Isolation and Identification of α-Amylase Producing Soil Microorganisms and Partial Characterization of α-Amylase Enzyme

By

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
Bachelor of Science in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

_____

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Approval

The thesis titled "**Isolation and Identification of α-Amylase Producing Soil Microorganisms and Partial Characterization of α-Amylase Enzyme**" submitted by Shams Tahsin Tamanna of Spring, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on December 5, 2019.

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Ethics Statement

The data presented in this thesis are acquired from conducted experiments. Information that are taken from other sources are appropriately cited through accurate referencing.

Abstract

Alpha (α)-amylase is an enzyme responsible for the breakdown of starch molecules. It hydrolyzes starch into reducing and fermentable sugars like maltose. The enzyme has various industrial applications; namely in food, detergent, leather, paper, chemical, and pharmaceutical industries. In this study, α-amylase producing bacteria were isolated from two soil samples taken from two different places of Dhanmondi, Dhaka. Out of 23 isolates from the soil samples, 10 could hydrolyze starch and were selected for identification and characterization. The selected isolates were gram-positive *Bacillus* and *Solibacillus;* and gram-negative *Raoultella* and *Klebsiella.* Antibiotic susceptibility of the 10 isolates was determined by the Kirby-Bauer test. Gram-negative isolates were more resistant to antibiotics than the gram-positive isolates. Among the selected isolates, *Bacillus cibi* showed the highest antimicrobial activity against pathogenic strains. The effects of temperature, pH and salinity on the growth of isolates were determined. The optimum growth temperature for the isolates ranged from 37^0C to 40^0C, and optimal pH for growth ranged from 7.0 to 7.5. The isolates were subjected to submerged fermentation for the production of α-amylase and the activities of extracted amylase enzymes were determined by the DNS test. Maximum amylase activity were displayed by *Bacillus subtilis* (0.49 unit/ml), *Bacillus amyloliquefaciens* (0.46 unit/ml), and *Bacillus sporothermodurans* (0.44 unit/ml). The isolates were further characterized by studying the effects of temperature, pH, and presence of various chemical agents and metallic salts on the activity of bacterial amylase. The enzymes showed maximum activity at temperature 37^0C~40^0C, and pH 6.0~6.7. Enzyme activity was increased in the presence of calcium. However, it was decreased in the presence of chemical agents like urea, EDTA and acetic acid.

Dedication

To everyone who prayed for my success.

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Shams Tahsin Tamanna

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List of Acronyms

Amt	Amount
Approx.	Approximately
Conc.	Concentration
DNA	Deoxyribonucleic Acid
DNS	3,5-Dinitrosalicylic acid
EDTA	Ethylenediaminetetraacetic acid
Et at.	et alia, which means “and others.”
GEN III	Generation 3
LB Broth	Luria Bertani Broth
MR/VP	Methyl Red / Voges-Proskauer
NADH	Nicotinamide Adenine Dinucleotide (NAD)+Hydrogen (H)
OD	Optical Density
TM	Transcendental Meditation

1. Introduction

The efficient and selective conversion of chemicals via catalysis is a fundamental condition for sustaining life. Enzymes are responsible for this conversion in biological systems. It is one of the central biochemical processes that degrade larger macromolecules, create complex molecules from simpler precursors and conserve and transform chemical energy. In today's world, the role of enzymes is not limited to sustaining the life of an organism. They play a pivotal role when applied in the study of environmental science, food technology, agriculture, and chemical engineering.

Among various sources, microbial enzymes play a significant role in the industrial application as it is more stable than plant or animal enzymes (Raveendran *et al.,* 2018). Some of the enzymes commonly used in industries include α-amylase, glucoamylase, protease, phospholipase, esterase, cellulase, xylanase, catalase, peroxidase, α—acetolactate dehydrogenase, asparaginase and nariginase (Raveendran *et al.,* 2018). Glucoamylase is majorly used in the food industry and is isolated from *Aspergillus oryzae, Aspergillus niger* and *Saccharomyces cerevisiae.* Cellulase is used in textile industries for cotton softening, denim furnishing; in food industries for fruit juice processing. It is isolated from *Aspergillus niger* and *Penicillium funiculosum*. *Aspergillus* sp.is widely used in industries in the production of various enzymes such as cellulase, lipase, catalase, amyloglucosidase, xylanase, pectinase, nariginase, uricase for dairy, baking, polymer, leather, detergent and cosmetic industries (Tamura *et al.,* 2016). Various enzymes produced by *Bacillus* sp.; such as pectate lyase, protease, laccase, neutral proteinase, β-glucanase are useful in textile and baking industries (Tamura *et al.,* 2016). Microbial enzymes such as sacrosidase, phenylalanine ammonia lyase are used in the medical treatment of disorders involving deficiency of human enzymes caused by genetic problems (Vellard, 2003, Anbu *et al.,* 2015).

Microbial amylase has almost completely replaced the chemical hydrolysis of starch in factories. (Pandey *et al.,* 2000). α-Amylase is an important industrial enzyme used in the starch industry for liquefaction of glucose, fructose and maltose, baked goods, brewing, paper, textiles, detergent, and sugar industries. (Crueger *et al.*, 1990).

α-Amylase (1,4-α-D-glucan glycanohydrolase) is responsible for hydrolyzing starch into monomeric compounds. The enzyme attacks glycosidic bond which is responsible for holding

monomeric subunits together. α-Amylase belongs to a large family of Ca^{2+} proteins that share several structural features. The three dimensional structures of α-amylases from various sources such as *Aspergillus oryzae, Aspergillus niger,* porcine pancreas, barley, human saliva, *Bacillus licheniformis, Bacillus stearothermophilus* and Alteromonas *haloplanctis* have been determined. α-amylase has been purified from various sources such as microorganisms (Takahashi *et al.*, 1981), poplar leaves (Sauter *et al.,* 1996) and pears (Pech *et al.,* 1973). The application of amylase in industrial reactions depends on its unique characteristics like its action pattern, substrate specificity, reaction products, optimal temperature and pH.

Microbial amylase is a preferred choice for industrial production of amylase due to the simplicity, availability of source and its action at a higher temperature. Different strains of bacteria are used for commercial production of α- amylase. *Bacillus licheniformis, Bacillus subtilis, Bacillus sporothermodurans* and *Bacillus amyloliquefaciens* are preferred strains for bacterial α-amylase production as they can operate at higher temperatures. *Bacillus amyloliquefaciens* is commonly used in syrup industries. The enzyme must be stable due to the high temperature of starch liquefaction and saccharification.

Soil contains diverse microbial population which contributes to its fertility (Metting, 1993). *Bacillus* sp. are economically important species utilized for their ability to synthesize a variety of antibiotics, enzymes, amino acids and sugars (Lyngwi, 2014) and are present in abundance in soil.

Each of these strains require specific environmental condition and nutrition for enzyme production. Extracted amylase requires optimum temperature and pH for maximum activity (Sivaramakrishnan *et al.,* 2006). The role of other factors such as metal ions, carbon and nitrogen source, surface acting agents, phosphate, and agitation have been studied for α-amylase production. The properties of each α-amylase such as thermostability, pH profile, pH stability, and Ca^{2+}-independency must be matched to its application. For example, α-amylases used in the starch industry must be active and stable at low pH (Couto *et al.,* 2006), but at high pH in the detergent industry. (Sajedi *et al.,* 2005). Most notable parameters are the composition of growth medium, pH of the medium, phosphate concentration, inoculum age, temperature, aeration, carbon source, and nitrogen source.

In Bangladesh, α -amylase is used for the production of high glucose and maltose syrups that are used in food and pharmaceuticals. α-Amylase is used in the textile industry for smoothening the fabric. Garment industries also use a huge amount of amylases for the removal of starch

from clothes. Unfortunately, there is no industry for amylase production in this country. Amylases are imported from other countries which is very costly (Islam *et al.,*2014). According to previous studies, α- amylase producing *Bacillus* sp. (Nusrat & Rahman, 2007) and *Streptomyces* sp. (Islam, 2014) have been isolated from Bangladeshi soil samples; and amylase producing *B. subtilis* and *B. amyloliquefaciens* have been isolated from tannery wastes (Rahman, 2018). Isolation of amylase producing microorganism strains from various sources, purification, and determination of activity of α- amylase from various microbial sources have not been extensively studied or reported on.

This study focused on the isolation of α- amylase producing microorganisms from appropriate local soil sample and characterization of the isolated microorganisms and their secreted α-amylase by optimizing growth conditions. The study can be useful in further research on the commercial production of α-amylase in local factories.

2. Literature Review

2.1 Definition of Enzyme:

Enzymes are highly specialized proteins with extraordinary catalytic power that can speed up biological and biochemical reactions. They work in an extremely substrate-specific manner. Enzymes are active in aqueous solutions and work most efficiently at mild temperature and pH.

The term "biocatalyst" is used to coin enzymes because it acts as chemical catalysts to accelerate the time of reactions internal and external of a cell (Gurung *et al.,* 2013). Biological reactions are carried out in several steps via the enzyme's catalysis process which converts substrate into products, conserving and transforming chemical energy.

2.2 History of enzymes:

Biological catalysis was fist described in the late 1700s in the study of meat by stomach secretions. During the 1800s, scientists continued their research on enzyme by studying amylase from saliva that converted starch to sugar, along with various enzymes extracted from plants. In 1850, Lewis Pasteur concluded that fermentation of sugar into alcohol was carried out by molecules called 'ferment' which are only active inside living cells (yeast). The view was termed 'vitalism.'

In 1897, Edward Buchner proved 'ferments' can work outside of a living system by demonstrating fermentation of sugar to alcohol by yeast extract. The term 'Enzyme' was given by Fredrich W. Kuhn from the Greek word "*enzymos"* (leavened). In 1926, James Sumner revolutionized enzyme study by isolating and crystalizing urease. Studying the structure of Urease, Sumner postulated that all enzymes are proteins. Later, John Northrop and Moses Kunitz crystalized pepsin, trypsin and other digestive enzymes and reached a similar conclusion regarding enzyme's protein nature. J. B. S. Haldane suggested that weak bonds interact in the enzyme-substrate reaction which helps in catalysis (Nelson *et al*., 2000).

2.3 Classification of enzymes:

Enzymes catalyze about 4000 biochemical reactions (Gurung *et al.,* 2013). Internationally, Enzymes are classified into six groups: Oxidoreductase, Transferase, Hydrolase, Lyase, Isomerase, and Ligase.

Table: Classification of enzymes

Class no.	Class name	Type of Reaction Catalyzed
1	Oxidoreductase	Catalysis of oxidation reduction reaction; hence involved in electron transfer. Example: alcohol dehydrogenase.
2	Transferase	Transfer of functional group. Example: aminotransferase.
3	Hydrolase	Catalysis of hydrolysis of various bonds. Functional groups are transferred into water. Example: amylase.
4	Lyase	Cleavage of C-C, C-O, and C-N bonds by means other than hydrolysis and oxidation. Bonds are cleaved by eliminating, leaving double bonds/rings and adding groups to double bonds. Example: pectate lyase.
5	Isomerase	Transfer of groups in a molecule to yield isomers. Example: mutase.
6	Ligase	Formation of C-C, C-S, C-O, and C-N in a condensation reaction which is coupled with cleavage of cofactors like ATP. Example: DNA ligase.

2.4 The mechanism and conditions for enzyme action:

Enzyme reactions are summarized by the following overall reaction (Pelczar *et al.*, 1958):

$$\text{Enzyme E} + \text{Substrate S} \leftrightarrows \text{Product P} + \text{Enzyme E}$$

At the initial phase of the reaction, enzyme and substrate form an Enzyme-Substrate complex which is broken down to yield Product P. The enzyme remains unchanged and can catalyze another substrate's transformation.

Enzymes have certain surface areas with the high affinity of the substrate. These areas are called the active sites. Enzyme binds to substrates in the active site and forms an unstable state which requires less activation energy for the chemical reaction to proceed.

The conditions affecting the enzyme's activity are:

(1) Concentration of the enzyme
(2) Concentration of the substrate
(3) pH
(4) Temperature

There is an optimum relation between the concentration of enzyme and substrate for maximum stability. If the amount of enzyme is kept constant and substrate concentration is increased gradually, the reaction rate will increase and reach a maximum value. After this, the reaction rate will no more depend on the substrate concentration.

Similar to the necessity of optimal temperature and pH for cellular growth, optimal temperature and pH are required for maximum enzyme activity. But the values may vary from one enzyme to another in the same cell. An optimal condition is determined in terms of what is the best for the entire cell itself. The enzymes, when extracted and purified, are no longer in their natural condition and no longer involved in the multitude of reactions that occur in a cell. Hence, the optimum condition in vitro may not be the same as that of in vivo (Pelczar *et al*., 1958).

2.5 Amylase Enzyme: Definition and History:

Amylase in a hydrolase enzyme involved in the hydrolysis of starch into simple sugar molecules. It is considered as glycosidic hydrolases (Kaur *et al.,* 2012). Amylase is present in human saliva where carbohydrate digestion initiates.

Amylase was first discovered in 1811 by Kirchhoff in wheat. Kuhn named α-Amylase in 1925 due to the alpha configuration of the hydrolyzed products. In 1930, Ohlsson discovered another form of amylase involved in degradation in β-mannose. The enzyme was named β-amylase. Amylase was the first enzyme to be discovered and isolated in the form of diastase by Anselme Payen in 1833. It was also the first industrially produced enzyme, from a fungal source in 1894. Initially, fungal strains were extensively produced and used by SSF techniques in the United States. Boidin and Effront pioneered the use of bacterial strains for commercial production. They used *Bacillus subtilis* and *Bacillus mesentericus* to produce α-amylase in a commercial scale in the submerged fermentation method in 1917.

2.6 Structure of Amylase:

Amylse is a smaller enzyme molecule stable in unfavorable conditions. Human α-amylase has 512 amino acid molecules in a single oligosaccharide chain (MW=57.6 kDa). The largest domain is barrel shaped and holds the other two domains via disulfide bond. (Souza *et al.*, 2010). The active site of α-Amylase contains three acidic groups that are involved in most of the works. Glutamate 233, aspartate 197 and aspartate 300 together hydrolyze two adjacent sugar molecules in starch. Five short sugar chains are bound to the active site of the α-amylase molecule. A calcium ion is present to stabilize the structure and for allosteric activation. A chloride ion is bound underneath the active site and it assists the reaction.

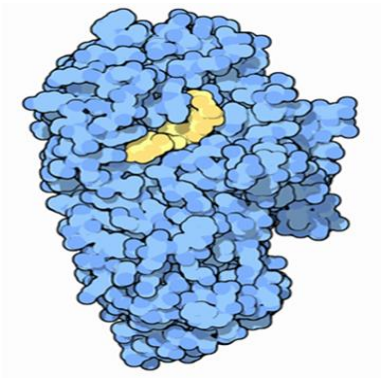


Figure: α-Amylase Molecule with Short Sugar Chains (yellow)

2.7 Structure and Breakdown of Starch by Amylase:

Starch is a polymer of glucose linked by α-1,4 and α-1,6 glycosidic bonds. Due to two different types of bonds, starch molecules can have various structures. Amylase is an unbranched chain of 500 to 2000 glucose molecule subunits held together by α-1,4-glycosidic bond only. Amylopectin is a branched form of starch is referred as amylopectin and has α-1,6 glycosidic bonds forming branches along the long chain. The degree of branching in in amylopectin is one per 25 glucose units. The degree of branching is proportionate to solubility.

Amylase is a glycoside hydrolase enzyme that attacks α-1,4 and α-1,6 glycosidic bonds and breaks them into smaller subunits. α-Amylase requires the presence of calcium ion for its activity. Depending on the bond that is attacked by amylase, end product varies in nature. The products can be dextrin, maltotriose, maltose, glucose, etc. Dextrins are the short-chained D-glucose polymers which are produced by random cleavage along the starch chain. If the third bond from one end of a starch chain is cleaved, it produces a form of tetrasaccahride called maltotriose. If the second bond from the starch chain is cleaved, it gives maltose. Glucose is a product of terminal bond cleavage.

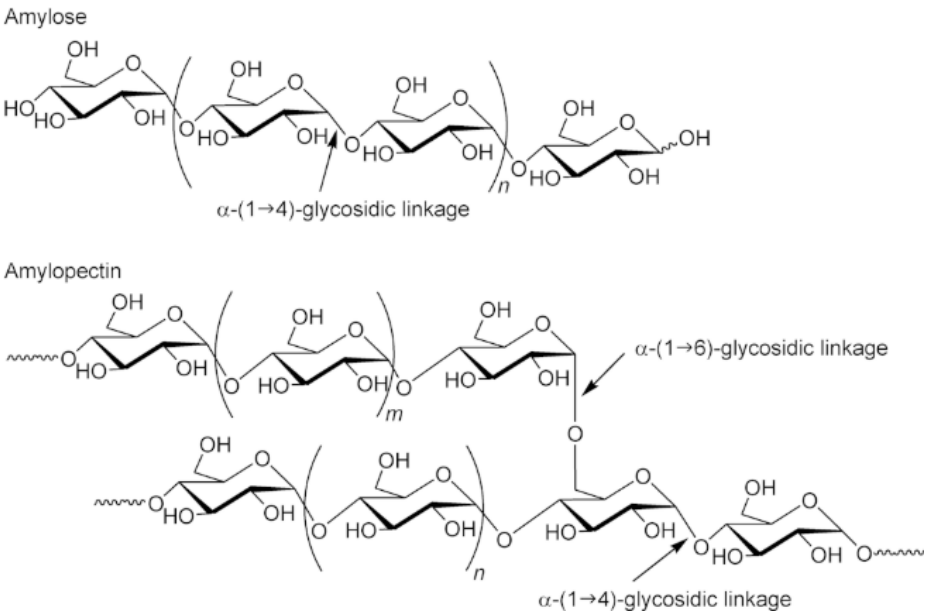


Figure: Molecular Structure of Amylose and Amylopectin

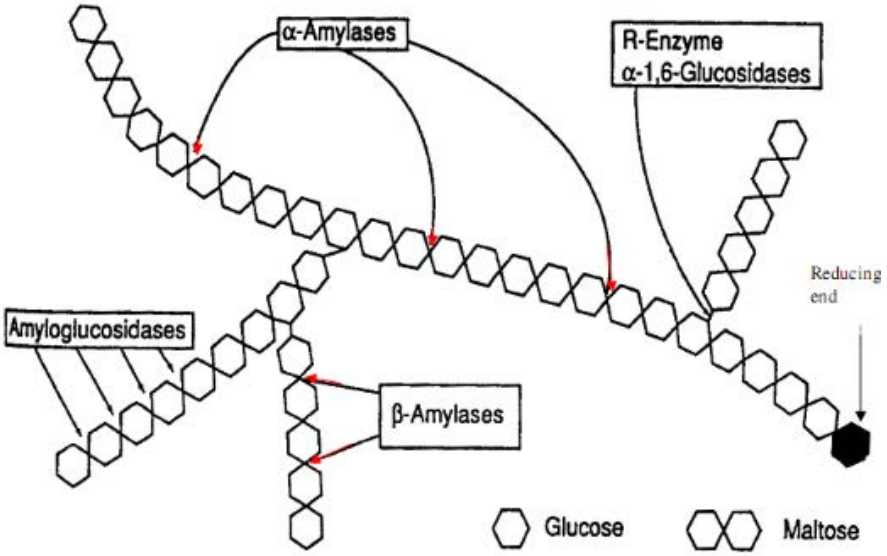


Figure: Reaction Sites of Amylase Molecules

The process of starch hydrolysis highly depends on temperature effect, hydrolysis condition and enzyme's origin.

2.8 Classification of amylases:

Amylases are classified based on the glycosidic bonds they attack for the breakdown of starch molecules. α- amylase, or 1,4-α-D glucan glucanohydrolase breaks the starch chains at random locations and produces large chain saccharides. β-amylase, or 1,4-α-D glucan maltohydrolase hydrolyzes the second α-1-4 glycosidic bonds from non-reducing end and produces two maltose molecules. γ-amylase (1,4-α-D glucan glucohydrolase) cleaves the α-1-6 glycosidic linkages and the last α-1-4 glycosidic linkage to produce glucose molecules.

The classification of Amylases is described below.

Table: Classification of amylases

	α- amylase	β-amylase	γ-amylase
Site of Action	Acts at random -1-4 glycosidic bonding sites in starch chain	Acts at the second α-1-4 glycosidic bond from non-reducing end	Acts at γ-amylase cleaves the α-1-6 glycosidic linkages and the last α-1-4 glycosidic linkage
Source	Present in humans (pancreatic and salivary amylase), mammals, plants and some bacteria (*Bacillus)* and fungi (*ascomycetes* and *basidiomycetes)*	Present in Bacteria, Fungi and seeds and fruits of plants	Present in the small intestine of animals and in microbes
Reaction Product	Dextrin, maltose, maltotriose, glucose and pectin	Maltose	Glucose
Optimum pH	6.7 -7.0	4.0-5.0	3.0
Optimum Brewing Temperature	63-70^{0}C	55-65^{0}C	-
Applications	Widely known for industrial application (i.e. glucose and fructose syrup production, detergent formulation)	Research-oriented application (i.e. structural study of glycogen and starch molecules) and industrial application (i.e. brewing and distilling, maltose syrup production)	Research-oriented application

2.9 Production of α-amylase from bacteria

For industrial production, α-amylase is acquired from barley, rice plants, animals or microorganisms. Microorganisms are a source of interest due to rapid growth pattern as well as easy handling and manipulation. Besides, manipulating the nature of amylase (i.e. thermostability, pH stability, and calcium ion independence) is easier when produced from microorganisms. The microbial sources involve fungi, bacteria and some genetically modified microbial species (Sundarram *et al.*, 2014).

Production method can be two types:

(1) Submerged fermentation (SmF)

(2) Solid State fermentation (SSF).

In submerged fermentation, free flowing liquid substrate is used and the product accumulates in broth media. Due to the rapid mature of fermentation, the substrate is supplied constantly. A major advantage of this method is easier downstream processing. The method is more suitable for genetically modified organisms and microorganisms that require higher moisture level for their growth. SmF is used for the production of secondary metabolites.

Solid State fermentation is a newer method used in bagasse, bran and paper processing. Due to slow utilization, a constant supply of substrate is not required. The waste materials can be recycled and reused. It is suitable for microorganisms that require lower moisture content. Simple equipment, a higher concentration of product and less effluent generation are some advantages of this method.

Due to lower cost, SSF is a preferred method for the production of bacterial α- amylase. *Bacillus subtilis, B. polymixa, B. mesentericus, B. vulgaris, B. megaterium, B. licheniformis* are some strains suitable for SSF. Some halophilic species i.e. *Chromolaobacter sp., halobacillus sp., Haloarcula hispanica* are used to produce stable and thermotolerant amylase.

Enzymes used in industries are crude preparations that are obtained without extensive downstream processing. Simpler techniques like filtration, precipitation, centrifugation, and dialysis are used to obtain crude enzyme. Enzymes used for clinical purposes, however, must be purified and require more instruments. Chromatographic techniques like ion exchange, affinity chromatography, gel filtration; liquid-liquid extraction, precipitation are used for purification of amylase.

After purification, the enzyme is characterized by studying their rate of activity, determining molecular weight and iso-electric point. SDS PAGE is a suitable method for enzyme characterization.

2.10 Optimum Environmental Parameters for Production of α- amylase

Temperature: For the production of microbial amylase, two different temperatures are taken into consideration: a temperature for optimal growth of microorganisms and a temperature suitable for the production of stable amylase. Studying *Bacillus licheniformis* and *Bacillus subtilis*, the optimal growth and the enzyme production condition was determined to be 45^0 - 46^0C and 50ºC respectively. In *B. subtilis,* temperature for growth was found to be 5^0 higher than that of enzyme production (37^0C). *Thermococcus* produced α- amylase is most active at 80^0C. *Clostridium acetobutylicum* produced α- amylase is usually more active in temperature range 30^0-60^0C. (Sundarram *et al,* 2014).

pH: The enzymes are pH sensitive so the pH of the production process must be controlled. *Pyrococcus furiosus* produces α-amylase which shows activity at an optimum pH of 6.5–7.5. *Bacillus amyloliquefaciens* produces the enzyme with an optimum pH of 7.0. *Halomonas meridiana*has maximum activity at pH 7.0 and is stable in alkaline conditions. The optimum pH for the production of the enzyme by *Bacillus* sp. isolated from dhal industry waste was found to be 6.5.

Duration: The enzyme activity increases with an increase in incubation time till it reaches the optimum duration. The production of the 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. *Bacillus subtilis* gives a high yield of alpha-amylase after 48 hours of fermentation.

Carbon and nitrogen sources: Common carbon sources used as substrates include maltose, sucrose and glucose. The nitrogen source may be organic or inorganic. Few of the inorganic nitrogen sources include ammonium sulfate, ammonium chloride, and ammonium hydrogen phosphate. Most commonly used organic sources of nitrogen include peptone, yeast extract, and soybean meal.

2.11 Application of α- Amylase:

Starch industry: The most widespread applications of α-amylases are in the starch industry. Amylase is used for starch hydrolysis that converts starch into fructose and glucose syrups. Initially, the α-amylase of *Bacillus amyloliquefaciens* was used but it has been replaced by the α-amylase of *Bacillus stearothermophilus* or *Bacillus licheniformis. Bacillus* is a preferred species for its thermostability and efficient expression.

Detergent industry: Amylases have activity at lower temperatures and alkaline pH, maintaining the necessary stability under detergent conditions and the oxidative stability of amylases is one of the most important criteria for their use in detergents where the washing environment is very oxidizing. Examples of amylases used in the detergent industry are derived from *Bacillus* or *Aspergillus.*

Paper and textile industry: Starch is a preferred sizing agent as it is easily available, cheaper and can be easily removed from the fabric. The layer of starch is subjected to hydrolysis in the de-sizing process where α-Amylase is employed to cleave starch particles randomly into water soluble components that can be removed by washing. Like textiles, paper is also treated with sizing agents to protect it from mechanical strain during processing. The sizing also contributes to a better quality of the paper in terms of strength, smoothness, and writing. Starch is commonly used as the sizing agent. The role of α-Amylase in the paper industry is the partial hydrolysis of starch to make it less viscous in a batch or a continuous process.

Food industry: Amylases are extensively employed in the processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups. The addition of α-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product. Moreover, it generates additional sugar in the dough, which improves the taste, crust color, and toasting qualities of the bread.

In Bangladesh, α-amylase is industrially used for the production of maltose and glucose syrup for food and pharmaceuticals and removal of starch in garment and textile industries. The enzyme is imported as there is no local factory for the production of α-amylase. (Rahman *et al.,* 2014).

Aims and Objectives

- Isolating potent α- amylase producing microorganisms from a local soil sample.
- Screening out α- amylase producing microorganisms by Starch Hydrolysis Test.
- Identifying the selected strains by BioLog^{TM} System.
- Characterizing the isolate microorganisms with:
    - Biochemical test analysis, morphology analysis, and microscopic analysis.
    - Antibiotic sensitivity and antipathogenic activity analysis.
    - Determination of growth patterns in different temperature, pH and salinities.
- Extracting α- amylase from isolates via submerged fermentation.
- Determining and comparing activities of α- amylase secreted by the selected strains.
- Characterizing α- amylase by determining the optimum temperature, optimum pH and stability in different chemical agents & metallic salts.

3. Materials and Methods

3.1 Sample collection:

Soil samples were collected from a local potato mill and a flower garden in Bangladesh Council of Scientific & Industrial Research (BCSIR), Dhanmondi, Dhaka. One gram of each sample was serially diluted in physiological saline and transferred to LB agar media in the spread plate method. After 24 hours of incubation, growth was observed and 23 distinct colonies were selected for the study.

Single colonies were isolated by streaking onto separate LB agar media plates and were named A-1 to A-23. A-1 to A-13 were isolated from potato mill's sample and A-14 to A-23 were isolated from flower garden's sample.

3.2 Screening and selecting potent amylase producers:

Potent amylase producer isolates were screened and selected using the starch hydrolysis test. The selected isolates were subcultured by streaking onto separate starch media plates. After incubating at 37^0C for 48 hours, the plates were flooded with Gram's iodine and the formation of clear zone was observed.

Ten isolates (A-2, A-7, A-13, A-14, A-15, A-16, A-18, A-19, A-20, and A-21) were found to produce α-amylase; which were then subcultured on LB agar plates for identification and further studies.

3.3 Identification of selected bacterial isolates:

The identification of amylase producing bacterial isolates was carried out based on morphological characteristics, Gram staining, and spore staining characteristics, biochemical characteristics and Biolog^{TM} identification system.

3.3.1 Observation of colony characteristics:

The cell shape, size, color and other colony characteristics of the selected isolates were observed and tabulated.

3.3.2 Endospore staining:

Endospore staining was carried out to detect endospore producers among isolates. Smears were prepared on glass slides which were subsequently flooded with malachite green dye. The slides were heated for 30 minutes. After cooling down, the slides were washed thoroughly to remove excess dye. Safranin was added and kept for 1 minute. The dye was washed with water. The slides were observed under oil immersion lens. Endospores uptake and retain Malachite green dye, hence appear green under a microscope. Vegetative cells appear red or pink.

3.3.3 Biochemical reactions:

All required media for the biochemical tests were prepared in respective test tubes, Petri plates, and vials.

All biochemical tests were performed with specific requirements for each test as outlined in Bergey's Manual of Determinative Bacteriology (1989).

a) Casein hydrolysis test:

Casein Agar or skim milk agar is a growth medium used for the detection of hydrolytic microorganisms. The agar is supplemented with skim milk as the casein source. The medium is opaque due to the casein in colloidal suspension. The hydrolysis reaction causes the milk agar, normally the opacity of real milk, to clear around the growth area as the casein protein is converted into soluble and transparent end products, such as small chains of amino acids, dipeptides, and polypeptides.

The isolates were inoculated on Casein agar in a zig-zag pattern and incubated overnight at 37^0C. Presence of clear area around growth was observed and noted.

b) Egg yolk agar test:

Egg Yolk test or Lecithinase test is used to identify organisms that liberate phospholipase (lecithinase). Lecithin is a normal component in egg yolk. Bacterial lecithinase breaks down lecithin to insoluble diglycerides resulting in opaque halo surrounding the colony when grown in egg yolk agar medium.

Basal egg yolk media was prepared by dissolving the standard amount of egg yolk agar powder in distilled water, in which 10% egg yolk was added after autoclaving and cooling the

media before dispensing into petri-plates. Isolates were inoculated on the plates in a zig-zag pattern in 35^0C. The plates were examined after 48-72 hours. Plates were kept up to 7 days before regarding them as lipase negative.

c) Motility test:

Motility test confirms the bacterium's ability to move by itself. Motility medium was prepared in test tubes and was inoculated with isolates by stabbing. The tubes were incubated overnight and observed for a positive result. Motility media is semisolid in nature and has soft consistency that allows motile bacteria to migrate freely and cause cloudiness whereas non-motile bacteria only grow around the site of inoculation.

d) Citrate utilization test:

Citrate utilization test is used for differentiating among enteric bacteria on the basis of their ability to utilize Citrate as their sole carbon source. The utilization of citrate depends upon the presence of enzyme 'citrate permease' produced by organisms that help its transport to the cell. Citrate media was prepared in vials in a given composition and sterilized. After inoculating the media with cultures, the vials were incubated at 37^0C for 48 hours. Color change from green to blue was observed. The vials were kept up to 7 days before regarding them as negative results.

e) Methyl red test:

Methyl red test detects an organism's ability to perform mixed-acid fermentation. MR-VP media contains glucose, peptone and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth and decreasepH. Other kinds of fermentation cannot overcome the buffering capacity of the broth. Isolates were inoculated in 10 ml of sterilized MR-VP broth and were incubated for 24 hours at 37^0C. Five drops of Methyl red indicator was added into each inoculated and uninoculated tube (control). The appearance of bright red color indicates positive test.

f) Lysine decarboxylase test:

Lysine decarboxylase test detects microbe's ability to utilize Lysine as a source of carbon and energy for growth. Microbes with Lysine Decarboxylase enzyme show positive results. Lysine Decarboxylase broth is used where 0.5% Lysine is added. Glucose and pH indicator bromocresol purple are present as a media component. When lysine is utilized by the

bacteria, the pH of the media increases. Due to the presence of the indicator, the media's color changes from purple to yellow.

Inoculum of the pure cultures were transferred to the broth and incubated at 35-37^{0}C for 24 hours and preliminary results were determined. The cultures were incubated an additional 24 hours to obtain the final result.

g) Gelatin hydrolysis test:

The test was performed to determine bacteria's capability to produce gelatinase enzyme and use gelatin as a media source. Degradation of Gelatin indicates the presence of gelatinase enzyme (Aneja *et al.,* 2007). The test organisms were inoculated into sterilized test tubes containing media. One uninoculated gelatin tube was kept as control. The tubes were incubated at 37^{0}C for 24-48 hours. After incubation, the tubes were kept on ice bath for 30 minutes. The solid or liquid condition of the media was noted down afterwards. The liquid form indicates gelatin has been broken down and the result is positive.

h) Catalase test:

The test determines the presence of catalase in microbe which mediates the breakdown of toxic Hydrogen peroxide into Oxygen and water. Bacterial colony was transferred to a glass slide and a drop of 3% Hydrogen Peroxide reagent was added. The positive result is a rapid evolution of bubbles due to the production of Oxygen.

i) Nitrate reduction test:

Nitrate reduction test differentiates microorganisms based on the presence of the enzyme nitrate reductase that hydrolyzes Nitrate to Nitrite, which can then be degraded into various Nitrogen products, depending on the enzyme system of microbe.

Heavy inoculums of isolates were inoculated and incubated (37^{0}C for 24 hours) in Nitrate broth. After incubation, the presence of nitrogen gas is detected in Durham's tube. 6-8 drops of reagent A (sulfanilic acid) was added, followed by 6-8 drops of reagent B (α-naphthylamine). The change of color was observed for a minute.

In presence of Reagent A, Nitrite is converted to nitrate sulfanilic acid, which is converted to red precipitates of protonsil in addition of reagent B. The broth turns red in a positive result and remains colorless in case of a negative result.

j) Oxidase test:

Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase. Both of these catalyze the transport of electrons from donor compounds (NADH) to electron acceptors (usually Oxygen). The test reagent N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochioride acts as an artificial electron acceptor for the enzyme oxidase. The oxidized reagent forms the colored compound indophenol blue.

2-3 drops of reagent were directly added to suspect colonies on agar plate. The color change was observed in 10 seconds.

3.3.4 Identification by Biolog^{TM} System:

Biolog^{TM} System is the latest generation redox chemistry that enables testing and identification of Aerobic Gram negative and Gram positive bacteria in the sample test panel. A simple, one-minute setup protocol is used for each sample, the expanded GEN III database is designed to meet the needs. Biolog^{TM} System's powerful carbon source utilization technology accurately identifies environmental and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" from discrete test reactions performed within a 96 well microplate. Culture suspensions are tested with a panel of pre-selected assays, then incubated, read and compared to extensive databases of environmental organisms, human pathogens, veterinary pathogens and plant pathogens. The scope of 96 assay reactions, coupled with sophisticated interpretation software, delivers a high level of accuracy that is comparable to molecular methods but simpler and faster than DNA sequencing.

Procedure: Before starting, microplates and IF are pre-warmed to room temperature. Organisms were cultured on LB agar media. Single colony was taken after 24 hours (the target cell density must be in the range of 90-98%T). The inoculum was prepared at the desired density. To inoculate the microplate, the cell suspension was poured into the multichannel pipette reservoir. 8 sterile tips were securely added to 8-channel repeating pipette set to the "100-multi" mode. All wells were filled with precisely 100 microliters. Care is required to avoid carrying over one chemical or splash one well into another.

The inoculating fluid forms a soft gel shortly after incubation. The microplates were covered with lid. Finally, the microplate was placed into an incubator for 3 to 36 hours at 33^0C. The

reaction pattern was observed and entered in Biolog^{TM} System machine to determine the result.

3.4 Antibiotic sensitivity test:

The sensitivity of the isolates to different antimicrobial agents was measured *in vitro* by employing the modified Kirby-Bauer. This method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from the formation of the agent into the medium surrounding the disk.

The medium of choice is Mueller Hinton agar (pH 7.3) which was poured into plates to a uniform depth of 5 mm. Commercially available microbial disks were used for the test. The list of antibiotics used for the test is given below.

Table: List of Antibiotics used in antibiotic sensitivity test

Name of Antibiotic	Dose (µg)
Doxycycline (DO)	30
Gentamicin (G)	10
Nalidixic Acid (NA)	30
Nitrofurantoin (F)	30
Amikacin (AK)	30
Neomycin (N)	10
Imipenem (IMP)	10
Ceftazime (CAZ)	30
Metronidazole (M)	30
Ciprofloxacin (CIP)	5

Isolates were inoculated into LB broth and incubated overnight at 37^0C. A sterile cotton swab was dipped into the broth. By pushing and rotating the swab by pushing firmly against the tube, excess fluid was removed from the swab. The swab was used to heavily inoculate over the entire surface of the agar surface to obtain a confluent growth of the organism. Antibiotic disks were applied aseptically to the surface of the inoculated plates at an appropriate distance in a special arrangement with the help of sterile forceps. Plates were then incubated at 37^0C for 24 hours. After incubation, the plates were examined and diameters of the zone of

inhibition were measured in mm. The zone diameters for individual antimicrobial agents were translated into susceptible, intermediate and resistant categories by referring to an interpretation table.

3.5 Antimicrobial activity against pathogens:

Antimicrobial properties of the isolates against various pathogens were tested in the agar well diffusion method (Magaldi *et al.*, 2004).

Pathogenic organisms were inoculated into LB broth and incubated overnight (37^0C). A sterile cotton swab was dipped and by pushing and rotating the swab by pushing firmly against the tube, the excess fluid was removed from the swab. 0.1 mL of diluted inoculum (2 x 10^8 CFU/ml) of pathogenic organism was inoculated on Mueller Hinton agar plates. Wells of 6 mm diameter was punched into the agar and filled with 10 µL of isolate suspension. After 24 hours of incubation at 37^0C, the formation of clear zones was observed and the diameters of the zones were measured in millimeters.

3.6 Influence of physiochemical parameters on the growth of isolates:

3.6.1 Effect of temperature:

To study the effect of temperature on the growth of isolates, organisms were cultured at different temperatures. The growth pattern was observed by measuring absorbance at 600 nm in different time intervals using a spectrophotometer.

Isolated organisms were inoculated into LB broth and incubated overnight (37^0C). Fresh LB broth was transferred to nine sets of Eppendorf tubes for culturing the organisms at nine different temperatures. For each organism, 100 ml of sample suspension was inoculated into 1000 ml fresh broth in an Eppendorf tube from each set. Different sets of Eppendorf tubes were incubated at different temperatures (20^0C, 25^0C, 30^0C, 35^0C, 37^0C, 40^0C, 45^0C, 50^0C, and 55^0C). The control tubes were left uninoculated.

After 24 hours, cell density was determined by measuring absorbance at 600 nm using a spectrophotometer.

3.6.2 Effect of pH:

To study the effect of pH on the growth of isolates, organisms were cultured at different pH. The growth pattern was observed by measuring absorbance at 600 nm in different time intervals using spectrophotometer.

Sample organisms were inoculated in nutrient broth and incubated overnight at 37^0C. Flasks with fresh LB broth containing optimum concentration of substrate and carbon source were taken and the pH was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 in different flasks using HCl (to increase acidity) and KOH (to increase alkalinity). The flasks containing media are sterilized by autoclaving. The cultures were inoculated and incubated at 37^0C for 24 hours. At the end of the incubation period, cell density was determined by measuring absorbance at 600 nm using a spectrophotometer.

3.6.3 Effect of salinity:

To study the effect of saline concentration on the growth of isolates, the organisms were cultured at different saline concentrations. The growth pattern was observed by measuring absorbance at 600 nm in different time intervals using a spectrophotometer.

Sample organisms were inoculated in the nutrient broth and incubated overnight at 37^0C. Flasks with fresh LB broth containing an optimum concentration of substrate and carbon source were taken and the salinity was gradually increased to 1%, 2%, 3%, 4%, 5%, 6%, and 7% in different flasks by adding sodium chloride (NaCl). The flasks containing media were sterilized by autoclaving. The cultures were inoculated in the method described and were incubated at 37^0C for 24 hours. At the end of the incubation period, cell density was determined by measuring absorbance at 600 nm using a spectrophotometer.

3.7 Characterization of α- amylase extracted from isolates

α-Amylase was extracted from the isolates to determine their activity and to characterizeα-amylase by optimizing the temperature and pH. The enzyme was further characterized by observing the its stability in different chemical agents & metallic salts.

3.7.1 Isolation of crude enzyme from bacteria:

Selective media was used for the proper growth of amylase producing microbes and fermentation was carried out in favorable conditions for bacteria to produce amylase and release the enzyme in the extracellular media. The media was collected and centrifuged to

separate supernatant and solid components. The supernatant contained amylase enzyme produced by the bacteria.

3.7.2 Inoculum preparation:

Isolates were grown on LB agar media plates. The plates were incubated overnight at 37^0C. One loop full of organism was inoculated in a conical flask containing 50 ml liquid media prepared for amylase production. The media had following composition: Starch: 10 g/1000ml, Peptone: 10 g/1000ml, Yeast extract: 20 g/1000 ml, KH_2PO_4: 0.05 g/1000ml, $MnCl_2.4H_2O$: 0.015 g/1000ml, $MgSO_4.7H2O$: 0.01g/1000ml, $CaCl_2.2H_2O$: 0.05 g/1000ml, $FeSO_4.7H_20$: 0.01 g/1000ml.

The flasks were incubated in a rotary shaker incubator for 150 rpm at 37°C for 48 hours. After incubation, the fermented broth was centrifuged at 7000 rpm for 15 min in a cooling centrifuge. The supernatant was collected and used for the estimation of amylase.

3.7.3 Assay of enzyme activity:

Amylase activity was assayed following the method described by Jayaraman (1981). One percent starch solution was used as a substrate (1 gm in 100 ml 0.1 M phosphate buffer, pH 6.7). The amylase activity was measured by estimating the release of maltose. One unit of amylase activity was defined as the amount required for liberating 1 mg of maltose in 15 minutes at 37°C. The required reagents are:

1. 0.1 M phosphate buffer (pH 6.7)
2. 1% starch solution in 0.1 M phosphate buffer, pH 6.7
3. 1% NaCl in distilled water
4. 2 N NaOH
5. Dinitrosalisylic acid (DNS)

Sodium sulfite was added to DNS reagent just before use.

3.7.4 Procedure:

Three sets of experiments (Blank, control and sample) were performed for the measurement of amylase activity. The following different solutions were taken in different test tubes:

Table: Composition of DNS test solution

Substance	Blank (ml)	Control (ml)	Sample (ml)
0.1 M Phosphate buffer (pH 6.7)	2.5	2.5	2.5
1% Starch solution	2.5	2.5	2.5
1% NaCl	1.0	1.0	1.0
Distilled water	1.0	0.5	0.5
Enzyme extract	-	0.5	0.5

The contents in the test tube were mixed uniformly and test tubes were incubated in a water bath at 37°C for 10 minutes. Then, 0.5 ml of crude enzyme extract and 0.5 ml distilled water were added to sample and control tubes respectively; whereas 1 ml distilled water was added to blank test tube. Immediately after the addition of crude enzyme extract and distilled water, 0.5 ml of 2N NaOH was added to the control test tube.

The rest of the tubes were incubated at 37°C for 15 minutes and the reaction was then stopped by addition of 0.5 ml of 2N NaOH. Then 0.5 ml of DNS reagent was mixed to all the tubes. The tubes were heated in a water bath for five minutes. After cooling at room temperature, absorbance was measured at 520 nm.

3.8 Effect of environmental parameters on activity of amylase producing isolates:

3.8.1 Determination of temperature stability:

The activities of α- amylase at different temperatures were measured following a similar procedure of amylase activity determination. The incubation temperatures were changed to 10^0C, 20^0C, 30^0C, 37^0C, 40^0C, 45^0C, 50^0C, 60^0C, 70^0C, 80^0C, and 90^0C. The absorbance was measured and documented for calculation of activity.

3.8.2 Determination of optimum pH and pH stability:

The activities of α- amylase at different pH values (2.0-10.0) were measured at 37^0C following a similar procedure of activity determination. The stability of enzymes at various pH was determined by preparing buffers of different pH values.

The compositions of the buffers are given below:

Table: Compositions of the buffers for testing the pH stability of extracted enzymes

Buffer Mixture	pH range
KCl-HCl	2.0-2.5
NaOAc-HCl	3.0-5.5
NaOAc-CH_3COOH	4.5-5.5
NaH_2PO_4- Na_2HPO_4	6.0-8.0
$Na_2B_4O_7$-HCl	8.5-9.0
$Na_2B_4O_7$-Na_2CO_3	9.5-10.0

3.9. Determination of the effect of metallic salts and chemical agents:

3.9.1 Treatment with various metallic salts:

α-Amylase solution (0.5 ml) was mixed with different metallic salts at 20^0C for 10 minutes. The mixture was incubated at 37^0C and the remaining enzyme activity was assayed.

3.9.2 Effect of calcium:

Calcium Chloride (0.5 ml, 0.25-0.30 mg/ml) was added to enzyme solutions and the mixture was kept for at 20^0C 10 minutes. Then it was incubated at 37^0C and the remaining activity was assayed.

3.9.3 Treatment with urea:

α-Amylase solution (0.5 ml) was mixed with different concentrations of urea for 10 minutes at 20^0 C. The mixture was incubated at 37^0C and the remaining activity was assayed.

3.9.4 Treatment with EDTA:

α-Amylase solution (0.5 ml) was mixed with different concentrations of EDTA for 10 minutes at 20^0 C. The mixture was incubated at 37^0C and the remaining activity was assayed.

3.9.5 Treatment with acetic acid:

α-Amylase solution (0.5 ml) was mixed with different concentrations of acetic acid for 10 minutes at 20^0 C. The mixture was incubated at 37^0C and the remaining activity was assayed.

After performing the experiments, the results were compiled and analyzed.

4. Results

4.1 Isolation and selection of α-amylase producer microorganisms from soil sample:

From 23 isolates found in soil samples, the isolates that showed positive results were A-2, A-7, A-13, A-14, A-15, A-16, A-18, A-19, A-20, A-21 (10 isolates in total). Among them, A-16 formed the largest clear zone. Figure 01 shows the result of the starch hydrolysis test.

4.2 Colony and staining characteristics:

The 10 selected isolates were subcultured onto LB agar plates and their morphological features were observed after 24 hours.

Most of the isolated colonies were white in color, irregular in shape and smooth in texture. In the Gram's test, except A-2 and A-19, all strains were positive. Table 01 summarizes the colony characteristics of isolates and Figure 2.2 shows the results of Gram's staining.

Table 01: Colony and staining characteristics of α-amylase producing isolates on LB agar medium

Isolates	Color	Size	Shape	Elevation	Surface texture	Opacity	Gram Test	Microscopic morphology
A2	White	Large	Irregular	Flat	Smooth	Opaque	-ve	Short rods
A7	White	Large	Wavy	Flat	Smooth	Translucent	+ve	Long rods, presence of spore
A13	White	Large	Irregular	Flat	Smooth	Translucent	+ve	Small rod, Chain-like structure, presence of spore
A14	White	Large	Irregular	Flat	Smooth	Opaque	+ve	Small rod, chain-like structure
A15	White	Large	Irregular	Raised	Smooth, Glistening	Opaque	+ve	Medium-sized rods, presence of spore
A16	White	Large	Irregular	Flat	Smooth	Opaque	+ve	Short rods
A18	White	Large	Irregular	Flat	Smooth	Opaque	+ve	Short rods
A19	White	Small	Circular	Convex	Shiny, Smooth	Opaque	-ve	Short rods
A20	White	Small	Circular	Convex	Smooth	Opaque	+ve	Long rods
A21	White	Large	Irregular	Raised	Smooth	Opaque	+ve	Thin and chained rods

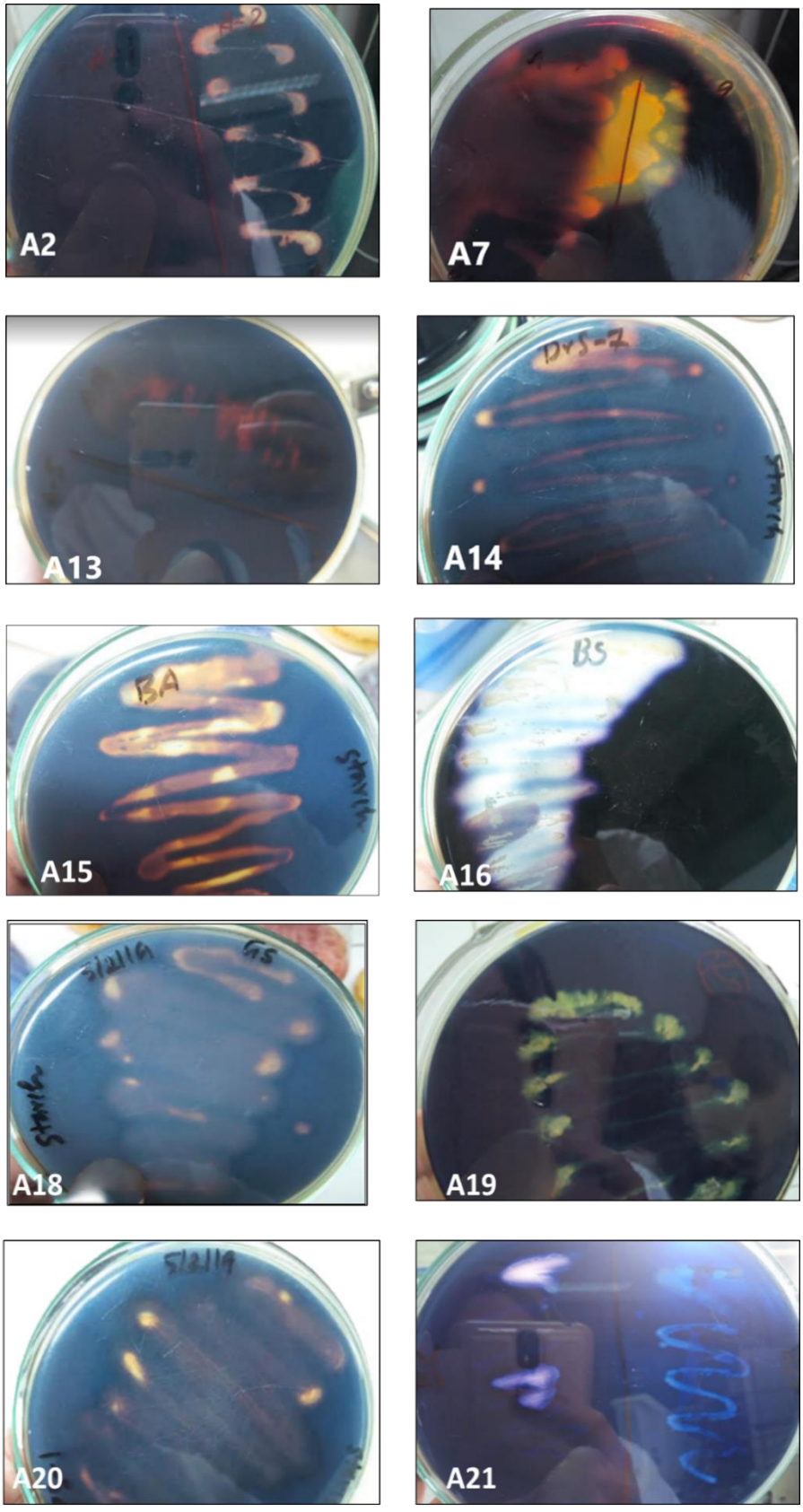
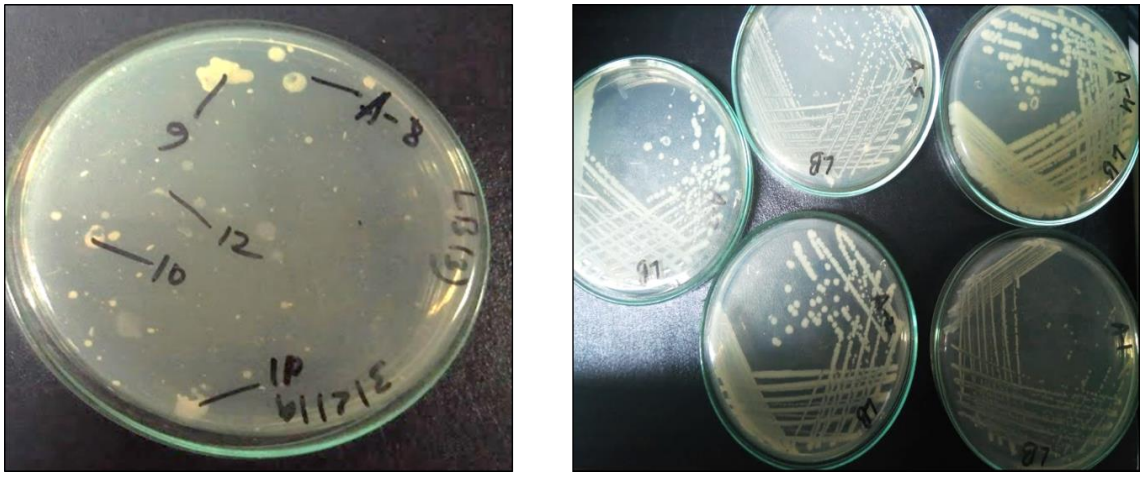
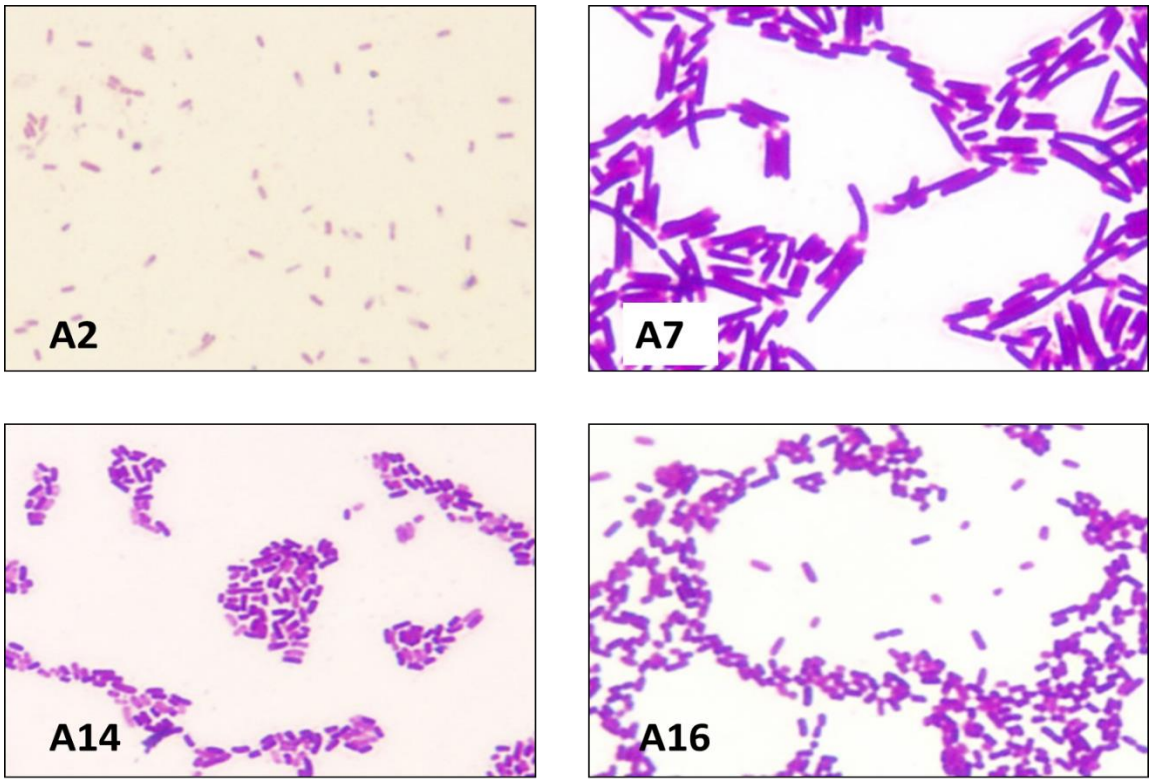


Figure 01: Starch hydrolysis test of the isolates.



(2.1) Isolation ofα-amylase producer microorganisms



(2.2) Microscopic observation of isolated microorganisms.
A-7, A-14 and A-16 are gram-positive and A-2 is gram-negative.

Figure 2: Isolation and microscopic observation of α-amylase producing isolates.

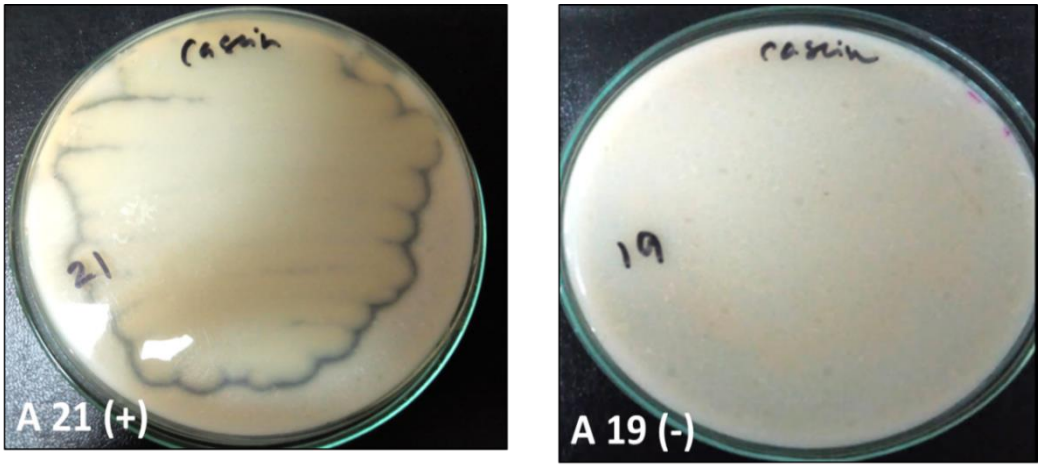
4.3 Biochemical tests:

For identification and analysis of biochemical characteristics, numerous biochemical tests (casein hydrolysis test, egg yolk agar hydrolysis test, motility test, methyl red test, lysine decarboxylase test, gelatin liquefaction test, catalase test, nitrate reduction test, oxidase test and endospore staining) were carried out for the 10 selected isolates. Comparing the result to the standard biochemical results, possible organisms were interpreted. All isolates gave positive result in the nitrate test. Except A-19, all isolates were motile and displayed presence of spores in the endospore staining. Majority of the isolates gave positive result in the oxidase test and the citrate test. Negative results were more frequent in the methyl red test. It was interpreted that the majority of the test isolates belonged to *Bacillus* spp. The isolates were later identified using the Biolog^{TM} system.

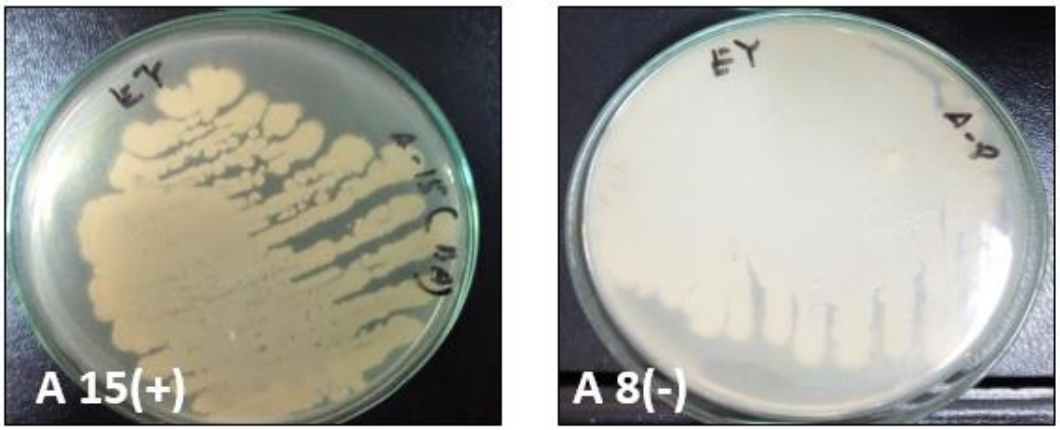
Table 02 and Figure 03 show the results of biochemical tests.

Table 02: Biochemical characteristics of isolates

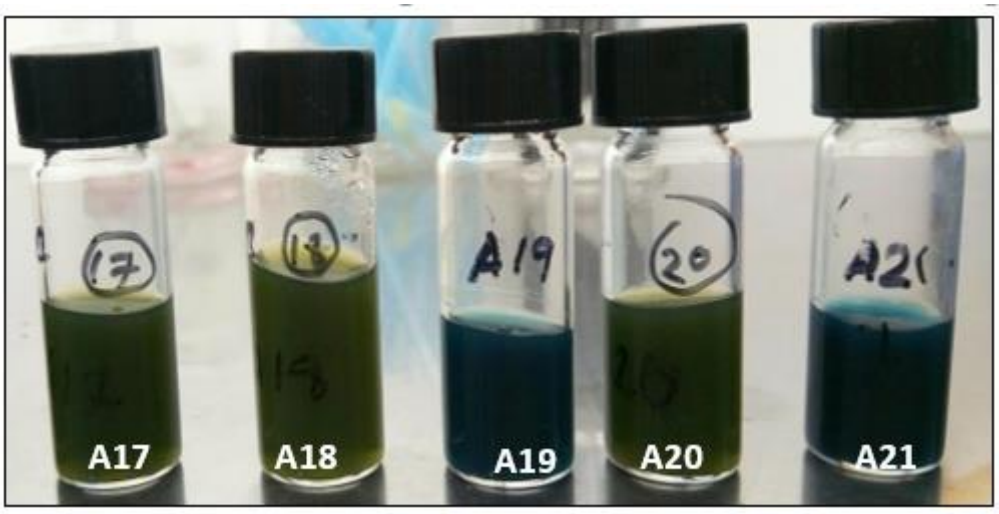
Isolates	Casein Hydrolysis	Egg Yolk agar	Motility	Citrate	Methyl red	Lysine	Gelatin	Catalase	Nitrate	Oxidase	Endo-spore	Possible Organism Interpretation
A2	-	+	+	+	+	+	+	-	+	+	+	*Raoultella planticola*
A7	+	-	+	+	-	+	-	-	+	+	+	*Bacillus* sp.
A13	+	-	+	+	-	-	-	+	+	+	+	*Bacillus* sp.
A14	+	-	+	+	-	+	+	+	+	+	+	*Bacillus* sp.
A15	+	+	+	+	-	+	+	+	+	+	+	*Bacillus* sp.
A16	+	-	+	+	-	+	-	-	+	+	+	*Bacillus* sp.
A18	+	+	+	-	+	+	+	-	+	+	+	*Solibacillus sp.*
A19	-	+	-	+	-	+	-	+	+	-	-	*Klebsiella oxytoca*
A20	+	-	+	-	-	+	-	-	+	+	+	*Bacillus* sp.
A21	+	+	+	+	-	+	-	-	+	-	+	*Bacillus cereus*



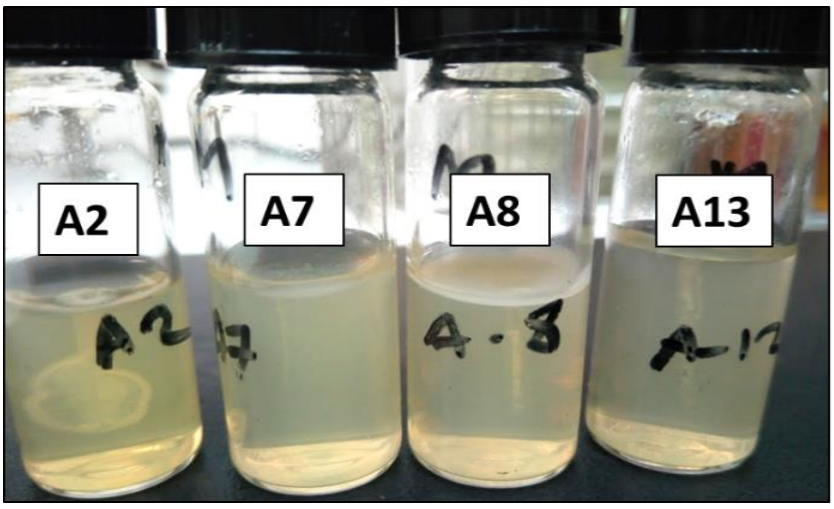
(3. 1) Casein hydrolysis test



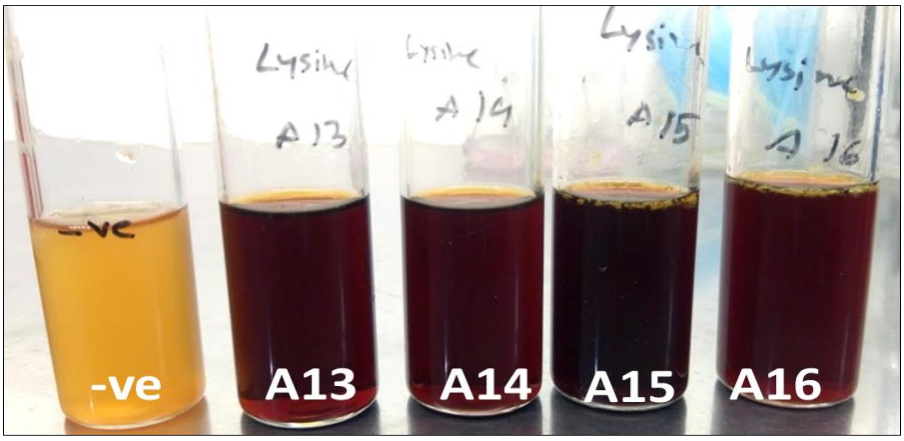
(3.2) Egg yolk agar hydrolysis test



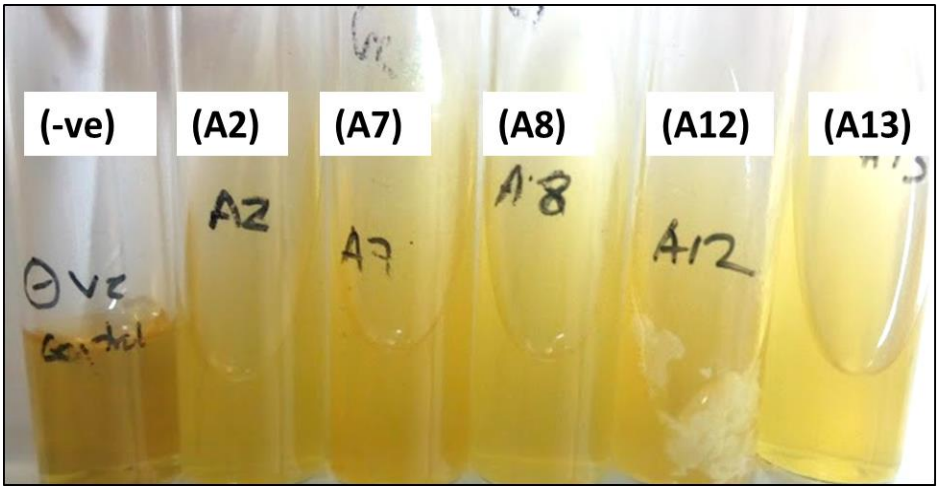
(3.3) Citrate utilization test



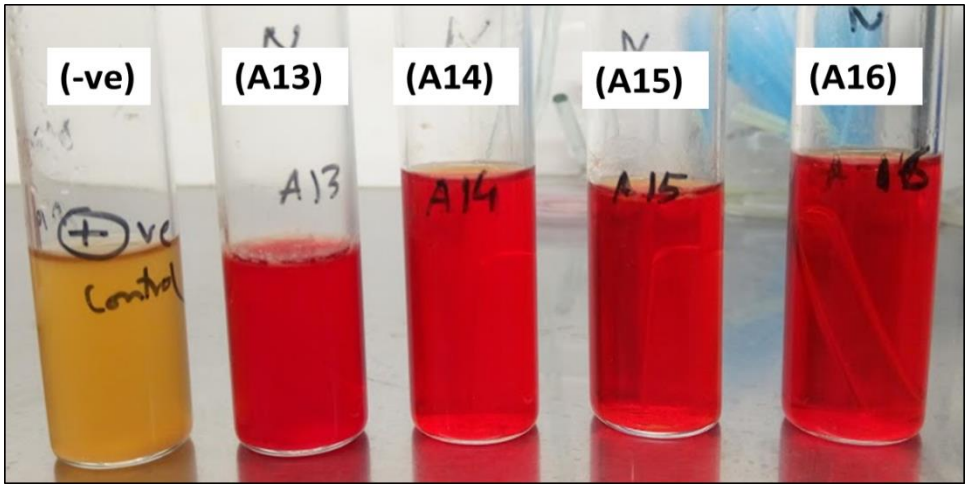
(3.4) Motility test



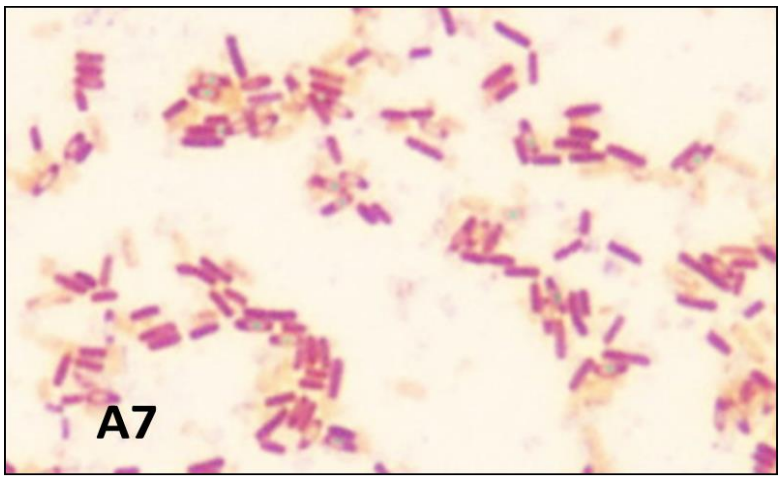
(3.5) Lysine decarboxylase test



(3.6) Gelatin liquefaction test



(3.7) Nitrate reduction test



(3.8) Endospore staining

Figure 3: Biochemical test results of α-amylase producing isolates.
Majority of the isolates were endospore forming, casein hydrolysis positive, citrate positive, nitrate reduction positive, motility positive and gelatin liquefaction positive. Comparing the results of the biochemical tests to the standard chart, it was assumed that majority of the test isolates belonged to *Bacillus* sp.

4.4. Identification of isolates by Biolog™ System:

The selected isolates were identified using Biolog™ System. Fresh culture suspension was inoculated into Biolog™ microplate and incubated at 33^0C for 36 hours. The reaction pattern was observed and ID results were obtained using Biolog™ Microplate reader.

According to ID results, all isolates except A-2, A-18 and A-19 belonged to *Bacillus* spp.

A-2, A-18 and A-19 were identified to be *Raoultella planticola, Solibacillus silvestris* and *Klebsiella oxytoca,* respectively. The identified isolates are listed in Table 03.

Table 03: Identification of α-amylase producingisolates by Biolog™ System

Isolated organism	Name of Microorganism
A2	*Raoultellaplanticola*
A7	*Bacillus cibi*
A13	*Bacillus subtilis*
A14	*Bacillus sporothermodurans*
A15	*Bacillus amyloliquefaciens* sp. *amyloliquefaciens*
A16	*Bacillus subtilis*
A18	*Solibacillus silvestris*
A19	*Klebsiella oxytoca*
A20	*Bacillus vallismortis*
A21	*Bacillus cereus*

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	19.02.19.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	A2
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Feb 19 2019 9:51 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

Result	Species ID: Raoultella planticola
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.589	0.589	5.955	GN-Ent	Raoultella planticola/ornithinolytica
2	0.145	0.145	6.698	GN-Ent	Klebsiella oxytoca
3	0.074	0.074	7.515	GN-Ent	Klebsiella pneumoniae ss ozaenae
4	0.072	0.072	7.571	GN-Ent	Enterobacter aerogenes (Kleb. mobilis)

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	127	< 236	{ 182	{ 207	{ 208	< 229	{ 210	{ 174	< 225	< 306	< 294	< 256
B	{ 178	{ 166	{ 196	{ 196	{ 172	< 258	< 235	< 230	< 234	< 292	< 260	{ 204
C	{ 194	{ 203	< 235	{ 215	{ 213	< 227 -	< 256	{ 209	< 243	< 303	< 276	{ 250
D	{ 207	{ 170	< 235	{ 185	< 254	< 263	< 266	138	< 264	< 298	< 310	88
E	12	< 243	< 251	{ 169	{ 208	< 246	< 224	{ 187 -	< 248	< 272	< 281	< 279
F	46	< 251	< 257	< 252	< 251	< 246 -	< 263	{ 149	< 273	< 305	< 309	< 317
G	10 +	{ 164	{ 162	< 261	< 269	141	{ 143 +	< 261	{ 211 -	151	< 272	{ 170
H	10	84	107	74	98	{ 156	96	{ 184	67	< 256	{ 216	77

Report Date Feb 19 2019 9:51 AM

Figure 4.1: Identification of A-2 *(Raoultella planticola)*

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	19.02.19.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	A7
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Feb 19 2019 9:55 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

Result	Species ID: Bacillus cibi
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.575	0.575	6.248	GP-Rod-SB	Bacillus cibi
2	0.140	0.140	6.370	GP-Rod-SB	Bacillus endophyticus
3	0.138	0.138	6.376	GP-Rod-SB	Bacillus mojavensis/subtilis
4	0.120	0.120	6.459	GP-Rod-SB	Bacillus subtilis ss spizizenii

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, (x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	191 +	< 268	< 262	< 261	< 263	< 266	< 260	< 263	< 253	< 262	(209	12
B	(233	< 254	< 257	< 262	< 259	< 267	< 254	(227	(233	< 240	< 223	27 +
C	< 268	< 270 -	< 265	< 249	(240	< 245	(241	(240	< 254	< 268	51	46
D	< 266	< 267	(239	< 256	< 252	(242	(241	(243	91	43	48	52
E	< 259	< 254	< 254	< 251	< 259	< 269	< 254	< 255	< 249	49	< 228 -	46
F	< 252	< 257	< 254	< 262	< 250	(219	< 253	(234	< 257	47	109	90
G	180	< 255	< 249	< 263	< 267	< 245	115 +	< 260	< 252	47	< 243	< 255
H	(210	< 258	(200	(215	120	< 252	178 +	< 255	< 257	87	71	111

Report Date Feb 19 2019 9:55 AM

Figure 4.2: Identification of A-7 (*Bacillus cibi)*

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	san17-1018.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	BS
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Oct 17 2018 11:02 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

A13

Result	Species ID: Bacillus subtilis
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	---	0.574	4.948	GP-Rod-SB	Bacillus subtilis ss subtilis
2	---	0.082	6.029	GP-Rod-SB	Bacillus subtilis ss spizizenii
3	---	0.046	6.374	GP-Rod-SB	Bacillus mojavensis/subtilis
4	---	0.013	7.150	GP-Rod-SB	Bacillus atrophaeus/subtilis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	101	< 257	< 244	< 247	< 254	< 259	< 245	< 231	{ 182	< 280	< 273	< 243
B	< 206	109	< 219	< 260	< 258	< 208	{ 151	110	104	< 270	< 272	{ 194
C	< 205	< 246	< 242	122	{ 131	{ 142	{ 138	{ 184	{ 129	< 287	49	50
D	< 250	< 232	85	{ 182	{ 188	115	{ 165	{ 156	32	52	< 250 -	65
E	{ 156	100 +	< 252	< 237	< 264	< 263	< 249	{ 137	< 218	55	47	53
F	{ 147 +	< 253	< 225 -	< 246	{ 158	{ 149	{ 145 +	104	< 223	57	{ 115	87
G	71	< 224	101	< 254	< 254	{ 151	87	< 275	< 228	57	< 267	< 282
H	125	120	73	104	71	< 242	117	< 210	{ 187	{ 103	< 271	{ 211

Figure 4.3: Identification of A-13 *(Bacillus subtilis)*

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	29.01.19.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	44s
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Jan 29 2019 4:22 PM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

A19

Result	Species ID: Bacillus sporothermodurans
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.704	0.704	4.250	GP-Rod-SB	Bacillus sporothermodurans
2	0.147	0.147	4.751	GP-Rod	Lactobacillus oris
3	0.143	0.143	4.776	GP-Rod	Lactobacillus parabuchneri
4	0.129	0.129	4.868	GP-Rod	Lactobacillus vaginalis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, (x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	27	(45	< 199	26	27	38	23	28	23	< 194	< 167	48
B	26	23	35	22	25	23	25	23	21	< 177	51	51
C	< 115	41	31	31	34	(45	(53	41	22	< 172	51	(130
D	23	21	27	23	23	< 120 -	(68	23	22	48	52	58
E	25	22	23 +	22	23	25	31	25	22	55	49	52
F	31	31	38	< 109	35	(68	26	22	22	< 187	(110	(84
G	26	25	23	23	22	25	28	23	23	< 177	43	(117 -
H	23 +	23	23	40	26	34	27	28	25	< 181	57	53

Report Date Jan 29 2019 4:22 PM

Figure 4.4: Identification of A-14 *(Bacillus sporothermodurans)*

A15

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	supplement cheese lemon.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	BC-2
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Jan 29 2019 11:41 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

Result	Species ID: Bacillus amyloliquefaciens ss amyloliquefaciens
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.646	0.646	5.056	GP-Rod-SB	Bacillus amyloliquefaciens ss amyloliquefaciens
2	0.132	0.132	5.635	GP-Rod-SB	Bacillus mojavensis/subtilis
3	0.107	0.107	5.762	GP-Rod-SB	Bacillus subtilis ss subtilis
4	0.043	0.043	6.308	GP-Rod-SB	Bacillus vallismortis/subtilis

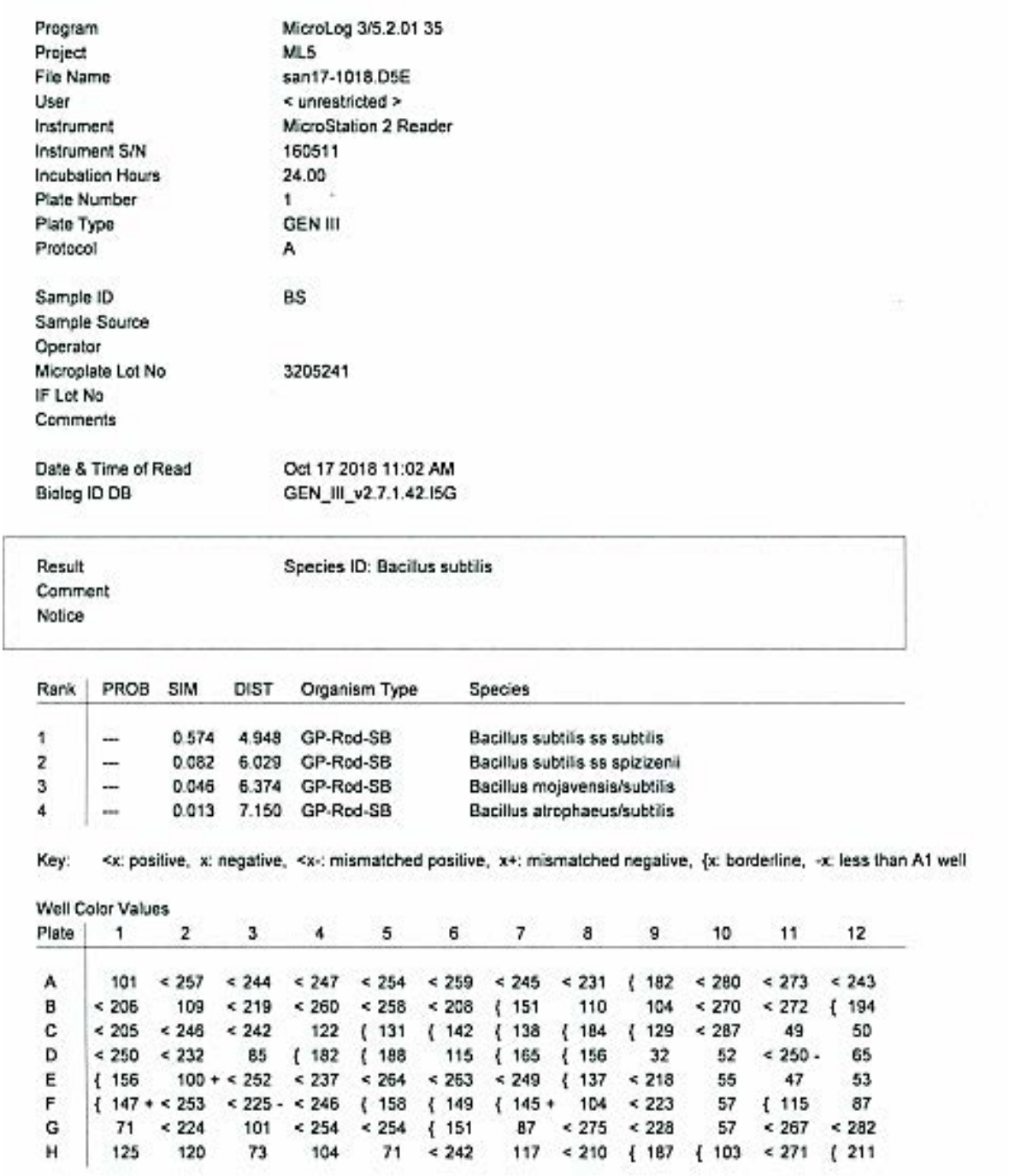
Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	109	< 225	< 242	< 251	< 255	< 263	< 253	< 248	{ 192	< 285	< 271	< 245
B	{ 213	< 254	{ 216	< 259	< 253	< 266	< 218	{ 166	140	< 292	< 275	< 261
C	< 245	< 248	< 253	{ 200	{ 175	{ 188	{ 163	{ 152	< 227	< 300	52	{ 120
D	< 253	< 243	{ 183	< 249 -	< 244	{ 172	{ 208	< 223	128	48	54	57
E	< 225	< 223	< 254	< 254	< 268	< 275	< 258	< 250	< 238	54	< 273	52
F	< 235	< 235	{ 205 -	< 256	136	{ 163	{ 182	< 224 -	{ 161	51	{ 115	86
G	117	< 245	{ 163	< 255	< 256	{ 180	66 +	< 261	< 233	50	< 293	< 279
H	129 +	{ 174	63	138	54	< 245	97	< 233	< 247	96	< 257	92

Report Date Jan 29 2019 11:42 AM

Figure 4.5: Identification of A-15 (*Bacillus amyloliquefaciens)*



Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	san17-1018.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	BS
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Oct 17 2018 11:02 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

Result	Species ID: Bacillus subtilis
Comment	
Notice	

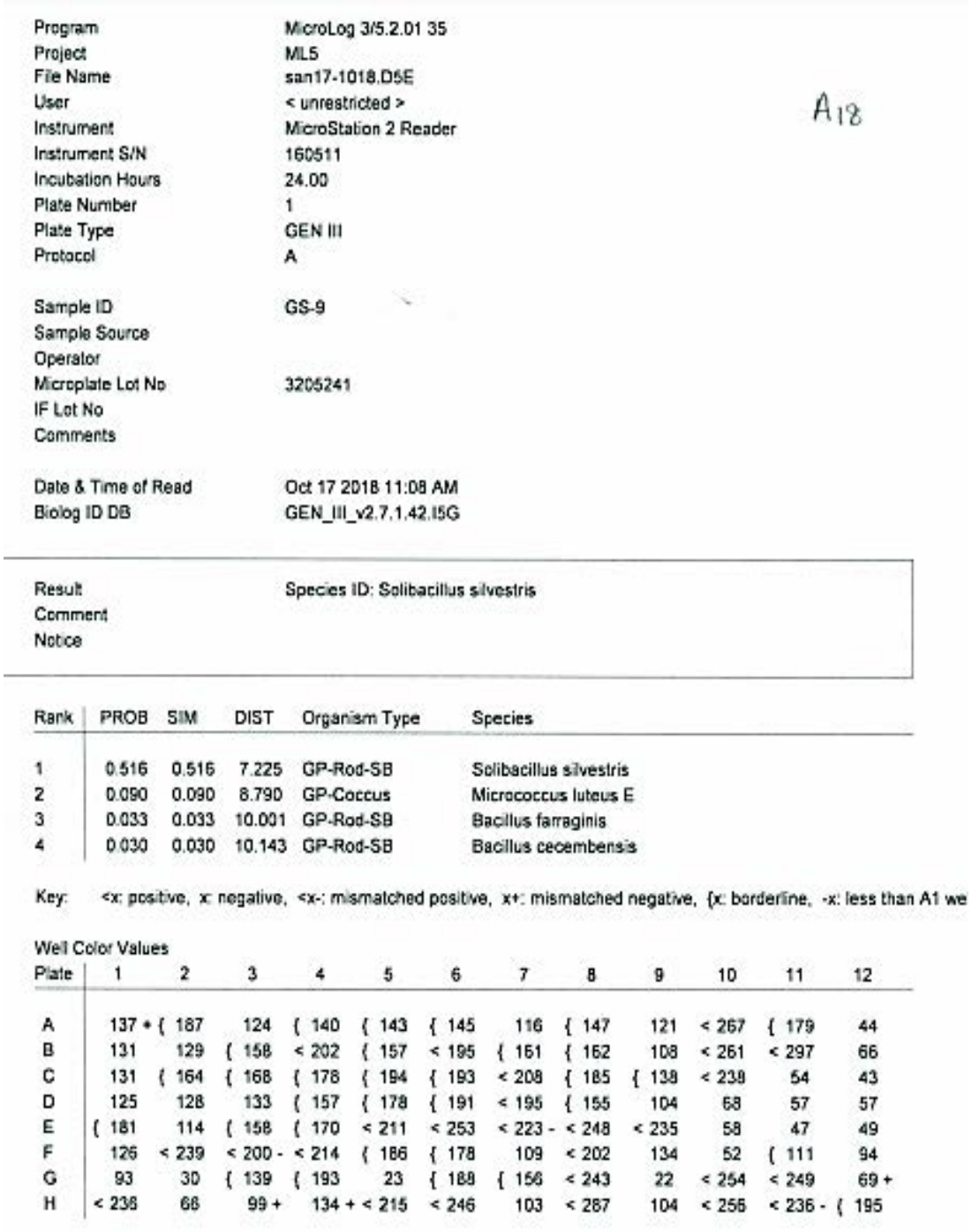
Rank	PROB	SIM	DIST	Organism Type	Species
1	---	0.574	4.948	GP-Rod-SB	Bacillus subtilis ss subtilis
2	---	0.082	6.029	GP-Rod-SB	Bacillus subtilis ss spizizenii
3	---	0.046	6.374	GP-Rod-SB	Bacillus mojavensis/subtilis
4	---	0.013	7.150	GP-Rod-SB	Bacillus atrophaeus/subtilis

Key: <x: positive, x: negative, <x+: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	101	< 257	< 244	< 247	< 254	< 259	< 245	< 231	{ 182	< 280	< 273	< 243
B	< 206	109	< 219	< 260	< 258	< 208	{ 151	110	104	< 270	< 272	{ 194
C	< 205	< 248	< 242	122	{ 131	{ 142	{ 138	{ 184	{ 129	< 287	49	50
D	< 250	< 232	85	{ 182	{ 188	115	{ 165	{ 156	32	52	< 250 -	65
E	{ 156	100 +	< 252	< 237	< 264	< 253	< 249	{ 137	< 218	55	47	53
F	{ 147 +	< 253	< 225 -	< 246	{ 158	{ 149	{ 145 +	104	< 223	57	{ 115	87
G	71	< 224	101	< 254	< 254	{ 151	87	< 275	< 228	57	< 267	< 282
H	125	120	73	104	71	< 242	117	< 210	{ 187	{ 103	< 271	{ 211

Figure 4.6: Identification of A-16 (*Bacillus subtilis)*



Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	san17-1018.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	GS-9
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Oct 17 2018 11:08 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

A18

Result	Species ID: Solibacillus silvestris
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.516	0.516	7.225	GP-Rod-SB	Solibacillus silvestris
2	0.090	0.090	8.790	GP-Coccus	Micrococcus luteus E
3	0.033	0.033	10.001	GP-Rod-SB	Bacillus farraginis
4	0.030	0.030	10.143	GP-Rod-SB	Bacillus cecembensis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 we

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	137 +	{ 187	124	{ 140	{ 143	{ 145	116	{ 147	121	< 267	{ 179	44
B	131	129	{ 158	< 202	{ 157	< 195	{ 161	{ 162	108	< 261	< 297	66
C	131	{ 164	{ 168	{ 178	{ 194	{ 193	< 208	{ 185	{ 138	< 239	54	43
D	125	128	133	{ 157	{ 178	{ 191	< 195	{ 155	104	68	57	57
E	{ 181	114	{ 158	{ 170	< 211	< 253	< 223 -	< 248	< 235	58	47	49
F	126	< 239	< 200 -	< 214	{ 186	{ 178	109	< 202	134	52	{ 111	94
G	93	30	{ 139	{ 193	23	{ 188	{ 156	< 243	22	< 254	< 249	69 +
H	< 238	68	99 +	134 +	< 215	< 246	103	< 287	104	< 256	< 236 -	{ 195

Figure 4.7: Identification of A-18 (*Solibacillus silvestris)*

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	san17-1018.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	PR-9
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Oct 17 2018 11:07 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

A19

Result	Species ID: Klebsiella oxytoca
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.653	0.653	4.999	GN-Ent	Klebsiella oxytoca
2	0.101	0.101	6.125	GN-Ent	Raoultella planticola/ornithinolytica
3	0.078	0.078	6.349	GN-Ent	Klebsiella variicola
4	0.073	0.073	6.396	GN-Ent	Klebsiella pneumoniae ss pneumoniae

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 w

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	95	{ 189	< 254	< 248	< 245	< 239	< 256	{ 203	< 253	< 268	< 284	< 260
B	< 259	< 223	< 245	< 247	< 258	< 281	{ 156	93	86 +	< 280	< 270	< 287
C	< 241	< 243	< 235	< 250	121 +	{ 162	< 219	< 241	< 249	< 298	< 254	53 +
D	{ 142 +	< 252	< 234	{ 196	{ 204	< 266	< 268	92	< 259	< 288	< 291	61
E	120	< 235	< 253	< 250	< 236	< 246	< 252	92	< 220	< 282	< 288	< 317
F	{ 203	< 258	< 238	< 261	< 259	{ 155	< 240	< 244	< 234	< 281	< 297	< 321
G	< 247	{ 184	94	< 255	< 245	79	98	< 252	{ 176	60	{ 250	57
H	60	< 267	87	{ 132	54	101	66	121 +	120	{ 196	< 264	98

Figure 4.8: Identification of A-19 *(Klebsiella oxytoca)*

A20

(1)

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	mojahid 25.09.18.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	An 1
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Sep 25 2018 11:14 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

Result	Species ID: Bacillus vallismortis/subtilis
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.535	0.535	6.835	GP-Rod-SB	Bacillus vallismortis/subtilis
2	0.165	0.165	6.940	GP-Rod-SB	Bacillus subtilis ss subtilis
3	0.101	0.101	7.541	GP-Rod-SB	Bacillus amyloliquefaciens ss amyloliquefaciens
4	0.075	0.075	7.920	GP-Rod-SB	Bacillus mojavensis/subtilis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	122	< 252	8 +	{ 172	< 253	< 256	< 253	< 253	< 243 -	< 294	< 259	73
B	{ 189	147	< 243	< 254	< 253	< 250	{ 198	136	< 226 -	< 290	< 289	< 269
C	< 241	< 248	< 251	{ 179	{ 183	{ 182	{ 184	{ 166 +	< 234	< 305	56	58
D	{ 189	< 250	{ 171	< 249	< 244	{ 177	{ 197	< 221	146	44	61	77
E	< 229	{ 181	< 239	< 247	< 257	< 254	{ 177	{ 168	< 247	55	{ 178 -	49
F	< 228	{ 180	{ 201 -	< 253	< 225	{ 168	{ 176	{ 161	< 250	55	{ 131	{ 102
G	132	< 246	< 233	< 256	< 258	{ 204	{ 181	< 279	< 243	64	< 247	< 278
H	{ 167	{ 176	118	138	73	< 244	{ 158	< 247	< 242	82 +	{ 98	{ 121

Figure 4.9: Identification of A-20 (*Bacillus vallismortis)*

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	mojahid 25.09.18.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	An 3
Sample Source	
Operator	
Microplate Lot No	3205241
IF Lot No	
Comments	
Date & Time of Read	Sep 25 2018 11:18 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G

Result	Species ID: Bacillus cereus
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.781	0.510	5.040	GP-Rod-SB	Bacillus cereus
2	0.107	0.061	6.314	GP-Rod-SB	Bacillus thuringiensis/cereus
3	0.068	0.038	6.601	GP-Rod-SB	Bacillus weihenstephanensis/cereus
4	0.044	0.023	6.891	GP-Rod-SB	Bacillus mycoides/cereus

Key: <x: positive, x: negative, <x+: mismatched positive, x+: mismatched negative, (x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	196 +	< 235	< 241	< 243	(220	(204	< 242	(215	(210	< 245	< 255	42
B	179	(204	(204	< 245	< 232	< 251	(217	193	(220	< 261	< 261	< 252
C	< 236	196	< 245	(200	(219	(219	< 230	(213	< 238	< 254	48	< 244
D	(204	(210	(224 -	< 240	< 242	< 246	< 248	170	< 230	44	51	54
E	< 243	< 232 -	< 241	< 238	< 244	< 246	< 248	< 234	< 243	50	< 257	48
F	< 231	< 228	< 229 -	< 243	< 231	(202	< 237	< 236	< 235	54	118	91
G	163	< 234	< 230	< 243	< 236	< 236	< 231	< 247	< 240	104	< 252	< 249
H	(213	(213	(220	(204 +	(217	< 243	< 240	< 244	< 248	< 240	< 280	(144

Figure 4.10: Identification of A-21 (*Bacillus cereus)*

Figure 04: Identification of α-amylase producer isolates by Biolog^{TM} System.

4.5 Antibiotic susceptibility test of the isolates (KB method):

The antibiotic susceptibility of isolates was determined by the Kirby Bauer method.

The zone of inhibition corresponds to a microorganisms' sensitivity to antibiotics. Larger and clear zones indicate that the test microorganism is sensitive to the antibiotics. Conversely, smaller and cloudy zones are indicative of test microorganism's resistance. Ten different antibiotics were used for the test and susceptibility to the antibiotics varied among isolates. Most of the applied antibiotics formed clear zones of inhibition on culture plates. The diameter of the zone of inhibition was measured in millimeters.

The majority of the isolates displayed resistance against imipenem, ceftazidime and metronidazole. Eight out of 10 isolates were resistant to ceftazidime, 7 were resistant to imipenem and almost all isolates were resistant to metronidazone. Conversely, almost all isolates were sensitive to doxycycline, gentamicin, nalidixic acid, amikacin, and ciprofloaxin; the last two caused formation of the largest clear zones (diameter: 26 mm).

A-2 *(R. planticola)* was the most resistant to antibiotics (resistant to 8 out of 10 antibiotics). A-13 (*B. subtilis)* and A-16 (*B. subtilis*) were the most sensitive and only showed resistance against neomycin, ceftazidime and metronidazole.

Figure 05 and Table 04 illustrate the results of the antibiotic susceptibility test.

Table 04: Effect of antibiotics on α-amylase producing isolates (Kirby-Bauer Test)

Test isolates	Zone of inhibition (in millimeters)									
	Doxycycline	**Gentamicin**	**Nalidixicacid**	**Nitrofurantonin**	**Amikacin**	**Neomycin**	**Imipenem**	**Ceftazidime**	**Metronidazole**	**Ciprofloxacin**
A2	11.1 R	12.0 R	19.3 S	0.0 R	12.4 R	0.0 R	0.0 R	0.0 R	0.0 R	20.2 S
A7	25.0 S	27.0 S	26.2 S	18.3 S	24.1 S	12.0 R	0.0 R	0.0 R	0.0 R	23.0 S
A13	26.3 S	24.3 S	23.1 S	14.0 I	26.0 S	10.0 R	15.4 I	10.0 R	0.0 R	22.2 S
A14	27.0 S	21.2 S	22.0 S	13.4 R	21.5 S	14.1 I	0.0 R	16.3 I	13.5 I	23.2 S
A15	23.0 S	21.1 S	24.5 S	12.0 R	20.0 S	09.5 R	0.0 R	0.0 R	0.0 R	19.0 S
A16	25.2 S	19.0 S	21.2 S	14.1 I	17.3 S	12.0 R	14.4 I	10.0 R	0.0 R	23.2 S
A18	15.0 I	24.0 S	26.2 S	12.0 R	22.0 S	0.0 R	16.2 S	16.1 I	0.0 R	26.0 S
A19	15.1 I	14.0 I	17.1 I	0.0 R	15.0 S	11.0 R	0.0 R	0.0 R	0.0 R	19.0 S
A20	20.0 S	22.2 S	20.0 S	10.0 R	25.1 S	15.4 R	0.0 R	0.0 R	08.4 R	26.2 S
A21	21.0 S	25.1 S	21.5 S	16.1 I	26.3 S	08.5 R	0.0 R	13.0 R	0.0 R	17.0 I

All zone of inhibition was measured in millimeter. The results were interpreted in accordance to Performance Standards for Antimicrobial Disk Susceptibility Test. “S”=Sensitive, “I”=intermediate, and “R”=Resistant.

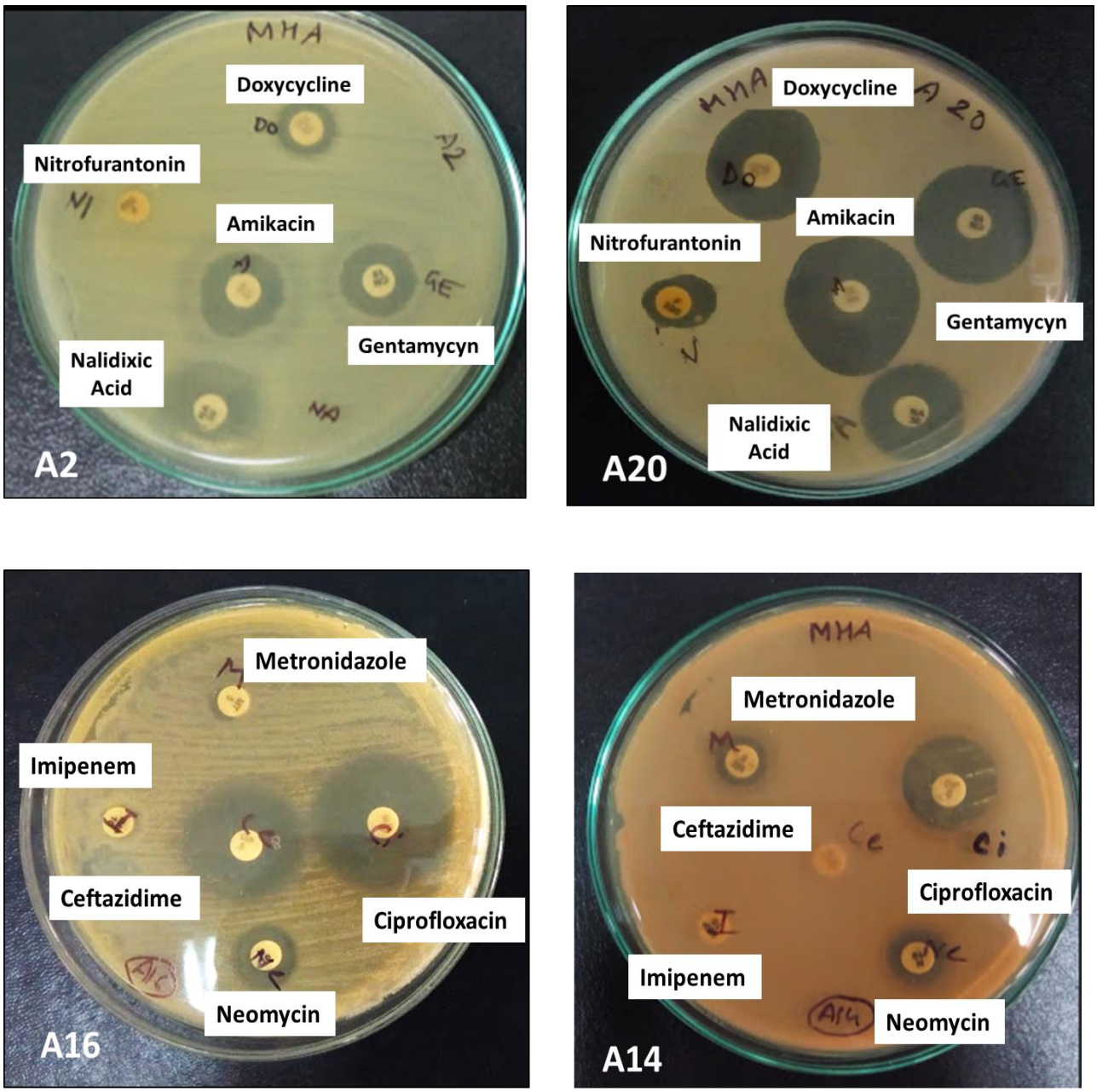


Figure 05: Effect of Antibiotics on α-Amylase Producing Isolates (Kirby-Bauer Test)

The figure shows antibiotic susceptibility of *R. planticola* (A-2), *B. vallismortis* (A-20), *B. subtilis* (A-16), and *B. sporothermodurans* (A-14).

4.6 Antimicrobial activity of α-amylase producing isolates against pathogens:

The isolates displayed antimicrobial activity against various pathogens in well diffusion method. The zone of inhibition corresponds to the degree of antimicrobial activity of the isolated microorganisms. Zone diameters were measured in millimeters and strains with zones having diameter smaller than 12 mm were considered to be resistant to the isolate.

Sixteen different pathogenic strains were used to test the antimicrobial activity of isolates. The degree of antimicrobial activity varied among isolates. It was observed that 14 out of 16 pathogens were sensitive to the culture suspension of test isolate A-7 (*B. cibi)*; which is indicative of the test isolate's strong antimicrobial activity. A-13 (*B. subtilis)* and A-16 (*B. subtilis)* formed the largest clear zones (~28 mm zone diameter against *Trichosporum)*, implying their strong antimicrobial activity. Conversely, only 4 out of 16 pathogen strains were sensitive to the culture suspension of A-19 *(K. oxytoca)*.

Figure 06 and Table 05 summarize the results of antimicrobial activity of isolates against pathogens.

Table 05: Antimicrobial activities of α-amylase producing isolates against pathogens

Pathogenic Strains	Zone of inhibition (in millimeters)									
	Test isolates									
	A2	A7	A13	A14	A15	A16	A18	A19	A20	A21
Listeria monocytogenes	0.00 ®	16.1 S	13.5 S	15.4 S	17.6 S	13.1 S	15.2 S	0.00 ®	10.1 ®	11.3 ®
Bacillus subtilis	0.00 ®	12.6 S	12.6 S	14.2 S	15.4 S	12.5 S	12.6 S	0.00 ®	11.5 ®	12.3 S
Bacillus thuringiensis	0.00 ®	12.3 S	13.1 S	13.4 S	16.4 S	14.0 S	12.8 S	0.00 ®	11.3 ®	12.2 S
Pseudomonas aeruginosa	08.4 ®	08.5 ®	09.0 ®	09.4 ®	14.5 S	10.0 ®	15.6 S	0.00 ®	15.1 S	12.4 S
Vibrio parahaemolyticus	09.5 ®	12.0 S	13.3 S	10.0 ®	09.3 ®	14.0 S	12.5 S	08.5 ®	09.10 ®	10.0 ®
Salmonella typhi	13.0 S	12.3 S	10.6 ®	15.4 S	15.6 S	10.1 ®	15.4 S	10.1 ®	10.0 ®	09.8 ®
Salmonella enteritis	13.2 S	24.4 S	16.8 S	12.9 S	14.6 S	17.2 S	23.4 S	0.00 ®	11.0 ®	12.1 S
Enterobacter sp.	08.4 ®	21.0 S	18.0 S	15.6 S	17.8 S	17.5 S	17.7 S	0.00 ®	12.1 ®	08.3 ®
Bacillus cereus	0.00 ®	12.8 S	22.8 S	15.4 S	25.4 S	25.0 S	10.0 ®	0.00 ®	10.1 ®	0.00 ®
Klebsiella pneumonia	10.0 S	12.7 S	17.2 S	11.1 ®	18.8 S	17.1 S	14.3 S	08.1 ®	08.2 ®	0.00 ®
Bacillus sphaericus	12.3 I	17.8 S	12.9 S	10.0 ®	17.8	11.0 ®	13.2	0.00 ®	0.00 ®	09.5
Staphylococcus aureus	09.7 ®	10.1 ®	12.8 S	12.4 S	18.9 S	12.6 S	10.0 ®	0.00 ®	12.2 S	0.00 ®
Enterobacter galinia	18.6 S	21.0 S	21.5 S	21.5 S	26.6 S	21.2 S	17.8 S	15.5 S	13.4 S	16.7 S
Campylobacter sp.	16.5 S	27.8 S	20.5 S	18.9 S	17.5 S	19.6 S	23.2 S	17.3 S	17.8 S	20.0 S
Endomyces fibuliger	20.0 S	17.8 S	05.7 ®	15.6 S	13.4 S	0.00 ®	15.4 S	13.2 S	0.00 ®	0.00 ®
Trichosporum beigelli	13.5 S	20.0 S	27.8 S	17.8 S	21.0 S	27.9 S	20.0 S	17.7 S	21.0 S	15.0 S

Zone of inhibition was measured in millimeter. "®" = Resistant to the test isolate (0.00 to <12.00 mm zone diameter), "S"= sensitive to the test isolate (≥12 mm zone diameter)

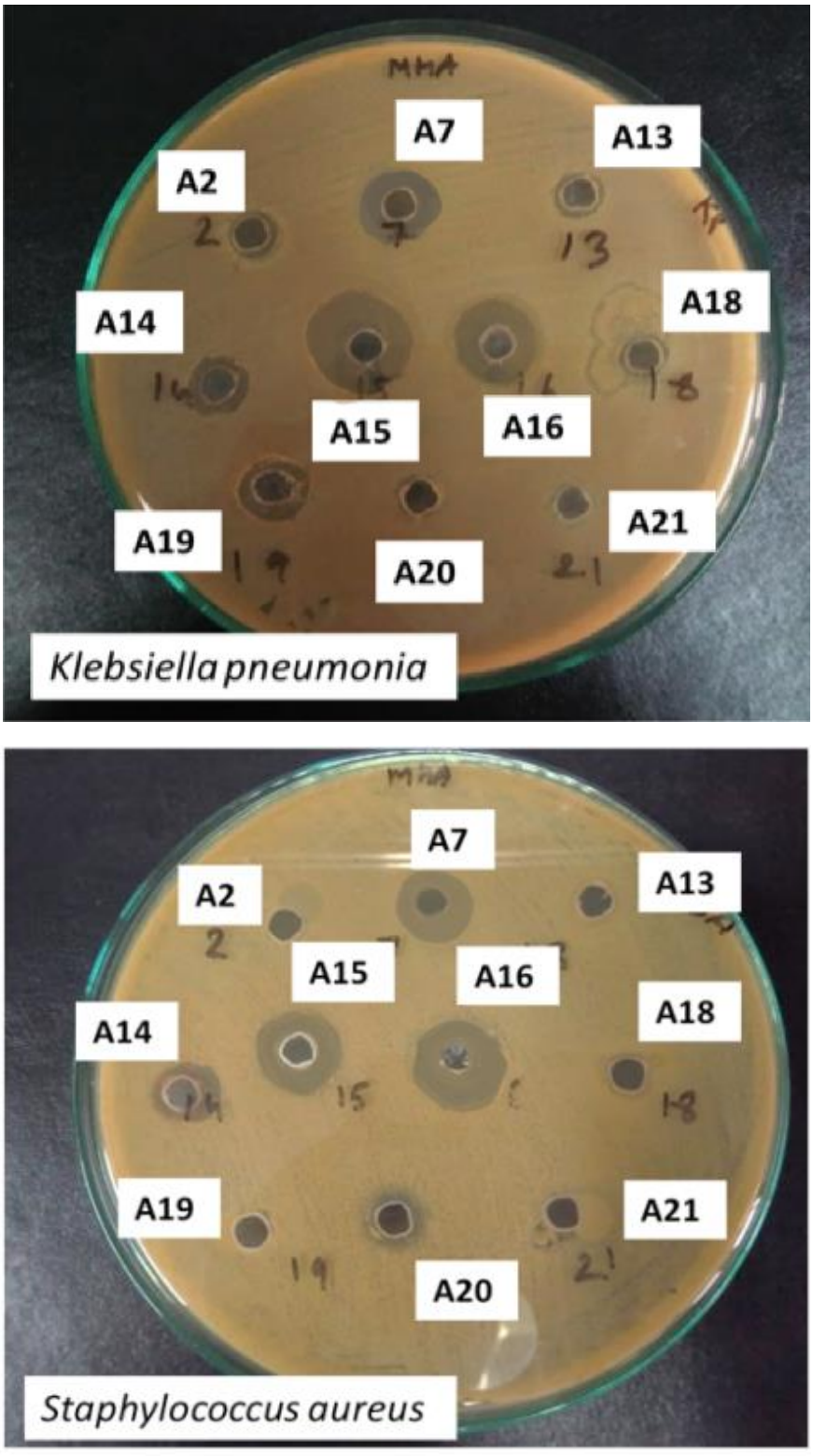


Figure 06: Antimicrobial activity of the isolates against pathogens *K. pneumonia* and *S. aureus* (well diffusion method)

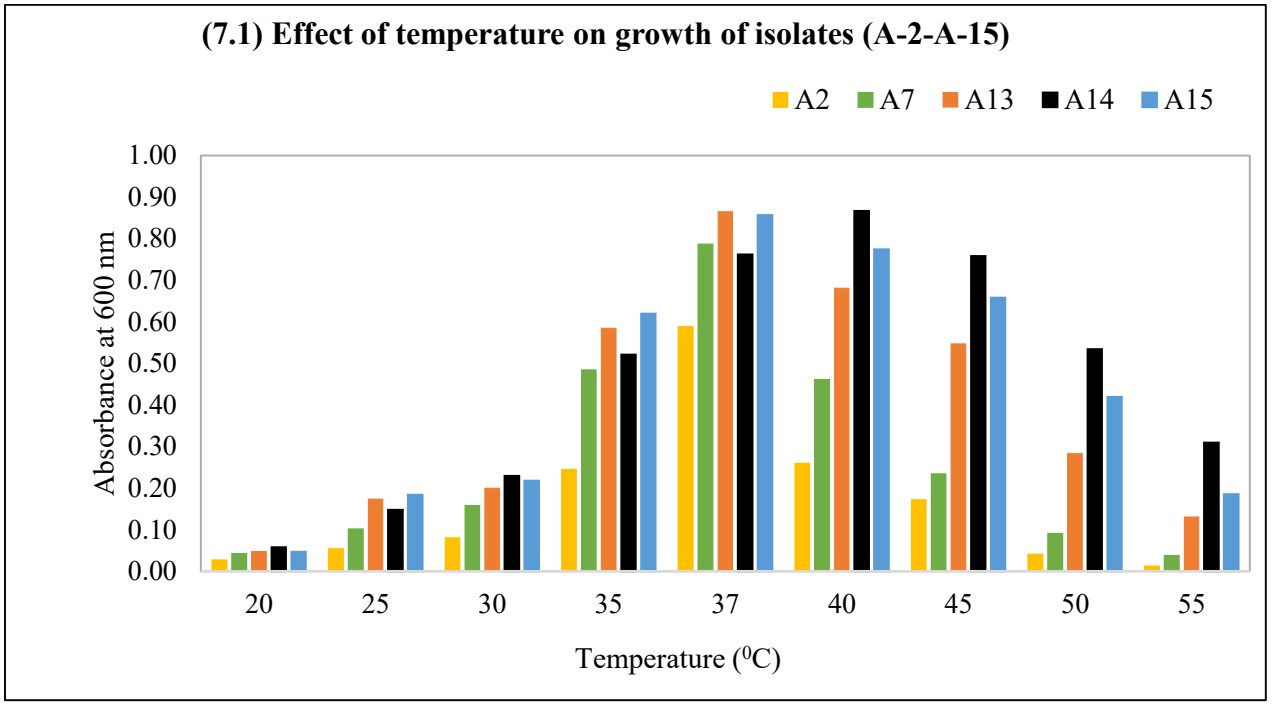
4.7 Effect of environmental parameters on growth of isolates:

4.7.1 Effect of temperature:

The growth of isolated microorganisms was observed by measuring the absorbance at 600 nm of the culture medium after overnight incubation and comparing to a negative control. The peak value of absorbance was found at 37^0C for 8 isolates (A-2, A-7, A-13, A-15, A-16, A-18, A-19, A-20) and 40^0C for 2 isolates (A-14, A-21). Table 06 and Figure 07 illustrate the growth of 10 isolates in different temperatures.

Table 06: Effect of temperature on growth of α-amylase producing isolates

Test isolates	Absorbance (600 nm)								
	Temperature								
	20^0C	**25^0C**	**30^0C**	**35^0C**	**37^0C**	**40^0C**	**45^0C**	**50^0C**	**55^0C**
A2	0.027	0.054	0.080	0.245	0.588	0.260	0.172	0.041	0.013
A7	0.043	0.102	0.158	0.484	0.787	0.461	0.234	0.091	0.038
A13	0.048	0.174	0.200	0.585	0.865	0.681	0.547	0.283	0.131
A14	0.058	0.148	0.230	0.521	0.762	0.867	0.758	0.534	0.310
A15	0.047	0.184	0.218	0.620	0.857	0.774	0.658	0.420	0.186
A16	0.048	0.124	0.268	0.585	0.865	0.708	0.527	0.283	0.131
A18	0.041	0.104	0.205	0.482	0.764	0.451	0.279	0.077	0.033
A19	0.024	0.045	0.181	0.314	0.440	0.255	0.153	0.063	0.017
A20	0.031	0.062	0.190	0.339	0.628	0.377	0.172	0.130	0.026
A21	0.046	0.183	0.248	0.561	0.687	0.838	0.569	0.222	0.100



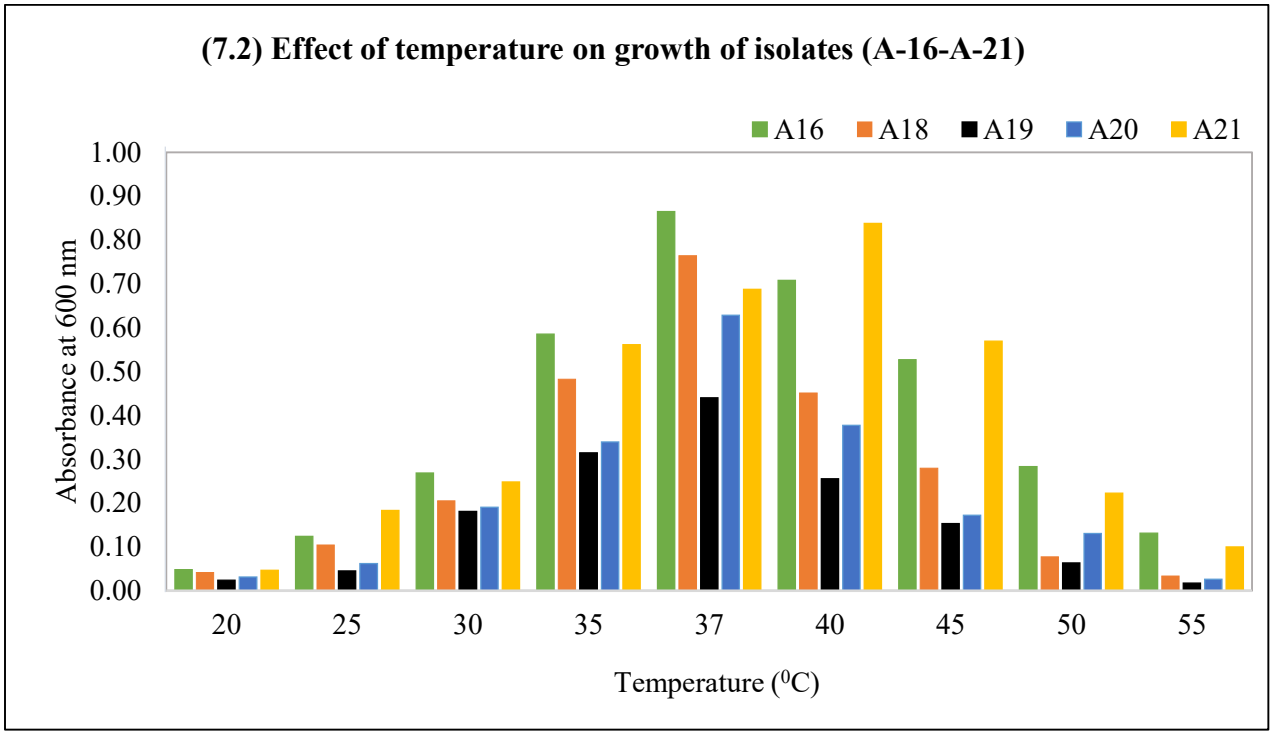


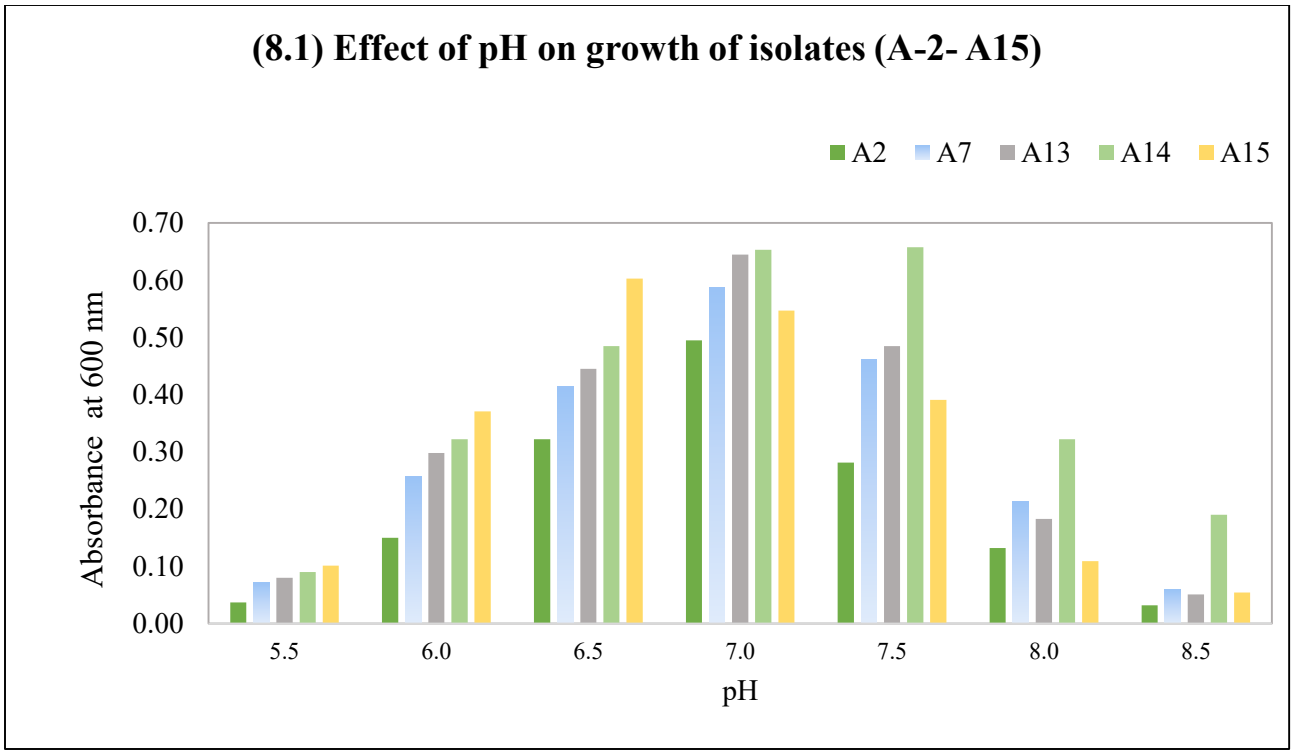
Figure 07: Effect of Temperature on Growth of α-Amylase producing Isolates. Figure 7.1 summarizes the result for isolates A-2 to A-15 and Figure 7.2 Summarizes the result for isolates A-16 to A-21. For all isolates except A-14 and A-21 (*B. sporothermodurans* and *B. cereus)*, the peak value was obtained at 37^{0}C. A-14 (*B. sporothermodurans)* and A-21 *(B. cereus)* reached the peak value at 40^{0}C.

4.7.2 Effect of pH:

The growth was observed by measuring the absorbance of the overnight cultured medium at 600 nm and comparing to a negative control. The peak value of absorbance was found at pH 7.0 for 9 isolates (A-2, A-7, A-13, A-15, A-16, A-18, A-19, A-20, A-21) and pH 7.5 for one isolate (A-14). Table 07 and Figure 08 illustrate the growth of 10 isolates in different pH values.

Table 07: Effect of pH on growth of isolated α-amylase producing microorganisms from soil sample

Test isolates	Absorbance (600 nm)						
	pH						
	5.5	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**
A2	0.037	0.150	0.322	0.495	0.281	0.132	0.032
A7	0.072	0.258	0.414	0.587	0.461	0.214	0.060
A13	0.080	0.298	0.445	0.645	0.485	0.183	0.051
A14	0.090	0.322	0.485	0.653	0.658	0.322	0.19
A15	0.101	0.371	0.603	0.547	0.391	0.109	0.054
A16	0.077	0.222	0.431	0.645	0.485	0.183	0.101
A18	0.071	0.150	0.34	0.592	0.364	0.151	0.079
A19	0.051	0.132	0.262	0.54	0.302	0.134	0.032
A20	0.062	0.173	0.267	0.569	0.37	0.146	0.078
A21	0.073	0.190	0.307	0.624	0.401	0.181	0.088



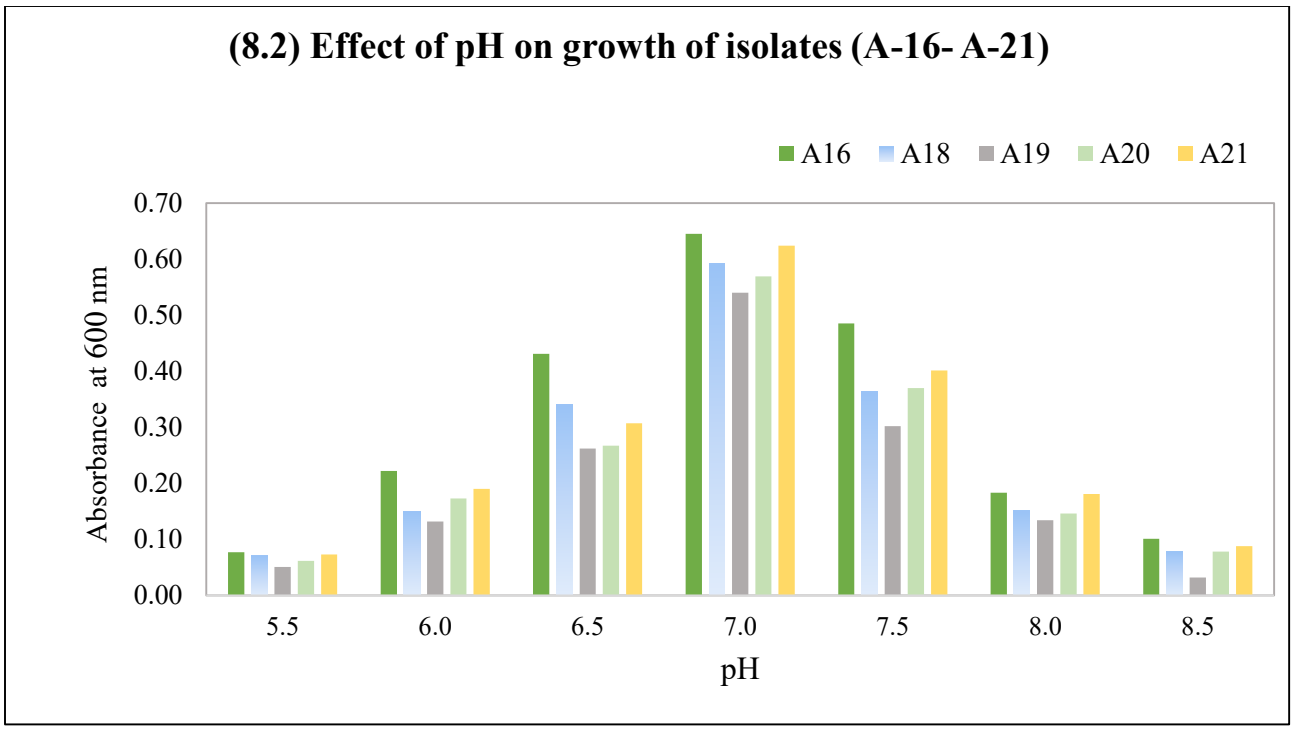


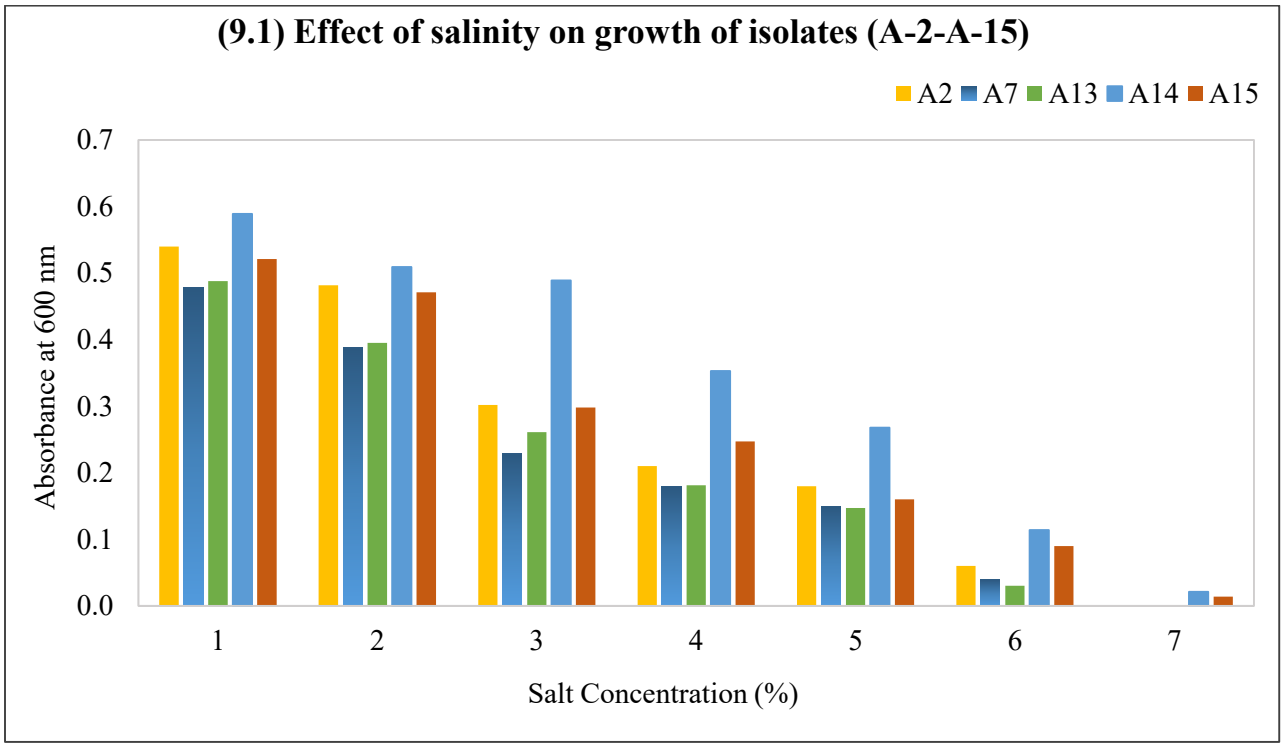
Figure 08: Effect of pH on growth of α-amylase producing isolates.
Figure 8.1 summarizes the result for isolates A-2 to A-15 and figure 8.2 for isolates A-16 to A-21. The peak value of the absorbance was found at pH 7.0 for all isolates except A-14 (*B. sporothermodurans,* peak absorbance recorded at pH 7.5).

4.7.3 Effect of Salinity:

All isolates showed growth in different degrees of salinity after overnight incubation. The growth was observed by measuring the absorbance of the culture medium at 600 nm and comparing it to a negative control. For all isolates, the value of absorbance decreased with increase of salt concentration, indicating the decline of cell concentration. The value was 0.0~0.1 for all isolates at 7% salt concentration. Table 08 and Figure 08 illustrate the growth of 10 isolates in different salinities.

Table 08: Effect of salinity on growth of isolated α-amylase producing isolates

Test isolates	Absorbance (600 nm)						
	Salt concentration						
	1%	2%	3%	4%	5%	6%	7%
A2	0.540	0.280	0.302	0.510	0.381	0.142	0.038
A7	0.078	0.288	0.424	0.680	0.465	0.200	0.072
A13	0.080	0.195	0.461	0.781	0.547	0.193	0.061
A14	0.089	0.200	0.466	0.653	0.668	0.514	0.100
A15	0.121	0.271	0.498	0.647	0.491	0.209	0.064
A16	0.083	0.189	0.355	0.588	0.350	0.184	0.048
A18	0.071	0.150	0.340	0.592	0.364	0.151	0.079
A19	0.051	0.132	0.262	0.540	0.302	0.134	0.032
A20	0.062	0.173	0.267	0.569	0.370	0.146	0.078
A21	0.073	0.190	0.307	0.624	0.401	0.181	0.088



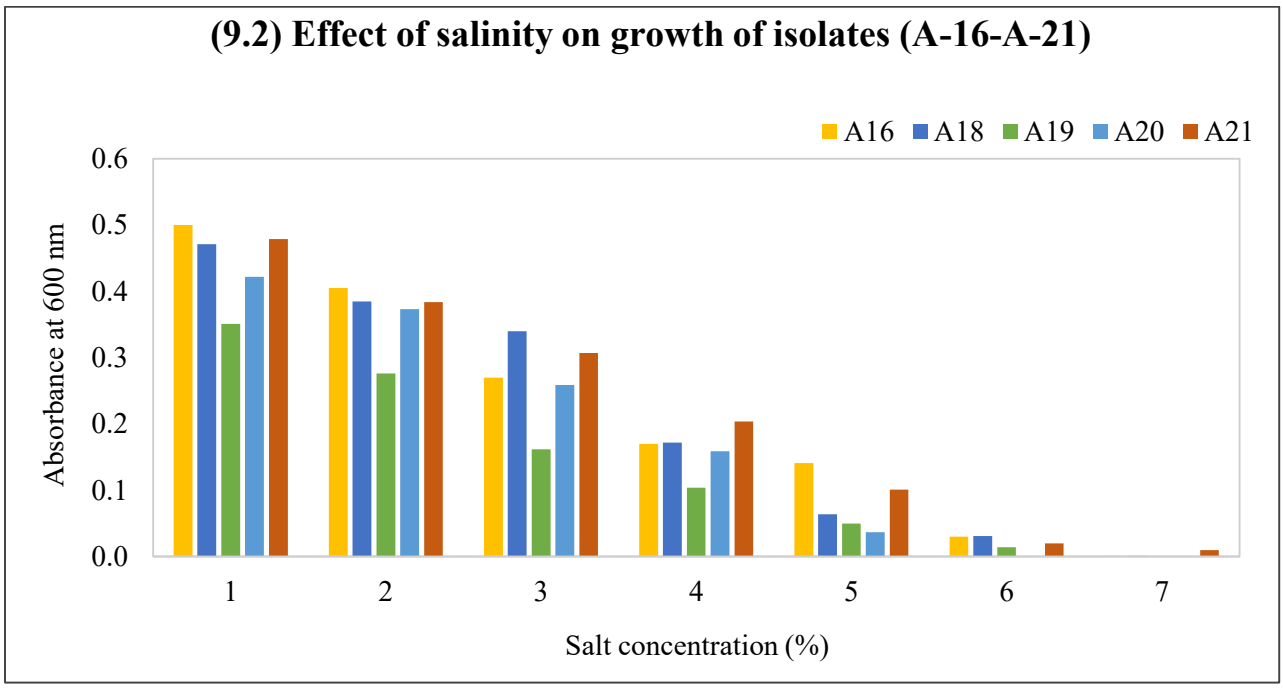


Figure 09: Effect of salinity on growth of α-amylase producing isolates.
Figure 9.1 summarizes the result for isolates A-2 to A-15 and Figure 9.2 for isolates A-16 to A-21. The growth gradually declined with increase of the salt concentration. A-14 (*B. sporothermodurans*) was more salt tolerant than other strains.

4.8 Activity of α-Amylase secreted by Soil microorganism samples

The specific activity of the enzymes was calculated according to the method described by Jayaraman (1981). Specific activity of an enzyme is expressed as unit per ml of protein, where 1 unit is equivalent to 1 mg maltose per 1 ml enzyme extract, released in 15-minute reaction at 37^0 C.

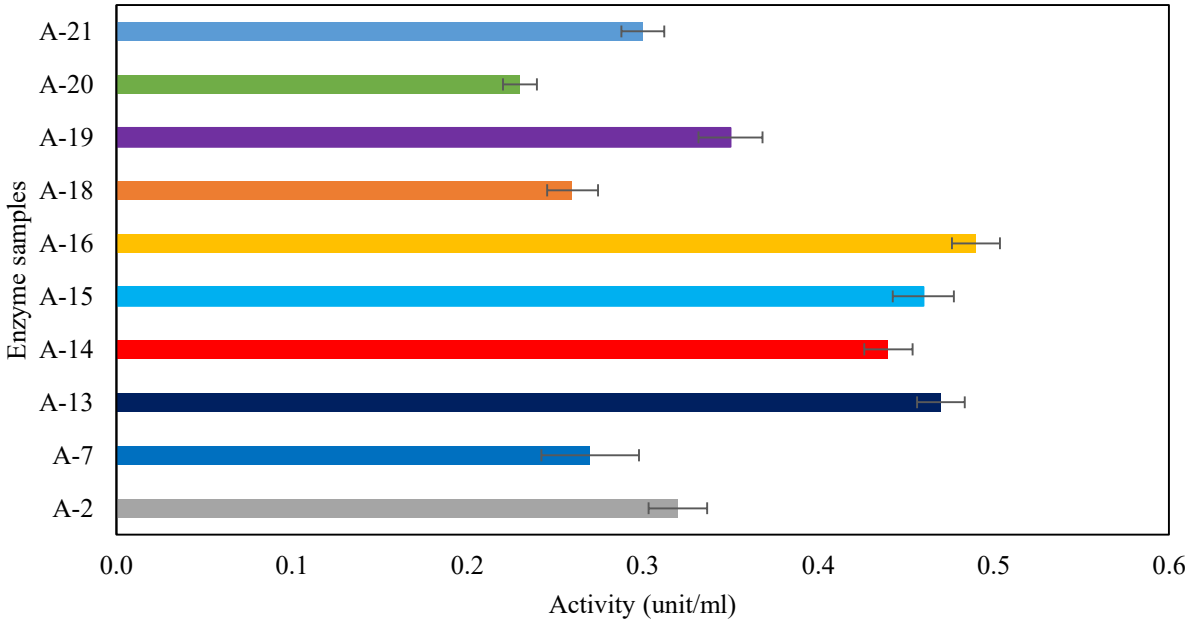
Analyzing to the result, A-13, A-14, A-15, and A-16 showed the highest activity. A-13 and A-16 were both *Bacillus subtilis* and displayed similarity in their enzyme activity. Further characterization of the enzymes was carried out for A-14, A-15, and A-16.

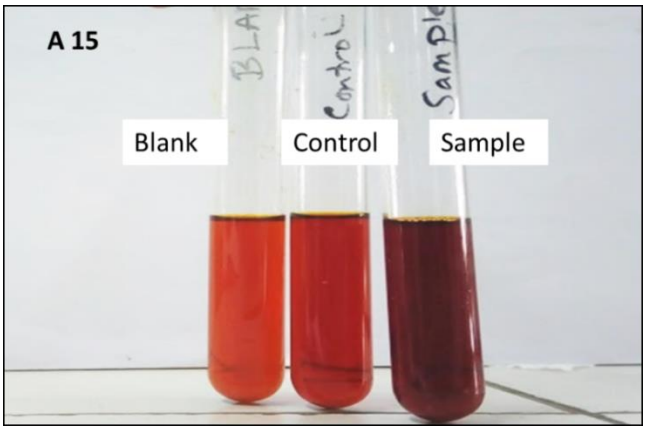
Table 09 and figure 10.1 show the activities of enzymes secreted by 10 bacterial isolates.

Table 09: Activities of α-amylase enzyme secreted by isolated samples

Enzyme samples	Activity (unit/ml)
A-2	0.32 ±0.017
A-7	0.27 ±0.028
A-13	0.47 ±0.014
A-14	0.44 ±0.014
A-15	0.46 ±0.017
A-16	0.49 ±0.014
A-18	0.26 ±0.015
A-19	0.35 ±0.018
A-20	0.23 ±0.009
A-21	0.30 ±0.012

Figure (10.1): Activity of α-amylase enzyme secreted by soil microorganism samples





(10.2) DNS test result for α-amylase activity determination (A-15)

Figure 10: α-Amylase activity determination of isolates using DNS test.

Comparing the enzyme activity derived from the results of DNS test (Figure 10.1), it was observed that A-13, A-14, A-15, and A-16 showed the most α-amylase activity. Figure 10.2 summarizes the result of the DNS test. The dark color of starch solution indicates the presence of a high concentration of maltose which was produced due to the activity of α-amylase enzyme.

4.9 Effect of environmental parameters on activity of α-amylase producing isolates from soil sample:

4.9.1 Effect of temperature on the activity of α-amylase:

The effect of temperature on enzyme activities of A-14, A-15, and A-16 was determined by incubating the reaction mixture at various temperatures during the DNS test. The peak value was considered as 100% and relative activities in other temperatures were calculated accordingly.

The enzyme activity gradually increased with the increase in temperature. The peak value of activity was obtained at 37^0 C, 38^0 C, and 40^0 C; for A-14, A-15, and A-16 respectively. With a further increase in temperature, the activity slowly declined. At 90^0C, the enzymes showed minimum activity. Table 10 and figure11 illustrate the effect of temperature on the activity of the enzymes.

Table 10: Effect of temperature on the activities of α-amylase extracted from isolates

Temperature (^{0}C)	Activity (%)		
	A14	A15	A16
10	40.0	25.0	20.0
20	70.0	56.0	55.0
30	91.0	83.0	81.0
37	100.0	98.0	96.0
38	99.0	100.0	98.0
40	96.0	97.0	100.0
45	86.0	84.0	93.0
50	74.0	70.0	82.0
60	53.0	46.0	60.0
70	34.0	29.0	40.0
80	18.0	15.0	23.0
90	6.0	3.0	7.0

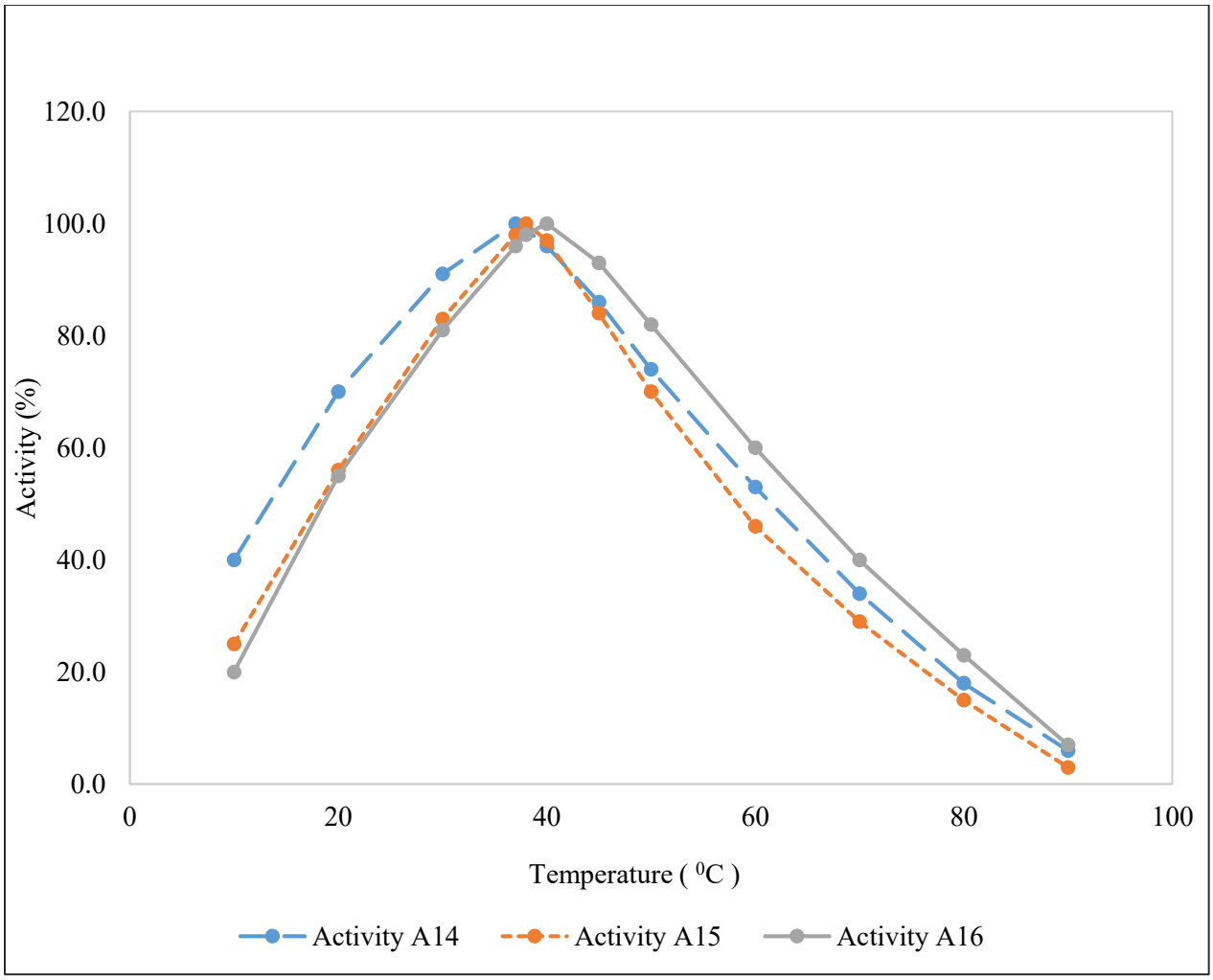


Figure 11: Effect of temperature on the activity of α-amylase secreted by isolates A-14, A-15, and A-16.

The enzyme activity gradually increased with the increase in temperature. The peak value of activity was obtained at 37^{0}C, 38^{0}C, and 40^{0}C; for A-14, A-15, and A-16 for *B. sporothermodurans, B. amyloliquefaciens,* and *B.subtilis* respectively. With a further increase in temperature, the activity slowly declined. At 90^{0}C, the enzymes showed minimum activity.

4.9.2 Effect of pH on the activity of α-Amylase:

The effect of pH on enzyme activities of A-14, A-15, and A-16 was determined by carrying out DNS test in buffers of different pH values. The peak value was considered as 100% and relative activities in other pH were calculated accordingly.

The enzyme activity gradually increased with the increase of pH. The peak values of activity were obtained at pH 6.2, 6.5 and 6.7; for A-14, A-15, and A-16 respectively. With the increase of pH value, the activity slowly decreased. At pH 10.0, the enzymes A-14, A-15, and A-16 showed minimum activity (15%, 22% and 28%, respectively). Table 11 and Figure 11 demonstrate the effect of pH on activities of the enzymes.

Table 11: Effect of pH on the activities of α-amylase extracted from isolates

pH	Activity (%)		
	A14	A15	A16
2.0	0.0	2.0	3.0
2.5	29.0	22.0	25.0
4.0	56.0	42.0	45.0
4.5	78.0	63.0	62.0
6.0	95.0	82.0	78.0
6.2	100.0	94.0	90.0
6.5	89.0	100.0	98.0
6.7	70.0	87.0	100.0
8.0	49.0	63.0	82.0
9.0	29.0	38.0	53.0
10.0	15.0	18.0	25.0

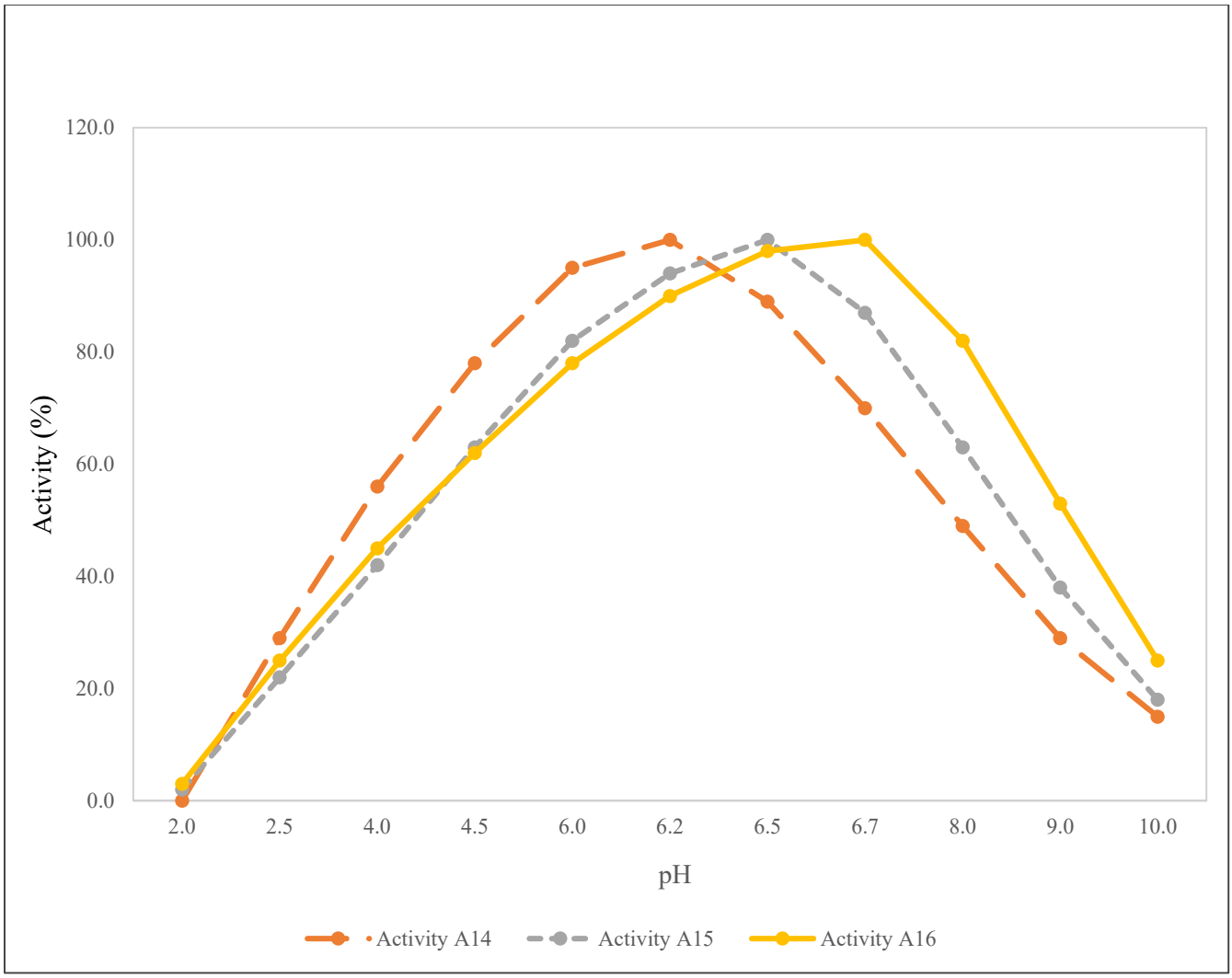


Figure 12: Effect of pH on the activity of by isolates A-14, A-15, and A-16.

The enzyme activity gradually increased with the increase of pH. The peak value of activity was obtained at pH 6.2, 6.5, and 6.7; for A-14, A-15, and A-16 for *B. sporothermodurans, B. amyloliquefaciens,* and *B.subtilis* respectively. With further increase of the pH, the activity slowly declined. At 90^0C, the enzymes showed minimum activity.

4.10. Effect of various metallic salts and chemical agents on the activity of α-amylase

4.10.1 Effect of various metallic salts:

The chloride salts were used for observing the effect of metallic salt on enzyme activity of isolates. For each metallic salt, two salt solutions of different concentrations (0.001 M & 0.002 M) were prepared. Because of its high activity in the standard environment, A-16 was chosen for observing salt effect. The activity of α-amylase in absence of additional salts was considered as 100% and relative activities of the enzyme were calculated accordingly.

Analyzing the results, it was concluded that Na^+, and K^+ and Mg^{2+} had little effect on activity of the enzyme. Mn^{2+} notably increased activity of the enzyme. Conversely, Zn^{2+} was able to exhibit inhibitory function to some extent. The activity decreased dramatically in presence of Fe^{2+}; indicating its inhibitory function. When the salt concentration was raised to 0.002 mol/L, Fe^{2+} reduced enzyme's activity by almost 50%. Effect of various metallic salt on α-amylase activity is presented in Table 12 and Figure 13.1.

Table 12: Effect of various metallic salts on the activity of α-amylase from sample A-16

Test Salts	Concentration (mol/L)	α-Amylase activity (%)
None	-	100.00
$MgCl_2$	0.001	103.48
	0.002	107.52
$ZnCl_2$	0.001	84.36
	0.002	75.68
NaCl	0.001	100.00
	0.002	98.86
KCl	0.001	100.00
	0.002	99.28
$MnCl_2$	0.001	114.76
	0.002	127.16
$FeCl_2$	0.001	66.26
	0.002	54.42

4.10.2 Effect of calcium on the activity of α-Amylase:

The effect of calcium on the enzyme activities of A-14, A-15, and A-16 was determined. The activity of α-amylase in absence of additional calcium salt was considered as 100% and relative activities of the enzymes were calculated accordingly.

The enzyme activity gradually increased with increasing concentration of calcium. In presence of 0.50 M Ca^{2+}, the activities of A-14, A-15, and A-16 were 124.85%, 126.52%, and 137.82%, respectively. Table 13 and Figure 13.2 summarize the effect of calcium on sample enzymes.

Table 13: Effect of calcium on the activities of α-amylase extracted from isolates

Concentration Of $CaCl_2$ (M)	Activity (%)		
	A14	**A15**	**A16**
0.00	100.00	100.00	100.00
0.001	100.00	100.00	102.58
0.002	103.34	103.82	106.36
0.005	105.86	106.46	109.68
0.010	108.23	109.33	113.42
0.050	112.56	114.16	119.28
0.100	115.48	116.74	123.86
0.200	118.62	120.38	127.20
0.300	121.60	123.85	132.45
0.500	124.85	126.52	137.82

4.10.3 Effect of urea on the activity of α-Amylase:

The effect of urea on the enzyme activities of A-14, A-15, and A-16 was determined. The enzyme activity gradually declined with the increase of urea concentration. The activity of A-14 was completely inhibited when urea concentration was raised to 6.00 M. Similarly, the activities of A-15 and A-16 were completely inhibited when urea concentration was raised to 8.00 M.

Table 14 and Figure 13.3 show the effect of urea on the activities of α-amylase enzymes extracted from A-14, A-15 and A-16.

Table 14: Effect of urea on the activities of α-amylase extracted from theisolates

Concentration Of Urea(M)	Activity (%)		
	A14	**A15**	**A16**
0	100.00	100.00	100.00
1	77.85	86.52	92.36
2	53.68	70.55	78.65
4	18.62	39.72	46.72
6	0.00	17.28	20.52
8	0.00	0.00	0.00

4.10.4 Effect of EDTA on the activity of α-Amylase:

The effect of EDTA on enzyme activities of A-14, A-15, and A-16 was determined. The enzyme activity gradually declined with the increase of EDTA concentration.

Activities of A-14, A-15, and A-16 were completely inhibited when EDTA concentration was raised to 0.50 M. A-16 showed the most stability in presence of the chemical agent; while A-14 showed the least stability and rapid decrease of activity.

Table 15 and Figure 13.4 show the effect of EDTA on the activities of α-amylase enzymes isolated from A-14, A-15 and A-16.

Table 15: Effect of EDTA on the activities of α-amylase extracted from isolates

Concentration Of EDTA(M)	Activity (%)		
	A14	**A15**	**A16**
0.00	100.00	100.00	100.00
0.001	57.48	61.50	71.30
0.002	52.32	56.28	61.85
0.005	43.28	49.72	52.48
0.010	25.55	30.15	31.20
0.100	8.62	12.68	14.62
0.500	0.00	0.00	0.00

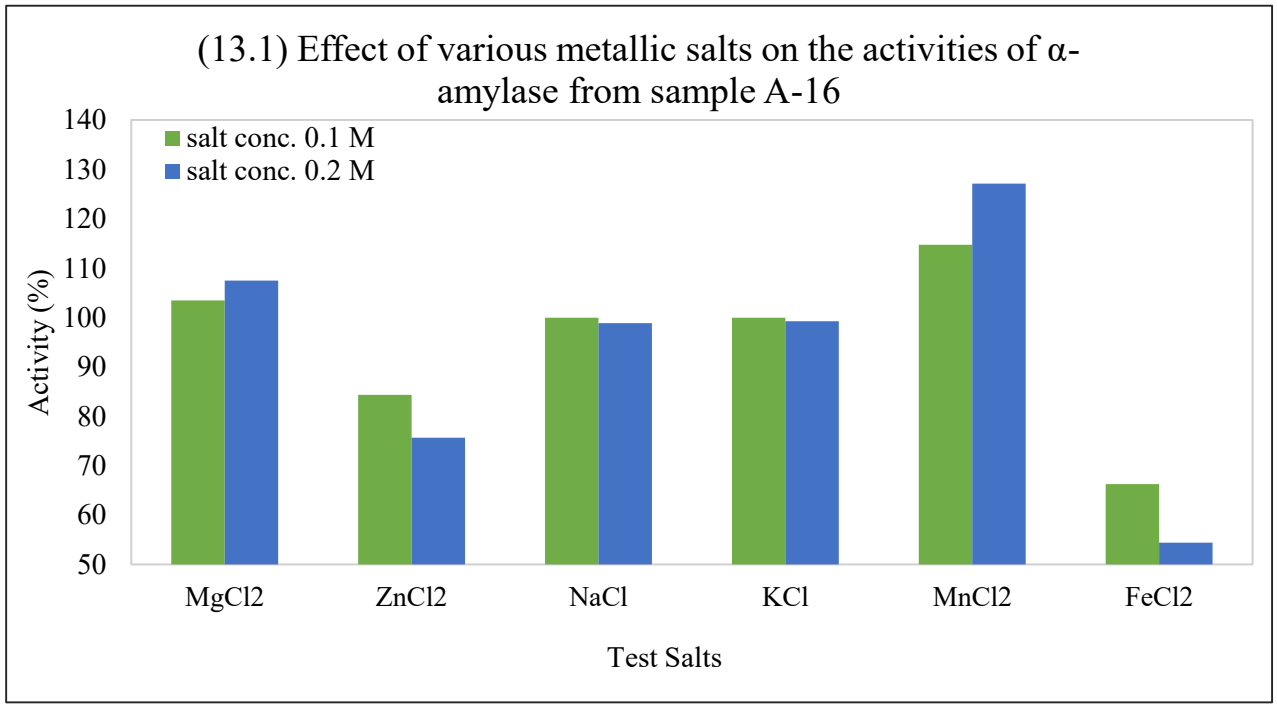
4.10.5 Effect of acetic acid on the activity of α-amylase:

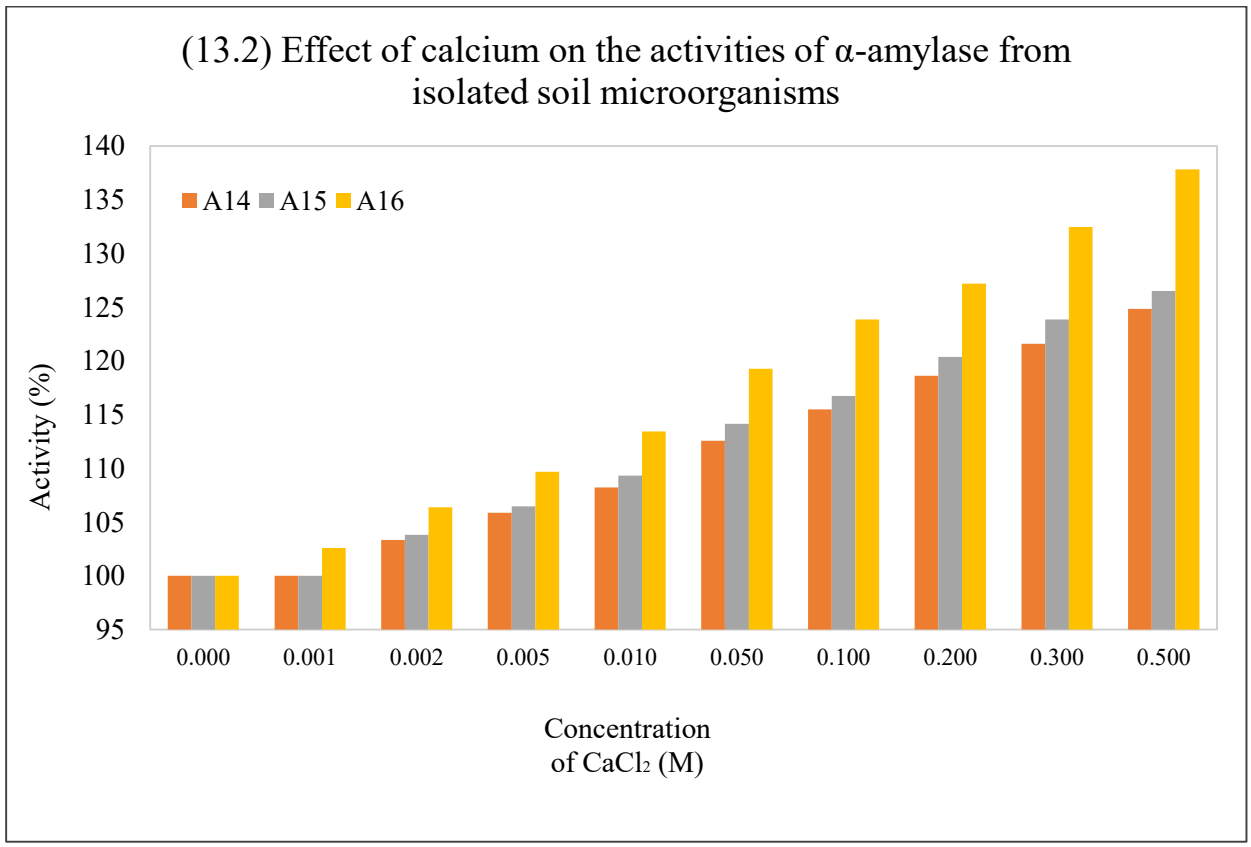
The effect of acetic acid on the enzyme activities of A-14, A-15, and A-16 was determined. The enzyme activity gradually declined with the increase of concentration of Acetic acid.

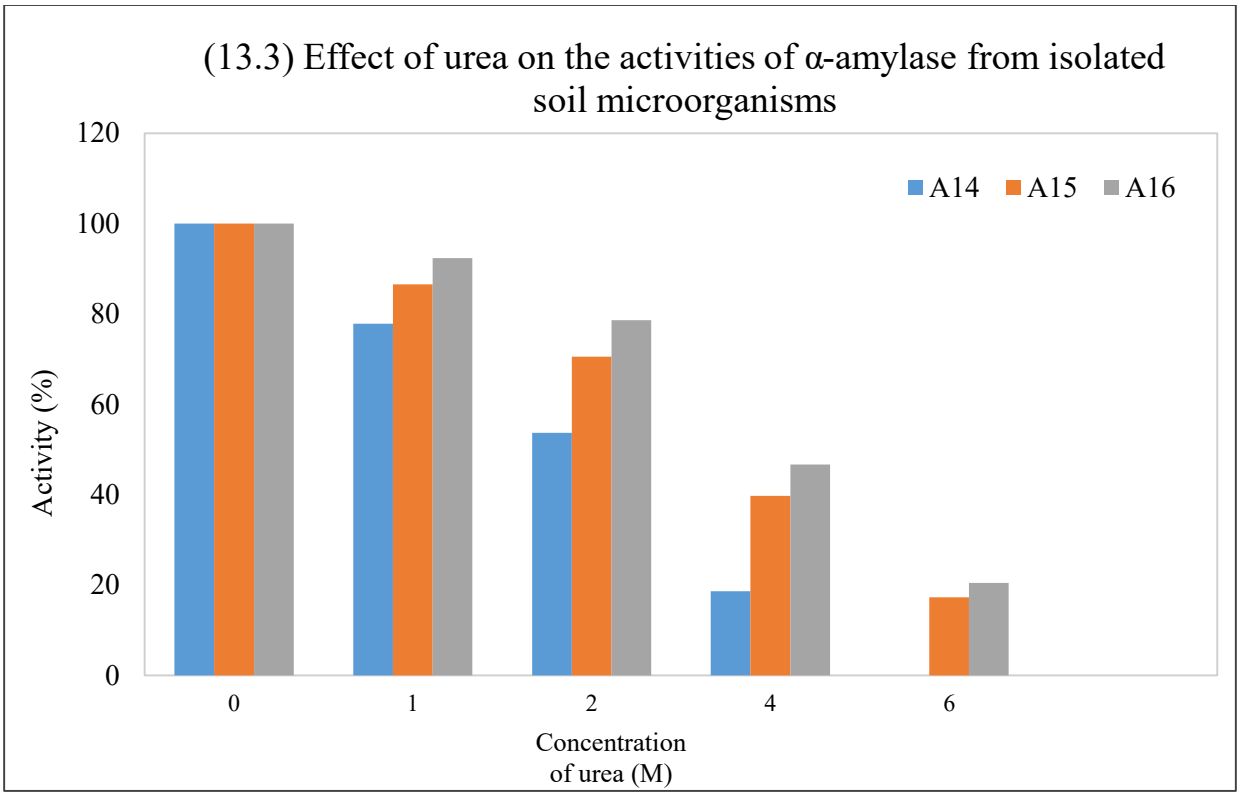
A-16 showed the most stability in the presence of the chemical agent; whereas A-14 showed least stability and rapid decrease of activity. The activity of A-14 was completely inhibited when acetic acid concentration was raised to 20%. The activities of A-15 and A-16 were completely inhibited when acetic acid concentration was raised to 30%. Table 16and Figure (13.5) show the effect of acetic acid on the activities of α-amylase enzymes isolated from A-14, A-15, and A-16.

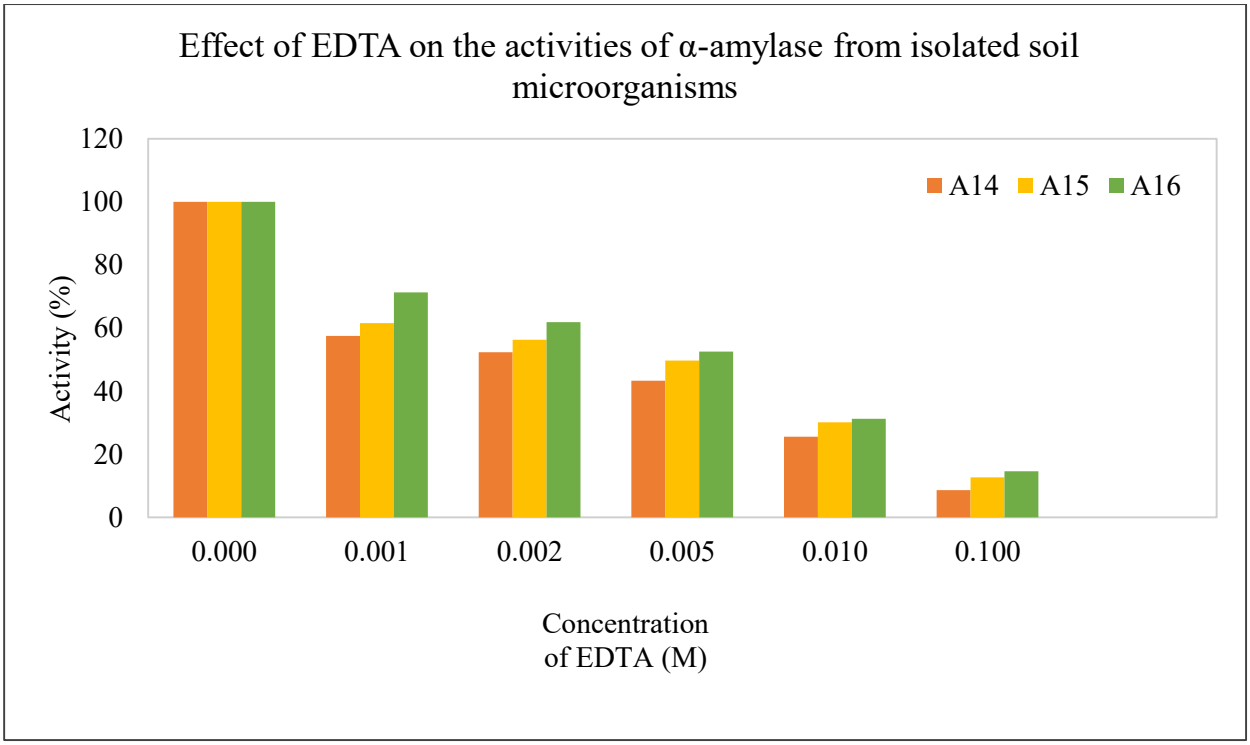
Table 16: Effect of acetic acid on the activities of α-amylase extracted from isolates

Concentration Of acetic acid(%)	Activity (%)		
	A14	**A15**	**A16**
0.00	100.00	100.00	100.00
2.50	74.58	82.65	89.16
5.00	47.63	66.36	75.53
10.00	18.32	33.12	45.32
20.00	0.00	6.88	18.62
30.00	0.00	0.00	0.00


(13.1) Effect of various metallic salts on the activities of α-amylase from sample A-16
salt conc. 0.1 M
salt conc. 0.2 M
Activity (%)
140
130
120
110
100
90
80
70
60
50
MgCl2
ZnCl2
NaCl
KCl
MnCl2
FeCl2
Test Salts


(13.2) Effect of calcium on the activities of α-amylase from isolated soil microorganisms
A14
A15
A16
Activity (%)
140
135
130
125
120
115
110
105
100
95
0.000
0.001
0.002
0.005
0.010
0.050
0.100
0.200
0.300
0.500
Concentration of CaCl2 (M)


(13.3) Effect of urea on the activities of α-amylase from isolated soil microorganisms
120
100
80
60
40
20
0
Activity (%)
A14
A15
A16
0
1
2
4
6
Concentration of urea (M)


Effect of EDTA on the activities of α-amylase from isolated soil microorganisms
120
100
80
60
40
20
0
Activity (%)
A14
A15
A16
0.000
0.001
0.002
0.005
0.010
0.100
Concentration of EDTA (M)

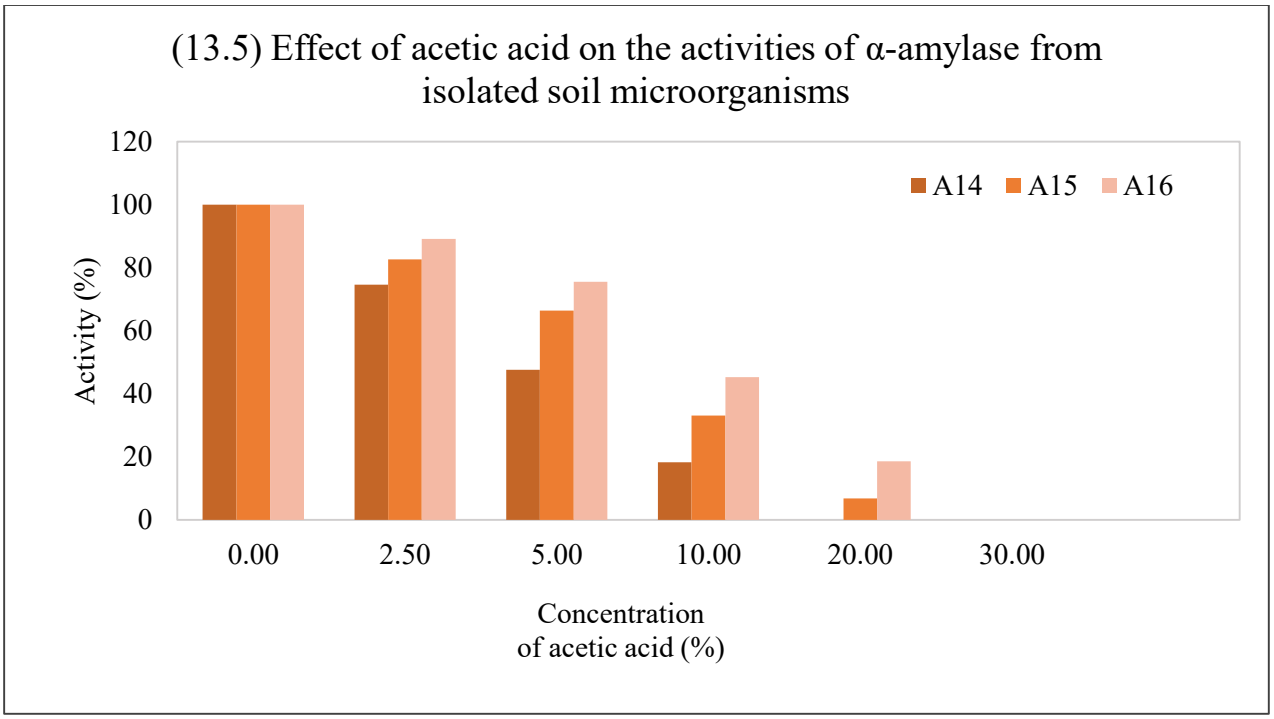


Figure 13: Effect of various metallic salts and chemical agents on the activity of α-amylase secreted by microorganisms from soil sample.

The activities of enzymes decreased dramatically in presence of Fe^{2+}; indicating Fe^{2+} ion's inhibitory function (Figure 13.1). The enzyme activities increased in the presence of calcium (Figure 13.2). However, it decreased in presence of chemical agents such as urea (Figure 13.3), EDTA (Figure 13.4), and acetic acid (Figure 13.5).

5. Discussion

In the present study, two different soil samples were collected from the root-adjacent soil of rose plants and a local potato starch factory. The isolated microorganisms exhibited a various degree of starch degradation in starch hydrolysis test. Only 23% (3 out of 13) of the total number of isolates found in starch factory soil were found to produce amylase. Meanwhile, 70% (7 out of 10) of the total isolates from flower garden soil were found to produce amylase. Enzyme activity depends on the bacterial strain, soil properties and available biomass (Frankenberger,1983). Plant root exudates and senescent parts of plants play an important source of nutrition for soil microorganisms (Atlas *et. al.,* 1998). It is assumed that the humid and nutrient rich soil from the flower garden provided a better environment for the nourishment of amylase producing soil microbes.

Isolates were identified based on their morphological characteristics, Gram's staining results, staining reaction, different biochemical reactions and Biolog^{TM} System identification. Biolog^{TM} identification results identified the isolates as *Raoueltola planticolla (*A-2*), Bacillus cibi (*A-7), *Bacillus sporothermodurans (*A-14*), Bacillus amyloliquefaciens (*A-15*), Solibacillus silvestris (*A-16*), Klebsiella oxytoca (*A-18*), Bacillus vallismortis (*A-19), and *Bacillus cereus (*A-20*).* Both A-13 and A-16 were identified as *Bacillus subtilis. Bacillus* is a gram-positive, rod-shaped bacterium (Gordon *et al.,* 1973). *Solibacillus silvestris* is a gram-positive, aerobic, rod-shaped bacterium from the genus *Solibacillus* (Rheims *et al.,* 1999). *Raoueltola planticola* is gram-negative, rod-shaped bacteria commonly found in water, soil, and fish and is capable of causing various infections. (Kalaria *et al.,* 2017). *Klebsiella oxytoca* is a rod-shaped gram-negative bacterium which is present in water, soil, and animal & human bowel (Singh, 2017). The result of biochemical tests corresponded to the standard biochemical test results for respective species.

Antibiotic susceptibility of the selected isolates was determined by the Kirby-Bauer test. Analyzing the result, it was noted that the majority of the strains were resistant to antibiotics that impair cell wall synthesis (imipenem, ceftadizime). conversely, antibiotics that bind to the 30S ribosomal subunit (doxycycline, amikacin, gentamycin) and antibiotics that inhibit DNA gyrase (ciprofloxacin, nalidixic acid) formed the largest zones of inhibition on plates. Both *B. subtilis* strains (A-13 and A-16) were more sensitive to antibiotics than other strains. However, the degree of resistance to specific antibiotics varied from each other. The gram-

negative strains *R. planticola* and *K. oxytoca* displayed less antibiotic sensitivity compared to the gram-positive strains. *R. planticola* (A-19) was resistant to 50% of the applied antibiotics. The largest zone of inhibition was formed in the presence of ciprofloxacin. According to previous studies, *R. planticola* has been proven to be susceptible to third and fourth generation ciprofloxacin. However, it is capable of acquiring the plasmid mediated resistance (Yu *et al.,* 2015).

The antimicrobial activity of the isolates against pathogens was determined by agar well-diffusion method. The culture suspension of test isolates displayed various degrees of antibacterial activity against pathogenic strains. Fourteen out of 16 pathogens were sensitive to the culture suspension of test isolate A-7 (*B. cibi). B. subtilis* (A-13 and A-16) formed large zones of inhibition on culture plates. Conversely, the pathogenic strains were most resistant to *K. oxytoca* (A-19). *B. subtilis* produces various antimicrobial compounds and enzymes including Cyclic lipopeptides such as surfactins, fengycins, iturins (Falardeau *et al.,* 2013), anti-fungal lytic enzymes such as cellulase, glucanase, protease, chitinase and antibiotics such as subtilin, polymyxin, difficidin and mycobacillin. (Ariffin *et al.,* 2006, Caulier *et al.,* 2018)

The effect of environmental parameters was determined for optimizing growth condition of isolates. The parameters were temperature, pH and salinity. All isolates were subjected to overnight growth at several temperatures ranging from 20^0C to 55^0C and pH values ranging from 5.5 to 8.5. At low pH and temperature, the isolates showed minimum growth which gradually increased with the increase in pH and temperature. After reaching a peak, the growth of isolates started declining with a further increase in temperature and pH. The absorbance reached a peak value at 37^0C for all isolates except A-14 and A-21 (*B. sporothermodurans and B. cereus,* respectively); for which the peak values were obtained at 40^0C. pH 7.0 was optimum for all isolates except *B. sporothermodurans;* which displayed the most growth at pH 7.5. In several studies, gram-positive *Bacilli* have shown optimum growth at 37^0C (Khan *et al,* 2011) and pH 7.0 (Teodoro *et al.*, 2000), which was similar to the result of the present study. *K. oxytoca* was reported to grow at an optimum temperature of 32 to 37.8^0C (Tondo *et al.*, 2014, Cheung *et al.,* 1983). Mesophilic *B. cereus* grow at a temperature range of 7 to 46ºC (Francis *et al.,* 1998). Observing the effect of salinity, the growth of isolate declined with the increase of salt percentage. *B. sporothermodurans* (A-14) and *B. subtilis* (A-16) were most salt tolerant and showed more growth at a high salt concentration.

B. subtilis can survive high salt concentration by forming spores which lack salt sensing system. (Nagler *et. al.,* 2013).

α-Amylase was extracted from the isolated starch degrading soil microorganisms and the enzyme was partially characterized. Comparing the activities of the isolates' extracted amylases, maximum enzyme activity was obtained from *B. subtilis, B. sporothermodurans* and *B. amyloliquefaciens*. *B. subtilis* showed the most activity (0.49 unit/ml), followed by *B. amyloliquefaciens* (0.46 unit/ml) and *B. sporothermodurans* (0.44 unit/ml). The effects of temperature, pH, various metallic salts and chemical agents on the activities of *B. subtilis, B. sporothermodurans* and *B. amyloliquefaciens* were studied. Enzyme activity decreased at higher temperatures due to destruction of primary and secondary enzyme structures. The optimum temperatures for the enzyme activities of *B. subtilis, B. amyloliquefaciens* and *B. sporothermodurans* were 40^0C, 38^0C and 37^0C, respectively. Optimum *B. subtilis* amylase activity was acquired at 40ºC in a previous study (Raul *et al.,* 2014). Optimum *B. amyloliquefaciens* amylase activity was acquired at 37ºC in multiple studies (Torres *et al,* 2017, Deb *et al.,* 2013). No report was found on the activities on *B. sporothermodurans* amylase. The optimum pH for *B. subtilis, B. amyloliquefaciens* and *B. sporothermodurans* were 6.7, 6.2 and 6.0, respectively. *B. subtilis* amylase with the highest enzyme activity at pH 6.7 was found in a previous study (Paul *et al.,* 2013). Optimum amylase activity was acquired at pH 6.5 in a separate study but for *B. cereus* SB2 strain (Raplong *et al.,* 2014). The decline of enzyme activity at higher pH might be associated with ionization of groups located at the active site of the enzyme responsible for substrate binding.

The activities of the enzymes were influenced by the presence of metallic salts. The enzyme activities increased significantly in the presence of divalent cations Ca^{2+} and Mn^{2+}. It suggests the involvement of divalent ions in maintenance of active conformation of the enzyme. *B. subtilis* isolated from soil had increased activity in presence of Ca^{2+} ion in a previous study (Alariya *et al.,* 2013). The amylase from fermented cassava (Oboh, 2016) and the amylase from soybean (Prakash, 2011) were reported to increase their activities in presence of chloride salts such as Na^{2+}, K^{+}, and Mg^{2+}. Chemical agents such as EDTA and acetic acid in relatively higher concentrations inhibited the enzyme activities completely. The metal ions essential for enzyme binding might be released completely during EDTA and acetic acid treatment. EDTA was able to decrease amylase activity in starch degrading *E. coli* in a previous study (Alariya *et al.,* 2013).

Conclusion

Amylase has significant contribution in production and study of enzymes. Due to its biodegradable nature, bacterial amylase is a preferred source of the enzyme to limit environmental pollution. Researchers are developing advanced techniques to make them more thermoresistant and stable in higher pH to increase storage life.

Amylase producing microorganisms are present in abundance in the soil of Bangladesh. However, the country is dependent on imported amylase because there is no local factory for enzyme production. (Rahman *et al,* 2014). Commercial α-Amylase enzymes which are currently available in market cost about BDT 250-350 per liter. (Ferdaus *et al,* 2018). It is essential to take initiatives for producing the enzyme locally and to research on available resources and substrates for cost effective production of the enzyme. In the present study, numerous microorganism species from soil sample showed stable and consistent growth and enzyme activity in a wide range of temperature, pH and salinity. Crude enzymes extracted from *B. sporothermodurans, B. amyloliquefaciens* and *B. subtilis* had a satisfactory enzyme activity which can make them potential candidates for mass production. Further research should be carried out to determine the capability of the enzymes on industrial scale.

This study was an attempt to encourage commercial production of α-amylase locally in order to reduce the cost and take our country one step closer to self-reliance.

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Appendix A

Reagents and Buffer

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 temeperature.

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 tartarate. 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. 66

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

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

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Oxidase Reagent (100 ml)

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediaminedihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solutions was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

Nitrate Reagent A (100 ml)

5N acetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water. In a reagent bottle, 0.6 g of N,N-Dimethyl-α-naphthylamine was added

along with 100 ml of acetic acid (5N) and mixed until the colour of the solution turned light yellow. The reagent was stored at 4°C.

Nitrate Reagent B (100 ml)

In a reagent bottle, 0.8 g of sulfalinic acid was added along with 100 ml acetic acid (5N) to form a colourless solution and stored at 4°C.

Ethidium Bromide (100 ml)

To 100 ml distilled water, 1 g ethidium bromide was mixed. The solution was carefully stored at room temperature.

Lysozyme solution (20 ml)

To 20 ml of distilled water, 0.2 g of lysozyme powder was added and dissolved. The solution was stored at 4°C.

Tris Boric Acid EDTA (TBE) Buffer (500 ml)

To 500 ml distilled water, 5.4 g Tris HCL powder and 2.75 g boric acid were dissolved. The pH of the buffer was adjusted to 8, autoclaved and stored at room temperature.

Citrate Buffer (0.05 M 50 ml)

To make 1 M 50 ml citrate buffer, 14 g citric acid was added to 50 ml distilled water. From there, 2.5 ml buffer was added to 47.5 ml distilled water to make 50 ml of 0.05 M buffer. The pH was adjusted to need (3,4,5) using NaOH and HCl. The buffer was autoclaved and stored at 4°C.

Sodium Phosphate Buffer (0.05 M 50 ml)

For 50 ml of 0.05 M NaH_2PO_4, 0.35 g was added to 50 ml distilled water. For 50 ml of 0.2 M Na_2HPO_4, 0.67 g was added to 50 ml distilled water. To make sodium phosphate buffer of pH 6, 1.28 ml of Na_2HPO_4 was mixed with 3.73 ml NaH_2PO_4 and the volume was adjusted to 50 ml using distilled water. For pH 7, 2.89 ml $Na_2HPO_4$was added to 2.12 ml NaH_2PO_4 and the volume was adjusted to 50 ml by adding distilled water. The buffer was autoclaved and stores at 4°C.