# Isolation and characterization of Amylase producing bacteria from Chitu Soda Lake East Shewa, Oromia, Ethiopia

**Zekarias Mantegaftot**

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Sciences

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in

Biotechnology

Office of Graduate Studies

Adama Science and Technology University

May, 2024

Adama, Ethiopia

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## DECLARATION

I hereby declare that this Master Thesis entitled **"Isolation and Characterization of Amylase Producing Bacteria from Chitu Soda Lake East Shewa, Oromia, Ethiopia"** is my original work. Thus, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All materials used for this thesis have been duly acknowledged through citation.

| Zekarias Mantegaftot | _____ | _____ |
|---|---|---|
| Name of student | Signature | Date |

## RECOMMENDATION OF THE ADVISOR

I, the advisor of this thesis, hereby certify that I have read the revised version of the thesis entitled **"Isolation and Characterization of Amylase producing Bacteria from Chitu Soda Lake East Shewa, Oromia,Ethiopia",** prepared under my guidance by Zekarias Mantegaftot submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Biotechnology. Therefore, I recommend the submission of revised version of the thesis to the department following the applicable procedures.

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| Dr.Seid Mohammed (PhD) | _____ | _____ |
| Advisor | Signature | Date |

## APPROVAL SHEET

I, the advisors of the thesis entitled **"Isolation and characterization of Amylase producing bacteria from Chitu Soda Lake East Shewa, Oromia, Ethiopia"**and developed by Zekarias Mantegaftot, hereby certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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| Dr.Seid Mohammed (PhD) | _____ | _____ |
| Advisor | Signature | Date |

We, the undersigned, members of the Board of Examiners of the thesis by Zekarias Mantegaftot have read and evaluated the thesis entitled **"Isolation and characterization of Amylase producing bacteria from Chitu Soda Lake East Shewa, Oromia, Ethiopia**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfilment of the requirement of the degree of Master of Science in Biotechnology.

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| | |
|---|---|
| DNS | 3, 5- Dinitrosalicylicacid |
| EDTA | Ethylenediaminetetraaceticacid |
| nm | nanometer |
| OM | Optimized medium |
| pH | Negative logarithm of Hydrogen ion concentration |
| Rpm | Revolutions per minute |
| SMF | Submerged fermentation |
| APBI | Amylase producing bacteria isolates |
| MALDI TOF | Matrix-assisted laser desorption ionization time flight mass-spectroscopy |

## ABSTRACT

Enzymes are biological catalysts, which controls certain biochemical reactions. Amylases are among the most important enzymes that are widely utilized in different industries. Amylase could be produced from plant, animal, and microorganisms. Alkaliphilic Amylase producing bacteria were isolated from sediment soil samples obtained from Ethiopian soda lake in the Rift Valley area Lake Chitu. Starch-hydrolyzing isolates were selected on the basis of their activity on starch agar plate assay. Five isolates were chosen, characterized, and subjected to MALDI -TOF analysis. All the isolates were gram positive, urease, and catalase positive. All isolates except one were motile endospore forming rods and were found to be closely related to the Bacillus , being grouped with Bacillus cereus. The one exception had non- motile, non-endospore rod cells and was closely related to Cutibacterium acnes. The isolates were characterized for incubation period, effect of temperature, pH tolerance, nitrogen source preference, and effect of salt. The amylase activities were also characterized for effect of buffer, temperature, metal ions, and EDTA. All isolates grew optimally within 48 hours at temperature of 45°C and pH 9 when they grew on peptone as a nitrogen source that was supplemented with 15(w/v) NaCl. The maximum amylase activity was found at phosphate buffer pH 6. The amylase activities varied significantly across the presence of different metals.The amylase activity of all isolates inhibited in the presence of ethylenediaminetetraacetic acid (EDTA).



Key words: Alkaliphiles, Biocatalyst , Soda lake, Starch hydrolysis.

# 1. INTRODUCTION

Enzymes are biological catalysts which controls certain biochemical reactions (Tiwari *et al.*, 2015). Amylases are one of the most important industrial enzymes that catalyze the hydrolysis of internal α- 1-4 glycosidic bonds of starch molecules into low molecular products of glucose, maltose and maltotriose units (Irfan *et al.*, 2016). Amylases share about 25% of world enzyme market with major industrial applications (Lal *et al*., 2016b). Amylases are amongst the most studied worldwide attractions to exploit their physiological and biotechnological applications. In biotechnology, they are the most important enzymes and widely distributed in microbial, plant and animal kingdoms (Kalyani and Rajesh, 2018). Moreover, they have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. Fungal and bacterial amylases could be potentially useful in the pharmaceutical and fine-chemical industries (Souza and Magalhães, 2010). The use of amylase is increasing from time to time globally including Ethiopia. Despite this, there is enormous interest in exploring enzymes with better properties such as thermostable raw starch degrading amylase suitable for industrial applications and cost-efficient production methods. In an industry that works at mild conditions, contamination and product batch-to-batch inconsistency is a major challenge that necessitates us to perform the fermentation activity at extreme conditions where the contamination is almost nil (Irfan *et al.*, 2016). For this reason, most of starch-processing industries are designed to operate at elevated temperature, but the enzymes from microbes that survive only in mild environmental conditions easily get denatured in industrial conditions where the temperature, pH, and other parameters are higher or lower (Kalyani and Rajesh, 2018). Research is still undergoing to find out the bacterial strain that produces more stable amylase enzyme with higher production rate within low cost. Therefore, the presence of starch-degrading enzymes that have appreciable stability at high temperatures is crucial. One of the important options is studying microbial populations that can live and reproduce in extreme environments.

Ethiopia is endowed with a range of natural habitats like geothermal sites, hot water springs and alkaline environment. Those sites are good sources of microbes that are potential for the production of polyextremophilcs biomolecules (enzymes).

Thus, today, a large number of microbial amylases are recovered from such environments and are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. Microbial amylases have broad applications in industrial sectors to meet industrial demands. Microbial amylases are more stable than plant and animal amylases, because of their ability to produce extracellular enzymes that are active and stable at high pH values. The unusual properties of these enzymes offer a potential opportunity for their utilization in processes demanding such extreme conditions (Aguilar et al. 1998). Many of these enzymes find numerous applications in various industrial sectors.They are also easy to manipulate and obtain the desired characteristics of enzymes (Mathew *et al.,* 2016). Enzyme production from microbial sources is very important for industrial well competitiveness, our health, prosperity and well-being (Fiorilli *et al.,* 2019).

Starch hydrolysis by amylases is the basis of several industrial practices under the conditions of different temperature, pH and metal ions (Vaidya et al., 2015).The growing demand and wide application of amylase in different industrial sectors, makes to improve the potential productivity of amylases with typical strain selection (Dehkordi and Javan, 2012). The selection of microbes to industrial production of amylase and the characterization of rhizobia as amylolytic activity contribute diverse metabolic profiles of the root nodule bacteria ( Júnior et al., 2012). Amylase improves and compensates for the nutritional deficiencies of several food grains (Al-Maqtari et al., 2019).Wheat bread is an important source of energy in human diets and one loaf of bread consists of about 50% starch, 40% water and 7% protein by weight (Freitas et al., 2017).

Bakery industry uses a large variety of enzymes to optimize dough properties and improve the quality of baked products. Among the enzymes, amylases are the most popular and commonly employed enzymes in dough leavening process (Barrera *et al*., 2016). In food industry, amylases have significant role in today‘s baking industry and their market is increasing day by day (Singh *et al.*, 2011a). Amylase improves the rate of dough fermentation and reduces its viscosity. In addition, it is very important for the hydrolysis of starch to prevent polymer crystallization and hardening of bread, increase qualities and shelf life of breads (Singh *et al.*, 2016). Moreover, it increases the resistance, elasticity, and softness of the dough (Onyango, 2016).

Soda lakes are widely distributed (Grant and Jones 2000); however, as a result of their inaccessibility, few such lakes have been explored from the microbiological point of view. The East African Rift Valley is a volcanically active region with an abundance of soda lakes, many of which are fed by hot springs of varying temperature. In recent years, much effort has been dedicated to biological and geochemical studies of Kenyan soda lakes in the Rift Valley (Grant and Horikoshi 1992). The microbial population of these lakes, which is considerably diverse phylogenetically, includes Archaea, Cyanobacteria, gram-negative organisms and gram-positive organisms that are mostly related to members of the genera Bacillus and Arthrobacter ( Grant *et al.*, 1990).

This thesis reports a study on screening and characterization of starch-degrading alkaliphiles isolated from samples around the soda lake Lake Chitu , Rift Valley area of Ethiopia. Ethiopian soda lakes have been characterized with respect to chemical and algal compositions (Kebede *et al*. 1994); however, reports on the prokaryotic population of the lakes are limited. The few reports concerned with the enzymes from microorganisms isolated from these soda lakes reveal rather exceptional catalytic features that render them promising candidates for biotechnological applications (Gessesse *et al.,* )

## 1.1 Statement of the problem

Presently, nutrition security problem is becoming the major concern worldwide, mostly for Asian and African countries (Serventi *et al.,* 2016). In human nutrition, starch plays a vital role by providing the required energy for normal functioning of cells. Even though cooking improves digestibility of starch, all of the starch present in a food is not digestible (Raigond *et al*., 2014, Sahnoun *et al.,* 2016) and hence there is a need to include amylase for the complete degradation of starch (Paucean *et al.,* 2016). Currently, there is an increasing interest in microbial amylase in food industries mainly for starch processing activities (Andualem and Gessesse, 2020). Microbial amylases have potential roles in starch hydrolysis process and ferment the flour without the presence of yeast by increasing the rate of fermentation. In addition, Microbial amylases are very important to reduce the leavening time of dough and replace the conventional yeasts with better performance (Alemayehu *et al*., 2017). Although Ethiopia has abundant microbial diversity, in general and to the best of my knowledge, there is dearth of information about amylase from Ethiopian soda lake isolates and these initiates to

look for potential amylase and the enzymes from microorganisms isolated from this soda lake reveal rather exceptional catalytic features that render it promising candidates for biotechnological applications . Therefore, the present study had been initiated to screen potential isolates and characterize their amylase.

### 1.2 Significance of the study

After additional purification of the enzyme, the results of the current study was contributed in food industries, primarily for starch processing, bakery industries, laundry, tannery, pharmaceutical, paper and textile industry. Moreover, it helps to save the currency of importing amylase enzyme by producing large amount of amylase enzayme in local industries. Amylase production from bacteria is limited and hence the findings of the present study was given basic information to the scientific community.

### 1.3 Scope of the study

The general overview of this thesis was to isolate and characterize amylase producing bacteria from Chitu Soda Lake that found in East Shewa Zone. Accordingly, the following issues were addressed: Isolation and characterization of amylase producing bacterial isolates from Chitu Soda Lake and determining growth potential from selected samples.

### 1.4 Objectives

#### 1.4.1 General objective

To isolate and characterize amylase producing bacteria from Chitu Soda Lake, East Shewa, Oromia, Ethiopia

#### 1.4.2 Specific objectives

- To identify potential amylase producing bacteria isolates using diferent grouth parameter
- To characterize potential amylase producing bacteria isolates using MALDI TOF MS techniques
- To optimize growth condition for newly identified amylase producing bacterial isolates

# 2. LITERATURE REVIEW

## 2.1 Enzymes and their economic role

Enzymes are highly efficient biological catalysts which start and speed up thousands of biochemical reactions in living cells (Tiwari *et al*., 2015), and perform all synthetic and degradative responses in living organisms. Enzymes are biological catalysts that have changed the way we prepare our food and improve nutritional value of different food products (Al-Maqtari *et al*., 2019). Enzymes are the complex protein molecules which are produced by living cells to increase the rate of an immense and diverse set of chemical reactions required for life. They are involved in all processes essential for life such as, DNA replication and transcription, protein synthesis, metabolism, cell regulation and signal transduction often via kinases and phosphatases (Chaudhary *et al.*, 2015). Enzymes are highly specific both in the reactions that they catalyze and in their choice of reactants, which are known as substrates. Enzymes have high catalytic rates and work in aqueous solution. An enzyme typically catalyzes a single chemical reaction or a set of closely related reactions (Bhatia, 2018). Enzymes are proteins that act as catalysts which can speed up chemical reactions that occur in the cells. An enzyme can add atoms to a molecule, move atoms from a molecule, split a large molecule into smaller molecules, or join together smaller molecules to form a large molecule. Enzymes have unique shapes and reactive sites that allow them to bind with specific molecules namely substrate molecules (Sahni and Goel, 2015).

## 2.2 Types of amylase

Amylases are the most important enzyme groups within the field of biotechnology. They are a class of enzymes that are capable of digesting glycosidic linkages in starch components and glycogen molecules (Bedan *et al*., 2014). Enzymes belonging to amylases, endoamylases and exoamylases are able to hydrolyse starch. These enzymes are classified according to the manner in which the glycosidic bond is attacked (Tiwari *et al*., 2015). Alpha amylase Alpha amylase is a typical calcium containing enzyme which catalyze the hydrolysis of starch and related carbohydrates by randomly cleaving internal α-1, 4-glycosidic linkage to produce glucose, maltose, maltotriose, and other oligosaccharides. It is completely unable to function in the absence of calcium (Singh *et al*., 2011b). Alpha Amylases are extracellular enzymes that are important in starch processing industries. The optimal pH of alpha amylases ranges

from 2 to 12 and they are thermostable. The application of these enzymes has been established in starch liquefaction, paper, food, sugar and pharmaceutical industries. In the food industry, amylolytic enzymes have a large scale of applications, such as the production of glucose syrups, high fructose corn syrups, maltose syrup, and reduction of viscosity of sugar syrups, reduction of turbidity to produce clarified fruit juice for longer shelf-life, solubilization and saccharification of starch and delay the staling of baked products (Christopher and Kumbalwar, 2015).

### 2.2.1 Structural characteristics of α-amylase

The α-amylase catalyzes the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-1, 4-glycosidic bonds belongs to endo-amylases family. Neither α-1, 6-linkages nor terminal glucose residues can be broken by α-amylase. The end products of α-amylase activity are oligosaccharides with different length of α-configuration and α-limit dextrin, which form a mixture of smaller oligosaccharides consisting of maltose, maltotriose and a number of αl, 6 and α-1, 4 oligoglucans. Different other enzymes participate in starch breakdown process, but among them, α-amylase is the most important for the initiation of this process (Fiorilli *et al.*, 2019).

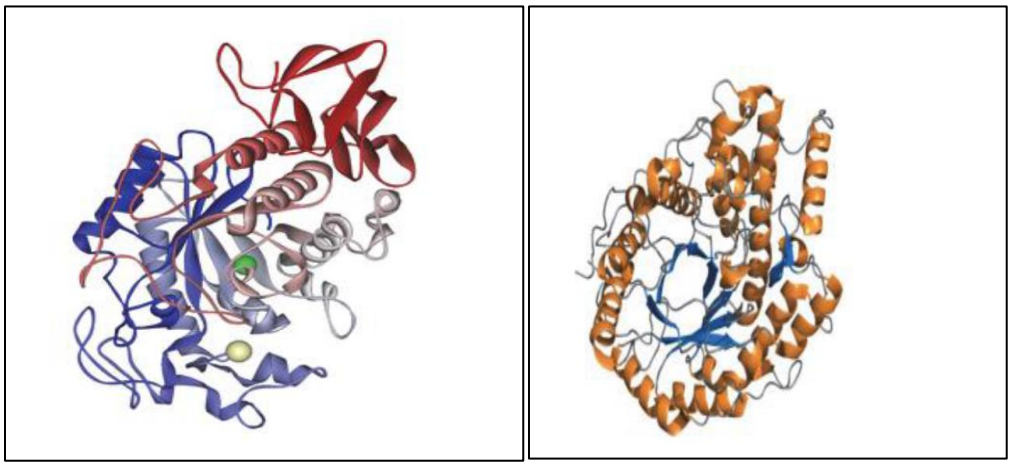


Figure 1 Three dimentional structure of α- amylase and β- amylase (Anbu et al., 2017)

### 2.2.2 Beta amylase

Beta amylase is an exo-hydrolase enzyme that acts from the non-reducing end of a polysaccharide chain by hydrolysis of α-1, 4-glucan linkages to yield successive maltose

units. Since it is unable to cleave branched linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and dextrin units remain. Primary sources of βamylase are the seeds of higher plants and sweet potatoes. During ripening of fruits, β-amylase breaks down starch into maltose resulting in the sweetness of ripened fruit. The optimal pH of the enzyme ranges from 4.0 to 5.5. Beta-amylase can be used for different applications on the research as well as industrial front. It can be used for structural studies of starch and glycogen molecules produced by various methods. Additionally, it is used to produce high maltose syrups and has crucial role in brewing and distilling industry (Sundarram *et al.*, 2014b).

### 2.3 Microbial source of amylase

Amylase can be produced by different species of microorganisms. The level of amylase production differs depending on the microbe‘s origin. Strains isolated from starch- or amylaserich environments can produce higher amounts of enzyme. Hence, for the commercial production of specific enzymes, effective microbial strains which have the capacity to produce the highest amounts of particular enzymes are desired (Sahni and Goel, 2015).

Microorganisms are the primary source of amylase, because they are cultured in large quantities in short period of time and genetic manipulation can be done to enhance the enzyme production (Anbu *et al*., 2017). In addition, microbial amylases are the most produced and used in industry, due to their cost effectiveness, consistency, short life span and high productivity rate (Garg et al., 2016); Andualem and Gessesse, (2020) Microbial amylases obtained from bacteria, fungi, and yeast has been used predominantly in industrial sectors and scientific research (Gopinath *et al.*, 2017).

The use of amylase obtained from fungal and bacterial sources have dominated in different industrial sectors. But bacterial enzymes are the cheapest sources and widely used in different Industries such as food, textile, and beverage, as well as in waste treatment (Banerjee *et al.*, 2016). The production of microbial amylases from bacteria depends on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements (particularly carbon and nitrogen), incubation period, pH, temperature and metal ions.

Therefore, the nature and concentration of nitrogen source in the culture medium affects bacterial growth and amylase production and acts as pH stabilizer (Lal *et al.*, 2016b).

Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications (Sinha and Vakilwala, 2016). Utilization of the potential amylase producing fungal isolate is vital to be used in starch degradation process ( Asrat and Girma, 2018). Yeasts are unicellular fungi with ubiquitous distribution in many ecosystems. In recent years the capability of some yeast species to degrade starch has increased its potentiality in biotechnological applications, such as a source of hydrolytic enzymes. Although yeasts are not among the industrial producers of amylases, the enzyme is widely distributed in many yeast species (Wanderley *et al.,* 2004). Moreover, yeast isolates have the capability to produce cold active amylase (Dikmen *et al*., 2018).

## 2.4 Process Optimization for Amylase Production

Optimization is the process which is used to maintain a balance between the medium components and minimize the utilization of unused products at the end of the processing. The optimum process control parameters vary depending on the microbial source, desired end product, method of fermentation employed and many other such factors. The major factors influencing the enzyme production are pH, temperature, nitrogen and carbon sources, incubation period, starch and its nutrient medium. The formation of clear zones around the colonies refers to the hydrolysis process of amylase production. The enzymes maintain high stability over a particular setting of temperature and pH. Temperature is said to be the prime aspect for almost all enzyme activities. It is also essential for the enzymes to be active and stable even at peak temperature levels (Shalinimol, 2016).

### 2.4.1 Effect of temperature, pH and incubation period

Optimum temperature ranges are required for the growth of the microbial source and amylase production. On the other hand, optimum hydrogen ion concentration is a critical factor for the stability of enzyme production. Enzymes are pH sensitive and hence care must be taken to control the pH of the production process. Incubation time play a very crucial role in bacterial metabolic activity and growth. Production of amylase may not take a lot of time, but considerable increase in its yield is noted after its submission to an incubation period of

several minutes. This is a crucial factor in fermentation process. If the process is carried out for a time period shorter than the optimum duration, maximum yield cannot be obtained. Enzyme activity increases when incubation time increase till it reaches the optimum duration. In most cases, the production of enzyme begins to decline if the incubation time is further increased. This could be due to the depletion of nutrients in the medium or release of toxic substances (Sundarram *et al*., 2014a).

### 2.4.2 Effect of carbon and nitrogen sources

Different carbon and nitrogen sources present in the fermentation medium are the major factors affecting microorganism‘s growth and amylase production as well. The effect of different carbon source such as glucose, galactose, maltose, sucrose, lactose, fructose and dextrose can be used as carbon sources. On the other hand, organic and inorganic nitrogen sources like peptone, casein, urea, yeast or beef extract, tryptone or soybean meal, ammonium sulphate, ammonium nitrate and ammonium chloride, can be used as sole nitrogen source for the growth and production of amylase (Shalinimol, 2016).

For the production of amylase, inoculums size plays a significant role. It is one of the most important parameter which affects the enzyme activity by affecting substrate utilization rate. It is analyzed that by increasing the overall size of the inoculums, its production is enhanced. An accurate inoculums size is not usually followed (Shalinimol, 2016).

## 2.5 Industrial Applications of Microbial Amylases

Applications of bacterial amylases at industrial level have inspired the interest to explore several microbes as bio-resources for their amylolytic activity. Amylases have potential application in a number of industrial processes such as in the food, textiles, paper industries, bread making, glucose and fructose syrups, detergents, fuel ethanol from starches, fruit juices, alcoholic beverages, sweeteners, pharmaceutical, chemicals and digestive aid. The potential industrial applications of enzymes are determined by the ability to screen new and improved enzymes, their fermentation and purification in large scale, and the formulations of enzymes. Bacterial αamylases are now also used in areas of clinical, medicinal, and analytical chemistry. It is extensively used in pharmaceutical industries in digestive tonics, for hydrolysis of starch to produce different sugars like glucose and maltose which have several applications. The most widespread applications of α-amylases are in the starch based food and

non-food industry, which are used for starch hydrolysis in the starch liquefaction process that alters starch into fructose and glucose syrups (Saini *et al*., 2017).

### 2.5.1 Food industry

The beginning of modern biotechnology has brought many changes in the food industry. The application of enzymes in the food industry is diverse, and most often, they are applied as processing agents. Amylases are widely utilized in the processed food industry, including baking, beverages, starch syrups, etc. Amylases are utilized in the clarification of juices to maximize the production of clear juice (Singh *et al*., 2016). In addition, α-amylase is added to the dough, and hence, it plays a vital role in the bread-making process. This causes the starch to degrade into small dextrins, which allows yeast to work continuously during dough fermentation. Hence, amylase can increase bread volume and crumb texture. Furthermore, small oligosaccharides and sugars can increase the Maillard reactions responsible for the browning of the crust and the development of an attractive baked flavour (Saini *et al*., 2017).

The addition of amylase to the dough increases the rate of fermentation and the reduction of the viscosity of the dough, resulting in improvements in the volume and texture of the product. Moreover, it also generates additional sugar in the dough, which improves the bread's taste, crust colour and toasting qualities. Besides generating fermentable compounds, amylases also have an anti-staling effect in bread baking, improving the softness retention of baked goods and increasing the shelf life of these products (Sahni and Goel, 2015).

### 2.5.2 Starch processing industry

Starch is an essential food ingredient used as a substrate for producing many industrial products. Starch is the second main polysaccharide food store in nature, and it is the primary constituent of most staple foods and is used in various food and non-food industries. Starch is utilized in many industries including food, alcohol, paper, textiles etc. Enzymatic starch hydrolysis was the first industrial enzyme-driven process, established in the 1840s in France (Božić *et al*., 2017). Starch is a widely used renewable resource. It is a storage compound in many plants' leaves, tubers, seeds and roots. The starch is usually modified chemically or enzymatically to various derivatives. However, starch degradation in the industrial sector is

generally initiated by α-amylases, ubiquitous enzymes in microorganisms (Chaudhary *et al*., 2015).

The most comprehensive applications of α-amylases are in the starch industry, which is used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups. Chemical processing of starch has disadvantages such as the requirement for high temperature, low pH, lower yield of glucose, unpleasant taste of compounds, and synthesis of undesired colour. In contrast, enzyme-mediated hydrolysis can overcome these unfavourable features. The enzymatic conversion of starch to syrups is widely used in the starch industry, which includes gelatinization, which involves the dissolution of starch granules to form a dense suspension. The following process provides liquefaction, which involves partial hydrolysis of starch to reduce viscosity, and the third is saccharification, involving the production of glucose and maltose as end products via further hydrolysis (Sahni and Goel, 2015).

### 2.5.3 Detergent industry

Amylase plays a vital role in local detergent industries (Simair *et al.*, 2017). The use of enzymes in detergents has increased with the changing methods of dishwashing and laundry. Amylases are added to liquid laundry and dishwashing detergent formulations to remove residues from starch-based food products. Consumers prefer to use cold water and mild conditions, which requires the detergent to work in those activities. The enzymes are environmentally safe and work in mild conditions. Alpha amylase is used to digest the starch-containing food particles into more minor water-soluble oligosaccharides. Hence, removing starch is also essential to maintain the whiteness of clothes. The stability of α-amylase at low temperatures and alkaline pH contributes to its extensive use in detergents (Sundarram *et al.*, 2014a).

### 2.5.4 Paper industry

The consumption of microbial source enzymes has developed progressively in the paper and pulp industry due to increasing awareness of sustainability issues and the reduction of adverse effects on the environment. The use of enzymes in this industry diminishes energy consumption, processing time, and the quantity of chemicals required for processing. The

applications of amylases in this industry include coating starch, improving drainage, and cleaning paper. The primary application of amylase in the paper industry is to produce high molecular weight starch with low viscosity through modification of starch of coated paper. The coating treatment makes the paper smooth and robust to enhance the writing quality. Amylase is important in degrading polymer partly in a batch or continuous process and maintaining the starch viscosity for paper sizing. Starch is a good sizing agent for finishing paper, improving the quality and erasability. Paper sizing improves the finished product's quality, strength, and stiffness in papers. Low-temperature active amylases are crucial to reducing starch viscosity for the proper coating of paper (Sahni and Goel, 2015).

### 2.5.5 Fuel alcohol industry

Ethanol is the most utilized liquid biofuel. Starch is the most abundant substrate for ethanol production due to its low price and easy availability as a raw material. Starch is solubilized and subjected to two enzymatic steps to obtain fermentable sugars during this process. The first step is the bioconversion of starch into ethanol through the liquefaction and saccharification process. The second step is the hydrolysis of starch into sugar using an amylolytic enzyme such as α-amylase, followed by fermentation. After that, sugar is converted into ethanol using an ethanol-fermenting microorganism like Saccharomyces cerevisiae (Sahni and Goel, 2015).

### 2.5.6 Textile industry

Textile industries extensively use alpha-amylases to hydrolyze and solubilize the starch, which then washes out of the cloth to increase the stiffness of the finished products. In this industry, starch is usually removed by applying alpha-amylase during the design process. That means alpha-amylase is used as a desizing agent for removing starch from the grey cloth before its further processing in bleaching and dyeing. Hence, the removal of starch improves uniform wet processing. Starch is a low-price and readily available sizing agent, added for a fast and secure weaving process. In textile weaving, starch paste is used for warping to give strength and prevent the loss of strings. After weaving, amylase is added, which specifically catalyzes the hydrolysis of starch to water-soluble dextrins. Amylase efficiently removes starch without damaging the fabric. The enzymatic desizing of cotton with amylases has been

state-of-the-art for many decades. Many garments, especially the ubiquitous 'Jean' are designed after mashing, and the desired fabrics are finally laundered and rinsed (Saini *et al*., 2017).

## 2.6 Alkaline Soda lakes in Ethiopia

Ethiopia is rich in soda lakes, which are well-known for their unique diversity, scientific benefits, economic significance and scenic attractions. Presently, these lakes are under threat from both anthropogenic activities and natural events (Tesfaye ,2006).

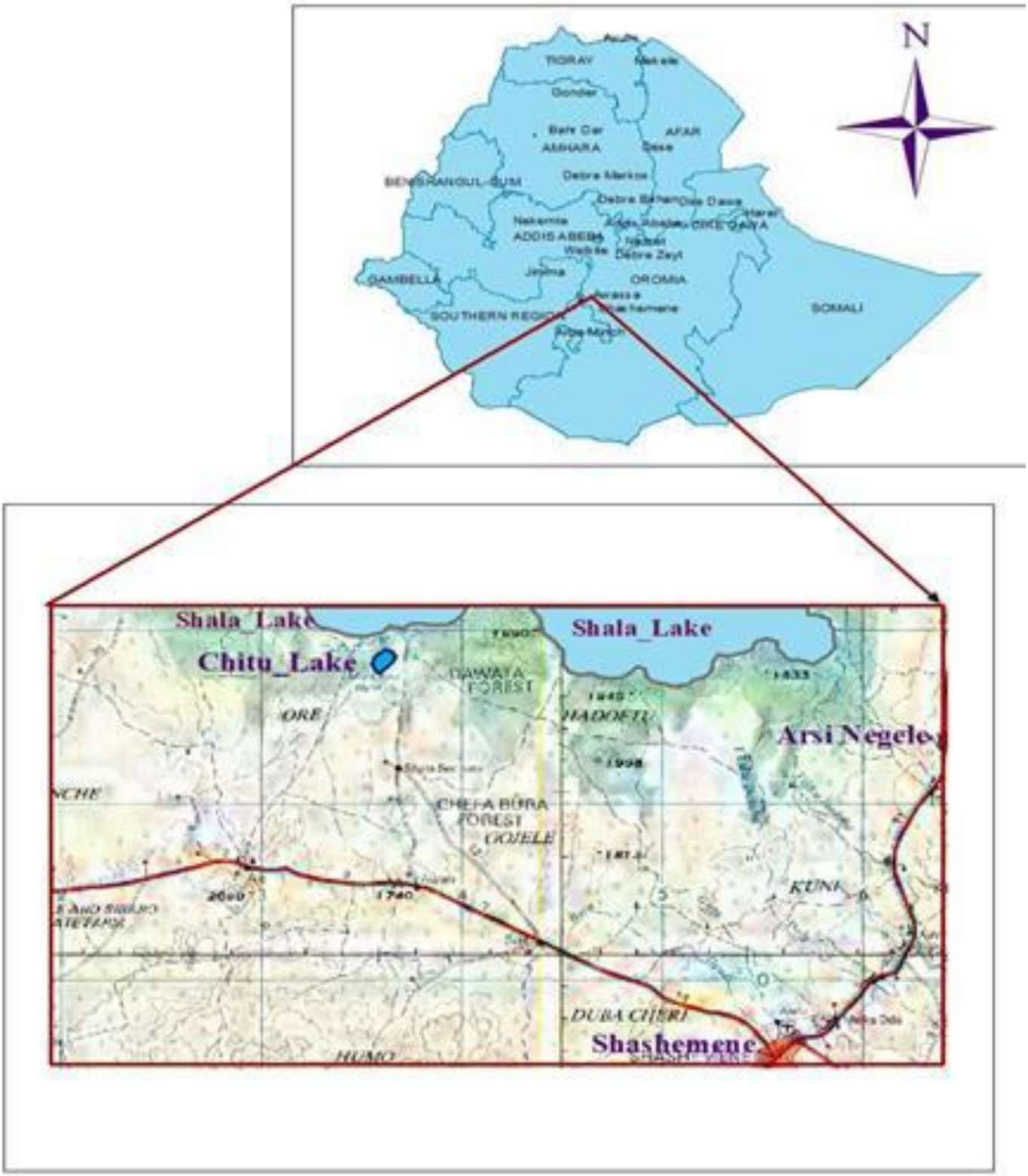
For instance, the water level of Lake Abijata is dropping due to soda ash extraction and upstream diversion for irrigation purposes, resulting in high salinities, change in biotic structure, and collapse in fish stock( Solomon *et al* .,2019). Furthermore, there is a plan to expand the Abijata soda ash manufacturing plant to Lake Shala. This will have a negative impact on the Limnology of Lake Shala ( Solomon *et al* ., 2021). In Lake Arenguade, dilution in lake chemistry, particularly salinity, conductivity, and alkalinity, have resulted in a dramatic decline in phytoplankton biomass and productivity. Underwater explosions for seismic experiments are considered to be major factor in the lake's general limnological change . Lake Beseka has grown tenfold increase in size over the last 30 years, indicating a consistent change in the lake's chemistry with a decrease in total ionic concentration and electrical conductivity . The primary cause of the lake's water level expansion was hypothesized to be discharge from hot springs climate variability, ground water input and irrigation return water from adjacent farms (Alemayeh *et al* ., 2006).

Amylases are enzymes that originated from fungi and bacteria.There are many microbial sources that can produce amylases such as *Bacillus, Pseudomonas* and *Clostridium* family. Nowadays, and at industrial level *Bacillus licheniformis* and *B stearothermophillus* are the probable bacteria used to produce amylases (Ploss *et al.*, 2016).

# 3. MATERIALS AND METHODS

## 3.1 Descriptions of Study Area

The study was conducted in East Shewa Zone at Chitu Lake in south-central Ethiopia, lying in the Great Rift Valley. It is a tropical soda lake located some 287 km south of Addis Ababa at a geographical position of 7º24′ N 38º25′ E and altitude of 1600 m above sea level. It is situated at about 1 km southwest of Lake Shala in the Abijata-Shala Lakes National Park of the Ethiopian Rift Valley. It is a small (0.8 km2) and fairly deep (maximum depth 21 m) cup shaped Crater Lake surrounded by a crater rim. Characterized by high pH, saline-alkaline conditions and high phosphate, but frequently limiting level of nitrogen compounds ( Kifle, 2014). Its water is rich in $Na^+$, $CO_3^{2-}$ $HCO_3^+$ and $Cl^-$, which are related to their presence in large concentrations in the trachytic and rhyolitic rocks that formed the Ethiopian rift (Klemper and Cash, 2007). The lake lies in a depression within a closed basin (Ziway-Shala basin) lacking obvious surface outflow and inflows, and is fed by direct precipitation and a few hot springs located at its shores. There is no information available on the exact size of the catchment area and the theoretical renewal time of Lake Chitu. The lake region has semi arid to sub-humid type of climate with mean annual precipitation of 600 mm and air temperature of 25 °C (Legesse *et al.*, 2002). The high evaporation rate that exceeds the mean annual rainfall characterizes the lake's region (Ayenew and Legesse, 2007) and contributes to the saline-alkaline nature of the lake. The region has two seasons: dry (October to February) and wet (March to September) seasons. The wet season is characterized by a bimodal pattern of rainfall, with minor rainy period extending from March to May and major rainy period from June to September.




7°24'11"N 38°25'10

Figure 2 Location map of the study area. Source:www.researchgate.net

### 3.2 Sample collection and transportation

Sediment Soil samples (approximately 60 g) collected in labelled sterile polyethylene bags from Lake Chitu, Ethiopia. The soil samples were labelled as LCS (Lake Chitu Samples) and transported in a cold transportation icebox to Adama Science and Technology University, Biology laboratory for enrichment and cultivation.

### 3.3 Isolation of amylase producing bacteria from Chitu soda Lake

The samples were enriched in basal media contains 28 chemical ingredients composition and incubated for 15 days; 45 $^{0}$C; 150 rpm (Beshah and Assefa, 2019). 1ml of enriched culture was Transferred into 9 ml of 0.9% sterile saline solution with dilution factors from $10^{-1}$ up to $10^{-6}$. Then a 0.1 ml from odd dilution factors were transferred in to pleats containing alkaline media (NaCl-1g/l, KH2PO4-2g/l, CaCl2-0.1g/l, MgSO4.7H2O-0.2g/l,FeSO4.H2O-0.15g/, nutrient agar-20g/, peptone-5g/l,starch-5g/l and trace mineral solution-20g/l). Then it was spread using sterile "L" shaped glass spreader . Finally, the plates were incubated at 37$^{o}$C for 48 h. (Beshah and Assefa, 2019).

### 3.4 Purification and preservation of isolates

Single colonies were picked up with a sterile inoculating loop and streaked on sterile nutrient agar media and incubated at 28 $^{o}$C for 24 h. The purity of the colony was carefully examined through repeated re-streaking and a single well-isolatedcolony was picked and incubated at 37 $^{o}$C for 48 h. After enough growth was observed, cultures were preserved at 4 $^{o}$C for further analysis ( Legesse, 2017).

### 3.5 Primary screening of potential amylase producing bacteria using starch hydrolysis test

Primary screening of the bacterial isolates for amylase production was carried out qualitatively. The isolated colonies were sub-cultured in freshly prepared Petridis containing starch agar using sterile inoculating needles by dot method. After incubation at 33°C for 48 h, each plate was flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5 mg potassium iodide solution and 125 ml of water, stored at room temperature) to produce a deep blue-colored starch-iodine complex. The clear zone diameter was measured using a ruler (Singh & Kumari, 2016).

### 3.6 Morphological and cultural characteristics for amylase producing bacteria isolates

The morphological characteristics of isolates were determined according to the method of Hassan *et al*. (2015). A loopful of isolates from 24 h old broth culture were inoculated by streak plating onto nutrient agar and incubated at 45 $^{o}$C for 48 h. After 2 days of incubation period, a motility test, Gram-staining test, colony shape, color and structure of colonies were recorded (Ahmed and Abdelmageed, 2015).

#### 3.6.1 Gram staining

Gram stain reactions of the isolates were done to check whether the isolates were gram-positive or negative.. Various isolates were subjected to gram staining tests. Pure colonies were picked up and prepared on sterile glass slides separately and fixed using a flame. Then it was stained with crystal violet, for one minute. After washing with tap water, it was immersed in gram‘s iodine for one minute. Again it was washed with tap water and decolorized with 95% ethyl alcohol for 30 sec. Thereafter, it was rinsed with water and stained with safranin. Finally, it was washed with tap water and examined under an oil immersion objective on the microscope (Tyagi *et al.,* 2017).

#### 3.6.2 Motility test

The active movement of amylase producing bacteria was evaluated on (NaCl-1g/l, KH2PO4-2g/l, CaCl2-0.1g/l, MgSO4.7H2O-0.2g/l,FeSO4.H2O-0.15g/, peptone-5g/l,starch-5g/l and supplemented with 0.2 – 0.5% of agar per litre. Then, a loopful of isolates were picked up and stab cultured in test tubes with motility medium and incubated at 30 $^{o}$C for 24 h of incubation. After the 24 h incubation period, the movement of isolates in each test tube were recorded (Karel et al., 2017).

#### 3.6.3 Spore staining

In spore staining, a bacterial smear was made under sterile conditions. The smear was air-dried and heat-fixed. The slide was flooded with green malachite, placed on top of a beaker containing boiling water, and left for 3 minutes. After washing, it was stained with safranin for 30 seconds and washed. The smear was air-dried and observed under the microscope (Islam, 2016).

### 3.7 Biochemical characterization of amylase producing isolates

**Catalase test:** The catalase test was done to study the presence of the enzyme catalase, which hydrolyzes hydrogen peroxide ($H_2O_2$) into $H_2O$ and $O_2$. Soda sediment soil sample colonies (48 h old) were taken on a clean glass slide and one drop of 3% (v/v) of $H_2O_2$ was added. The bubble appeared when the isolates produced catalase enzyme (Tyagi *et al*., 2017).

**Citrate utilization test:** Citrate utilization as a carbon source was checked using Simmons citrate agar medium (Christensen's citrate sulfide medium .Ingredient Amount Yeast extract 0.5 g Glucose 0.2 g Sodium citrate dihydrate 3.0 g Monopotassium phosphate 1.0 g Cysteine-HCl 0.1 g Iron (III) ammonium citrate (50/50) (optional) 0.4 g Sodium chloride 5.0 g Sodium thiosulfate pentahydrate (optional) 0.08 g Agar 15.0 g Phenol red 0.012 g). The medium was dispensed into sterile test tubes and one loop full of pure isolates were inoculated into the slant and incubated at 28$^{o}$C for 48 h. After 2 days incubation period, color changes from green to blue were recorded and taken as a positive result (Kumari *et al*., 2018).

**Indole test :** Appearance of bright red and yellow color which composed after added 0.5 ml of Kovac's reagent to incubated bacterial culture at 35 $^{0}$C for 24 h on SIM media indicated a positive and negative results respectively ( Ardhi et al.,2020).

**Methyl red (MR) test :** After adding methyl red indicator solution (TSBA, Himedia) to inoculated culturing media and incubation at 35 $^{0}$C for up to 4 days, changing color to red indicate MR test positive- appearance of tested bacteria( Ardhi et al.,2020).

**Urease test :** Slanted two millilitres of urea medium which placed in bijou bottles applied for the incubated bacterial colony at room temperature. Red-pink colour in the medium was considered as a positive test for urease induction( Ardhi et al.,2020).

### 3.8 Secondary screening of isolates using submerged fermentation

Minimal broth medium (0.4 g/L yeast extract, 0.2 g/L peptone, 0.25 g/L NaCl, 0.4 g/L $K_2HPO_4$, 0.1 g/L $KH_2PO_4$, 0.2 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl_2.2H_2O$ and 10 g/L soluble starch) was prepared. From the broth medium, 50 ml was transferred into 250 ml Erlenmeyer flasks and sterilized at 121°C for 15 min. Then, 1 ml (approximately 8.37 log CFU/mL MBRI1; 8.25 log CFU/mL MBRI2 and 8.22 log CFU/mL CPRI3) of active inoculums were transferred in to 50 ml of production medium (Oliveira *et al*., 2007). The medium was

adjusted at pH 7 and placed in a shaker incubator at 28°C, 120 rpm for 48 h ( Andualem, 2014a, Ayansina *et al.*, 2017).

### 3.9 Optimization and Culture condition for Amylase producing bacterial isolates (APBI)

**Effect of incubation period on APBI**: To determine the effect of incubation time on bacterial growth, the cultures were grown in 250 mL flasks with 50 mL of minimal media containing 1% starch broth (w/v) under submerged. The incubator was adjusted in the range of 12 h ,24h ,36h ,48h and 72h. Then, the growth was measured by a spectrophotometer at 600 nm (Demirkan et al., 2017).

**Effect of pH on APBI**: The pH tolerance of bacterial isolates was examined by growing in 50 mL of minimal media containing 1% starch broth (w/v). The pH of the medium was adjusted in the range of 9.0, 10.0, 11.0, 12.0, and 13.0 using 1 M HCl or NaOH in 250 mL Erlenmeyer flask and incubated at 37 $^0$C for 24 h. Then, the growth was measured by a spectrophotometer at 600 nm (Demirkan et al., 2017).

**Effect of temperature on APBI**: The optimum growth temperature of the isolates was examined by growing in 50 mL of minimal media containing 1% starch broth (w/v). The fermentation medium containing bacterial isolates was incubated at varying temperatures from 30 , 40, 50 and 60 °C for 48 h. Then, the growth was measured by a spectrophotometer at 600 nm (Kalyani and Rajesh, 2018).

**Effect of nitrogen source on APBI:** Different microorganisms have different nitrogen source preferences. Therefore, the isolates' growth was evaluated by supplementing organic and inorganic nitrogen sources in the growth media. The nitrogen content [yeast extract (0.4 g/L) + peptone (0.2 g/L)] of basal fermentation medium were replaced with different extract concentrations of organic and inorganic nitrogen sources i.e. peptone, urea, tryptone and yeast extract, ammonium sulphate and sodium nitrate (0.1, 0.2, 0.5, 1.0, 2.0% w/v) (Lal *et al.*, 2016a). It was carried out in 250 mL of Erlenmeyer flasks containing 50 mL of sterile starch broth media. After inoculation, the isolates were incubated at 37 °C for 24 hours. Then, the growth was measured by a spectrophotometer at 600 nm (Silpa *et al.*, 2018).

**Effect of salt (NaCl) concentration on APBI:** Salt concentration may have detrimental effects on microbial growth because of direct toxicity as well as through osmotic stress. In

order to determine the optimum salt concentration, isolates were cultured in 250 mL of Erlenmeyer flasks containing 50 mL of minimal media containing 1% starch broth (w/v) supplemented with different amounts of NaCl such as zero (control), 5, 10, 15 ,20 and 25% (w/v) and incubated at 28 °C for 24 h (Khaitov *et al.*, 2016). After completion of 24 h incubation, the growth rate of bacterial isolates were measured by spectrophotometer at 600 nm (Patil *et al*., 2014).

### 3.10 Amylase extraction to obtain crude enzyme

Selected isolates were separately cultured at 28°C temperature, pH 10, and 250 rpm for 48 h in 100 mL starch broth (1% starch, 0.5% peptone, and 1.5% yeast extract) after inoculated with 4 mL of an overnight bacterial culture. 6 mL of the sample was withdrawn from flasks after 48 and 72 hrs of incubation.The cell-free supernatant obtained after centrifuging the culture broth at 10,000 rpm for 10 min was used as the crude enzyme source. The crude extract was used for characterization of the enzyme activity and stability under different conditions (Demirkan *et al*., 2017).

### 3.11 Characterization of amylase activity

**Effect of different buffers:** To evaluate the solubilization capacities of each buffer, crude amylase was solubilized by acetate, phosphate and citrate buffer at pH 3.0, 4.0, 5.0, 6.0, 7.0 ,8 .0 and 9.0. The activity of the enzyme was measured at 540 nm using spectrophotometer (Fikade and Tesfaw, 2019).

**Effect of metal ions**: The effect of metal ions on amylase activity were studied by adding 0.5 ml of different metal salts ($CaCl_2$, $MgCl_2$ and NaCl) to the assay mixture. Then various concentrations of the best salts were incorporated in the assay mixture and amylase activity was measured at 540 nm using spectrophotometer (Fikade and Tesfaw, 2019).

**Effect of inhibitors on enzyme activity**: Different inhibitors had different inhibition patterns. To examine the effect of inhibitors on enzyme activity, Ethylene Diamine Tetra Acetic acid (EDTA) was prepared at different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3 g/L). One ml of 1% soluble starch, 0.5ml of 0.05M phosphate buffer at pH 6.5 and 1 ml crude enzyme was mixed in test tubes. Then 0.5 ml of EDTA was added to the mixture and incubated in a water

bath at 37 $^{o}$C for 30 minutes (Divakaran *et al.,* 2011). The activity was mesured following the DNS method as modified by Senaite Leykun and Amare Gessesse (2017).

### 3.12 Amylase activity assay

Amylase activity was determined following the DNS method as modified by Senaite Leykun and Amare Gessesse (2017). One ml soluble starch (1%) in 0.5 mM phosphate buffer at pH 6 was mixed to 3ml enzyme source and incubated for 30min at 37°c in a heating water bath. After 30 minute incubation, the reaction was stopped by adding 2ml DNS and heated in a boiling water bath. After 10 minute incubation, the reaction was stopped by adding 0.5ml sodium potassium tartrate (1g/100ml). After cooling in running water, absorbance was measured at 540nm against a blank reagent. DNS reagent was composed of (%): 3, 5-Dinitrosalicylic acid, 1; sodium hydroxide, 1; Sodium bi sulphate, 0.05 and phenol, 0.2. Each assay was done in triplicate and averages were taken.

### 3.13 Identification of Amylase producing bacterial isolates using MALDI TOF MS techniques

Pure isolates of the amylase producing bacterial isolates (APBIs) were identified following the methods of Toubal et al., (2018). Briefly, all isolates were initially purified and screened based on their ability to clear zone prior to identification using Matrix-assisted laser desorption ionization time flight mass-spectroscopy (MALDI-TOF MS). These isolates classifications were performed using the direct transfer method (MALDI Biotyper 3.1. User Manual, Bruker Daltonics Inc.). The representative single colonies of the possible APB was smeared as a thin film directly into a spot on MALDI targeted plate using tooth applicator. The MALDI-TOF MS target plate was overlaid with 1µl of 70% (v/v) of formic acid and allowed to dry at a room temperature. Immediately the spot was overlaid with 1µl of matrix solution α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile (HCCA) solution and allowed to dry at room temperature. The resulting spectra was compared with reference spectra by using the Biotyper 3.1 software (Bruker MALDI Biotyper, UK). The identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Isolates with a score of ≥2.0 for a given species were considered adequately identified to the species level as prescribed by Tomazi et al. (Tomazi et al., 2015). *E. coli* ATCC 25922$^{T}$ was employed as a standard for calibration and quality control.

### 3.14 Data Analysis

The data was analyzed by descriptive data analyzed and was used line graph and bar graph by excelling 2016 and each assay was done in triplicate and averages were take

# 4. RESULTS AND DISCUSSION

## 4.1 Isolation and screening of amylase producing bacteria from soda lake sample

In this study, based on colony morphology, a total of 79 different colonies were isolated and grown individually on a solid alkaline medium and subjected to iodine staining;Thirty (30) (38%) were observed to give a zone of clearance around their colony. The isolate shows a variation in the size of clear zone hydrolysis on starch agar plates ranging from 1 mm to 4.5 mm (Table 1). Similar method has been used for isolation of soda lake microorganism by some researcher(Jones et al.,1998) and (Martins et al.,2001) .

**Table 1 Clear zone of starch hydrolyzing soda lake isolates**

| Isolate NO; | Isolate code | Halozone diameter (centimeter) |
|---|---|---|
| 1 | LCI-12 | 3 .5 |
| 2 | LCI-13 | 1.9 |
| 3 | LCI-14 | 1.2 |
| 4 | LCI-15 | 1.3 |
| 5 | LCI-17 | 1.8 |
| 6 | LCI-18 | 1.4 |
| 7 | LCI-19 | 1.3 |
| 8 | LCI-22 | 1.1 |
| 9 | LCI-23 | 1.4 |
| 10 | LCI-24 | 1 |
| 11 | LCI-25 | 1.2 |
| 12 | LCI-26 | 4 .5 |
| 13 | LCI-27 | 1.6 |
| 14 | LCI-28 | 1.9 |
| 15 | LCI-29 | 1.6 |
| 16 | LCI-30 | 1.2 |
| 17 | LCI-39 | 1.5 |
| 18 | LCI- 41 | 4.1 |
| 19 | LCI-43 | 1.7 |

| | | |
|---|---|---|
| 20 | LCI- 44 | 1.2 |
| 21 | LCI- 49 | 1.1 |
| 22 | LCI-50 | 2 |
| 23 | LCI-51 | 1.3 |
| 24 | LCI-54 | 1.4 |
| 25 | LCI-55 | 1.8 |
| 26 | LCI-58 | 1.7 |
| 27 | LCI-68 | 1.2 |
| 28 | LCI-73 | 1.4 |
| 29 | LCI-78 | 4 .3 |
| 30 | LCI-79 | 4. |

Where : L - Lake
C - Chitu
I - Isolate

### 4.2 Morphological characteristics of amylase producing bacteria

Cultures grown in agar media for 24 hours at 28 $^{0}$c were used for characterization for gram staining , Endospore test and motility test . All isolates showed gram-positive and rod-shaped cells, with the exception of isolate LCI-18 and LCI-27, which showed coccus cells With the exception of LCI-18 , LCI-27 and LCI- 41, all isolates were endospore forming, in normal gram staining process the result shown as (Table 2).

Table 2 : Result of gram staining for selected isolates

| Isolate NO | Isolate code | Gram staining | Endospore test | Motility test |
|---|---|---|---|---|
| 1 | LCI-12 | +ve | +ve | Motile |
| 2 | LCI-13 | +ve | +ve | Motile |
| 3 | LCI-14 | +ve | +ve | Motile |
| 4 | LCI-15 | +ve | +ve | Motile |
| 5 | LCI-17 | +ve | +ve | Motile |
| 6 | LCI-18 | -ve | -ve | Non -motile |
| 7 | LCI-19 | +ve | +ve | Motile |
| 8 | LCI-22 | +ve | +ve | Motile |
| 9 | LCI-23 | +ve | +ve | Motile |
| 10 | LCI-24 | +ve | +ve | Motile |
| 11 | LCI-25 | +ve | +ve | Motile |
| 12 | LCI-26 | +ve | +ve | Motile |
| 13 | LCI-27 | -ve | -ve | Non -motile |
| 14 | LCI-28 | +ve | +ve | Motile |
| 15 | LCI-29 | +ve | +ve | Motile |
| 16 | LCI-30 | +ve | +ve | Motile |
| 17 | LCI-39 | +ve | +ve | Motile |
| 18 | LCI- 41 | +ve | -ve | Non -motile |
| 19 | LCI- 43 | +ve | +ve | Motile |
| 20 | LCI- 44 | +ve | +ve | motile |
| 21 | LCI- 49 | +ve | +ve | Motile |
| 22 | LCI-50 | +ve | +ve | Motile |
| 23 | LCI-51 | +ve | +ve | Motile |
| 24 | LCI-54 | +ve | +ve | Motile |
| 25 | LCI-55 | +ve | +ve | Motile |
| 26 | LCI-58 | +ve | +ve | Motile |
| 27 | LCI-68 | +ve | +ve | motile |
| 28 | LCI-73 | +ve | +ve | Motile |
| 29 | LCI-78 | +ve | +ve | Motile |
| 30 | LCI -79 | +ve | +ve | Motile |

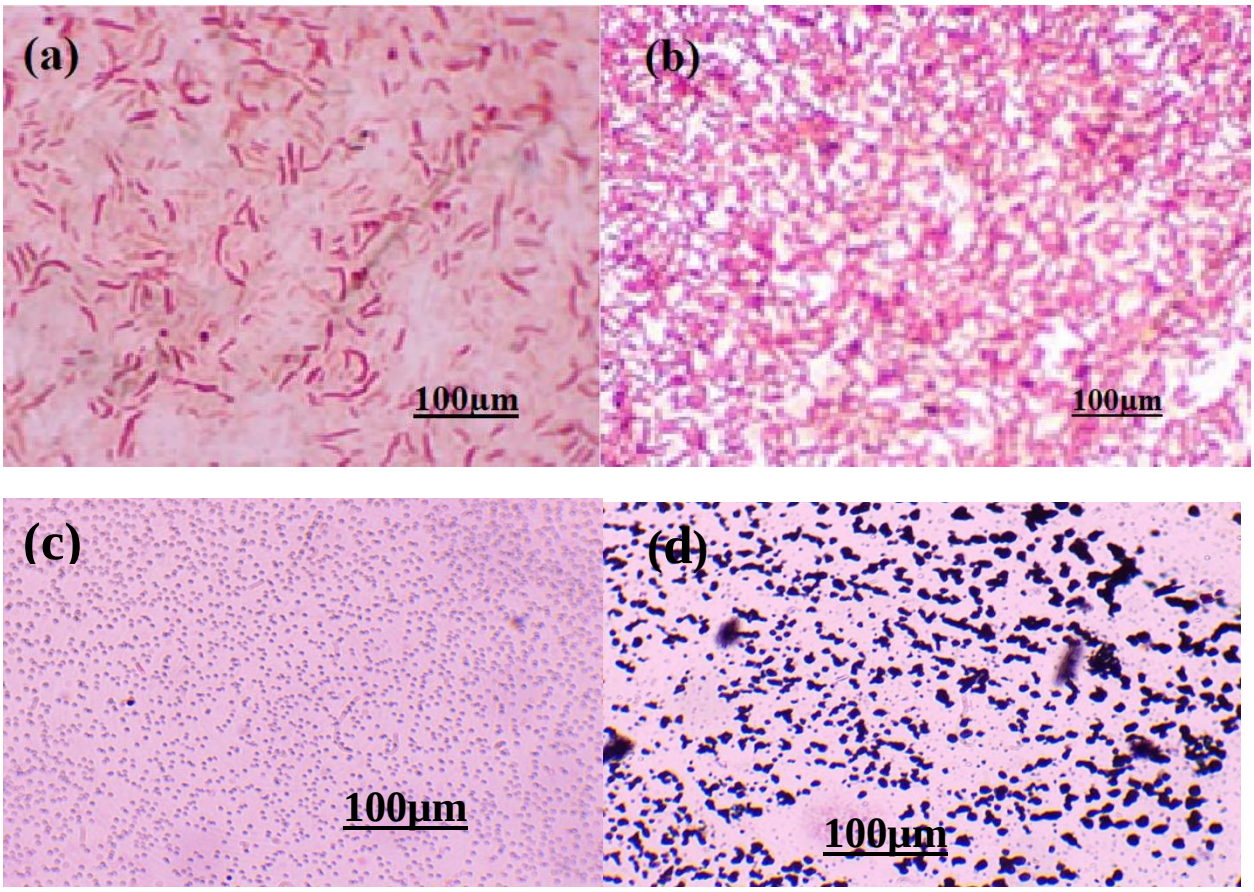



Figure 3 : potential Gram negative and positve Amyalase producing bacteria isolates (a) LCI-18, potential Gram-negative amylase produicn bacterial isolate. (b) LCI-27, Gram-neagtive APBI. (c) LCI- 12, Gram-postive APBI. (d) LCI-26, Gram-postive APBI. The magnification power was 100x using oil immersion.

### 4.3 Biochemical characteristics of Amylase producing bacteria

Table 3 : Result of biochemical test performed for 30 isolates

| Isolate NO | Isolate code | Catalase test | Indole test | Methyl red test | Voges Proskauer test | Citrate test | Urease test | Hydrogen Sulphide test | Triple Sugar test |
|---|---|---|---|---|---|---|---|---|---|
| 1 | LC-12 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 2 | LC-13 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 3 | LC-14 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 4 | LC-15 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 5 | LC-17 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 6 | LC-18 | -ve | +ve | -ve | +ve | -ve | -ve | +ve | +ve |
| 7 | LC-19 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 8 | LC-22 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 9 | LC-23 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 10 | LC-24 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 11 | LC-25 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 12 | LC-26 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 13 | LC-27 | -ve | +ve | -ve | -ve | -ve | -ve | +ve | +ve |
| 14 | LC-28 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 15 | LC-29 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 16 | LC-30 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 17 | LC-39 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 18 | LC- 41 | +ve | -ve | +ve | +ve | +ve | +ve | -ve | +ve |
| 19 | LC- 43 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 20 | LC- 44 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 21 | LC- 49 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 22 | LC-50 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 23 | LC-51 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 24 | LC-54 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 25 | LC-55 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 26 | LC-58 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 27 | LC-68 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 28 | LC-73 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 29 | LC-78 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| 30 | LC-79 | +ve | -ve | -ve | +ve | +ve | +ve | -ve | +ve |

### 4.4 Enzyme assay to select the highest amylase producing bacteria

The results of enzymatic activities of 30 isolates were shown in (Table 1) . Isolate LCI -26 showed maximum enzymatic activities where as Isolate LC – 24 the lowest. The activities of Isolate LC I-78 ,LCI -79 , LCI - 41 and LCI -12 were potential amylase producing bacteria. Five potential amylase producing bacteria isolates were selected for further study MALDI – TOF was used. In line with this study, Formation of clear zone around the bacteria colony after addition of drop of iodine solution indicated the amylase soda lake strains hydrolyzed the starch present in the media the use of starch alkaline medium containing nutrient agar for the isolation of amylase producing bacteria has earlier been reported by some researchers ( Spanka and Fritze .,1993) and (Agnew et al.,1995). The lower amylase positive isolates might be due to the extensive dilution of the sample during sample processing and thus many bacterial isolates might be lost in theprocess(MamoandGessesse,1999). Glucose was used to formulate standard curve to determine the amount of reducing sugar liberated by the amylase that was produced by bacterial isolates. One ml of different glucose solutions ( 0.1, 0.22, and 0.36g/L) were added to 2ml DNS in test tubes and heated in 55°C boiling water bath. After 10 min incubation, the reaction was stopped by adding 0.5ml sodium potassium tartrate. Soon

after cooling in running water, absorbance was measured at 540 nm against a blank reagent. A graph was plotted where x-axis was OD reading at 540 nm and Yaxis was glucose concentration (g/L) (Mogess ,(2015).Isolates LCI-26, LCI-41, and LCI-78 were shown maximum enzymatic activities on glucose reduction as shown in figure 4.

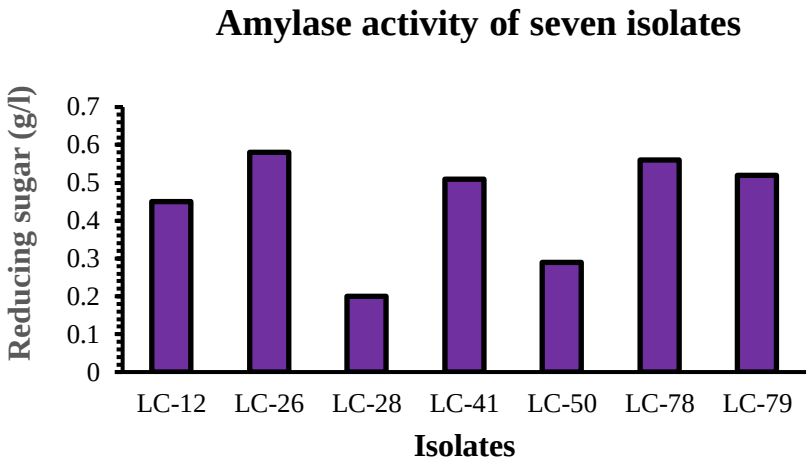



Figure 4 : Amylase activity of seven isolates incubated at 37°C for 30 minutes.

### 4.5 Identification of amylase producing isolates based on protein profile by MALDI – TOF

These APBIs were identified based on Protein profiles (MALDI – TOF MS). As indicated in (Table 5). the higher number of these APBIs were belonged to *Bacillus* isolates. Similary study showed that maximum number of *Bacillus* species were identified using MALDI – TOF MS anlaysis and follwedby *Pseudomonas* specie (Pandey et al., 2019)s. Since m/z ration for LCI -26, LCI – 41, and LCI -79 were less than 2, these ioslates could be new species. It was desinagted as *Bacillus* sp. LC -26, *Cutibacterium sp.* LCI- 41 and *Bacillus* sp. LCI -79. In this study, the recent Amylase prodcung isolates which belonged to *Bacillus cereus* was also is ceened. These isolates were desinaged as *B. cereus* LCI -12, and *B. cereus* LCI -78 with more than 2 m/z ration (Table 5). The previous study disp;ayed that (Pascon et al., 2011) amylase producing isolates were identified as *Arthrobacter arilaitensis, Acinetobacter calcoaceticus Isoptericola variabilis,* and *Solibacillus silvestris,* based MALDI – TOF MS analysis. The

previous study further confirmed that MALDI TOF MS tools were employed for identification of various genera of bacterial species. For instance, *Alcaligenes*, *Carnobacterium*, *Lysinibacillus*, *Microbacterium*, *Paenarthrobacter*, *Rhodococcus*, *Serratia* and *Stenotrophomonas* were other frequently identified based protein profiles and 16SrRNA gene analysis (Pandey et al., 2019).

Table 4: Result of amylase producing isolates by MALDI-TOF

| **S.N.** | **Isolates** | **Possible isolates** | | **Score** | **Most likely APBIs after designated based on m/z ration** |
|---|---|---|---|---|---|
| | | Genus | Species | | |
| **1** | LCI -12 | *Bacillus* | *cereus* | 2.02 | *Bacillus cereus* LC -12 |
| **2** | LCI -26 | *Bacillus* | NI | 1.76 | *Bacillus sp.* LC -26 |
| **3** | LCI - 41 | *Cutibacterium* | NI | 1.73 | *Cutibacterium sp.* LC - 41 |
| **4** | LCI - 78 | *Bacillus* | *cereus* | 2.10 | *Bacillus cereus* LC-78 |
| **5** | LCI -79 | *Bacillus* | NI | 1.77 | *Bacillus* sp. LC -79 |

All *Bacillus* species are known to be alkaliphilic (Nielsen *et al*.,1995 )and (Martins *et al*.,2001). B. cereus the most common aerobic in many types of soil and in sedimented dust and plants, *B . cereus* is also frequently present in food production, pharmaceutical , agriculture and industrial processes (Farrow et al.,1991).

### 4.6 Optimization and Culture condition for Amylase producing bacterial isolates (APBIs)

#### 4.6.1 Effect of incubation period on APBI

The enzymatic activities across incubation time are displayed in (Figure 5a). The enzymatic activities increased when the incubation time increased until 24 h, when optimal activity was obtained. Then, after 24 h, their activities declined abruptly, even if how they decreased differed among the three isolates. The amylase activities against these APBIs were found to be effective at 12 h. Similarly, it was shown that (Mishra & Behera, 2008) amylase activity was the highest for these bacterial species; these were isolated from soil receiving kitchen wastes and can degrade starch at 12-23 h .

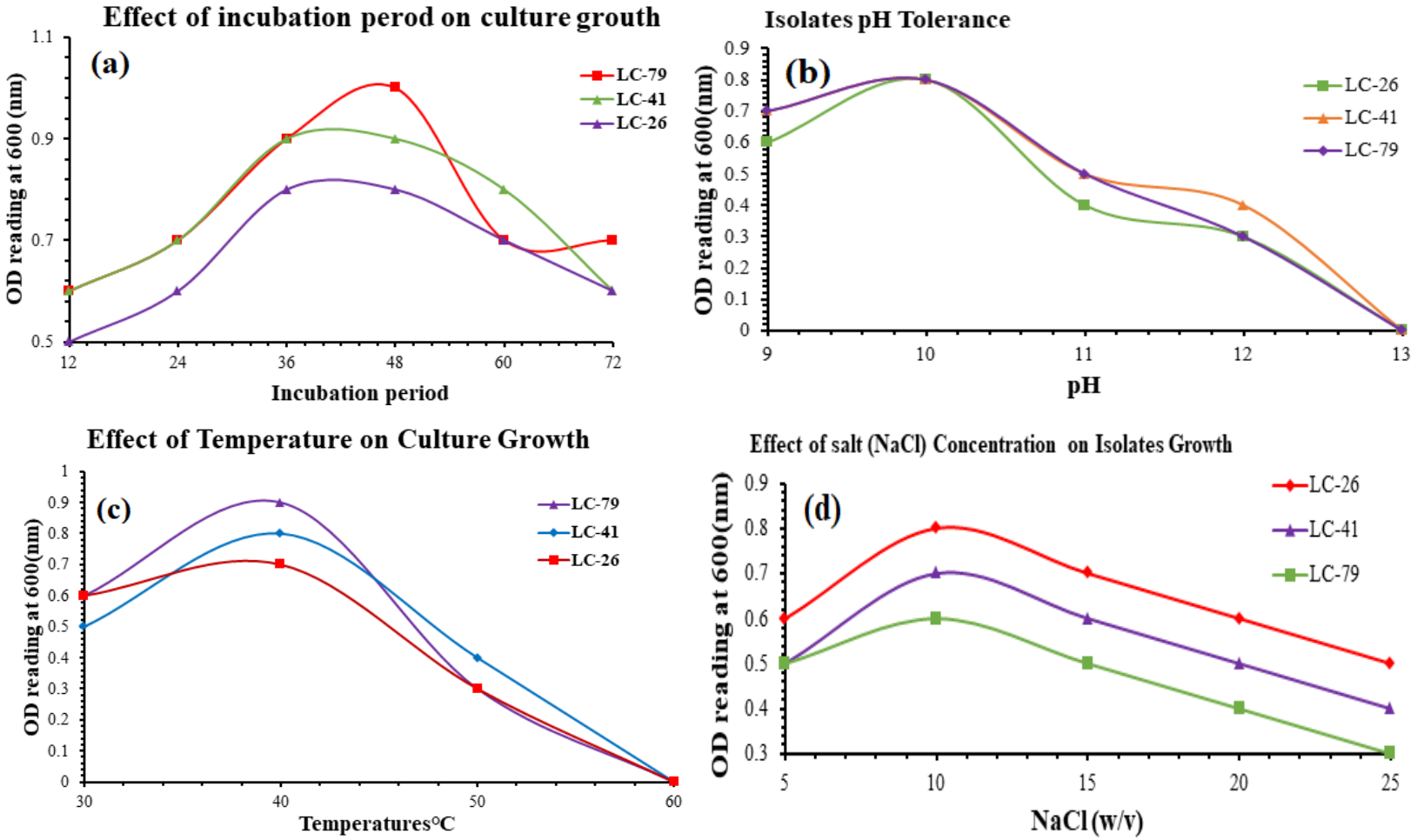



**Figure 5 :** Optimazation of amylase producing bacteria where (a) Effect of incubation period on amylase producing bacteria isolates. (b) Effect of pH on amylase producing bacteria isolates. (c) Effect of temperature on amylase producing bacteria isolates. (d) Effect of salt (NaCl) concentration on amylase producing bacteria isolates.

4.6.2 Effect of pH on APBI

A pH range of 9 to 13 was used to affect the growth of these APBIs, as shown in Figure 5b. The isolates' growths were higher at pH 9 and 10 and then grew slower when the pH rose to 11 from 10. The highest growth was observed around pH 10, and no growth was observed at pH 13. Previous study (Amutha & Priya, 2011) exhibited the the effect of pH, and other growth factors like metal ions and temptaure on amylase activity against *Bacillus subtilis* KCX 006.

4.6.3 Effect of temperature on APBI

As indicated in (Figure 5c). the growth of isolate increase with increased temperature until a temperature of 50 $^{o}$C. The highest growth of isolates was observed in a range of 30 -50°C and reached maximum at 40°C.Thier growth declined when the incubating temperature was further increased beyond 40°C. In line with this study, (Amutha & Priya, 2011) the amylase activities (38 U/mL, 37$^{o}$C) was recorded to be the maximum at stationary phase against *B. subtilis* KCX006, a lower temperature range against the recent isolates. The barly malt α-amylase isoenzyme activities have also detcetd against pH and other growth condition like calcium ions and temprtaures (Bertoft et al., 1984).

4.6.4. Effect of salt (NaCl) concentration on APBI

Figure 5d , display the effect of sodium chloride on the growth of amylase producing isolates. The NaCl concentration until 15(w/v) promoted the growth of the isolates and further of the salt concentration sharply declined the growth of the isolates. Isolate *LCI- 26* grew better than the remaining isolates at the presence of the salt. The growths of the isolates were almost inhibited at 25 (w/v) NaCl. NaCl had significant effect on the growth of amylase producing isolates. The highest relative growth of isolate was observed in a range of 1 and 1.5g/L and reached its maximum activity at 1.5g/L. Similar observations were reported earlier by Hanan et al., (2016) and (Sankaralingam., 2012). There was a decrease in growth of isolate when the salt concentration was further increased beyond 1.5g/L. This might be due to its impact on water movement. Similarly, (Reyed 2007) and (Hanan, et al.,2016) reported that the growth of bacterial organism was lost when NaCl concentration increased more than 2 g/l.

4.6.5 Effect of nitrogen source on APBI

In peptone, the highest (1.5nm) bacteria growth was recorded against LCI-79 isolate, while the lowest (1.2 nm) was recorded for *LC-26* in isolate . In urea, the highest(1.1nm ) growth was recorded against LCI-79 isolate, while the lowest growth (0.8nm) was recorded against LCI-26 isolate, amylase producing bacterial isolate (APBI). In this study, the sodium nitrate was also employed for the growth of these APBIs. The highest $OD_{600nm}$ was recorded against

LC-26 isolate for sodium nitrate, while the lowest growth was recorded against *LC-41* isolate . In Ammonium phosphate, the highest $OD_{600nm}$ was recorded against *LCI –41* for isolate while the lowest growth was obtained from LCI-79 ioslate. Generally, nitrogen source preference by amylase-producing isolates was investigated, and the results are shown in (Figure 6). Isolate LCI-79 grew higher than the other isolates in the media that contained peptone and urea compared to ammonium sulphate and sodium nitrate. Peptone and urea, these of organic nitrogen source, supported the growth of these all APBIs than the inorganic nitrogen sources. In line with this study, it was noticed that (Damisa et al., 2013) the nitrogen source such as Cotton seed and Peptone have considerable amount enffulence against amylase activities. Cotton seed gave an amylase activity of 1.282 IU/ml and peptone had shown 0.962 IU/ml amylase activity(Damisa et al., 2013). Various agricultural wastes such as green gram husk, rice bran, and wheat bran can exhibit amylase activities against *Aspergillus* sp.MK07(Chimata et al., 2010).

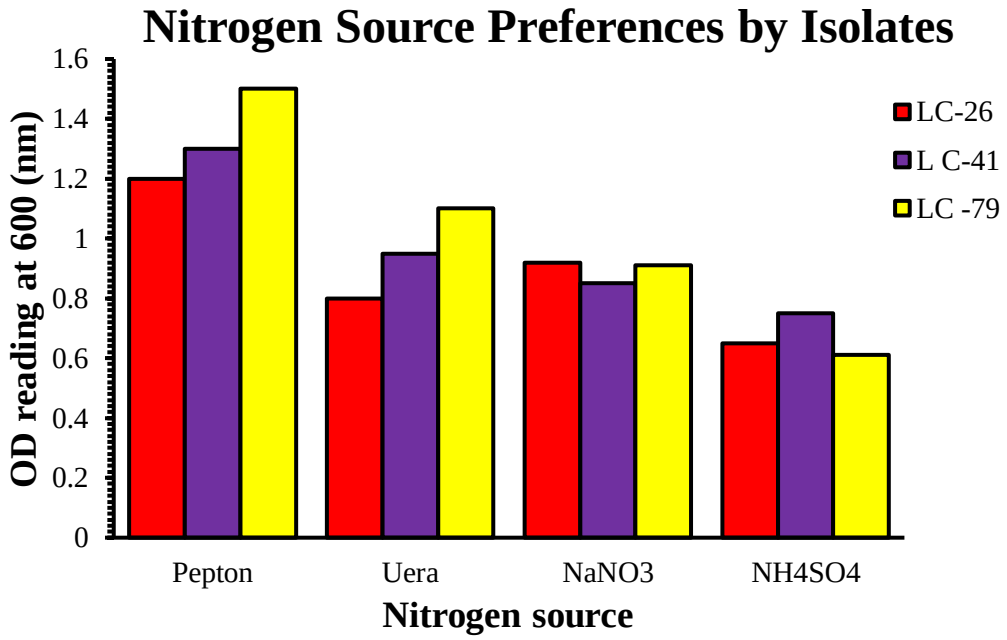



**Figure 6 : Effect of nitrogen source on amylase producing bacteria isolates**

### 4.7 Characterization of amylase activity

4.7.1 Determination of optimum acetate buffer pH on amylase activities

Figure 7a , shows the impact of acetate buffer pH on amylase activities. The optimal acetate buffer pH range was found to be 6 and their activities were lower beyond this range in both sides. The enzymatic activity of isolate *LCI – 26* and *LCI- 41* was the highest where as isolate

*LCI – 79* was the least across all acetate buffer pH range. The manner of acetate buffer pH effect was almost the same for all isolates. In line with this study, different enzymes have different buffer pH optima and decrease or increase in buffer pH on either side of the optimum value results in poor production of the amylase. Similar observations were reported earlier by Negussie and Mesfin (2015) and Shah et al. (2014).

4.7.2 Determination of optimum phosphate buffer pH on amylase activities

Figure 7b, shows the impact of phosphate buffer pH on amylase activities. The optimal phosphate buffer pH range was found to be 6 and their activities were lower beyond this range in both sides. The enzymatic activity of isolate LCI- 41 was the highest were as isolate LCI – 26 and LCI- 79 were the least across all phosphate buffer range. The manner of phosphate buffer pH effect was almost the same for all isolates.

4.7.3 Determination of optimum citrate buffer pH on amylase activities

Figure 7c, show the impact of Citrate buffer pH on amylase activities. The amylase activity was optimal at pH 6. Amylase from isolate *LCI- 41* liberated more sugar than isolate *LCI-26* and *LCI-79.* citrate buffer lad least impact on the amylase activity. The enzymatic activity of isolate LCI- 41 was the highest were as isolate LCI – 26 was the least across all Citrate buffer range. The manner of Citrate buffer pH effect was almost the same for all isolates.

4.7.4 Determination of optimum temperature on amylase activity

The effect of temperature on the amylase activity is illustrated in (Figure 7d). The enzymatic activity was increasing when the temperature rose to $50^0$c. The highest activity of amylase was observed in a range of 45°C to 50°C and reached its maximum at 45°C for isolate *LCI-41*. The highest reducing sugar was liberated by amylase which was produced by isolate *LCI-41* at 45 $^0$C. However, the remaining 2 isolates were shown similar manner of amylase activities. In line with this study, Then their activities decreased progressively with further increase in temperature. Like this study, similar observations were reported earlier by (Kalyani and Rajesh, 2018). Optimal amylase activity was observed at 50 and 55°C by (Kalyani and Rajesh, 2018).

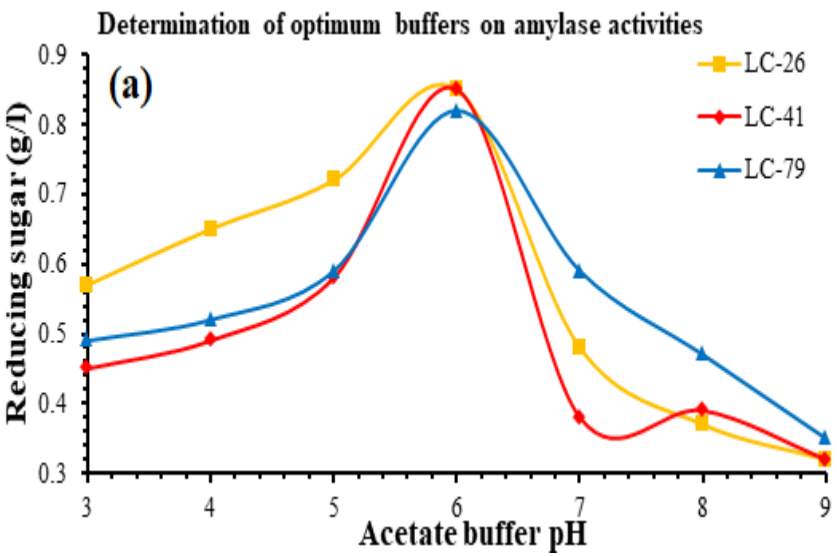
4.7.5 Sodium chloride (NaCl) impacts on amylase activity

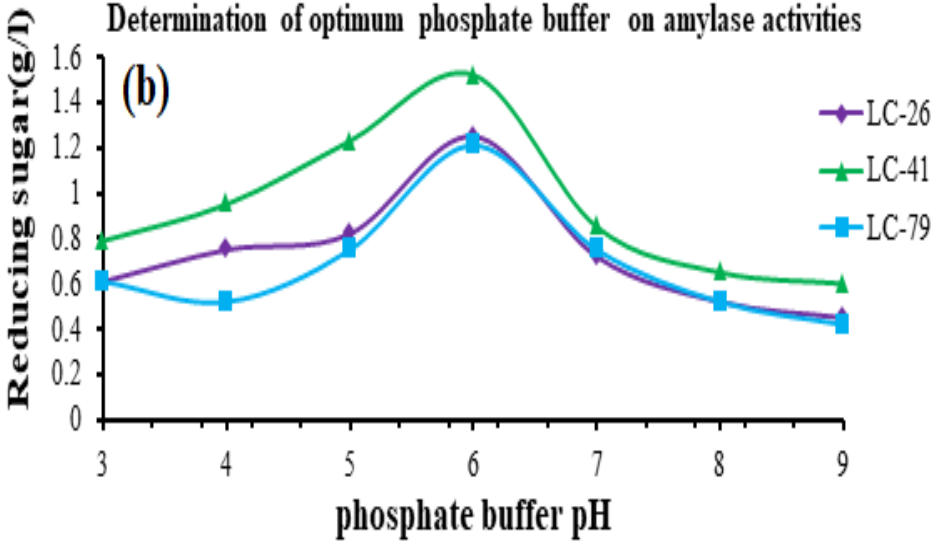
The enzyme activity in the presence of NaCl concentration was evaluated and the result is exhibited in (Figure7e). The enzymatic activity in the presence of NaCl corresponds with isolates growth in the presence of the same salt (Figure7e). The amylase activity was

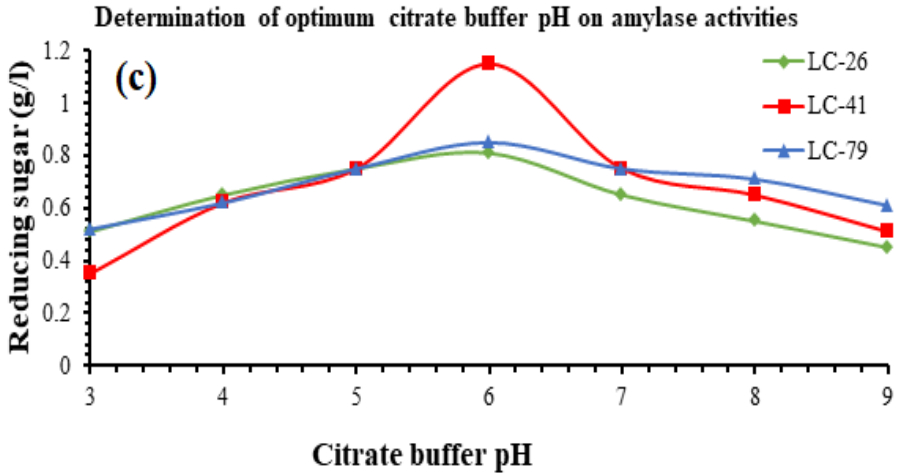
enhanced when the NaCl concentration was increased until 1g/l for isolate LCI - 26 and 1.5g/l for isolate LCI – 41 and LCI - 79.However the reducing sugar liberated was not significantly different at 1and 1.5g/l for the three isolates. In line with this study, metal ions (NaCl, MgCl2 and CaCl2) concentration increased the production of amylase by microorganisms (Fikade and Tesfaw., 2019). In this study, it was observed that all tested metal ions had increased the amylase activity. The optimal concentrations were different across metal ions used and isolate types that produced amylase. But CaCl2 and NaCl had higher yield than MgCl2.Similar observations were reported earlier by Anupama and Aparna.,(2012). Enhancement in amylase enzyme activity by 167%, 140% and 147% was recorded after addition of CaCl2, MgCl2 and NaCl respectively. There was a decrease in production of amylase when the metal ion (NaCl, MgCl2 and CaCl2) concentration was further increased beyond the maximum. Similarly, Reyed, (2007) and Hanan et al.,(2016) reported that the production amylase enzyme production was lost when metal ion concentration (NaCl, MgCl2 and CaCl2) further increased. There might be the distraction of ion linkages due to the interaction of growth media with metal ion concentration at the surface of their natural structure there by results inhibiting (Zambare, 2010).

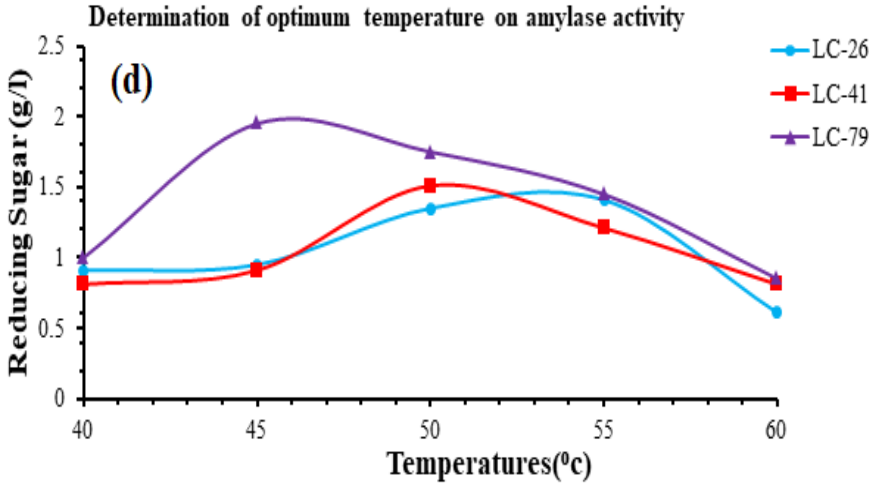
### 4.7.6 Effect of magnesium chloride ($MgCl_2$) on amylase activity

The amylase obtained from the three isolates required $MgCl_2$ to work properly (Figure7f). The result shows that when $MgCl_2$ concentration increased up to 0.5g/l, amylase activity of all isolates also increased sharply and then after declined slowly. Therefore, the optimum reducing sugar was found at 0.5g/l. The amylase activity of isolate *LC - 41* and *LC -79* was found to be higher at 0.5g/l than isolate LC- 26.


Determination of optimum buffers on amylase activities
(a)
LC-26
LC-41
LC-79
Reducing sugar (g/l)
Acetate buffer pH


Determination of optimum phosphate buffer on amylase activities
(b)
LC-26
LC-41
LC-79
Reducing sugar(g/l)
phosphate buffer pH


Determination of optimum citrate buffer pH on amylase activities
(c)
LC-26
LC-41
LC-79
Reducing sugar (g/l)
Citrate buffer pH


Determination of optimum temperature on amylase activity
(d)
LC-26
LC-41
LC-79
Reducing Sugar (g/l)
Temperatures(⁰c)

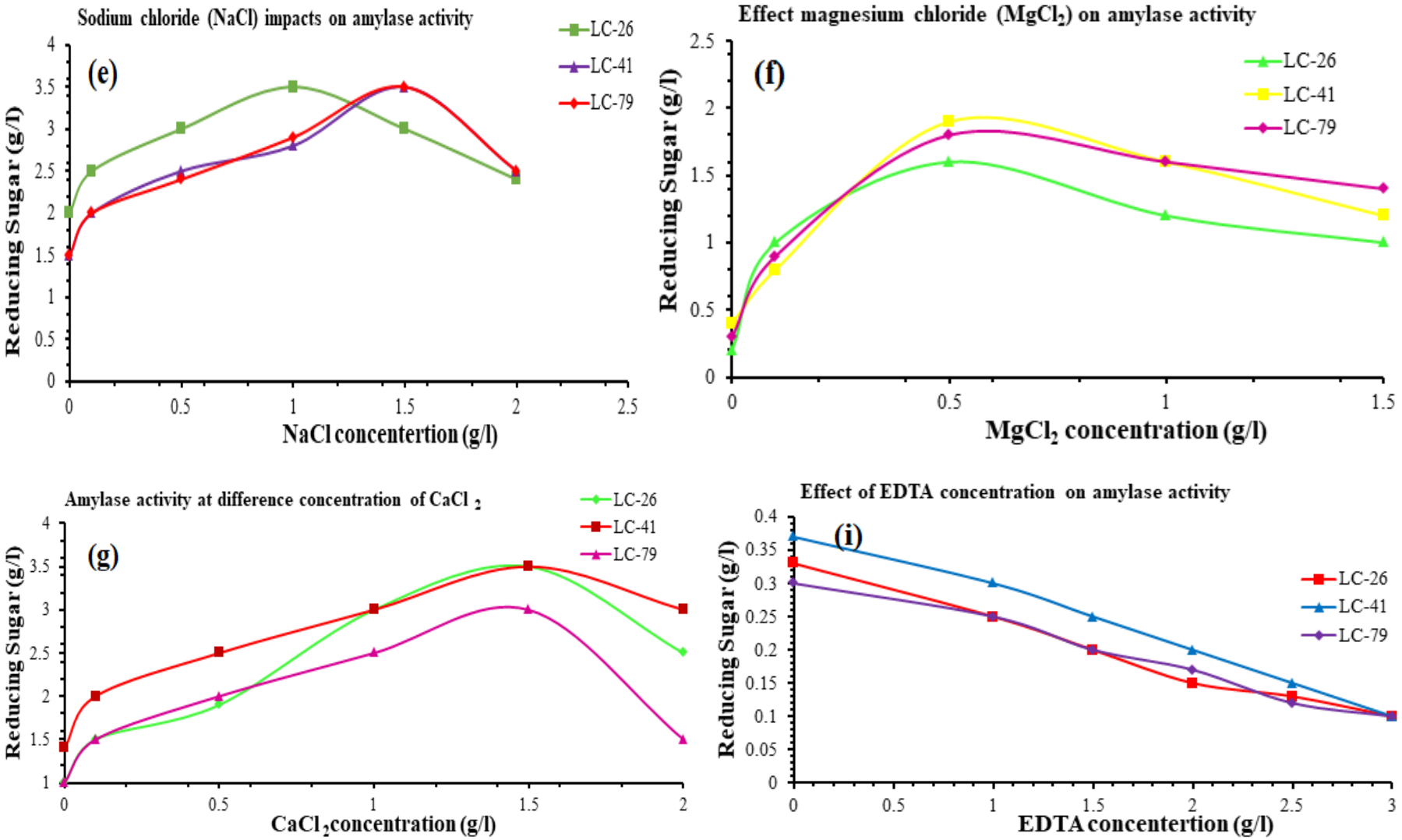



Figure 7 : Determination of optimum buffer on amylase activities where(a) Determination of optimum acetate buffer pH on amylase activities. (b) Determination of optimum phosphate buffer pH on amylase activities. (c) Determination of optimum citrate buffer pH on amylase activities. (d) Determination of optimum temperature on amylase activity. (e) Sodium chloride (NaCl) impacts on amylase activity. (f) Effect magnesium chloride ($MgCl_2$ on amylase activity. (g) Amylase activity at difference concentration of $CaCl_2$. (i) Effect of EDTA concentration on amylase activity.

4.7.7 Amylase activity at differente concentration of $CaCl_2$

The amylase activity in the presence of different $CaCl_2$ concentration was evaluated and the result is displayed in (Figure7g). The amylase activity increased almost step wise and it was highest at 1.5g/l $CaCl_2$. Amylase from Isolate LCI - 41 showed maximum enzymatic activity across all concentration and isolate LCI -79 exhibited lower enzymatic performance. The trend in which $CaCl_2$ affected the enzymatic activity was the same.

4.7.8 Effect of EDTA concentration on amylase activity

The EDTA had a negative impact on the amylase activity (Figure7i.The amylase activity in the absence EDTA concentration was higher than in its presence. The enzymatic activity decreased when the concentration rose to 3 g/L. In line with this study, the EDTA at various concentrations on amylase had decreased the activity of amylase and the decrement became more pronounced at higher concentration of EDTA. This might be due to cell membrane permeability problems since EDTA disintegrate phospholipids (Divakaram., 2011).

# 5. CONCLUSIONS AND RECOMMENDATIONS

## CONCLUSIONS

Chitu soda lake, East Shewa Oromia is a good source of Alkaliphilic amylase producing bacteria. The ability to grow at higher pH and temperature, the endospore testing, the Gram reactions, morphological features and MALDI -TOF analysis enable to group them under Bacillus spp. The amylase activity produced by different isolates had been affected differently by pH, incubating temperature, metal ions, and The amylase production was optimal at very short incubation time 12 h, since enzymes are primary metabolites. Hence, it might reduce the production cost at commercial level.

## RECOMMENDATIONS

Therefore based on the above conclusion, the following recommendations are forwarded:

- Extra optimization and scale up of enzyme production.
- Extra optimization of divalent metal ion on the activity and stability of amylase.
- Purification should be carried out to determine the produced crude enzyme is whether it is alpha amylase or beta amylase
- Evaluate the potential applications of the enzyme for beer production, paper industry, textile industry and alcohol production
- Study the feasibility of large-scale production for commercial purpose.

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# 7. APPENDIX

## Appendix- I- Media compositions

The composition of all media used in the study is given below

**Nutrient Agar**

| Component | Amount (g/L) |
|---|---|
| Nutrient Agar | 28 |
| Soluble starch | 10 |

**Nutrient Broth**

| Component | Amount (g/L) |
|---|---|
| Nutrient broth | 13.5 |
| Soluble starch | 5 |

**Trace mineral**

| Component | Amount (g/L) |
|---|---|
| $H_3BO_3$ | 3 |
| $NaNO_4.2H_2O$ | 3 |
| $ZnSO_4.7H_2O$ | 10 |
| $MnCl_2.4H_2O$ | 3 |
| $NiCl_2.6H_2O$ | 2 |
| $CoCl_2.6H_2O$ | 10 |
| $CuCl_2.2H_2O$ | 1 |

## Appendix – II- Reagents and Buffers

### Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

### Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

### Malachite green (100 ml)

To 20 ml distilled water, 5 g malachite green was dissolved in a beaker. The solution was transferred to a reagent bottle. The beaker was washed two times with 10 ml distilled water separately and a third time with 50 ml distilled water and the solution were transferred to the reagent bottle. The remaining malachite green in the beaker was washed a final time with 10 ml distilled water and added to the reagent bottle. The stain was stored at room temperature.

### Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was

mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

### 3, 5-Dinictrosalicyclic acid (100 ml)

To make 100 ml DNS, 1 g of DNS was added to 50 ml distilled water. Then, 20 ml of 2 M

NaOH was added along with 28.2 g of sodium potassium tartrate. The volume was adjusted to 100 ml by adding distilled water and mixed well. The solution was stored at room temperature in an amber bottle to prevent exposure to light.

### Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol

(95%). 200 ml of distilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

### Preparation of Buffers

**Acetate Buffer (100ml)**

To prepare 100ml of 0.2mM acetate buffers were used sodium acetate and acetic acid. They were procedurally prepared 100ml of 0.2mM acetate buffers was added 1.64g/m of sodium acetate and 1.2g/m acetic acid. Then adjusted the solution to final desired pH (3, 4, 5,6,7,8 and 9) were used HCI or NaOH.

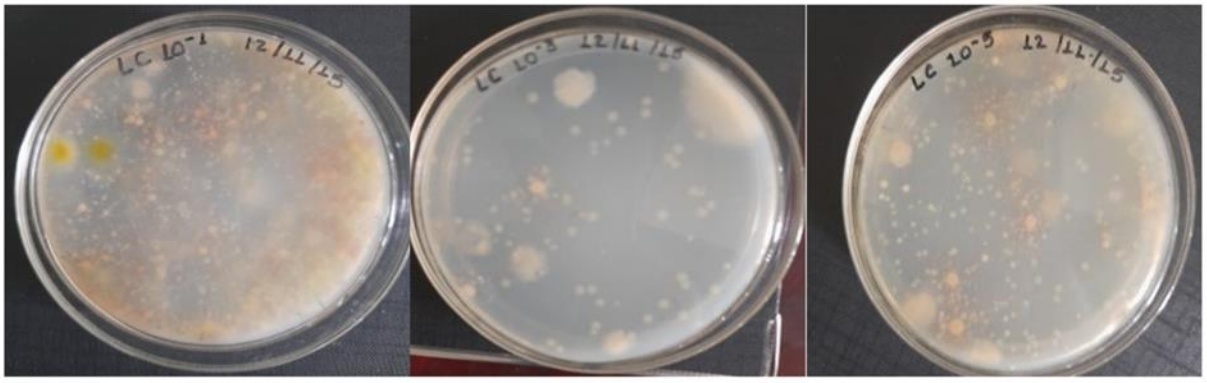
**Phosphate Buffer (100ml)**

To prepare 100ml of 0.2mM phosphate buffer buffers were used Na 2HPO 4.2H 2O and NaH 2 PO4.2H 2O. They were procedurally prepared 100ml of 0.2mM phosphate buffer was added 3.55 g/m of Na 2H PO 42H 2O and 3.12g/m NaH 2PO 4.2H 2O. Then adjusted the solution to final desired PH (3, 4, 5,6,7,8 and 9) were used HCI or NaOH.

**Citrate Buffer (100ml)**

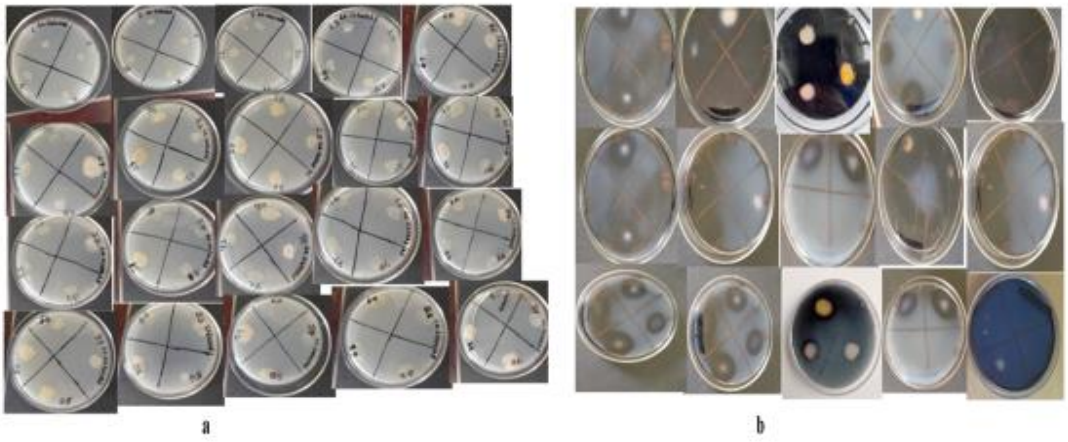
To prepare 100ml of 0.2mM Citrate buffer were used Tir-sodium citrate and citric acid. They were procedurally prepared 100ml of 0.2mM Citrate buffer was added 5.88g/m of Tir-sodium citrate and 3.84g/m citric acid. Then adjusted the solution to final desired PH (3, 4, 5,6,7,8 and 9) were used HCI or NaOH.

## Appendix –III-pictures

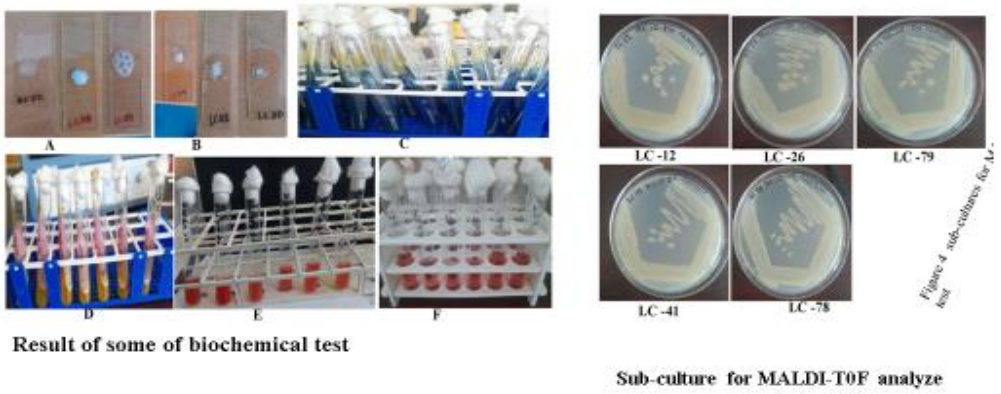


A B C

Picture : 1 colonies size ,color and shape for selected isolates




Picture : 2 zone of hydrolysis of starch on 79 isolates before (a) and after (b) the addition of iodine solution




Picture : 3 result of some of biochemical test and sub-culture for MALID-TOF analyze

Picture : 4 Isolation of amylase producing bacteria in the safety hood