

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE

DEPARTMENT OF BIOTECHNOLOGY

ISOLATION AND CHARACTERIZATION OF PROTEASE PRODUCING BACILLUS SPECIES FROM SOLID WASTES(SLUDGE) OF ZEBIDAR BEER FACTORY GUBREE,WOLKITE, ETHIOPIA

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APPROVAL SHEET

WOLKITE UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE

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LIST OF ABBREVIATIONS

MR	Methyl red test
P^H	Power of hydrogen
RPM	Revolution per minute
SL-E	Sludge sample at the end
SL-M	Sludge sample at the dehydrating machine
SL-S	Sludge sample at the surface
Spp	Species
TSI	Triple sugar iron test
TSIA	Triple sugar iron agar
VP	Voges proskauer test

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ABSTRACT

Microbes are ubiquitous and live in familiar settings such as soil, water, food and animal intestines as well as in more extreme setting such as rocks, hot springs and deep-sea vents. They are essential component of the biodiversity as they account half percent of the living biomass of this planet. Bacillus species are a source of proteases, having a long history of application in environmental bioremediations, food and detergent industries and currently this enzyme has different role in medical and different industries. The objective of the present study was to isolate and characterize protease producing bacteria from sludge wastes collected from Zebidar beer factory. The proteolytic activities were determined by blood hydrolysis. The isolated bacteria were characterized by morphological and biochemical methods. The morphological appearances of isolate were varied in color, form and margin. The isolate were gram positive short rod and long rod. The strain SL-E1, SL-E2, SL-S1, SL-S2, SL-M1, SL-M2 of the isolate were positive for biochemical test of TSI, catalase, VP, MR, motility. From the isolates the sludge sample at the end was show better performance to produce protease enzyme. The isolates were positive results for citrate test. The highest proteolytic activity on blood spot was obtained at optimum temperature of 37^0C and pH 9This temperature and pH was expectation. Bacillus species from the Zebidar beer factory waste soil sample could be alternative for industrial and commercial use. The isolated types of microbes need to be fully characterized using molecular techniques.

Key-words*:* *Bacillus Spp*, Characterization, Protease, Proteolytic activity

1. INTRODUCTION

1.1. Background

Proteases are also called peptidase or proteinase or proteolytic enzymes and they are belonging to the class of hydrolases and they can hydrolyze peptide bonds in proteins (Arun Kumar *et al*., 2015). Proteases have great industrial and commercial applications including food, detergent, leather and pharmaceutical industries (Sikander *et al.,* 2017). The enzyme proteases classified in to two types according to sites at which they catalyze the cleavage of proteins, exopeptidases and endopeptidases. On the other hand, based on their acid base behavior, proteases are classified in to three groups; acid, neutral and alkaline proteases (Rhagunath *et al,* 2010). Acid proteases performed best at pH range of 2.0-5.0 and are mostly produced by Fungi. Proteases having pH optimum in the range of 7.0 or around are called neutral proteases and many of them are plant origin while proteases having optimum activity at pH range 8 and above are classified as alkaline proteases (Alnahdi, 2012). Alkaline protease in fact the first enzyme produced in bulk (Gupta *et al*., 2002). Also, proteases are classified into six types based on the functional groups in their active sites. They are aspartic, cysteine, glutamic, metallo, serine, and threonine proteases.

Proteases have physiological role in all living organisms and therefore, they are present in a wide range of sources such as animals, plants and microorganisms (Rao *et al*., 1998). Bacterial proteases are preferred as they grow rapidly, needless space, can be easily maintained and are accessible for genetic manipulations (Odu *et al*., 2012). Currently, a large proportion of commercially available proteases are derived from *Bacillus* strain (Gupta *et al.,* 2002). *Bacillus*-species are Gram- positive, endospore-forming, chemoheterotrophic rod-shaped bacteria which are usually motile with peritrichous flagella; they are aerobic or facultative anaerobic and catalase positive (Waites *et al*., 2008).

Chemicals used in leather industry are carried out by toxic chemicals such as sodium sulphide, solvents, lime and salts also peptide synthesis through chemical methods has disadvantages, such as, low yield, racemization issues and health and environmental concern due to toxic nature of solvents and reagents used in the processes (Kumar, 2005). So, substitution of chemicals by enzyme is decrease the risk to health as wellas they are safe to environment. In current situation of industries in our country they need more supply of enzymes like protease.

The goal of this research is isolation and characterization of protease producing *Bacillus*

species from sludge. This study has so many importance's to our country by upgrading it to industrial scale for the production of high amount of protease enzyme to use as detergent, diagnostic and therapeutic purposes and modification of properties of food proteins.

Waste that we see in our surroundings is also known as garbage. Garbage is mainly considered as a solid waste that includes wastes from our houses (domestic waste), wastes from schools, offices, (municipal wastes) and wastes from industries and factories (industrial wastes). Biodegradable waste: These are the wastes that come from our kitchen and it includes food remains, garden waste, etc. Biodegradable waste is also known as moist waste. This can be composted to obtain manure. Biodegradable wastes decompose themselves over a period of time depending on the material. Non-biodegradable wastes are most highly damaged the environment. It takes longer time, to decompose their components, their components may contain toxic chemicals that toxic chemicals are threaten human life.

These are the wastes which include old newspapers, broken glass pieces, plastics, etc. Non-biodegradable waste is known as dry waste. Dry wastes can be recycled and can be reused. Non-biodegradable wastes do not decompose by themselves and hence are major pollutant. Any unwanted materials that are attributed as unusable and are discarded after primary use without considering their secondary or tertiary use. Waste, as a term, is sometimes considered subjective as a particular material that is being discarded for one reason could not be waste for another use) whereas often considered objectively wrong for instance, it is not suitable to classify the scrap as "waste" when we send leftover metallic waste for dumping in a landfill because they are still valuable and recyclable. Concrete waste type of community including domestic debris, chemical/harmful waste, and polluted water containing sewage (comprises feces and urine), Surface runoff and radioactive waste, all of these falls under the category of industrial waste.

During industrial activities in mills and factories or mining operations, some of the materials are discarded after declaring as invaluable or waste and this practice is being done from the time of Industrialization. Sludge is any type of industrial waste that is specific for that industries were used the raw materials as byproduct. Sludge is must be managed before release to the environment. Industrial wastes have diverse range such as metals, by-products, radioactive wastes, sandpaper, paper products and gaseous, liquid, solid wastes like effluents. Rates of globalization were intensified when the rural population started shifting from rural to urban areas.

During exerting pressure on resources available per capita. This intense emerging caused mismanagement in waste management because of economic reasons cities were not equipped to tackle waste at this level (Oteng-Ababio et al., 2013).

1.2. Statement of the Problem

Every chemical has its own bundle of side effects that leads to emerge negative impact on environment and health. Processed leather is obtained by going through these steps: soaking, bating, liming, deliming, degreasing, dehairing, and pickling (Sumantha *et al.,* 2005). All these steps are carried out by toxic chemicals such as sodium sulphide, solvents, lime and salts and these chemicals cause environment pollution (Sikander *et al.,* 2017). Due to these effects, some of leather industry used alkaline proteases as an alternative to pollutant chemicals (Park *et al.,* 2014). On the other hand, Photographic and X-ray films contain gelatine layer and around 2% silver (Hira *et al*., 2016). This silver able to be recovered, and retrieved by burn the films which has dire environmental hazards (Hira *et al*., 2016). However, proteases from *Bacillus* species have been successfully used in the recovery of silver and minimize environmental hazards (Abdalall *et al*., 2016). Washing with detergents also require lot of energy particularly when done at high temperature (peroxide-based bleaches need higher temperature, 60°C, to work properly). These can be solved by using biocatalysts and helps to saves lots of energy (Kirti *et al.,* 2011).

Change of chemical processes by enzymatic hydrolysis processes is now a day an attractive option to produce safe environment and maintain good health of the individuals. The presently available proteases are not sufficient to meet industrial demands. Hence, there is a need to search new proteases with novel characteristics for industrial application. Microbes from varied habitats have been examined by many researchers to obtain the industrially suitable proteases. Here, after the intention of the present study is to find out the best protease enzyme from *Bacillus* species isolated from Zebidar beer factory solid waste in Gubree sub-city.

1.3 Objectives of Study

1.3.1. General objective

The overall objective of this study is to isolate and characterize and assessing activity of enzyme produced by bacillus spp. from final solid wastes of Zebidar beer factory in Gubree, Ethiopia.

1.3.2. Specific objectives

- ✓ To isolate and characterize protease producing *Bacillus* spp.

  To produce protease and assess activity of enzyme produced by bacillus spp.

1.4. Significance of the Study

The result of the current study has great important to the Ethiopian industries by upgrading the use of chemicals to protease enzyme in detergent, diagnostic and therapeutic purposes and modification of properties of food proteins to improve nutritional value, solubility, digestibility, flavor, palatability and minimizing allergenic compounds. This enzyme has decreases environmental pollution by replacing chemicals used in leather industries and saves different aquatic lives which are exposed to the waste of leather industries that contain toxic chemicals. As we know, most of detergent in our country are not effective in removing stains from clothes as a result we lost a lot of electric power and time: so, this enzyme is needed for effectively removing stains and save a lot of time and power. Chemicals used in food industries are changes the taste, flavor and cause allergies as a result of this enzyme is needed to maintain natural taste and flavor in foods and also decrease allergies. The result of the current study will be providing an input regarding the situation for policy makers to use alternative enzymatic compounds in the country. Moreover, this study will serve as a source of information for other researchers at national and international level.

1.5. Scope of the Study

This study was conducted at Wolkite University, particularly in the biotechnology department molecular biology laboratory of Gubree main campus. The study was focus on the isolation and characterization of protease producing bacillus species from final sludge wastes of Zebidar beer factory in Gubree, Ethiopia.

2.LITERATURE REVIEW

2.1. Bacillus Species

Bacillus species are Gram-positive, endospore-forming, chemoheterotrophic rod-shaped bacteria which are usually motile with peritrichous flagella; they are aerobic or facultative anaerobic and catalase positive (Waites *et al*., 2008). *Bacillus* species are ubiquitous and widely diffused in the environment including soil, alkaline environments, paintings, hydrothermal vents, insect gut and seawater (Yazdani*et al*., 2009). The presence of thebacteria in these very diverse environments reflects their broad and versatile metabolic capabilities, which makes of them very important microorganisms with a high potential forthe industry (Daffonchio*et al*., 2000). *Bacillus*sp are one among the best protease producers studied so far since they exhibit properties like broad substrate specificity, significant activity, stability, simple downstream purification, short period of fermentation and low cost (Haddar*et al*., 2009). These properties make the bacterial proteases most suitable for a wider application in industries. Several *Bacillus*species involved in protease production are *B.sterothermophilus*, *B. cereus*, *B. megaterium, B.mojavensis,* and *B. subtilis* (Anwar.2000). Common physiological traits important to their survival include production of a multilayered cell wall structure, formation of stress-resistant endospores and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes (Gardener.,2004). *Bacillus*is the most abundant genus in the rhizosphere of soil is naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion (Gutierrez *et al*., 2003). The characterization of *Bacillus* species is done bymorphological, biochemical test, and different molecular methods (Gregersen, 1978). *Bacillus* species are play major roles in the mineralization of plant-derived materials, humus, pesticides and hydrocarbons in soil (Prescott *et al*., 2002).

2.2. Protease

Proteases can hydrolyze peptide bonds in proteins and they are also called peptidase or proteinase or proteolytic enzymes (Arun Kumar *et al*., 2015). Protease enzymes can produce eco-friendly products and so they play a vital role in modern biotechnology industries (Abebe*et*

al., 2014). Proteolytic enzymes are ubiquitous in nature and they are found in all living organisms such as plants, animals and microbes (Srinivas Naik *et al*., 2013). Microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications and their protease was lacks pathogenicity (Anwar *et al*., 1997). Proteases are one of the mostimportant groups of industrial enzymes and account for nearly 60% of the total enzyme sale (Brown and yada, 1991). Protease enzymes are majorly used in various industries such as in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestives and for certain medical treatments of inflammation and virulent wounds (Van der Oost *et al*., 2003).

2.2.1. Classification of protease

Proteases are broadly classified as endo or exoenzymes on the basis of their site of action on protein substrates. They are further categorized as serine protease, aspartic proteases, cysteine proteases, or metalloproteases depending on their catalytic mechanism (Geethanjali and Subash, 2011). Based on site of action on protein substrates

2.2.1.1. Exopeptidases

Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate. Based on the site of action at the N or C terminus, they are classified as aminopeptidases and carboxypeptidases (Vishalakshi*et al*., 2009). The former act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide while the later act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide (Rao *et al*., 1998).

2.2.1.2. Endopeptidase

Endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, endo-peptidases are further classified into four prominent groups, i.e, serine proteases, aspartic proteases, cysteine proteases and metallo-proteases (Jaswal*et al*., 2008).

2.2.2. Sources of proteases

Proteases have physiological role in all living organisms and therefore, they are present in a wide range of sources such as animals, plants and microorganisms (Rao *et al*., 1998).

2.2.2.1. Plant Proteases

Proteolytic enzymes in plants are involved in key physiological process like photoinhibition, photomorphogenesis in seeds, and senescence (Estelle *et al*., 2001). Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin (Mala *et al*, 1998).

2.2.2.2. Animal Proteases

The most widely used animals derived proteases are pancreatic trypsin, chymotrypsin, pepsin and rennin (Rao *et al*., 1998).

2.2.2.3. Microbial Proteases

Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996). Fungi and bacteria are the major source of production of this enzyme and they show catalysis in broad range of pH and temperature (Kinda et l., 1995). Microbial proteases, especially from *Bacillus* Species have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergent formulations (Beg *et al*., 2003). The protease producing bacterial strains are as *Bacillus* subtilis, *Bacillus* licheniformis, and *Bacillus* thuringiensi (ArunKumar *et al*., 2015).

2.2.3. Applications of Proteases

Proteases prove to be of great importance in a wide variety of industries. These include food industries, detergent, pharmaceutical industries. Digestive proteases are part of many laundry detergents. Alkaline proteases are used to remove stains (food, blood, grass, and body secretions) and other dirt by breaking down the macromolecules (Kalisz., 1998). Washing powders containing enzymes are used in dry cleaning industries as stain and spot removers. Activity and stability at high pH and temperature, and compatibility with other chelating and oxidizing agents added to the detergent are among the major prerequisites for the use of proteases in detergents (Godfrey. 1996). Most of the detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains (Deshpande *et al*., 2004).

2.2.3.1. Leather Industry

The major building blocks of skin and hair are proteinaceous (Hameed *et al*., 1999). In order to overcome the hazards caused by the tannery effluents, the use of enzymes as a viable alternative to chemicals has successfully resorted to in improving leather quality and in

reducing species, is environmental pollution (Grbavčid *et al*., 2001). Proteases are used for selective hydrolysis of non-collagenous constituents of the skin and for removal of non-fibrillar proteins such as albumins and globulins, in the several pre-tanning operations. The purpose of soaking is to swell the hide. Traditionally, this step was performed with alkali. The use of non-ionic and, to some extent, anionic surfactants to accelerate the process is compatible with the use of enzymes. (Rhagunath *et al*, 2010).

2.2.3.2. Food Industry

Proteases are used in food industry for a wide range of applications. These enzymes are efficiently involved in the modification of properties of food proteins to improve nutritional value, solubility, digestibility, flavor, palatability and minimizing allergenic compounds (Pardo *et al*., 2000). Besides, their basic function, they are also used to modify functional properties, such as coagulation, emulsification, foaming, gel strength, fat binding etc. of food proteins (Pardo *et al*., 2000). The catalytic function of proteases is used in the preparation of protein hydrolysate of high nutritional value, which is used in infant food products, medicinal dietary products, fortification of fruit juice and soft drinks (Heredia-Sandoval *et al*., 2016).

In dairy industry, proteases are primarily used in cheese manufacturing to hydrolyze specific peptide bonds to produce casein and macro peptides (Rao *et al*., 1998). The ability of proteases to hydrolyze connective tissues and muscle fiber proteins is used for tenderization of meat (Gupta *et al*., 2002). The alkaline proteases play an important role in the production of soy sauce and other soy products.

In baking industry, they are added to ensure dough uniformity, reduce dough consistency, maintain gluten strength in bread and, improve flavor and texture in bread (Heredia-Sandoval*et al*., 2016). These hydrolytic enzymes are utilized for degradation of the turbidity complex resulting from protein in fruit juices & alcohol-based liquors; in gelatin hydrolysis and recovery of meat proteins (Pardo *et al*., 2000).

2.2.3.3. Therapeutic application

The wide diversity and specificity of microbial proteases are extensively used in diagnostic and therapeutic purposes. Bacterial and fungal proteases have contributed equally in developing effective therapeutic agents, such as anticancer, clot dissolving, antimicrobial, anti-inflammatory (Ellaiah *et al*., 2002). Protease mediated treatment of acute and chronic inflammation is cost effective without any reported side effect. Natto kinase from *Bacillus* subtilis is used as cardiovascular disease nutraceutical and helps to lower risk of the disease also prevents blood coagulation and dissolves thrombus (Phadatare, 1993). Several proteases from bacterial and fungal sources have also been reported for anti-microbial properties(Gupta *et al*., 2002). As trauma medicine, these hydrolytic enzymes are applied to remove scar, regenerate epithelia, and faster healing processes (Hsia *et al*., 2009).

3. MATERIALS AND METHOD

3.1. Description of the Study Area and Period

Gubree town is found in central Ethiopia, 166km away from south Western direction Addis Ababa. The town has 8 11` 42.59`` North latitude and 37 47` 22 43``East longitude with an elevation of 1932 meters above sea level. An experimental study was conducted from February to May, 2024 at Wolkite University, in main campus, college of natural and computational science, department of biotechnology, molecular biology Laboratory.

3.2. Study Design

The study design was a completely randomized design (CRD) based on experimental procedure and sample collection for isolation bacillus species from waste sludge, for characterize protease producing bacillus species, biochemical characterization was determined for produce protease enzyme. This research design was given in case of randomly sample collection. It is experimental design that is observation the results.

3.3. Sample Collection

The samples were collected from Gubree on Zebidar beer factory final solid wastes (sludge), That samples were collected from treated sludge (solid wastes) at the surface of artificial pond, at the bottom artificial pond, at dehydrating machine and final place of waste sludge accumulation. The sludge samples were collected aseptically and each sample was kept in different clean sterile plastics bags for each sample and transfer to molecular biology laboratory and store at 4°C (Richard, 2011).



Figure 1: sample collection site from Zebidar beer factory

3.4. Media preparation

Nutrient agar was prepared in the amount of 5.6 grams of lab prepared nutrient agar based on packed bottle instruction and it was added on 300 ml of distilled water in Erlenmeyer flask. The suspension was heated to boiling to dissolve the medium completely. The dissolved medium and any necessary materials (petri dish, test tube, tips) were autoclaved at 15 lbs pressure (121^0c) for 15min. The autoclaving process was completed the Erlenmeyer flask taken out and left to cool. The media was poured into sterile petri dishes under sterile conditions bacterial culture were incubated at 37^0cfor 24 to 48 hr.

3.5. Isolation of protease producing Bacillus species

The techniques used for isolation of bacteria was serial diluted and spread plate method.1gmof soil samples were weighed and serial dilution ($10^{-1\ to}10^{-6}$) of each soil sample were carried out. 0.1ml of each aliquot sludge samples (10^{-4}to10^{-6}) was spread on nutrient agar plate at temperature 37^{o}c for 24 to 48hr. After incubated time subculturing process on nutrient agar by Streak Plate: a sterile loop or inoculating needle were used to transfer a small or individual pure colony of the bacteria from the original culture to the surface of a fresh nutrient agar plate. The bacteria were streaked across the plate in a zig-zag pattern to isolated pure colony bacterial. Then cultures were incubated at 37^{o}C for 24-48hrs (Soumya Vaidya and Pragya Rathore.,2015).

Again, the subculture process was done on skim milk agar in two parts (i.e. part A,3.52 grams and part B,4.2grams there was separately boiled on each two flasks. Then, there was autoclaved and poured part A to part B and mixed well after this process poured on sterilized petri dish

under sterilize condition. The incubation period was 24 to 48hr at 37^0c.after incubation time the clear zone was formed that was the sign of protease producing bacillus species. Skim milk agar was used to isolate protease producing bacillus species. The zone of hydrolysis was noted for each sample. The colony showing highest zone of inhibition was selected for further study. The colony was grown on nutrient agar plate repeatedly and preserved on nutrient agar slant at 4^oc.

3.6. Characterization of protease producing Bacillus Species

Selected isolates were gram stained and characterized using different biochemical tests Include Catalase, motility, Simmon citrate, VP, MR, Starch hydrolysis, TSI (Rajeswari and Bhuvaneswari, 2016).

3.6.1. Reaction of the organism in the triple sugar iron (TSI) agar slant

The appropriate amount of TSIA powder media was measured at 5.44 grams and dissolved in 100 ml of distilled water of the required volume in a conical flask. The media was boilled and dissolved entirely in distilled water. Dispensed about 5 to 10 mL of the medium in each test tube and loosely put on the cap or cotton plug the opening. Then autoclaved the tubes at 121°C and 15 lbs pressure for 15 minutes. Let it cooled and solidify at a slanting position (around 30° inclinations) to form an agar slant with a deep butt of about 3 to 5 cm. Touch a well-isolated colony from a fresh culture of the test bacterium were 18 to 24 hours old using a sterile inoculating wire. Incubated the tube aerobically (with a loose cap) at 37°C for about 24 hours. Examine for color change of the slant and butt, recorded and reported the color within 24 hours of incubation, but read sugar fermentation and color change within the first 24 hours of inoculation and incubation (Wind R.D et al.1994).

3.6.2 Catalase test

A small amount of bacterial colony was transfered to a surface of a clean, dry glass slide used a loop or sterile wooden stick. Be sure the colony were visibled to the naked eye on the slide. Place a drop of 30% H2O2 onto the slide and mixed. A positive result was the rapid evolution of oxygen (within 5-15 seconds), as evidence by bubbled. A negative result was no bubbled or only a few scattered bubbles. Dispose of your slide in the biohazard glass disposal container.

3.6.3 Methyl red and voges proskauer test

The MR and VP agar were formulated in the amount of 3.4 g dissolved in 200ml distilled water, boiled and 10ml were added to each test tube. The test tubes were autoclaved to sterilized the broth. After that, for each test tube aloop of 24 hours fresh culture were inoculated. The test tubes were incubated at 37°C for 24 hours.

3.6.3.1 Voges proskauer (vp) test

After incubated at 37°C for 24-48 hours added 6 drops of 5% alpha-naphthol, and mixed well to aerate. Added 2 drops of 40% potassium hydroxide, and mixed well to aerate. Observed for a pink-red color at the surface within 30 min. Shaked the tube vigorously during the 30-min period. Positive Reaction observed pink-red color at the surface, Negative Reaction observed lack of a pink-red color.

3.6.3.2 Methyl red test

After 72 hours of incubated the MR-VP medium were added five drops of the methyl red indicator solution. Finally, observed for changed in color in each test tubes. The development of a stable red color on the surface of the medium with MR reagent indicates sufficient acid production to lower the pH less than six for a positive test. Because other organisms may produce smaller quantities of acid from the test substrate, an intermediate yellow color. It was indicated a negative test.

3.6.4 Simmon's citrate test

The media were prepared in the amount of 2.4g was dissolved in 100ml distilled water and 10 ml medium was added to each test tube. Each test tubes were autoclaved and then left to cool at a slant position in order to create a butt and slant. An inoculated needle was used to pick up a pure 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 tube was incubated at 37°C for 24-48 hours. The color of the media was observed after incubated. Positive Reaction were Growth with color change from green to intense blue along the slant. A negative reaction was evidenced by the absence of growth to a trace of growth with no color change.

3.7. Protease Production from Bacillus species

A 1000ml of casein broth will be prepared in conical flask. The conical flasks containing medium will be autoclaved at 121ºC for 15 min at 15 LBS pressure. After the isolation and screening of protease producing microorganism, efficient protease producer microorganism will be inoculated in small flasks and it was then incubated at 37^0C for 72 hrs. After 3 days of incubation, content of flasks was centrifuged at 3500 rpm for 30 minutes at 4ºC. A clear supernatant was recovered after centrifugation. The crude enzyme supernatant was subjected to further studies.

3.8. Purification of Enzyme

3.8.1. Ammonium Sulphate Precipitation

At first 2ml of crude enzymes will be measured and then it was treated with 50% (NH4)2SO4. The salt was slowly added to the volume of crude enzymes with continuous-stirring, after completing the addition, the enzyme will be kept at 4ºC overnight for precipitation.

3.8.2. Effect of Temperature and pH

To study the effect of temperature and pH, the Medias pH will be adjusted at pH of 5, 7 and 9 by using hydrochloric acid and sodium hydroxide these Medias was then incubated for 72 hours at temperature of 30^0C, 37^0C and 60ºC. After that the produced enzyme will be done by centrifugation and dispensed at cloth which would have blood spot then the effect of temperature and pH will be assessed.

3.9. Washing Test

Application of protease enzyme by isolated organisms a detergent additive will be studied as per (SidraAftabet.al2006). For this three white cotton clothes (5*5cm) will be stained with blood. Following sets will be prepared and studied:

- Blood-stained cloth will be dipped in flask with distilled water (100ml).
- Blood-stained cloth dipped in flask with distilled water (100ml) +1 ml detergent (7mg/ml)
- Blood-stained cloth dipped in flask with distilled water (100ml) +1 ml detergent

(7mg/ml) +2ml enzyme solution.

All three flasks were incubated at 60oC for 15 min. After incubation, cloth pieces will be taken out, rinsed with water and dried. Visual examination of cloth pieces will be exhibited the effect of enzymes in removal of stains. Untreated cloth pieces stained with blood as a control.

3.10. Data Analysis

Data collected from this research was analyzed via Microsoft 2010. Tabulation and figures were used for representation.

4. RESULTS

4.1. Isolation of Bacillus species

The current study was on the basis of morphological characterization (color, form, shape, and elevation of pure colonies) and biochemical tests. Most colonies were able to grow within 24h-48h of incubation at 37ºC. The colony morphology of the isolates ranged form,, flat and filamentous or branching (**Table 1**). They were smooth or rough and the color ranged from white to cream. SL-E sample was formed white colony that sample was collected from the sludge sample at the end accumulation.SL-S and SL-M samples were formed cream and white colony separately that samples were collected from sludge sample at the surface of artificial pond and compressed(dehydrating) machine respectively. All samples were showed gram positive reaction.

Table 1: Morphological characterization of isolates obtained from Zebidar beer factory in Gubree, Ethiopia.

Isolate	Colony characterization				Gram reaction	
	Colony color	Colony form	Colony elevation	Colony margin	Gram reaction	Cell arrangement
SL-E1	White	Regular	Flat	Ciliate	Positive	Large Rod
SL-E2	White	Regular	Flat	Ciliate	Positive	Large Rod
SL-S1	cream	Irregular	Flat	Branching	Positive	Short Rod
SL-S2	White	Regular	Flat	Branching	Positive	Short Rod
SL-M1	cream	Irregular	Flat	Ciliate	Positive	Large Rod
SL-M2	White	Regular	Flat	Undulate	Positive	Large Rod



Figure 2: Colony of Bacillus species

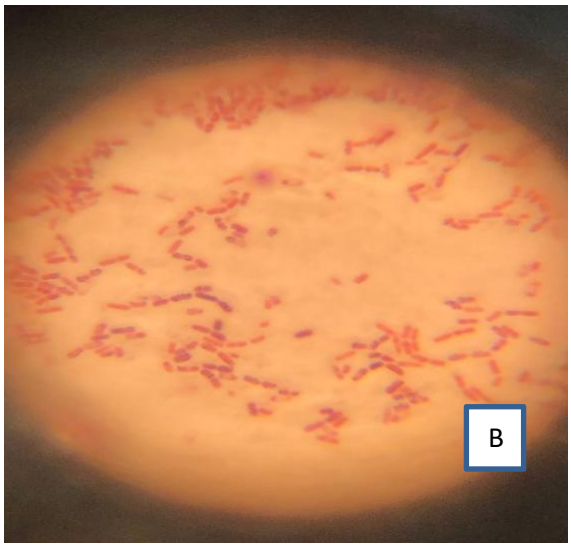


Figure 3: Gram reaction result (+Ve)

The pure colony was formed by primary screening by subculturing initially isolated bacteria. In figure 4 was showed the single colony of bacteria but in figure 5 was showed the clear zone of bacteria that cultured on skim milk agar. This was the sign of protease producing bacillus species.



Figure 4: pure colony by subculture streak method



Figure 5: protease producing bacteria show clear zone on skim milk agar

4.2. Biochemical Test

The ability of the isolates to excrete extracellular enzymes was tested through hydrolysis of starch and the ability of the isolates to excrete intracellular enzymes was determined through tests on catalase reaction; citrate utilization, motility and triple sugar- iron test. The isolates differed greatly on their ability to excrete various enzymes (Table 2).

Table 2: Biochemical characterization of isolates obtained from Zebidar beer factory in Gubree sub city, Ethiopia.

Isolate	Tests						
	Mot	TSI	Cit	Cat	sta	MR	VP
SL-E1	+	K/A	+	+	+	+	+
SL-E2	+	K/A	+	+	+	+	+
SL-S1	+	K/A	+	+	+	+	+
SL-S2	+	K/A	+	+	+	+	+
SL-M1	+	K/A	_	_	_	+	_
SL-M2	+	K/A	_	_	_	+	_

Key: (+)positive.(-)negative, sta: starch, TSI: triple sugar iron, cat: catalase , mot: motility, Cit: simmon citrate, MR: methyl red test, VP: Voges Proskauer test , SL-E1: end sludge sample 1, SL-E2: end of sludge effluent sample 2, SL-S1: sludge sample at the surface 1, SL-S2: sludge sample at the surface 2, SL-M1: sludge sample at the machine 1,SL-M2:sludge sample at the machine 2 ,K: alkaline =no fermentation, A:acidic =sugar fermentation.

A) Simmon's Citrate Test

Growth on the slant and change in colour to blue of the medium indicates positive result and colour to green of the medium indicates negative results.Four samples were simmon`s citrate positive wheteas two samples of sludge at the machine were negative.

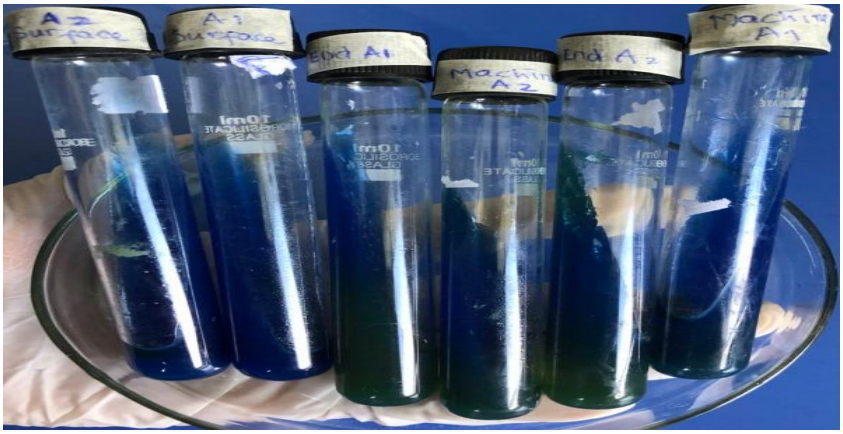


Figure 6: simmon`s citrate after incubation

B) Triple sugar iron (TSI) agar test

TSI agar was used for the presumptive identification of based on their fermentation of glucose patterns. This specific result (yellow butt/red slant) on TSI agar was suggested that the bacteria fermented only glucose. It served as a crucial indicator for further identification tests to pinpoint the specific bacterial species within the family. All test isolates showed result for triple sugar iron (TSI) test as there were bacteria fermented only glucose there were formation.all samples were TSI positive.



Figure 7: TSI after incubation

C) Methyl red (MR) test

The test isolates were confirmed a positive result, with the color of the culture medium changing from bright yellow to red, while negative result no color changed.all samples were positive.



Figure 8: MR test

D) Vogues proskauer (VP) Test:

The Voges-Proskauer (VP) test was performed on bacterial isolates were gave clear results. A reaction between alpha-naphthol and potassium hydroxide produced a red color for isolates, confirming a positive VP test. Four samples were positive where as two samples were negative that was sludge sample at the machine.



Figure 9: VP test

E) **Starch hydrolysis**

As indicated on the Figure 10 below, that isolated from sludge sample at the end accumulation was showed positive result starch hydrolysis While the other isolated from sludge sample from compressed machine (dehydrating machine) showed negative test indicating non- hydrolyzed regions exhibited by blue-black zone around the colonies for starch hydrolysis negative isolates (A. Shyam, S. S. Sonia, and G. Lal, 2013).



Figure 10: starch hydrolysis test

F) Catalase Test

Catalase test isolates were produced bubbles, some isolates were showed negative results but isolates were confirmed to be catalase positive result by formed bubble. Four samples were catalase positive because form bubble during the reaction.other sample especially sludge sample at the machine was catalase negative. It was shown below the figure 11.



Figure 11: catalase test

5. DISCUSSION

Proteolytic bacteria are wide spread in nature and able to isolate them from any ecological entities like soil, water, leaves, marine environment and waste and wastewater (Temam, 2018). So, the current study was dealing with isolation and characterization of protease producing bacteria from soil through morphological and biochemical tests according to the method described in Bergey's Manual of Determinative Bacteriology (Buchana *et al.,* 1974).

The morphological features of *Bacillus* species are diverse such as being large, dry, flat, or sticky, characteristics typical for the *B. cereus-B. Thuringiensis* group and based on the colony morphology those organisms classified into four, A (white, round, flat, and with wavy/scalloped edged margin), B (white, round, slightly raised center, and with fried egg appearance), C (white, shiny, round, little raised Centre and with irregular but entire margin), and D (white, round, mucoid, slightly raised Centre, and spread out with irregular spike-like margin (El-kersh*et al.,*2012). This is in agreement with the result of the current study indicated that the morphological features of *Bacillus* species were different in colony color, form, elevation and margin. The gram reaction of *Bacillus* spp are gram positive as indicated by different studies (Vinod and More, 2013). This is similar with the present study in which the gram reaction indicated that some of the isolated strains were gram positive short rod whereas others were gram positive long rod. The biochemical tests were positive for citrate, sucrose, starch, catalase (Padama*et al.,*2015). Triple sugar iron (TSI) agar was was inoculated with an unidentified culture and displays a red slant and a yellow butt, which indicates that glucose was fermented with acid production. Based upon the differences in fermentation products, these two groups of enteric can be differentiated by the Methyl Red-Voges Proskauer (MR-VP) test. Methyl red is a pH indicator. In the presence of highly acidic conditions, as generated by mixed acid fermenters and as the pH rises, i.e., becomes alkaline, methyl red turns yellow.

When methyl red was added to a culture of a mixed-acid fermenter previously grown in MR-VP broth, the culture appeared red, indicating a positive MR test. Conversely, a non-mixed acid fermenter grown in MR-VP broth would have turned the dye yellow, resulting in a negative MR testg the Voges-Proskauer (VP) test. In this test, alpha-napthol and potassium hydroxide was added to a culture of a grown in MR-VP broth. The potassium hydroxide causes acetoin to form diacetyl, which in the presence of, alpha-napthol forms a red complex. The latter, is indicative of a positive VP reaction. The absence of a red colour is considered a negative VP result. The proteolytic activities of *Bacillus* spp vary in different pH and temperature. One of the reports indicated that the expected optimum temperature for the production of protease has been reported between 30-70^{o}C (Sevine*et al.,* 2011). This is parallel with the present study in which the expected best proteolytic activity was obtained at 37^{o}C.

Whereas others reported that the optimum temperature for protease production from *Bacillus* species was at 60^{o}C (Muhammad *et al.,* 2013). This disagrees with the current study in which there were no any significant bacterial growth at 60^{o}C.pH is another factor which affect the proteolytic activity. Other investigators recorded optimum proteolytic activity at different pH values such as pH 7.8 (Tsujibo*et al.,* 1990), 8.0 (Anwar and Saleemuddin, 1997), 9.8-10.2 (Kumar *et al.,* 1999) and 12-13 (Takami*et al.,* 1989). The highest proteolytic activity on blood spot was expected to obtained at optimum temperature of 37^{0}C and at pH of 9.

6. CONCLUSION

Protease producing bacteria is still explored to achieve high quality industrial grade enzyme to produce goods. Although many strains of protease enzymes are on market for enzyme production researchers are looking for more efficient and effective because they could be alternative for commercial use. The morphological features of *Bacillus* species are diverse such as being large, dry, flat, or sticky, characteristics typical for the *B. cereus-B. Thuringiensis* group and based on the colony morphology was showed in each isolated sample. Proteolytic activity was measured for enzyme producing *Bacillus species* in which the protease enzyme production was isolated from sludge wastes. which were grown in acidic, neutral and alkaline medium at temperature of 30, 37 and 65^0c. However, the production was highly observed in SL-E1 and SL-S1 grown in alkaline medium at temperature of 37^0c high protease producing bacillus species were isolated by showing of clear zone of hydrolysis. Proteases have great industrial and commercial applications including food, detergent, leather and pharmaceutical industries (Sikander *et al.,* 2017). However, the supply of this enzyme is low and the cost of procurement by developing countries can be even higher, hence the new isolated protease producing *Bacillus* species from the local soil sample could be alternative for industrial and commercial use. From others isolated sample the surface of the pond and at the end of effluent was better performance for production of protease enzyme. Such inherent stable protease is very helpful during various applications because they don`t need any modification to be stabilized when used as an additive to some commercial detergents .it showed prominent compatibility and destaining performance. In addition, it was able to hydrolyze waste x ray films. Efficiently releasing silver and could allow the reuse of the bedding polyester materials. Furthermore, it showed potent dehairing activity of animal skin with a short time collectively. The present investigation highlights the possible use of the alkaline protease from the local isolate bacillus species.

7. LIMITATION

During the laboratory session time our research was not finished. Because of unavailability of basic chemicals like casein broth agar, due to the lack of this liquid media this study was not made head way, this liquid media was used to culture secondary screening of protease production. By these constraints our study was not fulfilled. Then our results were unfulfilled like optimization of enzyme, Protease production, enzyme purification, analyze the effect of temperature and P^H but the expected result was producing protease by given procedure and that produced protease enzyme will be checked the performance by washing the white blood rinsed cotton clothes. Other constraints were the lack of Spectro-photometer microscope.by this limitation our research was not accomplished.

8. RECOMMENDATIONS

Depend on the results obtained from this study the following recommendations should be considered for future studies:

- The isolated types of microbes need to be fully characterized using molecular techniques. It is important to use shaking incubator for better protease production.
- Protease from *Bacillus* spp is performing better at temperature of 37^0c and at pH of 9. The further investigation should be studying to purified crude protease enzyme.
- Further study showed the morphological characterization and some biochemical test. After that further optimization and characterization of protease should be studied.

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