Screening and Characterization of Industrially Important Lipase Producing Bacterial Isolates from Soil and Water Samples Collected from Lake Ziway, Oromia Regional State, Ethiopia

By

Tseday Temesgen Teferi

A Thesis Submitted to the Department of Applied Biology in Partial Fulfillment of the Requirement of the Award of Degree of Master of Science in Applied Biology (Applied Microbiology)

School of Applied Natural Science

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

June, 2022

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DECLARATION

I hereby declare that this Master Thesis entitled "**Screening and Characterization of Industrially Important Lipase Producing Bacterial Isolates from Soil and Water Samples Collected from Lake Ziway, Oromia Regional State, Ethiopia**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation

_____ _____ _____

Name of the student Signature Date

RECOMMENDATION

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Screening and Characterization of Industrially Important Lipase Producing Bacterial Isolates from Soil and Water Samples Collected from Lake Ziway, Oromia Regional State, Ethiopia**" prepared under our guidance by Tseday Temesgen submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Applied Biology (Applied Microbiology). Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

Major Advisor	Signature	Date
Co-Advisor	Signature	Date

APPROVAL PAGE

We, the advisors of the thesis entitled "**Screening and Characterization of Industrially Important Lipase Producing Bacterial Isolates from Soil and Water Samples Collected from Lake Ziway, Oromia Regional State, Ethiopia**" and developed by Tseday Temesgen; hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-Advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Tseday Temesgen have read and evaluated the thesis entitled "**Screening and Characterization of Industrially Important Lipase Producing Bacterial Isolates from Soil and Water Samples Collected from Lake Ziway, Oromia Regional State, Ethiopia**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Microbiology.

Chairperson	Signature	Date
Internal Examiner	Signature	Date
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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LISTS of ACRONYMS

ABIS: Advanced Bacterial Identification Software

CDW: Cell dry weight

EC: Enzyme Commission

FO: Fried oil

LPBI: Lipase producing bacteria isolates

MALDI-TOF MS: Matrix assisted laser desorption ionization-time of flight mass spectrometry

SCoRR: Sediment-bound Contaminant Resiliency and Response

SIM: Sulfide indole motility

T-20: Tween 20

T-80: Tween 80

WWT: Waste water treatment

YE: Yeast extracts

ABSTRACT

Lipases are group of enzymes which have various applications in different industries such as food, detergent, leather, pharmaceutical, paper and cosmetics. Production of these enzymes from microbial sources will have more advantages compared to those produced from plant and animal sources. Economic and environmental issues are major problems that can be reduced by using these microbial enzymes. Therefore, the purpose of this study was to screen and characterize industrially important lipase producing bacteria from water and soil samples collected from Lake Ziway. To this end, soil, water and sludge samples were collected from Lake Ziway and Adama Science and Technology University wastewater treatment plant and screened for lipase producing bacterial isolates. Tributyrin agar and phenol red agar were used for the screening purpose. Gram staining and biochemical tests such as citrate utilization, motility test, sulfur production and other tests were performed for the purpose of characterization of the isolates. MALDI-TOF MS and ABIS bacteria identification software (version 12) were used for identification purpose. A total of 11 lipase positive bacterial isolates were obtained depending on their growth characteristics on the growth medium. Among those, four isolates were selected for further analysis. The four isolates were characterized as LPBI-1 (Pseudomonas aeruginosa), LPBI-2 (Aeromonas veronii), LPBI-3 (Aeromonas veronii) and LPBI-4 (Pseudomonas aeruginosa). LPBI-4(Pseudomonas aeruginosa) was selected to determine its physio-chemical requirements. The isolate showed its best growth when olive oil and Tryptone was used as a carbon and nitrogen source respectively. Its best growth also recorded when pH value 6.5 and temperature at 37^{o}C. Submerged fermentation method was used for the production of lipase and the enzyme was collected by centrifugation process. Hydrolysis of milk fat by the enzyme was examined in order to check the efficiency of the enzyme. Disappearance of pink color of the solution was taken as positive result of the test. It was concluded that Pseudomonas aeruginosa and Aeromonas veronii species were best candidates for lipase production.

Keywords: *Aeromonas*, fat hydrolysis, microbial lipase, *pseudomonas*

1. Introduction

1.1. Background of the study

Enzymes are highly efficient biological catalysts studied for their industrial scale catalysis because of their numerous advantages (Chapman *et al*., 2018). Enzymes can be found in practically all naturally existing organisms, from microorganisms to plants and animals. Due to their presence in very small quantity in plants and animals, they cannot be exploited for industrial applications (Singh *et al*., 2019). In contrast, the production of enzymes from microorganisms has a number of advantages, such as easy handling, rapid replication under controlled conditions, easy genetic manipulation, high production yield, and so forth (Bharathi & Rajalakshmi, 2019).

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are hydrolytic enzymes that catalyze the hydrolysis of insoluble triacylglycerol to glycerol, acylglycerols, and free fatty acids (Bharathi & Rajalakshmi, 2019). They are the most versatile biocatalyst, capable of a wide range of bioconversion processes, including hydrolysis, esterification, acidolysis, and aminolysis (Faiz, 2021). Claude Bernand discovered lipase in pancreatic juice in 1856 as an enzyme that hydrolyzes insoluble oil droplets and turns them to soluble molecules (Ilesanmi *et al*., 2020).

Lipase are produced by many microorganisms include bacteria, fungi, yeasts and actinomyces (Hasan *et al*., 2018). Among these, microbial lipases, particularly those produced by bacteria, have significant benefits over those produced by plants and mammals due to a number of important properties. Microbial lipases are high in demand due to their specificity of reaction, low energy consumption, hydrolytic and synthetic activities, high yield and ease of genetic manipulation (Aktar *et al*., 2016). Lipase enzyme have been reported to be produced by some microbial species which include *Bacillus sp., Pseudomonas sp., Burkholderia sp., Candida rugosa, Candida antarctica, Galactomyces geotricum, Saccharomyces cerevisiae, Yarrowia lipolytica, Trichosporon ermantans, Cryptococcus albidus, Aspergillus flavus, Thermomyces lanuginosus* and *Rhizopus oryzae* (Ramnath *et al*., 2017).

Lipase-producing microbes have been discovered in a variety of environments, including industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds and decaying food, compost heaps, coal tips and hot springs. Oily environments (oil mill effluent) may be favorable for the isolation of lipase-producing bacteria (Qamsari *et al*., 2011).

Lipases from diverse microbiological sources have different 3-D structures and a wide range of sequence diversity. As a result, these enzymes are unique and specific to the type of bioconversion processes they catalyze, finding relevance in a wide array of industrial processes. They are found to be important in catalyzing different reactions related to food, pharmaceuticals, medical and diagnosis, dairy, fatty acid, leather, cosmetic, detergent, beverage and paper industries (Ilesanmi *et al*., 2020).

In order to determine a lipase positive bacterium by growing it, three factors must be present that include (i) growth of the organism, (ii) production of lipase by the organism under suitable growth conditions and (iii) the presence of sensitive method to detect lipase activity.

Several growth parameters influence the ability of bacteria to synthesize lipase. Carbon and nitrogen sources, the presence of activators and inhibitors, incubation temperature, pH, inoculum amount, and oxygen tension are all factors that might affect lipase synthesis. Because lipases are inducible enzymes, the carbon supply has been identified as a primary determinant of lipase production. The production of these enzymes depends on the presence of a lipid, such as olive oil or any other inducer, such as triacylglycerols, fatty acids and tweens (Muthumari *et al*., 2016). Olive oil is the most suited lipid substrate since it contains a high concentration of oleic acid and is less expensive (Hamied *et al*., 2017).

1.2. Statement of the problem

Enzymes are now being used in a growing number of industrial operations. As a result, the global industrial market is rapidly expanding, with an estimated worth of US$7 billion at the moment. Although enzymes are found in all living organisms, most industrial enzymes currently in use are obtained from microorganisms. Ethiopia has a lot of potential when it comes to discovering new enzymes that might be very useful in many industrial operations. The East African region has a distinct microbial biodiversity that could provide new enzymes for industrial applications. Furthermore, because of the availability of extremely unique habitats, such as alkaline environments, hot springs, etc. with huge

microbial diversity, the region could be, in the long term highly competitive in the global industrial enzyme market (Abdissa, 2018).

Ethiopia is working to boost sectors such as textiles, leather, detergents, pulp, and paper. Enzymes like amylase, lipase, and protease are required as an input in these industries. Various sectors in the region currently import large amounts of enzymes for usage in the leather tanning, textile, and brewing industries. Other industries hesitate to import enzymes due to high cost. For example in Ethiopia more than 150,000 kg of bating enzyme is imported annually with a cost of US$ 900,000 -1,000,000 (Gessesse *et al*., 2011). This is a severe economic burden that is having a negative impact in the country's economy. Environmental effect is another impact which is posed by importing those synthetic enzymes. In Ethiopia there are about 25 leather tanning industries, many of them engaged in the production of finished leather. In Tanzania more than a dozen tanneries are currently in operation. In these factories hundreds of thousands of people work and export of leather and leather goods is an important source of foreign currency. However, the leather tanning industry is also well known for its negative impact on the environment. The leather tanning process uses a variety of chemicals, including sulfides, chromium, lime, salts, and others, and produces large amounts of solid and liquid waste. As a result leather tanning industries are negatively associated with severe environmental pollution (Gessesse *et al*., 2011). As a result, potential strains capable of filling those gaps must be identified. The isolates are capable of reducing the costs associated with the importation of those enzymes and provide an environmentally friendly manufacturing technique.

1.3. Significance of the study

Because of their viability for large-scale synthesis in fermenters, recent advances in enzymology have piqued the interest of many researchers all over the world in producing industrial enzymes from microbes, particularly bacteria. Hydrolytic enzymes such as proteases, amylases and lipases have important role with wide range of applications in many industries particularly food processing, detergent, pharmaceutical, dairy, animal feed and cosmetics. Lipases are a type of hydrolytic enzyme that catalyzes the conversion of triacylglycerol to diacylglycerol, fatty acids, and glycerol (Soleymani *et al*., 2017). It has been estimated that about 13 billion tons of detergents are using about 1000 tons of lipase enzymes annually. In a country like Ethiopia, producing lipase enzyme from lipolytic bacteria will have different advantages. The first and most significant benefit is that the cost of importing those enzymes will be reduced. This is due to the fact that we can readily

generate enzymes from microorganisms such as bacteria in a simple and cost-effective manner, and we do not need to spend money on enzymes from other countries. Another advantage is that we can use those environmentally friendly enzymes to manage and minimize environmental pollutions. Because the enzymes are derived from natural or biological sources, such as microorganisms, they lessen pollution caused by synthetic enzymes. As a result, the current investigation has isolated and identified bacteria that are capable of producing this crucial enzyme.

1.4. Objectives of the study

1.4.1. General objective

The general objective of this study was to screen and characterize industrially important lipase producing bacteria from water and soil samples.

1.4.2. Specific objectives

The specific objectives of the study were:

- ✓ To isolate lipase producing (lipolytic) bacteria from soil and water sample.
- ✓ To screen potential lipase producing bacterial isolates from isolated bacterial strains.
- ✓ To characterize potential lipase producing isolates using microscopic and biochemical tests.
- ✓ To determine the nutritional and physicochemical factors (temperature, pH, carbon source and nitrogen sources) for lipase producing bacteria.
- ✓ To extract crude lipase enzyme from the screened isolates and determine its activity on milk.

2. Literature review

2.1. Lipase enzymes

Lipases (Triacyclglycerol acylhydrolases EC 3.1.1.3) belong to a class of hydrolases that hydrolyze fats into fatty acids and glycerol at water lipid interface (Figure-1). They are also capable of reversing the reaction in non-aqueous media (Ilesanmi *et al*., 2020). Claude Bernard discovered lipases in 1856 while researching the role of the pancreas in fat digestion. Many different lipases have been discovered and isolated from bacteria, fungi, plants, and mammals since then. The presence of lipases has been noticed as early as in 1901 for *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* which are now called *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively (Patel *et al*., 2016). They are serine-hydrolases of the alpha-beta hydrolase fold superfamily whose catalytic triad is formed by Ser-Asp/Glu-His. They do not need cofactors for their activity, although some of them exhibit increased activity in the presence of Ca^{+2} and other divalent cations. Most known lipases are active between 30°C and 60°C and generally show alkaline or close to neutral optimal pH, with some exceptions like a lipase from *Aspergillus niger* which has higher activity at pH 2.5 (Atalah *et al*., 2019).

These enzymes are adapted to operate at the interfaces of biphasic systems, which is a phenomenon known as interfacial activation. The characteristic substrate is an aggregate, micelle, or monomolecular film formed by ester molecules. In an organic medium, lipases catalyze esterification, transesterification and inter esterification reactions. The substrates of lipases are diversified and can be composed by neutral lipids, phospholipids and lyso phospholipids, or ether lipids (Melani *et al*., 2020).

These microorganisms have been discovered in a variety of environments, including oil refineries. These enzymes are employed in a variety of biotechnological processes, including cosmetics, food, leather, detergents, and pharmaceuticals. Microbial lipases production has increased for the past one decade, because of its potential application in industries. When compared with plant and animal lipases bacterial lipase were well studied. The microorganisms are usually grown in nutrient medium supplemented with carbon source, nitrogen and phosphorous. Glycerol, triglycerides and bile salts are usually used as inducer for the production of lipases (Rajeshkumar *et al*., 2013).

Lipases are ubiquitous enzymes which are found in animals, plants, fungi and bacteria (Hasan *et al*., 2018). Lipases from microbes, on the other hand, are more interesting for industrial applications because (1) they can be produced in high yields (2) there are many different catalytic activity that can be used in many applications (3) genetic manipulation is simple (Verma & Sharma, 2014). Most lipases investigated are from bacterial sources, such as enzymes from the genera *Bacillus, Geobacillus, Pseudomonas, Streptomyces, Burkholderia, Chromobacterium, Achromobacter, Arthobacter* and *Alcaligenes*. Among them, *Bacillus* lipases are the most explored enzymes, having stable activity at high temperatures in a broad range of pH conditions, besides their tolerance to organic solvents (Contesini *et al*., 2020).

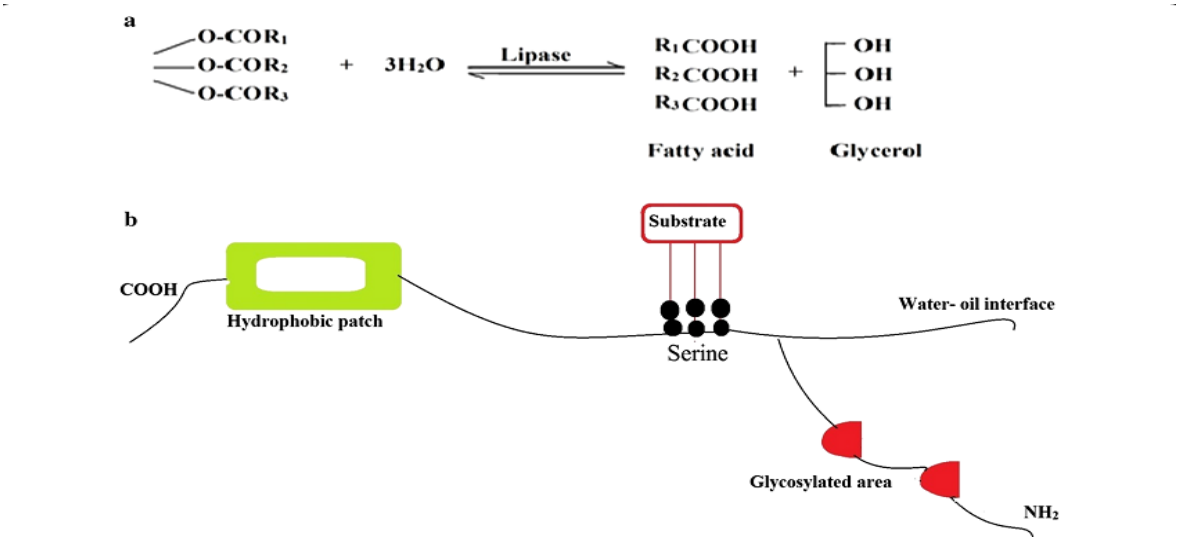

Figure 1: (a) Hydrolysis of triglyceride converts in to glycerol and fatty acid. (b) Representation of a molecule of lipase with its features (Chandra *et al*., 2020).

Unlike esterases that preferentially hydrolyze "simple" esters and triglycerides composed by short chain fatty acids (shorter than C6), lipases are mostly active against water-insoluble substrates, such as triglycerides composed by long-chain fatty acids. Consequently, lipases have specific kinetic properties, which are related to their catalytic site conformation conferred by a flexible subdomain lid or flap located over the active site. In the presence of insoluble substrates, this structure undergoes a structural change-called interfacial activation-conferring an open conformation to the catalytic site and increasing the lipase activity (Salihu &Alam, 2012). In a more aqueous condition, the lid assumes a closed structure, making the lipase inactive. Lipases, in general, act in a wide range of pH conditions. Typically, bacterial lipolytic enzymes tend to act in alkaline pH conditions,

while fungal lipases prefer acidic conditions. Apart from those aspects, lipases can be investigated based on their substrate selectivity, including regio- and enantio selectivity, which makes these enzymes excellent choices for the production of enantio pure compounds with high added value (Contesini *et al*., 2020).

2.1.1. Structure of lipase enzyme

Lipases or triacylglycerol acyl ester hydrolases belong to the serine hydrolase family; they are called carboxylic acid esterases, numbered as EC 3.1.1.3, and are a group of enzymes that are abundantly present in nature. Majority of lipases are built on α/β hydrolase fold (Figure-2) consists of a core of predominantly eight parallel β strands forming a super-helically twisted central β sheet surrounded by varying number of α helices. However, the quantity as well as organization of β strands may vary (Sarmah *et al*., 2018). For instance, *Bacillus subtilis* lipase lacked the β1 and β2 strands n the canonical fold (Contesini *et al*., 2020).

The catalytic triad, which is conserved among lipases, consists of a serine as nucleophile, an aspartate/glutamate as the acidic residue, and a histidine. It is similar to the one observed in serine proteases but with a different order in the sequence. In the alpha/beta-hydrolase fold (Figure-2), the catalytic serine is located in a γ-like turn, after the sheet-β5 and before the following α-helix, in a highly conserved structural feature of the fold. The aspartate or glutamate is found in a loop after the β7-sheet, and the histidine is located in a loop after the β8-sheet. Another subclass of esterase/lipase has been reported, in which the G-X1-S-X2-G consensus sequence containing the catalytic serine is replaced by a GDSL sequence located close to the protein N-terminus (Godoy *et al*., 2018).

The key structural components of lipase include lid, binding pocket, oxyanion hole, and disulfide bond. Lipases entail a lid or flap like structure, which is made up of one or more α helices of varying length with two hinge segments on both of its ends and it is essential for exposing a hydrophobic patch in the presence of a substrate. The binding pocket of lipases is present on the central β sheet, which can be a hydrophobic, crevice-like binding site located near the protein surface or funnel-like or tunnel-like binding sites.

Another crucial component that affects the enzyme's catalytic effectiveness is the oxyanion hole. . During hydrolysis, a negatively charged tetrahedral intermediate is generated, and the oxygen ion thus formed gets stabilized by hydrogen bonding in which the role of oxyanion hole residues is crucial. The oxyanion hole residues are important for hydrogen

bonding to stabilize this oxygen ion. . Always, one of the oxyanion hole's residue is positioned next to the nucleophilic serine residue, whereas the second residue is positioned between β3 strand and α helix. In addition, lipases are cysteine-rich proteins that contain one to four disulfide bonds to maintain their structure. Disulfide bridges formed between cysteine residues significantly contribute to conformational stability by decreasing the entropy of protein. Recently, a study has been reported on the conformational changes occurring in digestive lipases, fungal lipases, and cutinases in the presence of surfactants or inhibitors through X-ray crystallographic studies (Sarmah *et al*., 2018).

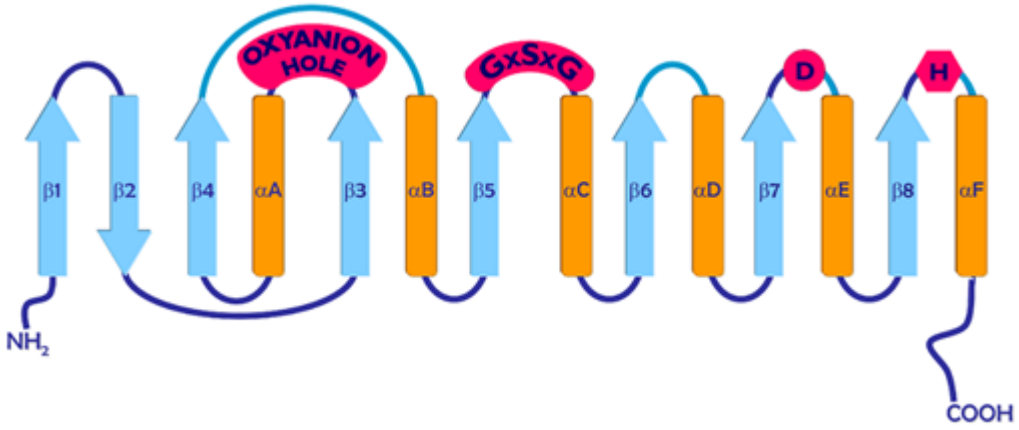

Figure 2: Canonical fold of α/β hydrolases. Strands are indicated by arrows and helices by cylinders. The positions of the histidine (H) and aspartate (D) residues, as well as the GXSXG and the oxyanion hole are shown (Ugo *et al*., 2017).

2.1.2. Major sources of lipase enzyme

Lipases are ubiquitous esterase enzymes produced by animals, plants and microorganisms (Melani *et al*., 2020). Plant lipases have been identified from oleaginous plants and cereals' leaves, oils, stems, latex, and seeds. The most appropriate approach for preparing biocatalysts from plant biomass is determined by the part of the plant and the desired degree of purification. The majority of plant lipases are available from seed sources including castor bean, African bean, elm, sunflower, physic nut, lupin, linseed, coconut almond, black cumin, wheat grain, rice, corn, oat, barley, sesame, sorghum, etc. Seeds have more lipase activity than other sources because they contain a high concentration of triacylglycerols and so serve as a source of energy for plant growth. Animal cells synthesize lipases that enable the digestion of fats and lipids (Melani *et al*., 2020). However, Because of complexities such as culture handling and product separation, animal lipases are more commonly utilized in clinical diagnosis than commercial production.

When compared with the lipases from plants and microbes, these lipases are much less studied. Amongst the various animal lipases, pancreatic lipase has been extensively used as a research tool in the fields of lipid chemistry as well as biochemistry as it effectively catalyzes the hydrolysis of primary alcohol esters (Sarmah *et al*., 2018).

2.1.2.1. Lipase production by microbial cells

Among various lipases, microbial lipases have gained superior industrial attention due to their selectivity, stability, and broad substrate specificity. Many microorganisms are known as potential producers of lipases, including bacteria, fungi and yeast (Bharathi & Rajalakshmi, 2019). Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. They are also widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications (Vasconcelos, 2018). Moreover, the higher stability of microbial enzymes than corresponding plant and animal enzymes, and the convenience and safety of their production are advantageous (Baki, 2017).

Microbial enzymes should perform well under extreme temperature and pH conditions. At the commercial scale, thermally stable enzymes reduce the risk of microbial contamination over long fermentation periods. Various new microbial enzymes have been designed for many bioprocesses with the input of rDNA technology, protein engineering, and metagenomics to improve the yield and performance of many microbial enzymes in food, pharmaceuticals, textiles, paper, leather, biotechnological (Singh *et al*., 2019).

2.1.2.1.1. Lipase production by bacterial cells

Lipase is produced by a variety of Gram-positive and Gram-negative bacteria. Some of the most commercially important lipase producing bacteria is recognized as belonging to the genera of *Bacillus*, which includes *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus alcalophilus*, *Bacillus coagulans*, *Bacillus stearothermophilus* and also some other bacterial strains such as *pseudomonas* sp., *Burkholderia* sp. and *Staphylococcus* sp. are also reported as better bacterial lipase producer. Lipase-producing bacterial strains have been discovered in a variety of environments, including oil industrial waste, vegetable oil processing plants, dairy plants, paper industries, and oil-contaminated soil (Bharathi & Rajalakshmi, 2019).

Bacterial lipases are mostly extracellular which are greatly influenced by nutritional and physico-chemical factors such as temperature, pH, nitrogen and carbon sources, inorganic salts, agitation and dissolved oxygen concentration (Aktar *et al*., 2016). Moderately halophilic bacteria which can grow optimally in media containing 3–15% NaCl are a valuable source of such enzymes. Therefore, screening and isolation of moderate and extreme halophiles from hypersaline environments, able to produce these enzymes would be useful for further industrial applications (Ghasemi *et al*., 2011). Extremophile bacteria, such as thermophiles and psychrophiles, can survive in hostile settings such as hot springs, deep marine sediments, and extreme cold, such as Antarctica, and produce a variety of lipases with distinct tolerance capabilities. The lipases from extremophiles have been the focus of many studies and according to the Genomes Online Database, there are currently 1364 metagenomics studies of environmental samples covering more than 10,000 strains. Those data bring evidence of the great potential of the uncultured microbial communities as sources for prospecting new lipases with catalytic activity for a broad array of substrates, as well as being pH and temperature tolerant (Contesini *et al*., 2020).

2.1.2.1.2. Lipase production by fungal cells

Most commercially important lipase-producing fungi are recognized as belonging to the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp. The strain, the content of the growth medium, cultivation conditions, pH, temperature, and the kind of carbon and nitrogen sources all influence lipase production in fungi. Among microbial sources, filamentous fungus are good lipase producers and the extraction, purification, and processing steps are relatively simple (Bharathi & Rajalakshmi, 2019). The isolation and selection of novel strains is prompted by the industrial desire for new sources of lipases with various catalytic properties. Lipase producing microorganisms have been found in different habitats such as industrial wastes, vegetable oil processing factories, dairy plants, and soil contaminated with oil and oilseeds among others (Thakur, 2012). Yeasts are frequently employed in food applications and a variety of industrial biotransformations since they have the GRAS (Generally Recognized as Safe) designation and do not contain toxins or other pathogenic compounds. The major genera of lipase producers are *Candida, Rhodotorula, Yarrowia, Geotrichum* and *Trichosporon* (Melani *et al*., 2020).

2.1.3. Lipase catalyzed reaction

Lipases are the most adaptable biocatalysts, capable of carrying out a wide range of biotransformation processes. (Figure-3), i.e., hydrolysis, esterification, interesterification, aminolysis, alcoholysis and acidolysis (Joshi & Kuila, 2018). (I)Hydrolysis: This reaction occurs in the presence of large amount of water, where breakdown of ester bond take place. This technique is currently used in the formation of fatty acids, monoglycerides, diglycerides, flavoring substance for detergents and dairy products. (II) Esterification: It happens under little water environment (anhydrous solvents). High quantity of the products (esterified compounds) is achieved under regulated environment. Formation of Primary (oleic acid esters) and Secondary (aliphatic and terpenic) are most common example. (III)Transesterification: It includes the exchange of acid moiety between two or more compounds. According to the type of substrates, lipases are able to catalyze acidolysis (it involve ester and carboxylic acid), alcoholysis (it involve ester and an alcohol), aminolysis (ester is allowed to react with amine) and interesterification (where two acyl groups are exchange between two esters).

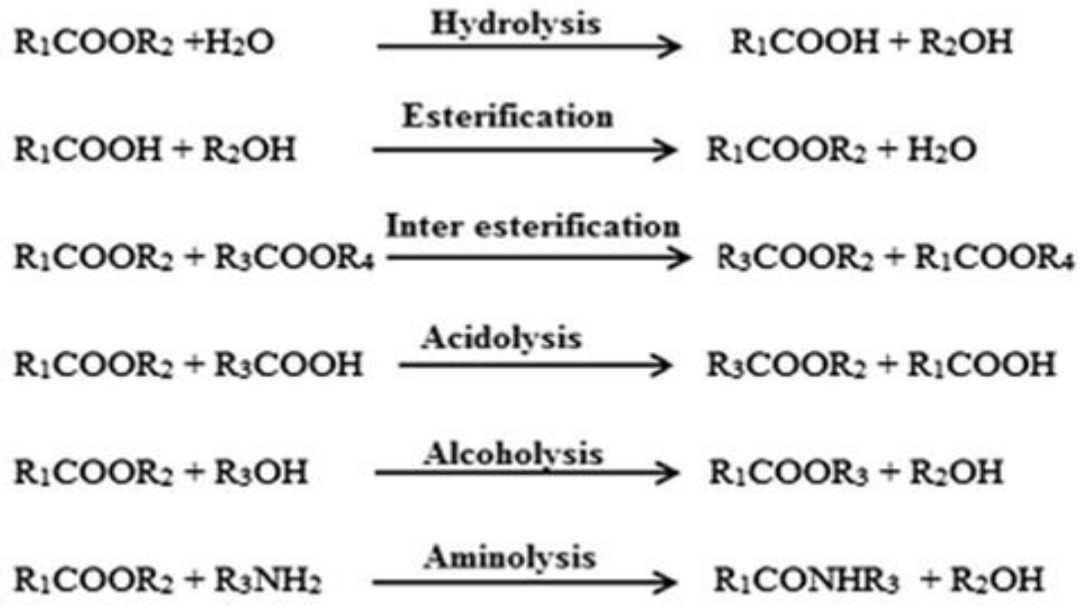

Figure 3: Lipase catalyzed different reactions (Chandra *et al*., 2020)

2.2.4. Major applications of lipase enzyme

Lipases play important role in the industries ranging from medicine, food, agrochemical, dairy and detergents to oleo-chemicals, cosmetics, leather, and tea industries and in numerous bioremediation procedures (Sharma *et al*., 2016). Lipase's versatility is due to a number of features, including their regiospecific, enantiospecific, and

chemospecific nature, as well as their capacity to catalyze reactions in both aqueous and non-aqueous conditions and to catalyze forward and backward reactions. Moreover, in immobilized form, lipase offers certain processing advantages including ease of separation from the product mixture, improved stability, and continuous operation that are essential requirements for any large scale production process (Sarmah *et al*., 2018).

2.2.4.1. Application of lipase enzyme in detergent industry

In industrialized countries, enzymes are widely utilized in detergent composition. Lipases, as well as other enzymes including amylases, cellulases, and proteases, are utilized in the detergent industry because they can break fats/oils, starch, cotton-fluff, and proteins (Sharma *et al*., 2016). These enzymes make the detergent safer for the environment by lowering the environmental load and saving energy by allowing for a lower wash temperature. Also, the products are mostly biodegradable and leave no harmful residues (Pascoal *et al*., 2018). Lipases for detergent formulation are selected to fulfill the following requirements: (i) ability to hydrolyze the fat of different compositions (lower specificity to substrate); (ii) ability to tolerate relatively tough conditions (pH 9.0-11.0, 30-60ºC) of washing; and (iii) ability to tolerate damaging surfactants (linear alkyl benzene sulfonates) and enzymes (proteases), both are chief constituents of several detergent formulations (Sharma *et al*., 2016).

Lower washing temperatures, which help to retain fabric quality while also saving energy, have become a major emphasis in these industries in recent years. *Pseudomonas* ADT3 purified lipases were found useful in the manufacturing of detergents. Cold-active lipases can be used as a detergent additive and are also utilized in laundry to wash textiles at a low temperature. Few lipases obtained from strain likes by *Pseudomonas aeruginosa* BUP2 have a high specific activity which makes them efficient to be significantly used in detergent industries. Frequently used lipases in the detergent industry are of *Bacillus licheniformis, Geobacillus sp., Serratia marcescens* DEPTK21, *Bacillus flexus* XJU-1, *Bacillus pumilus* SG2, *Staphylococcus arlettae, B. cepacia, P. fluorescensand Candida* species. A patent on the use of lipase in laundry detergent for cold-water cleaning was recently registered (Patel *et al*., 2018).

2.2.4.2. Application of lipase enzyme in food industry

These enzymes have long been employed in the food business, particularly in the dairy sector, to hydrolyze milk fat, modify fatty acid chain lengths to improve cheese qualities,

and, more recently, to expedite cheese ripening and lipolysis of butter fat and cream. Lipases have also been utilized to selectively hydrolyze fat, allowing it to be employed in the creation of flavored products, butter substitutes, and other additives for cereals, snacks, and chewing. The addition of these hydrolysates provides a variety of food sensory characteristics (Pascoal *et al*., 2018).

By liberating short chain fatty acids during esterification, lipases are employed to improve the flavor of bakery items. Lipases can help extend the shelf life of bakery items. Catalyzation of lipases helps in improving the softness and texture of the bakery products. Enzymes productivity can be increased by using yeast with bacterial lipase gene LIP A and these can be used as a technological additive in bread making industry (Patel *et al*., 2018).

2.2.4.3. Role of lipase enzyme in bioremediation

Bioremediation is the process employed to decontaminate samples from oil spills, oil-wet soils, industrial wastes, and wastewater tinged with lipids that can otherwise have hazardous consequences when they enter natural resources without prior treatment. Lipase-catalyzed bioremediation steps can be adopted to treat the wastes effectively from lipid processing factories and restaurants (Sarmah *et al*., 2018).

Employment of lipases in bioremediation process is a new aspect in lipase biotechnology. Lipases from various sources could be used to clean up oil spills in refineries, shore sand, and processing plants. It has been also used for the degradation of wastewater contaminants such as olive oil from oil mills. Another important application has been reported for the degradation of polyester waste and removal of biofilm deposits from cooling water systems (Patel *et al*., 2016). When compared to traditional approaches, using bacterial lipolytic in the bioremediation process is more energy intensive, environmentally favorable, and effective (Soleha & Retnaningrum, 2020).

2.2.4.4. Application of lipase enzyme in pulp and paper industry

Pitch, or hydrophobic components found in wood (mostly triglycerides and waxes), is one of the most significant difficulties related with the pulp and paper industry (Joshi & Kuila, 2018). Lipases are one of the most significant enzymes that can assist remove these pitches from pulp, which can then be utilized to make paper (Patel *et al*., 2018). Nippon Paper Industries in Japan developed a pitch control method that used a fungal lipase from *Candida rugosa* to hydrolyze up to 90% of the triglycerides. Hata and coworkers at Jujo Paper Company reported in 1990 that lipases could reduce pitch problems by lowering

the triglyceride content of ground wood pulp. A lipase obtained from *Candida cylindrica,* when added to the ground wood stock chest, reduced pitch problems and talc consumption considerably. *Candida antartica* lipase A (CALA) was used in pitch control in the paper industry (Mehta *et al*., 2017).

2.2.4.5. Role of lipase enzyme in fat and oil processing

One of the most important applications for lipase in the food processing sector is fat and oil modification, which results in better food products. Lipases are used to modify lipid properties vegetable oils like sunflower, coconut, olive, corn and rice bran oil that are rich in omegax-6 fatty acids and fish, linseed oil, walnuts and milk that are rich inx-3 fatty acids, both of which play an important role in an individual's health by maintaining the essential fatty acid levels within the permissible limits (Sarmah *et al*., 2018). Lipases are used to catalyze the hydrolysis, esterification, and inter-esterification of oils and fats. Lipases can be utilized in their pure form, cell-bound form, or immobilized form for hydrolysis of fats and oils. It helps in modifying the lipids property by altering the position of the fatty acids and replacing one or more of these to the new ones in the glycerides. This results in the production of lipids of higher value, which is cost effective simultaneously (Patel *et al*., 2018).

2.2.4.6. Role of lipase enzyme in pharmaceutical industry

Pharmaceutical companies are interested in some lipases because of their medicinal potential. Several medications for the treatment of cardiovascular disease, obesity, anxiety, inflammation, and pain have been authorized or are in clinical studies. The organic chemical and pharmaceutical use of lipases in the preparation of optically active compounds, such as pure alcohols, amines and carboxylic acids, demonstrate that these enzymes can be used in practical synthetic methods (Melani *et al*., 2020).

Lipase is the primary enzyme for the fat metabolism and the lack of which will pose a threat to the health directly and hence the pharmaceutical industries use lipase in productive processes. This is because of the excellent capability of lipase for specific regio, enantio, and chemo selective reactions in a variety of organic solvents with extensive substrate recognition, their use in resolution of racemic mixtures and their ability to catalyze reactions even in biphasic systems. Microbial lipases are used for the hydrolysis of racemic esters, for trans esterification and racemization in situ to yield optically pure enantiomers for the manufacture of chiral compounds. For example, Profens (2-aryl

propionic acids), an important group of non-steroidal anti-inflammatory drugs that are active only in a specific conformation are synthesized by enantio selective esterification catalyzed by lipase originating from *C. antartctica* and *C. rugosa* (Sarmah *et al*., 2018). Lipase can potentially be employed as a tuberculosis diagnostic tool (TB). Mycobacterium tuberculosis secretes lipase, which may be tested with high specificity and sensitivity to check for infection. Lipase levels in blood serum can be utilized as a diagnostic test for acute pancreatitis and pancreatic damage. Acute pancreatitis is usually caused due to misuse of alcohol or bile duct obstruction. Lipases are used in making hair waving, as a component of topical anti-obese creams. These are also used as digestive aids and for the treatment of malignant tumors because lipases are found as activators of tumor necrosis factor (Javed *et al*., 2018).

2.2.4.7. Application of lipase enzyme as biosensor

Estimation of lipids in analytical samples of patients in order to diagnose cardiovascular complaints is a critical use of microbial lipases. Lipase catalyzes the hydrolysis of triacylglycerol contained in the analytical sample into glycerol and free fatty acids, which is the core idea of quantitative estimate.. Then the amount of released glycerol or free fatty acids is determined by enzymatic and chemical method. In this way the level of lipids in the patient sample can be determined. *C. rugosa* lipase biosensor system has been developed. Because of its high specific activity, *C. rugosa* lipase is nonspecific and facilitates rapid liberation of glycerol and fatty acids from lipid (Sarmah *et al*., 2018).

2.2.4.8. Role of lipase enzyme in leather industry

Lipases have recently been used in the soaking, dehairing, bating, and degreasing processes in the leather industry. Hides and skins contain proteins and fat in the collagen fibers. Before the hides and skins are tanned, these substances must be removed partially or completely. Lipases specifically degrade fat and do not damage the leather itself (Mehta *et al*., 2017). Traditional methods for removing fat from animal skins, such as using organic solvents and surfactants, can be damaging to the environment since they produce hazardous end products such as volatile organic compound (VOC) emissions. Usage of lipases in association with other hydrolytic enzymes like proteases is a new approach in leather processing. Since the process in carried out at alkaline pH, alkalophilic lipases are used in combination with alkaline or neutral proteases and other necessary hydrolytic enzymes (Patel *et al*., 2016).

2.2.4.9. Role of lipase enzyme in biodiesel production

Biodiesel is a group of esters produced by transesterification reaction between fatty acids and an alcohol in presence of catalyst (Mehta *et al*., 2017). The process of producing biodiesel as an alternative fuel using natural oils and fats is environment friendly since these substrates are free of nitrogen and sulphur compounds. This method will significantly minimize the greenhouse effect and pollution caused by fossil fuels (Salihu, 2012). Lipases can make biodiesel in the presence of a lot of water, which is a good method when using waste oils because they usually have a lot of water molecules in them (Melani *et al*., 2020).

Immobilized *P. cepacia* lipase was used for the transesterification of soybean oil with methanol and ethanol. Fatty acid ethyl esters have also been prepared from castor oil using n-hexane as solvent and two commercial lipases, Novozym 435 and Lipozyme IM, as catalysts. In a solvent-free media, Novozyme 435 has also been utilized to catalyze the transesterification of crude soybean oils for biodiesel generation (Hasan *et al*., 2006).

2.2.4.10. Application of lipase enzyme in oleochemical industry

In the oleochemical business, reactions such as hydrolysis, alcoholysis, and glycerolysis using mixed substrates or immobilized lipases are used. Immobilized lipases can improve productivity and the continuous operating process (Patel *et al*., 2018). There are huge differences between the chemical and lipase-catalyzed reactions. The lipase-catalyzed reactions can be carried on in weaker conditions than the chemical reactions. This is advantageous because undesirable side reactions such as heat degradation of the substrates can be prevented. The use of lipases in the oleochemical industry reduces thermal deprivation during glycerolysis, hydrolysis, alcoholysis and acidolysis. Oil spills in processing factories, refinery and shore sand could be handled by the use of lipases (Sharma *et al*., 2016).

3. Materials and methods

3.1. Description of the study area

Lake Ziway (Figure-4) is a shallow freshwater lake located in the northern section of the Rift Valley. It is located in the Oromia regional state's East Showa zone, some 160 kilometers from Addis Ababa. Lake Ziway has a surface area of 434 km^2, a depth of 4 meters, and a height of 1636 meters above sea level. The Ziway Catchment is located in between 7° 15′N to 8° 30′N latitude and 38° E to 39° 30′E longitude, covering a total area of about 7300 km^2 (Teklu *et al*., 2016). Adama is located in east Shoa zone, Oromia regional state, located at 99 km south east of Addis Ababa, the capital city of Ethiopia. The town is located at an average elevation of 1712 meters above sea level and an average monthly temperature of 21^oC. The climate of this area is hot and arid climate type.

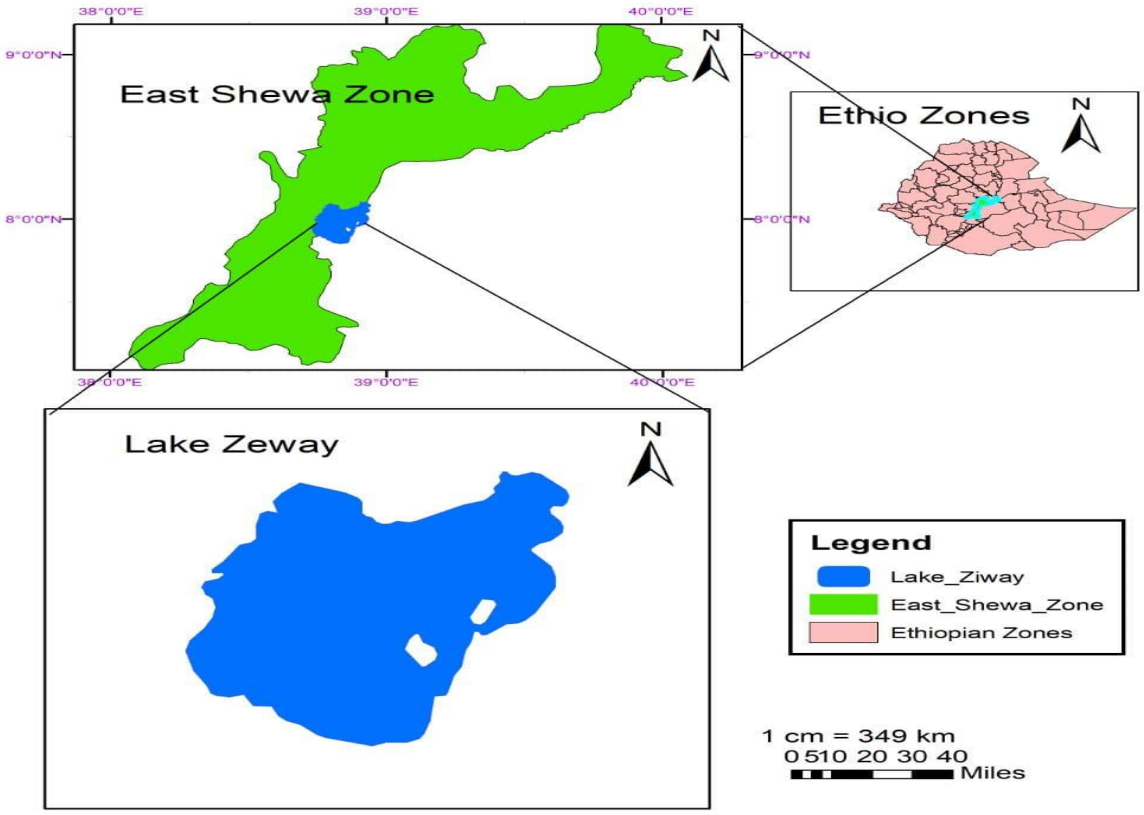

Figure 4: Location map of the study site

3.2. Sample collection

A total of 300g soil/sludge and 250ml water samples were collected from Lake Ziway and its surrounding area where more fishing activity is performed. The sludge and waste water

samples were also collected from ASTU's wastewater treatment plant. After removing the uppermost layer of 3-5cm, composite mixtures of sediment/soil samples were also collected. The sediment/soil was collected using sterile plastic bags. However, the water sample was collected using sterilized sampling bottles. The samples were labeled with date of collection, sample number and transported to ASTU Applied Biology laboratory for further analysis. This was performed according to Standard Operating Procedure for Collection of Soil and Sediment Samples for the Sediment-bound Contaminant Resiliency and Response (SCoRR) Strategy Pilot Study.

3.3. Isolation of lipase producing (lipolytic) bacterial isolates

A 1g of soil/sludge sample was dissolved in 9ml of distilled water (1ml of water sample in 9 ml of water) (w/v). A serial dilution was performed from 10^{-1} to 10^{6}. From the dilutions, a 0.1 ml sample was applied onto nutrient agar medium (Peptone, 5g/l; Beef extract, 3g/l; NaCl, 8 g/L; Agar, 15g/l and 6.8 ± 0.2 pH) and incubated at 37°C for 24h (Malik & Naz, 2019). During inoculation, spread plate technique was employed. Pure cultures were prepared by taking a single colony and transfer into other fresh nutrient agar medium. These pure cultures were maintained on slant agar at 4^{o}C until used for further analysis.

3.4. Screening of lipase producing potential isolates from collected soil, water and sediment samples

Pure isolated bacterial cultures were screened for lipase activity using tributyrin agar and phenol red agar. The tributyrin agar medium pH 7.5 was prepared with 5g/L peptone, 3g/L yeast extract, 15g/L agar,10ml/L tributyrin/olive oil in distilled water. The medium was sterilized for 15min by autoclaving with pressure at 121°C and cooled. The medium was transferred to petri dishes and allowed to solidify. A loopful of each pure culture was streaked each onto tributyrin agar plates separately and incubated at 37°C for 48h (Ilesanmi *et al*., 2020). The isolates that show the best growth will be selected for further analysis.

Phenol red olive oil agar plates were prepared as follows (g/L); 0.01% (w/v) phenol red, 0.1% (w/v) CaCl2, 1% (v/v) substrate, 2% (w/v) agar and pH adjusted to 7.3-7.4 with 0.1 N NaOH. Organisms were inoculated onto the phenol red agar plates supplemented with 1% substrate and incubated at 37°C for 2-4 days. The principle behind this assay is that a slight drop in pH from 7.3 (end point of the phenol red dye) to a more acidic pH will result in a change of color from red to orange (Ramnath *et al*., 2017).

3.5. Characterization of lipase producing isolates

3.5.1. Morphological and biochemical characterization

Identification and characterization of bacteria were carried out by microscopic characterization, biochemical tests, and Proteomic identification (MALDI–TOF MS) as follows.

3.5.1. Microscopic identification

Gram staining methods was used to distinguish the isolated strains as Gram positive or Gram negative bacteria. The cells were heated to adhere to the slide and stained with crystal violet for 1min before being mordant with an iodine solution for 1 min. The stained cells then washed with ethanol. Gram-positive cells retain the stain, whereas gram-negative cells do not. The preparation was counterstained with a contrasting red dye, safranin for 45sec, to make the contrast between the two results evident. Gram negative cells turned red and were easily seen. Finally, the slide was examined using a microscope with an oil emulsion objective set to 100X magnification (Gram, 1884).

3.5.2. Biochemical characterization

Different biochemical tests were performed to identify the isolated bacteria strains and described as follows;

3.5.2.1. Indole test

This test was used to identify microbes that can breakdown tryptophan to indole. Sterilized tubes containing tryptophan broth (4ml) were inoculated with a loopful of the isolates and incubated at 37^{o}C for 24-28 h. At the end of incubation period 0.5ml of Kovac's reagent was added. Presence/absence of ring was used to judge as positive/negative test for indole production ability (Dawodu & Akanbi, 2021).

3.5.2.2. Methyl Red Voges Proskauer test

The MR-VP test was performed as prescribed by Bhagya *et al*. (2019). Briefly, methyl Red and Voges Proskauer test (MR-VP) broth prepared in two sets was inoculated with a loopful of the bacterial isolates and incubated for 48h at 30°C. Two or three drops of methyl red alcoholic solution were added in the first tube. The appearance of distinct red color was used as indicative of positive reaction for MR test. In the second set of tube, α-naphthol solution (5 percent solution in 70% ethyl alcohol) (v/v) was added and shaken

gently for 15minute. The positive reaction of acetyl methyl carbinol production was indicated by development of red color. This indicated positive result for the VP test.

3.5.2.3. Citrate utilization test

Bacterial isolates were inoculated on Simmons citrate agar medium and slants were incubated at 37°C for 48 h. Discoloration of medium from green to blue color due to change in pH indicates positive reaction. Citrate present in the Simmons medium was used as its carbon and energy source (Bhagya *et al*., 2019).

3.5.2.4. Catalase test

A drop of 3% hydrogen peroxide solution was added to the sterile slide containing a loopful of the organism. Foaming or bubble indicates a positive result (Dawodu & Akanbi, 2021).

3.5.2.5. Urease test

The bacterial isolates were inoculated to the urea agar plates and incubated for 24 to 48 hours. The change of color of the medium to pink indicate positive test for Urease production (Bhagya *et al*., 2019).

3.5.2.6. Triple sugar iron (TSI) test

Slants of triple sugar iron medium were prepared in the test tubes by autoclaving. Using sterile technique; small amount of the experimental bacterium from 24h old pure culture was inoculated into the tubes by means of a stab and streak inoculation method with an inoculating needle. The screw caps were not fully tightened and the tubes were incubated for 24h at 37°C. Fermentation is indicated by yellowing of the butt and the slant of Triple Sugar Iron (TSI) agar medium. If gas was formed during the fermentation, it is shown in the butt either by the formation of bubbles or cracking of the agar (Aditi *et al*., 2017).

3.5.2.7. Motility test

Semisolid nutrient agar medium tubes were prepared and bacterial culture was stabbed into the center of tubes containing semi solid medium and motility was observed by its growth in medium (Bhagya *et al*., 2019).

3.5.2.8. Hydrogen sulfide producing test

Sulfide indole motility (SIM) medium was used to study hydrogen sulfide production. SIM agar stab was prepared; the stab was inoculated with bacterial isolates. After

inoculation, stabs were incubated at 30°C for 48h. After 48h of incubation, black coloration along the line of stab inoculation indicated H_2S production (Bhagya *et al*., 2019). Hence, it indicates the potential of the bacteria to reduce sulfur containing compounds.

3.5.3. Identification of Lipase producing bacterial isolate using MALDI–TOF MS

MALDI–TOF MS (specification) was used to assess all isolates. The samples were first inoculated into nutrient agar for 24 hours at 37°C. Each colony was transferred to a microplate (96 MSP, Bruker®– Billerica, USA). The bacterial sediment was covered with a lysis solution (70% formic acid; Sigma–Aldrich®). Then a 1-L aliquot of matrix solution (alpha-ciano-4-hidroxi-cinamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma–Aldrich®) was added. The spectra of each sample were generated in a mass spectrometer (MALDI–TOF LT Microflex, Bruker®) equipped with a 337 nm nitrogen laser in a linear path, controlled by the Flex Control 3.3 (Bruker®) program. The spectra were collected in a mass range between 2000 and 20,000 m/s, and then were analyzed by the MALDI Biotyper 2.0 (Bruker®) program, using the standard configuration for bacteria identification, which compared the spectrum of the samples with the references in the database.

3.6. Optimization of conditions for lipase producing bacteria

3.6.1. Determination of suitable carbon source for bacterial isolate

Different oils were used as carbon sources. Different oils like olive oil, fried oil, tween 80 and tween 20 were used. A 1% each these oils were added into mineral salt medium containing fresh bacteria. The media was incubated at 37°C for 48 hours and optical density (OD) was measured (Jaiswal *et al*., 2017).

3.6.2. Determination of suitable nitrogen source for bacterial isolate

In this study, various 1% (w/v) nitrogen sources like yeast extract, tryptone, ammonium chloride and ammonium sulphate were added to the mineral salt medium (Dipotassium hydrogen phosphate 1.73g/l, Potassium dihydrogen phosphate 0.68g/l, Magnesium sulfate heptahydrate 0.1g/l, Sodium chloride 4g/l, Ferrous sulfate heptahydrate 0.03g/l, Ammonium nitrate 1g/l, Calcium chloride 0.02 g/l and Glucose 5g/l) to evaluate the effect of nitrogen source on enzyme activity. Remaining parameters were unaltered (Sagar *et al*., 2013).

3.6.3. Determination of optimum pH value for bacterial isolate

The organism was grown in mineral salt medium; media were adjusted to different pH (4.5, 6.5, 8.8 and 12) and incubated at 37^{o}C for 48hrs. The OD_{660nm} was measured to identify the optimum growth of pH value for these isolates (Ilesanmi *et al*., 2020).

3.6.4. Determination of optimum temperature for bacterial isolate

The bacteria isolate were cultured for different temperatures; 25^{o}C, 30^{o}C, 37^{o}C and 45^{o}C to select the optimum temperature for maximum growth by keeping the remaining parameters constant (Sagar *et al*.,2013).

3.7. Submerged fermentation for extraction of lipase production

The lipolytic bacterial species were inoculated in to the lipid medium for the production of lipase enzymes. The medium consists of peptone 5g/l, yeast extract 3g/l, NaCl 0.5g/l, $CaCl_2$ 0.05g/l, olive oil 5 ml and pH adjusted using optimum pH value in 250ml flask. Inoculated flasks were incubated on a shaking incubator for 48 h at 37°C. After 48 hours of incubation, the inoculated bacterial isolates were centrifuged at 4000 rpm for 20 minutes to collect the supernatant as a crude lipase enzyme (Malik & Naz, 2019).

3.8. Application of crude lipase enzyme

Application of the crude lipase enzyme was done by testing the ability of the enzymes to hydrolyze the fat found in milk. For this purpose 5ml milk, 7ml sodium carbonate and phenolphthalein were prepared and shaken for mixing in two test tubes. A 1ml of distilled water was added to the first test tube and 1 ml of the prepared enzyme was added to the second test tube. The change in color seen in the two test tubes was recorded (Lucas, 1894).

3.9. Data analysis

Experiments were performed in triplicate and the optimization data was analyzed and interpreted using Microsoft Excel 2010 and SPSS version 26 Software. Identification of bacterial species was performed ABIS version 12 online and MALDI-TOF MS.

4. Results

4.1. Isolation and screening of lipase producing potential isolates

In this study, a total of 11 bacterial isolates (Table-1) showing their best growth on tributyrin agar and phenol red agar were selected to be potential lipase producers. The isolates were labeled as (LPBI-1 to LPB1-11). Four bacteria isolates were obtained from water sample collected from Lake Ziway. Four bacteria isolates were obtained from soil sample collected from nearby soil of Lake Ziway. One bacterium isolate was obtained from liquid sample which was taken from activated sludge of ASTU WWT plant. One isolate was also selected from ASTU WWT plant from the inlet part and the last isolate was retrieved from sludge sample collected from ASTU WWT plant. Among those eleven isolates four best isolates were selected for further studies (Figure-5a, & b).

Table 1: Lipase producing bacterial isolate code and their sources

S.N	Sample source	Lipase producing isolates
1	Water sample from Lake Ziway	LPBI-1, LPBI-2, LPBI-3 and LPBI-4
2	Soil sample from Lake Ziway	LPBI-5, LPBI-6, LPBI-7 and LPBI-8
3	Sample from ASTU WWT plant (sludge, activated sludge and from Inlet)	LPBI-9, LPBI-10, LPBI-11

Key Note: LPBI= Lipase producing bacteria isolate

ASTU= Adama Science and Technology University

WWT= Waste Water Treatment

As indicated in Figure-5 and Figure-6 certain lipase producing isolates were able to grow and produce lipase enzyme when they had inoculated on to tributyrin and phenol red agar. These isolates were typically including such as LPBI-2 which recognized to be *Aeromonas veronii* and LPBI-4 that identified as *Pseudomonas aeruginosa.*

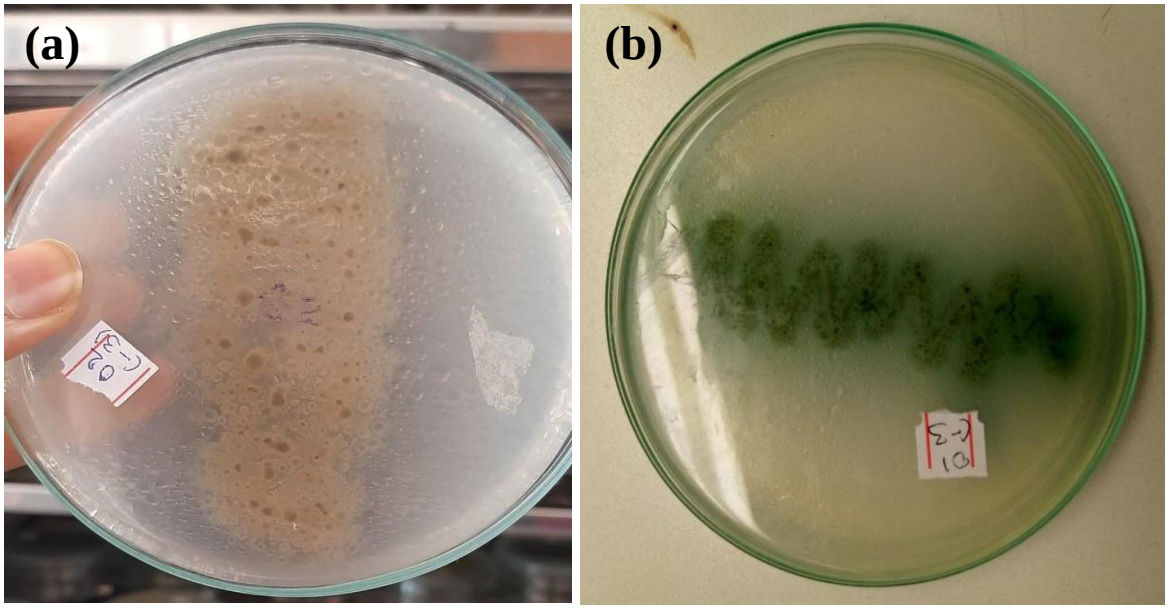

Figure 5: Growth of selected lipase producing bacterial isolates in tributyrin agar. (a) LPBI-2 that grown on tributyrin agar and found to be *Aeromonas veronii.* (b) LPBI-4 that grown on tributyrin agar found to be *Pseudomonas aeruginosa.*

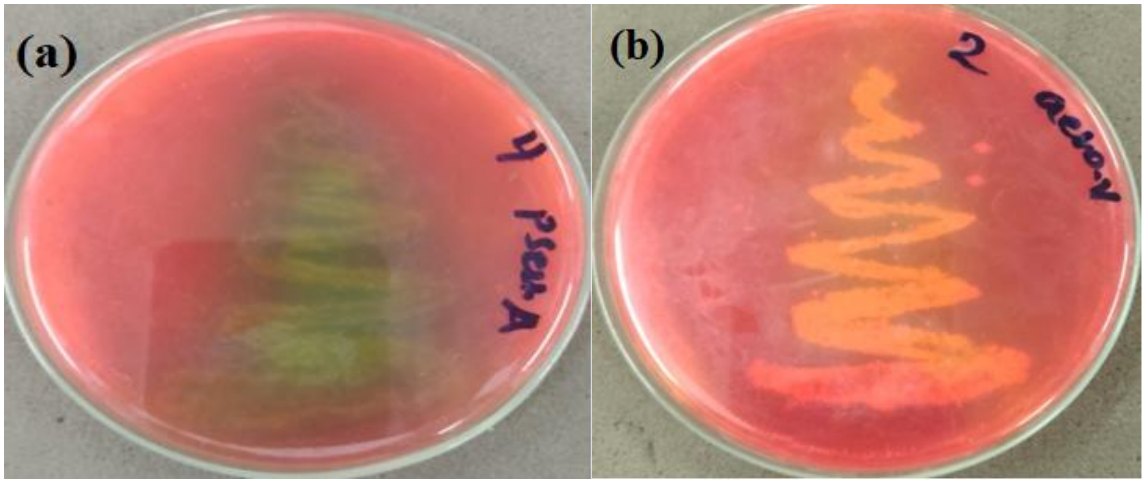

Figure 6: Growth of selected lipase producing bacterial isolates in phenol red agar. (a) LPBI-4 that grown on phenol red agar and found to be *Pseudomonas aeruginosa.* (b) LPBI-2 that grown on phenol red agar found to be *Aeromonas veronii.*

4.2. Characterization of lipase producing isolates

4.2.1. Microscopic characterization of lipase producing isolates

Gram staining technique was performed for all 11 isolates and the result for the selected four isolates revealed that all the four isolates were gram negative and rod shaped (Table-2 and Figure-7).

Table 2: Microscopic features of four selected bacterial isolates

S.N	Isolates	Gram reaction	Shape
1	LPBI-1	Negative	Rod shaped
2	LPBI-2	Negative	Rod shaped
3	LPBI-3	Negative	Rod shaped
4	LPBI-4	Negative	Rod shaped

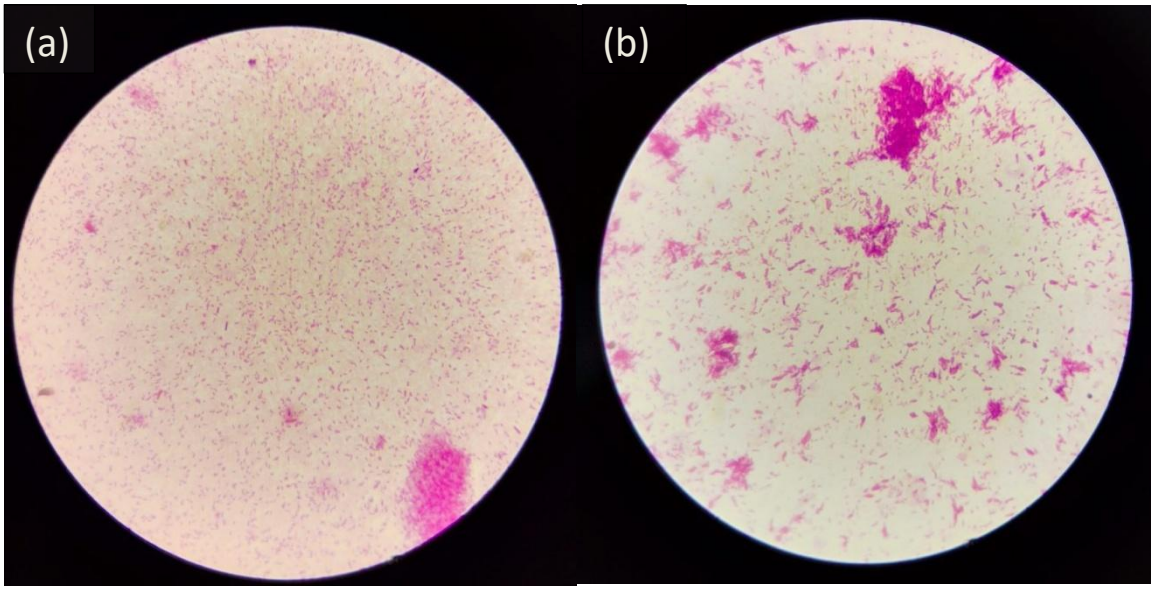

Figure 7: Microscopic characterization of lipase positive bacterial isolates. (a) LPBI-4 that isolated from Water sample from Lake Ziway and identified to be *Pseudomonas aeruginosa*. (b) LPBI-2 that isolated from water sample of Lake Ziway and identified as *Aeromonas veronii.*

4.2.2. Biochemical characterization of the four selected isolates

Biochemical characterization of the eleven isolates was performed and the result of the four selected isolates was described in Table-3. Isolate LPBI-1 showed positive result for most biochemical tests such as citrate utilization, urease, and catalase but showed negative for indole production, sucrose and lactose fermentation. Isolate LPBI-2 showed positive results for tests such as catalase, motility, methyl red and negative for citrate and urease tests. LPBI-3 and LPBI-4 have showed positive response for citrate, catalase, motility, and negative test for urease, hydrogen sulfide production, Voges-Proskauer and other tests.

Table 3: Biochemical features of four selected bacterial isolates

S.N	Biochemical tests	Results			
		LPBI-1	**LPBI-2**	**LPBI-3**	**LPBI-4**
1	Citrate utilization	+	+	+	+
2	Urease	+	-	-	+
3	Catalase	+	+	+	+
4	Indole production	-	+	+	-
5	Motility	+	+	+	+
6	MR(methyl red)	-	+	+	-
7	VP(voges proskauer)	-	+	+	-
8	Hydrogen sulfide production	-	+	+	-
9	Gas production	+	+	+	+
10	Glucose fermentation	+	+	+	+
11	Lactose fermentation	+	-	-	+
12	Sucrose	+	-	-	+
13	Nitrate reduction	+	+	+	+

Key note: + = positive, - = negative.

Depending on the result shown by microscopic and biochemical tests which are described in Table-2 and Table-3, the four isolates LPBI-1, LPBI-2, LPBI-3 and LPBI-4 were characterized using Advanced Bacteria Identification Software (ABIS version12) and MALDI-TOF MS. Accordingly, LPBI-1 and LPBI-4 were identified as *Pseudomonas aeruginosa* and the other LPBI 2 and LPBI 3 were identified as *Aeromonas veronii.*

4.2.3. Proteomic identification (MALDI–TOF MS)

The isolates are identified as *Pseudomonas aeruginosa* and *Aeromonas veronii.* In this study, these isolates were frequently obtained from Ziway lake water sample.

4.3. Optimization of conditions for lipase producing bacteria

4.3.1. Determination of suitable carbon source for bacterial isolate

Pseudomonas aeruginosa showed considerable growth on T-20, T-80, olive oil, and fried oil. However, the highest growth was seen when 1% olive oil was used as carbon source (Figure 8). Statistically, with respect to cell dry weight, significant difference was observed between tween 20 and olive oil but no significance difference was shown between olive oil and the other two carbon sources at ($p \leq 0.05$) significance level. This was also analyzed for optical density (OD_{600}) value and all carbon sources showed significance difference as indicated in figure 8.

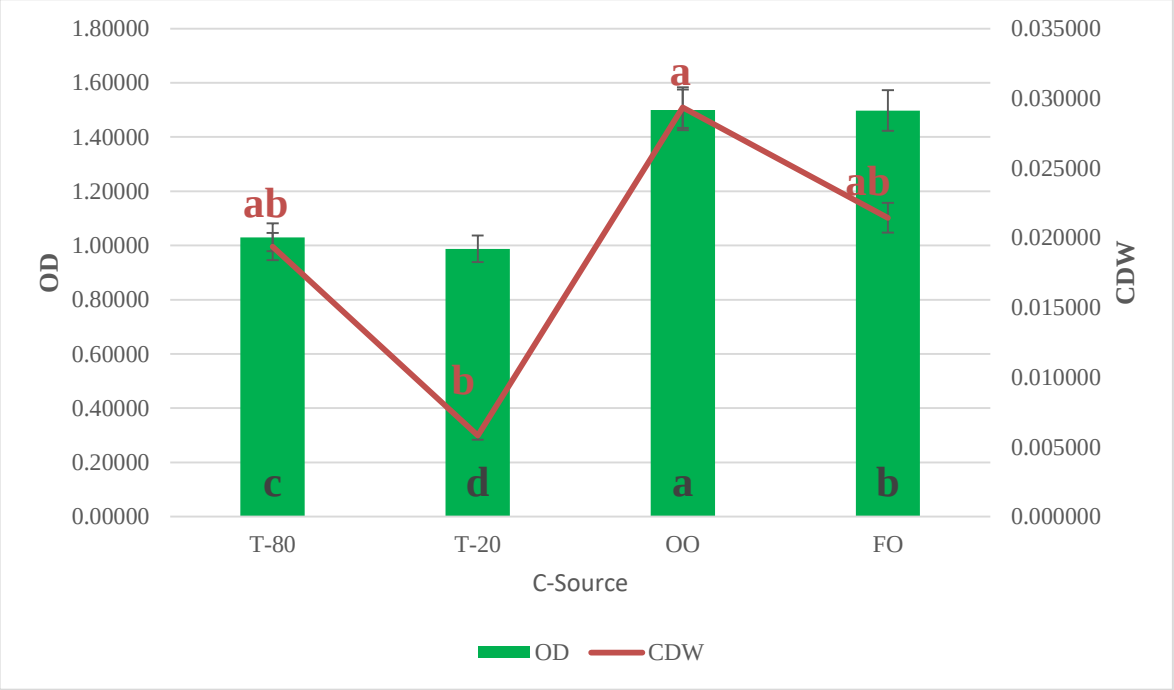

Figure 8: **Figure 9: effect of carbon source on growth of *Pseudomonas aeruginosa.* OD= optical density, CDW= cell dry weight, T-20= tween 20, T-80= tween 80, OO= olive oil, FO= fried oil. The same letters in the error bars shows no significance different values based on one-way ANOVA and Tukey's honestly significance difference (HSD) post hoc test ($p \leq 0.05$).**

4.3.2. Determination of suitable nitrogen source for bacterial isolate

Among the four tested nitrogen sources for *Pseudomonas aeruginosa*, Tryptone showed the highest growth followed by yeast extract, ammonium sulphate and ammonium chloride. Significance difference in growth conditions was seen among the four nitrogen

sources ($p \leq 0.05$) with respect to optical density (OD_{600nm}). Cell dry weight showed no significance difference among the four nitrogen sources (Figure 9).

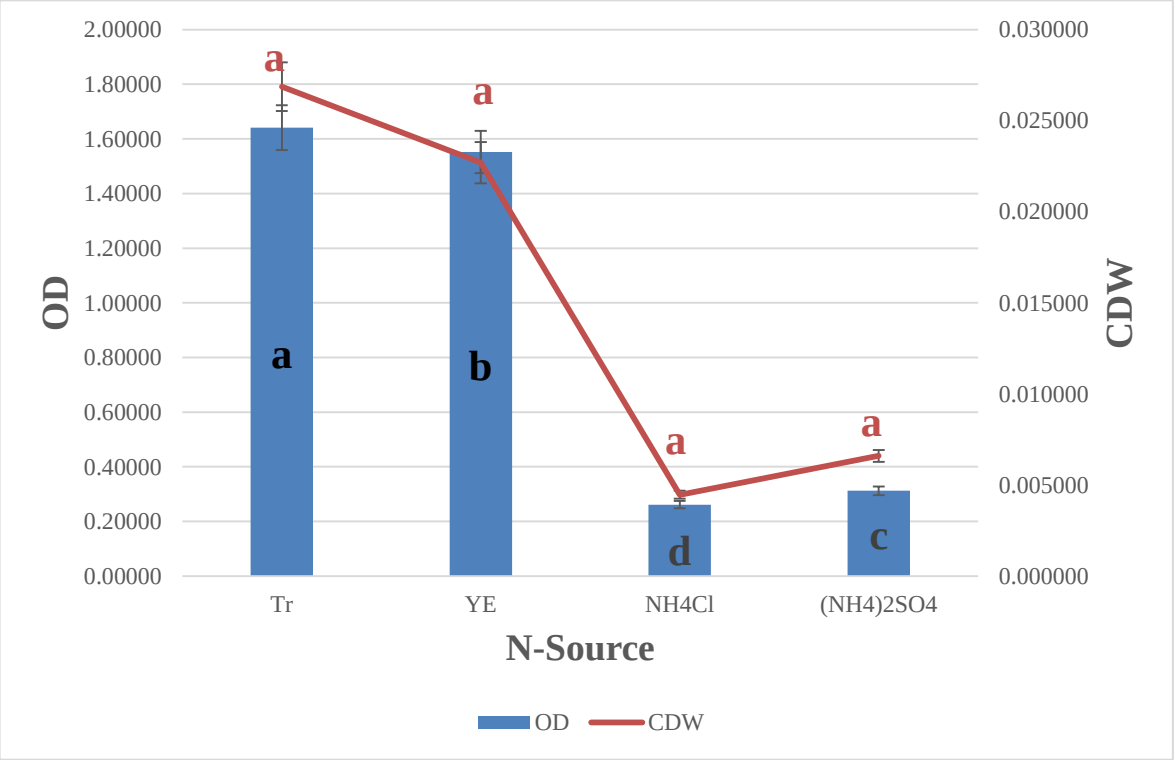

Figure 10: effect of nitrogen source on growth of *Pseudomonas aeruginosa*. OD= optical density, CDW= cell dry weight, Tr= tryptone, YE= yeast extract, NH_4Cl= ammonium chloride, $(NH_4)_2SO_4$= ammonium sulphate. The same letters in the error bars shows no significance different values based on one-way ANOVA and Tukey's honestly significance difference (HSD) post hoc test ($p \leq 0.05$).

4.3.3. Determination of optimum pH value for bacterial isolate

The effect of pH on the growth of *Pseudomonas aeruginosa* is shown in Figure 10. Using olive oil as a carbon source and tryptone as a nitrogen source, maximum bacterial growth was obtained at pH 6.5 and minimum growth was at pH of 12. Test of significance for the C and N sources was analyzed for both optical density and cell dry weight. Optical density values of the four pH values shows significance difference. Cell dry weight of at pH 6.5 was also showed significance difference with pH 12 and no significance difference with pH 4.5 and 8.8 (Figure 10).

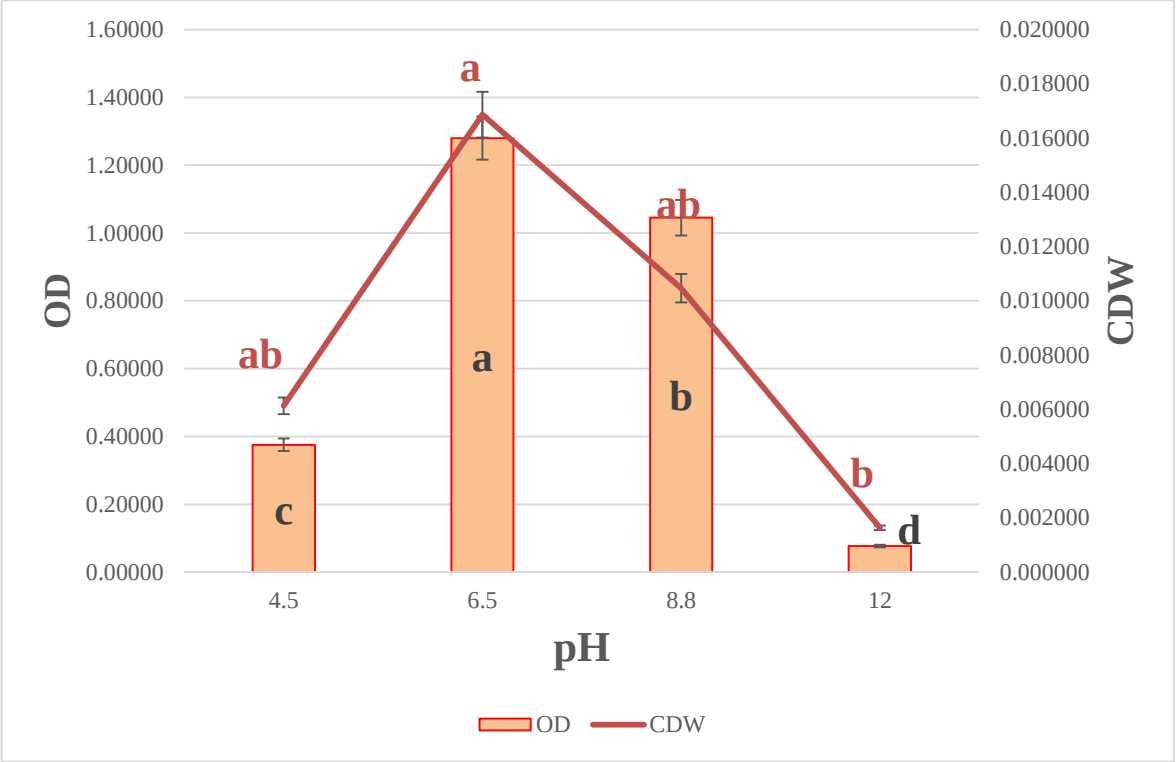

Figure 11: Effect of pH on the growth of *Pseudomonas aeruginosa.* OD= optical density, CDW= cell dry weight. The same letters in the error bars shows no significance different values based on one-way ANOVA and Tukey's honestly significance difference (HSD) post hoc test ($p \leq 0.05$).

4.3.3. Determination of optimum Temperature for bacterial isolate

The effects of different incubation temperatures on the growth condition of *P. aeruginosa* was investigated in a range of temperatures (25^{o}C- 45^{o}C) Figure-9b. The result indicated that *P. aeruginosa* showed good growth at 37^{o}C and 45^{o}C. The optimum pH for the growth of *P. aeruginosa* was obtained at 37^{0}C and least growth was observed at 25^{o}C. Significance difference was observed among optical density value of the four temperature values. However, no significance difference was examined among 37^{o}C, 45^{o}C and 30^{o}C during cell dry weight measurement. In addition significance difference was shown between 37^{o}C and 25^{o}C cell dry weight value (Figure 11).

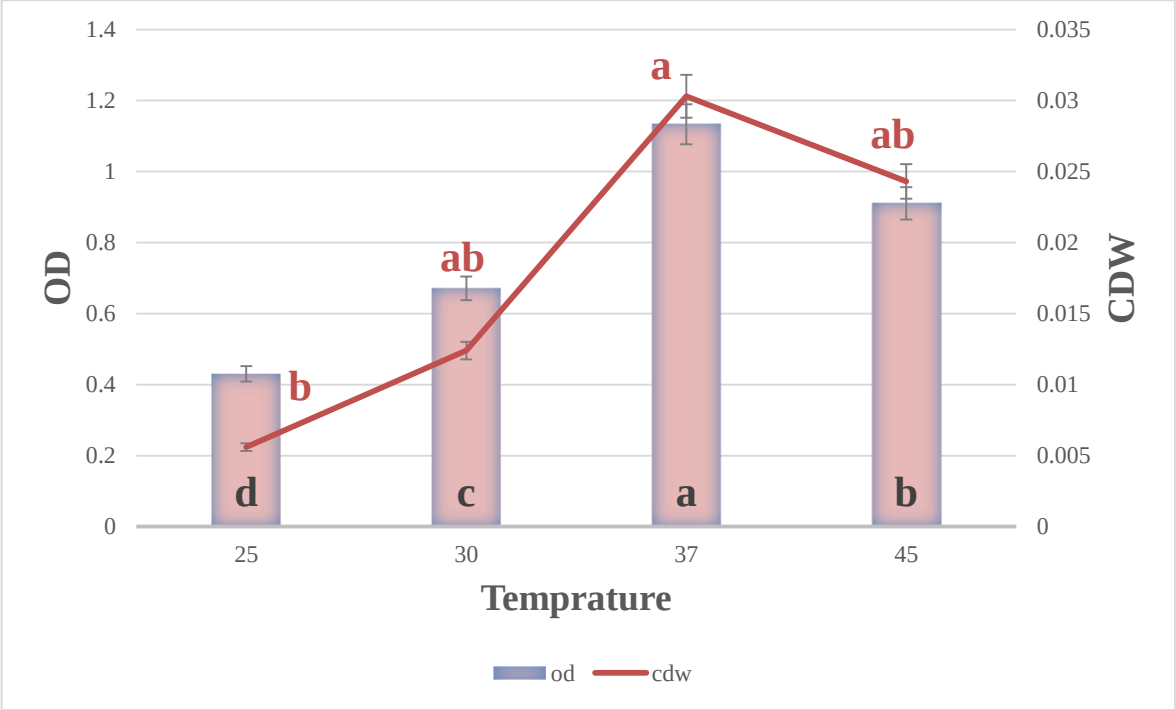

Figure 12: Effect of Temperature on the growth of *Pseudomonas aeruginosa.* OD= optical density, CDW= cell dry weight. The same letters in the error bars shows no significance different values based on one-way ANOVA and Tukey's honestly significance difference (HSD) post hoc test ($p \leq 0.05$).

4.4. Application of crude lipase enzyme

The result of crude enzyme assay showed that the crude enzyme collected from all four isolates (Figure-10) were effective in the hydrolysis of milk fat and LPBI-1 (*Pseudomonas aeruginosa)* hydrolyzed the lipid within 30min (Table 4). However, crude enzyme collected from LPBI-2 (*Aeromonas veronii)* was the most effective (hydrolyzed lipid in 15 minutes) followed by LPBI-4 (*Pseudomonas aeruginosa)* (hydrolyzed lipid in 18 min). The lipolytic ability was measured depending on the time it takes to change the color from pink to white (Table 4 and Figure-10).

Table 4: Fat hydrolyzing ability of selected isolates

S.N	Isolates	Bacterial species	Time taken to hydrolyze the fat
1	LPBI 1	*Pseudomonas aeruginosa*	30 minute
2	LPBI 2	*Aeromonas veronii*	15 minute
3	LPBI 3	*Aeromonas veronii*	25 minute
4	LPBI 4	*Pseudomonas aeruginosa*	18 minutes

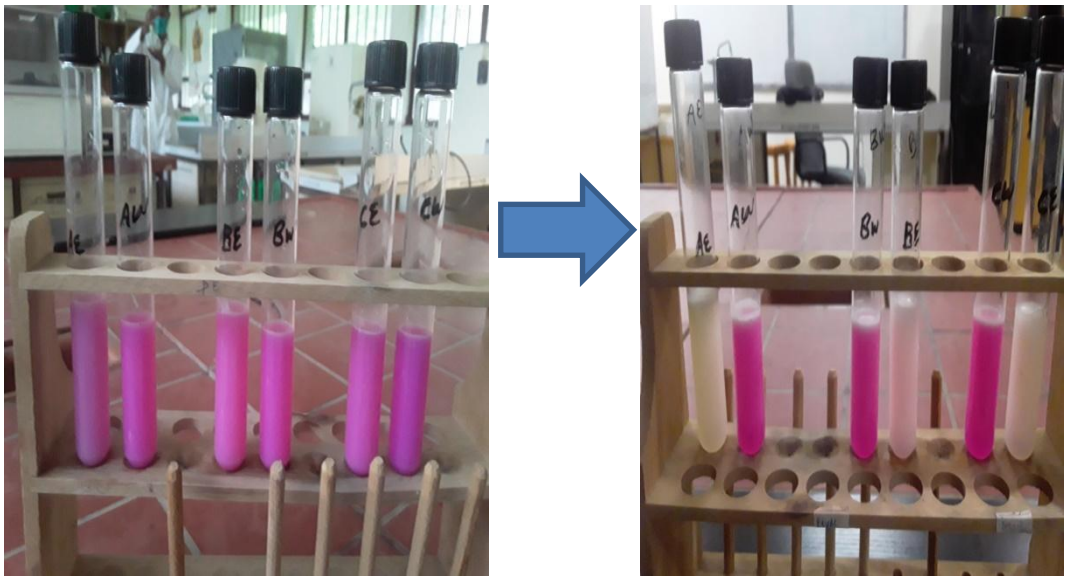

Figure 12: Fat hydrolysis application of crude lipase enzyme on milk

After some time of observation the second test tube which contains the enzyme shows a color change from pink to white. This is because the enzyme hydrolyzes the fat into fatty acid and glycerol which creates acidic condition for the solution. Due to this the indicator phenolphthalein were become colorless and the solution become white.

5. Discussion

Lake Ziway being one of the Lakes in the country with substantial fish resource and fishery activity it is an important commercial center as a major supplier of fish to the markets in Addis Ababa (Hailu *et al*., 2017). Due to high lipid content of the fishes, oil degrading microorganisms are capable of colonizing those areas. Lipase producing bacteria are capable of degrading those lipids because of the lipase enzyme they produce. The potential of this place for having these lipase producing bacteria is not reported.

Bacterial isolates showing best growth in the olive oil medium and changed the color from red to orange or yellow were considered as lipase positive isolates (Figure 5 and 6) in agreement with Muhsin *et al*. (2016). Four isolates were selected for further identification process and were studied for morphological characteristics, biochemical and proteomic characteristics. In this work all the selected four lipase producing bacterial isolates were selected from water samples collected from Ziway Lake. All the isolates were gram negative and rod shaped. The four isolates then identified as *Pseudomonas aeruginosa* (LPBI-1), *Aeromonas veronii* (LPBI-2), *Aeromonas veronii* (LPBI-3) and *Pseudomonas aeruginosa* (LPBI-4) on the basis of their morphological, biochemical and proteomic property. The result of MALDI-TOF MS and ABIS bacteria identification software was also confirmed the identification of the isolates. Muhsin *et al*. (2016) were reported that *Pseudomonas aeruginosa* was considered the best lipase producing bacteria which could withstand the different conditions. However there are no sufficient published articles that describe *Aeromonas veronii* as lipase producing agent. It could be the first report in Ethiopia for lipase enzyme production from *Aeromonas veronii.* Abd-el-malek (2017) has reported that lipase enzyme was present in 17.14% of *Aeromonas* species. In contrast escarpulli *et al* (2003) reported that *A. veronii* was the species with the least lipase producing capability gene.

Aeromonas veronii species, isolated and identified in this study are Gram-negative, rod-shaped bacterium, which was originally described by Hickman-Brenner *et al*. (1987). It is commonly isolated from environmental, clinical, and food samples. *A. veronii* strains have been isolated from diseased fish. The clinical symptoms of infected fish commonly comprised ulcer, fin rot/tail rot, abdominal distention, exophthalmia and hemorrhage. However, symptoms are not presented homogeneously across infected fish; different pathogenicity was observed depending on the particular bacterial isolates or strains. Thus

far, there is a shortage of research exploring their characteristics such as virulence, growth features, and histological lesions (Chen *et al*., 2019).

Pseudomonas aeruginosa is a Gram-negative, aerobic rod shaped bacterium, which belongs to the bacterial family *Pseudomonadacea* and it is one of the most clinically and epidemiologically important bacterium (Rocha, 2019). Many reports have shown the capacity of *Pseudomonas aeruginosa* in lipase production (Arora *et al*., 2015; Zouaoui & Bouziane, 2011 and Malook *et al*., 2018). In agreement with this study, Malik & Naz (2019) have reported that *Pseudomonas aeruginosa* showed the maximum lipase productions as compared to the other lipase producing bacterial strains. The production of extracellular lipases and growth of bacteria isolates are influenced by several nutritional and physico-chemical factors such as availability of carbon and nitrogen sources, incubation time, temperature, pH, presence of lipid as an inducer and/or substrate (Soleymani *et al*., 2017). In this study, the lipase producing bacteria *Pseudomonas aeruginosa* was tested for physical and chemical requirements for its growth.

The lipase producing isolate *Pseudomonas aeruginosa* showed maximum growth at pH of 6.5 (Figure 10). This agrees with the finding of Bharathi *et al* (2019) who reported that maximum lipase production achieved at pH at 6–7. Zouaoui & Bouziane (2011) also showed that the *Pseudomonas aeruginosa* was able to grow in the pH range from 6 to 8 and reached the maximum lipase activity at pH 7.0. A significance difference in optical density measurement was observed ($p \leq 0.05$) among the four pH values assayed in this study. The cell dry weight measurement showed the highest significant difference between pH 6.5 and pH 12. However, no significance difference in cell dry weight was observed among the rest of pH values assayed in this study.

Temperature is a critical parameter that has to be controlled and optimum temperature varies from organism to organism. Temperature influences secretion of extra cellular enzymes by changing the physical properties of the cell membrane (Veerapagu *et al*., 2013). Every enzyme producing bacterium has its own optimum temperature for its growth and maximum enzyme production (Haque *et al*., 2019). In this study the optical density was recorded while the bacteria were growing at different temperatures. The highest growth of *Pseudomonas aeruginosa* was observed at 37ºC. Mohan e*t al* (2008) have also reported that the influence of medium temperature indicated that the lipase production by the isolated strains was higher at 37 °C when compared to those at 27

and 47 °C. In contrast, the study done by Willerding *et al* (2011) showed that, the optimum growth and lipase activity of *Pseudomonas* were at 30^{o}C for 72 h at pH range 7–9. A significant difference was observed among all temperature values in case of optical density; hence cell dry weight shows significance difference only between the temperature value 25 and 37^{o}C.

Various oil types such as olive oil, fried oil, Tween-80 and Tween-20 were used as a carbon sources for the growth of the isolate LPBI-4. In this study, isolated *Pseudomonas aeruginosa* showed maximum growth in olive oil. This result agreed with the finding of Ilesanmi *et al* (2020) who reported that carbon was a major factor for the expression of lipase activity. The effect of different carbon sources showed higher activity on lipid substrate (olive oil) and its analogue (Tween-20 and Tween-80) with olive oil being the carbon source with the highest lipase activity. Among natural oils, olive oil has been referred as one of the best inductor and substrate for lipase production (Bharathi *et al*., 2019). As stated by Melani *et al* (2020) evaluation of the lipase production from a bacterial strain of *Pseudomonas* sp. using submerged fermentation with different carbon sources and concluded that the enzyme production was maximized when olive oil was used as the carbon source in the medium. Significance difference among the four carbon sources was observed when their optical densities were considered. In case of cell dry weight, olive oil and tween-20 showed a significance difference whereas the others did not.

Nitrogen source is well known to affect bacterial growth since it supply amino acids and many cell growth factors which needed for cell metabolism and protein synthesis (Hasan *et al*., 2018). In this study bacterial strain *Pseudomonas aeruginosa* was grown in growth medium with various nitrogen sources in order to determine the effect on the growth of bacterial isolate. The highest levels of bacterial growth were detected when growth medium supplemented with tryptone. Muhsin *et al* (2016) have reported that in addition to carbon source microbes used peptone and yeast extract as a nitrogen source, the type of nitrogen source in the culture medium also affected lipase secretion. In general, microorganisms produce high yield of lipase when organic nitrogen sources are used, like peptone and yeast extract, which utilized for lipase production by different thermophiles like *Bacillus* sp. and different *Pseudomonads* species. Kanimozhi & Perinbam (2010) have also reported the maximum lipase activity when yeast extract and peptone were used. Significance difference was observed in optical density among all nitrogen. In contrast, no significance difference was observed in cell dry weight. This may

be due to personal errors occurred when separation of pellet and supernatant process takes place.

In this study, the ability of the crude lipase enzyme was tested by applying the supernatant or crude lipase on raw milk in the presence of phenolphthalein. This was performed in order to check the ability of the crude lipase enzyme to hydrolysis the fat which was found in the milk. The presence of phenolphthalein was important to show a color change from basic to acidic. The prepared solution was basic due to the presence of sodium carbonate but when the crude lipase enzyme hydrolyzes the lipid to free fatty acids a color change was observed. This demonstrates the effectiveness of the enzyme extracted in this study. Lipases are extensively used in the dairy industry for hydrolysis of milk fat. Current applications include the flavor enhancement of cheeses, acceleration of cheese ripening and the lipolysis of butter fat and cream. The free fatty acids generated by the action of lipases on milk fat are essential for many dairy products, particularly for softening the cheeses, since these have specific flavor characteristics (Muthumari *et al*., 2016). Among the four lipolytic bacteria obtained in this study LPBI-2 (*Aeromonas veronii)* was the best lipid hydrolyzing bacteria and hence the best lipase producer. LPBI-4 (*pseudomonas aeruginosa*) was the second best lipid hydrolyzing bacteria.

6. Conclusion and recommendation

Lipases are versatile enzymes that are used widely and are becoming increasingly important in high-value applications in the food industry and the production of fine chemicals. Lipases are capable of region selective and stero selective biotransformation and allow resolution of racemic mixtures. The present study was aimed to isolate and characterize lipase producing bacteria from water and soil samples. It was confirmed that Lakes and sediment soils were good sources of lipolytic bacteria. Four bacterial isolates with lipase producing ability were revealed in this study. The identified bacterial isolates were LPBI-1 (*pseudomonas aeruginosa*), LPBI-2 (*Aeromonas veronii),* LPBI-3 *(Aeromonas veronii) and* LPBI-4 *(pseudomonas aeruginosa).*

From the four identified bacteria strains, *Pseudomonas aeruginosa* were selected to be studied for physiochemical requirements. The maximum growth was observed at 37^{o}C and pH of 6.5. Olive oil was the best carbon source which shows the best growth of the isolate. The isolate prefer tryptone over the other nitrogen source for growth. Submerged fermentation was used to extract crude lipase enzyme for the purpose of this study.

Finally, an in depth studies on the isolated *Aeromonas veronii* characteristics and physico-chemical requirements is highly recommended. Beside its importance as lipase producer, the isolated *Aeromonas veronii* is a fish pathogen. Therefore, it is recommended to study the effect of this isolate on fish species in Lake Ziway so that it won't affect the productivity of the lake.

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APPENDICES

Appendix 1: Representative Pictures of pure bacteria isolates on tributyrin agar plate with potential to produce lipase enzyme.

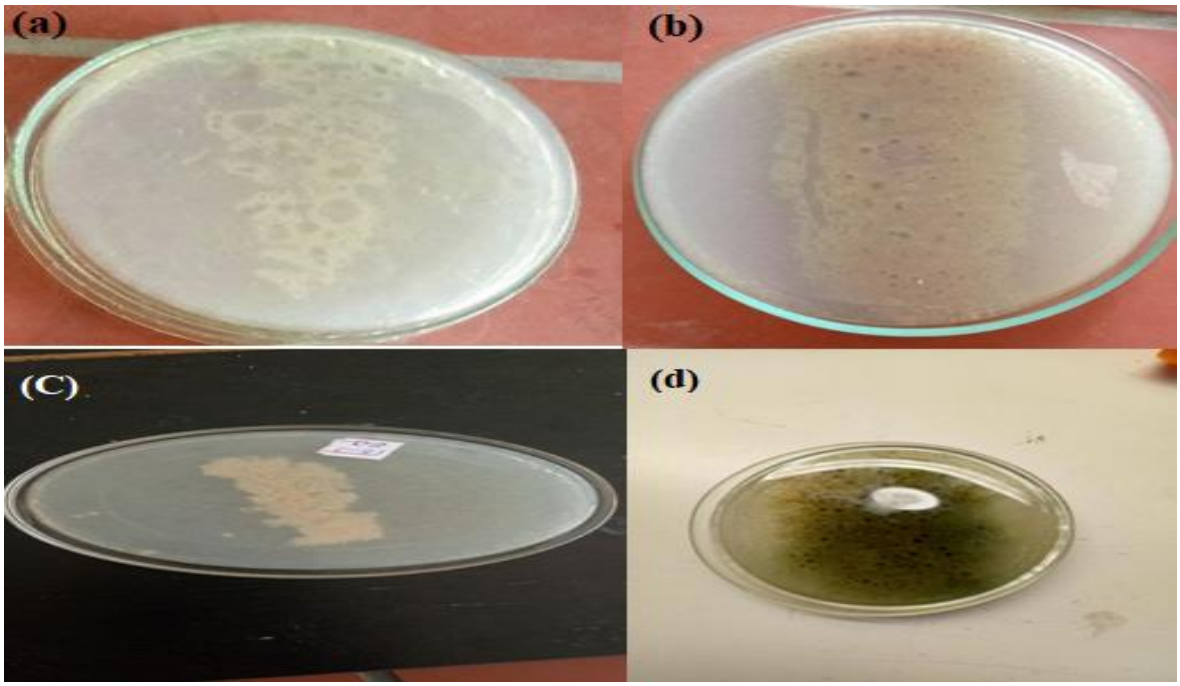


Figure-A1: Certain lipase producing bacterial isolate and respective pure colonies. (a) LPBI-1 that isolated from water sample. (b) LPBI-2 that identified as *A. veronii.* (c) LPBI-3 is a pure isolate that obtained from water sample. (d) LPBI-4 which characterized as *P. aeruginosa*

Appendix 2: Photo of biochemical profile of *pseudomonas aeruginosa* and *Aeromonas veronii*

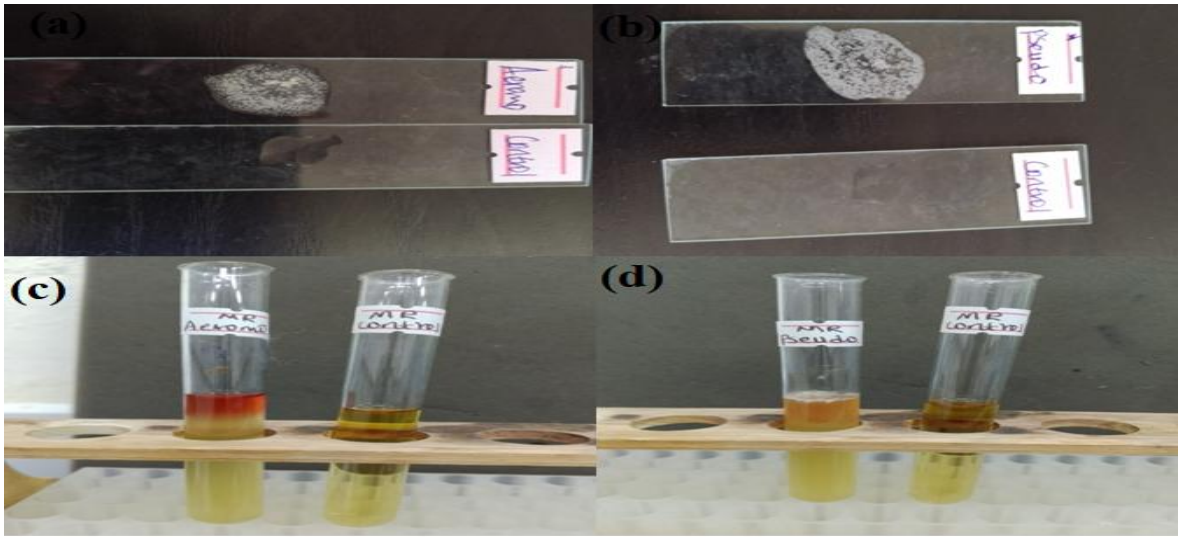


Figure-A_2: biochemical results of the two bacteria isolate *P. aeruginosa* and *A. veronii.* (a) Positive catalase reaction for *A. veronii*. (b) Positive catalase reaction for *P. aeruginosa.* (c) Positive methyl red reaction for *A. veronii.* (d) Positive methyl red reaction for *P. aeruginosa.*

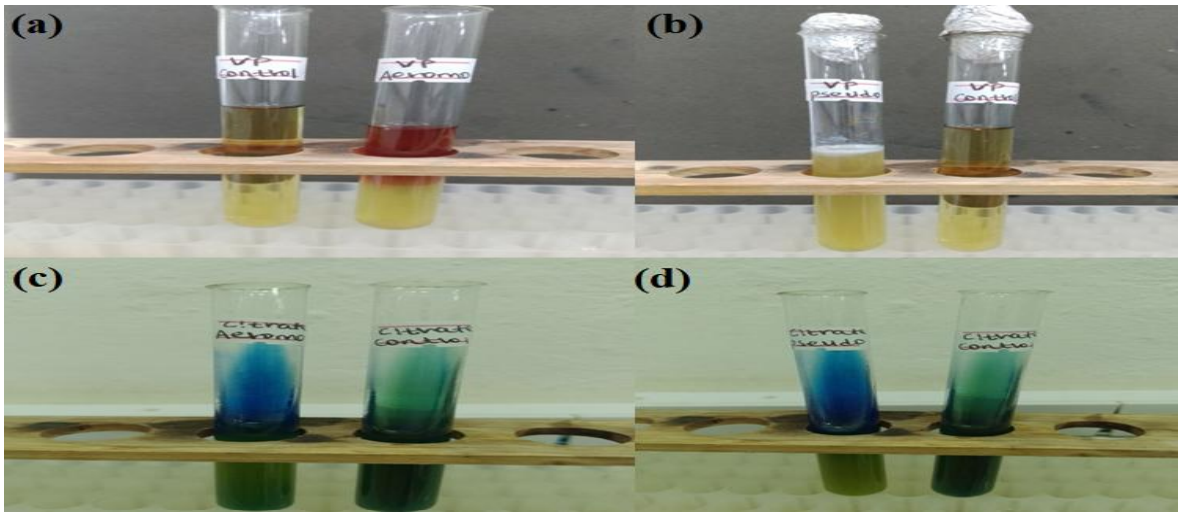


Figure-A_3: biochemical results of the two bacteria isolate *P. aeruginosa* and *A. veronii.* (a) Positive voges proskauer test reaction for *A. veronii*. (b) Negative voges proskauer test reaction for *P. aeruginosa.* (c) Positive result of citrate utilization for *A. veronii.* (d) Positive result of citrate utilization for *P. aeruginosa.*

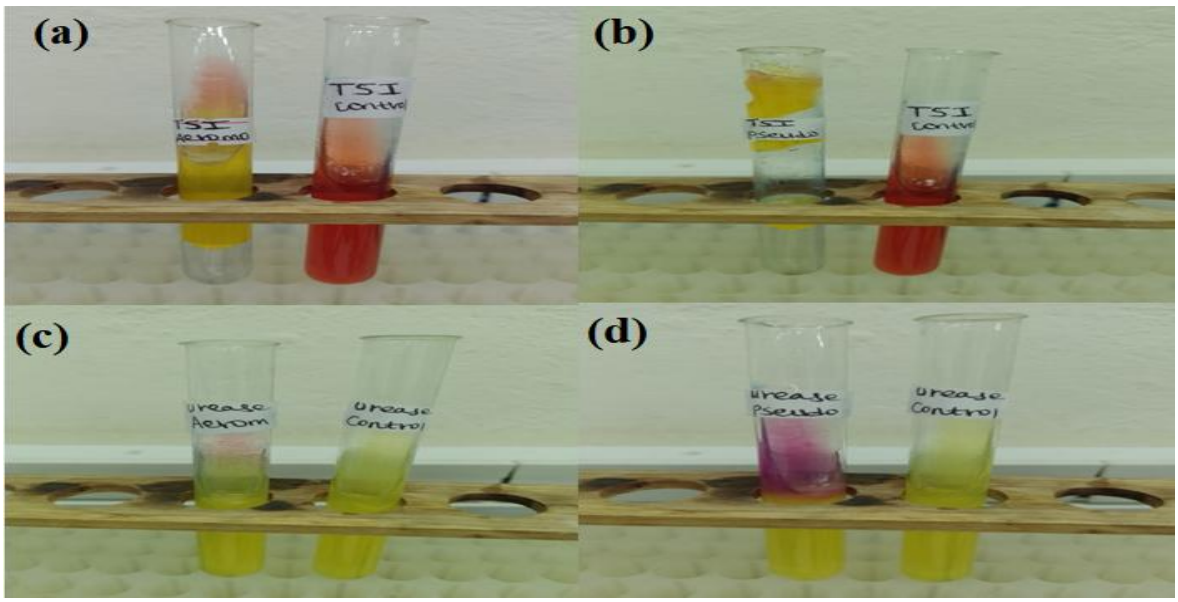


Figure-A_4: TSI and urease test result for the two bacteria isolate *P. aeruginosa* and *A. veronii.*

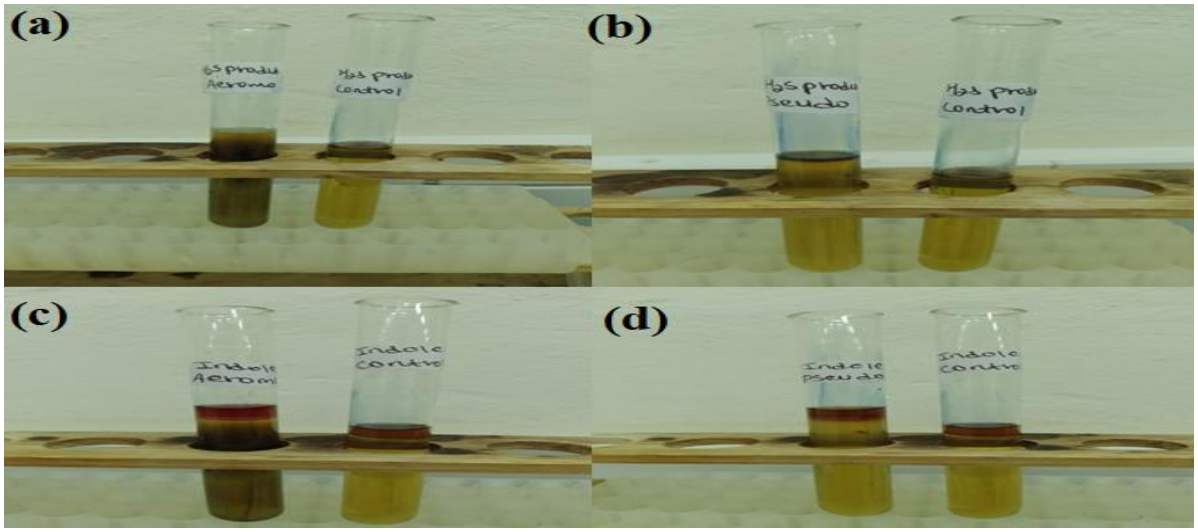
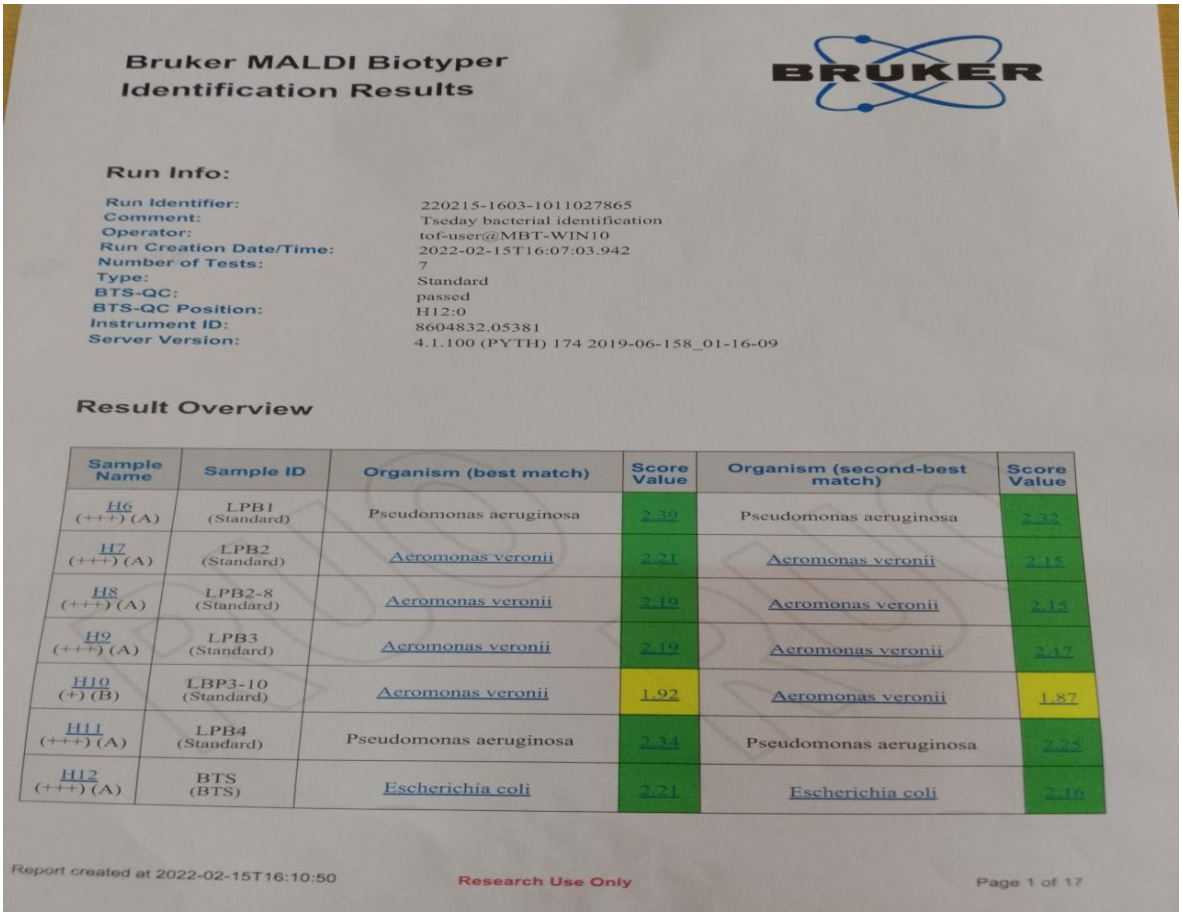


Figure-A$_5$: pictures showing the result of hydrogen sulfide production and indole production for *P. aeruginosa* and *A. veronii.*

Appendix 3: Photo that represent MALDI-TOF result



Bruker MALDI Biotyper
Identification Results

BRUKER

Run Info:

Run Identifier:	220215-1603-1011027865
Comment:	Tseday bacterial identification
Operator:	tof-user@MBT-WIN10
Run Creation Date/Time:	2022-02-15T16:07:03.942
Number of Tests:	7
Type:	Standard
BTS-QC:	passed
BTS-QC Position:	H12:0
Instrument ID:	8604832.05381
Server Version:	4.1.100 (PYTH) 174 2019-06-158_01-16-09

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
H6 (+++) (A)	LPB1 (Standard)	Pseudomonas aeruginosa	2.39	Pseudomonas aeruginosa	2.32
H7 (+++) (A)	LPB2 (Standard)	Aeromonas veronii	2.21	Aeromonas veronii	2.15
H8 (+++) (A)	LPB2-8 (Standard)	Aeromonas veronii	2.19	Aeromonas veronii	2.15
H9 (+++) (A)	LPB3 (Standard)	Aeromonas veronii	2.19	Aeromonas veronii	2.17
H10 (+) (B)	LBP3-10 (Standard)	Aeromonas veronii	1.92	Aeromonas veronii	1.87
H11 (+++) (A)	LPB4 (Standard)	Pseudomonas aeruginosa	2.34	Pseudomonas aeruginosa	2.25
H12 (+++) (A)	BTS (BTS)	Escherichia coli	2.21	Escherichia coli	2.16

Report created at 2022-02-15T16:10:50 Research Use Only Page 1 of 17

Figure-A$_6$: The isolates were identified as *A. veronii* and *P. aeruginosa* as determined by MALDI-TOF MS.

Appendix 4: One way ANOVA analysis of optimization data by SPSS software

A. analysis of data of carbon sources depends of optical density and cell dry weight values

Oneway

[DataSet0]

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OPTICAL DENSITY	tween 80	3	1.03000	.000000	.000000	1.03000	1.03000	1.030	1.030
	tween 20	3	.98767	.001155	.000667	.98480	.99054	.987	.989
	olive oil	3	1.50000	.001000	.000577	1.49752	1.50248	1.499	1.501
	fried oil	3	1.49767	.000577	.000333	1.49623	1.49910	1.497	1.498
	Total	12	1.25383	.256374	.074009	1.09094	1.41673	.987	1.501
CELL DRY WEIGHT	tween 80	3	.019367	.0100012	.0057742	-.005478	.044211	.0082	.0275
	tween 20	3	.005800	.0021932	.0012662	.000352	.011248	.0033	.0074
	olive oil	3	.029333	.0043616	.0025182	.018499	.040168	.0243	.0320
	fried oil	3	.021433	.0059475	.0034338	.006659	.036208	.0151	.0269
	Total	12	.018983	.0103558	.0029895	.012404	.025563	.0033	.0320

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OPTICAL DENSITY	Between Groups	.723	3	.241	361498.167	.000
	Within Groups	.000	8	.000		
	Total	.723	11			
CELL DRY WEIGHT	Between Groups	.001	3	.000	7.212	.012
	Within Groups	.000	8	.000		
	Total	.001	11			

Homogeneous Subsets

OPTICAL DENSITY

Tukey HSD[a]

carbon source	N	Subset for alpha = 0.05			
		1	2	3	4
tween 20	3	.98767			
tween 80	3		1.03000		
fried oil	3			1.49767	
olive oil	3				1.50000
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

CELL DRY WEIGHT

Tukey HSD[a]

carbon source	N	Subset for alpha = 0.05	
		1	2
tween 20	3	.005800	
tween 80	3	.019367	.019367
fried oil	3	.021433	.021433
olive oil	3		.029333
Sig.		.063	.287

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B. analysis of data of nitrogen sources depends of optical density and cell dry weight values

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
OPTICAL DENSITY	TRYPTON	3	1.64100	.001732	.001000	1.63670	1.64530	1.640	1.643
	YEAST EXTRACT	3	1.55233	.002517	.001453	1.54608	1.55858	1.550	1.555
	AMMONIUM CHLORIDE	3	.26167	.001528	.000882	.25787	.26546	.260	.263
	AMMONIUM SULPHATE	3	.31233	.001528	.000882	.30854	.31613	.311	.314
	Total	12	.94183	.684992	.197740	.50661	1.37706	.260	1.643
CELL DRY WEIGHT	TRYPTON	3	.026867	.0063066	.0036411	.011200	.042533	.0204	.0330
	YEAST EXTRACT	3	.022700	.0227719	.0131474	-.033869	.079269	.0063	.0487
	AMMONIUM CHLORIDE	3	.004467	.0055824	.0032230	-.009401	.018334	.0009	.0109
	AMMONIUM SULPHATE	3	.006600	.0034395	.0019858	-.001944	.015144	.0035	.0103
	Total	12	.015158	.0146076	.0042169	.005877	.024440	.0009	.0487

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OPTICAL DENSITY	Between Groups	5.161	3	1.720	491554.635	.000
	Within Groups	.000	8	.000		
	Total	5.161	11			
CELL DRY WEIGHT	Between Groups	.001	3	.000	2.538	.130
	Within Groups	.001	8	.000		
	Total	.002	11			

Homogeneous Subsets

OPTICAL DENSITY

Tukey HSD[a]

NITROGEN SOURCE	N	Subset for alpha = 0.05 1	2	3	4
AMMONIUM CHLORIDE	3	.26167			
AMMONIUM SULPHATE	3		.31233		
YEAST EXTRACT	3			1.55233	
TRYPTON	3				1.64100
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

CELL DRY WEIGHT

Tukey HSD[a]

NITROGEN SOURCE	N	Subset for alpha = 0.05 1
AMMONIUM CHLORIDE	3	.004467
AMMONIUM SULPHATE	3	.006600
YEAST EXTRACT	3	.022700
TRYPTON	3	.026867
Sig.		.193

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

C. analysis of data of temperature depends of optical density and cell dry weight values

Oneway

[DataSet1] C:\Users\SAMRAWIT\Documents\1\the whole spss file\MODIFIED ANAYSED DATA\ANALYZED DATA\Tseday Untitled1.sav

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
Optical density	25 0C	3	.4306667	.00115470	.00066667	.4277982	.4335351	.43000	.43200
	30 0C	3	.6713333	.00115470	.00066667	.6684649	.6742018	.67000	.67200
	37 0C	3	1.1333333	.00152753	.00088192	1.1295388	1.1371279	1.13200	1.13500
	45 0C	3	.9106667	.00057735	.00033333	.9092324	.9121009	.91000	.91100
	Total	12	.7865000	.27415739	.07914242	.6123087	.9606913	.43000	1.13500
Cell Dry Weight	25 0C	3	.0056667	.00355293	.00205129	-.0031593	.0144926	.00220	.00930
	30 0C	3	.0124000	.00867122	.00500633	-.0091405	.0339405	.00410	.02140
	37 0C	3	.0303000	.01224418	.00706918	-.0001162	.0607162	.01750	.04190
	45 0C	3	.0243000	.00857146	.00494874	.0030073	.0455927	.01450	.03040
	Total	12	.0181667	.01259663	.00363633	.0101631	.0261702	.00220	.04190

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Optical density	Between Groups	.827	3	.276	206693.583	.000
	Within Groups	.000	8	.000		
	Total	.827	11			
Cell Dry Weight	Between Groups	.001	3	.000	4.812	.034
	Within Groups	.001	8	.000		
	Total	.002	11			

Homogeneous Subsets

Optical density

Tukey HSD[a]

Temprature	N	Subset for alpha = 0.05: 1	2	3	4
25 0C	3	.4306667			
30 0C	3		.6713333		
45 0C	3			.9106667	
37 0C	3				1.1333333
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell Dry Weight

Tukey HSD[a]

Temprature	N	Subset for alpha = 0.05: 1	2
25 0C	3	.0056667	
30 0C	3	.0124000	.0124000
45 0C	3	.0243000	.0243000
37 0C	3		.0303000
Sig.		.119	.137

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

D. analysis of data of pH depends of optical density and cell dry weight values

Oneway

[DataSet0]

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Optical Density	4.5	3	.37567	.000577	.000333	.37423	.37710	.375	.376
	6.5	3	1.28000	.000000	.000000	1.28000	1.28000	1.280	1.280
	8.8	3	1.04533	.000577	.000333	1.04390	1.04677	1.045	1.046
	12	3	.07667	.000577	.000333	.07523	.07810	.076	.077
	Total	12	.69442	.508813	.146882	.37113	1.01770	.076	1.280
Cell Dry Weight	4.5	3	.006133	.0062939	.0036338	-.009502	.021768	.0024	.0134
	6.5	3	.016867	.0037873	.0021866	.007459	.026275	.0138	.0211
	8.8	3	.010467	.0086674	.0050041	-.011064	.031998	.0033	.0201
	12	3	.001633	.0004933	.0002848	.000408	.002859	.0013	.0022
	Total	12	.008775	.0076134	.0021978	.003938	.013612	.0013	.0211

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Optical Density	Between Groups	2.848	3	.949	3797067.889	.000
	Within Groups	.000	8	.000		
	Total	2.848	11			
Cell Dry Weight	Between Groups	.000	3	.000	3.907	.055
	Within Groups	.000	8	.000		
	Total	.001	11			

Homogeneous Subsets

Optical Density

Tukey HSD[a]

pH	N	Subset for alpha = 0.05			
		1	2	3	4
12	3	.07667			
4.5	3		.37567		
8.8	3			1.04533	
6.5	3				1.28000
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell Dry Weight

Tukey HSD[a]

pH	N	Subset for alpha = 0.05	
		1	2
12	3	.001633	
4.5	3	.006133	.006133
8.8	3	.010467	.010467
6.5	3		.016867
Sig.		.299	.174

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.