COLLEGE OF NATURAL SCIENCES

SCHOOL OF POSTGRADUATE STUDIES

DEPARTMENT OF BIOLOGY

Isolation and Characterization of Thermostable Enzyme-Producing Microbes from Selected Hot springs of Ethiopia

By: Genet Abebe

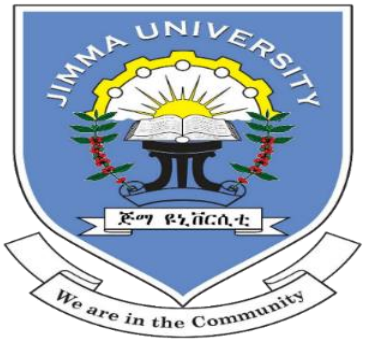
Principal advisor: Meseret Guta (MSc. Asst. Prof.)

Co-advisor: Lataa Lachisa (MSc.)

A Thesis Submitted to the Department of Biology, College of Natural Sciences, Jimma University, In Partial Fulfillments of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

November, 2021

Jimma, Ethiopia



COLLEGE OF NATURAL SCIENCES

SCHOOL OF POSTGRADUATE STUDIES

DEPARTMENT OF BIOLOGY

Thesis Title:

Isolation and Characterization of Thermostable Enzyme-Producing Microbes from Selected Hot Springs of Ethiopia

By: Genet Abebe

Approved by the Examining Board

Principal Advisor:	Signature	Date
Meseret Guta (MSc. Asst. Prof.)	_____	_____
Co-advisor:		
Lata Lachisa (MSc.)	_____	_____
Internal Examiner:		
Shiferaw Demisse (MSc. Asst. Prof.)	_____	_____
External Examiner:		
Mesfin Tafesse (PhD)	_____	_____

November, 2021

Jimma, Ethiopia

Declaration

I, the undersigned, declare that this Thesis is my original work, has not been presented for the award of any academic degree, diploma or certificate in any other institution and the materials used in the Thesis are duly acknowledged.

Name: ----------

Signature: ----------

Date: ----------

As research supervisor of the candidate, we have gone through the document and approved submission of the Thesis for public defense.

Main Advisor:

Name ----------

Signature ----------

Date----------

Co- Advisor:

Name----------

Signature----------

Date----------

Acknowledgments

First and foremost, I want to express my gratitude to Almighty God for blessing me with good health and giving me the patience and willpower to reach my goal.

I would like to express my deep sense of gratitude and gratefulness to my advisor Meseret Guta (MSc. Asst. Prof.) for her overall intellectual guidance, advices and unreserved interest to help me in a friendly approach throughout my research process. I also extend my gratitude to my co-advisor Lata Lachisa (MSc.), for his valuable comments, who directly contributed to the successful completion of my thesis research.

My deepest gratitude and respect also goes to Ketema Bacha (PhD, Prof.) who is principal investigator (PI) of the current research work for his sincer advice and help to cover all research costs and guidance during thesis work. I am also thankful to Jimma University, College of Natural Sciences, Department of Biology for the provision of the required materials: culture media, technical supports, laboratory facilities, and for all-round cooperation. I also thank my internal examiner Shiferaw Demissie (MSc, Asst. Prof.) who is a lecturer at Jimma University for his critical comments to enrich the proposal and thesis.

I would also like to thank Mr. Reda Nemo, PhD student for his suggestion and guidance during the thesis work and also other PhD students are acknowledged. I would also like to thank my district, Doyogena for giving me the chance to study MSc. I would also like to thank all my classmates.

Finally, I would like to express my sincer love, respect and gratitude to my parents Abebe Arficho and Etagegn Gebre, my husband Eshet handamo and my brothers and sisters for their help in prayer, encouragement, advice, love and care throughout my study.

Table of Contents

Contents **Page**

List of Tables

Tables **Pages**

List of Figures

Figures **Pages**

List of Appendices

Appendices **Pages**

List of Acronym

ATCC = American type culture collection

CAGR = Compound annual growth rate

CMC= Carboxymethyl cellulose

DO = Dissolved oxygen

mS = milli-Siemens

ppt = parts per thousand

REA = Relative enzyme activity

SKA = Skimmed milk agar

TDS = Total Dissolved Solids

Abstract

Hot springs are springs that occur naturally at elevated temperatures, originated when water seeps into the earth and come contact to the magma near to earth's surface and are potential sources of microbial diversity and thermostable hydrolytic enzymes. Cross sectional and experimental study design was conducted to isolate and characterize thermostable enzyme-producing microbes from selected hot springs of Ethiopia. A total of 90 samples 30 from each hot springs, Woliso, Wondo Genet, and Shalla were collected from three different locations and different depths. Isolation of thermophiles was done by enriching samples in peptone water for 24 hours and conducting serial dilution followed by spread plating the samples on nutrient agar supplemented with 1% agar and thermus agar for bacteria and potato dextrose agar supplemented with 1% agar was used for fungi. Then, the isolated bacterial and fungal isolates were screened for the production of thermostable hydrolytic enzyme (amylase, protease, cellulase, and lipase) using their respective enzyme screening media and then the formation of a clear zone and opaque zone around the colony was detected as enzyme production. A total of 252 bacterial isolates and 9 fungal isolates were obtained after sub-culturing. All of the 252 bacterial isolates were producers of 1 or more enzymes they were screened for but all the fungal isolates were not capable of producing a promising clear zone for all the tested enzymes. Based on the highest clear zone formation 45 bacterial isolates were selected for biochemical and physiological characterization. Based on their biochemical characterization, 39 of the bacterial isolates were tentatively classified under the genus Bacillus and the rest 6 isolates were identified to the genus Thermus. Among these, isolates JUWG84 (30.5 ± 0.71) and JUW103 (30 ± 0), JUS4 (29.5 ± 0.71) and JUS14 (29.5 ± 0.71), JUWG44 (30 ± 0) and JUWG9 (30 ± 0) obtained the higher inhibition zone and were recognized as excellent amylase, protease, cellulase and lipase producers respectively. Seven of the isolated fungal isolates were identified as genus Aspergillus and two were identified as genus Penicillium. It is concluded that the three hotsprings studied in this study are rich with microbial community and the microbes are an important source of thermostable enzymes for industrial application. So industries need to use microbial-based enzymes by applying extra screening, optimization, and scale-up.

***KEYWORDS**: Ethiopia, hot springs, thermophilic microbes, and thermostable enzymes.*

1. Introduction

1.1. Background of the Study

Hot springs are springs where water temperature is higher than that of the ambient temperature (Pentecost *et al.,* 2011). They are formed by the emergence of geothermally heated ground water from earth's crust (Jaffer *et al.*, 2019) and found in regions of young volcanic activity (Oarga, 2009). Hot springs are exceptional sites for thermophilic microbial life (Gupta *et al.,* 2014) and are potential sources of microbial diversity and thermostable hydrolytic enzymes (Sahay *et al.*, 2017). Thermophiles are organisms which grow beyond room temperature (Eze *et al.,* 2011) and are grouped into three groups based on growth temperature: moderate thermophiles (growth optimum, 45–60 ˚C), extreme thermophiles (growth optimum, 61–80 ˚C), and hyperthermophiles (growth optimum, 81–110 ˚C) (Gupta *et al.,* 2014). In addation to temperature microbial growth is also influenced by several factors including pH (power of hydrogen), NaCl, humidity, and other chemicals) (Jufri, 2020). Thermophilic microorganisms are sources of thermostable enzymes such as amylases, cellulases, chitinases, pectinases, xylanases, proteases, lipase, and DNA polymerases. The organisms with the highest growth temperatures within archaea are members of the genera *Pyrobaculum, Pyrodictium*, *Pyrococcus*, and *Melanopyrus*; with in fungal genera *Aspergillus, Myceliopthora* (Mohammad *et al.,* 2017), while, in case of bacteria, *Thermus* (Brock, 1997), *Thermotoga, and Aquifex* (Kumar *et al.,* 2014), and *Bacillus* exhibit the highest growth temperatures (Eze *et al.,* 2011).

Thermophiles have been isolated from different ecological zones of the earth, hot springs, deep sea hydrothermal vents, and geothermally heated oil petroleum reserves, (Mehta and Satyanarayana, 2013). *Flavobacterium* and *Anoxybacillus* isolated from Tatapani Hot spring of A North Western Himalayan State (Sharma *et al.,* 2018), *Bacillus* sp. and *Staphylococcus* sp. isolated from Maharashtra hot springs (Jaffer *et al.*, 2019), *Marinobacter, Nocardioides, Parvibaculum* isolated from deep-sea hydrothermal vents (Bertrand *et al.,* 2013), *Aspergillus* and *Penicillium* isolated from geothermal ecosystems Zhou *et al.,* (2015)

Thermostable hydrolytic enzymes are enzymes which have unique properties that can be suitable for performing industrial processes at elevated temperatures. Also, they have been reported to be more stable against many harsh environments (solvents, detergents, and acidic and alkaline pH)

(Thakur, 2017) and have been screened from different Hot Springs in the world, i.e., α-amylase, protease, and lipase were screened from Saudi hot springs (Alrumman *et al.*, 2018). Cellulase, gelatinase, and xylanase have been screened from Nepal Hot Springs (Yadav *et al.*, 2018). Amylase cellulase and protease have been screened from Jammu and Kashmir Hot Springs (Sharma, 2018). Amylase, protease, lipase, esterase, xylanase, and cellulase are enzymes screened from Kenyan Hot Springs (Salano *et al.*, 2018). Amylase has been screened from Hot Springs of Arba Minch Nech Sar National Park, Southern Ethiopia (Latebo and Abrar, 2017). Amylase and protease have been isolated from Hot Springs in the Koye Gore farmland of Addis Ababa City (Birhanu *et al.*, 2021).

Microbial enzymes are stable in harsh environments and they have been used in different industries for different purposes. For example, amylase is used in; food industry, textile industry (Souza *et al.*, 2010), the paper industry, and liquefaction (Saini *et al.*, 2017). Protease used in; food and feed industry (Pratush, 2013), detergent industry (Razzaq *et a.l*, 2019), leather industry (Sundus *et al.*, 2016), and waste *management* (Kudrya and Simonenko, 1994). Cellulase is used in; food industry, textile industry, paper processing, and biofuel production (Sahoo *et al.*, 2019; Acharya and Chaudhary, 2012).Whereas, Lipase is used in; textile industry (Hassan *et al.*, 2005), the leather industry (Lajis, 2018), food, pulp, and paper industries (Casa-Godoy *et al.*, 2018).

In Ethiopia, there are many hot springs available in different regions across the country including Woliso, Shala, and Wondo Genet Hot Springs. Although interest in studying thermophiles from hot springs in Ethiopia has been least demonstrated by previous microbial studies, still no sustained research had focused on the utilization of these thermophiles. Therefore, the present study was aimed to isolate and characterize thermostable enzyme-producing microbes from selected hot springs of Ethiopia.

1.2. Statement of the Problem

Thermozymes such as amylases, cellulases, xylanases, proteases, lipases, and Taq DNA polymerases show unique features that can be suitable for performing biotechnological processes at elevated temperatures. Moreover, they have been reported to be more stable against many solvents, detergents, and acidic and alkaline pH (Mohammad *et al.,* 2017). Ethiopia has many Hot Springs which found at different corners across the country. So far, Kassaye and Tilahun (2020) reported only microorganisms in Shala Hot Spring. Moreover, Mamo *et al.* (1999) isolated only amylase from wondogenet hotspring. However, there is no more scientific reports have been done on thermozymes and thermophiles from selected Hot Springs. The current study need to be studied to get novel thermophiles and their enzymes which will have importance in different industries. Therefore the present study aims to isolate and characterize thermostable enzyme-producing microbes from Woliso, Wondo Genet, and Shala hotsprings of Ethiopia as the main objective by having the following research questions:

- ✓ Which bacterial and fungal isolates dominate the selected hot springs?
- ✓ Which isolates can produce thermostable enzymes of all isolated microbes?
- ✓ To which genus/species the potent enzyme-producing isolates are grouped?
- ✓ What is the physiological parameter tolerance of the bacterial isolates?

1.3. Objectives of the Study

1.3.1. General Objective

- ✓ To asses thermo-stable enzyme producing microorganisms from selected hot springs of Ethiopia

1.3.2. Specific Objectives

- ✓ To isolate thermophilic microbes from the selected hot springs
- ✓ To screen their potential to produce thermostable hydrolytic enzymes
- ✓ To characterize the potent enzyme producing isolates to the genus level
- ✓ To evaluate the physiological parameter tolerance for potent enzyme-producing bacterial isolates

2. Literature Review

2.1. Extreme Environments and Extremophiles

The study of microbial communities from extreme environments is a interesting topic. Extremophiles are those organisms which thrive beyond 'normal' environmental parametres. Organisms from all three domains of life (Archaea, Eubacteria and Eukaryotes) are found at extreme conditions. Regarding extreme conditions, extremophiles are psychrophiles (thrive at low temperatures), thermophiles (high temperature), acidophiles (low pH), alkaliphiles (high pH), piezophiles (under extremes of pressure), xerophiles (desiccation), and halophiles (salinity) (Oarga, 2009). Regarding temperature, there are different types of thermophilic environments. The most common thermophilic areas are volcanic areas, geothermal areas, terrestrial fumaroles, terrestrial hot springs, deep-sea hydrothermal vents, geothermally heated oil petroleum reserves, and sun-heated soils/sediments (Mehta and Satyanarayana, 2013).

2.2. Hot springs and their Microbial Diversity

Geothermal areas have proven to be interesting environments for diverse scientific disciplines (Massello *et al.*, 2020). Hot springs are one of the geothermal areas and generally originated from precipitation, Be it rainfall or snowmelt, water seeps into the earth and at a certain point, it is heated up by magma close to the earth's surface. Heated water is much less dense than the rock that surrounds it, and thus it rises to the surface, in the form of a spring. The water is carried in openings; through the water table, cracks in sedimentary rock and other passages where the water can flow (Watson, 1997). There are several Hot springs found across the world. Some of the most popular Hot springs are the Yellowstone National Park (U.S.A.), the heliothermal Ekho Lake in Antarctica (Pathak and Rathod, 2017), the Shu Shu Hot Springs in South Africa (Olivier & Jonker, 2013), and Shalla abijata Hot spring (Ethiopia) (Kassaye and Tilahun, 2020). The Hots pring environmental factors influence microbial communities in Hot springs thus, different Hot springs have diffent microbial composition. Hot springs are acidic or alkaline innature and are one of potential habitats of thermophilic environments where diversity of the three domains of microbes found (Massello *et al.*, 2020). Some of the hot spring microbial genera include; *Sulfolobus*, *Acidiplasma*, *Caldivirga*, *Thermocladium*, and *Pyrobaculum* belonging to Archaea. within Fungi, *Aspergillus, Penicillium, Alternaria, Fusarium*, and *Trametes* isolated from hot-

spring of Bogoria, Kenya (Salano *et al.,* 2018). *Absidia*, *Acremonium, Aspergillus, Chaetomium, Cunninghamella*, *Penicillium* isolated from geothermal soils in yellow stone national park (Redman *et al.,* 1999), *Rhizomucor*, *Chaetomium*, *Talaromyces,* and *Aspergillus* (Mehta and Satyanarayana, 2013), *Determomyces*, *Myceliopthora* (Sharma *et al.,* 2013). With in bacteria; *Bacillus* and *Thermus* isolated from Shala hot spring (Belay and Tilahun, 202) *Streptomyces*, *Pseudoxanthomonas, Geobacillus, Termoanaerobacter, Anoxybacillus, and Bacillus* (Mehta and Satyanarayana, 2013), *Bacillus* and *Micrococcus* isolated from in Indian Himalayas (Sharma *et al.,* 2013)

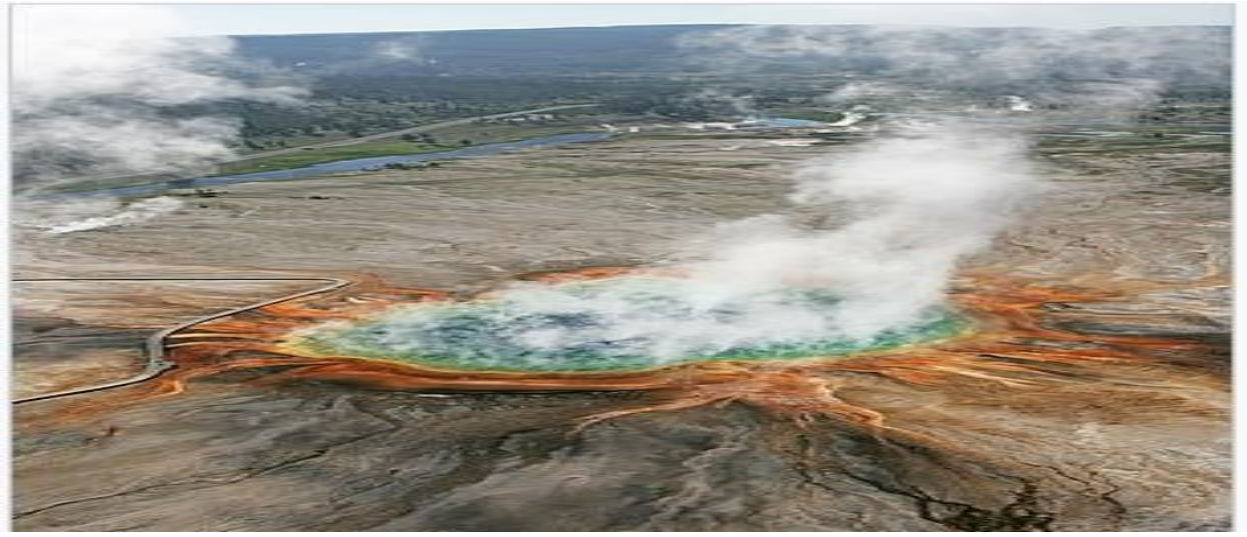


Figure 1: Grand prismatic spring and Midway Geyser Basin in Yellowstone National Park

2.3. Microbial Enzymes

Enzymes are complex protein molecules, often called biocatalysts, accelerate the rate of chemical reactions and are produced by living cells (Bhatia, 2018). The world industrial enzyme market in 2019 was valued at USD 9.9 Billion and is projected to grow at a CAGR of 7.1% from 2020-2027 (Alarcón, 2020). Diastase (a mixture of amylases) was the first enzyme to be discovered in 1883, quickly followed by other hydrolytic enzymes such as pepsin and invertase, but the term enzyme was only coined in 1877 by Wilhelm Kühne. There are six different classes of enzymes according to the type of chemistry being carried out. Oxidoreductases catalyze oxidation/reduction reactions (EC 1), transferases transfer a chemical group (EC 2), for example, a methyl or glycosyl moiety; hydrolases perform hydrolysis of chemical bonds (EC 3), lyases also cleave chemical bonds by other means than by oxidation or hydrolysis (EC 4), isomerases

catalyze geometric and structural changes between isomers (EC 5) and finally, ligases join two compounds with associated hydrolysis of a nucleoside triphosphate molecule (EC 6) (Cuesta 2014).

The most routine approach for discovering novel enzymes is the culture of microorganisms that express a protein of interest. This culture dependent methodology has been successful for the isolation and characterization of many biocatalysts (Santiago *et al.*, 2016). Microbial enzymes are known to be superior enzymes obtained from different microorganisms, particularly for applications in industries on commercial scales. The superiority was based on capability and appreciable activity under abnormal conditions, mainly of temperature and pH. Hence, certain microbial enzymes are categorized as thermophilic, acidophilic or alkalophilic. Selected microorganisms including bacteria, fungi and yeasts have been globally studied for the bio-synthesis of economically viable preparations of various enzymes for commercial applications. Some of enzymes with special characteristics in biotechnology include; protease, keratinase, amylase, xylanase, Laccase/Ligninase, cellulase, Pectinases, Lipases, and Phytases (Nigam, 2013; Rigoldi *et al.*, 2018).

2.4. Amylase

Amylases, hydrolytic enzymes, catalyze the hydrolysis of α-1,4 glycosidic linkages of starch into low molecular weight sugar molecules. In nature, starch is the most abundant polysaccharide food store after cellulose and the primary accessible source of carbon and energy on the Earth (Singh, 2016). Among industrially important enzymes, amylases sharing 25-30% of the world enzyme market are drawing more attention because of their wide commercial applications and economic benefits (Kiran *et al.*, 2018). Microbial amylases are of immense value because of characteristics like bulk production and easy genetic manipulation (Pranay *et al.*, 2019).

2.4.1. History of Amylase Discovery

The history of amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff in wheat and laid down the foundation for the discovery and research on Amylase. The α-amylases were named by Kuhn in 1925 because the hydrolysis products are in the alpha configuration. In 1930, Ohlsson discovered another amylase, which yielded a β-mannose. He

named it β-amylase. The crystal structure was established using a 3Å resolution structure which was further improved to a 1.5 Å resolution of α-amylases (Tiwari*et al.,* 2015). The three-dimensional crystal structures of each form were determined in the 1990s and found to be effectively identical. As diastase, amylase was the first enzyme to be discovered and isolated by Anselme Payen, 1833 (Tiwari *et al.,* 2015). Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (Singh *et al.,* 2016). Boidin & Effront, 1917 were the first to use *Bacillus subtilis* and *Bacillus mesentericus* for the production of α-amylases on a commercial scale using large fermentors in submerged fermentation (Tiwari *et al.,* 2015).

2.4.2. Types of Amylase

Amylases can be classified into two groups, endo and exo amylases based on their mode of action. The endo amylases catalyze random hydrolysis of α-1,4 glycosidic bonds present in the structural components i.e. amylose or amylopectin chain of starch. This catalytic activity results in the formation of linear and branched oligosaccharides of different chain lengths. The exo-amylases hydrolyze from the non-reducing end, successively resulting formation of short end products. Exo amylase such as β-amylase hydrolyze α-1,4 glycosidic and glucoamylase catalyze cleavage of α-1,4 and α-1,6 glycosidic bonds (Singh *et al.,* 2016). The starch degrading enzymes are found in the numerous glycoside hydrolase families 13 (GH-13 families) (Twari *et al.,* 2015). **α-Amylase (EC 3.2.1.1)**, a calcium metalloenzyme, is also known as glycogenase or 1,4-α-D-glucan glucanohydrolase. They hydrolyze α-1,4 glycosidic bonds randomly and cleave long-chain sugars to yield maltotriose and maltose from amylose, or maltose, glucose and limit dextrin from amylopectin (Singh *et al.,* 2016). α-Amylases are derived from plants, animals, bacteria (*Bacillus*), and fungi (ascomycetes and basidiomycetes) (Twari *et al.,* 2015).

β-amylase (EC 3.2.1.2) hydrolyze α-1,4 glycosidic linkages from non-reducing ends and cannot bypass α-1,6 linkages, unlike α-amylase. It is produced by plants, bacteria, and fungi. Though β-amylase is absent in animal tissues, it may be present in the microbes that live in the digestive tracts. β-amylase hydrolyze starch into maltose in ripened fruits resulting in sweet flavor (Singh *et al.,* 2016).

γ-Amylase (EC 3.2.1.3) cleaves α (1-6) glycosidic linkages, in addition to cleaving the last α (1-4) glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose. Unlike the other forms of amylase, γ-amylase is most efficient in acidic environments and has an optimum pH of 3 (Twari *et al.,* 2015). Figure 2 shows types of summary of amylase.

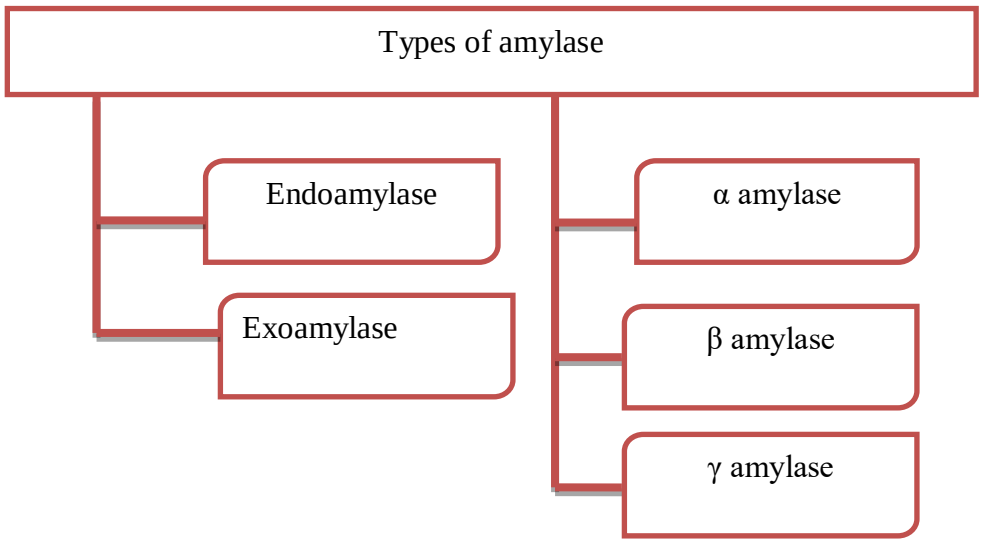


Figure 2: Summary of types of amylase enzyme (Saini *et al.,* 2017)

2.4.3. Structure of Amylase

Amylases have three-dimensional (3D) ribbon structure and is found in different organisms. Its protein is made up of 496 amino acids arranged in alpha helices and beta sheets. The structure also contains 170 water molecules, one calcium ion, and one chloride ion. Three water molecules and one of each amino acids asparagine, aspartate, arginine, and histamine are bound to the calcium ion. Calcium is needed for the enzyme to function. Also, two arginines, one asparagine, and one water molecule is attached to the chloride ion (Islam *et al.,* 2017).

2.4.4. Microbial Amylase

Amylases can be derived from several sources, such as plants, animals, bacteria, and fungi. Because of the short growth period, biochemical diversity, and the ease with which enzyme concentrations might be increased by environmental and genetic manipulation; the enzymes from microbial sources generally meet industrial demands (Naidu and Saranraj, 2013). Despite the wide distribution of amylases, microbial sources, mainly fungal and bacterial amylases, are used

for industrial production due to advantages such as cost-effectiveness, consistency, less time, and ease of process modification and optimization (Tiwari *et al.,* 2015).

Among bacteria, Several *Bacillus* sp. and thermostable Actinomycetes including *Thermomonospora* and *Thermoactinomyces* are versatile producers of the enzymes (Naidu and Saranraj, 2013). The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance. Highly thermostable and alkaline α-amylases are available from the mesophilic *Bacillus* sp. (Naidu and Saranraj, 2013). The thermophilic bacterium *Bacillus stearothermophylus* offers an alternative for commercial production of thermostable α-amylases (Naidu and Saranraj, 2013). Commercial production of thermostable and alkaline α-amylases was produced by mesophile *Bacillus* sp. such as *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* which have large applications (Al-Johani *et al.,* 2017).

Fungal species producing amylase efficiently include those of genus *Aspergillus* (*A. oryzae, A. niger, A. awamori, A. fumigatus, A. kawachii, and A. flavus*), as well as *Penicillium* species (*P. brunneum, P. fellutanum, P. expansum, P. chrysogenum, P. roqueforti, P. janthinellum, P. camemberti,* and *P. olsonii*), *Streptomyces rimosus, Thermomyces lanuginosus, Pycnoporus sanguineus, Cryptococcus flavus, Thermomonospora curvata, and Mucor* sp. (Sundarramand and Murthy, 2014). Fungi belonging to *Aspergillus* genus and thermophilic fungi such as *Talaromyces emersonii*, *Thermomonospora fusca* and *Thermomyces lanuginosus* have been mostly employed for a-amylase production (Al-Johani *et al.,* 2017).

2.4.5. Application of Microbial Amylase in Industry

In the 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 (Souza *et al.,* 2010). In the baking industry, these enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. 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 (Souza *et al.,*2010). Amylases are added to dishwashing detergents for the removal of spots containing food particles (Singh *et al.,* 2016). In the paper industry,α-amylases were

used for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch paste when used as a mounting adhesive modified with additives such as protein glue or alum, frequently, causes damage to paper as a result of its embrittlement (Souza *et al.*, 2010), used for sizing and coating the paper instead of expensive chemically modified starches (Saini*et al.*,2017).

In textile industry, amylases are exploited for designing processes i.e. removal of starch to improve uniform wet processing. Starch, a low price and easily available sizing agent, is added to yarn for a fast and secure weaving process (Souza *et al.*, 2010). In textile weaving, the starch paste is used for warping to give strength and prevent the loss of strings. After weaving, amylase is added that specifically catalyze the hydrolysis of starch to water-soluble dextrins. Amylase efficiently removes starch without damaging the fabric (Singh *et al.*, 2016). In liquefaction, thermostable amylases catalyze the dispersion of insoluble starch granules in an aqueous solution followed by partial hydrolysis. In industrial processes, the starch suspension for liquefaction is generally more than 35% (w/v). Therefore the viscosity is extremely high following gelatinization. Thermostable α-amylase is used as a thinning agent,which brings about a reduction in viscosity and partial hydrolysis of starch. Retro gradation of starch is thus avoided during subsequent cooling (Saini*et al.*, 2017).

2.5. Protease

Proteases are a group of enzymes known as peptidyl-peptide hydrolases and catalyze the cleavage of the peptide bond in proteins using proteolysis (Mikaela, 2018). Though the hydrolytic enzymes belong to the largest group of enzymes and are the most commercially applicable enzymes, among the enzymes within this group the microbial proteases have been extensively studied (Nigam,2013).

2.5.1. History of Protease Discovery

Early in his career, Theodor Schwann, who is often considered the father of modern cell biology, described the purification of an active component from gastric juices that was able to digest egg albumin. He named this compound pepsin, which is the first protease isolated in history (Sandra *et al.*, 2018). Nowadays, the discovery of pepsin is widely regarded as the beginning of modern

protease research. However, it was not until 40 years after his discovery when trypsin, one of the best-studied enzymes, was purified by physiologist Wilhelm Friedrich Kühne (Sandra *et al.*, 2018).

2.5.2. Types of Protease

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), proteases are classified in subgroup 4 of group 3 (Pratush *et al.*, 2013). Proteases were initially classified into endopeptidases, which target internal peptide bonds, and exopeptidases (aminopeptidases and carboxypeptidases), the action of which is directed by the NH2 and COOH termini of their corresponding substrates. Proteases of the different classes can be further grouped into families based on amino acid sequence comparison, and families can be assembled into clans (a group of interlinked families) based on similarities in their three-dimensional structures (Pratush*et al.,* 2013).

Depending upon the optimum pH microbial proteases are of three types: acidic, neutral, and alkaline (Nigam, 2013). Acid proteases, active in the pH range 2.0 – 3.5; neutral Proteases, active in the pH between 6.5 and 7.5 and alkaline proteases, active in the pH between 7.5 and 10.5 (Pratush *et al.,* 2013). Alkaline serine proteases have the largest applications in the bio-industry. Alkaline proteases are of particular interest being more suitable for a wide range of applications since these possess high activity and stability in abnormal conditions of extreme physiological parameters. Alkaline proteases have shown their capability to work under high pH, temperature and in presence of inhibitory compounds (Nigam, 2013; Mienda *et al.,* 2014).

2.5.3. Structure and Function of Protease

Proteolytic enzymes belong to the hydrolase class of enzymes (EC 3) and are grouped into the subclass of the peptide hydrolases or peptidases (EC 3.4). Depending on the site of enzyme action the proteases can also be subdivided into exopeptidases or endopeptidases. Exopeptidases catalyze the hydrolysis of the peptide bonds near the N- or C-terminal ends of the substrate. Aminopeptidases can liberate single amino acids (EC 3.4.11), dipeptides (dipeptidyl peptidases, EC 3.4.14), or tripeptides (tripeptidyl peptidases EC 3.4.14) from the N-terminal end of their substrates. Single amino acids can be released from dipeptide substrates by dipeptidases (EC

3.4.13) or from polypeptides by carboxypeptidases (EC 3.4.16-3.4.18), while peptidyl dipeptidases (EC 3.4.15) liberate dipeptides from the C-terminal end of a polypeptide chain. Endopeptidases cleave peptide bonds within and distant from the ends of a polypeptide chain (András *et al.*, 2013).

2.5.4. Microbial Protease

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases (Pratush, 2013). Microbial protease (peptidyl-peptide hydrolases, EC: 3.4.11-19) representing one among the three largest groups of industrial enzymes, accounts for 65% of total worldwide enzyme sales (Pathak,2015). Proteases have been successfully produced by researchers from different microbial sources. Microbes account for a two-thirds share of commercial protease around the globe (Razaq *et al.*, 2019). Since the beginning of enzymology, microbial proteolytic proteases have been the most widely studied enzyme. These enzymes have gained interest not only due to their vital role in metabolic activities but also due to their immense utilization in industries (Razaq *et al.*, 219).

Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus Bacillus. Viz., *Bacillus sphaericus* NRC 24, Mutant strain of *Bacillus subtilis, Bacillus cereus, Bacillus firmus* MTCC 7728, *Bacillus badius* MTCC 7727, *Bacillus subtilis* FP-133, *Bacillus subtilis* DM-04, *Bacillus aquimaris* VITP4, *Bacillus* strain HS 08, *Bacillus circulans* BM15, *Bacillus circulans, Bacillus licheniformis* MIR 29 (Pratush, 2013). *Bacillus brevis* (APP28), *Bcillus stearothermophilus* (APP29), *Pseudomonas oleovorans* (APP30), *Bacillus aminivorans* (APP38), and *Bacillus firmus* (APP39) are excellent producers of thermostable alkaline protease (Pathak and Rathod 2014).

Among fungus strains, *Mucor pussilus, Aspergillus oryzae, Mucor miehei, F. chibinensis, Penicillium citrinum, Candida albicans, Acremonium typhinum, Penicillium expansum, Rhizomucor pusillus, Pleurotus ostereatus* are protease producers (Pratush, 2013).

2.5.5. Application of Protease in Industry

In the food and feed industry, proteases are used for cheese making, baking, preparation of soy hydrolysates, and meat tenderization. The major application of proteases in the dairy industry is

in the manufacture of cheese (Pratush, 2013). During cheese production from milk, proteases are added to hydrolyze kappa casein to prevent coagulation by stabilizing micelle formation. In Detergent Industry, Proteases have been widely used at commercial scale. The various products in the detergent industry containing proteases as an essential component or ingredient have been used for cleaning household laundry, dentures, or contact lenses (Razzaq *et a.l,* 2019). In the leather industry processing proteases play vital roles such as soaking, dehairing, bating, and tanning, for example. Proteases play a vital role in the treatment of raw leather in tanneries (Sundus *et al.,* 2016). In Waste Management, alkaline proteases degrade keratin wastes from the poultry and leather industry (Kudrya and Simonenko, 1994). *Bacillus* species is the most widely reported bacterial source of keratinases for feather degradation (Razzaq *et al.,* 2019).

2.6. Cellulase

The third-largest industrial enzyme worldwide is cellulases, because of their utility in paper recycling, cotton processing, detergent industry, and food processing industry. To produce a wide variety of cellulases, both fungi and bacteria have been heavily exploited. Till then, the emphasis has been placed on fungal cellulases because of the large amount of less complex extracellular cellulases which used to be more readily cloned and produced via recombination in a rapidly growing bacterial host. However, recently the researchers have been shifted towards the bacterial cellulases, because of robust bacterial growth, survival in harsh conditions of bioconversion processes, stability, and presence of multi-enzyme complexes which provide increased function and synergy (Acharya and Chaudhary, 2012).

2.6.1. History of Cellulase

Cellulose has been utilized by man for centuries; however, its huge potential as a renewable source of energy was recognized only after the discovery of the cellulose-degrading enzymes or, “cellulases” had been identified by Mary Mandels and Elwyn T. Reese (Bhat and Bhat, 1997; Bischof *et al.,* 2016). During World War II, the U.S Army was worried at the rate of deterioration of cellulosic materials including clothing, tent, and sandbags, in the south pacific. Several organizations within the army set up laboratories to find an immediate solution to this problem. As a result, parent strain QM6a was isolated from shelter remains and identified as *Trichiderma viride* and later recognized as *Trichoderma resii*. The immediate benefits of the

army led to further research on the selection and characterization of hyper-cellulolytic *T. resii* strains. These projects not only improved the production of cellulase by *T. resii* but also aroused worldwide research activity. The work of Natick army laboratory and other places quickly led to the recognition that cellulosic wastes may be converted to glucose, soluble sugars, alcohols, single-cell protein, and other industrially useful chemicals through the agency of cellulases (Bhat and Bhat, 1997; Bischof *et al.,* 2016).

2.6.2. Types and Function of Cellulase in Cellulose Degradation

Cellulose is one of the main components of plant cell wall material and is the most abundant and renewable nonfossil carbon source on Earth. The degradation of cellulose to its constituent monosaccharides has attracted considerable attention for the production of food and biofuels. The degradation of cellulose to glucose is achieved by the cooperative action of endocellulases (EC 3.1.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91; glucanohydrolases, EC 3.2.1.74), and beta-glucosidases (EC 3.2.1.21) (Li, *et al.,* 2011; Acharya and Chaudhary, 2012; Rodríguez *et al.,* 2018).

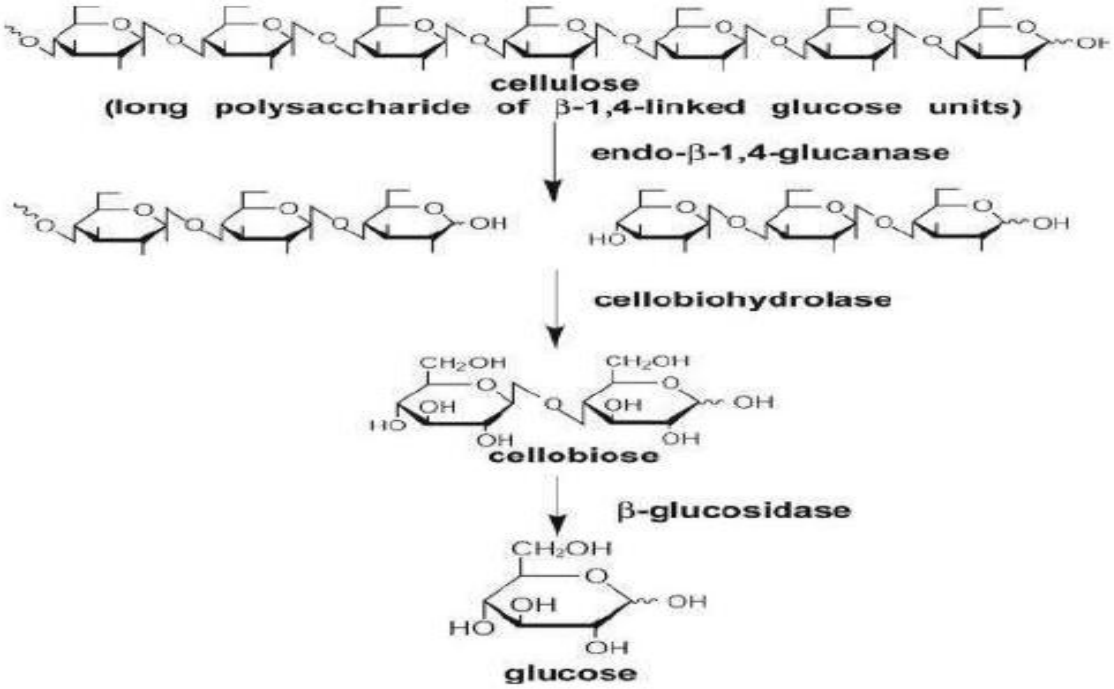


Figure - 1: - Cellulose degradation

Figure 3: Classes of enzymes involved in cellulose breakdown (Lakhundi *et al.,* 2015).

Endoglucanase produces random cuts at an internal position within cellulose fiber releasing cellooligosaccharides. Exoglucanases/cellobiohydrolase act on chain ends releasing cellobiose which is then acted upon by β glucosidase to release glucose.

2.6.3. Structure of Cellulase

A common characteristic of cellulases is their modular structure. Typically, endocellulases and cellobiohydrolases are composed of four domains or regions. A signal peptide that mediates secretion, a cellulose-binding domain (CBD) for anchorage to the substrate, a hinge region (linker) rich in Ser, Thr, and Pro residues, and a catalytic domain (CD) responsible for the hydrolysis of the substrate. The mature proteins are O- and N-glycosylated in the hinge region and the CDs, respectively. The effect of the glycosylation sites in the hinge region is not clear yet but they may play a role in the flexibility and disorder of the linker (Li*et al.*, 2011).

2.6.4. Microbial Cellulase

Among bacteria, the cellulolytic property has been reported in *Bacillus* strains and thermophilic anaerobic bacterium *Clostridium thermocellum*. Acharya and Chaudhary (2012) believed that thermophilic cellulolytic *Bacillus* strains are found in the hot springs and their optimal activity is 60^0C. Thermophilic bacteria such as, *Bacillus licheniformis* JK7, *Bacillus* sp. SMIA-2, *Geobacillus stearothermophilus* and *Bacillus megaterium* are thermostable cellulase producers. *Acidothermus cellulolyticus, Anaerocellum thermophilum, Aneurinibacillus thermoaerophilus WBS2, Anoxybacillus flavithermus* EHP2, *Anoxybacillus* sp. 527, *Bacillus licheniformis* 2D55, *Bacillus licheniformis* MVS1, *Bacillus subtilis, Bacillus* sp. C1AC5507 are cellulase producers (Sahoo *et al.,* 2019).

Unlike bacterial cellulase, fungal cellulases are active over a wide range of temperatures. Psychrotolerant fungus, *Aspergillus terreus* AKM-F3 produce optimum cellulase at 15 °C (Maharana and Ray, 2015), Aspergillus niger produce optimum cellulase at 30 °C (Mrudula and Murugammal,2011). *Nectria catalinensis* cellulase activity ranged from 50 to 55 °C (Pardo and Forchiassin,1999). *Aspergillus fumigatus* M.7.1 and *Myceliophthora thermophila* M.7.7 produce cellulase at 70 °C (Moretti *et al.,* 2012). Acid tolerant cellulase was reported by *Trichoderma reesei* (Onofre *et al.,* 2014). Fungus with dual tolerance such as acido-thermo-tolerant cellulase

by *Chaetomium thermophile* (pH 4.0-4.5 and 60°C) and *Penicillium* sp. CR-316 and *Penicillium* sp. CR-313 (65 °C and pH 4.5) (Picart *et al.,* 2007) was also reported. Thermophilic fungi such as *Chaetomium thermophile*, *Sporotrichum thermophile,* and *Thermoascus aurantiacus* are also sources of cellulase. *Trichoderma* sp. was considered as the best source of cellulases (Acharya and Chaudhary, 2012).

2.6.5. Use of Cellulase in Industry

In the food industry, improved extraction, clarification, yields, and bitterness removal can be done through the use of thermostable cellulases. Mainly involving sources such as *Bacillus* and *Paenibacillus* (Sahoo*et al.,* 2019). In Textile Processes, the textile industry uses thermophilic cellulases for creating the stone-washed look in jeans, biopolishing of cotton, and other cellulosic fabrics. Commonly the thermophilic cellulases are used in the stone washing of jeans to make them appear faded. (Acharya and Chaudhary, 2012). Decolorization and fuzzing effects after repeated washing can be decreased after the addition of cellulase in detergents (Sahoo *et al.,* 2019). In the Pulp and Paper mill, thermophilic cellulases lead to energy saving and also improve the mechanical strength of the pulp (Acharya and Chaudhary, 2012; Sahoo *et al.,* 2019). Cellulases can also be used in the production of paper towels, biodegradable cardboard, and sanitary paper (Sahoo *et al.,* 2019). In Biofuel production, cellulases are important in the conversion of lignocellulose to ethanol (Acharya and Chaudhary, 2012). *Bacillus* sp. SMIA-2,and *Geobacillus* sp. T1 has been involved in biofuel formation (Menendez *et al.,* 2015).

2.7. Lipase

Lipases (triacylglycerol acylhydrolases; EC3.1.1.3) catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Lajis, 2018). Thermostable lipases from microbial sources are highly advantageous, for biotechnological applications, since they can be produced at low cost and exhibit improved stability. In recent years there has been a great demand for thermostable enzymes in industrial fields. Thus thermostable lipases from various sources have been purified and characterized (Hasan, 2005).

2.7.1. History of Lipase

Eijkman was the first person to isolate lipases from *Bacillus prodigiosus*, *Bacillus pyocyaneus*, and *Bacillus fluorescens*, then they are named *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*, respectively (Eijkmann, 1901). Nowadays, it is recognized that lipases are produced by various organisms, including animals, plants, and microorganisms. Most animal lipases are obtained from the pancreas of cattle, sheep, hogs, and pigs (Casas-Godoy *et al.*, 2018). Unfortunately, lipases extracted from the animal pancreas are rarely pure enough to be used in the food industry. For example, pig pancreatic lipase is polluted by trace amounts of trypsin which generates a bitter taste. Other impurities include animal viruses and hormones. Therefore, due to the ease of production and abundance, most studied and industrially used lipases are obtained from microbial sources (Casas-Godoy *et al.*, 2018).

2.7.2. Types and Structure of Lipase

There are three types of lipase; pharyngeal lipase, hepatic lipase, and pancreatic lipase. All lipases display the same structural architecture, the so-called α/β- hydrolase fold and have identical catalytic machinery (Ugo *etal.*, 2017). The first lipase structures were obtained from *Rhizomucor miehei* and the pancreatic human lipase. Several hundreds of lipase sequences are listed in databases, in 2012 the Protein Data Base had among 100 three dimensional lipase structures; nowadays this database has 182 three-dimensional lipase structures (Casas-Godoy *et al.*, 2018).

2.7.3. Microbial Lipase

Lipases are ubiquitous and are produced by several plants, animals, and microorganisms. Lipases of microbial origin represent the most widely used class of enzymes in biotechnological applications and organic chemistry. Microbial lipases have gained special industrial attention due to their stability, selectivity, and broad substrate specificity (Thakur, 2012). Among bacteria, *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus coagulans, Bacillus stearothermophilus,* and *Bacillus alcalophilus* are the most common lipases producers (Thakur, 2012). Several extracellular thermoalkaline lipases have been identified from Gram-positive bacterial strains such as *Geobacillus thermoleovorans* (i.e., strain DA2, ID-1), *Geobacillus*

thermodenitrificans (i.e., IBRL-nra, AV-5), *B. stearothermophilus*, *Cohnella thermotolerans*, and *Thermosyntropha lipolytica* DSM 11003 (Lajis, 2018).

Major commercially important lipase-producing filamentous fungi are belonging to the genera *Rhizopus*, *Aspergillus*, *Penicillium*, *Mucor*, *Ashbya*, *Geotrichum*, *Beauveria*, *Humicola*, *Rhizomucor*, *Fusarium*, *Acremonium*, *Alternaria*, *Eurotrium* and *Ophiostoma* (Thakur, 2012; Chandra*et al.*, 2020). From species, *A. niger*, *C. rugosa*, *H. lanuginosa*, *M. miehei*, *R. arrhizus*, *R. delemar*, *R. japonicus*, *R. niveus* and *R. oryzae* are the principal manufacturers of commercial lipases (Chandra *et al.*, 2020).

2.7.4. Industrial Application of Lipase

In the textile industry, Lipases are used to assist in the removal of size lubricants, to provide a fabric with greater absorbency for improved levelness in dyeing (Hasan *et al.*, 2005). In the food industry, lipase is used for the production of dairy products, such as cheese, modification of fats and oils (e.g., manufacture of butter and margarine, new cooking oils), and production of baby food and structured lipids with unique properties (e.g., cocoa butter equivalent, human milk substitute, high or reduced-calorie fats, polyunsaturated fatty acid (PUFA)-enriched oils) (Casas-Godoy *et al.*, 2018). In the leather industry, lipases facilitatethe process and improve leather quality during different stages in leather manufacturing, such as dehiring, curing, soaking, bating, liming, pickling, tanning, and degreasing (Lajis, 2018). Lipases are also utilized in other industries i.e., Pulp and paper industry, Energy industry (Casas-Godoy *et al.*, 2018).

2.8. Factors Affecting the Growth of Microbes

2.8.1. Temperature

Temperature is one factor that affects the growth of microbial growth. Microorganisms that have optimum temperatures between 0º-20ºC are called psychrophilic. Microorganisms that grow rapidly in the temperature range of 20º-50º C are called mesophilic, while microorganisms that grow in the temperature range of 50º-100º C are called thermophiles. Some microorganisms can survive at high temperatures even though these temperatures cannot grow, this group is called thermoduric. Spore-forming microorganisms can survive boiling temperatures for 5-15 minutes because they are heat resistant (Jufri, 2020).

2.8.2. pH

pH is another factor that affects the growth of microbes. A closed environment such as nutrient broth in a tube quickly causes a change in pH, thereby inhibiting the growth of microorganisms. To prevent changes in pH, a buffer solution is often added to the media. In general, bacteria grow well at a pH of around 7.0, although they can grow in a pH range of 5.0 - 8.0. Microorganisms that carry out the fermentation process produce acid so that the pH can drop to 3.5. On the contrary, when the metabolism of proteins and amino acids is released ammonium ions so that the pH of the media gets wet (Amaliyah, 2017).

2.8.3. NaCl Concentration.

Extreme environments involve a wide range of extreme conditions (pH, temperature, pressure, Light intensity, oxygen, nutrient conditions, heavy metals, and salinity). Microorganisms can adapt to a wide range of NaCl concentrations. Many scholars did examine different optima of NaCl concentration of microbes. For example, mild halophiles grow at NaCl concentration of (1-6%, w/v), moderate halophiles grow at NaCl concentration of (7-15%, w/v) and extreme halophiles grow at NaCl concentration of (15–30%, w/v). At high concentrations of soluble salts, cytoplasm mainly of bacteria and archaea is exposed to high ionic strength and achieves osmotic equilibrium by maintaining a cytoplasmic salt concentration similar to that of the surrounding media (Cira-Chávez *et al.,* 2018).

3. Materials and Methods

3.1. Description of Study Sites

The study was conducted in three hot springs located in different geographical locations of Ethiopia. Namely, Woliso, Wondo Genet, and Shalla Hot Springs.

Woliso, Waliso (Afan Oromo name), also translated as Wolisso is a town located in the South West Shewa Zone of the Oromia Region, 114 km southwest of Addis Ababa, with an elevation of 2063 meters above sea level. The Hot Spring lies at global position of 8˚32’00.81N and 37˚58’53.11’’E. Woliso is the administrative center of this Zone. The average minimum, maximum and mean annual temperatures of Woliso are about 13.6, 25, and 19.3 ˚C, respectively. The amount of annual average rainfall is about 1404.76mm (Tekalign, 2015). About 1253 mm of precipitation falls annually.

Lake Shalla hot springs are some of the saline springs in central rift valley of Ethiopia, which are concentrated in the eastern and southwestern shores of Lake Shalla. The hot spring is the largest and hottest spring water with a temperature of 97˚C and a daily discharge rate of 50,000 liters. It is located in the lake’s northeastern corner with an elevation of 1556 meters and a global position of 07˚28.677’N 38˚38.085’E, some 286 km south of Addis Ababa. The hot springs are used by the local people and visitors for the treatment of dermatological, respiratory, and neurological diseases, recreation, sanitation, and livestock watering. The region where this hot spring and Lake Chitu are located has a semi-arid to a sub-humid type of climate with air temperature ranging from 25 to 28 ℃ and annual mean precipitation of 955 mm (Asmare, 2016).

Wondo Genet (also transliterated Wendo Genet) is a resort town in Ethiopia. Located at 236km south of Addis Ababa and 13km southwest of Shashemene in the Sidama Zone of the Southern Nations, Nationalities, and Peoples' Region, at an elevation of 1723 meters and its Hot spring lies on latitude and longitude of 7° 5'0.61"N and 38°38'15.53"E. The rain of Wondo Genet is characterized by a bimodal distribution with the main rainy season between July and October, which accounts for 50 % of the total, and a short rainy season between March and May. The mean annual rainfall is 1247mm and the mean monthly temperature is 19.5˚C with monthly maximum and minimum temperature of 26 and 12.4˚C respectively. Wendo Genet is one of the

most well-known nature-based recreational sites in Ethiopia. The main attractions of the site include the hot spring water for bathing. The attractive quality of the site for many of the users is its nature-based resources. The natural ecosystem of the area can be described as wetland which can be categorized under freshwater or geothermal spring or streams and creeks category according to RAMSAK (1971) classifications of wetland types.

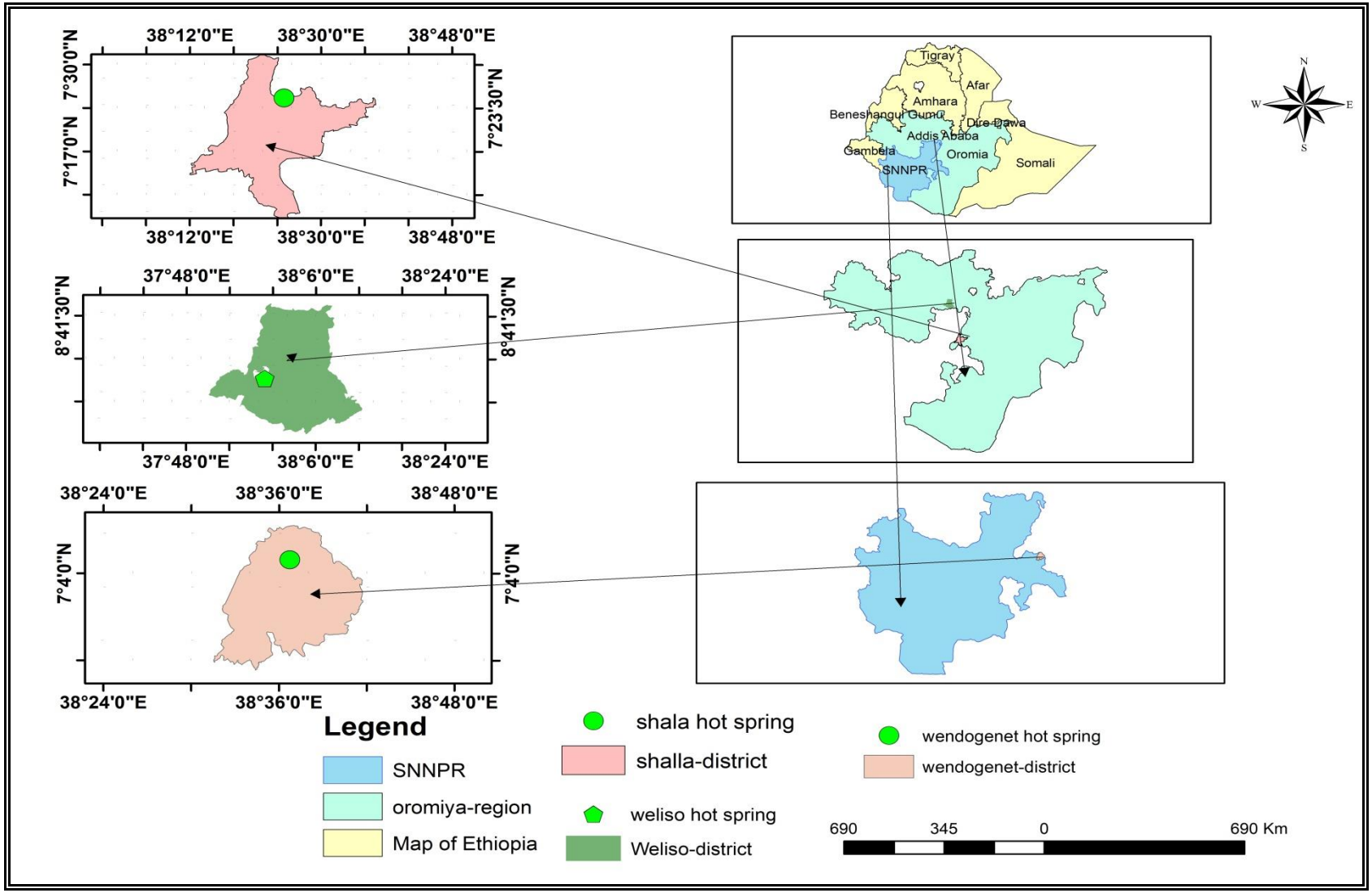


Figure 4: Map of study areas (ArcGIS)

3.2. Study Design and Period

Cross-sectional and experimental study was conducted to collect the sample for the isolation and characterization of thermostable enzyme-producing microorganisms between February 2020 and October 2021.

3.3. Sample Collection and Transportation

A total of 90 water and sediment samples were collected purposively from Woliso, Wondo Genet and Shalla hot springs. Thirty samples (30) each were collected from the three hot springs consisting of 15 water and 15 sediment samples. Three different sites were selected to collect the water and sediment samples five times (a total of 90 samples) from Woliso, Wondo Genet and Shala hot spring. Water (500 ml) and sediment (500g) were aseptically collected using sterile thermal glass containers and immediately placed in a thermoflask to maintain the temperature of the water samples. The water samples were collected considering three different depths: 10cm, 20 cm and 30cm below the surface and away from the margin in order to have representative samples and the sediment samples were collected at the bottom of the hotsprings using sterile ladle and placed in sterile polythene bags. Parameters such as pH, temperature, salinity, electric conductivity, and GPS were evaluated on-site using probing devices such as pH meter, thermometer, salinity meter, conductometer, and GPS measuring. Then, the samples were aseptically transported to Jimma University, Research and Postgraduate Laboratory for further processing.

3.4. Isolation of Thermophilic Bacteria and Fungi

After aseptically transporting the samples to the laboratory, 25 milliliter water samples from each hot springs were suspended in 225ml of peptone water. Sediment samples (25 grams) were suspended in 225ml of peptone water and both suspended water and sediment samples in peptone water were enriched by incubating for 24 hour at 45, 71.9 and 96.45 ˚C respectively simulating their natural environments and to be served as a stock sample also the enrichment was conducted at 45 ºC for all samples collected from each hot springs to increase the likely chance of the isolates to grow well (Sahay *et al.,* 2017; Mohammad *et al.,* 2017). Then a series of test tubes containing 9ml of peptone water were used for serially diluting the samples (10^{-1}- 10^{-5}). Aliquots of (0.1 ml) of dilutions of each enriched sample were spread on Nutrient agar (NA) and ATCC (Thermus medium) containing (%), (0.5% NaCl, 0.5% Peptone, 0.4% Beef extract, 0.2% Yeast extract, 2.0% Agar) for bacteria and Potato Dextrose agar medium (PDA) plates for fungi Welday *et al.,* (2014) and incubated at 45˚C for 24-48 hrs. for bacteria and 45˚C for 3-5 days for fungi. After incubation, morphologically distinct colonies in shape, color, and size were then purified by sub culturing and maintained at 4℃ for further studies (Patel *et al.,* 2019). Twenty (20%) glycerol broth was used for preservation purpose. One loop-full of bacterial inoculum was transferred to 5 ml nutrient broth medium and incubated at 45℃ for 24 hours. Then, 800 µl of bacterial suspension was mixed with 200 µl autoclaved glycerol in 1.5ml autoclaved eppendorf tubes. It was then sealed with parafilm tapes and stored in -20˚C refrigerator (Islam *et al.,* 2017).

3.5. Screening of Bacterial and Fungal Isolates for Hydrolytic Enzyme Production

All isolates of bacteria and fungi were tested for the production of amylase, protease, cellulase, and lipase in their respective enzyme screening medium by incubating at 45˚C for 24 to 48 hrs and 3-5 days for bacteria and fungi isolates respectively. For amylase activity, a starch agar medium was used. After incubation, the plates were flooded with 1% Lugol's iodine solution. For protease activity, Skimmed Milk Agar (SMA) medium was prepared and the nutrient broth culture of bacterium after 24 h of incubation was centrifuged at 10,000 rpm (4ºC) for 20 min and the cell-free supernatant obtained was spot inoculated following agar well method (Panda *et al.,* 2013). Nutrient agar supplemented with 1%Carboxymethyl cellulose (CMC) was used for the detection of cellulase-producing isolates. After incubation, the plates were flooded with an

aqueous solution of Congo red (1%w/v) for 15 minutes. The Congo red solution was then discarded, and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation. For lipase, nutrient agar medium was supplemented with 1% of Tween 80. Any formation of an opaque halo around the colonies that show the positive lipolytic activity was indicated (Mohammad *et al.*, 2017). Any formation of a zone of hydrolysis around the colonies was observed for each enzyme and isolates exhibiting the largest clear zones (15mm and above) around the colony were selected as potent enzyme producers (Sundarapandiyan and Jayalakshmi, 2014). Then the relative enzyme activity was calculated using the following formula (Latorre *et al.*, 2016).

$$Relative\ Enzyme\ Activity = \frac{Diameter\ of\ clear\ zone\ (mm)}{Diameter\ of\ colony\ (mm)}$$

3.6. Characterization of Potent Enzyme-Producing Bacterial Isolates

The selected isolates, which were chosen as potent thermostable amylase, protease, cellulase, and lipase producers, were further characterized by their morphology and biochemical properties.

3.6.1. Morphological Test

3.6.1.1. Gram Staining

To apply the Gram test, air-dried, heat-fixed smears of cells were flooded with crystal violate staining reagent for 1 minute. Then the slide was washed in a gentle and indirect stream of tap water for 2 seconds. Secondly, the slide was flooded with the mordant: Gram's iodine and Waited for 1 minute and washed in a gentle and indirect stream of tap water for 2 seconds. Thirdly, the slide was flooded with a decolorizing agent (95% ethyl alcohol) and waited for 5 seconds, and washed with water as above. Fourthly, the slide was flooded with counterstain, safranin and waited for 30 seconds. After that, the slide was washed in a gentile and indirect stream of tap water until no color appears in the effluent and let dry in the air. Finally, the slide was observed in the oil immersion objective under the compound light microscope found in the laboratory.The observations include the color and shape of the bacterial cell. Gram-positive bacteria are marked by purple color which indicates that the bacteria cell wall is capable of

binding to crystal violet dye, whereas gram-negative is characterized by the formation of pink color indicates that the bacteria cell wall are not able to bind the crystal violet dye (Gram, 1884)

3.6.1.2. Spore Formation

A bacterial smear was made in sterile conditions. The smear was air-dried and heat-fixed. The slide was flooded with malachite green and placed on top of a beaker containing boiling water and left for 3 minutes. After washing with water, it was stained with safranin for 30 seconds and washed. Then the smear was air-dried and observed under the compound light microscope. Endospores stained green while vegetative cells stained pink (Islam, 2016).

3.6.1.3. Motility Test

To test for motility, a sterile needle was inoculated with pure bacterial culture and is stabbed into the test tube containing the motility medium within 1cm of the bottom of the test tube. The needle was in the same line as it enters and was removed from the motility medium. Then the test tube was incubated at 45˚C until growth is evident. Any growth was observed after incubation. Non-motile bacteria had growth confined to the stab line with definite margins without spreading to the surrounding area while motile bacteria gave diffused growth extending from the surface and line of inoculation (Talaiekhozani *et al.,* 2015).

3.7. Biochemical Test

3.7.1. Catalase Test

The purpose of the catalase test is to determine the ability of bacteria to produce the catalase enzyme. A drop of 3% hydrogen peroxide was placed on a clean microscope slide. A visible amount of bacteria (colony) was streaked on the glass slide with the inoculating loop and observed for gas (bubble) production. Any formation of gas in the form of bubble was observed and recorded (Holt and Krieg, 2000).

3.7.2. Oxidase Test

A clean what man filter paper was placed on a Petri dish. A drop of 1% Kovács oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride) was added to the filter paper and let dry.

Using an inoculating loop, a small amount of bacterial culture was streaked on the drop of the oxidase reagent. Any formation of the dark purple color of the reagent was observed as oxidase positive (Yadav*et al.*, 2018).

3.7.3. Simon's Citrate Test

The media was prepared and 7 ml media was added to clean test tubes. The test tubes were autoclaved and then left to cool at a slanted position to create a butt and slant. An inoculating needle was used to pick up a single colony from a 24 hours fresh bacterial culture. The slant of the media was streaked from bottom to top using a zigzag motion with the needle. The test tubes were incubated at 45˚C for 48 hours. The color of the media was observed after incubation. A change from green to blue indicates utilization of the citrate (Hensyl, 1994).

3.7.4. Indole Test

To test for indole SIM medium was boiled and 8ml of the medium was dispensed to test tubes and autoclaved at 121°C under 15 psi pressure for 15 minutes. After autoclaving, the test tubes were allowed to cool in an upright position. Then the tubes were inoculated with bacterial isolate under test and incubated at 45°C for 24 to 48 hrs. After incubation, 5 drops of Kovac's reagent was added to 8 ml of a 24-48 hrs. old culture. Any formation of the red color ring was observed as indole positive (MacWilliams, 2016).

3.8. Physiological Test

3.8.1. Temperature and pH Tolerance

The optimum temperature and pH for the growth of thermophilic bacterial isolates were studied by streaking on nutrient agar medium for alkaline pH and nutrient broth medium was used for acidic pH. The cultures were grown at different temperatures in the range of 40-70 °C. The pH of the medium was adjusted to different pH ranges (3-11). Hydrocholorc acid was used to adjust the acidic pH 3-6; whereas, the alkaline pH of 7.0-11.0 was adjusted by 5N NaOH. After incubation, plates were observed for the growth of the bacterial isolates (Sharma *etal.*, 2018).

3.8.2. Salt Tolerance

Six batches of NA were prepared in 1 L of distilled water and each supplemented with 3, 5, 7, 10, 12, and 15% sodium chloride concentration. This was to determine the ability of the isolates to grow at different sodium chloride concentrations. Three sets of experiments for each concentration were conducted. These were incubated at 45°C then checked for growth after 24 hrs. (Salano *et al.*, 2018).

3.8.3. Identification of Fungal Isolates

Fungal isolates were characterized by morphological and microscopic characterization. For microscopic characterization Lacto phenol cotton blue staining was used. Any result showing different genus of fungus was observed and recorded after culturing and microscopic mounting.

3.9. Data Analysis

The experimental setup was prepared with three replications. The results were expressed as the mean ± SD of different independent replicates. Analysis of variance (ANOVA) followed by Duncan post hoc multiple comparison tests was done using SPSS software (version 20). The values of $P \leq 0.05$ were considered statistically significant.

4. Results and Discussion

4.1. Results

4.1.1. Physicochemical Properties of the Samples

The physical and chemical properties of water samples obtained from Hot springs were examined to evaluate their effect on the isolation of thermophilic microbes. The results showed that Woliso Hot spring had a minimum temperature gradient of 45 ± 0.00ºC and Shalla Hot spring had the highest temperature gradient of 96.45 ± 0.35ºC; Wondo Genet Hot spring had the minimum pH gradient of 7.6 ± 0 and Shalla Hot spring had the maximum pH of 8.1 ± 0.14 (table 1).

Table 1: In situ physicochemical analysis of hot spring water samples.

Parameters	Hotsprings			
	Woliso	Wondo Genet	Shalla	
			Site 1	Site 2
Temprature (˚C)	45 ± 0.00	71.9 ± 0.14	96.45 ± 0.35	88.15 ± 0.35
pH	7.75 ± 0.07	7.6 ± 0.00	8.1 ± 0.14	7.8 ± 0.14
EC (µS cm-1)	1225.5 ± 3.54	794.5 ± 3.54	9247 ± 0.00	9542.5 ± 3.54
TDS (mgl^{-1})	791.5 ± 2.12	521 ± 1.41	5094 ± 1.41	6202.5 ± 3.54
DO (mgl^{-1})	683 ± 2.83	676.5 ± 2.12	671.5 ± 2.12	657.5 ± 3.54
Salinity (ppm)	581.5 ± 2.12	341.5 ± 2.12	4781 ± 1.41	5380 ± 0.00

Where; EC = Electric conductivity, TDS= Total dissolved solids, DO= Dissolved oxygen

4.1.2. Thermophilic Bacteria and Fungi Isolated From the Hot Springs

A total of 252 bacterial and 9 fungal thermophilic isolates were isolated from the three Hot springs based on differences in colony morphological features. From the three Hot spring sites, the highest number of thermophilic isolates of bacteria 112 (44.44%) and fungi 5 (55.56%) were obtained from Woliso Hot spring; isolates of bacterial 85 (33.73%) and fungi 2 (22.22%) were isolated from Wondo Genet Hot spring; and the rest bacterial 55 (21.83%) and fingi 2 (22.22%) were isolated from Shalla Hot spring. From 252 (100%) thermophilic bacterial isolates, 246 (97.62%) were Gram-positive, rod-shaped, and endospore-forming, and the rest 6 (2.38%) isolates were Gram-negative, rod-shaped, and non-endospore-forming. The isolates showed

different types of colonies (large, matt, or rough) (Appendix 5). Optimum growth was between 50°C and 55°C (table 8).

Table 2: Frequency and Percentage of isolates from hot spring sites

Hot springs	Bacteria		Fungi	
	Frequency	Percentage	Frequency	Percentage
Woliso	112	44.44	5	55.56
Wondo Genet	85	33.73	2	22.22
Shalla	55	21.83	2	22.22
Total	252	100	9	100

4.1.3. Enzymatic Activities by the Isolates

All bacterial and fungal isolates were tested for hydrolytic enzymes (amylase, protease, cellulase, and lipase) production. Of 252 bacterial isolates, 240 (95.23%) were amylase producers, 212 (84.12%) were protease producers, 193 (76.58%) were cellulase producers and 164 (65.07%) were lipase producers but none of the fungal isolates produced tested enzymes. In the current study, the majority (95.23 %) of the bacterial isolates were found to be amylase producers followed by protease producers (84.12%) (Figure 5).

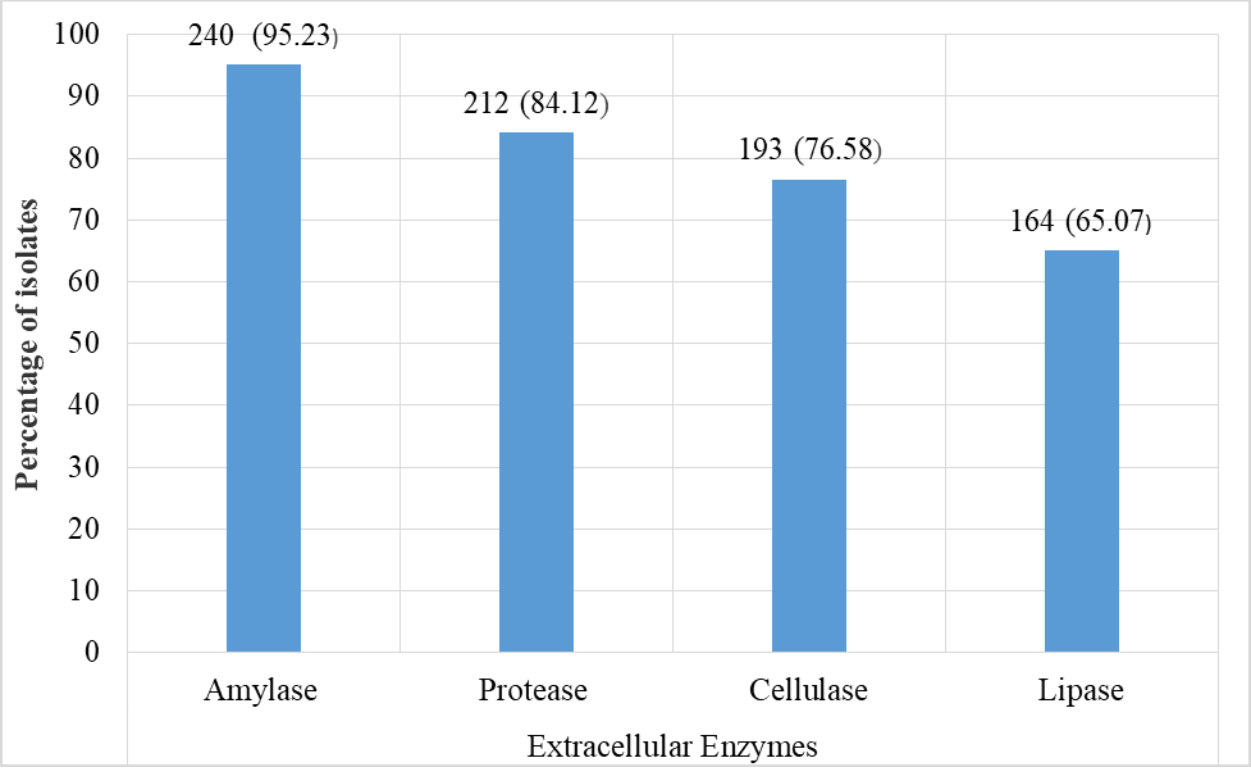


Figure 5: Percentage of the isolates producing extracellular enzymes from all Hot springs.

4.1.4. Multiple Enzyme Production by Bacterial Isolates from all Hot springs

The majority of the isolates 244 (96.8%) were found to produce more than one type of enzyme. Therefore, the isolates were further grouped according to the production of enzymes with their codes and the results are presented in (Table 3).

Table 3: Total number of enzyme groups formed by the bacterial isolates obtained from all Hot springs.

No	Enzyme group	Number of isolates	Name of isolates
1	Amylase, Protease, Cellulase, Lipase	105	WG2,WG3,WG6,WG8,WG9,WG10,WG11,WG13,WG14, WG16,WG18,WG19,WG20,WG21,WG22,WG23,WG,WG24,WG,25,WG26,WG28,WG29,WG30,WG32,WG39,WG42, WG44,WG45,WG47,WG48,WG49,WG50,WG55,WG56,WG57,WG58,WG60,WG61,WG62,WG66,WG67,WG69,WG70,WG72,WG76,WG77,WG83,S4,S7,S9,S12,S13,S14,S15,S16,S17,S18,S20,S21,S34,S35,S38,S40,S41,S42,S46,S47,S49, S51,W1,W4,W5,W9,W10,W12,W15,W17,W21,W23,W27, W28,W29,W32,W35,W38,W39,W41,W43,W49,W54,W55, W58,W59,W61,W62,W64,W66,W69,W71,W80,W83,W86, W88,W90,W95,W103
2	Amylase, protease, cellulase	57	WG7, WG27, WG35, WG36, WG52, WG63, WG68, WG71, WG74, WG75, WG79, WG82, S2, S8, S11, S22, S25, S27, S33, S37, S39, S44, SS45, W3, W8, W18, W22, W25, W26, W33, W34, W36, W37, W40, W42, W44, W48, W50, W53, W57, W60, W65, W68, W72, W73, W75, W85, W87, W91, W96, W97, W99, W100, W102, W104, W106, W112
3	Amylase, protease, lipase	25	WG1, WG31, WG34, WG53, WG59, WG65, WG73, WG81, WG85, S1, S5, S6, S10, S43, S50, W11, W19, W20, W31, W77, W79, W82, W89, W94, W101
4	Amylase, cellulase, lipase	15	WG4, WG5, WG40, WG41, WG51, WG64, WG80, S48, S53, S54, S55, W46, W76, W109, W110
5	Amylase, Protease	14	WG15, WG46, S23, S24, S26, W2, W6, W7, W16, W52, W70, W74, W92, W98
6	Amylase, Lipase	9	WG17, WG38, WG43, WG54, WG78, WG84, S3, W45, W56,
7	Amylase,Cellulase	8	WG37, S29, S31, S32, W63, W81, W93, W107
8	Amylase	7	WG33, S28, S30, S36, S52, W84, W111
9	Protease, cellulase, lipase	6	WG12, W13, W30, W51, W67, W78,
10	Protease, Lipase	3	W14, W47, W105
11	Protease, Cellulase	2	S19, W24
12	Lipase	1	W108

Where; W= Woliso, S=Shalla, and WG = Wondo Genet

Combined activity for more than one enzyme was observed by the majority of bacterial isolates, 41.6% of isolates exhibited all four hydrolytic enzyme activities, 40.8 % were positive for three enzymes, 14.4% showed two hydrolytic activities and 3.2% were positive for one enzyme (figure 6).

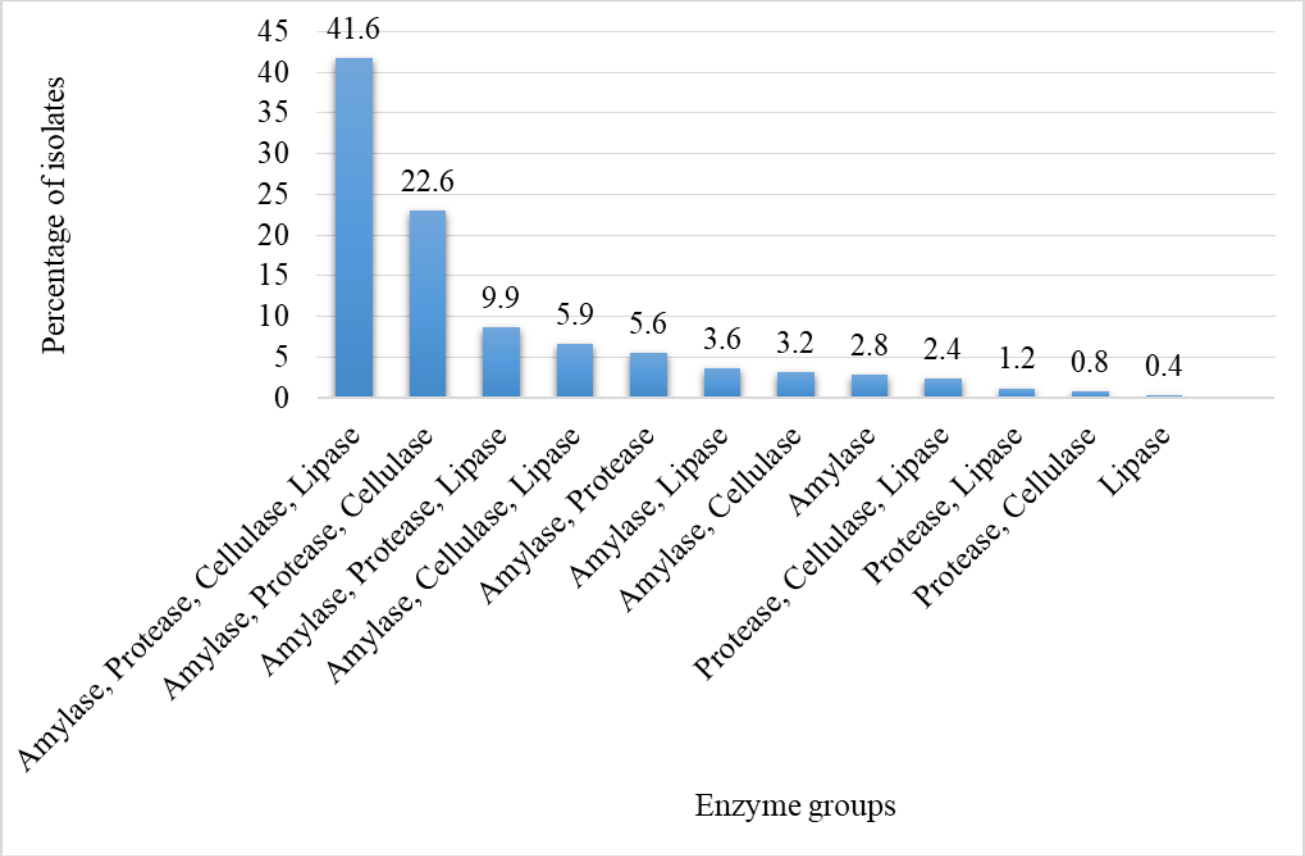


Figure 6: Grouping of the isolates obtained from all Hot springs according to the combined enzyme production.

4.1.5. Potent Isolates for Extracellular Hydrolytic Enzyme Production

Out of 252 isolates, 45 of the isolates were selected based on the higher clear zone. Isolates JUWG84 and JUW103 obtained higher clear zone values of 30.5 ± 0.71 and 30 ± 0 respectively and the isolates were recognized as excellent amylase producers. In the case of protease, isolates JUS4 (29.5 ± 0.71) and JUS14 (29.5 ± 0.71) showed the highest clear zone of all other screened isolates. Cellulase production was superior in the isolate JUWG44 (30 ± 0), while lipase

production was excellent in the isolate JUWG9 with a turbid halo zone around the colony value 30 ± 0 mm (Table 4).

Table 4: Clear zone in mm (mean ± SD) of potent enzyme producers

No.	Isolates code	Amylase	Protease	Cellulase	Lipase
1	JUW12	10.5 ± 0.71^{f}	29 ± 1.41^{ab}	21 ± 1.41^{def}	11 ± 0^{h}
2	JUS33	23.5 ± 0.71^{cde}	10 ± 0^{cde}	20 ± 2.83^{ef}	11 ± 1.41^{h}
3	JUS3	22 ± 1.41^{de}	29 ± 1.41^{ab}	23 ± 0cd^{ef}	10.5 ± 0.71^{h}
4	JUW29	21 ± 1.41^{d}	13.5 ± 0.71^{d}	24 ± 1.41^{cd}	10 ± 0^{h}
5	JUW38	29 ± 1.41^{abc}	11 ± 1.41^{abc}	25.5 ± 0.71^{bc}	12 ± 1.41^{h}
6	JUW43	23 ± 0de	14 ± 0.71^{de}	19.5 ± 0.35^{f}	19 ± 0.71^{fg}
7	JUW54	21 ± 1.41^{d}	21 ± 0^{def}	10 ± 0^{g}	11.5 ± 2.12^{h}
8	JUWG21	29 ± 1.41^{abc}	17 ± 1.41^{fgh}	11 ± 1.41^{g}	20.5 ± 0.71efg
9	JUWG22	24 ± 0^{bcde}	19 ± 1.41^{efg}	11 ± 0^{g}	10.5 ± 0.71^{h}
10	JUWG45	25 ± 1.41^{abcde}	11 ± 0^{j}	11.5 ± 2.12^{g}	23 ± 0^{cdefg}
11	JUS4	13.5 ± 0.71^{f}	**29.5 ± 0.71^{a}**	10 ± 0g	19 ± 1.41^{fg}
12	JUW64	12 ± 2.83^{f}	20 ± 0^{efg}	11.5 ± 0.71^{g}	22.5 ± 0.71^{cdefg}
13	JUW86	26 ± 1.41^{abcde}	29 ± 1.41^{ab}	11.5 ± 0.71^{g}	10.5 ± 0.71^{h}
14	JUS38	11 ± 1.41^{f}	22.5 ± 0.71^{cde}	24.5 ± 0.71^{cd}	11.5 ± 0.71^{h}
15	JUWG84	**30.5 ± 0.71^{a}**	21 ± 1.41^{def}	10 ± 0^{g}	11.5 ± 0.71^{h}
16	JUW28	11 ± 1.41^{f}	14 ± 1.41^{hij}	19.5 ± 0.71^{f}	25.5 ± 0.71^{abcd}
17	JUW62	12 ± 2.83^{f}	12 ± 0^{ij}	25.5 ± 0.71^{bc}	26 ± 0^{abc}
18	JUWG50	11 ± 1.41^{f}	11 ± 0^{j}	21 ± 1.41d^{ef}	18.5 ± 0.71^{g}
19	JUW103	**30 ± 0^{a}**	16 ± 1.41^{ghi}	10 ± 0^{g}	23.5 ± 2.12^{cdef}
20	JUS2	27 ± 1.41^{abcd}	11 ± 1.41^{j}	10 ± 0^{g}	21 ± 1.41^{defg}
21	JUS5	25 ± 0^{abcde}	26.5 ± 0.71^{abc}	23.5 ± 0.71^{cde}	11 ± 1.41^{h}
22	JUS42	29.5 ± 0.71^{ab}	29 ± 0^{ab}	10.5 ± 0.71^{g}	11 ± 1.41^{h}
23	JUS14	27 ± 0^{abcd}	**29.5 ± 0.71^{a}**	10 ± 0^{g}	11.5 ± 0.71^{h}
24	JUS27	12 ± 2.83^{f}	29 ± 1.41^{ab}	11.5 ± 0.71^{g}	12.5 ± 0.71^{h}
25	JUS10	11 ± 0^{f}	11.5 ± 2.12^{j}	22.5 ± 0.71^{cdef}	11 ± 1.41^{h}
26	JUS9	11 ± 1.41^{f}	11 ± 0^{j}	13 ± 1.41^{g}	24.5 ± 0.71^{bcde}
27	JUS28	10.5 ± 0.71^{f}	11 ± 1.41j	10 ± 0^{g}	21 ± 1.41^{defg}
28	JUS39	11.5 ± 0.71^{f}	10 ± 0j	13.5 ± 0.71^{g}	19 ± 1.41^{fg}
29	JUS53	29 ± 1.41^{abc}	11 ± 1.41^{j}	25.5 ± 0.71^{bc}	26.5 ± 0.71^{abc}
30	JUWG28	29 ± 1.41^{abc}	12 ± 1.41^{ij}	24 ± 0^{cd}	10 ± 0^{h}
31	JUS50	22.5 ± 0.71^{de}	10.5 ± 0.71^{j}	25 ± 0^{bc}	11.5 ± 0.71^{h}
32	JUS51	29.5 ± 0.71^{ab}	27.5 ± 0.71^{ab}	12.5 ± 0.71^{g}	11 ± 1.41^{h}
33	JUS52	23.5 ± 0.71^{cde}	11 ± 1.41^{j}	22 ± 0^{cdef}	13 ± 1.41^{h}
34	JUS49	26.5 ± 2.12^{abcde}	11 ± 1.41^{j}	10.5 ± 0.71^{g}	13.5 ± 0.71^{h}
35	JUS54	11 ± 1.41^{f}	25 ± 0^{bcd}	22.5 ± 0.71^{cdef}	13 ± 1.41^{h}

36	JUS55	10 ± 0^{f}	$21 \pm 1.41d^{ef}$	22.5 ± 0.71^{cdef}	11 ± 1.41^{h}
37	JUWG77	14.5 ± 0.71^{f}	26.5 ± 0.71^{abc}	28.5 ± 0.71^{ab}	11.5 ± 0.71^{h}
38	JUWG7	12 ± 2.83^{f}	25.5 ± 0.71^{abc}	12.5 ± 0.71^{g}	11.5 ± 0.71^{h}
39	JUWG44	11.5 ± 2.12^{f}	13.5 ± 0.71^{hij}	**30 ± 0^{a}**	12 ± 1.41^{h}
40	JUWG60	14 ± 1.41^{f}	13 ± 1.41^{hij}	25.5 ± 0.71^{bc}	10.5 ± 0.71^{h}
41	JUWG3	12.5 ± 0.71^{f}	11 ± 1.41^{j}	11 ± 0^{g}	28.5 ± 0.71^{ab}
42	JUWG8	14.5 ± 0.71^{f}	11.5 ± 0.71^{j}	12.5 ± 0.71^{g}	24.5 ± 0.71^{bcde}
43	JUWG9	13 ± 1.41^{f}	12.5 ± 0.71^{ij}	10.5 ± 0.71^{g}	**30 ± 0^{a}**
44	JUWG51	11 ± 1.41^{f}	13 ± 0^{hij}	11.5 ± 0.71^{g}	24 ± 1.41^{bcde}
45	JUW53	13 ± 1.41^{f}	10 ± 0^{j}	12 ± 1.41^{g}	20.5 ± 0.71^{efg}

Where; JU= Jimma University, S= Shalla, W= Woliso, and WG= Wondo Genet. Superscripts with similar letters indicate the absence of significant differences in measured values. Different letters indicate the presence of significant differences between measured values down the column.

4.1.6. Relative Enzyme Activity of the Potent Bacterial Isolates

The relative enzyme activity of the 45 potent bacterial isolates showed that isolates JUW103 and S2 obtained relative enzyme activity values of 4.92±1.13 and 4.58±0.73 respectively and the isolates were recognized as excellent amylase producers. In the case of protease, isolates JUS51 (4.86±0.48) got the highest relative enzyme activity values of all other screened isolates. A cellulase relative enzyme activity value was bigger in the isolate JUWG44 (4.35±0.63), while a lipase relative enzyme activity value was first-rate for the isolate JUWG9 (4.52±0.41) (Table 5).

Table 5: Relative enzyme activity (mean ± SD) of potent enzyme producer bacterial isolates

No.	Isolates Code	Amylase	Protease	Cellulase	Lipase
1	JUW12	1.63 ± 0.2^{j}	2.7 ± 0.3^{f}	2.7 ± 0.15^{f}	1.6 ± 0.15^{gh}
2	JUS33	2.96 ± 0.31^{hi}	1.50 ± 0.14^{jh}	1.24 ± 0.12^{g}	1.38 ± 0.14^{h}
3	JUS3	3.47 ± 0.19^{defghi}	4.19 ± 0.46^{bc}	3.51 ± 0.55^{bcde}	1.36 ± 0.12^{h}
4	JUW29	3.61 ± 0.36^{cdefgh}	1.57 ± 0.21^{gh}	3.81 ± 0.37^{abcd}	1.59 ± 0.14^{h}
5	JUW38	3.78 ± 0.19^{cdefg}	1.66 ± 0.21^{gh}	3.83 ± 0.44^{abcd}	1.72 ± 0.1^{gh}
6	JUW43	4.09 ± 0.44^{bcde}	1.6 ± 0.23^{gh}	2.96 ± 0.51^{ef}	3.74 ± 1.22^{bcd}
7	JUW54	4.3 ± 0.75^{abc}	3.57 ± 0.6^{cde}	1.51 ± 0.14^{g}	1.63 ± 0.24^{h}
8	JUWG21	3.98 ± 0.42^{bcdef}	1.59 ± 0.35^{gh}	1.46 ± 0.23^{g}	3.44 ± 0.51^{cd}
9	JUWG22	3.14 ± 0.25^{ghi}	2.19 ± 0.07^{g}	1.39 ± 0.18^{g}	3.33 ± 0.48^{cde}
10	JUWG45	2.85 ± 0.17^{i}	1.66 ± 0.15^{gh}	1.4 ± 0.13^{g}	3.47 ± 0.32^{cd}
11	JUS4	1.73 ± 0.14^{j}	4.65 ± 0.32^{ab}	1.45 ± 0.21^{g}	2.6 ± 0.24^{ef}
12	JUW64	1.44 ± 0.16^{j}	3.4 ± 0.57^{def}	1.53 ± 0.29^{g}	3.64 ± 0.2^{cd}

13	JUW86	3.76±0.47^{cdefg}	3.67±0.52^{cde}	1.4±0.3^{g}	1.5±0.3^{h}
14	JUS38	1.75±0.23^{j}	3.24±0.55^{ef}	3.38±0.54^{cdef}	1.44±0.25^{h}
15	JUWG84	3.88±0.55^{bcdefg}	2.81±0.35^{f}	1.25±0^{g}	1.6±0.31^{h}
16	JUW28	1.42±0.16^{j}	1.58±0.08^{gh}	3.33±0.17^{cdef}	3.51±0.24^{cd}
17	JUW62	1.41±0.26^{j}	1.5±0^{jh}	3.93±0.7^{abc}	4.13±0.36^{abc}
18	JUWG50	1.5±0.07^{j}	1.66±0.15^{gh}	3.56±0.51^{bcde}	2.45±0.24^{fg}
19	JUW103	**4.92±1.13^{a}**	1.38±0.14^{h}	1.64±0.2^{g}	4.47±0.97^{ab}
20	JUS2	**4.58±0.73^{ab}**	1.89±0.49^{gh}	1.45±0.21^{g}	3.24±0.45^{de}
21	JUS5	3.62±0.52^{cdefg}	3.71±0.14^{cde}	3.33±0.08^{cdef}	1.6±0.31^{h}
22	JUS42	4.22±0.91^{abcd}	4.07±0.81^{bcd}	1.44±0.48^{g}	1.96±0.7^{fgh}
23	JUS14	3.91±0.56^{bcdefg}	4.11±0.43^{bc}	1.59±0.14^{g}	1.39±0.28^{h}
24	JUS27	1.72±0.1^{j}	3.8±0.42^{cde}	1.68±0.31^{g}	1.75±0.07^{gh}
25	JUS10	1.51±0.11^{j}	1.51±0.38^{gh}	3.32±0.33^{cdef}	1.6±0.35^{h}
26	JUS9	1.6±0.31^{j}	1.59±0.23^{gh}	1.81±0.32^{g}	3.86±0.25^{abcd}
27	JUS28	1.81±0.34^{j}	1.59±0.22^{gh}	1.45±0.21^{g}	3.33±0.33^{cde}
28	JUS39	1.6±0.31^{j}	1.48±0.08^{gh}	1.81±0.22^{g}	3.61±0.35^{cd}
29	JUS53	3.8±0.42^{cdefg}	1.33±0.2^{h}	3.2±0.35^{def}	3.68±0.67^{cd}
30	JUWG28	3.27±0.29^{fghi}	1.7±0.47^{gh}	3.74±0.94^{abcd}	1.37±0.1^{h}
31	JUS50	3.42±0.36^{efghi}	1.56±0.12^{gh}	3.47±0.6^{bcde}	1.66±0.21^{gh}
32	JUS51	3.79±0.44^{cdefg}	**4.86±0.48^{a}**	1.75±0.4^{g}	1.69±0.39^{gh}
33	JUS52	3.32±0.33^{efghi}	1.38±0.14^{h}	3.14±0^{def}	1.9±0.16^{fgh}
34	JUS49	4.06±0.51^{bcdef}	1.66±0.21^{gh}	1.33±0.2^{g}	1.7±0.06^{gh}
35	JUS54	1.51±0.24^{j}	3.62±0.52^{cde}	3.72±0.1^{abcd}	1.7±0.31^{gh}
36	JUS55	1.51±0.14^{j}	3.67±0.63^{cde}	3.98±0.54^{abc}	1.75±0.29^{abc}
37	JUWG77	1.92±0.2^{j}	4.02±0.42^{bcd}	4.1±0.53^{ab}	1.44±0.17^{h}
38	JUWG7	1.38±0.13^{j}	3.63±0.63^{cde}	1.73±0.2^{g}	1.52±0.17^{h}
39	JUWG44	1.71±0.24^{j}	1.88±0.4^{gh}	**4.35±0.63^{a}**	1.32±0.1^{h}
40	JUWG60	1.85±0.33^{j}	1.77±0.07^{gh}	3.67±0.08^{abcde}	1.5±0.22^{h}
41	JUWG3	1.68±0.3^{j}	1.44±0.31^{h}	1.43±0.35^{g}	3.83±0.43^{abcd}
42	JUWG8	1.65±0.08^{j}	1.5±0.07^{gh}	1.62±0.28^{g}	3.27±0.15^{de}
43	JUWG9	1.65±0.28^{j}	1.61±0.11^{gh}	1.63±0.23^{g}	**4.52±0.41^{a}**
44	JUWG51	1.47±0.32^{j}	1.96±0.18^{gh}	1.45±0.21^{g}	3.46±0.36^{cd}
45	JUW53	1.64±0.19^{j}	1.45±0.21^{h}	1.71±0.37^{g}	3.76±1.08^{bcd}

Where JU= Jimma University, S= Shalla, W= Woliso, and WG= Wondo Genet. Superscripts with similar letters indicate the absence of significant differences in measured values. Different letters indicate the presence of significant differences between measured values down the column.

4.1.7. Characterization of Potential Enzyme Producing Bacterial Isolates

4.1.7.1. Morphological and Biochemical Tests of Bacterial Isolates

The biochemical test results showed that all potent bacterial isolates were positive for catalase, 36 were oxidase-positive, and 2 isolates were indole producers. Only four isolates were utilized citrate as a carbon source. Out of 45 isolates, 39 isolates were gram-positive, endospore formers, and motile while the rest 6 isolates were gram-negative, non-endospore forming, and non-motile. So based on the biochemical test results 39 of the bacterial isolates were tentatively identified to the genus *Bacillus* and the rest 6 isolates were identified as genus *Thermus* spp. (Table 6).

Table 6: Morphological and biochemical characteristics of potent bacterial isolates

No.	Isolates Code	Sample Source	Location	Morphological and Biochemical test of bacterial isolates								
			City/ Town/ District	Shape	Gram R.	Endospore	Motility	Catalase	Oxidase	Citrate	Indole	Suggested Genus
1	JUW12	Water	Woliso	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
2	JUS33	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
3	JUS3	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
4	JUW29	Water	Woliso	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
5	JUW38	Water	Woliso	Rod	-	-	-	+	+	-	-	*Thermus* spp.
6	JUW43	Water	Woliso	Rod	+	+	+	+	-	-	-	*Bacillus* spp.
7	JUW54	Sediment	Woliso	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
8	JUWG21	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
9	JUWG22	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
10	JUWG45	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
11	JUS4	Sediment	Shalla	Rod	+	+	+	+	-	-	-	*Bacillus* spp.
12	JUW64	Sediment	Woliso	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
13	JUW86	Water	Woliso	Rod	+	+	+	+	+	-	+	*Bacillus* spp.
14	JUS38	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
15	JUWG84	Water	Wondo G.	Rod	+	+	+	+	-	-	-	*Bacillus* spp.
16	JUW28	Water	Woliso	Rod	+	+	+	+	+	-	+	*Bacillus* spp.
17	JUW62	Sediment	Woliso	Rod	+	+	+	+	+	+	-	*Bacillus* spp.
18	JUWG50	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
19	JUW103	Water	Woliso	Rod	+	+	+	+	-	-	-	*Bacillus* spp.
20	JUS2	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
21	JUS5	Sediment	Shalla	Rod	+	+	+	+	-	-	-	*Bacillus* spp.
22	JUS42	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.

23	JUS14	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
24	JUS27	Water	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
25	JUS10	Sediment	Shalla	Rod	+	+	+	+	+	+	-	*Bacillus* spp.
26	JUS9	Sediment	Shalla	Rod	-	-	-	+	+	-	-	*Thermus* spp.
27	JUS28	Sediment	Shalla	Rod	-	-	-	+	+	-	-	*Thermus* spp.
28	JUS39	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
29	JUS53	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
30	JUWG28	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
31	JUS50	Water	Shalla	Rod	+	+	+	+	-	-	-	*Bacillus* spp.
32	JUS51	Sediment	Shalla	Rod	+	+	+	+	-	-	-	*Bacillus* spp.
33	JUS52	Sediment	Shalla	Rod	-	-	-	+	+	-	-	*Thermus* spp.
34	JUS49	Sediment	Shalla	Rod	+	+	+	+	+	+	-	*Bacillus* spp.
35	JUS54	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
36	JUS55	Sediment	Shalla	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
37	JUWG77	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
38	JUWG7	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
39	JUWG44	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
40	JUWG60	Water	Wondo G.	Rod	-	-	-	+	+	-	-	*Thermus* spp.
41	JUWG3	Water	Wondo G.	Rod	-	-	-	+	+	+	-	*Thermus* spp.
42	JUWG8	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
43	JUWG9	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.
44	JUWG51	Water	Wondo G.	Rod	+	+	+	+	-	-	-	*Bacillus* spp.
45	JUW53	Water	Wondo G.	Rod	+	+	+	+	+	-	-	*Bacillus* spp.

Where; JU= Jimma University, S= Shalla, W= Woliso and WG= Wondo Genet, "+" = Positive reaction (presence), "-" = Negative reaction (absence)

4.1.8. Identification of Fungi

Even if the fungal isolates are not capable of forming a promising inhibition zones around the colonies, the characterization was conducted because they are few in number and to evaluate the existence of fungal isolates in the three hotsprings. So the isolated 9 fungi were characterized, and identified to two genera (*Aspergillus* and *Penicillium*). Moreover, the majority of fungi were deep black, circular with a fluffy-white margin (Table 7).

Table 7: Morphological and Microscopic Identification of fungal species

No	Isolates Code	Sites	Morphological Characterization		Suggested genus
			Colonial morphology	Microscopic morphology using lactophenol cotton blue staining	
1.	JUW10	Woliso	Deep black, circular shape with fluffy white margin.	Conidia heads are large, globose, dark brown, biseriate and, rough walled. Smooth walled Conidiophores.	*Aspergillus* spp.
2.	JUW7	Woliso	Deep black circular shape with fluffy white margin		
3.	JUS3	Shalla	Deep black circular shape with fluffy white margin		
4.	JUW11	Woliso	Deep black circular shape with fluffy white margin		
5.	JUW4	Woliso	Deep black circular shape with fluffy white margin		
6.	JUWG5	Wondo Genet	Deep black circular shape with fluffy white margin		
7.	JUS1	Shalla	Deep black circular shape with fluffy white margin		
8	JUWG12	Wondo Genet	Greenish, circular shape with a white and smooth margin.	Conidiophores are smooth and short. mycelia arranged irregularly with branches of various lengths.	*Penicilliu m* spp.
9.	JUW9	Woliso	Greenish, circular shape with a white and smooth margin.		

Where; JU= Jimma University, S= Shalla, W= Woliso, and WG= Wondo Genet.

4.1.9. Physiological Test of Bacterial Isolates

Different physiological parameters (Temperature, pH, and NaCl) were conducted for the potent bacterial isolates. The temperature ranges (45-55 ˚C) were conducted on nutrient agar and 60-70 ˚C conducted in nutrient broth and the optical density was read in spectrophotometer. It was observed that 50-55˚C was optimal growth temperature; 7-8 was optimal growth pH, and 3-10 was optimal growth NaCl concentration. Isolates JUW103, S2, JUS51, JUWG44, and JUWG9 shown growth at extremes of temperature (65-70 ˚C), and pH (3-4). Isolates JUS3**,** JUWG22**,** JUS28**,** and JUWG60 shown growth at extreme pH 11. Isolates JUS2, JUS5, JUS28, JUS39, JUS53, JUWG28, and JUS50 shown growth at extreme 15% NaCl concentration (Table 8).

Table 8: Physiological characteristics of the potent thermophilic bacterial isolates

No.	Isolates code	Temperature tolerance						pH tolerance									NaCl tolerance					
		45	50	55	60	65	70	3	4	5	6	7	8	9	10	11	3	5	7	10	12	15
1	JUW12	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
2	JUS33	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
3	JUS3	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	+	+++	+++	+++	+++	+	-
4	JUW29	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
5	JUW38	++	+++	+++	+	+	+	-	+	+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
6	JUW43	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
7	JUW54	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
8	JUWG21	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
9	JUWG22	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	+	+++	+++	+++	+++	+	-
10	JUWG45	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
11	JUS4	++	+++	+++	+	+	+	-		+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
12	JUW64	++	+++	++	-	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
13	JUW86	++	+++	++	-	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
14	JUS38	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
15	JUWG84	++	+++	+++	+	+	+	-	+	+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
16	JUW28	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
17	JUW62	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
18	JUWG50	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
19	JUW103	++	+++	+++	+	+	+	-	+	+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
20	JUS2	++	+++	+++	+	+	+	-		+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	+
21	JUS5	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	+
22	JUS42	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
23	JUS14	++	+++	+++	+	+	+	-		+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
24	JUS27	++	+++	++	-	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	++	-	-
25	JUS10	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	++	-	-
26	JUS9	++	+++	+++	+	+	+	-	+	+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
27	JUS28	++	+++	+++	+	+	+	-		+	+	+++	+++	++	+	+	+++	+++	+++	+++	+	+

28	JUS39	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	+
29	JUS53	++	+++	++	-	-	-	-	-	-	+	++	++	++	+	-	+++	+++	+++	+++	+	+
30	JUWG28	++	+++	++	-	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	+
31	JUS50	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	+
32	JUS51	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
33	JUS52	++	+++	+++	+	+	+	-		+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
34	JUS49	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
35	JUS54	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
36	JUS55	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	++	-	-
37	JUWG77	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
38	JUWG7	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
39	JUWG44	++	+++	+++	+	+	+	-	+	+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
40	JUWG60	++	+++	++	+	+	+	-	+	+	+	++	++	++	+	+	+++	+++	+++	+++	+	-
41	JUWG3	++	+++	+++	+	+	+	-	+	+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
42	JUWG8	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
43	JUWG9	++	+++	+++	+	+	+	-	+	+	+	+++	+++	++	+	-	+++	+++	+++	+++	+	+
44	JUWG51	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	-
45	JUW53	++	+++	+++	+	-	-	-	-	-	+	+++	+++	++	+	-	+++	+++	+++	+++	+	+

Where; JU = Jimma university, S = Shalla, W = Woliso, WG = Wondo Genet. (+++) = Highly positive reaction (++) = Very Positive reaction; (+) = Positive reaction and (-) = Negative reaction

4.2. Discussion

In the present study, the physical and chemical properties of water samples obtained from three Hot springs were examined on *situ*. Accordingly, Shalla Hot springs had a temperature gradient of 88.15 ± 0.35℃ and 96.45 ± 0.35℃ while Wondo Genet and Woliso Hot springs had a temperature gradient of 71.9 ± 0.14 and 45±0℃ respectively. All of the Hot springs had slightly alkaline pH and fell within a small alkaline range of values (7.6 ± 0-8.1 ± 0.14); the lowest value of pH was 7.6 ± 0 in the Wondo Genet Hot spring site and the highest was 8.1 ± 0.14 in the Shalla Hot spring site 1. Sahay *et al.,* (2017) and Alrumman *et al.,* (2018) examined similar gradients of temperature (63-95℃ and 55-70℃) and pH (6.5-8.2 and7.2–8.1) of Hot spring waters of Indian Himalayas and Saudi. In lower to the present study, Weldey *et al.,* (2014) examined 36.46-48.33℃ temperature gradient and 7.49-8.07 gradient of pH from Geothermal Sites of Afar, Ethiopia. The increment and the decrement of temperature and pH in Hot springs could be due to the difference in the physical condition and chemical composition. Moreover, the highest temperature observed in Shalla Hot spring is detected to obtain the extreme thermophiles. The variation in temperature of Hot spring water is governed by the physical condition, chemical composition, cultural and social activities associated with spiritual values (Kiran *et al.,* 2018). The different ranges of temperature and pH in Hot springs, therefore, have a high chance of supporting the survival of thermophilic bacteria (Alrumman *et al.,* 2018).

In the current study, the Electric conductivity and TDS values were highest in Shalla site 2 Hot springs ranging 9545± 3.54 µS cm-1 and 6202.5 ± 3.54 mg l^{-1}, respectively. However, the highest ratio of DO (683 ± 2.83) was observed in Woliso Hot spring. The high electrical conductivity could be due to the presence of high mineral concentrations and organic materials. According to Alrumman *et al.,* (2018) report, mineral content has a direct relationship with EC, whereas, the highest TDS may be due to the presence of inorganic salts. Elements such as calcium, magnesium, potassium, sodium, bicarbonate, chlorides, and sulfates as well as small amounts of dissolved organic materials increase the TDS of Hot spring water (Bamsaoud and Saeed, 2021). The highest DO in Woliso Hot spring could be due to the mixing of cold Water found near the Hot spring. The mixing of the cold spring existing in the vicinity of the Hot spring has the effect of increasing DO (Jaffer*et al.,* 2019). The increase in TDS and EC shows a high

number of microorganisms found in the Hot spring while the increase in DO shows a low number of microorganisms in the Hot spring (Luo *et al.,* 2019).

In the current study, a total of 261 microbes (252 bacterial and 9 fungal isolates) were screened for enzyme production. However, only bacterial isolates were positive for enzyme production. The reason for the inability of fungi to produce enzymes could be due to the effect of substrate concentration. Some fungal isolates cannot produce enzymes due to the effect that is caused by concentration of substrates of growth medias (Fentahun and Vijaya, 2017).

In this study, from the total of 252 bacterial isolates, 240 (95.23%) isolates were amylase producers, 212 (84.12%) isolates were protease producers, 193 (76.58%) isolates were cellulase producers, and 164 (65.07%) isolates were lipase producers. In line with the present study, Lele and Deshmukh (2016) reported that thermophilic bacteria isolated from Hot springs in Ganeshpuri, Maharashtra, India produced a high quantity of amylase and protease enzymes. According to their report, all bacterial isolates, 5 (100%) were amylase producers, 3 (60%) were protease producers, 1 (20%) isolate was cellulase producer, and 1 (20%) isolate was lipase producer. In addition to that, Mohammad *et al.,* (2017) studied the amylase enzyme-producing ability of bacteria isolated from Jordanian Hot Springs was very high. In their report, among the 10 identified isolates, 7 (70%) isolates were amylase producers but no isolates produced lipase. Furthermore, Yadav *et al.,* (2018) reported a similar study, out of 150 bacterial isolates; only 15 (10%) bacterial isolates were lipase producers while the number of isolates that produced other enzymes was high. On the contrary, Elsharayidi *et al.,* (2020) investigated a high number of bacterial isolates that showed activity for cellulase. According to their report, of 20 bacterial isolates subjected to enzyme screening, the majority 19 (95%) of the isolates were cellulase producers. The reason for the difference in enzyme production proposed that, the physical and chemical properties of hot spring waters. The environmental conditions and the chemical properties of hot springs cause a significant difference in enzyme production (Alrumman *et al.,* 2018). The increased production of amylase enzymes from the bacteria makes it to be needed by different industries. The bulk production of amylases from microorganisms is economical and this indicates them to be used in different industries (Abel-Nabey and Farag, 2016).

In the current study, combined activity for more than one enzyme was observed by the majority of bacterial isolates, 41.6% of isolates exhibited all four hydrolytic enzyme activities, 40.8 % were positive for three enzymes, 14.4% showed two hydrolytic enzyme activities. In lower to the current study, Mohammad *et al.,* (2017) reported that only 1 (10%) isolate produced 5 of the screened enzymes, 4 (40%) isolates produced 3 of the extracellular enzymes, and another 1 (10%) isolate produced 2 enzymes from Saudi Hot Springs. In addition Ranawat and Rawat (2017) reported that 6.45%, 13.60% produced two enzymes; 16.12%, 27.27% produced three enzymes; 22.58%, 22.72% produced other 3 enzymes from Tapovan kund and Soldhar Hot Springs repectively. Microbes with the different enzyme can survive different extreme environments. The property of production of two or more enzymes by microorganisms helps them to survive in hostile environments so that they can acquire nutrition by breakdown of variety of nutrients (Ranawat and Rawat, 2017)

In the present study, a total of 45 bacterial isolates (39 *Bacillus* spp. and 6 *Thermus* spp.) were identified as potent enzyme producers.Thus *Bacillus* spp. were dominant over *Thermus* spp. Similarly, Alrumman *et al. (*2018) confirmed that the most promising thermophilic bacterial isolates isolated from Saudi Hot Springs producing amylase, lipase, and protease belonged to the genus *Bacillus*. In addition, Yadav *et al.,* (2018) reported that most of the enzyme producer potent isolates isolated from hot springs of Myagdi, Nepal were *Bacillus* spp. The dominance of *Bacillus* in all samples could be due to the resistance to harsh environmental conditions and versatility of metabolism. So the genus *Bacillus* move at high rates in hot springs (Mohmmad *et al.,* 2017).

In the current study, of the 45 potent bacterial isolates, 2 isolates (JUWG84 and JUW103) showed significantly considerable difference in the clear zone (30.5 ± 0.71 and 30 ± 0) for amylase. Similarly, Sundarapandiyan and Jayalakshmi (2017) reported that 5 (17±0, 18±0, 15±0, 20±0, and 27±0) isolates isolated from Vellar estuary, Tamil Nadu, showed a higher clear zone for amylase. In lower with the current study, Yassin *et al.,* (2021) reported that 3 isolates isolated from soil samples of Afdera, Afar Region, and Ethiopia produced the largest ratio of halo diameter (5.96 ± 0.057, 4.96 ± 0.057, and 4.73 ± 0.115mm) for amylase. On the other hand,a statistically significant difference in relative enzyme activity was also observed for amylase in

two isolates JUW103, and S2 (4.92±1.13 and 4.58±0.73) respectively in the current study. Similarly, Latorre *et al.,* (2016) reported a higher relative enzyme activity for amylase in three isolates. According to them, of 31 *Bacillus* species isolated from different sources, a significant difference was observed in 3 (6.3±0, 6.1±0, and 5.8±0,) of the other isolates.

In the present study, the colony feature of most of the potent bacterial isolates was creamy white and brown, medium-sized, rough consistency with erose and undulate margin. Similar studies carried out by Mohammad *et al.,* (2017) and Patel *et al.,* (2019) revealed that bacterial isolates isolated from Jordanian Hot Springs and Dholera thermal springs of Gujarat, India were creamy white and brown, medium-sized, rough consistency with erose and undulate margin.

Most of the fungal isolates in the current study were identified to the genus *Aspergillus* (77.77%). Silmilar with the current study, Salano *et al.,* (2018) reported the dominance of *Aspergillus* spp. (29.1%) isolated from a hot-spring on the shores of Lake Bogoria, Kenya, and also it is indicated that the genus *Aspergillus* and *Penicillium* were the most commonly observed genera. In addition to this the current result is in agreement with Zhou *et al.,* (2015) report which indicated the dominance of *Aspergillus* spp. (8.13%) from geothermal ecosystems and found that they are dominant fungal genera in their study along with the other dominant fungal spp. The dominance of the genus *Aspergillus* could be due to the presence of resistant spores that enable it to survive in harsh environmental conditions including high temperatures (Salano *et al.,* (2018).

In the present study, all the 45 potent bacterial isolates were subjected to different temperature ranges. As the temperature increases the growth potential of bacterial cells increased up to 55 ˚C but bacterial cells growth potential was declined as the temperature is elevated to 60-70˚C.The optimum temperature for the growth of the isolates was 50-55°C. Similarly, Aanniz *et al.,* (2015) reported that the optimum growth of bacterial isolates isolated from Moroccan Hot springs, salt marshes, and desert soils was 55°C. In addition, Lele and Deshmukh (2016) reported that the optimum temperature of the *Bacillus* sp. isolated from the Hot spring of Ganeshpuri, Maharashtra, India was 45-55°C. On the otherhand, Pandey *et al.,* (2015) reported that the optimum temperature preference for all the isolates isolated from Hot springs of Uttarakhand, India was observed between 55 to 65 °C which is higher than the present study. The ability of

bacteria to grow at high temperatures may be due to the ability to produce spores that help to grow and survive at high temperatures. Members of the genus *Bacillus* and *Thermus* are probably the most frequently isolated thermophiles in hot environments because of their resistance to harsh environmental conditions (Kassaye and Tilahun, 2020).

In this study, the optimum pH range for isolates' growth was 7-8 and the optimal pH pick was found to be 8. Further increase in the pH beyond pH 8 resulted in a decrease in the growth of the organism. None of the isolates grew at 3.0 and 11.0 pH. Similarly, Kassaye and Tilahun (2020) studied the optimum pH for *Bacillus* and *thermus* isolates isolated from Shalla Hot Springs, Ethiopia was 7-7.5. In addition, Ifandi and Alwi (2018) reported that the optimum growth pH of thermophilic bacteria isolated from Bora Hot Springs in Central Sulawesi was 6-8.5. The ability of bacteria to grow at high pH could be due to the bacterial isolates having alkalophilic nature. The thermophilic bacteria which grow at high pH are able to manage different alkaline pH ranges and are termed thermoalkalophilic (Alrumman *et al.,* 2014)

In the present study, optimum growth for NaCl concentration was from 3-10%. Singh *et al.* (2010) reported that thermophilic *B. cereus* SIU1 strain isolated from slightly alkaline soils of Uttar Pradesh, India was highly halotolerant, which was able to grow in the presence of 0.0-10% NaCl. This report is similar with present findings concerning salt tolerance of thermophilic *Bacillus and thermus*. On the contrary, Elnasser *et al.* (2007) reported that thermophilic bacterial strain *Bacillus justea* isolated from hot springs of Jordan grew optimally at a NaCl concentration of 0.5%. The ability of bacteria to grow in different NaCl concentrations could be due to the isolates are also halophilic. The bacteria that grow at the different high NaCl ranges are called halophiles (Cira-Chávez *et al.,* 2018).

5. Conclusion and Recommendation

5.1. Conclusion

Based on the current study findings the following conclusions are forwarded.

- ✓ A total of 252 bacterial and 9 fungal isolates were obtained from this study. Out of the 252 bacterial isolates, 240 of the bacterial isolates were producers of two or more enzymes. However none of the fungi isolates were producers of the screened enzymes.
- ✓ From the total of 252 isolates of bacteria, 45 of them were selected as a potent producers of amylase, protease, cellulase and lipase enzymes. Of 45 potent bacterial isolates, JUWG84 (30.5 ± 0.71) and JUW103 (30 ± 0) were excellent amylase producers, JUS4 (29.5 ± 0.71) and JUS14 (29.5 ± 0.71) were excellent protease producers, JUWG44 (30 ± 0) was excellent cellulase producer and JUWG9 (30 ± 0) was excellent lipase producer and theses isolates had statistically different inhibition zone values compared to the other isolates.
- ✓ Isolates JUW103, JUS2, JUS51, JUWG44, and JUWG9 has got a higher REA value of 4.35±0.63 and above. The first two isolates had statistically significant relative enzyme activity values compared to the others for amylase production.
- ✓ The 45 potent bacterial isolates were characterized in to two genus, out of that 39 of the isolates were identified as *Bacillus* spp. and theresyt 6 were identified as *Thermus* spp.
- ✓ The optimum temperature, pH and NaCl concentration of the potent bacterial isolates were 50-55,7-8 and 3-10 respectively.

5.2. Recommendations

Based on the findings obtained from this study, the following recommendations are forwarded.

- ✓ The thermophilic bacterial isolates from this study were capable of producing extracellular hydrolytic enzymes such as amylase, protease, cellulase, and lipase. So the isolates obtained from the current study are the best candidates for producing these enzymes on large scale and using the enzymes for different industrial applications.
- ✓ The result of the present study will contribute to the future applications and utilization of the bacterial isolates for the production of other extracellular and intracellular enzymes including Taq DNA polymerase.
- ✓ Since the studied Hot springs are rich in microbial diversity, and identification of the microorganisms were done by using the conventional method in the current study. Thus, better identification of the isolates using molecular methods and also investigating the microbial diversity of the hot springs using metagenomics technique is recommended.
- ✓ The potent bacterial isolates identified in this study could be further used for producing the enzymes using submerged fermentation and also purification of the enzymes using chromatographic techniques for their better industrial application is recommended.

6. References

Abel-Nabey, H., & Farag, A. (2016). Production, optimization and characterization of extracellular amylase from halophilic *Bacillus lichineformis* AH214. *African Journal of Biotechnology*, **15**: 670-683.

Acharya, S., & Chaudhary, A. (2012). Bioprospecting thermophiles for cellulase production: a review. *Brazilian Journal of Microbiology*, **43**: 844-856.

Alarcón, J. (2020). State-of-the-art evaluation of lignocellulosic biomass pretreatments. Case of study: mechanocatalytic processing of rice husk. Msc. Thesis, National university of Colombia, Bogota, Colombia.

Letebo, A., & Abrar, T. (2017). Isolation and Characterization of Thermostable Amylase Producing Bacteria from Hot Spring at Arba Minch Nech Sar National Park, Southern Ethiopia. *International Journal of Novel Research in Interdisciplinary Studies*, **4**:9-16.

Al-Johani, N., Al-seeni, M., & Ahmed, Y. (2017). Optimization of alkaline α-amylase production by thermophilic *bacillus subtilis*. *Afr J Tradit Complement Altern Med.* **14**:288-301.

Alrumman, S., Yasser, S., Mostafa, Y., Eifan, S., Saad, A., Alamri, S., & Hesham, A. (2014). Isolation of Thermoalkalophilic-α-amylase Producing Bacteria and Optimization of Potato Waste Water Medium for Enhancement of α-amylase Production. *Advances in Life Science and Technology*, **20**:2224-7181.

Alrumman, S., Mostafa, Y., Al-Qahtani, S., & Taha, T. (2018). Hydrolytic Enzyme Production by Thermophilic Bacteria Isolated from Saudi Hot Springs, *Open Life Science*, **13**: 470-480.

András, J., Tóth, M., & Tőzsér, J. (2013). Research Applications of Proteolytic Enzymes in Molecular Biology. *Biomolecules,* **3**:923-942.

Aanniz, A., Ouadghiri, M., Melloul, M., Jean Swings, J., Elfahime, E., Ibijbijen, J., Ismaili, M., & Amar, M. (2015). Thermophilic bacteria in Moroccan Hot springs, salt marshes and desert soils. *Brazilian Journal of Microbiology,* **46**:443-453.

Asmare, E. (2016). Evaluation of The Potential of Shala Hot Spring Water For The Biomass Production of *Arthrospira Fusiformis* (Voronikhin). M.Sc thesis. Addis Ababa university, Addis Ababa, Ethiopia.

Bamsaoud, S., & Saeed, F. (2021). Physical and Chemical Characteristics of Assaiq and Senah Hot Springs Water in Hadhramout-Yemen and the Assessment of Water Quality for Drinking and Irrigation Purposes, *Journal of Physics: Conference Series,* **19**:012-011.

Bertrand, E., Keddis, R., Groves, J., Vetriani, C., & Narehood, R. (2013). Identity and mechanisms of alkane-oxidizing metalloenzymes from deep-sea hydrothermal vents. *Frontiers in microbiology,* **4**: 109.

Bhat, M. & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnology advances,* **15**:583-620.

Bischof, R., Ramoni, J., & Seiboth, B. (2016). Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. *Microbial Cell Factories*, **15**:106.

Birhanu, A., Alemayehu, A., Ali, A., Tesfaye, D., Farid, S., Kebede, M., and Masi, C. (2021) Hot Spring Derived Bacterial Hydrolytic Thermozymes for Biotechnological Application. *International Journal of Scientific & Engineering Research*, **12**:2229-5518

Brock, T. (1997). The value of basic research: Discovery of *thermus aquaticus* and other extreme thermophiles. *Genetics*, **146**:1207-1210.

Casas-Godoy, L., Gasteazoro, F., & Duquesne, S. (2018). Lipases: An Overview. In: *Methods in molecular biology*. Georgina Sandoval (ed.), Springer Science+Business Media, LLC, pp. 1-28.

Chandra, P., Enespa, Singh, R., & Arora, P. (2020). Microbial lipases and their industrial applications: a comprehensive review. *Microbial Cell Factories.***19**:169.

Christian, M., & Paradisi, F. (2020). Looking back: A short history of the discovery of enzymes and how they became powerful chemical tools. *The European Journal for catalysis,* **12**:6082–6102.

Cira-Chávez, L., Guevara-Luna, J., Soto-Padilla, M., Román-Ponce, B., Vásquez-Murrieta M., & Estrada-Alvarado, M. (2018). *Kinetics of Halophilic Enzymes.* In tech open.

Cuesta, S., Rahman, S., Furnham, N., & Thornton, J. (2014). The Classification and Evolution of Enzyme Function. *Biophysical Journal,* **109**:1082–1086.

Elnasser, Z., Maraqua, A., Owais, W., & Khraisat, A. (2007). Isolation and characterization of new thermophilic bacteria in Jordan. *The International Journal of Microbiology,* **3**:1-10.

Elsharayidi, M., Dewedar, A., Shafik, H., & Elkazzaz, W. (2020). Screening for Hydrolytic Enzyme Production by Thermophilic Microbes Isolated from Egyptian Hot Spring. *Journal of Plant Production Sciences,* **9**:17-22.

Fentahun, M., & Vijaya, P. (2017). Isolation and screening of amylase producing thermophilic spore forming Bacilli from starch rich soil and characterization of their amylase activity. *African Journal of Microbiology Research,* **11**: 851-859.

Gram, H,C. (1884). Uber die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten" . *Fortschritte der Medizin,* **2**: 185-189.

Kassaye, E., & Tilahun, B. (2020). Isolation and Characterization of Extremophilies from Shalla/Abidjata Hot Springs, Ethiopia. *acta scientific microbiology*. **3**:01-05

Eze, U., Okonji, R., Ibraheem, O., & Shonukan, O. (2011). Isolation and characterization of a bacterial thermostable protease from poultry dung. *Life Journal of Science,* **13**:289-297

Gupta, G., Srivastava, S., & Khare, S. (2014). Prakash,"Extremophiles: an overview of microorganism from extreme environment," *International Journal of Agriculture, Environment and Biotechnology*, **7**:371–380.

Hasan, F., Shah, A., & Hameed, A. (2005). Industrial applications of microbial lipases. *Enzyme and Microbial Technology,* **39**:235–251

Ifandi, S., & Alwi, M. (2018). Isolation of Thermophilic Bacteria from Bora Hot Springs in Central Sulawesi. *Biosaintifika,* **10** :291-297.

Islam, T., Choudury, N., Hossain, M., & Khan, T. (2017). Isolation of Amylase Producing Bacteria from Soil, Identification by 16S rRNA Gene Sequencing and Characterization of Amylase. Bangladesh Journal of Microbiology, **34**:1-6.

Jaffer, Y., Vinothkumar, R., Irfan, A., Ishfaq, N., Ganie, P., Bhat, R., & Vennila, A. (2019). Isolation and characterization of thermophilic bacteria from Maharashtra hot springs: *Bacillus* sp. and *Staphylococcus* sp. *Journal of Entomology and Zoology Studies,* **7**:691-695.

Jufri., R. (2020). The Effect of Environmental Factors on Microbial Growth. *Journal of life science,* **01**:012-017

Khalil, A. (2011). Screening and characterization of thermophilic bacteria (lipase,cellulase and amylase prducers) from Hot springs in Saudi Arabia."*Journal of Food, Agriculture and Environment*, **9**:672–675.

Kiran, S., Singh, A., Prabha, C., Kumari, S., & Kumari, S. (2018). Isolation and Characterization of Thermostable Amylase Producing Bacteria from Hot Springs of Bihar, India. *International Journal of Pharma Medicine and Biological Sciences*, **7**:28-34.

Kudrya, V., & Simonenko, I. (1994). Alkaline serine proteinase and lectin isolation from the culture fluid of *Bacillus subtilis*. *Applied microbiology and biotechnology*, **41**:505–509.

Kumar, A., Yadav, N., Tiwari, R., Prasanna, R., & Saxena, A. (2014). "Deciphering the diversity of culturable thermotolerant bacteria from Manikaran Hot springs," *Annals of Microbiology*, **64**:741–751.

Lajis, A. (2018). Realm of Thermoalkaline Lipases in Bioprocess Commodities. *Journal of Lipids,* **2018**: 22

Lakhundi, S., Siddiqui, R., & Khan, N. (2015). Cellulose degradation: a therapeutic strategy in the improved treatment of Acanthamoeba infections. *Parasites & Vectors*. **8**:23

Lasekan, A., Bakar, F., & Hashim, D. (2013). Potential of chicken byproducts as sources of useful biological resources. *Waste Manage*ment. **33**:552–565.

Latorre, J., Hernandez-Velasco, X., Wolfenden, R., Vicente, J., Wolfenden, A., Menconi, A., Bielke, L., Hargis, B., & Tellez, G. (2016). Evaluation and Selection of *Bacillus* Species Based on Enzyme Production, Antimicrobial Activity, and Biofilm Synthesis as Direct-Fed Microbial Candidates for Poultry. *Frontiers in Veteternary Science*, **3**:95.

Lele, O., & Deshmukh. P. (2016). Isolation and characterization of thermophilic *Bacillus* sp. with extracellular enzymatic activities from hot spring of Ganeshpuri, Maharashtra, India. *International Journal of Applied Research* **2**:427-430.

Li, D., Li, A., & Papageorgiou, A. (2011). Cellulases from Thermophilic Fungi: Recent Insights and Biotechnological Potential. *Enzyme Research,* **2011**:9.

Li, G., & Yung, K, (2015). A new suite of tnaA mutants suggests that Escherichia coli tryptophanase is regulated by intracellular sequestration and by occlusion of its active site. *BMC Microbiology*, **15**:14.

MacWilliams, M. (2016). *Indole Test Protocol*. American Society for Microbiology, USA

Mamo, G., Gashe, B., & Gessesse, A. (1999). A highly thermostable amylase from a newly isolated thermophilic *Bacillus* sp. WN11 *Journal of Applied Microbiology*, **86**:557–560.

Maharana, A., & Ray, P. (2015). Optimization and characterization of cold-active endoglucanase produced by *Aspergillus terreus* strain AKM-F3 grown on sugarcane bagasse. *Turkish Journal of Biology*, **39**:175-185.

Massello, F., Chan, C., Chan, K., Goh, K., Donati, E., & Urbieta, M. (2020). Meta-Analysis of Microbial Communities in Hot Springs: Recurrent Taxa and Complex Shaping Factors beyond pH and Temperature. *Microorganisms*, **8**:906

Menendez, E., Fraile., P, & Rivas, R. (2015). Biotechnological applications of bacterial cellulases. *Bioengineering*, **2**:163-182.

Mehta, D., & Satyanarayana, T. (eds.), Thermophilic Microbes in Environmental and Industrial Biotechnology. In: *Biotechnology of Thermophiles*, pp. 5-1.

Mienda, B., Yahya, A., Galadima, I., & Shamsir, M. (2014). An Overview of Microbial Proteases for Industrial Applications. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, **5**:388.

Mikaela, J. (2018). Metaproteogenomics guided enzyme discovery. M.Sc. thesis, Likopng University, Sweden.

Mohammad, B., Al Daghistani, H., Jaouani, A., Latif, S., & Christian Kennes, C. (2017). Isolation and Characterization of Thermophilic Bacteria from Jordanian Hot Springs:

Bacillus licheniformis and *Thermomonas hydrothermalis* Isolates as Potential Producers of Thermostable Enzymes. *International Journal of Microbiology*, **2017**:12

Moretti, M., Bocchini-Martins, D., Da, S., Andre, R, Sette, L., & Eleni, G. (2012). Selection of thermophilic and thermotolerant fungi for the production of cellulases and xylanases under solid-state fermentation. *Brazilian Journal of Microbiology*, **43**:1062-1071.

Mrudula, S., & Murugammal, R. (2011). Production of cellulose by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate. *Brazilian Journal of Microbiology*, **42**:1119-1127.

Musa, H., Kasim, F., & Arbain, D. (2018). Isolation, molecular identification and screening of halophilic and thermophilic lipase producing bacterial strains from extreme environmental conditions. *Malaysian Journal of Microbiology*, **14**:413-423.

Naidu, M., & Saranraj, P. (2013). Bacterial Amylase: A Review. *International Journal of Pharmaceutical & Biological Archives*, **4**:274 – 287.

Nigam, P. (2013). Microbial Enzymes with Special Characteristics for Biotechnological Applications. *Biomolecules*, **3**:597-611.

Oarga, A. (2009) . Life in extreme environments. *Revista de biologia e ciências da terra*, **9**:1-10.

Olivier, J., & Jonker, N. (2013). *Optimal utilisation of thermal Springs in South Africa*. Water Research Commission, South Africa.

Ovando-Chacon, S., Tacias-Pascacio, V., Ovando-Chacon, G., Rosales-Quintero, A., Rodriguez-Leon, A., Ruiz-Valdiviezo, V., & Servin-Martinez, A. (2020). Characterization of Thermophilic Microorganisms in the Geothermal Water Flow of El Chichón Volcano Crater Lake, *Water*, **12**:2172.

Panda, M., Sahu, M., & Tayung, K. (2013). Isolation and characterization of a thermophilic *Bacillus* sp. with protease activity isolated from hot spring of Tarabalo, Odisha, India. *Iranian Journal of microbiology*, **5**:159-165.

Pandey, A., Dhakar, K., Sharma, A., Priti, P., Sati, P., & Kuma, B. (2015). Thermophilic bacteria that tolerate a wide temperature and pH range colonize the Soldhar (95 °C) & Ringigad (80 °C) Hot springs of Uttarakhand, India. *Annals of Microbiology*, **65**:809–816.

Pardo, A., & Forchiassin, F. (1999). Influence of temperature and pH on cellulase activity and stability in *Nectria catalinensis*. *Revista Argentina De Microbiologia*, **31**:31-35

Patel, D., Dave, S., Braganza,V., & Modi, H. (2019). Assessment of culturable microbial diversity of dholera thermal springs of gujarat, India. *Journal of Experimental Biology and Agricultural Sciences*, **7**:57-64.

Pathak, A., & Rathod, M. (2017). Exploration of a hot spring for thermostable protease producers. *Journal of Microbiology. Biotechnology and Food Sciences*, **7**:101-109.

Pentecost, A., Jones, B., & Renaut., R. (2011). what is Hot spring? *Canadian journal of earth science*. **40**:1443-1446

Pratush, A ., Gupta, A., & Bhalla, T. (2013). *Microbial Proteases: Prospects and Challenges*. Har Krishan Bhalla & Sons, India.

Rawat, R., Rautela, R., Rawat, S., & Bhatt, A. (2018). Hydrolytic enzyme production potential of bacterial population from sahastradhara cold sulfur spring, uttarakhand. *Plant Archives*, **18**:1313-1316.

Razzaq. A., Shamsi, S., Ali, A., Ali, Q., Sajjad, M., Malik, A. & Ashraf, M. (2019). Microbial Proteases Applications. In. *Bioengineering and Biotechnology*. Ji, X., (ed.). Frontiers, China, pp. 1-20.

Redman, R., Litvintseva, A., Sheehan, K., Henson, J., & Rodriguez, R. (1999). Fungi from Geothermal Soils in Yellowstone National Park. *Applied and Environmental Microbiology*, **6**:55193–5197.

Rehman, S., & Shash, A. (2005). *Geothermal resources of Saudi Arabia country update report*. Proceedings World Geothermal Congress; Antalya, Turkey

Rigoldi, F., Donini, S., Redaelli, A., Parisini, E., and Gautieri, A. (2018). Review: Engineering of thermostable enzymes for industrial applications. *Application of bioengineering,* **2**:2473-2877.

Sahay, H., Yadav, A., Singh, A., Singh, S., Kaushik, R., & Saxena., A. (2017). Hot springs of Indian Himalayas: potential sources of microbial diversity and thermostable hydrolytic enzymes. *Biotechnology,* **7**:118.

Sahoo, K., Sahoo, R., Gaur, M., and Subudhi, E. (2020). Cellulolytic thermophilic microorganisms in white biotechnology: a review. *Folia Microbiologica*, **65**:25-43.

Saini, R., Saini, H. & Dahiya, A. (2017). Amylases: Characteristics and industrial applications. *Journal of Pharmacognosy and Phytochemistry,* **6**:1865-1871.

Salano, O., Makonde, H., Kasili, R., Boga, H. (2018). Isolation and characterization of fungi from a hot-spring on the shores of Lake Bogoria, Kenya. *Journal of Yeast and Fungal Research*, **9**:1-13.

Salman, T., Kamal, M., Ahmed, M., Siddiqa, M., Khan, A., & Hassan, A. (2016). Medium optimization for the production of amylase by *Bacillus subtilis* RM16 in Shake-flask fermentation. *Pakistan journal of pharmaceutical sciences,* **29**:439-44.

Santiago, M., Ramírez-Sarmiento, C., Zamora, R., and Parra., L. (2016). Discovery, Molecular Mechanisms, and Industrial Applications of Cold-Active Enzymes. *Frontiers in Microbiology*, **7**:1408.

Singh, K., Tripathi, R., Jain, K., Vikram, S., & Garg, K. (2010). An antibiotic, heavy metal resistant and halotolerant *Bacillus cereus* SIU1 and its thermoalkaline protease. *Microbial Cell Factories,* **9**:59.

Singh, R., Mitta, A., Kumar, M., & Mehta, P. (2016). Amylases: A Note on Current Applications. *International Research Journal of Biological Sciences,* **5**:27-32.

Shajahan, S., Moorthy, I., Sivakumar, N., & Selvakumar, G. (2017). Statistical modeling and optimization of cellulase= production by *Bacillus licheniformis* NCIM 5556 isolated from the Hot spring, Maharashtra, India. *Journal of King Saud University-Science,* 29:302–310.

Sharma, N., Vyas, G., & Pathania, S. (2013). Culturable Diversity of Thermophilic Microorganisms Found in Hot Springs of Northern Himalayas and to Explore Their Potential for Production of Industrially Important Enzymes. *Scholars Academic Journal of Biosciences,* **1**:165-178.

Sharma, S., Sharma, P., Sourirajan, A., avid J. Baumler, D., & Dev, K. (2018). Identification of Thermophilic *Flavobacterium* and *Anoxybacillus* in Unexplored Tatapani Hot spring of Kishtwar District of Jammu and Kashmir: A North Western Himalayan State.*Current Trends in Biotechnology and Pharmacy*,**12**:245-25.

Souza, P & Magalhães, P. (2010). Application of microbial α-amylase in industry-a review. *Brazilian Journal of Microbiology,* **41**:850-861.

Sundarraman, A. KrishnaMurthy, P. (2014). "α-amylase production and applications: a review," *Journal of Applied & Environmental Microbiology*.**2**:166-175.

Sundarapandiyan., B. & Jayalakshmi, S. (2017). Isolation, Screening and optimization of amylase production by a marine bacterium *Bacillus subtilis* SJ33. *International Journal of Advanced Research in Biological Sciences*, **4**:8-14.

Sundus, H., Mukhtar, H., & Nawaz, A. (2016). Industrial applications and production sources of serine alkaline proteases: a review. *Journal of Bacteriology & Mycology*, **3**:191–194.

Thakur, S. (2012). Lipases, its sources, Properties and Applications: A Review. *International Journal of Scientific & Engineering Research,* **3**:2229-5518

Welday, T., Haki, G., Abera, S., & Baye, K. (2014). Isolation and Characterization of Thermo-stable Fungal Alpha amylase from Geothermal Sites of Afar, Ethiopia, *International journal of advances in pharmacy, Biology and chemistry*. **3**:2277-4688.

Tiwari, S., Srivastava, R., Singh, C., Shukla, K., Singh, R., Singh, P., Singh, R., Singh, N., & Sharma, R. (2015). Amylases: an overview with special reference to alpha amylase. *Journal of Global Biosciences,* **4**:1886-1901.

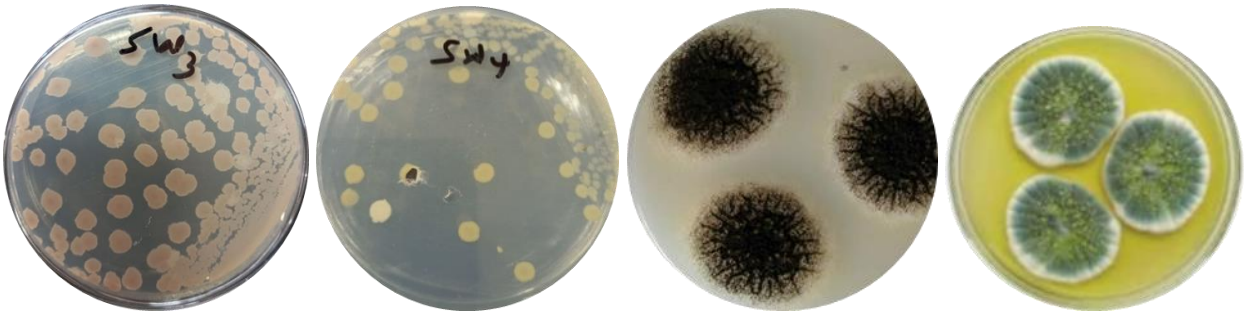
Zhou, W., James, F., White, Jr., Soares, M., Torres, M., Zhou, Z & Li, H. (2015). Diversity of fungi associated with plants growing in geothermal ecosystems and evaluation of their capacities to enhance thermotolerance of host plants. *Journal of Plant Interactions*, **10**:305-314.

Worthington, C., Worthington, V., & Worthington, A. (2019). *Introduction to Enzymes*. Worthington biochemical corporation, USA.

Yadav, P., Korpole, S., Prasad, G., Sahni, G., Maharjan, J., Sreerama, L., Bhattarai, T. (2018). Morphological, enzymatic screening and phylogenetic analysis of thermophilic bacilli isolated from five Hot springs of Myagdi, Nepal. *Journal of Applied Biology & Biotechnology*, **6**:1-8.

7. List of Appendices

Appendix 1. Cultural characteristics of bacterial and fungal isolates



Bacterial isolate 1 Bacterial isolate 2 Fungal isolate 1 Fungal isolate 2

Appendix 2. Diameter of zone of clearance of potent enzyme producer bacterial isolates

No.	**Isolates code**	**Diameter of halo (Dh)**			
		Amylase	Protease	Cellulase	Lipase
1.	JUW12	10	30	22	11
		10	31	23	11
2.	JUS33	24	10	22	10
		23	10	18	12
3.	JUS3	21	30	23	10
		23	28	23	11
4.	JUW29	20	13	25	10
		23	12	24	10
5.	JUW38	30	10	26	11
		29	11	25	12
6.	JUW43	23	12	19	20
		23	10	19	23
7.	JUW54	20	21	10	10
		21	21	10	11
8.	JUWG21	30	13	10	21
		28	12	12	20
9.	JUWG22	24	20	11	10
		24	18	11	11
		24	19	11	12
10.	JUWG45	26	11	10	23
		24	11	13	23
11.	JUS4	13	30	10	20
		14	29	10	18

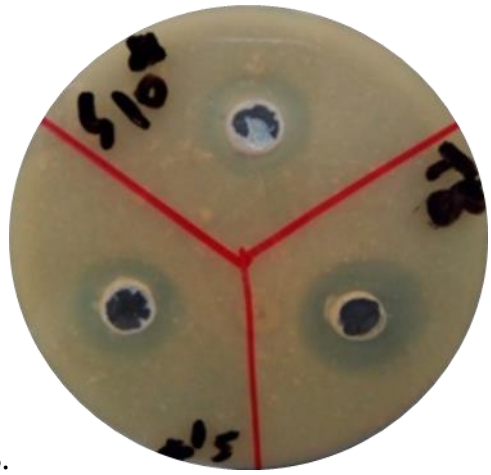
		11	29	10	19
12.	JUW64	10	20	11	23
		14	20	12	22
13.	JUW86	27	30	11	10
		25	28	12	11
14.	JUS38	10	22	25	11
		12	23	24	12
15.	JUWG84	30	20	10	11
		31	22	10	12
16.	JUW28	10	11	20	26
		11	12	19	25
17.	JUW62	10	12	25	26
		12	12	26	26
18.	JUWG50	10	11	20	19
		12	11	22	18
19.	JUW103	30	11	10	25
		30	12	10	22
20.	JUS2	28	10	10	25
		26	12	10	22
21.	JUS5	25	27	23	10
		25	26	24	12
22.	JUS42	30	29	11	10
		29	29	10	12
23.	JUS14	27	30	10	11
		27	29	10	12
24.	JUS27	10	30	11	12
		11	28	12	12
25.	JUS10	11	10	23	10
		11	13	22	12
26.	JUS9	10	11	12	25
		12	11	14	24
27.	JUS28	10	10	10	20
		11	12	10	22
28.	JUS39	11	10	14	20
		12	10	13	20
29.	JUS53	30	10	25	27
		28	12	26	26
30.	JUWG28	25	11	24	10
		26	13	24	10

31.	JUS50	23	10	25	11
		22	11	25	12
32.	JUS51	30	28	12	10
		29	27	13	12
33.	JUS52	24	10	22	12
		23	12	22	14
34.	JUS49	28	10	10	13
		25	12	11	14
35.	JUS54	10	25	23	12
		12	25	22	14
36.	JUS55	10	20	23	10
		10	22	22	12
37.	JUWG77	15	27	29	11
		14	26	28	12
38.	JUWG7	10	26	13	11
		14	25	12	12
39.	JUWG44	10	14	30	11
		13	13	30	13
40.	JUWG60	13	12	26	10
		15	14	25	11
41.	JUWG3	13	10	11	28
		12	12	11	29
42.	JUWG8	14	11	12	25
		15	12	13	24
43.	JUWG9	12	12	11	30
		14	13	10	30
44.	JUWG51	10	13	11	25
		12	13	12	23
45.	JUW53	12	10	11	21
		13	10	12	23

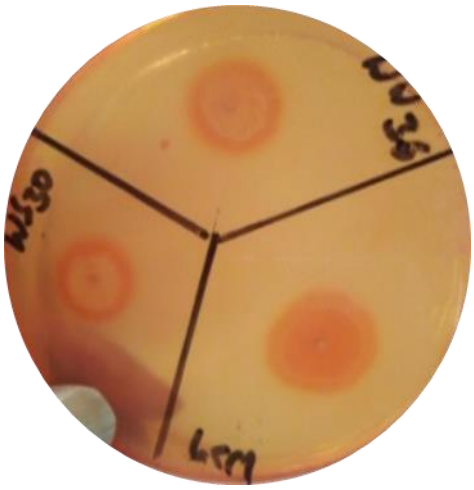
Where; JU=Jimma university, W=Woliso, WG=Wondo Genet, S=Shalla

Appendix 3. Figures of different types of enzymes screened

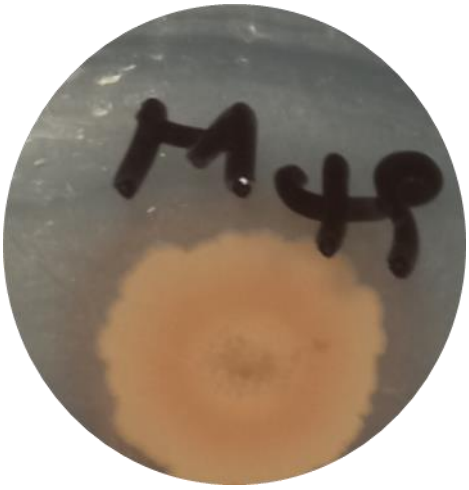
A.



B.



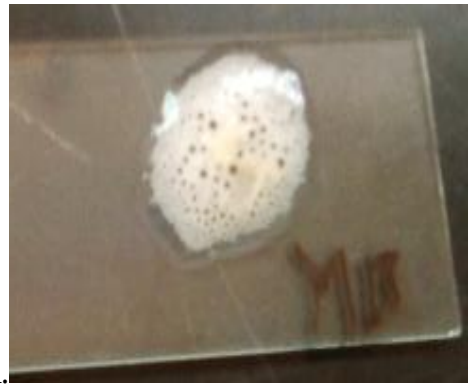
C.



D.

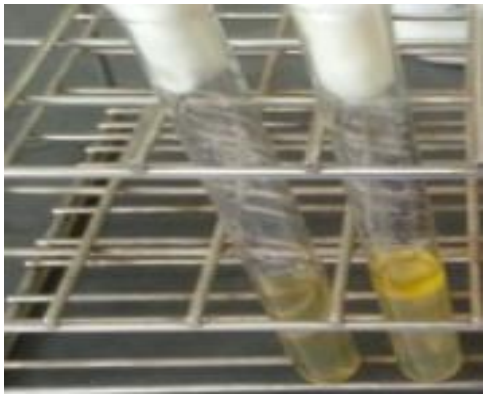
A. amylase screening B. protease screening C. cellulase screening and D. lipase screening

Appendix 4. Figures of biochemical tests performed

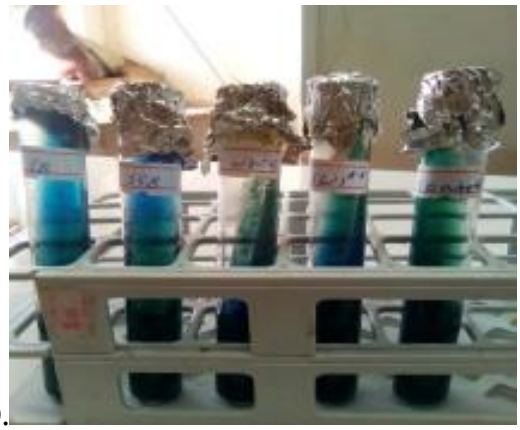
A. 

B1. 

B2. 

C1. 

C2. 

D. 

A= Catalase test, B1= Oxidase negative, B2=Oxidase positive, C1=Indole negative, C2= Indole positive, D= Citrate test

Appendix 5. Colony feature of bacterial isolates

Morphologically, the potent enzyme producing bacterial isolates showed some variation in the shape, size, elevation, consistency, color, opacity, degree of growth, and margin. They were regular, irregular, round, punctiform; small, medium, large; flat, raised, pulvinate; smooth, smooth-mucous, smooth-shiny, smooth-shiny-mucous, rough, rough-dry, rough-mucous, rough-shiny-mucous; creamy-white, whitish-yellow, brown, light-brown, whitish-grey, yellow, grey; opaque, translucent; scanty, moderate, profuse with smooth, undulate, lobate, erose, wavy and finger-like projections margin. The following (appendix 5) illustrates the colony morphology of all 45 isolates.

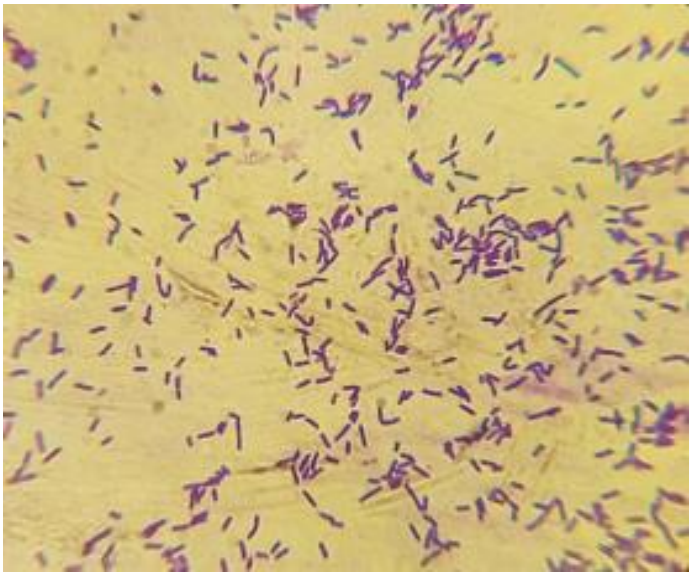
No.	Isolates code	Shape	Size	Elevation	Consistency (surface)	Margin	Color	Opacity	Degree of growth
1.	JUW12	Irregular	large	Raised	Smooth	Lobate	Creamy-white	Opaque	Profuse
2.	JUS33	Round	Large	Flat	Smooth	Smooth	Creamy-white	Opaque	Profuse
3.	JUS3	Round	Small	Flat	Smooth, shiny Mucous	Smooth	Brown	Opaque	Moderate
4.	JUW29	Round	Large	Flat	Smooth, mucous	Smooth	Creamy-white	Translucent	Profuse
5.	JUW38	Round	Small	flat	Smooth, mucous	Smooth	brown	Translucent	Scanty
6.	JUW43	Irregular	Medium	Flat	Rough, dry	Undulate	Creamy-white	Opaque	Moderate
7.	JUW54	Round	Small	Flat	Smooth, shiny, mucous	Smooth	Whitish-yellow	Opaque	Moderate
8.	JUWG21	Irregular	Large	Raised	Smooth mucous	Lobate	Creamy-white	Translucent	Profuse
9.	JUWG22	Irregular	Large	Flat	Rough, mucous	Undulate	Brown	Opaque	Moderate
10.	JUWG45	Punctiform	Medium	Flat	Rough, mucous	Erose	Creamy-white	Opaque	Moderate
11.	JUS4	Irregular	Medium	Flat	Smooth mucous	Lobate	Creamy-white	Translucent	Moderate
12.	JUW64	Punctiform	Medium	Rased	Rough	Wavy	Creamy-white	Opaque	Moderate
13.	JUW86	Round	Small	Flat	Smooth, shiny, mucous	Smooth	Whitish-yellow	Translucent	Moderate
14.	JUS38	Round	Small	Flat	Smooth mucous	Smooth	Creamy-white	Translucent	Scanty

15.	JUWG84	Round	Small	Flat	Smooth mucous	Smooth	Creamy-white	Translucent	Scanty
16.	JUW28	Round	Small	Flat	Smooth, Shiny, mucous	Smooth	Light-brown	Opaque	Moderate
17.	JUW62	Irregular	Medium	Flat	Rough	Erose	Light-brown	Opaque	Moderate
18 .	JUWG50	Irregular	Medium	Flat	Rough	Erose	Whitish-gray	Opaque	Moderate
19.	JUW103	Punctiform	Medium	Flat	Rough, Dry	Erose	Brown	Opaque,	Moderate
20.	JUS2	Irregular	Large	Pulvinate	Smooth Shiny, mucous	Lobate	Creamy-white	Translucent	Profuse
21.	JUS5	Irregular	Medium	Flat	Smooth Mucous	Lobate	Creamy-white	Translucent	Moderate
22.	JUS42	Regular	Medium	Flat	Rough	Finger like projections	Creamy-white	Translucent	Profuse s
23.	JUS14	Irregular	Large	Flat	Rough	Finger like projections	Creamy-white	Translucent	Profuse
24.	JUS27	Round	Large	Flat	Smooth shiny	Smooth	Yellowish-white	Translucent	Profuse
25.	JUS10	Round	Small	Flat	Smooth, Shiny	Smooth	Yellow	Translucent	Scanty
26.	JUS9	Irregular	Medium	Flat	Rough	Erose	Brown	Opaque	Moderate
27.	JUS28	Irregular	Medium	Flat	Smooth	Erose	Creamy-white	Opaque	Moderate
28.	JUS39	Irregular	Medium	Flat	Rough	Undulate	Brown	Opaque	Moderate
29.	JUS53	Irregular	Medium	Flat	Rough	Smooth	Whitish-gray	Opaque	Profuse
30.	JUWG28	Irregular	Large	Raised	Smooth, shiny mucous	Lobate	Brown	Opaque	Profuse
31.	JUS50	Irregular	Medium	Raised	Rough	Finger like projections	Creamy, white	Translucent	Profuse
32.	JUS51	Regular	Medium	Flat	Rough	Finger like projection	Creamy-white	Translucent	Profuse
33.	JUS52	Irregular	Large	Pulvinate	Smooth shiny, mucous	Lobate	Light-yellow	Translucent	Moderate
34.	JUS49	Irregular	Medium	Flat	Rough	Undulate	Whitish-grey	Opaque	Moderate
35.	JUS54	Round	Large	Flat	Smooth-mucous	Lobate	Yellow	Translucent	Moderate
36.	JUS55	Round	Small	Flat	Smooth, shiny, mucous	Smooth	Yellow	Translucent	Scanty
37.	JUWG77	Irregular	Medium	Raised	Rough	Erose	Creamy-White	Translucent	Moderate
38.	JUWG7	Irregular	Large	Raised	Rough shiny mucous	Lobate	Creamy-white	Opaque	Profuse

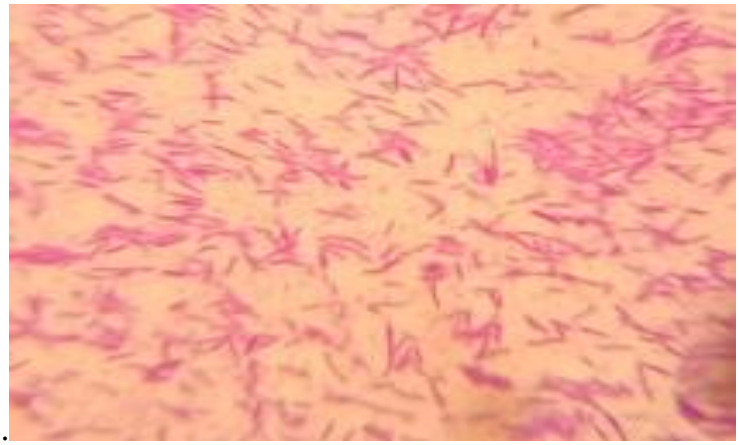
39.	JUWG44	Irregular	Medium	Raised	Rough	Erose	Brown	Opaque	Moderate
40.	JUWG60	Round	Small	Flat	Smooth shiny	Smooth	Whitish-yellow	Transparent	Scanty
41.	JUWG3	Irregular	Medium	Flat	Smooth mucous	Lobate	whitish-yellow	Translucent	Moderate
42.	JUWG8	Irregular	Medium	Flat	Smooth mucous	Lobate	whitish-yellow	Translucent	Moderate
43.	JUWG9	Irregular	Medium	Raised	Rough dry	Erose	Brown	Opaque	Moderate
44.	JUWG51	Irregular	Medium	Flat	Smooth dry	Erose	Brown	Opaque	Moderate
45.	JUW53	Irregular	Small	Flat	Smooth shiny	Erose	Whitish-yellowish	Translucent	Moderate

Appendix 6. Gram staining

The selected bacteria were observed and they were Gram-positive and Gram-negative rods arranged in single, pairs and chains when visualized under the microscope after Gram staining. The following appendix 6 shows the appearance of one for each of the gram-positive and gram negative bacteria under the microscope.

A. 

Representative isolate for Bacillus

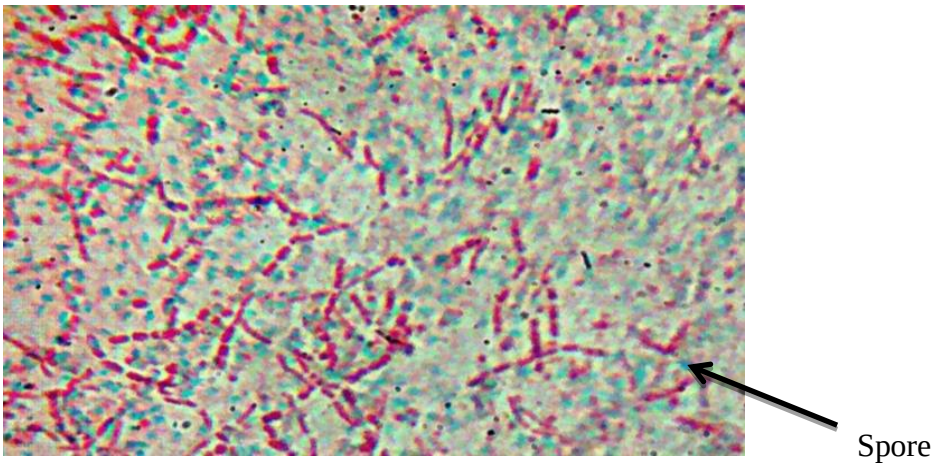
B. 

Representative isolate for Thermus

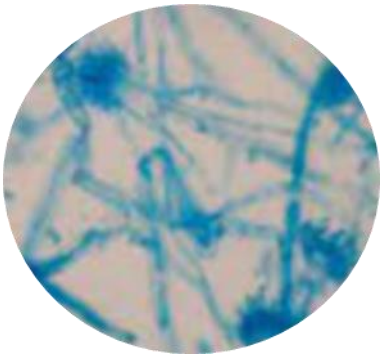
Appendix 7. Spore staining

The spore staining results showed that 39 of the isolates were endospore-forming bacteria. In (appendix 7) the red color indicates vegetative cells of bacteria, whereas the green color indicates an endospore.

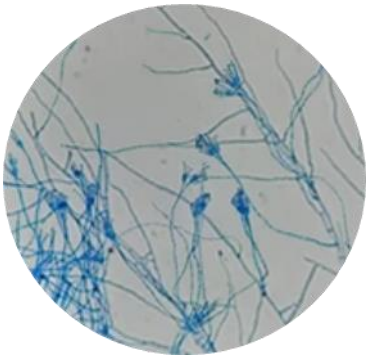
Appearance of one of the endospore forming bacteria under the microscope.



Appendix 8. Lacto phenol cotton blue staining results of fungi



Aspergillus spp.



Penicillium spp.

Appendix 9. Sampling Sites

A.  **B.**  C. 

A. Woliso hot spring B. Wondo Genet hot spring C. Shalla hot spring site 1 D. Shalla hot spring site 2

A.  B. 

A. During media preparation B. During enzyme screening