

Isolation and Characterization of Industrially Important Alkaline Protease Producing Bacteria from Soil of Sludge disposal site and Sediment around Adama City, Ethiopia

By: Yeshaneh Adimasu Lemenh



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

School of Applied Natural Sciences

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

June, 2018

**Isolation and Characterization of Industrially Important Alkaline
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APPROVAL SHEET

This is to certify that the thesis prepared by Yeshaneh Adimasu Lemenh entitled as "Isolation and Characterization of Industrially Important Alkaline Protease Producing Bacteria from Soil of Sludge disposal site and Sediment around Adama City, Ethiopia" submitted in partial fulfillment of the requirement for the master of degree of science in Applied Biology (Applied Microbiology) complies with the regulation of the university and meets the accepted standards with respect to originality.

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DEDICATION

This work is dedicated to my beloved parents Adimasu Lemenh and Tigared Adimasu

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LIST OF ABBREVIATIONS

ABIS	Advanced Bacterial Identification Software
API	Analytical Profile Index
EDTA	Ethylene diamine tetraacetic acid
GPS	Global Position System
H ₂ O ₂	Hydrogen peroxide
H ₂ S	Hydrogen Sulfide
Kg	Kilogram
mL	Milliliter
pH	Potential of Hydrogen
SIM	Sulfur Indole Motility
SmF	Submerged Fermentation
SSF	Solid State Fermentation
TSIA	Triple Sugar Iron Agar

LIST OF ACRONYMS

APBIS	Alkaline Protease producing Bacteria Isolated from Sludge disposal site
APBIT	Alkaline Protease producing Bacteria Isolated from Tannery sediment soil
ASTU	Adama Science and Technology University
MTSC	Modjo Tannery Share Company
OPHRCBQAL	Oromia Public Health Research Capacity Building and Quality Assurance Laboratory
rpm	revolution per minute

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Abstract

There are various microorganisms which are capable of producing the alkaline protease enzyme. Among them, bacterial proteases are most important when compared with plants and animals proteases due to their short generation time, cultivate in small space and easy for genetically manipulated to generate new enzymes. Bacterial proteases are the most important enzymes used in laundry, food, leather, and silk industry due to high production capacity and catalytic activity. The aim of this study was to isolate and characterize industrially important alkaline protease producing bacteria from soil of sludge disposal site and sediment around Adama City, Ethiopia. Five hundred gram of each soil samples were collected separately from sludge disposal sites at Adama Science and Technology University waste water treatment plant site and sediment soil at Modjo Tannery Share Company. One gram of soil sample was taken and carried out with serial dilution techniques and spread plate method using alkaline media (Skim milk agar). A total of 16 bacteria species were isolated the soil and screened for proteolytic activity on skim milk agar based on the zone of proteolysis. Four bacterial isolates showed high proteolytic activity were selected for enzymatic study. The isolates were identified by biochemical characterization using bioMerieux API kits and ABIS online software. The results indicated that the isolated bacteria were E.coli (99.69%), Pseudomonas putrefaciens (Shewanella putrefaciens) (91.61%), Bacillus carboniphilus (92.78%), and Lysinibacillus sphaericus (98.4%). Bacillus spp. were the dominant source for alkaline protease production. The crude alkaline protease enzyme produced by different bacterial isolates could promising for the application in the dehairing activity and destaining as detergent additives of which Bacillus carboniphilus was shown best activity. Based on this finding Bacillus carboniphilus was selected as most potent protease producing bacteria for both dehairing activity and destaining ability.

Keywords: dehairing, destaining, hydrolysis, protease, sediment,

1. INTRODUCTION

1.1. Background of the study

Microorganisms are being used for thousands of years to supply fermented products, Such as enzymes, amino acids, nucleotides, vitamins, organic acids, solvents, vaccines and polysaccharides (Gupta *et al.*, 2002). Proteases have a pivotal role in application areas ranging from domestic to leather processing, food, chemical, environmental pollution abatement to nutraceuticals applications, healthcare product to diagnostic kit development and value-added product production to clinical applications (Rao *et al.*, 2009). Alkalophilic enzymes are stable and active at high pH and temperature hence their application in processes could increase efficiency and reduce production cost. Studies relating to such properties are imperative and the biochemical parameters should determine the potential application in industries and medical therapeutics fields (Jisha *et al.*, 2013).

Microbes are preferred source of enzymes than plants and animals, due to their short generation time, cultivate in small space and easy for genetically manipulated to generate new enzymes that are desirable for various applications (Kocher and Mishra, 2009). Alkaline protease have attracted worldwide attention in attempts to achieve their physiological and biotechnological applications due to diversity and the specificity of action, then isolation and characterization of new bacteria strains are best ways to increase the diversity and yield of such enzymes. Even if the same species of alkaline protease producing microorganisms especially bacteria are isolated from different environments but their effectiveness are completely different. High amounts of protein wastes from plants and animals are released annually, one such protein waste is keratin that released from leather tanning industries in the form of hair. Over 90% of keratins are protein, and are highly crystalline and cannot digested by most enzymes. But some microorganisms can hydrolyzing keratin (Gupta *et al.*, 2002). Therefore, bioprospecting for new microorganisms that can be used for alkaline protease production is necessary continuous process for industrial development and environmental conservation. Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. Nonspecific reactions may result in poor product yields. High temperatures and/or high pressures needed to drive reactions lead to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous

processes involving high temperatures, pressures, acidity, or alkalinity need high capital investment, and specially designed equipment and control systems. Unwanted by-products may prove difficult or costly to dispose of. High chemicals and energy consumption as well as harmful by-products have a negative impact on the environment. In a number of cases, some or all of these drawbacks can be effectively eliminated by using enzymes. Industrial enzymes originate from biological systems (bacteria); they contribute to sustainable development through being isolated from microorganisms which are fermented using primarily renewable resources. In addition, as only small amounts of enzymes are needed in order to carry out chemical reactions even on an industrial scale, both solid and liquid enzyme preparations take up very little storage space.

In 20th century has observed remarkable growth and expansion in industrial output which provided jobs and income, goods and services, and opportunities to improve the standard of living for millions of people in many countries. Mostly, Africa participate in the global economy is largely confined to supplying raw materials. Adding value to these raw materials to have enormous economic contribution. However, from developed countries known that waste generated from industrial activities result in pollution of air, water, soil, and emit massive amounts of greenhouse gases which are responsible for climate change. To reduce further deterioration of the global environment it is important to reduce the amount of waste and pollution generated through industrial activities. Developing enzyme technology has offered an alternative approach for pollution reduction from many industrial sectors and increase production efficiency as well as product quality (Amare G. *et al.*, 2011).

1.2. Statement of the problem

Ethiopia is on the track of expanding various industries, like textile, leather, detergent, pulp and paper, food and dietary industry. Most of these industries are highly associated with chemical aids which has environmental pollution; in other way the use of enzymes or microorganisms for industrial processes has offered a viable option to decrease or avoid environmental pollution and leads to improve the product quality or process efficiency. Most industrial microorganisms are patented and may not be available (or must be purchased) for use outside their country of origin. There is a huge demand for alkaline proteases enzymes for use in leather tanning, textile, and brewing industries. Thus, the existing market for bating

enzymes in the East African region (Ethiopia, Tanzania) is estimated to be about US\$ 3.8 million per annum (Amare G *et al.*, 2011). This is a serious economic concern as it does not allow for rapid expansion of fermentation industries. So it indicates that there is the gap for production of enzyme especially alkaline protease, hence it need to isolate suitable microorganisms for sustainable industrial important alkaline protease production. Production of alkaline protease enzymes to locally is expected to have significant contribution for lowering environmental pollution; replacing several imported chemicals as processing aids, and could encourage local industries to adopt enzyme technology.

1.3. Significance of the study

Production of alkaline protease enzymes from bacteria have several advantages to leathers, detergents, and food and textile industries. The availability of alkaline protease enzymes could encourage local industries to adopt enzyme technology and release less/no waste which could help to reduce or avoid environmental pollution. And also play an important role in laying the foundation for the development of industrial biotechnology in the country. This is important when one considers the fact that Ethiopia is still in the process of growing its industrial manufacturing sector.

1.4. Objectives

1.4.1. General objective

The general objective of this research was to isolate efective alkaline protease producing bacteria from soil of sediment and sludge disposal site around Adama City, Ethiopia.

1.4.2. Specific objectives

The specific objectives of the study were to:-

- ✓ Isolate alkaline protease producing bacteria from soil of sediment and sludge disposal site.
- ✓ Characterize the isolated bacteria by morphological and biochemical tests using commercial kits (bioMerieux API kits).
- ✓ Evaluate dehairing and destaining potential of crude enzymes extracted from bacteria culture.

2. LITERATURE REVIEW

2.1. Enzyme

All known enzymes are proteins that catalyze or speed up a chemical reaction. The basic characteristics of enzymes are sensitive and labile to heat, water soluble, and enzymes could be precipitated by protein precipitating agents such as ammonium sulfate and trichloroacetic acid. Enzymes occur in all microorganisms, which bring specific biochemical reactions generally forming parts of the metabolic processes of the cells. Enzymes are highly specific in their mode of action on substrates and required to bring action in living cell. Organisms produce enzymes inside their cells called intracellular enzymes and may also produce enzymes that are secreted into the medium and function outside the cells called extracellular enzymes. Isolation of intracellular enzymes is difficult when compared to extracellular enzymes. Cells need to be broken for the release of intracellular enzymes and is not required for extracellular enzymes. Extracellular enzymes are more stable than intracellular enzymes. Separation from cell debris, other enzymes and unwanted chemicals is needed in the case of intracellular enzymes and not needed for extracellular enzymes. Purification procedures and downstream processing is difficult and expensive for intracellular enzymes when compared to extracellular enzymes (Kumar and Hiroshi, 2002).

Enzymes speed up a chemical reaction by decrease activation energy of a reaction, like any catalyst, enzyme remains unaltered by the completed reaction and can therefore continue to function. Because enzymes, do not affect the relative energy between the products and reactants, they do not affect equilibrium of a reaction (Karthik *et al.*, 2011). Although all enzymes have a biological role, some enzymes are used commercially for other purposes. Enzymes have various advantages in industrial processes: They are of natural origin and nontoxic, are highly specific, active at relatively low concentrations, and the rate of reaction could controlled by adjusting temperature, pH, and amount of enzyme employed, easily inactivated when reaction completed as far as desired. Because of these inherent advantages, many industries are intensely interested in adapting enzymatic methods to the requirements of their processes. Examples of some applications under intensive investigation of different sectors include food processing, textile, leather, pulp and paper, fruit juice and beverages etc. Nowadays advances in microbiology, isolation, identification and industrial exploitation of

extremophiles i.e. microbes adapted to exist and grow at extreme environments, are getting considerable attention (Viikari *et al.*, 2007).

2.2. Alkaline protease enzyme

Proteases are enzymes having various application in both physiological and commercial fields. Proteases are hydrolyase enzymes which catalyze and break peptide bonds of proteins. Since enzymes are specific, proteases are also highly specific and selective towards a chemical reaction (Rao *et al.*, 1998). Proteases have major role in food, detergent and leather industries (for dehairing and bating of hides) to substitute currently than chemicals methods which is a relatively new development in biotechnological application. Protein have high molecular weight made up of chains of amino acids linked together by peptide bonds as describe (Figure-1) bellow.

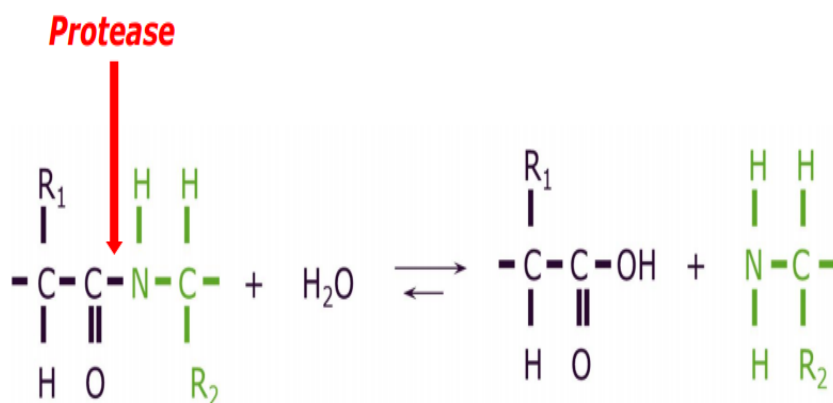


Figure 1. Chemical nature of protein structure

Alkaline protease enzymes are active at high temperature, these properties decrease the possibility of microbial contamination for prolonged duration in large scale industrial production (Wang *et al.*, 2012). The quality of thermo-stability in enzymes promotes the breakdown and digestion of raw materials and enhances the penetration of enzyme, mass-transfer and reduction of substrate viscosity during hydrolysis of substrates or raw materials in industrial processes (Berka *et al.*, 2011). Proteases are the most important industrial enzymes which accounts for about greater than 46% of which greater than 25% are alkaline proteases (Figure-2) and that execute a wide variety of functions and have various important biotechnological applications (Mohen *et al.*, 2005). Two thirds of the total enzymes used in

various industries are protease and it account for at least a quarter of the total global enzyme production (Kumar and Hiroshi, 2002). For industrial applications, alkaline proteases must possess activity and stability under relatively hostile conditions, often comprising extremes in temperature, pH and presence of inhibitors and oxidizing agents. Thus, candidate enzymes must have both process suitability and prolonged stability if intended for industrial applications. The ultimate aim of any research activity is to find out an enzyme which is robust in nature and versatile in applications (Akel *et al.*, 2009). Alkaline proteases are useful for detergent and are mostly active at pH range 8 - 12 and temperatures of 40°C - 70°C (Alshehri *et al.*, 2004). The other important feature of alkaline proteases is their ability to discriminate among competing substrates and the utility of these enzymes often depends on their substrate specificity. Proteases can hydrolyze natural substrates like casein, bovine serum albumin, keratin, and collagen as well as on synthetic substrates like N-benzoyl-L-tyrosine ethyl ester, N-benzoyl-L-arginine ethyl ester, N-benzoyl-D Larginine- p-nitroanilide, etc...(Shankar *et al.*, 2011).

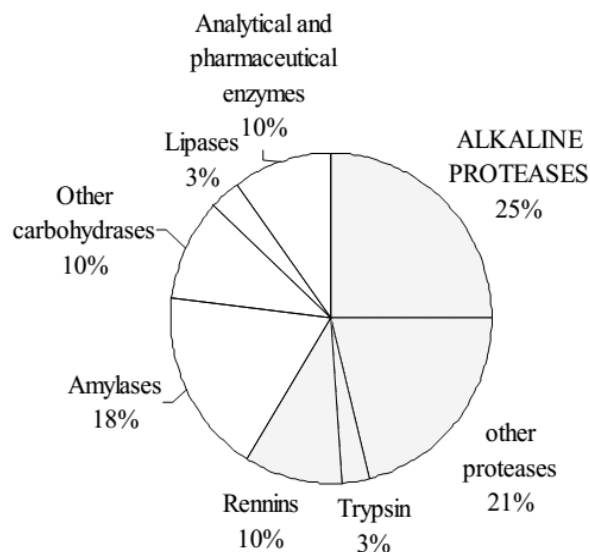


Figure 2. The contribution of different enzymes to the total sale of enzymes (Rao *et al.*, 1998).

2.3. Sources of protease enzymes

Globally protease enzymes are used by various industries worldwide in large amount. It found in a wide range of sources such as animals, plants and microorganisms and has physiological role in all living organisms.

2.3.1. Animal proteases

Some of the animal's origin of protease are chymotrypsin, pepsin, pancreatic trypsin, and rennin. The production of protease depend on the availability of livestock for slaughter, governed by political and agricultural policies.

Chymotrypsin: It is found in the pancreatic extract of animals. It cleaves peptides at the carboxyl group of tyrosine, tryptophan and phenylalanine. Pure chymotrypsin is used in analytical and diagnostic field and deallergenizing of milk protein hydrolysates (Rao *et al.*, 1998).

Pepsin: It is a digestive protease released by the chief cell in the stomach of almost all vertebrates that function to degrade food proteins into peptides. Pepsin functions best in acidic environments between pH 1- 2 and is inactivated above pH 6. Pepsin cleaves preferentially after the N-terminal of aromatic amino acids such as phenylalanine and tyrosine (Adinarayana and Ellaiah, 2002).

Trypsin: It is a serine protease found in the digestive system and is responsible for the breakdown of food proteins. Trypsin has an optimal operating pH and temperature of about 8 and 37°C respectively and predominantly cleaves proteins at the carboxyl side of the lysine and arginine. Trypsin is used for the preparation of bacterial media, to dissolve blood clots, treat inflammation and to dissociate dissected cells. These protease have the ability to inhibit the enzyme from the insect gut, trypsin is used for biocontrol of insect pests (Rao *et al.*, 1998).

2.3.2. Plant proteases

The problem associated with the production of plant proteases lies in the selection of suitable climatic areas for cultivation. As the concentration of enzyme in plant tissue is generally low processing of large amounts of plant material is necessary (Schechler and Berger, 1967). Papain, bromelain, keratinases, and ficin are some of the well-known plant origin proteases. However, their production from plant sources is a time consuming process.

Bromelain: Bromelain is a crude extract from the pineapple (*Ananas comosus*) plant. It is a mixture of sulfur-containing proteases. It is active between pH 5- 9 and stable up to 70°C beyond which it is inactivated. It is used as meat tenderizer, anti-inflammatory agent and in debridement (Secor *et al.*, 2005).

Papain: It is a cysteine protease extracted from latex of papaya (*Carica papaya*). The crude enzyme has broad specificity due to mixture of several proteases. The enzyme is active between pH 5 - 9 and is stable up to 50°C - 60°C in presence of substrates. It is extensively used in tenderization of the meat (to break down the tough meat fibers), preparation of highly soluble and flavoured protein hydrolysates (Kim *et al.*, 2004).

2.3.3. Microbial proteases

Microorganisms are an important source of alkaline proteases enzyme, they obtained in large quantities using cultural techniques within a short period of time and produce a regular and abundant supply of the desired product. Furthermore, microbial proteins have a longer shelf life and can be stored under less than ideal conditions for weeks without significant loss of activity (Gupta *et al.*, 2002b). Microbial proteases are extracellular in nature and directly express in the fermentation medium. The producers of these enzymes for commercial exploitation are non-toxic, nonpathogenic and designated as safe (Gupta *et al.*, 2002b). Microorganisms can degrade polysaccharide molecules and utilize the degraded products as nutrients for their survival. Proteases are produced by various microorganisms and depending on the species of bacteria, even belonging to the same species their effectiveness are different. For instance neutral and alkaline proteases hold great potential for application in the detergent and leather tanning industries due to the increasing trend in developing environment friendly technologies (Rao *et al.*, 1998).

Enzymes were discovered from microorganisms in the 20th century and their application in bio-industry have continuously progressed, and the knowledge has regularly updated to isolate and characterize the producer microorganism. Three forth (3/4) of commercial protease in the enzyme market across the world are microbe's origin (Kumar and Takagi, 1999). Microbial enzymes are well known enzymes obtained from different microorganisms. The producer microorganisms globally for the bio-synthesis of various enzymes for commercial applications are bacteria, fungi and yeasts (Pandey *et al.*, 1999). Most of the commercially industrially applicable proteases are alkaline protease which is bio-synthesized mainly by bacteria and some fungi (Kumar *et al.*, 2012).

2.3.3.1. Fungal proteases

Fungal proteases are active over a wide pH range (pH 4- 9). Fungal keratinases obtained from *Aspergillus flavus*, *A. fumigates*, *A. niger*, *A. oryzae*, *A. parasiticus*, *Fusarium oxysporum*, *Graphium putredinis*, *Penicilium camemberti*, *P. citrinum* and *Trichoderma harzianum* have also been studied towards the degradation of keratin (Ellaiah *et al.*, 2002; Kumar and Takagi, 1999 and Gupta, 2002). The advantages of fungal proteases are conveniently produced by solid-state fermentation. It offers an advantage of separation of mycelium by simple filtration. Fungal proteases are also used in for modifying food proteins but the enzymes are less tolerant to heat (Ahmed *et al.*, 2009).

2.3.3.2. Bacterial proteases

Bacterial proteases are the most important enzymes used in laundry, food, leather, and silk industry due to high production capacity and catalytic activity. Most of the commercial proteases are of bacterial origin (Prakasham *et al.*, 2006). Alkalophilic bacteria are known to produce alkaline proteases of which genus *Bacillus* being the most prominent source for alkaline protease production. The most potential alkaline protease producing *Bacillus* species are *Bacillus cereus*, *B. licheniformis*, *B. subtilis*, *B. stercorophilus*, *B. mojavensis*, *B. megaterium*, *B. brevis*, *B. thuringiensis*, *B. circulans*, *B. coagulans*, *B. marmarensis*, *B. firmus*, *B. alcalophilus*, *B. amyloliquifaciens*, *B. subtilis* and *Bacillus pumilus* (Kumar and Takagi 1999; Gupta 2002; Asha and Palaniswamy, 2017; Mohammed *et al.*, 2016 and Preeti *et al.*, 2016). Another bacterial source known as a potential producer is *Pseudomonas* sp. as reported by (Bayoudh *et al.*, 2000; Gupta *et al.*, 2002b) and *Enterobacteriaceae* sp. (Hamid *et al.*, 2003). Both alkaline proteases and neutral proteases are used in laundry and leather industries but neutral proteases have lower thermo-tolerance.

2.4. Distribution of alkaline protease producing microorganism

Alkaline proteases are essential enzymes hence abundant in nature. They are isolated from various sources by surface plating on an alkaline medium and subsequent screening for the desired characteristics. Natural habitats (dumping site, Soda Lake, and hot springs) have been explored for isolation of protease producing microorganisms (Gupta 2002). Soil samples from leather factories, detergent industry, wood factory, and industrial waste such as tannery waste, food processing industrial effluent have also suitable environment. Protease producers have

been isolated also from soil samples collected at disposal wastes from university (campus), sugarcane molasses, and vegetable waste. Other sources used for isolation of alkaline protease producer include mangrove sediment sample, compost containing dead animal's remnants, sewage sludge sample, contaminated soil, and soil from poultry waste site (Kumar *et al.*, 2014).

2.5. Classification of proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in hydrolases (subgroup 4 of group 3). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria: (I) type of reaction catalyzed, (II) chemical nature of the catalytic site, and (III) evolutionary relationship with reference to structure. On the basis of site of action on protein substrates, proteases are broadly classified as exopeptidases and endopeptidases enzymes (Barrett 1994).

2.5.1. Exopeptidases enzymes

Exopeptidases catalyze the hydrolysis of the peptide bonds near the amino (*N*) or carboxyl (*C*) terminal ends of the substrate. Exopeptidases are further classified into amino peptidases and carboxypeptidases proteases which act at the free N-terminus of the polypeptide substrate and free C-terminal of the polypeptide chain respectively.

Amino-peptidases: Aminopeptidases act on free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi. In general, aminopeptidases are intracellular enzymes, but there has been a single report on an extracellular aminopeptidase produced by *A. oryzae* (Barrett 1994). The substrate specificities of the enzymes from bacteria and fungi are distinctly different in that the organisms can be differentiated on the basis of the profiles of the products of hydrolysis.

Carboxypeptidases: Carboxypeptidases act on C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. The Action of exopeptidases on a polypeptide substrate are shown (figure-3) bellow.

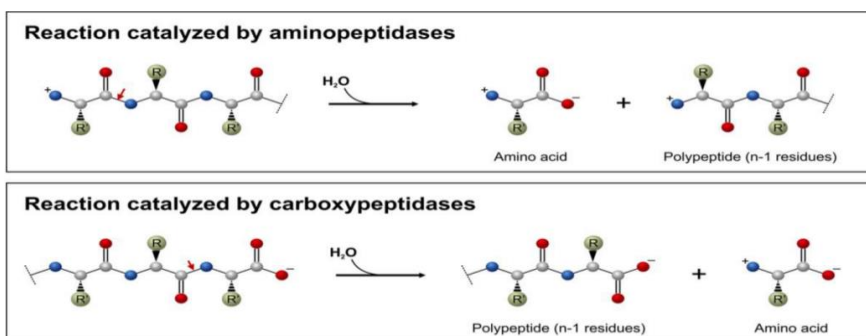


Figure 3 Reaction catalyzed by exopeptidases (Barrett 1994).

Note: The arrow show the peptide bonds to be cleaved.

2.5.2. Endopeptidases enzymes

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C terminal. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The Action of endopeptidases on polypeptide substrate are shown (Figure-4) below.

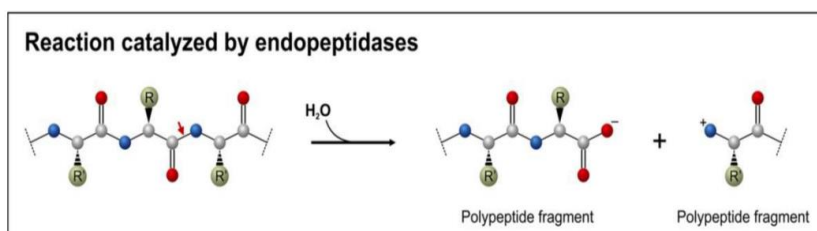


Figure 4. Reaction catalyzed by endopeptidases (Barrett 1994).

Note: The arrow show the peptide bonds to be cleaved.

Endoproteases are also classified based on their side chain specificity and functional group present in characteristic active site. Endopeptidases divide into four subgroups, (I) Serine proteases, (II) Aspartic proteases, (III) Cysteine proteases and (IV) Metalloproteases.

(I). **Serine proteases:** Serine proteases are proteases having a hydroxyl group (-OH) in their active site. Serine protease are the most widely distributed group of proteolytic enzyme of both microbial and animal origin. This are generally active at neutral and alkaline pH, with an optimum pH between 7-11. They have broad substrate specificity (Salvesen and Nagase, 1989). The reactive hydroxyl group are inhibited by diisopropyl fluorophosphates (DFP) and phenyl methyl sulphonyl fluoride (PMSF). The mechanism of action of serine for protein degradations are shown as (Figure 5) bellow.

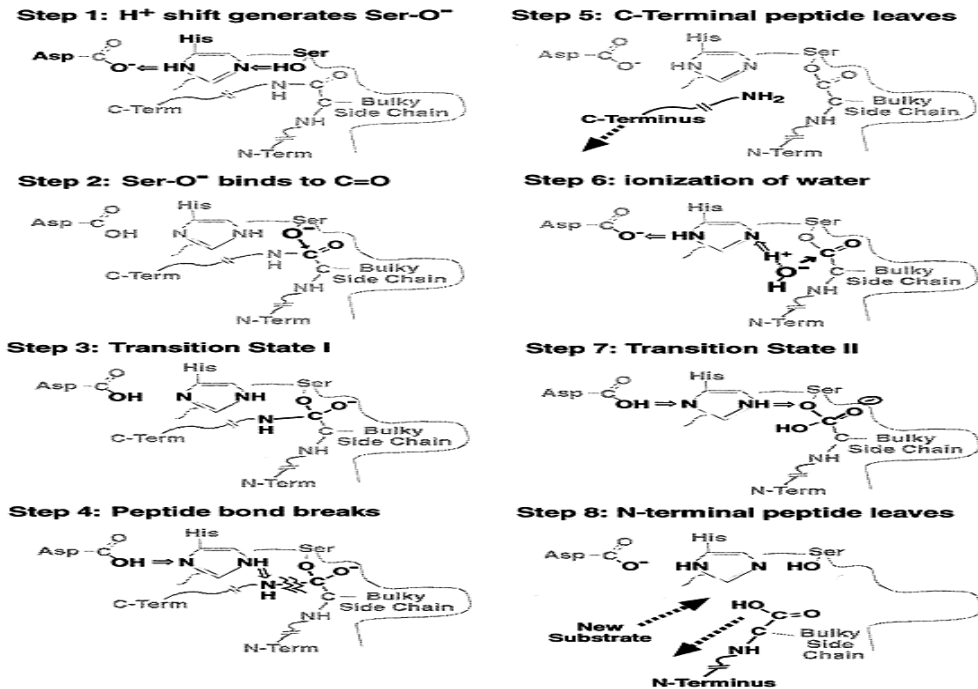


Figure 5: Enzymatic mechanism of protein hydrolysis by serine protease (Salvesen and Nagase, 1989).

(II). **Cysteine proteases:** The activity of these depends on the presence of an intact-SH group in their active site. Cysteine proteases are sensitive to sulfhydryl reagents, (Na-tosyl- L- lysine chloromethyl ketone) and activated by reducing agents (potassium cyanide or cysteine, dithiothreitol, and EDTA). The occurrence of cysteine proteases have been reported only a few fungi. Extracellular cysteine enzyme observed in *Microsporium* species, *Aspergillus oryzae*, and *Sporotrichum pulverulentum*. Most of these enzymes are active at pH 5-8 (Kalisz, 1988). The Enzymatic mechanism of protein hydrolysis by Cysteine proteases is shown as (Figure 6) bellow.

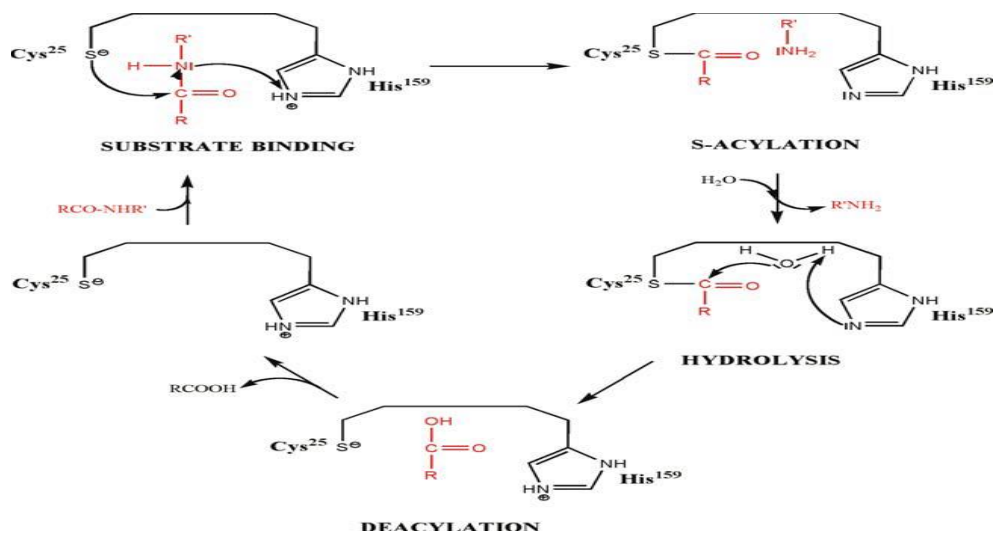


Figure 6 . Enzymatic mechanism of protein hydrolysis by cysteine proteases (Barrett 1994)

(III). **Metalloproteases:** These depend on the presence of more or less tightly bound divalent cations for their activity. These enzymes are sensitive to metal- chelating reagents (EDTA) but unaffected by serine protease inhibitor (sulfhydryl). EDTA inhibited enzymes can be activated by ions (zinc, calcium, and cobalt). These are widespread but only a few have reported in fungi. Most of the bacterial and fungi metalloprotease are zinc containing enzyme, with one atom of zinc per molecule of enzyme. The zinc atom are essential for enzyme activity. Calcium is required to stabilize the protein structure (Salvesen and Nagase, 1989). However, in some enzymes this function is undertaken by manganese, cobalt, nickel or even copper ions. In some metalloproteases two metal ions act co-catalytically. The metal ion is complexed by three conserved amino acid residues that can be His, Asp, Glu or Lys. The active site of protein hydrolysis by thermolysin amino acids is shown as (Figure 7) bellow.

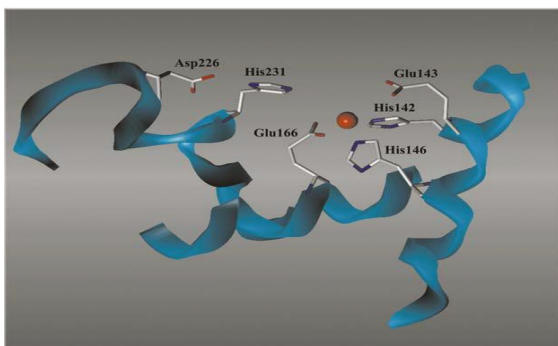


Figure 7. The active site of thermolysin amino acids involved in catalysis (Salvesen and Nagase, 1989).

The Enzymatic mechanism of protein hydrolysis by metalloproteases is shown as (Figure 8) below.

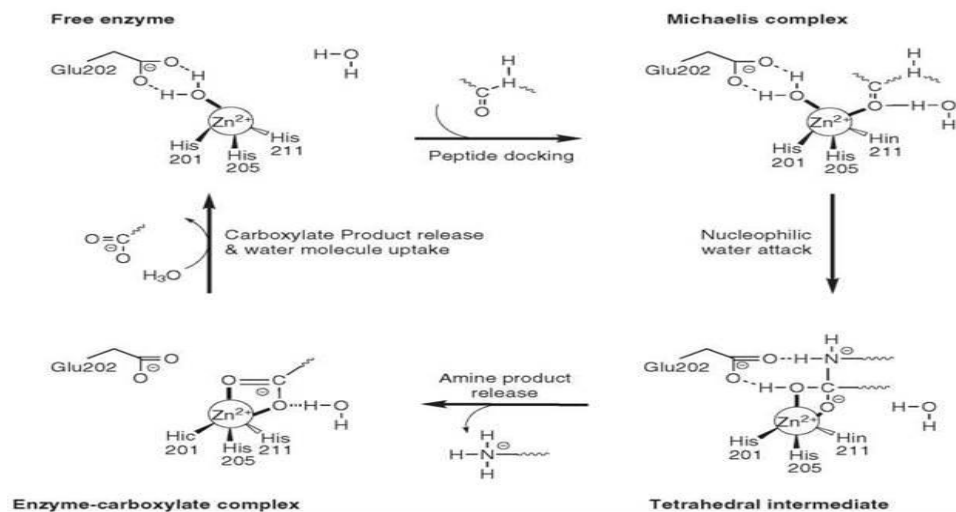


Figure 8. Enzymatic mechanism of protein hydrolysis by metalloproteases (Matthews, 1988)

The first mechanism to be proposed (Matthews, 1988) was based on the polarization of the zinc-bound water molecule by the glutamate residue and its subsequent nucleophilic attack on the carbonyl carbon atom of the scissile bond. A protonated His residue which is bound via a hydrogen bond to an Asp residue in the active site cleft is claimed to stabilize the transition state by forming a hydrogen bond to the carbonyl oxygen atom. The breakdown of the tetrahedral intermediate in the transition state is accomplished by protonation of the amide nitrogen of the scissile bond via glutamate residue.

(IV). **Aspartic proteases:** Acid proteases contain one or more side chain carboxyl groups in their active site. Commonly known as acid protease (Nduka, 2007). Aspartic proteases have maximum activity at low pH (3-4) and insensitivity to inhibitor of the other three group of enzymes (Kalisz, 1988). They are high distributed in fungi, but are rarely found in bacteria or protozoa. Most aspartic enzyme are sensitive to epoxy and diazo- ketone compounds in the presence of copper cations and also by pepstatin or Streptomyces pepsin inhibitor. The catalytic mechanism of aspartic proteases requires the initial binding of water molecule at the active site before nucleophilic attack on the substrate peptide bond. Most of the fungal aspartic protease are unstable above neutral pH and are not found in cultures growing at neutral or

alkaline pH (Kalisz, 1988). The Enzymatic mechanism of protein hydrolysis by Aspartic proteases is shown as (Figure 9) bellow.

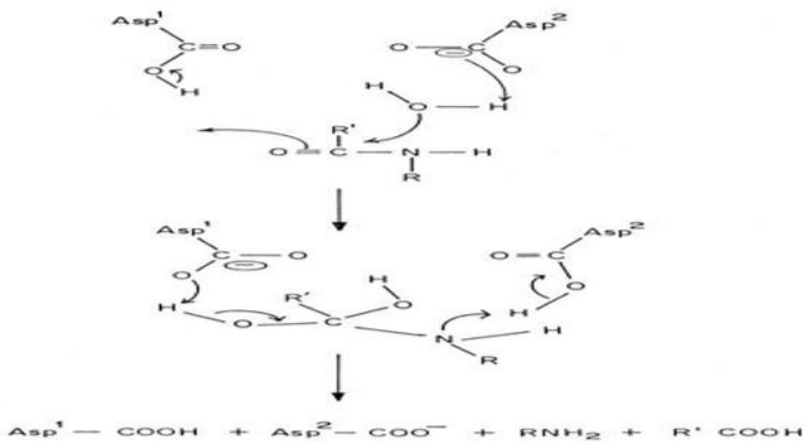


Figure 9. Mechanism of action of Aspartic proteases

Further endoproteases proteases are classified according to their maximal activity in a particular pH range as:

(I). **Acidic proteases.** Acid proteases are proteases which are active at pH ranges of 2-6

(II). **Neutral proteases.** Neutral proteases are proteases which are active at neutral, weakly alkaline or weakly acidic pH.

(III). **Alkaline proteases.** Alkaline protease can be defined as the protease those are active in alkaline range of pH 8- 12 (Rao and Narasu, 2007).

The overall classification of protease enzymes based on different criteria are generalized and shown as (figure 10) bellow.

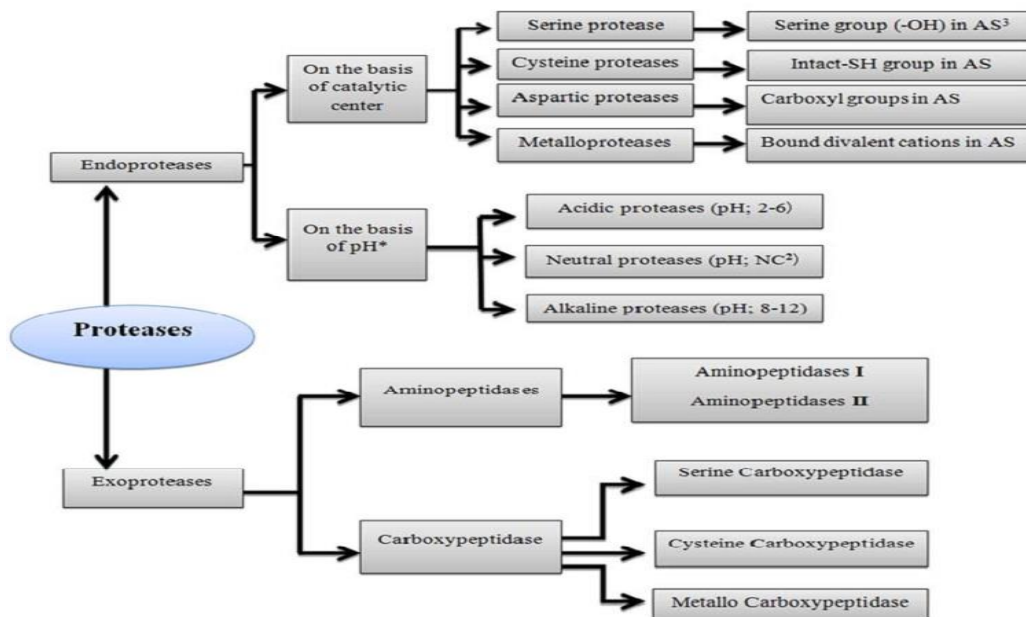


Figure 10. Diagram summarizing of classification of proteases (Nduka, 2007).

2.6. Production alkaline protease

There are two types of cultivation methods for all microbial enzymes: submerged fermentation (SmF) and solid state fermentation (SSF). Submerged fermentation involves the cultivation of microorganisms in high oxygen concentrated liquid nutrient medium. Viscosity of broth is the major problem associated with the fungal submerged fermentations. When fungal cells grow and a mycelium is produced, this hinders impeller action, due to this limitation occurring in oxygen and mass transfer. SSF has major advantages: high volumetric productivity, relatively higher concentration of the products, and less effluent generation, requirement for simple fermentation equipment. The exploration of protease producing bacteria are used to achieve high quality industrial grade enzyme to produce goods (Priya *et al.*, 2016). Production of protease from bacteria using cheap and readily available substrate like cow dung, wheat bran, groundnut shell, rice husk, cotton stalk, and wastes released from processing industries like dairy sludge and molasses were found to be promising industrial application and help to mitigate environmental pollution (Verma and Agarwa, 2016).

2.7. Application of alkaline proteases

Alkaline proteases from microbial systems are efficient under alkaline temperature and pH conditions and consist of a serine residue at their active site (Gupta *et al.*, 2002). Alkaline

proteases have shown their capability to work even in the presence of inhibitory compounds (Johnvesly *et al.*, 2001). Alkaline proteases are important group of enzyme used in physiological and commercial mostly in leather and detergent additives. They are also widely used in medical diagnostic, silk degumming, food and feed industries, etc. Several alkaline proteases from different sources are currently available in the market, from these almost all are products of microbial origin. Alkaline proteases are produced by both neutralophilic and alkalophilic microorganisms. Alkalophilic microorganisms are widely distributed in nature almost all environments without restriction of alkalinity (Pathak *et al.*, 2012). Some application of alkaline proteases are listed below as:-

2.7.1. Detergent industry

Alkaline proteases contribute for the development and improvement of modern household and industrial detergents. They are effective at high temperature and pH that characterize modern laundering conditions in industrial & institutional cleaning. Various enzymes used in laundry industry are proteases, lipase, Cellulases, amylases etc. (Ito *et al.*, 1998). Of these, alkaline protease find a major application as detergent additives because of their ability to hydrolyze and remove proteinacious stains like blood, egg, milk etc. in high pH conditions (Saeki *et al.*, 2007). Today detergent enzymes account about 89% of the total protease sales in the world. Alkaline proteases are produced from *Bacillus* species (*Bacillus cereus*, *Bacillus pumilus* strain CBS, *Bacillus licheniformis*, *Bacillus brevis*, *Bacillus subtilis* (Kwon *et al.*, 1994) for detergent industries.

2.7.2. Leather industry

Leather processing involves soaking, dehairing, bating, and tanning. The conventional methods of leather processing involve hazardous chemicals (sodium sulfide), which create pollution and effluent disposal. The use of enzymes as alternative to chemicals can successful improve leather quality and reduce environmental pollution. Proteases can selectively hydrolysis non-collagenous constituents of skin and non-fibrillary proteins (albumins and globulins). *Bacillus sp.* produced proteases with keratinolytic activity that replace sodium sulfide in dehairing process of leather industry. Alkaline proteases with keratinolytic activities are used for dehairing and bating process to obtain a desired grain, softness and tightness of leather in a short time (Giongo *et al.*, 2007). Traditionally, pancreatic enzyme containing

trypsin are used for bating which is expensive. Today, microbial proteases have replaced the use of trypsin. The quality of the finished leather mainly depends upon the bating efficiency (Deng *et al.*, 2010).

2.7.3. Textile industry

The commercial application of alkaline proteases in textile industry gives elegant texture (Dunaevsky, 2014). Alkaline proteases are used in removing gum and impurities present in the core protein fiber, especially in silk used worldwide as most precious fiber. Traditionally, degumming is performed using alkaline solution containing soap. This is a harsh treatment because the fiber itself is attacked, high energy and time consumption and also loss the luster of silk due to the amounts of water used in this process. The use of alkaline proteolytic enzymes are a better method because they remove the sericin without attacking the fiber. Tests with high concentrations of enzymes show that no fiber damage. Silk threads are stronger when it treated by alkaline protease enzyme than with traditional treatment (Gulrajani *et al.*, 2000).

2.7.4. Waste treatment

Alkaline protease are important for the treatments of waste from different industries, like food processing and domestic activities. These have ability to make proteins soluble into the wastes via several step process to recover the concentrates of liquids or the dry solids of great nutritional assessment for the fish or the livestock (Deng *et al.*, 2010). Likewise, various keratinolytic enzyme, alkaline proteases are utilized in food technology for making amino acids and peptides, degradation of waste keratinous material in the household and depilatory agent to eliminate hair in the bath tub drains that caused foul odors in public areas and houses. *Bacillus* species is the most widely reported bacterial source of keratinases for protein degradation. Other reported bacterial sources of keratinases are *Pseudomonas* sp. MS21, *Microbacterium* sp., *Chryseobacterium* sp. and *Streptomyces* sp. Fungal sources of keratinase are *Aspergillus Oryzae*, *Chrysosporium indicum*, *Microsporum* sp., *Trichophyton* sp., *Aspergillus terreus*, and *Fusarium oxysporum* for degradation of keratin protein (Ellaiah *et al.*, 2002).

2.7.5. Therapeutic application

Proteases are generally associated for the development of anticancer, anti-inflammatory, and antimicrobial. Proteases play an important role in upkeep of ordinary physiology and enzymes like “caspase” is principally required in degradation of unusual cells. Caspase is a proteolytic enzyme produced by natural means and important part of our immune system (Chiplonkar *et al.*, 1985). A number of proteolytic enzymes are being tested for “anti-cancer” treatment by therapy. Proteases obtained from many biological origins have become important key enzymes in formation of new generation anti-cancer drug molecules. Advantages of using enzymes in cancer treatment over chemotherapeutic agents are to reduce toxic effect (Białas and Kafarski, 2009).

2.7.6. Food industry

Proteases are used in food industry for the modification of proteins to improve nutritional value, solubility, digestibility, flavour, palatability and minimizing allergenic compounds (Tavano, 2013). The function of proteases in hydrolyze of protein gives high nutritional value which are used in infant food products, medicinal dietary products, fortification of fruit juice and soft drinks (Gupta, 2002). Lactic acid bacteria are used for milk coagulation and yogurt production. These bacteria are dependent on proteolytic enzymes which hydrolyze casein as nitrogen source for growth. 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, 2016). The enzymes hydrolyze complex protein molecule in gelatin hydrolysis, recovery of meat proteins, fruit juices and alcohol based liquors (Souza, 2015).

3. MATERIALS AND METHODS

3.1. Description of sampling area

Adama is a city in the central Ethiopia. It is located at 8.54 °N 39.27 °E at the elevation of 1712 m.a.s.l. 99 km southeast of Addis Ababa. Around Adama city leather tannery are found in Modjo as modjo tannery Share Company). Modjo is a town, named after the nearby Modjo River. Modjo is located in East Shewa Zone of Oromia region. It has a latitude (8° 35' 12.44" N) and longitude (39° 07' 16.00" E) and with an elevation between 1788 and 1825 meters above sea level. Other studying site is Adama Science and Technology University is one of the two Science and Technology University found in Adama, Ethiopia has Latitude 8°33'43.56" Longitude 39°17'23.28".

3.3. Sample collection

Five hundred gram of soil samples were collected from sludge disposal site of ASTU's waste water treatment plant located in the university campus and sediments from Modjo tannery Share Company with the aid of sterile spatula from 5cm depth into sterile plastic bags. The samples were labeled with site name, date of sample collection, and type of sample and transported to Oromia Public Health Research Capacity Building and Quality Assurance Laboratory (OPHRCBQAL) at bacteriology laboratory. The samples were air dried at room temperature for a week and maintained at 4°C for further analysis.

3.4. Isolation of protease producing bacteria

Isolation of bacteria were carried out with serial dilution techniques and spread plate method (Sneath *et al.*, 1986). One gram soil samples (sediment and sludge) were aseptically sub-sampled from 500g samples after thorough mixing and then transferred to 9mL distilled water and serially diluted up to 10⁻⁶ (i.e. 10⁻¹ - 10⁻⁶). Serially diluted aliquot (1 ml) was spread on skim milk agar. The plates were incubated at 37°C for 48 hours and zones of hydrolysis were recorded. The colonies showing highest zone of clearance were selected for further study and purified on nutrient agar plates repeatedly and preserve at room temperature.

3.5. Identification and characterization of protease producing bacteria

Identification and characterization of bacteria were carried out by macroscopic characterization, microscopic characterization, and biochemical tests as follows.

3.5.1. Cultural characterization

The bacteria culture grow on blood agar were characterized based on colony morphology like Colony type, shape, margin, and elevation.

3.5.2. Microscopic characterization

Microscopic examination was carried out from slide preparations and Gram staining methods to distinguish the isolated strains as Gram positive or Gram negative bacteria. Purified single colony from 24 hr. old culture on agar plate was taken with loop and put on the center of clean slide and made to smear. The smear was fixed on the slide with heat and stained with crystal violet for one minute. Then iodine was added, awaited for one minute, and 2 drop of alcohol was added as decolorizing agent. Finally, a counter stain, safranin was added and rinsed with water after one minute. The slide preparation was viewed under a microscope using oil emulsion objective 1000X magnification power. The results were recorded as purple color for Gram positive or pink color for Gram negative bacteria (Murray *et al.*, 2005).

3.5.3. Biochemical tests

The biochemical characterization of bacteria culture that grow on nutrient agar and/or nutrient broth were carried out using commercial kits (bioMerieux API kits) and manual methods. The preparation of bacteria suspension and setting up of API strips were done based on the procedure of Global Health Network Laboratory (Microbiology Standard Operating Procedure) as stated in (Appendix 6). The biochemical tests includes Catalase test, Oxidase test, Indole production test, Citrate utilization test, Carbohydrate fermentation, Urease test, H₂S production test and Nitrate reduction test.

3.5.3.1. Catalase test

Three drops of 3% of H₂O₂ solution was added on bacteria cultures grown on nutrient broth medium. The formation of bubble was indicated as positive reaction and no bubble formation considered as a negative reaction for catalase test Harley *et al.*, (2002).

3.5.3.2. Oxidase test

Oxidase test was conducted following the method described in Harley *et al.*, (2002). One drops of 1% p-amino dimethylaniline oxalate (Gaby and Hadley reagents) was flooded on bacteria culture on oxidase strip paper. The color was changed to deep purple-blue/blue colour within 30sec as positive results.

3.5.3.3. Urease test

Urea hydrolysis test was conducted according to the method described by Harley *et al.*, (2002) as follow. A loopful of bacterial culture was inoculated into a test tube containing urea broth and then incubated for 24hours at 37°C. The development of bright pink color was indicated as positive test for urea hydrolysis.

3.5.3.4. Citrate utilization test

Citrate utilization test was conducted according to the method described by Harley *et al.*, (2002). The isolated colony was streaked into the sodium citrate slant and incubate aerobically at 37°C for up to 24hr. A color change from green to blue along the slant was indicated as positive result.

3.5.3.5. Indole production test

According to the method described by Harley *et al.*, (2002). A loop full bacteria culture was inoculated in a test tubes containing 8 ml Sulfur Indole Motility (SIM) broth and incubated at 37°C for 24hr then 1mL of Kovac's reagent was added to the cultures. The development of cherry (red) color on the top layer was indication of positive test for indole production and the formation of a yellow color was a negative test and also if there was diffusion away from the site of inoculation after stabbing the bacteria culture was indicated as motile.

3.5.3.6. Carbohydrate fermentation test

Carbohydrate fermentation patterns of microorganisms were studied using media containing different carbohydrates as carbon source and phenol red as the pH indicator. The fermentation of carbohydrates such as glucose, lactose, sucrose, mannitol, inositol, sorbitol, arabinose etc. were observed by color change from red to yellow after 24 hours of incubation as described by Harley, (2002).

3.5.3.7. H₂S production test

Based on the method described by (Harley *et al.*, 2002); two milliliter of bacteria culture was inoculated in test tube containing Triple Sugar Iron Agar (TSIA) and incubated at 37°C for 24 hr. and in the last day of incubation the color changes red to yellow was indicates positive test for hydrogen sulfide production.

3.6. Enzymatic study

3.6.1. Crude enzyme extraction

Loop full of protease producing bacterial culture from broth was inoculated into a taste tube containing 30mL of sterilized skim milk broth (pH 9.0) and incubated at 37°C for 48 hr. After incubation period the culture was subjected to centrifugation at 10,000rpm for 10 minutes. The cell free supernatant was separated from residues and the solids were removed. The supernatant was used as crude enzyme for enzymatic activity.

3.6.2. Washing test

Application of alkaline protease enzyme by isolated bacteria as a detergent additive was studied according to a method described by (Sidra *et al.*, 2006). For these six white cotton clothes (5 x 5cm) were stained with sheep blood separately and then waited until it become well dried. The following set ups were designed to test enzymatic activity in the supernatant obtained in (section 3.6.1) to washing tests.

- ❖ Blood stained cloth dipped in plate with distilled water (10mL).
- ❖ Blood stained cloth dipped in plate with distilled water (10mL) + 1mL detergent (7mg/mL).
- ❖ Blood stained cloths dipped in plates with distilled water (10mL) + 2mL crude enzyme solution (supernatant of APBIT-3, APBIS-4, APBIT-6, and APBIT-6) in separate plates. All plates were incubated at 40°C for 20min. After incubation cloth pieces were taken out and were rinsed with distilled water without scrubbing and then dried.

3.6.3. Dehairing activity

Dehairing activity was performed by the method described by (Pravin *et al.*, 2014). Sheep skin was cut 3x2cm pieces and washed gently with tap water and then rinsed with distilled water to remove chemicals from the skin, which may hinder enzyme activity during dehairing activity. Four (3 x 2cm) piece of skins were inserted into 15mL centrifuge tubes separately. Five milliliter of crude enzyme solution (supernatant of APBIT-3, APBIS-4, APBIT-6, and APBIT-7) were added sequentially and incubated at 40° for 48 hrs. After incubation skin pieces were taken out and peel with swab stick and then observed the dehairing ability of the crude enzymes.

3.7. Data analysis

All experiments were performed in triplicate and the data was analyzed and interpreted, using Microsoft Excel 2013. Using Bergey's Manual of Determinative Bacteriology 7ed. and ABIS version 12 online were used for identification of bacterial species.

4. RESULTS

4.1. Screening protease producing bacteria

A total of 16 bacterial isolates showing various degree of zone of proteolysis were obtained in this study on skim milk agar plates inoculated with the soil samples (Table 1). The isolates were designated as (APBIS-1, APBIS-2, APBIS-3, APBIS-4, APBIS-5, APBIS-6, APBIS-7 and APBIS-8) for sludge soil from Adama Science and Technology University (ASTU) campus and (APBIT-1, APBIT-2, APBIT-3, APBIT-4, APBIT-5, APBIT-6, APBIT-7 and APBIT-8) for sediment soil from Modjo Tannery Share Company (MTSC). Among the sixteen isolates obtained through screening by growing on skim milk agar four bacterial cultures were selected which shown zone of hydrolysis $\geq 25\text{mm}$. From these APBIS-4 from soil of sludge disposal site at ASTU campus and APBIT-3, APBIT-6 and APBIT-7 from sediment soil collected from MTSC showed promising zone of hydrolysis ($\geq 25\text{mm}$). These four isolates were subsequently selected for further study. The proteolytic activity of the selected bacteria species were shown in Appendix 3.

Table 1. Zone of hydrolysis of isolated bacteria on skim milk agar

Sample sources	Isolates	Zone of hydrolysis (mm)
Sludge disposal site	APBIS-1	13
	APBIS- 2	20
	APBIS-3	16
	APBIS- 4	36
	APBIS-5	17
	APBIS-6	12
	APBIS-7	6
	APBIS-8	5
Sediment soil	APBIT-1	19
	APBIT-2	14
	APBIT-3	55
	APBIT-4	12

	APBIT-5	20
	APBIT-6	29
	APBIT-7	32
	APBIT-8	15

Note: - APBIS= alkaline protease producing bacteria isolated from sludge soil

APBIT= alkaline protease producing bacteria isolated from sediment

4.2. Cultural characterization

As can be seen from Table 2 bellow, the four selected bacteria isolates (APBIT-3, PBIS-4, APBIT-6 and APBIT-7) were shown different morphological characteristic (like colony type, shape, texture, elevation, margin and pigmentation or color) as described in Table 2 bellow. The morphological characteristics of the selected bacterial colonies on blood agar were shown in Appendix 4.

Table 2. Morphological characteristics of 24 hours colony on blood agar

Selected Colonies	Type	Shape	Texture	Elevation	Margin	Pigmentation
APBIT-3	Mucoid	Flat	Smooth	Convex	Entire	Greenish
APBIS-4	Mucoid	Circular	Smooth	Convex	Entire	White
APBIT-6	Dry	Circular	Rough	Pluvinate	Entire	Deep White
APBIT-7	Mucoid	Circular	Smooth	Flat	Entire	Gray

Note: APBIS= alkaline protease producing bacteria isolated from sludge soil

APBIT= alkaline protease producing bacteria isolated from sediment

4.3. Microscopic characterization (Gram staining)

The morphological features of the isolated bacteria were determined by Gram staining technique. Two of the isolates (APBIT-6 and APBIT-7) retained purple color of crystal violet stain implying that they were Gram positive and the remaining two of the isolates (APBIT-3 and APBIS-4) retained pink color of safranin stain implying that they were Gram negative bacteria. The images obtained from microscopic (Olympus CX21) observations were indicated in Appendix 4. Isolates APBIS-4 and APBIT-3 were rod shaped Gram negative bacteria but

APBIS-4 was non-motile and APBIT-3 was motile. APBIT-6 and APBIT-7 were motile rod shaped Gram positive bacteria (Table 3).

Table 3. Microscopic and motility results of isolated bacteria

Selected Colonies	Gram Staining	Motility
APBIT-3	Gram negative, Rod	Motile
APBIS-4	Gram Negative, Rod	Non-motile
APBIT-6	Gram positive, Rod	Motile
APBIT-7	Gram positive, Rod	Motile

Note: APBIS= alkaline protease producing bacteria isolated from sludge soil

APBIT= alkaline protease producing bacteria isolated from sediment

4.4. Enzyme tests of the isolated bacteria

Isolates (APBIT-3, APBIS-4, APBIT-6 and APBIT-7) showed positive results for catalase test and negative results for urease test. APBIT-3, APBIT-6 and APBIT-7 were oxidase positive but APBIS-4 was shown oxidase negative. APBIT-3 and APBIT-6 were shown positive results on L- Arginine dihydrolase but APBIS-4 and APBIT-7 shown negative results. The Enzyme characteristics of the selected bacteria were stated in Table 4 below and the observed color changes were indicated in Appendix 6.

Table 4. Results of enzyme test of isolated bacteria

Enzyme tests	Results			
	APBIT-3	APBIS-4	APBIT-6	APBIT-7
Catalase	+	+	+	+
Oxidase	+	—	+	+
URE (Urea hydrolysis)	—	—	—	—
ADH (L- Arginine dihydrolase)	+	—	+	—

Note: + Positive _ Negative

4.5. Biochemical profile of the isolated bacteria

Biochemical characteristics of the selected four bacterial isolates were indicated in Table 5 below. Isolates APBIT-3, APBIS-4, APBIT-6 and APBIT-7 were showed positive result to casein hydrolysis. APBIS-4 was carbohydrate fermenter which gives positive results (yellow color) after fermentation of carbohydrates (glucose, lactose, D- mannitol, inositol, D- sorbitol, L- rhamnose, D- sucrose, D- maltose and L- arabinose). APBIT-3, APBIT-6 and APBIT-7 were non-carbohydrate fermenter which couldn't ferment carbohydrates (Glucose, Lactose, D- mannitol, inositol, D- sorbitol, L- rhamnose, D- sucrose, D- maltose and L- arabinose) gave negative results. All four isolates were given negative results on 2-Nitro Phenyl galactopranoside, L- lysine decarboxylation and Voges Parkour's. The overall biochemical characteristics of the selected bacteria according to the biochemical tests within ABIS database were shown in Table- 5 below and the observed color change on API kite were stated in Appendix 7 and 8.

Table 5. Biochemical characteristics of isolated bacteria

No	Biochemical tests	Results			
		APBIS-4	APBIT-3	APBIT-6	APBIT-7
1	O/PNPG (O/P-Nitro Phenyl Galactopranoside)	–	–	–	–
2	LDC (L- Lysine decarboxylation)	–	–	–	–
3	ODC(L-Omithine decarboxylation)	–	+	–	–
4	CIT (Citrate utilization)	–	–	–	+
5	H ₂ S production	–	+	–	–
6	No ₃ reduction	+	–	–	–
7	TDA(L-Tryptophan deamination)	+	–	–	–
8	IND (Indole production)	+	–	–	–
9	VP (Voges parkour's)	–	–	–	–
10	GEL (Gelatine hydrolysis)	–	+	+	–
11	Casein hydrolysis	+	+	+	+
12	GLU (Glucose fermentation)	+	–	–	–

13	Lactose fermentation	+	—	—	—
14	MAN (D- Mannitol)	+	—	—	—
15	INO (Inositol)	+	—	—	—
16	SOR (D- Sorbitol)	+	—	—	—
17	RHA (L- Rhamnose)	+	—	—	—
18	SAC (D- Sucrose)	+	—	—	—
19	MEL (D- Maltose)	+	—	—	—
20	ESC (Esculin ferric citrate)	+	+	+	—
21	ARA (L- Arabinose)	+	—	—	—

Note: + Positive _ Negative

APBIS= alkaline protease producing bacteria isolated from sludge soil

APBIT= alkaline protease producing bacteria isolated from sediment

As indicated in the table above (Table 4 and Table 5) the isolated bacteria from sludge disposal site at Adama Science and Technology University and sediment soil at Modjo Tannery Share showed different biochemical characteristics. Both APBIS-4 and APBIT-3 were Gram negative bacteria and APBIT-6 and APBIT-7 were Gram positive but their biochemical profile and enzymatic activity were different. Using Bergey's Manual of Determinative Bacteriology 7ed. and Advanced Bacteria Identification Software (ABIS version12) online database. APBIS-4 was *E.coli* (99.69%), APBIT-3 was *Pseudomonas putrefaciens* (*Shewanella putrefaciens*) 99.99%). APBIT-6 was *Bacillus carboniphilus* (92.78%) and APBIT-7 was *Lysinibacillus sphaericus* (98.4%)

4.6. Application of crude alkaline protease enzymes

The cell free culture supernatant (crude enzyme) extracted from bacteria culture using skim milk broth was indicated at Appendix 9.

4.6.1 Washing ability of crude enzymes extracted from bacteria

The results of evaluation of enzyme for washing performance revealed that the blood stains on white cloth pieces (Appendix 10). Blood stains were completely removed from the cloths after rinsing them with a combination of detergent and crude enzyme in proportion of 1:5 mL after

incubation for 20 minutes. All crude enzymes obtained from four isolates could remove blood. APBIT-3 (*Pseudomonas putrefaciens* (*Shewanella putrefaciens*)), APBIS-4 (*E.coli*) and APBIT-6 (*Bacillus carboniphilus*) showed nearly similar efficiency on removal of blood stain. APBIT-7 (*Lysinibacillus sphaericus*) showed moderate effectiveness at 40⁰C with in 20 minute. These results clearly indicated that the enzymes were active for destaining activity as detergent additives.

The overall washing ability of all crude enzymes obtained in this study were summarized in Table 6 bellow.

Table 6. Washing ability of crude enzymes obtained from bacterial isolates

Codes	Isolated bacteria species	Site of isolation	Washing ability
APBIT-3	<i>Pseudomonas putrefaciens</i> (<i>Shewanella putrefaciens</i>)	MTSC	Excellent
APBIS-4	<i>E.coli</i>	ASTU campus	Excellent
APBIT-6	<i>Bacillus carboniphilus</i>	MTSC	Excellent
APBIT-7	<i>Lysinibacillus sphaericus</i>	MTSC	Very good

Note: Excellent= Stained blood completely removed

Very Good= Stained blood not completely removed

4.6.2. Dehairing ability of crude enzyme extracted from bacteria

All crude enzymes obtained from four isolated bacteria could remove hair. The effectiveness of the crude enzyme extracts obtained from APBIT-3 (*Pseudomonas putrefaciens* (*Shewanella putrefaciens*)) and APBIT-6 (*Bacillus carboniphilus*) to remove hairs from skin were almost similar efficiencies but the quality (softness) of skin treated with crude enzyme obtained from APBIT-6 (*Bacillus carboniphilus*) was tremendous. Crude enzyme obtained from APBIS-4 (*E.coli*) was moderately effective and crude enzyme extract from APBIT-7 (*Lysinibacillus sphaericus*) was less effective at 40⁰C within 48 hrs. The dehairing ability of the crude enzyme were shown in Appendix 11

The overall dehairing ability of all crude enzymes obtained from the isolated bacteria were summarized in table 7 bellow.

Table 7. Isolated bacteria and dehairing ability of their respective crude enzymes

Code	Isolated bacteria species	Sites of isolation	dehairing ability	Softness of skin
APBIT-3	<i>Pseudomonas putrefaciens</i> (<i>Shewanella putrefaciens</i>)	MTSC	Excellent	Very good
APBIS-4	<i>E.coli</i>	ASTU campus	Very good	Excellent
APBIT-6	<i>Bacillus carboniphilus</i>	MTSC	Excellent	Excellent
APBIT-7	<i>Lysinibacillus sphaericus</i>	MTSC	Good	Very good

Note: Excellent= completely removed hair and highly soften the skin

Very Good= much hair removed and soften the skin

Good= little hair removed

5. DISCUSSIONS

In this study, alkaline protease producing bacteria species isolated from sludge disposal site and tannery waste were assessed for potential dehairing and destaining abilities. All the isolated bacteria could hydrolysis casein protein on skim milk agar, which indicates that all bacteria could produce the enzyme casease that degraded casein protein. Alkaline protease enzymes could hydrolyze high molecular weight or complex substances (proteins) which cannot invade the bacterial cell membrane, because of their large size. The extracellular enzymes hydrolyze protein into their respective simple building blocks (amino acids) which can invade and easily pass through bacteria cell membrane. The extracellular enzyme produced by bacteria are specific among species, which help for the identification of bacteria.

In this finding potentially alkaline protease producing bacteria were isolated from sludge disposal site at ASTU campus and sediment soil in Modjo Tannery Share Company based on classical methods. From total of 16 bacterial isolates obtained from sludge disposal site and tannery waste four isolates showed promising proteolytic activity on skim milk agar. This implies that sludge disposal sites and soil sediments around tannery waste are good sources for protease producing bacteria. The potential of sludge disposal site soils in ASTU Campus and sediment at Modjo Tannery Share Company waste were not reported as a source of alkaline protease producing bacteria so far. However, several reports are available on the potential of soda lakes as a potential source for alkaline protease producing bacteria (Amare G. and Gizachew, 2012). Currently there are two possible ways to obtain enzyme with the desired property. The first one is by improving the existing enzyme by protein engineering and the second method is by screening of new microbial strains from their natural habitat with the required property for the desired purpose. Since the number of useful microorganisms characterized so far were extremely low < 1%, the searching for new potential microorganism in the environment may be the best choice. In line with this fact, the attempt to search for potential protease producing bacteria in unexploited sites such as sediments and sludge disposal sites yields four commercially exploitable species of bacteria.

They were identified and characterized based using Bergey's Manual of Determinative Bacteriology 7ed. and ABIS version 12 online advanced bacteria identification software. Isolate coded as APBIS-4 was *E.coli* APBIT-3 was *Pseudomonas putrefaciens* APBIT-6 was

Bacillus carboniphilus and APBIT-7 was *Lysinibacillus sphaericus*. This species of bacterial were already reported by some authors (Hamid et al., 2003; Bayoumi et al., 2011) from various ecosystems. However, their dehairing and destaining abilities were not clearly reported. These isolated bacterial for extracellular enzyme activities from these two isolates were *Bacillus* spp. (*Bacillus carboniphilus* and *Lysinibacillus sphaericus*) and others were *Enterobacteriaceae* sp. (*E.coli*) and *Pseudomonas* sp. (*Pseudomonas putrefaciens* (*Shewanella putrefaciens*)). From these the most dominant group of alkaline protease producers' bacteria were the genus *Bacillus* spp. This finding was confirmed with various reports regarding to alkaline protease producing bacteria which is isolated and characterize by classical methods. The potential of *Bacillus carboniphilus* as a potential for alkaline protease producer has already been reported by Mohammed *et al.* (2016). As already reported by the previous author *Bacillus carboniphilus* is Gram-positive, rod-shaped bacteria, obligate aerobes, or facultative anaerobes. Under stressful environmental conditions, these bacteria can produce endospores and remain at dormant state for long periods. *Bacillus carboniphilus* was isolated from soil and can be used for protease production. The enzyme produced by *Bacillus carboniphilus* has an enormous applications in different industries (leather and detergent industries). This bacteria showed promising activity for removal of blood stained on the white cloth and also had excellent dehairing activity. *Bacillus carboniphilus* was shown tremendous activity in both dehairing and removal of proteinacious (blood) stained. These bacteria species was very effective and encouraging to use in both detergent and leather industries for dehairing activity.

Lysinibacillus sphaericus (Preeti *et al.*, 2016) is Gram-positive, mesophilic, rod-shaped bacterium commonly found in soil. It is spore forming bacteria and is tolerant to high temperatures, chemicals and ultraviolet light and can remain viable for long periods of time in soil. *Lysinibacillus sphaericus* having the ability of the pure bacterial cultures to produce extracellular protease enzyme was carried out by growing the cultures on skim milk agar. *Lysinibacillus sphaericus* was well known bacteria used in the biological control of mosquitoes. It is of particular interest to the World Health Organization due to the larvicidal effect of some strains against two mosquito's genera (*Culex* and *Anopheles*) (Wirth, 2014). Additionally *Lysinibacillus sphaericus* had the ability to tolerate and reduces heavy metals and were commercially important in leather and detergent industries (Preeti *et al.*, 2016). Form this finding However *Lysinibacillus sphaericus* had good activity in both

removal of blood stained and hairs from skin it was less effective to dehairing activity. These bacteria specie was effective in detergent and better used as detergent additives.

Pseudomonas putrefaciens (*Shewanella putrefaciens*) (Bayoumi *et al.*, 2011) is Gram negative, facultative anaerobic, non- lactose fermenter, on blood agar plate the colony is typically convex and large, with a brown pigment, and cause “greening” of the agar around the colonies. *Pseudomonas putrefaciens* (*Shewanella putrefaciens*) are widespread that have been isolated from many different habitat. *Shewanella* species had the ability to reduce mercury, iron, uranium, and plutonium metabolically. These heavy metals especially mercury was released from leather industries and toxic to the organisms which live in water bodies (Bayoumi *et al.*, 2011). These bacteria was isolated from the sediment soil from the leather industry and have the ability to the removal of hair from the skin and also had excellent ability for removals of proteinacious (blood) stain from white cloth. From the current finding shown that *Shewanella putrefaciens* had promising activity in both dehairing and removal of blood stain next to *Bacillus carboniphilus*.

Escherichia coli (Hamid *et al.*, 2003) is Gram negative, rod, and mesophilic, facultative anaerobic and lactose fermenter bacteria. *E. coli* produce alkaline protease enzyme into alkaline culture media (Skim milk agar). This protease enzyme helps to hydrolysis protein (casein) which found in the culture media (Skim milk agar) for their carbon and nitrogen source. Reports shows that the protease enzyme release from *E. coli* is commercially important, mostly in laundry detergent for removal of pretentious stained. Similarly from the current finding shown that *E.coli* had great activity in blood stained removal. In dehairing activity *E.coli* had shown respectable activity and the softness of skin was promising. This indicated that this bacteria was commercially important in both leather and detergent industry (most appreciable). These results were encouraging as the isolated bacterial cultures had novel applications and also gave an emerging view of the bacterial diversity to the studying area. Since, there was no/ not much information available on bacterial diversity from sediment released from Modjo Tannery Share Company and sludge disposal site at ASTU.

Unlike the previous reports of using classical biochemical tests to characterize bacterial isolates this study utilized a modern standard kit ((bioMerieux API kits)) method of biochemical activities and ABIS data base system. API kite strips consist of microtubes containing dehydrated substances to detect the enzymatic activity or fermentation of sugar by

the inoculated microorganism during incubation metabolisms produce color changes. From these microtubes containing dehydrate substances some of the tubes containing ADH, LDC, ODC, H₂S, and urea test are required to add paraffin in order to create anaerobic environment to prevent the end gaseous product of the reaction. These gaseous substance would react with the other substance from the adjacent tubes and consequently they would affect the final results.

Alkaline proteases have contributed greatly to the development and improvement of modern household, leather and industrial detergents. Alkaline protease find a major application as detergent additives because of their ability to hydrolyze and remove proteinacious stains like blood (Saeki *et al.*, 2007). These enzymes are highly beneficial because they reduce activation energy of a reaction thereby making a reaction process more efficient with reduced energy consumption. From the current finding show that all crude enzymes extracted from the isolated bacteria could remove blood and since important for detergent industry. Their effectiveness to remove proteinacious stain (blood) were $ABPIT-6 \geq APBIT-3 \geq APBIS-4 > APBIT-7$; which mean that APBIT-3, APBIS-4 and APBIT-6 were have almost similar efficient for blood stain removal and APBIT-7 moderately effective at 40⁰C for 20minute.

Alkaline proteases with keratinolytic activity are used for dehairing and bating process to obtain a desired grain, softness and tightness of leather (Giongo *et al.*, 2007). In leather industry the toxic chemicals released from leather processing (lime, sulphide, etc.) are not only hazardous to environment but also has directly exposed (tannery workers, sewer men) as well as different life forms (macroscopic and microscopic) of contaminated water bodies and other ecosystems. Using alkaline protease enzymes rather than conventional methods in leather processing are eco-friendly, cost effective and reduce energy waste. So that keratinolytic proteases are such alternative option for dehairing activity. These enzymes are non-toxic, biodegradable and do not generate any toxic residues and also successful improve leather quality and reduce environmental pollution (Paul *et al.*, 2014b).

In this investigation for removal of hair from sheep skin undergoes the process without adding any additive of hazardous chemicals, thus, making it a potential candidate for possible biotechnological applications towards development of smart, clean, pollution free, green leather processing technology as compared to conventional chemical methods. Similarly the

crude enzyme from all isolated bacteria were removed hair from sheep skin but their effectiveness were different from one another. Crude enzyme from *Bacillus* sp. (APBIT-6) and *Pseudomonas* sp. (APBIT-3) were successfully removed hair from sheep skin than *Enterobacteriaceae* (APBIS-4) followed by *Bacillus* sp. (APBIT-7) after 24hr at 40°C. From the current study the crude alkaline protease enzyme from *Bacillus* sp. (*Bacillus carboniphilus*) could show potentially active in both dehairing and removal of blood stained and then selected as most potent bacteria for alkaline protease production.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

From this study it can be concluded that the soils of sludge disposal sites and sediments around tannery wastes could be good sources for isolating alkaline protease producing bacteria. The alkaline proteases obtained from bacterial species adapted in the sludge disposal sites and tannery sediments have the potential to maintain their activities up to 40°C and this signifies the promising potential application in detergent industries. From four bacteria isolates having high proteolytic activity two isolates were *Bacillus* species called *Bacillus carboniphilus* and *Lysinibacillus sphaericus*, the remaining isolates were *Enterobacteriaceae* sp. (*E.coli*) and *Pseudomonas* sp. (*Pseudomonas putrefaciens* (*Shewanella putrefaciens*)). Alkaline protease produced from *Bacillus* sp. APBIT-6 (*Bacillus carboniphilus*) in the present study had shown high potential in removal of hair from animal skin with improved quality and also in removal of proteinacious stain (blood). Finally, alkaline protease produced from *Bacillus* sp. APBIT-6 (*Bacillus carboniphilus*) was selected as most potent bacteria species due to potential industrial application for both washing ability in detergent and softness and dehairing purpose in leather processing.

The limitation of this study was due to unavailability of Folin's and Ciocalteu's phenol reagent (are expensive according to the given budget) the protein assay was not done and the amount of protein present in the crude enzyme extracted from bacteria were not done.

6.2. Recommendations

Therefore, beside this study the following recommendations were forwarded

- Further investigation of the isolates for other types of enzymes and detail characterization using molecular methods
- Optimizing all the parameters to better understand the isolated bacteria for better alkaline protease production and increase the efficiency of the activity for tannery and detergent industries.
- Producing alkaline protease in pilot scale and validate dehairing (tannery) and washing (detergents) capability at experimental level.

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APPENDICES

Appendix 1. Plating media and plating methodology used

Plating of media	Plating method employed
Skim milk agar	Pour plate method
Soya Casein digest agar	Pour and streak plate method
Nutrient agar	Streak plate method
Nutrient broth	Streak plate method
Blood agar	streak plate method

Appendix 2. Composition of media used in the investigation

1. Composition of Skim milk agar

Skim milk powder	28 (g/l)
Peptone	5 (g/l)
Yeast extract	1.5 (g/l)
Beef extract	1.5(g/l)
Sodium chloride	5 (g/l)
Agar	15 (g/l)
Distilled Water	1000mL
PH	7.0±0.2 at 25°C

2. Composition of Soya Casein digest agar

Pancreatic digestive casein	17 (g/l)
Sodium chloride	5 (g/l)
Soya peptone	3 (g/l)
Dipotassium phosphate	2.5 (g/l)
Dextrose	2.5 (g/l)
Distilled Water	1000mL
pH	7.3±0.2 at 25°C

3. Composition of Blood agar

Beef extract	500 (g/l)
Tryptose	10 (g/l)
Sodium chloride	5 (g/l)
Agar	15 (g/l)
Distilled Water	1000mL
pH	7.3±0.2 at 25°C

4. Composition of Nutrient agar

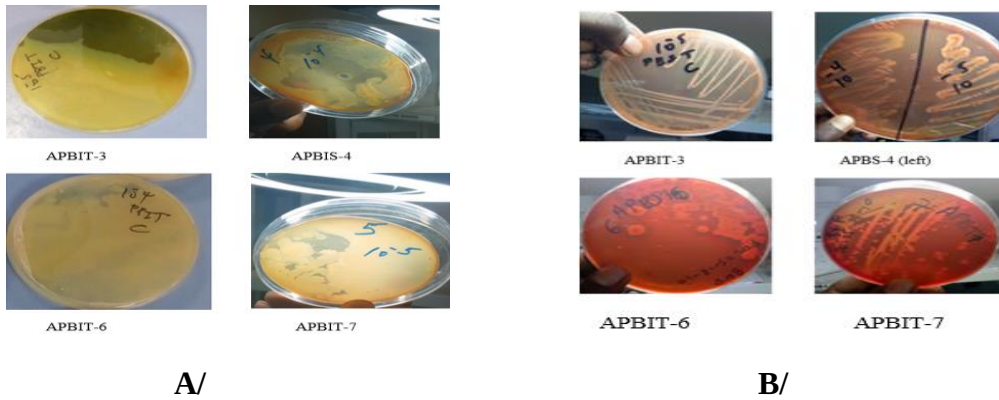
Agar	15 (g/l)
Peptone	5 (g/l)
Yeast extract	1.5 (g/l)
NaCl	5 (g/l)
Beef extract	1.5 (g/l)
Distilled Water	1000mL
pH	7.4 ±0.2 at 25 °C

5. Composition of Nutrient broth

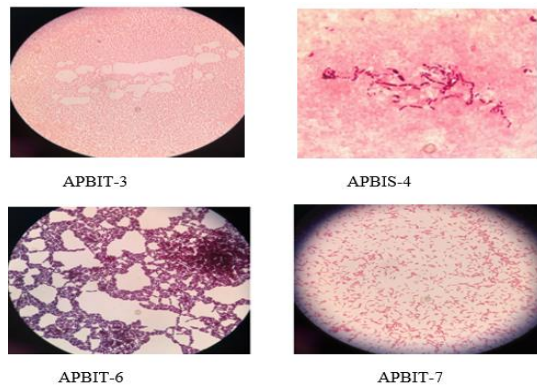
Peptone	15 (g/l)
D (+)-glucose	1 (g/l)
Yeast extract,	3 (g/l)
NaCl	6 (g/l)
Distilled Water	1000mL
pH	(7.5±0.2 (25 °C))

Appendix 3. Preliminary screening of bacteria and pure culture of isolated bacteria

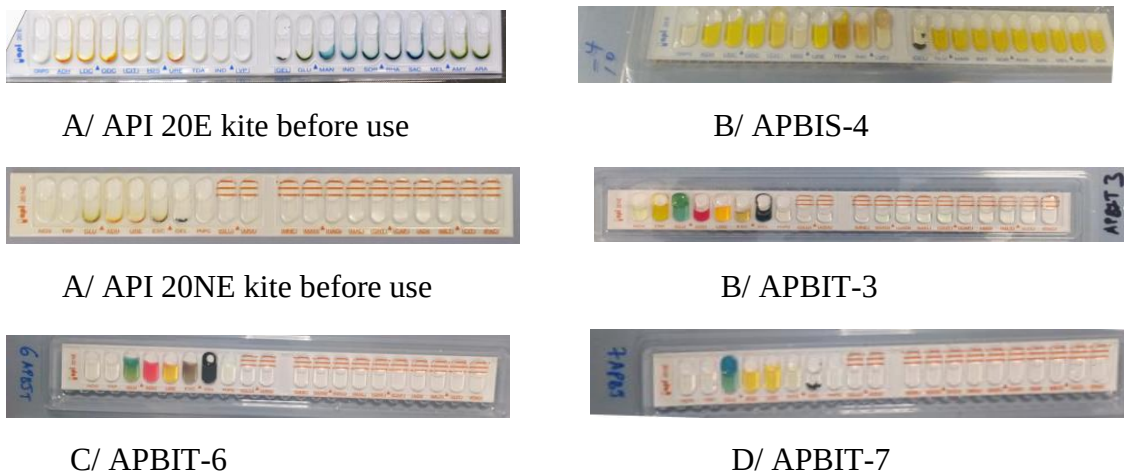
The proteolytic activity appeared as clear zone of hydrolysis on skim milk agar after incubated at 37°C for 48hrs. The primary screening of alkaline protease producing bacteria (A) and pure culture of the selected bacteria (B) on blood agar were shown below.



Appendix 4. Photograph of Gram staining of each isolated bacteria



Appendix 5. Biochemical profile of the isolated bacteria using API kites



Appendix 6. Observed color change in enzyme test of isolated bacteria

Enzyme tests	Observed Color change from the isolates			
	APBIS-4	APBIT-3	APBIT-6	APBIT-7
Catalase	Foaming	Foaming	Foaming	Foaming
Oxidase	Colorless	Purple	Purple	Purple
Urease test	Yellow	Yellow	Yellow	Yellow

Note: - APBIS= alkaline protease producing bacteria isolated from sludge soil

APBIT= alkaline protease producing bacteria isolated from sediment

Appendix 7. Preparation of bacteria suspension and setting up of API strips

1. Colonies of bacteria were taken from blood agar culture and then transferred into 5mL of saline solution.
2. The bottom try of the API kite strip holder was filled with distilled water in order to prevent dehydration of the strip during incubation time
3. The API strips was elevated at angle of 30 degree and the 50 μ L of bacterial suspension was added on one side of the tube by touching the inside wall of the cupule with the end of the pipette to avoid bubble formation.
4. Each of the tubes on the API strip were filled with the bacteria suspension from left to right
5. Mineral oil was added on the top of the cupule area of tubes containing dehydrated substance (ADH, LDC, ODC, H₂S, Urea in API 20E kite; Urea and Glucose in API 20NE kite) in order to stop the end gaseous products from a certain reaction to be released that affect final results by react with substances from adjacent tubes and incubated at 37⁰C for 24hr.

Appendix 8. Observed color change from biochemical tests of isolated bacteria

No	Biochemical tests	Observed color			
		APBIT-3	APBIS-4	APBIT-6	APBIT-7
1	ONPG (2-Nitro Phenyl Galactopranoside)	Colorless	Colorless	Colorless	Colorless
2	ADH (L-Arginine dihydrolase)	Red	Yellow	Red	Yellow
3	LDC (L-Lysine decarboxylation)	Yellow	Yellow	Yellow	Yellow
4	ODC (L-Omithine decarboxylation)	Yellow	Yellow	Yellow	Yellow
5	CIT (Citrate utilization)	Light green	Light green	Light green	Blue
6	H₂S production	Black	Colorless	Colorless	Colorless
7	No₃ reduction	Colorless	Pink	Colorless	Colorless
8	TDA (L- Tryptophan)	Yellow	Reddish brown	Yellow	Yellow
8	IND (Indole production)	Yellow	Pink	Yellow	Yellow
10	VP (Voges parkour's)	Colorless	Colorless	Colorless	Colorless
11	GEL (Gelatin hydrolysis)	Diffusion of black pigment	No diffusion	Diffusion of black pigment	No diffusion
12	Casein hydrolysis	Show Clear zone	Show Clear zone	Show Clear zone	Show Clear zone
13	GLU (Glucose fermentation)	Green	Yellow	Green	Green
14	Lactose fermentation	Green	Yellow	Green	Green
15	MAN (D- Mannitol)	Transparent	Yellow	Transparent	Transparent
16	INO (Inositol)	Transparent	Yellow	Transparent	Transparent
17	SOR (D- Sorbitol)	Transparent	Yellow	Transparent	Transparent
18	RHA (L- Rhamnose)	Transparent	Yellow	Transparent	Transparent
18	SAC (D- Sucrose)	Transparent	Yellow	Transparent	Transparent
20	MEL (D- Maltose)	Transparent	Yellow	Transparent	Transparent
21	ESC (Escuilin)	Black	Black	Black	Black
22	ARA (L- Arabinose)	Transparent	Yellow	Transparent	Transparent

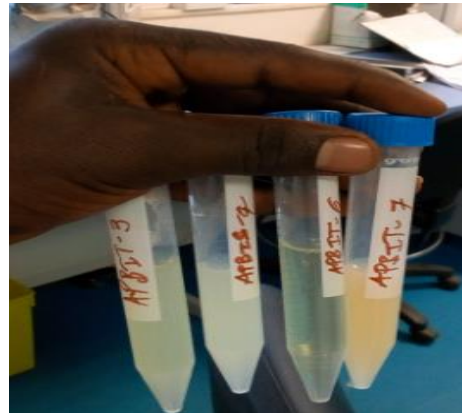
Note: - **APBIS**= alkaline protease producing bacteria isolated from sludge soil

APBIT= alkaline protease producing bacteria isolated from sediment

Appendix 9. Proteolytic activities (A) and crude enzyme (B) of the isolated bacteria on Skim milk broth

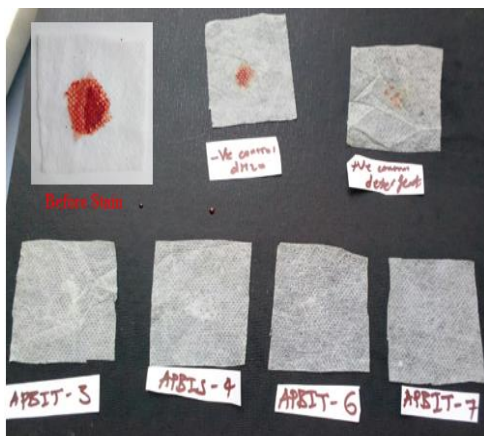


A



B

Appendix 10. Destining (A) and dehairing (B) performance of crude enzymes



A/



B/