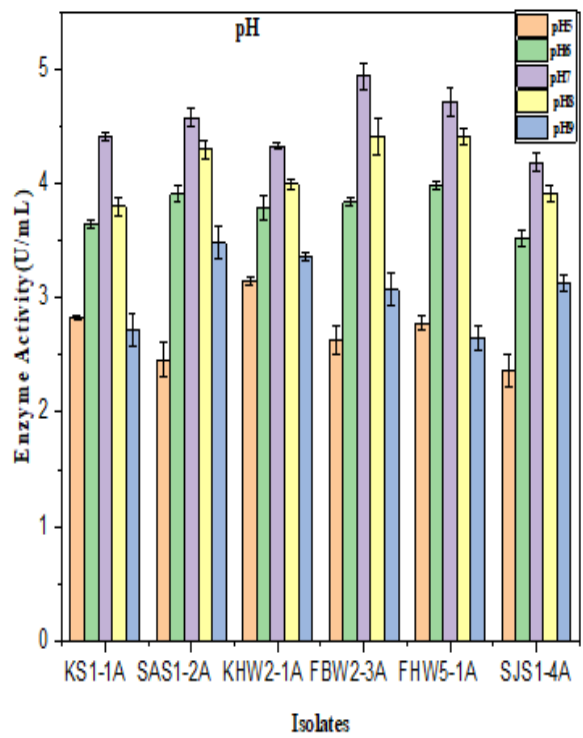


Figure (12 b)

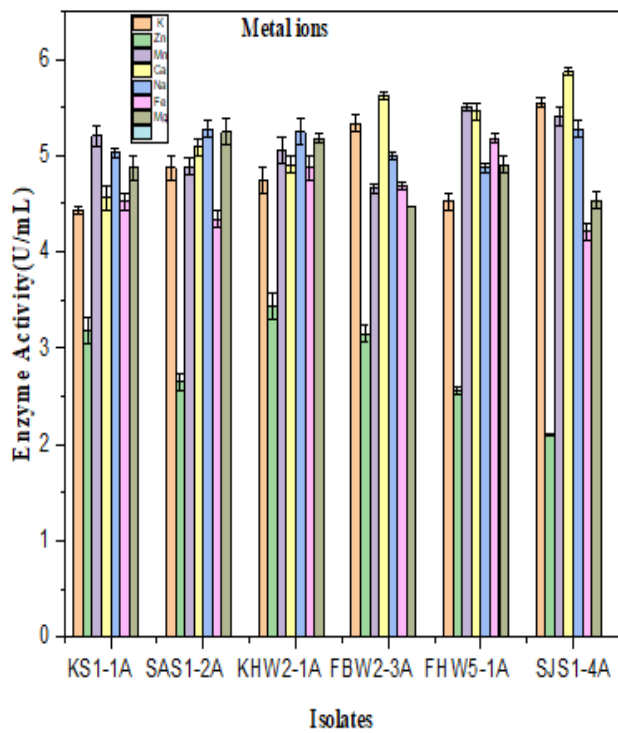


Figure (12c)

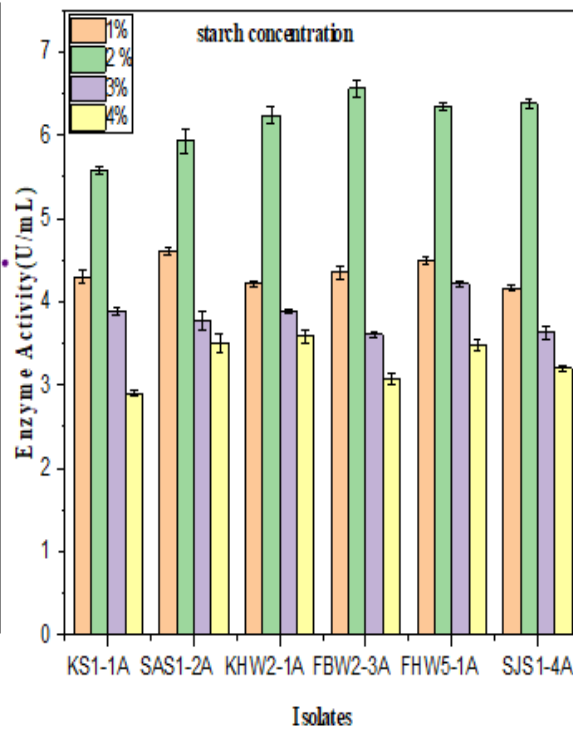


Figure (12d)

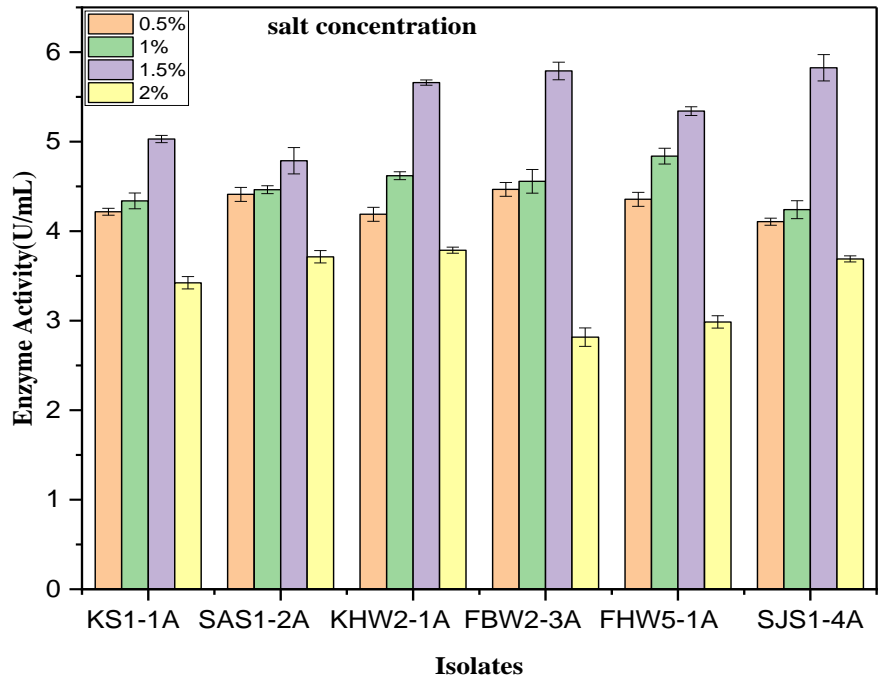


Figure: 12(e)

Figure: 12 Alpha-amylase characterizations

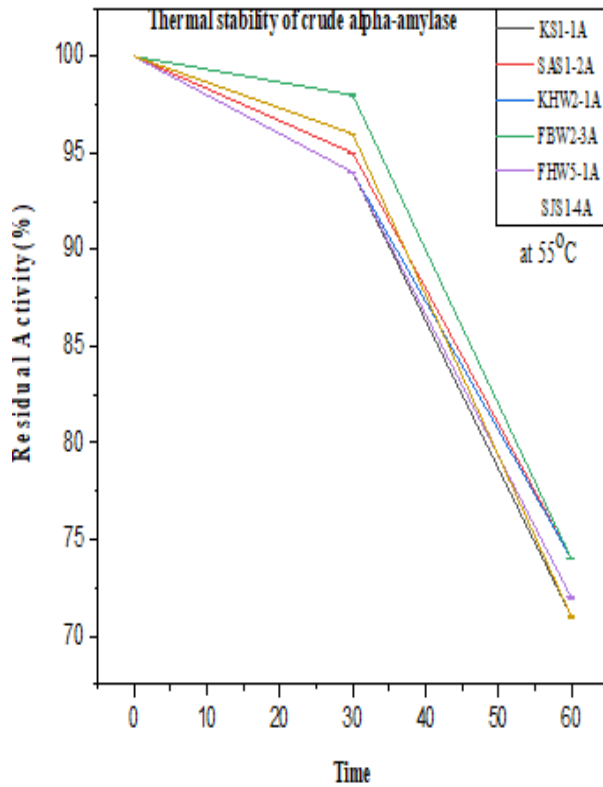


Figure (13f)

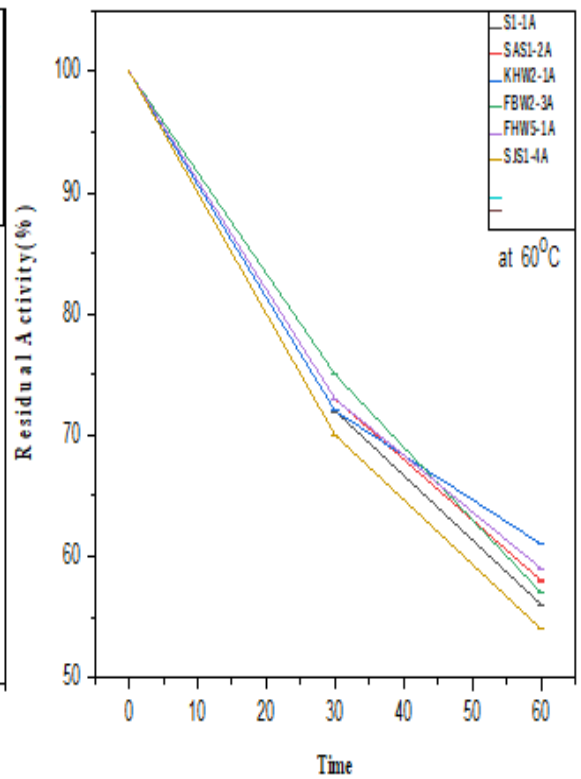


Figure (13g)

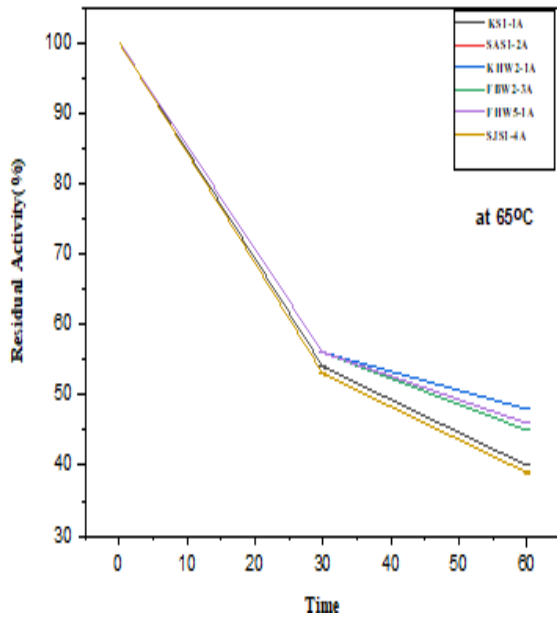


Figure (13h)

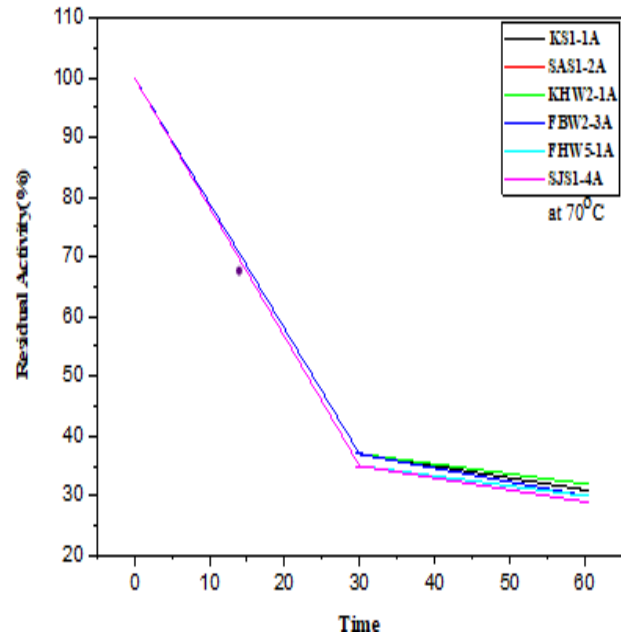


Figure (13i)

Figure: 13 Thermal stability of crude alpha-amylase

4.5. Partial purification and Specific activity of alpha-amylase

In the current study the partial purification of the α -amylase was conducted for isolate FBW2-3A (*Bacillus cabrialesii*) via ammonium sulphate precipitation method followed by dialysis. In which 50mL of crude alpha-amylase was participated and the best active fraction was obtained in 60% - 80% of ammonium sulphate concentration and followed dialysis. After dialysis and SDS- PAGE, specific activity and molecular weight of alpha amylase was determined by egg albumin (ovalbumin) protein in comparison with marker protein. The specific activity of crude alpha-amylase was determined by plotting the protein standard curve (appendix: D). As results indicted in Table 10, the total protein was decreased started from 30% ammonium salt precipitation to 80% while specific activity was showed increasing from 5.5 U/mg in the crude sample to 10.12 U/mg in the partially purified fraction, increasing the purity by 1.8-fold. Approximately 20kDa of amylase protein was detected after sample loading (Figure 12).

Table 10 : Partial purification steps and alpha-amylase activity

step	Volume 50mL	Total protein (mg/mL)	Activity (U/mL)	Total activity U/mg	Specific activity (U/mg)	fold	Yield (%)
crude	50	42.28	6.63	279.04	5.5	1	100
30-60%	6	5.94	6.66	39.59	6.5	1.1	13.9
60-80%	3	4.5	6.75	30.37	10.12	1.8	10.88

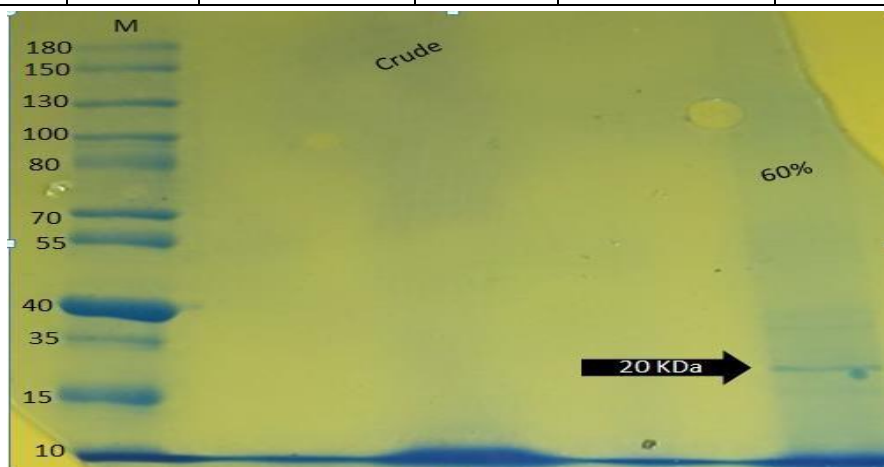


Figure 14: SDS PAGE

4.6. Application of crude alpha-amylase in in food industry

In this study the biotechnological application of the crude alpha amylase of *Bacillus carbialessi* was tested by making bread leavening. After 2h of blending, the rising height of dough was measured and compared with the yeast raising capacity in which the raising height of yeast plus crude alpha-amylase and yeast alone was 5.5 cm and 4.5 cm, respectively. The wheat flour that contained only crude amylase did not rise, remaining at the same height as the negative control. In contrast, the combination of crude alpha-amylase and yeast resulted in a higher dough rise compared to the control (negative), which included only crude alpha-amylase and yeast. This demonstrates the enhanced effectiveness of combining crude alpha-amylase with yeast in promoting dough fermentation and rising (the result indicated in the picture (appendix: D)).

5. Discussion

Due to high selectivity and catalytic capabilities, several enzymes are essential to carrying out biochemical reactions within the cells and outside of the cells and creating a broad range of products in industry. Among those enzymes amylase is the common one, especially thermostable alpha-amylase is the most interesting enzyme which can apply at high temperature in various industrial sectors to hydrolyses starch based complex molecule in to its smaller fragment (P. M. de Souza, 2010). Since most industrial alpha-amylase is extracted from microorganisms like bacteria, it is important to isolate a local strain with a high alpha-amylase production capacity and they can be isolated from a different of environments, like hot springs, and extreme environments. Hot springs and other hot environments were selected as the source of bacterial isolation (Fossi et al., 2005). In Ethiopia there are a number of hot springs and other hot environment that likely to good source of thermostable alpha-amylase synthesizing bacterial strains. From different hot springs that found in different parts of the country, Addis Ababa is one of which contain many hot environments such as Finfine hot spring, Leghar Jone Holy Tsebel, Hyat Medihaniaylem Holy Tsebel, Koyefech, Gelan Saint Gebreal and Saint George and also other hot environment such as Yeha Bio-gass and Qoshe damping sites (Masi et al., 2023) which was proposed as source of thermostable alpha-amylase producing bacterial strains. From ten sample sites six most potent isolates KS1-1A, SAS1-2A, KHW2-1A, FBW2-3A, FHW5-1A and SJS1-4A alpha- amylase producers identified and selected for further optimization. All the six selected bacterial isolates were identified and characterized through morphological, biochemical and also molecular method. The 16S rRNA gene sequencing analysis result indicated that all six isolates showed above 96% identity.

The their species type of the six most potent isolates belonged as FBW2-3A (*Bacillus cabrialesii*), a novel endophytic plant growth promotor bacterium which was discovered in 2018 from wheat (*Triticum turgidum* L. subsp. durum) plants found in Mexico's Yaqui Valley (de los Santos Villalobos et al., 2019). In the current study, this strain was isolated from Finfinnee hot spring bathroom outlet. As the outlet water were contained different derbies, soap bubble and particles that discharged from bathroom service. This environment indicated that there were starch contain substrate in which the strain we obtained in this environment produce amylase to degrade as an energy sources and can grow best at 50°C with highest amylase producer compared to the other selected isolates.

FHW5-1A isolates is belonged to *Acinetobacter haemolyticus*. This strain is reported to be ubiquitous, saprophytic, and become a significant nosocomial pathogen as a result of their ability to survive in hospital settings on different dry and wet surfaces(Gupta et al., 2015). In study, this strain was also isolated from four Finfnie hot spring combining bridge ground drain pipe surrounding which make as small pond. As the hot spring were contained different decayed grass, animal dung and other materials, in which this the strain may be obligate to produce amylase for breaking down of these molecules as their source of energy and other physiological functions.

SJS1-4A isolate is belonged to the *Enterobacter quasiharmachei*. This strain reported as a novel strain which was isolated from sputum samples of patients at a hospital in PR China in 2017 and is considered as opportunistic pathogens that can cause many kinds of diseases in humans (C. Wang et al., 2020). In the current study, this strain was obtained from Saint Jon Holy Tesbel surrounding moist hot soil. This soil condition mud and hot likely contains starch molecules in which the strains had experiences to produce amylase.

KHW2-1A isolate is belonged to *Bacillus cereus*, have demonstrated promise as bio-control agents for controlling plant diseases, lowering the demand for chemical pesticides, and promoting sustainable farming (SUKMAWATI et al., 2024). Other finding also show that this strain used to degrading of phenol compound and used for treatment of contaminated water by Hydrocarbon (Faculty, 2019) .In current study, this strain was isolates from Koye fech hot spring canal shore found in Lemi Kura sub city around ICT Park. As the hot spring water was filled with variety of derbies, suspended materials, grass, plastics probably contain starch and the strain might be experienced to produce amylase.

SAS1-2A isolate is belonged to *Pseudomonas plecoglossicida*, this strain are reported to cause illnesses in aquaculture especially in fish (Sukenda & Wakabayashi, 2000). In the current study, this strain was isolated from Saint Angle Gebreal Holy Tsebel surrounding soil it was hot and moist. As it surrounded by farm land in which plant residues generated towards the Holy Tsebel compound especially from consequent stream or steep land as it is sunken place in which the strain may be experienced for the production of amylase.

KS1-1A isolate was unable to determine by common molecular techniques. In this study, this strain was obtained from Qoshe damping site and the site is artificially made by human as a land fill with

different solid wastes were deposited over centuries. The soil contained huge organic and inorganic compounds and it was hot due to microbial decomposition of the material. Due to the presence of such starch-containing materials, the strain might have experience for the production of amylase for breaking down of such available compound.

The production of alpha-amylase by bacterial strains during submerged state fermentation is significantly influenced by a different of physicochemical factors (Yassin et al., 2021). There are factors that influence how quickly amylase breaks down starch. Therefore, determination of best fermentation conditions for alpha-amylase producing bacteria is necessary. Additionally, it is possible to determine the inhibitory amounts of metal ions and salts for that specific alpha-amylase production. It is imperative to determine its ideal circumstances to ensure the quality and quantity of alpha-amylase, which can be achieved by characterization (Islam, 2016). In current study, the impacts of physicochemical parameters on alpha-amylase synthesis on the selected bacterial isolates were exposed to find optimal growth condition.

Temperature has significantly impact on alpha-amylase synthesis. The selected bacterial isolates were subjected to various temperatures between 30 to 60°C for 24 h. The production of alpha-amylase was increased started from 30 until 50 °C being the optimal temperature and the highest result was recorded at 50°C for all isolates. While at 60°C drastic decreases of the amylase activity was observed, these might be associated with two reasons, due to direct impact on the reaction rate constant, and the thermal denaturation of the enzyme by high temperatures that cause the breaking of hydrogen and disulphide bond (Elmansy et al., 2018). Supportive study reported that bacterial strains from Turkish hot springs called *B. licheniformis* (Adiguzel et al., 2009) has the best alpha-amylase activity at 50°C. In contradiction to the study reported by (Gebreselema, 2015), showed that the range of 35 to 40°C was the greatest amylase synthesis for *Bacillus* sp. from the poultry source and soil, respectively. Variations between the current finding and the earlier research could be caused by different strain types and sources of bacterial isolation. Determining the duration of bacterial growth is important for maximizing alpha-amylase synthesis. In the current study, the maximum rate of alpha-amylase synthesis in all isolates was obtained at 24 h incubation time. However, the activity of amylase was decreased with further increasing incubation time above 24h. This is because when the bacterial isolates were incubating for long period of time without further addition of nutrient, they exploited all the nutrients and other resource that leads to depletion of nutrients and may resulted in

synthesis of harmful metabolites that causes autolysis, and slow growth of isolates as suggested by previous study (Ikram-Ul-Haq et al., 2005)..

Another important parameter that significantly influences the synthesis of amylases is pH (Alva et al., 2007). In this study, the maximum amylase production was recorded at pH 7 while decreasing amylase activity was observed as further increasing of the pH. This is because too high or low pH from the optimal conditions might cause denaturation of enzyme and such fluctuation of pH disrupt the hydrogen and disulphid bonds that keep the three dimensional structure of (Pan et al., 2007).

To increase the synthesis of alpha-amylase, the selected isolates were further optimized by different nutrient sources. The isolates showed different preferences for carbon sources in the production of amylase. This is a common phenomenon that different microbes have different carbon source requirement as their energy source, biosynthesis, and regulation of metabolism pathway (Lowe et al., 1993). Due to this the three isolates FBW2-3A, SJS1-4A and KHW2-1A having highest alpha-amylase synthesis was revealed when lactose was used as carbon source. This study is supported by other published finding report (P. E. et al., 2022b) on strain *B. circulans*. While isolate KS1-1A showed the best alpha-amylase in the presence of maltose as carbon source. In isolate FHW5-1A the highest alpha-amylase yield was achieved by the presence of fructose as carbon source. But, isolate SAS1-2A had the best alpha-amylase activity by glucose as carbon source.

The effect of organic and inorganic nitrogen sources had significant impact on the growth and production of alpha-amylase (Hayet et al., 2021). In this study all organic sources used as supplementation of the basal media were showed positive effect on the synthesis of alpha- amylase. This is because; organic nitrogen sources are essential for amino acid synthesis, cellular regulation, energy source and metabolic pathway (Mara & Horan, 2003). But all isolates has different alpha-amylase production performance which able to degrade certain starch based substrate (Sundarram & Murthy, 2014) In which the best alpha-amylase production yield was recorded from the isolate SAS1-2A and FBW2-3A when urea was used as nitrogen sources. This is because urea is a chemical compound that is made up of carbon dioxide and ammonia. Which was simple to use and supplement with the easily available nitrogen source found in the ammonia, which forms the urea, resulting in the induction of increased synthesis of alpha- amylase. While isolate KS1-1A and SJS1-

4A referred tryptone as nitrogen sources. Whereas isolate FHW5-1A has been performed in the presence of soybean as organic nitrogen source.

Inorganic nitrogen sources had significant role for energy synthesis of the cells, and synthesis of cellular components as well as for the synthesis of alpha-amylase (Sankaralingam et al., 2012). In this study, all inorganic nitrogen sources were showed relatively positive effect for production of alpha-amylase in all isolates and different performance among isolates. But the best alpha- amylase activity was obtained by isolates KS1-1A, SJS1-4A and KHW2-1A in the presence of ammonium sulphate as inorganic nitrogen source. While in isolates FBW2-3A the highest alpha-amylase was recorded in the presence of KNO₃ and other two isolates, KHW2-1A and FHW5-1A also showed the best alpha-amylase activity in NaNO₃ as an inorganic nitrogen source.

Starch is the basic substrate for synthesis of alpha-amylase (El-Fallal et al., 2012) in which its optimum concentration is necessary to initiate the synthesis of highest alpha-amylase and yielded best enzyme synthesis. In this study from four different investigated starch concentrations, the maximal alpha-amylase activity was yielded by all isolates at 2%. However, when the starch concentration increases the activity of alpha-amylase was decreased. This might impute fast starch consumption that resulted in the release of toxic metabolites and accumulation of hazardous metabolic byproducts may be the cause of the decrease production of α -amylase (Macneill et al., 2017). Moreover, elevated starch concentrations lead to increased viscosity in the broth and caused obstruction of oxygen transport and causing restriction of dissolved oxygen necessary for microbial growth (Elmansy et al., 2018). In other point of view, high amount of starch concentration induced stress response in bacterial cell which may divert resource away from enzyme production to stress mitigation mechanism (Csonka, 1989) and low metabolism capacity of isolates with short period of time. Metal ions are playing an important role in the synthesis of alpha-amylase. Because it's used as cofactor, stabilization of enzyme structure, radiation resistance, energy source and gene regulation (Lehninger, 1950). However, some metal ions are inhibitory and mask the activity of enzymes by binding the active site of the enzyme in which the activity of the enzyme decreases (Zohra et al., 2016). In the current study, from four different metal ions except Zn all metal ions had positive effect on the synthesis of alpha-amylase. Because zinc ion may have induced conformational change of the active site of the enzyme and interfere the enzyme substrate interaction and had high inhibitory properties (Maret, 2013),(Kiran et al., 2018)and (L. Lin et al., 1998).In this study, the

three isolates KS1-1A, KHW2-1A and FHW5-1A the maximal α -amylase activity was obtained when Mn used as metal ions. In isolate FBW2-3A and SAS1-2A the best α -amylase activity was achieved in the presence of K. While isolate SAS1-2A had highest α -amylase activity in the presence of Na. For maximal α -amylase production, optimal culture cell is necessary in a given fermentation condition. In current study, the optimal inoculum size for synthesis of α -amylase was obtained at 1mL of inoculum concentration with the highest and lowest activity was achieved by FHW5-1A and SJS1-4A, respectively. Beyond the optimal cell concentration and without supplementation of additional nutrient that lead depletion of resources and the growth of bacteria entered in death phase which leads to the accumulation of metabolic waste and caused the decrease of the production of α -amylase (Gonzalez & Aranda, 2023). Not only the increasing of cell culture but also the decreasing the amount of the cells caused decreasing of the α -amylase production due to unproportional enzyme substrate combination (Maier, 2009).

Salt is an important component of microbial nutrient, pH regulator, cell integration, enzyme activity and osmotic regulation of the cell and the surrounding environment (Csonka, 1989) which helps for the synthesis of best α -amylase synthesis. In this study, the optimal salt concentration for the synthesis of α -amylase was found at 1.5% and which resulted between maximum and minimum activity achieved by isolate FHW2-3A and SAS1-2A, respectively. But out of this optimal concentration the production of α -amylase became decreased. Because increasing or decreasing salt concentration from the optimum leads to hypertonic or hypotonic conditions, and ultimately dropped out or in the water from bacterial cell through osmosis and cause dehydration and turgidity of cells, respectively. This phenomenon creates the inhibition of bacterial growth and cause decreased α -amylase production (Taormina, 2010).

Enzymological characterization of the crude α -amylase is one of the crucial steps to determine the enzyme quality with different physical and chemical parameters which used for industrial application (Ivanova et al., 1993). In current study the optimal α -amylase activity, was revealed at 55°C in all isolates. But when the temperature increased out of the optimal, the activity of α -amylase was decreased. This is because when the incubation temperature increased beyond optimal, which causes the distraction of the tertiary structure of protein molecules and bring conformational change which make them the enzyme unable to fit properly with the substrate or don't form an enzyme-substrate complex due to the destruction of active site of the enzyme, this results to decrease

enzyme activity and finally diminished (Daniel et al., 1996). In this study, the optimal pH of all isolates for the synthesis alpha-amylase was at pH 7. But further increase of pH showed the activity of alpha-amylase decreased. This might result from a shift in the side chains on enzymes caused change of electrical charge on the active site, which would alter the enzyme's tertiary structure. Because when too few H⁺ ions or high protons) present, results the protein chains that comprise the enzyme's variable R-groups interact differently like ionic bonds, Van der Waals forces, and hydrogen bonds interaction which preserve and stabilize the enzyme's tertiary three-dimensional structure, affected (Nimisha et al., 2019).

The effect of metal ions on alpha-amylase activity was assessed by incubating the crude alpha amylase with various metals including K⁺, Na⁺, Zn⁺, Mn⁺, Fe⁺², Ca⁺² and Mg⁺. In this study, except Zn all metal ions showed positive effect on the activity of enzymes and the highest results in was achieved by isolate FBW2-3A and SJS1-4. Also other two isolates, KS1-1A and FHW5-1A obtained when Ca⁺² and Mn⁺ was used as metal ions sources (Figure 12c). While, other two isolates, SAS1-2A and KHW2-1A when Mg⁺ and Na⁺ was used as metal ion sources respectively. Because these metal ions could function as a co-factor required to boost the activity of the enzyme. On the other hand, Zn had significant inhibitory impact on most amylase activity. This is because of zinc ion has an ability to bind the active site of the enzyme that block the activity of reaction and also induced structural change of the enzyme (Liao et al., 2019). Zinc ion especially zinc oxide has nanomaterial or particles that has unique physical and chemical properties which might have binding capacity on the active site that inhibit the activity of amylase (Q. Lin et al., 2023).

In this study, the optimal starch concentration for all isolates' alpha-amylase was obtained at 2% level. As the result indicated in Figure 13d when the concentration of starch increased from the optimal, the activity of crude alpha-amylase was showed a decreasing trend. In contrast of this finding on another report conducted by Patel et al., 2005 showed that the maximum alpha- amylase activity was achieved in at 1% of starch concentration. This is because of enzymes are specific in terms of bond, substrate, cofactor, group, and optical formation and also the source of the enzyme where might the aviation the previous and the current study (Gebreselema 2015).

The effect NaCl concentrations on alpha-amylase activity was measured by incubating the crude alpha-amylase with various NaCl concentrations. The optimum alpha-amylase activity was obtained

at the concentration of 1.5% which resulted, the highest and the lowest activity KHW2-1A and KS1-1A, respectively. Optimal salt concentration is essential for stabilization of enzyme structure, ionic strength reduction of inhibitors and enhancement of enzyme activity (Park & Raines, 2001). However when the concentration of NaCl increase beyond the optimal the alpha- amylase activity decreased because variations in salinity can alter the enzyme's molecular structure, solubility, intermolecular hydrogen bonds, binding, and stability, resulting in denaturation, it reduces amylase activity at higher concentrations (Schneyer, 1952).

Thermal stability of alpha-amylase is the basic feature for the application alpha-amylase in starch hydrolysis industries (Rasooli et al., 2008). In current study, the stability of the alpha- amylase was obtained at 55°C for 30 and 60 min. When the enzyme was exposed to more with high temperature above the optimal, the enzyme lost its originality above 70% at 70 and 65°C at 30 and 60 min, respectively. This is because of further heating of enzyme lead to breaking of hydrogen bond and brings conformational change which resulted denaturation of enzyme (Kumar Srivastava, 2015). The crude alpha amylase activity during the submerged state fermentation was tested by selecting of nonpathogenic isolate's crude alpha-amylase and application of the enzyme in wheat flour dough formation was evaluated by blending 4mL of crude alpha-amylase plus the addition of fermentable commercial yeast. After 2h, the wheat flour dough was showed a rising height of dough compared with the yeast alone. The raising height wheat dough in yeast plus crude alpha- amylase is higher than the yeast containing wheat flour flask. This is because the enzyme breaks down the starch into simple sugar and creates conducive condition to facilitate yeast fermentation in which increase the dough raising capacity (Yamakawa et al., 2012).

Partial purification of the α -amylase was conducted the crude alpha-amylase from isolate FBW2-3A which obtained by ammonium sulphate precipitation followed by dialysis. The best activity was obtained in 60% ammonium sulphate concentration. After purification the specific activity was showed increasing from 5.5U/mg in the crude sample to 10.12 U/mg with 1.8-fold increment. This because of crude enzyme contains various impurities and related material that inhibit the activity of the enzyme and thus after purification, the impurities were removed and leads to increasing its activity than the crude once (Hansen et al., 2022). After dialysis the molecular weight of purified alpha amylase was determined by loading the sample in SDS-PAGE in which the band

approximately 20kDa was detected. This because it formed a single line in protein ladder as compared to the standard protein(Masci et al., 1998).

6. Conclusion

The current study was conducted by collecting samples from selected hot springs and other hot environment found in Addis Ababa for isolation of alpha-amylase producing bacteria. As the results showed that the current study areas were a source of a huge number of thermostable alpha-amylase producing bacteria. From many alpha-amylase producing isolates, the six were most potent alpha-amylase producing strain. The identified isolates were belonged to *Bacillus cabrialesii*, *Acinetobacter haemolyticus*, *Enterobacter quasiharmachei*, *Pseudomonas plecoglossicida*, and *Bacillus cereus*. The alpha-amylase produced by these isolates was thermostable at 55°C which can apply in different starch processing industries.

7. Recommendation

- Most isolates identified in the current study are opportunistic pathogen, so to apply in food and food related industries it needs further investigation such as specific gene that caused diseases for human unless the alpha-amylase extracted from such strains can only be applied in nonfood industry.
- Even though isolate KS1-1A was showed good performance for alpha-amylase production but it is difficult to use it for industrial application especially in food industry because it was not identified at molecular level whether it is pathogenic or not. Thus, we recommended it to find ways to identify for future investigation at species level.
- Addis Ababa is the fountain of many hot spring and other extreme environments and most likely considered as best source of thermostable-alpha-amylase producing microorganisms, so further investigation on amylase and other microbial enzyme are suggested.
- The current study was focused on bacterial alpha-amylase producing isolates but other microorganisms such as fungal amylase did not investigate so, we suggest microbiologist to focus on other alpha-amylase producing microorganisms for future study.

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9. Appendices

Appendix: A Sampling sites

Sampling area(Amylase from hot springs and extreme environments)

Yeha S & T. Biogas Qoshe Damping Koyefech hot spring fihwoha ((1)



Koyefech hot spring(2)

Fihwoha bath outlet

Fihwoha hot spring

Fihwoha hot spring



Hayat medhanialem siol sample



Appendix: B different laboratory activity picture

Countable colony morphology

streak plate (pure colony)



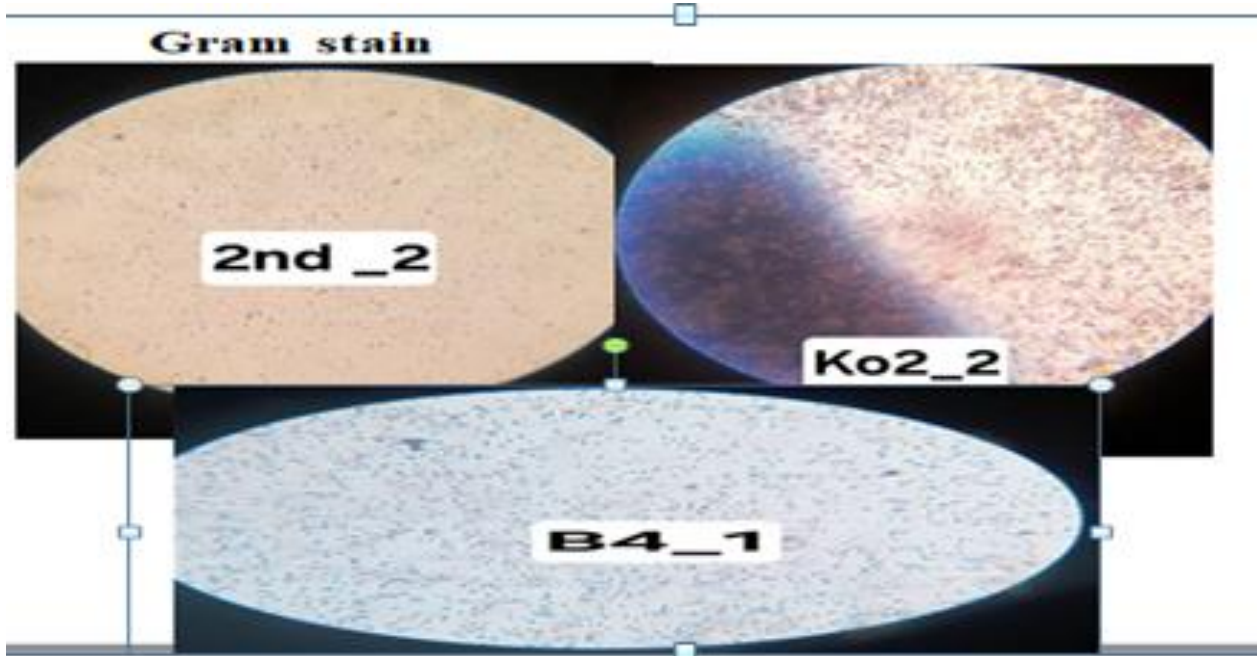
Spectrophotometer



Starch culture & hydrolysis test Agar culture & SmF media



Appendix C: gram stain and standard curves



Appendix: D Application of alpha-amylase in food industry

