JIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCES

SCHOOL OF GRADUATE STUDIES DEPARTMENT

THE EFFICIENCY OF SOME PROTEOLYTIC BACTERIA ISOLATED FROM MODJO TANNERY WASTE FOR APPLICATION IN LEATHER AND DETERGENT INDUSTRY

BY

TAYACHEW DESALEGN

A Thesis Submitted to the School of Graduate Studies of Jimma University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

July, 2015

Jimma, Ethiopia

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Principal advisor: Ketema Bacha (PhD)

Co advisor: Mesfin Tafesse (PhD)

August, 2015

Jimma, Ethiopia

APPROVAL SHEET

School of Graduate Studies

As thesis research advisor, we hereby certify that we have read and evaluated this thesis prepared, under our guidance, by Tayachew Desalegn Bekele entitled The Efficiency of some Proteolytic Bacteria isolated from Modjo Tannery Waste for Application in Leather and Detergent industry. We recommend that it be submitted as fulfilling thesis requirement.

Ketema Bacha (Assoc. Prof.)	----------	----------
Principal Advisor	Signature	Date
Mesfin Tafesse (PhD)	----------	----------
Co-Advisor	Signature	Date

As member of Board of Examiners of the MSc Thesis Open Defense Examination, We certify that we have read, evaluated the thesis prepared by Tayachew Desalegn Bekele and examined the candidate. We recommended that the thesis could be accepted as fulfilling the thesis requirement for the Degree of Master of Science in Applied Microbiology.

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DECLARATION

I declare that the thesis hereby submitted for the MSc. degree at the Jimma University, College of Natural sciences is my own work and has not been previously submitted by me or others at another University or institution for any degree. I concede copyright of the thesis in favor of the Jimma University, Collage of Natural sciences.

Name: Tayachew Desalegn Bekele

Signature; ----------

Place: Jimma University, Collage of Natural sciences

Date of Submission: ----------

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LISTS OF ABBREVIATION

BOD	biological oxygen demand
CAGR	Compound annual growth rate
COD	chemical oxygen demand
E.C	Enzyme Commission
IUBMB	International Union of Biochemistry and Molecular Biology
PMOJ	protease producing isolates
rpm	revolution per minute
ΔA	average absorbance
F.C	Folin Ciocalteu's reagent

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ABSTRACT

The extracellular proteases have great commercial value and multiple applications in various industrial sectors since they posses almost all characteristics desired for their biotechnological applications. In Ethiopia, all tanneries use many chemicals, despite the lack of survey on the particular types of optional technology. But enzyme technology serves as an immediate solution for such crucial problems beyond reducing environmental pollution. The objective of this work was to isolate efficient proteolytic bacteria from tannery wastes and production of protease enzyme from them. In this work samples of Leather sludge were collected from Modjo Tannery, South East of Addis Ababa. Five isolates were screened for protease production by culturing on skim milk agar, out of which two isolates showed efficient enzyme production. The bacterial isolates with prominent zone of clearance and showing efficient enzyme production were further characterized to genus level following standard procedures. Moreover, the growth conditions for the highest protease production were optimized with different pH, temperature and NaCl concentration. Enzyme activity was assayed using tyrosine-casein method. The proteases from PMOJ-01 (*Micrococcus)* and PMOJ-05 (*Bacillus)* were active at pH 8, pH 7, and temperature of 37 and 35^{o}C, respectively. The total protein contents of PMOJ-01 and PMOJ-05 was 0.45 μmole and 0.48 μmole, respectively. The enzyme activity and the total solid protease sample of crude enzyme of PMOJ-01 (*Micrococcus)* and PMOJ-05 (*Bacillus)* were 0.299 U/ml and 0.289 U/ml and 1.37 U/mg and 1.199 U/mg respectively. The partially purified enzyme of PMOJ-01 and PMOJ-05 had total solid protease of 2.05 U/mg and 11.55 U/mg and Enzyme activity of 0.242 U/ml and 0.231 U/ml respectively. The result of scum removal, hair removal, egg stain removal and blood stain removal revealed that the purified protease enzyme of PMOJ-01 and PMOJ-05 can be used in detergent and leather industries. Thus, the use of protease in leather processing could potentially minimize the use of hazardous chemicals such as sodium, lime and solvents and greatly minimize environmental pollution.

Key words: *Micrococcus sp., Bacillus sp.,* protease, partial Purification, leather and detergent Industries

1. INTRODUCTION

Enzymes are one of the most important biomolecules which has a wide range of applications in industrial field. Today it is one of the most important molecules which are widely used since the ancient human civilization. With the growing population and rising need enzymes for solving economical problems seem to be one of the most vital molecules that have a great impact in every sector that may be dairy, industrial, agriculture, or medicine (Gram *et al*., 2001).

Previously in the 19^{th} up till mid 20^{th} century, the world has seen great industrial expansions which we all know as industrial revolution that has created a steep rise in population and its demand for survival thus created a great impact in the agricultural, industrial, dairy, and medicinal fields. To meet the rising demand, many scientists had put their great effort to develop many chemical processes but in later years the harmful effects of using chemical catalysts to fast up the process have come in front of the mankind.

Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view (Gram *et al*., 2001).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. High chemicals and energy consumption as well as harmful by-products have a negative impact on the environment. Thus, a need for environment friendly process/biocatalyst came in light which again created a great research innovation in different scientific communities thus leading to a new field called “Biotechnology” where different live organisms were utilized to obtain desirable products in an eco-friendly way (Raven, 2002).

In a number of cases, some or all of these drawbacks can be virtually eliminated by using enzymes. Interestingly, enzyme reactions may often be carried out under mild conditions; they are highly specific and involve high reaction rates (Raven, 2002; Hasan *et al*., 2006). Industrial enzymes originate from biological systems (microorganisms) which can effectively contribute to sustainable development by fermenting renewable resources (Oyeleke *et al*., 2011). In addition, small amounts of enzymes are needed in order to carry out chemical reactions even on an industrial scale, and both solid and liquid enzyme preparations take up very little storage space. Mild operating conditions enable uncomplicated and widely available equipment to be used, and enzyme reactions are generally easily controlled. Enzymes also reduce the impact of synthetic chemicals on the environment by reducing chemicals, water, and energy and the subsequent generation of waste (Raven, 2002).

Microbial enzymes are currently acquiring much attention with rapid development of enzyme technology. Microbial enzymes are preferred due to their economic feasibility, high yields, consistency, ease of product modification and optimization, regular supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity (Hasan *et al*., 2006).

The demand for industrial enzymes, particularly microbial origin, is ever increasing owing to their applications in a wide variety of processes (Oyeleke *et al*., 2011). The current estimated value of the worldwide sales of industrial enzymes is $1 billion. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of total worldwide enzyme sales. This dominance of proteases in the industrial market is expected to increase further (Rao *et al*., 1998). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since it can posses almost all characteristics desired for their biotechnological applications (Gupta *et al.* 2002). Among the various proteases, bacterial protease was the most significant compared with animal, fungi and plant protease. Bacillus species were specific producers of extracellular protease (Gupta *et al.* 2002).

Proteases have extensive applications in industries like laundry detergents, pharmaceutical, food industry, leather processing and proteinaceous waste bioremediation (Bayoudh *et al*., 2000). Recently, bacterial proteases have received attention as a viable alternative for bioremediation of protein rich tannery waste and their use in treatment of raw hide by replacing the hazardous

chemicals especially involved in soaking, dehairing and bating of hides prior to tanning to produce quality leather without causing environmental pollution (Sudha *et al*., 2010). Although proteases have been identified from different sources and many of these have found their ways into industrial and biotechnological applications, but still the proteases exploited is not sufficient to meet all the demands.

A major cause for this is that many of the available enzymes could not resist the changes in environmental conditions in which they are supposed to work in industries. Thus, new promising bacterial strains that could survive under harsh environmental conditions could be isolated from environments to increase the yield of such enzymes. Effluent from leather industry might give us a good source of potential bacteria that could produce proteases that are active at extreme pH and temperature since the tannery effluent discharged is alkaline in nature.

Gupta *et al.* (2002) have reported bacterial protease as an important enzyme to be used in leather processing and laundry detergents. Mukhtar and Haq (2008) have also reported the production of protease by *Bacillus subtilis* and its application as a depilating agent. Several bacterial species, belonging to a variety of genera such as *Bacillus*, *Pseudomonas*, *Aeromonas*, *Staphylococcus*, *Micrococcus*, etc. are reported to produce protease having diverse industrial applications. There are still many unknown potential bacteria in the industrial waste dumping sites that can be a good source of protease (Gupta *et al.*, 2002). Therefore, the present study was initiated to isolate and identify bacteria from the tannery sludge that could produce extracellular protease. The availability of this enzyme is expected to help local industries, lower environmental pollution and enable these industries to be competitive in the global market.

1.1. Statement of the problem

Proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries (Gupta *et al*., 2002a). According to Leather Development Institute (2013), there are about 25 leather tanning industries in Ethiopia, many of them engaged in the production of finished leather. In these factories, thousands of people are working and export leather and leather goods as an important source of foreign currency. However, the leather tanning industry is also well known on the environmental pollution. Leather tanning process involves using different chemicals, such as sulfides, chromium, lime, salts, etc., and releases huge quantities of solid and liquid waste. As a result, leather tanning industries are negatively associated with severe environmental pollution.

According to Indian Standards Institution on Treatment and Disposal of Effluents of Tanning Industry, The use of protease not only improves yield but also results in a more environmentally friendly process and improves the quality of the leather (cleaner and stronger surface, softer leather, less spots). Here too industrial enzymes, such as amylases, protease, cellulases, peroxidases, and catalases proved in bringing about process efficiency and reduction in the amount of environmental pollution.

At present, different industries in the Ethiopia import substantial quantities of enzymes for use in leather tanning, textile, and brewing industries. Other industries stop from importing enzymes because of cost factor. For example more than 150,000 kg of bating enzyme is imported annually with a cost of US$ 900 - 1000 thousand in Ethiopia (Leather Development Institute, 2013). To date no tannery in the region is using enzymes for soaking and dehairing applications, mainly because of cost and lack of availability of such enzymes with affordable price. If enzymes are used for these processes, substantial quantity of chemicals imported could be replaced thus saving foreign currency expenditure. In this research Modjo tannery was selected since it is located at the upper catchment and if it uses enzyme technology than chemical method, the pollution of the environment will be reduced. Nowadays not only the effluent affects the society but also the sludge which is removed from the leather by sulphides and chromium. Therefore, this research introduces enzyme technology towards leather and detergent industries.

1.2. Objectives

Main Objective

The general objective of this study is to isolate, screen and characterize protease producing bacteria from tannery waste.

Specific Objectives

The specific objectives of the current work were to;

- isolate and Characterize proteolytic bacterial strains from tannery waste.
- screen the isolates for their extracellular protease enzymes production
- investigate the effect of temperature and pH on protease enzyme
- evaluate de-hairing effect of protease on sheep skin
- evaluate de-staining effect of protease on egg yolk and animal blood

2. REVIEW OF LITERATURE

In the present biotechnological era, many industries involve microbial activities. The microorganisms have been realized for their potential to be compatible with industrial set up and conditions. Bacterial exo-enzymes are not only retaining their stability; rather they work better at different pH (Prakasham *et al.,* 2006). These characteristics have rendered such enzyme's applications in detergent, leather and other industries. Following is a review of Enzymes, protease and its application.

2.1. Historical Outline of Enzymes

Enzymes are the biological substance or biological macromolecules that are produced by a living organism which acts as a catalyst to bring about a specific biochemical reaction. These are like the chemical catalysts in a chemical reaction which helps to accelerate the biological or biochemical reactions inside as well as outside the cell. These are generally known as "Biocatalyst." In 1877, Wilhelm Friedrich Kuhn a professor of physiology at the University of Heidelberg first used the term enzyme, which comes from a Greek word meaning "in leaven" (Kuhn *et al*., 2002).

Even many centuries ago enzyme and its use were well known to the mankind but Wilhelm Friedrich Kuhn was the first person to give a scientific terminology to this biomolecule. Use of enzyme has been seen in ancient Egyptians where they were used for the preservation of food and beverages. Cheese making has always involved the use of enzymes, and it goes as far as back in about 400 BC, when Homer's Iliad mentioned the use of a kid's stomach for making cheese. In 1783, the famous Italian catholic priest Lazzaro Spallanzani first mentioned the importance of this biomolecule in his work of biogenesis (spontaneous generation of microbes) where he mentioned that there is a life-generating force inherent to certain kinds of inorganic matter that causes living microbes to create themselves given sufficient time (Ullmann, 2007).

A few decades later in 1862, when studying the fermentation of sugar to alcohol by yeast, Louis Pasteur along with Ferdinand Cohn and Robert Koch came to the conclusion that this fermentation was catalyzed by a vital force contained within the yeast cells called "ferments," which were thought to function only within living organisms (Wang and Liu, 1996).

It was not known until 1926, however, that the first enzyme was obtained in pure form, a feat accomplished by James B. Sumner of Cornell University. Sumner in 1947 was able to isolate and crystallize the enzyme Urease from the jack bean. His work was to earn him the Nobel Prize. John H. Northrop and Wendell M. Stanley shared the 1947 Nobel Prize with Sumner. They discovered a complex procedure for isolating pepsin. This precipitation technique devised by Northropand Stanley has been used to crystallize several enzymes.

The development of fermentation processes was aimed specifically at the production of enzymes by use of particularly selected strains, due to which it is possible to produce purified, well-characterized enzymes on a large scale. This development allowed the introduction of enzymes into true industrial products and processes, for example, within the detergent, textile, and starch industries (Underkofler *et al*., 1957).

2.2. Global Market of Enzymes

According to Global Industry analysts' recent report on industrial enzymes, the global market for industrial enzymes was fairly immune to the recent disorder in the global economy and grew moderately during 2008-2009. Demand for industrial enzymes in matured economies such as the USA, Western Europe, Japan and Canada was relatively stable during the recent times, while developing economies of Asia-Pacific, Eastern Europe and Africa and Middle East regions emerged as the fastest growing markets for industrial enzymes. Increased demand for various specialty enzymes, polymerases and nucleases coupled with the robust growth in animal feed markets are likely to steer growth in industrial enzymes market. United States and Europe collectively command a major share of the world industrial enzymes market. On the other hand, Asia Pacific is poised to register the fastest compounded annual growth rate of more than 8.0% over the analysis period (Sarrouh *et al*., 2012).

Regarding the global market for the industrial enzymes, a recent report published by BBC Research (2011) states that the global market for industrial enzymes was estimated to reach a value of $3.3 billion in 2010. This market is expected to reach $4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% over the 5-year forecast period.

As described in the (Figure 1), Technical enzymes are valued at just over $1 billion in 2010. This sector will increase at a 6.6% compound annual growth rate (CAGR) to reach $1.5 billion in 2015. The highest sales of technical enzymes occurred in the leather market, followed by the bioethanol market. On the other hand, the food and beverage enzymes segment is expected to reach about $1.3 billion by 2015, from a value of $975 million in 2010, rising at a compound annual growth rate of 5.1%. Within the food and beverage enzymes segment, the milk and dairy market had the highest sales, with $401.8 million in 2009 (Sarrouh *et al*., 2012).

Another recently published research report by Norus (2006) on enzymes market highlighted the fact that Proteases constitutes the largest product segment in the global industrial enzymes market, and Carbohydrases market is projected to be the fastest growing product segment, with a CAGR of more than 7.0% over the analysis period. Lipases represent the other major product segment in the global industrial enzymes market with high growth potential.

According to Pitman (2011), a recent report on the use of enzymes in cosmetic industry appointed that this segment is estimated to grow by 5% CAGR every year up to 2015. Market researchers highlights the fact that industrial demands for enzymes is being driven by new enzyme technologies and increase use of organic compounds in place of petrochemical-based ingredients.

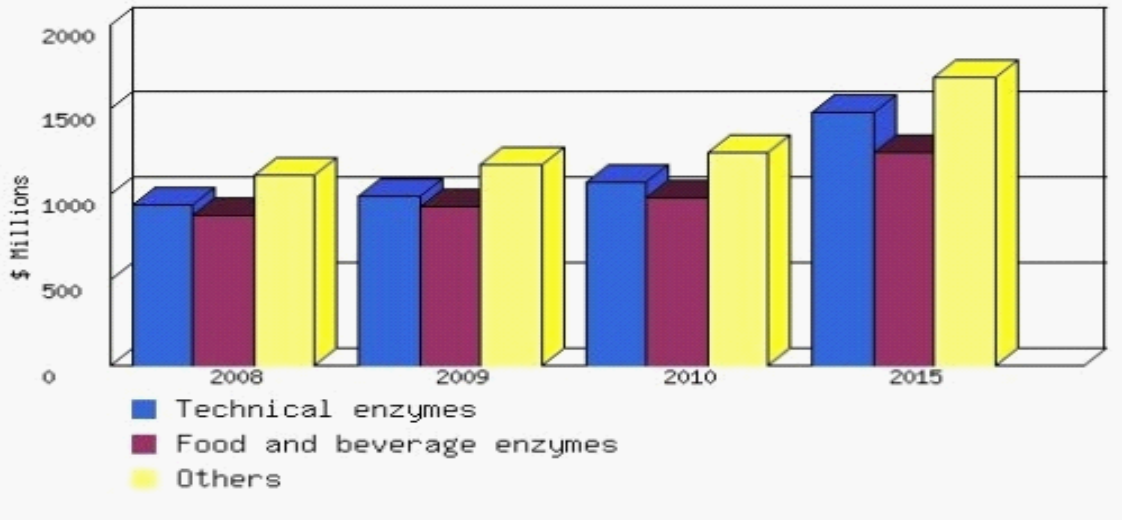

Figure 1: Global industrial enzymes market, 2008-2015 (Sarrouh *et al*., 2012)

2.2.1. How Enzyme Works

Enzymes act like tiny molecular machines to ensure that molecules come into contact with each other and react. Like a key fitting into a lock, chemical molecules fit into pocket-like structures located on an enzyme. These pockets hold the molecules in a position that will allow them to react with each other, ensuring that they are close enough together and aligned properly for a reaction to occur. In this way, enzymes speed up reactions (Rao *et al.*, 1998).

The enzymes are not changed themselves by the reaction. When the reaction is complete, enzymes release the product(s) and are ready to bring together more molecules and catalyze more reactions. The substrate is the substance that is present at the beginning of the reaction and the substance which is made by the enzyme is called the product (Rao *et al.*, 1998).

Furthermore, each enzyme has a particular temperature range and pH (acidity or alkalinity range) at which it works best. If an enzyme is subjected to extremes of pH or excessive temperatures, irreversible changes can occur in the 3-dimensional structure of the enzyme which can also affect the active site. If this happens, the enzyme is said to be denatured. It has lost its powers of catalysis (Rao *et al.*, 1998).

2.2.2. Classification of Enzymes According to Enzyme Commission

The IUBMB classified enzymes into six major groups (classes), according to the type of reaction they catalyze.

1. Oxidoreductases: All enzymes catalyzing oxidoreduction reactions belong to this class. The substrate that is oxidized is regarded as hydrogen donor.
2. Transferases: Transferases are enzymes which transfer a group, e.g. a methyl group or a glycosyl group, from one compound (generally regarded as donor) to another compound (generally regarded as acceptor).
3. Hydrolases: These enzymes catalyse the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds.
4. Lyases: Lyases are enzymes cleaving C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds.
5. Isomerases: These enzymes catalyse geometric or structural changes within one molecule and

6. Ligases: Enzymes that catalyze the joining together of two molecules coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate.

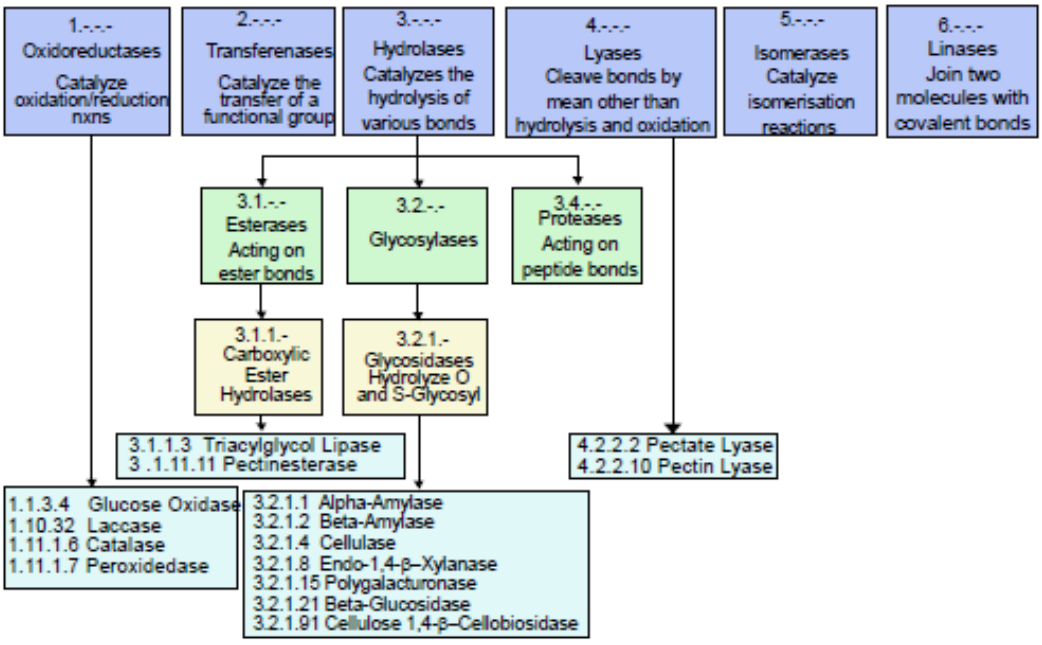

Figure 2: Classification of enzymes (source: E.C)

2.3. Protease Enzyme

Proteases are hydrolases that catalyze the hydrolysis of amide bonds within proteinaceous substrates. Proteolytic enzymes are ubiquitous in occurrence, found in all living organisms, and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta *et al*., 2002b).

These enzymes are by far the most commonly used types in detergents. Hydrolysis breaks the proteinaceous substrates down into smaller fragments (i.e., amino acids or oligopeptides), thereby increasing the ease with which the soils can be solubilized in the wash liquor by surfactants and the like. Proteases also help to prevent the redeposit ion of proteins on fabrics, particularly hydrophobic ones present in soils, such as blood, thereby also providing a whiteness benefit (Sekizaki *et al.*, 2008). Stains such as blood, grass, spinach, and keratin from collar and

cuff soil are most relevant for laundry applications, whereas baked-on egg soils are of interest for dishwashing applications. Proteases also are used for cleaning membranes and endoscopes in the industrial and institutional area.

Proteases represent one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market accounting for about 60% of total worldwide sale of enzymes (Figure 3) (Rao *et al*., 1998). This dominance of proteases in the industrial market is expected to increase further by the year 2015. The estimated value of the worldwide sales of industrial enzymes was $1 billion in 1998 (Rao *et al*., 1998).

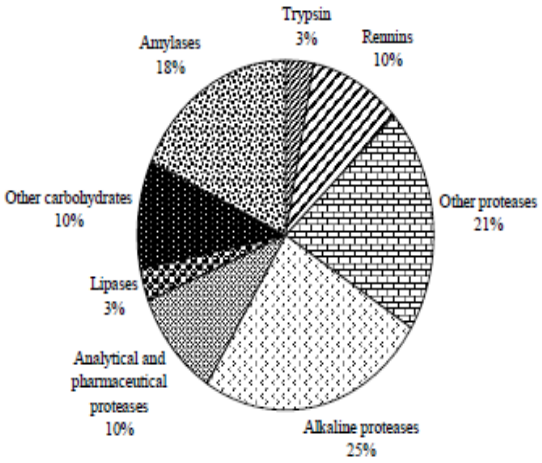

Figure 3: The contribution of different enzymes to the total sale of enzymes is indicated. The shaded portion indicates the total sale of proteases (Rao *et al*., 1998).

2.3.1. Sources of Proteases

Proteases are ubiquitous and found in all forms of life: plants, animals and microorganism including viruses.

2.3.1.1. Plant Proteases

Most common plant proteases are bromelain, ficin, papain and zingibain (Lee *et al*., 1986).

2.3.1.1.1. Ficin

It is extracted from latex of ficus and is a sulfhydryl proteinase with cysteine at the active site (EC 3.4.22.3). It preferentially cleaves at tyrosine and phenylalanine residues. Ficin has proven to be a versatile low cost biocatalyst useful in peptide synthesis (Sekizaki *et al.*, 2008).

2.3.1.1.2. Bromelain

Bromelain is a crude extract from the pineapple (*Ananas comosus*) plant. It is a mixture of sulfur-containing proteases. Bromelain is present in all parts of the pineapple plant but the stem is the most common commercial source. It is active between pH 5 to 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).

The major mechanism of action of bromelain appears to be proteolytic in nature, although evidence suggests an immunomodulatory hormone like activity acting via intracellular signaling pathways. Bromelain has also been shown to reduce cell surface receptors such as hyaluronan receptor CD44, which is associated with leukocyte migration and induction of proinflammatory mediators (Tochi *et al.,* 2008).

2.3.1.1.3. Papain

It is a cysteine protease (EC 3.4.22.2) 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 to 9 and is stable up to 80-90°C in presence of substrates. It consists of 212 amino acids stabilized by 3 disulfide bridges. Its catalytic triad is made up of 3 amino acids - cysteine-25 (from which it gets its classification), histidine-159, and asparagine-158. It is extensively used in tenderization of the meat (to break down the tough meat fibers), preparation of highly soluble and flavoured protein hydrolysates, dissociate cells in the first step of cell culture preparations, to make single cell preparation and an ingredient in some toothpastes and mints as teeth-whitener (Kim *et al.,* 2004).

2.3.1.2. Animal Proteases

The well-known proteases of animal origin are pepsin, trypsin, chymotrypsin and rennin.

2.3.1.2.1. Chymotrypsin

It is found in the pancreatic extract of animals (EC 3.4.21.1). The enzyme cleaves peptides at the carboxyl side of tyrosine, tryptophan and phenylalanine although over time it also hydrolyzes other amide bonds, particularly those with leucine-donated carboxyls. It is present in zymogen form and is activated on cleavage by trypsin into two parts that are still connected via an S-S bond. Pure chymotrypsin has main applications in analytical and diagnostic field. It is extensively used in deallergenizing of milk protein hydrolysates (Rao *et al*., 1998).

2.3.1.2.2. Pepsin

It is a digestive protease (EC 3.4.23.1) released by the chief cell in the stomach of almost all vertebrates that function to degrade food proteins into peptides. Pepsin is produced in its zymogenic form i.e. pepsinogen, whose primary structure has additional 44 amino acids. This zymogen is activated by hydrochloric acid (HCl), which is released from parietal cell in the stomach lining. HCl creates an acidic environment which allows pepsinogen to unfold and cleave itself in an autocatalytic fashion, thereby generating pepsin (Neklyudov *et al*., 2000).

Pepsin functions best in acidic environments between pH 1 to 2 and is inactivated above pH 6. Pepsin cleaves preferentially after the N-terminal of aromatic amino acids such as phenyalanine and tyrosine. It is an aspartyl protease and has resemblance with HIV-1 protease. Pepsin is commonly used in the preparation of fragments from antibodies.

2.3.1.2.3. Rennin

It is an aspartic acid protease (EC 3.4.23.4), produced as an inactive precursor, prorennin in stomachs of all nursing mammals but more specifically in the fourth stomach of cows. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in *k*-casein to generate insoluble para-*k*-casein and C-terminal glycopeptide. It cleaves the peptide bond between phenyalanine and methionine, the specific linkage between the hydrophobic (para-casien) and hydrophilic (acidic glycopeptide) group of casein in milk, since they are joined by phenylalanine and methionine (Rao *et al*., 1998).

The hydrophobic group would unite together and would form a three dimensional network to trap the aqueous phase of the milk resulting in the formation of calcium phosphocaseinate. This specificity is used to bring about the extensive precipitation and curd formation in cheese making.

2.3.1.2.4. Trypsin

It is a serine protease (EC 3.4.21.4) 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 commonly used in proteomics, since it has a very well defined specificity (Rao *et al*., 1998).

Trypsin is also used for the preparation of bacterial media, to dissolve blood clots, treat inflammation and to dissociate dissected cells. Trypsin has limited application in food because the protein hydrolysates generated have bitter taste. Based on the ability of protease inhibitors to inhibit the enzyme from the insect gut, trypsin is targeted for biocontrol of insect pests.

2.3.1.3. Microbial Proteases

Proteases are widely distributed in microbial population viz. bacteria, actinomycetes, viruses and fungi. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes and account for around two-thirds of commercial production worldwide. Alkaline serine proteases (EC 3.4.21) are the most important group of commercial enzymes (Kumar and Takagi, 1999).

Although proteases are wide spread in nature, microbes have been identified as preferred source of these enzymes because of rapid growth, limited space required for cultivation and ease of product separation from fermented broth (Rao *et al.,* 1998). Protease production is an inherent capacity of all microorganisms; and large numbers of bacterial species are known to produce alkaline proteases (Gupta *et al.,* 2002b). Among various bacteria, the *Bacillus* species are most significant and specific producers of alkaline proteases (Gupta *et al.,* 2002). Considering the richness of microbial diversity, there is always a chance of searching new organisms producing enzyme with better properties.

Current world demand for proteases has led to an interest in microbial proteases because of their rapid growth, cost effectiveness and the ease with which they can be genetically modified to generate high yielding strains with more efficient enzymes with desirable properties required for their diverse applications. Isolation of new and novel extremophiles organisms such as alkalophiles and thermophiles from diverse habitats secreting proteases that are active and stable under harsh conditions of industry has resulted in renewed interest in microbial proteases with novel properties (Kumar and Takagi, 1999).

2.3.1.3.1. Bacteria

Most of the commercial proteases are of bacterial origin. Though proteases are produced by variety of bacteria such as *Pseudomonas aeruginosa*, *Flavobacterium*, *Clostridium*, *Staphylococcus aureus*, *Micrococcus species*, *Achromobacter*, *Thermoactinomyces* and species belonging to *Streptomyce, Bacillus* is the major source which secretes a variety of soluble extracellular enzymes (Prakasham *et al.,* 2006).

Alkaline proteases from bacterial source are widely used in detergent formulations due to their activity and stability at high pH (9-11) and temperature (50-60°C). Neutral proteases of bacterial origin are active at pH 5-8 and between (35-40°C). Compared to alkaline proteases, neutral proteases have lower thermo-tolerance (Horikoshi, 1999).

Protein hydrolysate produced using neutral proteases of microbial origin has less bitterness compared to the one using animal trypsin and hence finds application in food industry. Neutral proteases are also used in brewing industry. Alkalophilic bacteria are also known to produce proteases. The first report of alkaline protease by an alkalophilic *Bacillus* sp. strain 221 was published in 1971 by Horikoshi, (1971). Purification and characterization of aqualysin 1 a thermo stable alkaline serine protease produced by thermophilic *Thermus aquatiens* YT-1 is reported by Matsuzawa *et al*. (1988). Subsequently many more proteases from alkalophiles with novel properties are also reported (Horikoshi, 1999).

2.3.1.3.2. Virus

Viral proteases are involved in processing of proteins that cause fatal diseases like AIDS and cancer. Mature enzymes encoded within the human immunodeficiency virus type 1 (HIV-1)

genome protease (PR); reverse transcriptase (RT) and integrase (IN) are derived from proteolytic processing of a large polyprotein (Gag-Pol). The viral PR catalyzes Gag-Pol processing, which is active as a homodimer (Olivares *et al*., 2007).

2.3.1.3.3. Fungi

Filamentous fungi can effectively secrete various hydrolytic enzymes and one of the main groups of secreted enzymes in fungi is protease. Submerged as well as solid state fermentation have been employed for protease production. They usually show better results when cultured in solid-state fermentation as compared to bacteria (Pandey *et al.*, 1999).

Fungi are known to produce acid, neutral, alkaline and metalloproteases. A single organism can produce more than one type of protease. Fungal proteases are active over a wide pH range pH (4 - 11) and exhibit broad substrate specificity. One of the first known representatives of proteases was proteinase K, an alkaline enzyme from *Engyodontium album* also known as *Tritirachium album* (Kotlova *et al*., 2007).

2.3.2. Classification of Proteases

The microbial proteases differ widely not only in their functions but also in properties, based on which they can be classified into various groups. Depending upon the optimum pH for activity they can be classified into acid, neutral and alkaline types. According to the presence of catalytic residue on the active sites, the proteases can be classified into four groups such as serine proteases, thiolproteases, aspartic proteases and metalloproteases (Kenny, 1999).

2.3.2.1. Classification of Proteases According to Enzyme Commission

According to the Committee of International Union of Biochemistry and Molecular Biology (1992), proteases are classified in subgroup 4 of group 3 (hydrolases) and assigned with EC.3 (hydroloases), 4 (proteases). The main classes of peptidases are defined by a third numeral (11 - 24) as listed in (Table 1).

The exopeptidases are classified mainly on the basis of their actions. Only peptides with unsubstituted terminus are attacked with the exception of a very small number, grouped as omega peptidases (3.4.19), which can release certain modified terminal residues. Proteases or

proteolytic enzymes are enzymes that break peptide bonds between amino acids of proteins. The process is called proteolytic cleavage, a common mechanism of activation or inactivation of enzymes. They use a molecule of water for this and are thus classified as hydrolases.

According to the Enzyme Commission there are currently six classes of proteases: Serine proteases, Threonine proteases, Cysteine proteases, Aspartic acid proteases, Metalloproteases and Glutamic acid proteases. The threonine and glutamic acid proteases were not described until 1995 and 2004, respectively and are therefore recent additions. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the character of a polarized peptide bond (serine, cysteine and threonine proteases) or a water molecule (aspartic acid, metallo and glutamic acid proteases) nucleophilic so that it can attack the peptide carbonyl group.

Rawlings *et al*. (2007), have devised a classification scheme based on statistically significant similarities in sequence and structure of all known proteolytic enzymes and term this database MEROPS. The classification system divides peptidases into clans based on catalytic mechanism and families on the basis of common ancestry. At present, over 66,000 peptidase protein sequences have been classified into 50 clans and 184 families (MEROPS release 7.90). Over 26,000 serine peptidases are grouped into 13 clans and 40 families (Page and Di Cera, 2008).

The MEROPS database is a manually curated information resource for peptidases (also known as proteases, proteinases or proteolytic enzymes), their inhibitors and substrates. The principle of the database is a hierarchical classification in which homologous sets of peptidases and protein inhibitors are grouped into protein species, which are in turn grouped into families, which are grouped into clans (Rawlings *et al*., 2010).

Table1. Classification of Proteases (E.C, 1992)

E.C. number	Peptidase type	Action
Exopeptidases		
3.4.11-	Aminopeptidase	N-terminal residue released
3.4.13-	Dipeptidase	Acts only on dipeptides
3.4.14-	Dipeptidyl peptidase Tripeptidyl peptidase	N-terminal dipeptide released N-terminal tripeptide released
3.4.15-	Peptidyl dipeptidae	C-terminal dipeptide released
3.4.16	Carboxypeptidase (serine)	C-terminal residue released (serine at active site)
3.4.17-	Carboxypeptidase (metallo)	C-terminal residue released (metal requiring protease)
3.4.18-	Carboxypeptidase (cysteine)	C-terminal residue released (cysteine at active site)
3.4.19-	Omega peptidase	Releases modified residue from N- or C- termini
Endopeptidases		
3.4.21-	Serine endopeptidase	Serine at active site
3.4.22-	Cysteine endopeptidase	Cysteine at active site
3.4.23-	Aspartic endopeptidase	Aspartate at active site
3.4.24-	Metallo endopeptidase	Metal requiring protease
3.4.99-	Endopeptidase of unknown Catalytic mechanism	

2.3.3. Mechanism of action of protease

Studies of the mechanism of proteases have revealed that they exhibit different types of mechanism based on their active-site configuration.

2.3.3.1. Serine Proteases

Serine proteases are characterized by the presence of serine group at their active site. They are abundant and widespread among viruses, bacteria and eukaryotes, suggesting that they are vital to organisms. Serine proteases are inactivated by organic phosphate esters which acylate the active serine residue. Serine proteases are generally active at neutral and alkaline pH, with an optimum between 7 and 11 (Rao *et al*., 1998).

2.3.3.2. Cysteine/Thiol Proteases

Cysteine proteases occur in both prokaryotes and eukaryotes. They have cysteine at their active site and have optima in the range of pH (6-8). They are activated by reducing agents such as hydrogen cyanide and inhibited by oxidizing agents. They are susceptible to sulfhydral (-SH) agent such as *p*-CMB but are unaffected by DFP and metal-chelating agents. The activation by reducing agents is due to regeneration of SH group. They have temperature optima between 50-70^{o}C (Doke and Ninojoor, 1987).

2.3.3.3. Aspartic Proteases

These proteases are widely distributed in animal, yeast and mould cells but rarely found in bacteria. They are commonly known as acidic proteases having aspartic acid residues at their active sites. They show specificity towards aromatic or bulky amino acids residues on both sides of the peptide bond and have pH optima between pH (3-4). The aspartic proteases are inhibited by pepstatin. Microbial aspartic proteases are further divided into two groups, (i) Pepsin like proteases and (ii) rennin-like proteases produced by moulds and yeasts.

2.3.3.4. Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases (Barret, 1995). They are characterized by the requirement for a divalent ion for their activity. These enzymes are sensitive to chelating agents but insensitive to sulfhydral agents and phosphate esters have metal ion

involved in the catalytic mechanism. Neutral and alkaline proteases from many microbial sources belong to this group of enzymes. All the above referred types of proteases have found applications in different sectors.

2.4. Microbial Protease Production by Fermentation

Fermentation is an ancient process used by humans to produce many food and food products. Ancient Egyptian regarded fermentation as a gift from gods whereas Roman regarded the appearances of mushroom to lightning bolt cast to the earth by the god Jupiter (Souza *et al*., 2004). Initially the word fermentation was used to describe any biological process occurring in the anaerobic conditions (without oxygen). Now the researchers define that fermentation is biological process to produce valuable specific products by the action of microbes on substrates. To produce a specific product such as protease enzyme, the mode of fermentation (solid or submerged) is very much crucial for obtaining maximum yield. Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth, whereas in solid state fermentation the microbes require limited water contents for their growth. Since bacteria generally require higher quantity of water for their growth. It has also been reported that protease activity in submerged fermentation is higher than in the solid state fermentation, using identical growth medium composition. The chance of cross contamination is also minimized in submerged fermentation process (Aguilar *et al*., 2002).

2.5. Characteristics of protease

A fermentation medium forms the environment in which the microorganisms live, reproduce and carry out their specific metabolic reactions to produce useful products. It is well established that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions and physical factors such as pH, temperature, dissolved oxygen (DO) and incubation time (Oberoi *et al.*, 2001).

2.5.1. Effect of Incubation Period

Incubation period for maximum yield of enzyme varies among various species or even in the same species isolated from various sources. Many workers have reported a broad incubation period ranging from 36 to 96 hrs for the maximum yield of protease enzyme by *Bacillus* strains

(El-Enshasy, *et al*., 2008). Johnvesly and Naik (2001) reported maximum yield of protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemical defined medium after 24 hrs of incubation.

2.5.2. Effect of Medium pH

The pH requirements vary from species to species or even in different strains of the same species isolated from different habitats. Some bacterial cultures in unbuffered media have been observed to exert a change in pH following the growth and protease production. Mao *et al*. (1992) documented importance of controlling pH of the culture at required level, favor higher yield of alkaline protease by a strain of *Bacillus licheniformis*.

Initial pH of the medium required for obtaining maximum production depends not only upon the bacterium but also upon the ingredients of the medium. Maximum protease production has been reported from different pH ranges (7-11) by different *Bacillus* species (Khosravi-Darani *et al*., 2008). Media with slightly acidic pH have been reported for protease production by bacteria such as *Bacillus coagulans* (Gajju *et al*., 1996) and *Bacillus licheniformis* (Mao *et al*., 1992). The optimum pH for alkaline protease is in the range between (9-11) (kumar and takagi, 1999). Exceptional cases of extreme pH stability were reported in the case of proteases with an optimum pH of 12.0 (Kumar *et al*., 1999), optimum pH 11.5 (Kumar, 2002).

2.5.3. Effect of Incubation Temperature

Temperature is one of the most critical parameters that have to be controlled in any bioprocess (Chi and Zhao, 2003). The optimum temperature requirement reported for protease production by different bacteria differs widely. Optimum production temperature for different *Bacillus* species have been reported as, 25^{o}C for *Bacillus circulans* (Jaswal and Kocher, 2007).

The optimum temperature for thermostable protease ranges from (50-70^{o}C) (Kumar, 2002). Extreme thermostable proteases that are stable up to 80^{o}c are reported (Fu *et al*., 2003). Protease from haloalkaliphilic bacterium O.M.A$_{18}$ optimally catalyzed the reaction over a wide of temperature from (50-90^{0}C), with a half life of 36 hrs at 90^{0}C (Purohit and Singh, 2011).

2.5.4. Effect of Agitation

Agitation speeds generally affect the bacterial growth as well protease production. The enhancing effect of agitation on protease production has been reported in *Bacillus licheniformis* which required agitation at 200 rpm for the maximum production. Takami *et al*. (1989) reported agitation speeds of 200 and 300 rpm for enzyme production by *Bacillus* species and maximum protease activity from alkalophilic *Bacillus* species has been reported at 100 rpm (Genekal and Tari, 2006).

2.6. Enzyme Purification

Crude preparations of proteases are usually employed for commercial use. Nevertheless, purification of the enzyme is important from the perspective of developing better understanding of the functioning of the enzyme (Takagi, 1993). There are no set rules for the purification of the proteases. After separating the cells from the fermentation broth by filtration or centrifugation, the culture supernatant is concentrated by means of ultra filtration (Kang *et al*, 1999), salting out by solid ammonium sulphate (Kumar, 2002), or solvent extraction methods using acetone (Kumar *et al.*, 1999; Thangam and Rajkumar, 2002) and ethanol (El Shanshoury *et al*., 1995). In addition, other methods, such as the use of PEG-35 (Larcher *et a*l., 1996) activated charcoal (Aikat *et al*., 2001), heat treatment of enzyme (Rahman *et al*., 1994) and lyophilization (Manonmani and Joseph, 1993) are also used for concentration of proteases.

2.7. Applications of Microbial Proteases

The role of enzymes in many processes has been known for a long time. Their existence was associated with the history of ancient Greece where they were using enzymes from microorganisms in baking, brewing, alcohol production, cheese making etc. With better knowledge and purification of enzymes the number of applications has increased many folds, and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged (Haki and Rakshit, 2003).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. The application of proteases scores highest among enzyme markets. There is renewed interest in the study of proteolytic enzymes, mainly due to

their cognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Figure 4). Only those microbes that produce substantial amounts of extracellular enzyme are of industrial importance. Several products based on bacterial proteases have been launched successfully in the market in past few years. Microbial proteases in general have a large variety of applications, in various industries. These include food industries, detergent, pharmaceutical industries. The application of these enzymes varies considerably (Gessesse, 1997).

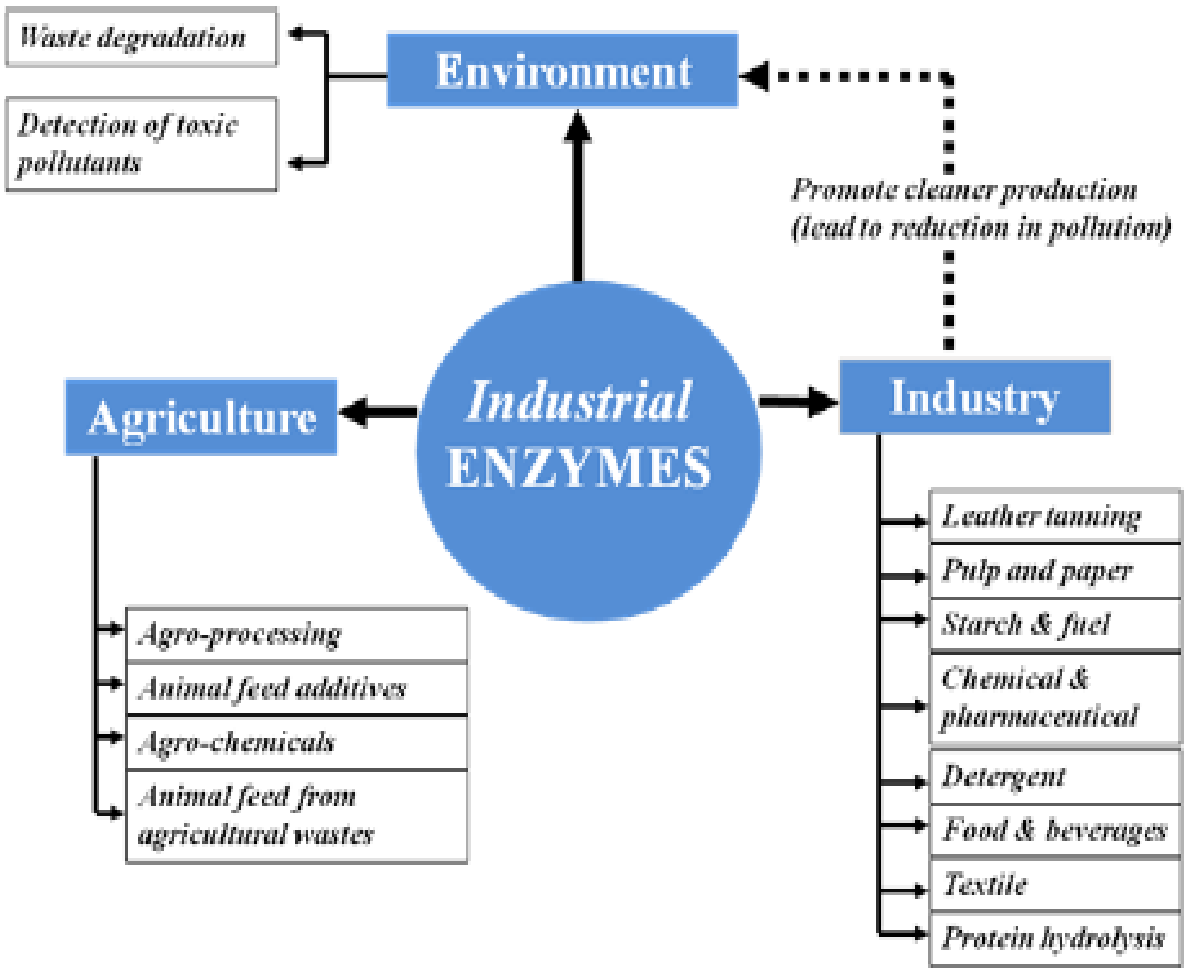

Figure 4: Application of industrial enzymes (Gessesse, 1997).

2.7.1. Applications in Leather industry

Leather manufacture is one of the highly polluting industries and generates solid wastes as well as liquid effluents. The major source of pollution is from dehairing step in the pretanning operations due to the use of hazardous chemicals like lime and sulphide. Leather processing involves number of steps such as soaking, dehairing, bating, degreasing and tanning etc. Though proteases have been used for bating for more than a century, their use for soaking and dehairing is more recent. The purpose of soaking is to swell the hide and addition of a small amount of protease to soaking liquor was found to facilitate water uptake and reduce the time required for swelling. Since skin and hair consist of proteins, their selective removal with proteases is environmentally friendly.

Conventional dehairing involves treatment with lime and sulphide to solubulize the proteins at the hair root and loosen the hair for removal. However, the hair is destroyed and removed in the form of pulp leading to high BOD and COD in the effluents. Crude proteases are being used increasingly to remove non-collagenous and globular proteins since they act under milder conditions and hair can be recovered as a valuable by-product (Rao *et al*., 1998; Kumar and Takagi, 1999; Gupta *et al*., 2002).

2.7.2. Applications in Food and Feed industry

Microbial proteases have been useful in the dairy industry in the process of cheese formation. Protease enzymes are added to milk during cheese production, to hydrolyze caseins (specifically kappa casein) which stabilize micelle formation preventing coagulation (Abhijit, 2012). Proteases are also used in the bakery industry. Dough may be prepared more quickly if its gluten is partially hydrolysed. A heat-labile fungal protease is used so that it is inactivated early in the subsequent baking (Abhijit, 2012). Microbial proteases have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks (Neklyudov *et al*., 2000).

2.7.3. Applications in Waste Management

Chemical treatment of wastes that can be very hazardous for the environment and its surrounding leads researchers to find an alternate way or an eco-friendly way for the treatment of wastes. The proteases solubilize proteinaceous waste and thus help in lowering the biological oxygen demand of aquatic systems. Recently, the use of protease in the management of wastes from various food-processing industries and household activities opened up a new era in the use of proteases for waste management. Proteases can be effectively used for degradation of protein containing waste and help in clearing pipes and to remove clogs in blocked drainage pipes.

Waste feathers make up approximately 5% of the body weight of poultry and are considered to be a high protein source for food and feed, provided their rigid keratin structure is completely destroyed. The use of keratinolytic protease for food and feed industry waste, for degrading waste keratinous material from poultry refuse and as depilatory agent to remove hair from the drains has been reported (Takami *et al*., 1992).

They have immense application in converting wastes generated from poultry, fish, meat industries and slaughter house to value added products like fish meal, feather meal and feed. Large quantities of waste are generated in the form of feather, hair, left over protein rich solids from meat industry. For example, feathers constitute around 5% of the body weight of the animal and proteases with keratinolytic activity are being successfully used for hydrolysis of keratin rich products like hair and feather to prepare animal feed and food, amino acids and peptides (Kumar and Takagi, 1999; Gupta *et al*., 2002).

A formulation containing proteolytic enzymes from *B. subtilis*, *B. amyloliquefaciens* and *Streptomyces sp.* and a disulfide reducing agent (thioglycolate), that enhances hair degradation and helps in clearing pipes clogged with hair-containing deposits, is currently available in market. It was prepared and patented by Genex (Jacobson *et al*., 1985).

2.7.4. Applications in Detergent Industry

Proteases in detergent industry have been used at large scale at commercial level. Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. The preparation of the first enzymatic detergent, "Burnus," dates back to 1913; it consisted of sodium carbonate and a crude pancreatic extract**.** The first detergent containing the bacterial enzyme was introduced in 1956 under the trade name BIO-40. In 1960, Novo Industry A/S introduced alcalase, produced by *Bacillus licheniformis*; its commercial name was BIOTEX. This was followed by Maxatase, a detergent made by Gist-Brocades (Bayoudh *et al*., 2000).

One of the important parameters for selection of detergent proteases is the pH value. It is known that detergent proteases perform best when the pH value of the detergent solution in which it works is approximately the same as the pH value for the enzyme. However, there are many more parameters involved in the selection of a good detergent protease, such as compatibility with detergent components, e.g. surfactants, perfumes and bleaches good activity at relevant washing pH and temperature, compatibility with the ionic strength of the detergent solution, stain degradation and removal potential, stability and shelf life (Gupta *et al.,* 1999 and Oberoi *et al.,* 2001). Conventionally, detergents have been used at elevated washing temperatures, but at present there is considerable interest in the identification of proteases which are effective over a wide temperature range (Oberoi *et al.,* 2001).

In general, the majority of the commercially available enzymes are not stable in the presence of bleaching/oxidizing agents. Hence, the latest trend in enzyme-based detergents is the use of recombinant DNA technology to produce bioengineered enzymes with better stability. Bleach and oxidation stability has been introduced through protein engineering by the replacement of certain amino acid residues (Yang *et al.,* 2000 and Wolff *et al*., 1996). However, apart from their use in laundry detergents, they are also popular in the formulation of household dishwashing detergents and both industrial and institutional cleaning detergents (Godfrey and West, 1996).

2.7.5. Medical usage

Microbial proteases are also used for developing products of medical importance. It was stated in Gupta *et al*., (2002b) that the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kim *et al.*, (2004) reported the use of protease from *Bacillus* sp. strain CK 11-4 as a thrombolytic agent having fibrinolytic activity (Gupta *et al*., 2002b). Furthermore, *Bacillus* sp. has been recognized as being safe to human (Kumar and Takagi, 1999).

2.7.6. Other Applications

Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure function relationship, in the synthesis of peptides, and in the sequencing of proteins. In essence, the wide specificity of the hydrolytic action of proteases finds an extensive application in the food, detergent, leather, and pharmaceutical industries, as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins.

2.8. Enzyme Immobilization

The use of enzymes in industrial applications has been limited by several factors, mainly the high cost of the enzymes, their instability, and availability in small amounts. Also the enzymes are soluble in aqueous media and it is difficult and expensive to recover them from reactor effluents at the end of the catalytic process. This restricts the use of soluble enzymes to batch operations, followed by disposal of the spent enzyme-containing solvent D´Souza (1999). Over the last few decades, intense research in the area of enzyme technology has provided many approaches that facilitate their practical applications. Among them, the newer technological developments in the field of immobilized biocatalysts can offer the possibility of a wider and more economical exploitation of biocatalysts in industry, waste treatment, medicine, and in the development of bioprocess monitoring devices like the biosensor D´Souza (1999).

D´Souza (1999) stated that, immobilization means associating the biocatalysts with an insoluble matrix so that it can be retained in adequate reactor geometry for its economic reuse under stabilized conditions. Immobilization helps in the development of continuous processes allowing more economic organization of the operations, automation, decrease of labor, and

investment/capacity ratio. Immobilized biocatalysts offer several other advantages; notable among them is the availability of the product in greater purity. The same author highlighted that the purity of the product is very crucial in food processing and pharmaceutical industry since contamination could cause serious toxicological, sensory, or immunological problems. The other major advantages include greater control over enzymatic reaction as well as high volumetric productivity with lower residence time, which are of great significance in the food industry, especially in the treatment of perishable commodities as well as in other applications involving labile substrates, intermediates or products (Sarrouh *et al.,* 2012).

A large number of techniques and supports are now available for the immobilization of enzymes or cells on a variety of natural and synthetic supports. The choice of the support as well as the technique depends on the nature of the enzyme, nature of the substrate and its ultimate application. Commercial success has been achieved when support materials have been chosen for their flow properties, low cost, non toxicity, and maximum biocatalysts loading while retaining desirable flow characteristics, operational durability, ease of availability, and ease of immobilization Gekas, Lopeiz-Leiva (1985).

Biocatalysts can be immobilized either using the isolated enzymes or the whole cells (Rao *et al.,* 1988). Immobilization of whole cells, due to operational facility, has been shown to be an easier alternative to immobilization of isolated enzyme. On the other hand, immobilized cells showed lower catalytic activity compared with immobilized enzymes. Enzymes are good catalysts in terms of high catalytic and specific activity with ability to function under mild conditions. However, they are not always ideal catalysts for practical applications because they are generally unstable and they inactivate rapidly through several mechanisms (Rao *et al.,* 1988).

Techniques for immobilization have been broadly classified into` four categories, namely entrapment, covalent binding, cross-linking and adsorption and the combination of one or more of these physical techniques together with chemical conjugation techniques. It must be emphasized that in terms of economy of a process, both the activity and the operational stability of the biocatalysts are important. They determine its productivity, which is the activity integrated over the operational time (D´Souza 1999).

Immobilization often stabilizes the structure of enzymes, thereby, allowing their applications even under harsh environmental conditions of pH, temperature and organic solvents, and thus enable their uses at high temperatures in nonaqueous enzymology, and in the fabrication of

biosensor probes. In the future, development of techniques for the immobilization of multi-enzymes along with cofactor regeneration and retention system can be gainfully exploited in developing biochemical processes involving complex chemical conversions (D´Souza 1999).

According to Ribeiro *et al.*, (2011) the use of free lipase for biodiesel production results in technical limitations, and it is practically unreliable, due to impossibility of their recovery and reuse, which in turn increases the production costs of the process, besides promoting the product contamination with residual enzyme. These difficulties can be overcome by the use of these enzymes in its immobilized form, allowing the reuse of biocatalyst several times, reducing costs, and further improving the quality of the product (Ribeiro *et al*.,2011) .

Alzohairy and Khan (2010) claimed that, recent advances in the design of immobilization supporting materials with tailor able pore size and surface functionality has enabled more precise control of immobilization of enzymes. New simulations of the surface characteristics of the target enzymes can be used to aid in the design of appropriate support materials. As the structure and mechanism of action of enzymes becomes available more controlled immobilization methods will be generated. The same authors concluded that, the development of cheaper and disposable array biosensors, bioreactors and biochips for the simultaneous detection of clinically important metabolites and rapid screening of diseases has attracted much attention during the recent past. The use of more and more immobilized enzymes in clinical, biotechnological, pharmacological and other industrial fields has great promise among future technologies.

3. MATERIALS AND METHODS

3.1. Sampling sites

Modjo (also write out as Mojo) is a town in central Ethiopia, named after the nearby Modjo River. It is located in the East Shewa Zone of the Oromia Region. It has a latitude and longitude of 8°39′N 39°5′E with an elevation between 1788 and 1825 m above sea level. It is the administrative center of Lome woreda. Modjo is located at 96 km from Addis Ababa to the south east direction. Based on figures from the Central Statistical Agency in 2005, Mojo has an estimated total population of 39,316 of whom 19,278 were males and 20,038 were females (CSA, 2010).

Modjo is a town where the first synthetic textiles company was introduced in August 1966 to Ethiopia with a capital of 2.5 million Birr by Japanese. Starting from that time many industries were opened in Modjo such as abattoirs, textiles, tanneries, paints and food processing industries. From 26 leather industries in Ethiopia 7 of them (Ethiopia Tannery, Kolba Tannery, Gelan Tannery, Mesako Global Tannery, East Africa Tannery, Mojo Tannery, Friendship Tannery, Farida Tannery and Vasen United Tannery) are found in Modjo. All this industries are established following Modjo River for the removal of their wastes and Modjo Tannery is located at the upper catchment of this river.

3.2. Collection of Samples and Isolation of Bacteria

One of the main concerns of this study was to isolate and identify bacteria having a vital tendency to secrete extra-cellular proteolytic enzyme. In order to isolate biofilm forming bacteria, samples were taken from more than 50 years much accumulated sludge.

Figure 5: Samples from Modjo tanning industry; A/ sludge accumulation; B/ effluent; C/ soil sample D/ sludge

A total of six sludge samples in two rounds of upper layer 0-5 cm, middle layer 50-55 cm and 100 cm depth were collected from Modjo Tannery Industry using a sterilized falcon tube in icebox. The samples were transported to microbiology laboratory, Addis Ababa Science and Technology University. A 1.0 g of it was transferred into 10 ml sterilized distilled water in 250 ml conical flask and agitated at 100 rpm for 15 min in a water bath shaker adjusted at 37^{o}C. The soil suspension was then diluted serially up to 10^{-6} dilutions. A 0.1 ml of 10^{-2}, 10^{-3} and 10^{-4} dilution was spread plated into the Petri plate containing nutrient agar medium. The inoculated plates were then incubated at 37^{o}C for 24 hrs.

3.3. Screening for Protease Production

3.3.1. Primary Screening for Protease Production

Purified bacterial colonies on nutrient agar plates were screened for their proteolytic activity on skim milk agar medium supplemented with carbon and nitrogen sources. The inoculated plates were then incubated at 37^{o}C for 48 hrs and were observed for zones of clearance which were considered indicative of proteolytic activities of the bacterial isolates (Venugopal and Saramma, 2006).

3.3.2. Secondary screening for protease producing potent strains

Five isolates which had shown the potential to produce protease in the primary screening were selected based on the diameter of the zone of clearance, and further screened to select the most potent isolates among them based on incubation period, size of clearance zone and efficiency (the ratio of diameter of zone of clearance to colony diameter). These colonies were labeled as PMOJ-01, PMOJ-02, PMOJ-03, PMOJ-04 and PMOJ-05 respectively, where PMOJ stands for protease production by isolates from Modjo tanning industry.

3.4. Culture preservation

Isolates with larger diameter these were preserved on nutrient agar slants in refrigerator at 4^{0}C for subsequent studies.

3.5. Identification of Proteolytic Isolates

The bacterial isolates which showed prominent zones of clearance on skim milk agar medium were identified morphologically (spore, morphology, gram characteristics and motility) and biochemically (citrate utilization, triple iron sugar test, Urease, VP test, catalase, indole test starch hydrolysis and oxygen requirement) and then identified in accordance with the methods recommended in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and Manual for the Identification of Bacteria (Cowan and Steel, 1993; Betty *et al.*, 2002). The isolates were also grown at different temperatures, pH and NaCl concentrations. The identified strains were maintained on nutrient agar slants in refrigerator till further use.

3.5.1. Gram Staining

Cultures were grown on solid media for 24 hrs and suspended on a microscopic slide with 2-5 µl of distilled water to a slight turbidity. Smear was prepared by spreading the specimen with drop of distilled water, it was then dried completely. The underside of the slide was passed five times over the flame of a Bunsen burner to induce adherence. After all treatments were performed, the slide was dried and the cells were observed under the light microscope. Purple cells were gram positive and pink colored cells were gram negative (Betty *et al.,* 2002). Cellular morphology such as cell shape and arrangement was also determined.

3.5.2. Spore Staining Method

Isolated microbe was smeared onto a clean slide. Slides with microbes were air dried and then flooded with malachite green and allowed to stay on the boiling for 5 min followed by washing with water, and then the smear was counter stained with Safranin for 30 s. Then, the stained smear was observed under microscope using oil immersion objectives (Betty *et al.,* 2002).

3.5.3. Motility Test

Motility was tested in soft agar medium consisting (g/l) of Beef extract 5, Peptone 5, NaCl 15, Agar 3, pH 7.5 and 1000 ml distilled water. The medium was melted and poured into tubes in 3 ml aliquots and autoclaved for 15 min. Stab inoculated the medium and incubated at 37^{o}C for 24 to 48 hrs. Rhizoidal growth from the line of inoculation towards the peripheral area was

considered as the sign of motility. A thick growth along the line of inoculation was considered as negative.

3.5.4. Biochemical tests

3.5.4.1. Catalase Test

Three percent hydrogen peroxide solution was poured onto the PMOJ-01 and PMOJ-05 colonies grown on nutrient medium for 24-48 hrs. Formation of air bubbles indicated the presence of catalase enzyme (Betty *et al.,* 2002).

3.5.4.2. Oxygen Requirement (Fluid Thioglycollate Medium)

Fluid thioglycollate medium containing Pancreatic digest of casein 15.000, Yeast extract 5.000, Dextrose (Glucose) 5.500, Sodium chloride 2.500, L-Cystine 0.500, Sodium thioglycollate 0.500, Resazurin sodium 0.001, Agar 0.750, Final pH (at 25°C) 7.1±0.2 was boiled and cooled with loose screw cap tubes. The medium was inoculated with the organism using a wire loop. Finally it was incubated at optimum temperature (37^oC). Aerobic colonies were grown at the top of the media but facultative microbes were grown throughout the medium (Betty *et al.,* 2002).

3.5.4.3. Mannitol Test

Mannitol salt slant agar was inoculated with isolated bacteria (PMOJ-01 and PMOJ-05). Then the inoculated slant was incubated at 37^oC for 24 hrs. It determines the ability of a bacterium to ferment the sugar as well as its ability to convert end products (pyruvic acid) into gaseous by-products. Phenol red indicator is used to show acid fermentation (yellow below pH 6.8) or alkaline fermentation (red above pH 8.4) (Cowan and Steel, 1993).

3.5.4.4. Methyl Red Test

The pre-prepared MR-VP broths containing (g/l) Meat peptone 7, Glucose 5 and Phosphate buffer 5 was inoculated with PMOJ-01 and PMOJ-05 bacterial colonies. It was incubated at optimum temperature (37^oC) for 3-5 days. Five drops of a methyl red solution (0.04 g/60 ml abs. ethanol, pH 5.0) to a part of the Methyl red Voges Proskauer Broth (MR-VP) to determine the methyl red reaction. Red Color was developed for positive tests while negative tests were colored yellow (Cowan and Steel, 1993).

3.5.4.5. Triple Sugar Iron test

A Triple Sugar Iron agar slant containing Lactose, Sucrose and Glucose in the concentration of 10:10:1 (i.e. 10 part Lactose (1%), 10 part Sucrose (1%) and 1 part Glucose (0.1%)) was prepared. fresh (24 hrs) pure culture as an inoculation source was used. A single isolated colony was picked and lightly streaked the surface of the slant. A needle was the preferred sampling tool in order to limit the amount of cell material transferred to the triple sugar iron agar slant. It was incubated at 37^{o}C for 48 hours.

3.5.4.6. Starch Hydrolysis

Nutrient agar with the composition of Peptone 5.0g, Beef extract 5.0g, Yeast extract 1.0g, NaCl 15.0g, Soluble starch 10.0g, Agar 15g, pH 7.5 in 1000 ml of Distilled water was used as basal medium for demonstrating amylase production. The test medium was prepared according to the above composition, autoclaved and poured into plates. The plates containing the medium were spot inoculated with the test organism and incubated until good growth was obtained at 37^{o}C for 24-72 hrs. The plates were then flooded with Gram's iodine solution (Iodine 1.0g, KI 2.0g and 100 ml Distilled Water).

3.5.4.7. Tryptophan Hydrolysis / Indole Test

Tryptone broth was inoculated with a small amount of a pure culture grown on nutrient agar. It was incubated at 37^{o}C for 24 to 48 hours. 5 drops of Kovác's reagent was added directly to the tube to test for indole production. A positive indole test was indicated by the formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent (Cowan and Steel, 1993).

3.5.4.8. Urease Test

A heavy inoculum from a 24 hours pure culture was used to streak the entire slant surface of urea agar containing Yeast extract 0.1, Dipotassium hydrogen phosphate 9.5, Potassium dihydrogen phosphate 9.1, Urea 20.0, Phenol red 0.01 and incubated overnight at 37^{o}C. The yellow colour of the phenol red in the agar changed into pink red colour due to the degradation of urea so that the pH increase changed the colour of the phenol red (Betty *et al.*, 2002).

3.5.4.9. Citrate Utilization

A fresh (24 hrs) pure culture as an inoculation source was used. A single isolated colony was picked and lightly streaked the surface of Simmons citrate agar slant containing(g/L) Magnesium sulphate 0.200, Ammonium dihydrogen phosphate 1.000, Dipotassium phosphate 1.000, Sodium citrate 2.000, Sodium chloride 5.000, Bromothymol blue 0.080, Agar 15.000 and final pH (at 25°C) 6.8±0.2. A needle was used to transfer cell material into the agar slant. It was incubated at 37^{o}C for 48 hrs. Citrate positive colonies were grown on the slant surface and the medium was an intense Prussian blue (Cowan and Steel, 1993).

3.5.4.10. Nitrate Test

Nitrate broth was boiled and cooled with loosen screw cap. A fresh (24 hrs) pure culture of isolates was inoculated and incubated at 37^{o}C for 24 hrs. the color change was analyzed after the addition of five drops of nitrate reagent A (sulfanilic acid) and five drops of nitrate reagent B (dimethyl alpha naphthalamine) to tube.

3.6. Preservation of Isolates

Glycerol stocks were prepared for long term preservation. Pure cultures grown on Nutrient agar were transferred in Nutrient broth until turbidity formed. Each culture broth (0.5 ml) was transferred into the test tubes and 0.5 ml broth containing 40% glycerol was added. Tubes were mixed gently and thoroughly and were stored at -20 °C in deep freezer (Frankena *et al*., 1985).

3.7. Characterization of Protease producing isolates

3.7.1. Effect of NaCl on microbial growth

The effect of NaCl concentration on the bacterial growth was analyzed by inoculating the organisms to nutrient broth having 0, 1, 2, 3, 4, 5%, 6 and 7% sodium chloride concentration.

3.7.2. Effect of temperature on microbial growth

The effect of temperature on growth was studied by inoculating the organisms in to nutrient broth and incubated at varying temperatures (4^{o}C, 25^{o}C, 35°C, 45°C, 55°C and 65°C) for 24 hrs.

3.7.3. Effect of pH on microbial growth

The effect of pH on growth was studied by inoculating the 24hrs old pure culture in nutrient broth having varying (pH 5, 6, 7, 8, 9 and 10). The cultures were incubated at 37^{o}C for 24 hrs.

3.8. Preparation of crude enzyme

The preserved cultures were inoculated onto nutrient agar slants to activate and incubated for 24 hrs and after incubation; the cells were harvested into sterile saline. Loopful of culture was added to 50 ml of nutrient broth in 250 ml conical flask and incubated at 37°C on a rotary shaker at 100 rpm for 24 hrs. 5% of it was taken for the actual fermentation. The actual fermentation was carried out in 250 ml Erlenmeyer flask containing 50 ml of the skim milk medium inoculated with 5% of the inoculum and incubated at 37ºC and 100 rpm for 48 hrs. After incubation, five ml samples were drawn from the 50 ml culture to determine growth, and the remaining culture broth was centrifuged at 6,000 rpm for 30 minutes at 4°C to remove the cells. The presence of protease enzyme in the crude was confirmed after clear zone formation from a drop of supernatant on a hole formed from skim milk agar within 24 hrs incubation at 37°C. This was assayed for protease activity.

3.9. Determination of Total Protein Content

A 500 µl of samples (cell-free supernatant of PMOJ-01 and PMOJ-05) were diluted to 5 ml distilled water so that the protein content would be within the range of the standards. A 5 ml Lowry reagent D solution was added to each tube and mixed well. The solutions were kept at room temperature for 10 min followed by the addition of 0.5 ml Folin and Ciocalteu's Phenol reagent working solution. Each tube was rapidly mixed, and incubated in dark for 30 min. Absorbances of the samples were measured at 660 ηm using spectrophotometer.

3.10. Protease Assay

3.10.1. Tyrosine standard graph

Tyrosine standard graph was prepared to estimate the unknown quantity in the experimental samples. In this assay, casein acts as a substrate. When the protease we are testing digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteu's Phenol or Folin's reagent primarily reacts with free tyrosine to produce blue colored

chromophores, which is quantifiable and measured as an absorbance value on the spectrophotometer. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. From the standard curve the activity of protease samples can be determined in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute.

For protease assay, the method adopted by Takami *et al.,* (1989) was used. Tyrosine standard solution, in the range of 0.2 mg/ml was prepared to obtain a standard curve. Five test tubes containing a volume of 0.05 ml, 0.10 ml, 0.20 ml, 0.40 ml, and 0.50 ml of standard solution was prepared but not standard blank. Then it was made up to 2 ml using distilled water including blank. Finally, one ml of Folin and Ciocalteu's reagent was added after the addition of 5 ml of Na_2CO_3 to each vial. After 30 minutes incubation at 37^oC, the blue colour developed was measured at 660 ηm using UV visible spectrophotometer (biochrom /libra S6, England).

3.10.2. Determination of Proteolytic Activity

Protease activity was determined by applying a modified form of the method given by Takami *et al.,* (1989). According to this procedure, 5ml of 0.65% casein taken from pH 7.5 (50 mM Potassium Phosphate buffer) equilibrated solution. The culture filtrate which was prepared after centrifugation of crude enzyme in skim milk broth serves as the source of enzyme. An aliquot of 1 ml, 0.7 ml, 0.5 ml and 0 ml for blank of the enzyme solution was added to this mixture and incubated for 10 minutes. The reaction was stopped by adding 5 ml TCA (tris-chloro acetic acid). Then 0 ml, 0.5 ml, 0.3 ml and 1 ml of enzyme solution were added to respective test tubes. After 30 more minutes' incubation at 37^OC, the whole mixture was centrifuged at 6000 rpm for 20 minutes. A 2 ml of the supernatant was mixed with 5ml of 500 mM sodium carbonate solution (Na_2CO_3) and 1ml of Folin-Ciocalteu's Phenol solution and was kept for 30 minutes at room temperature. The optical densities of the solutions were read against the sample blanks at 660 ηm using spectrophotometer. Readings were completed within 30 minutes. One protease unit (U) was defined as, the enzyme amount that could produce 1µg of tyrosine in one minute under the defined assay conditions.

3.11. Partial Purification of Protease Enzyme

A 10ml cell free enzyme solutions (supernatants) after centrifugation from crude enzyme were precipitated by adding ammonium sulphate at 80% saturation levels. After addition of the salt, the enzyme solutions were stirred for 1 hour at 4^{o}C. The precipitated proteins were collected by centrifugation at 6,000 rpm for 30 min at 4^{o}C. These precipitates in the form of pellets were re-dissolved in 10 ml volume of 0.05 M Tris-HCl buffer, pH 7.5 to get the concentrated enzyme suspension.

3.12. Characterization of Protease activity

Optimal conditions required for maximum protease activity by the two test isolates were determined by subjecting them to various incubation temperatures and pH.

3.12.1. Effect of pH on protease activity

The effect of pH on enzyme production was studied by inoculating the organisms in nutrient broth supplemented with 1% skim milk, having varying pH (pH 6, 7, 8, 9 and 10). The cultures were incubated at 37^{o}C for 12 hrs and enzyme production was measured using 660ηm spectrophotometer.

3.12.2. Effect of temperature on protease activity

Effect of temperature on enzyme production was determined by inoculating the cultures in nutrient broth supplemented with skim milk and incubating them at different temperatures (25, 35, 45, 55 and 65°C) and enzyme production were determined after 12hrs of incubation using 660ηm spectrophotometer.

3.13. Experiments to Evaluate Commercial Applications of Proteases

3.13.1. Removal of Stain

The application of proteases for removing blood stains were observed by the method of Najafi *et al*., (2003). A clean piece of pure white cotton cloth was flooded in 100% human blood for 15 min and then allowed to dry at 80^{o}C for 5 min in dry oven. The dried cloth was cut into equal sizes (4 x 4 cm^{2}) and soaked in 10 ml of the enzyme solutions. The soaked pieces were incubated at 37^{o}C for 30 min. After a given incubation, the cloth pieces were rinsed with tap water for a minute without scrubbing and then dried in open air. The same procedure was applied for the control but in the absence of enzyme solution.

3.13.2. De-hairing Effect

De-hairing property of proteases was observed following the method of (Wang *et al.,* 2007). Salted sheep skin was obtained from local market, washed with distilled water and cut into small pieces (5 x 5 cm^{2} sizes). The skin pieces were soaked in 10 ml of crude protease produced by two isolates (PMOJ-01 and PMOJ-05) and incubated at 37^{o}C in shaker at agitation speed of 100 rpm for 30 min. After required period, the skin pieces were observed. The dehairing efficiency was assessed according to the depilated area of the skin and the quality of the de-haired skin was estimated according the physical appearance observed by naked eyes. The de-haired skin with high quality showed clear grain structure and no collagen damage. The result of dehairing by the protease enzyme was compared with dehairing effect of 3% of sodium hydroxide solution (lime).

3.14. Method of data analysis

The data obtained for efficiency and size of clear zone were calculated by Microsoft excel. Statistical analyses of microbial load and enzyme characterization were performed using SPSS version 20 in order to setup mean, standard deviation and standard error of the laboratory result. Enzyme activity to some physicochemical factors comparisons with standard was done by one-way ANOVA of variance, LSD and tukey to determine significant difference of the enzyme at $p \leq 0.05$. Finally the results were displayed using graph.

4. RESULTS

4.1. Isolation and screening of protease-producing bacteria

A total of twenty different bacteria were isolated from the sludge samples collected from Modjo Tannery industry. Out of these only twelve of them were potent in clear zone formation in 24 hrs on 1.0 % skim milk agar as shown in the Figure 6 A. These bacteria were different in pigmentation, size, margin and elevation (Figure 6 B).

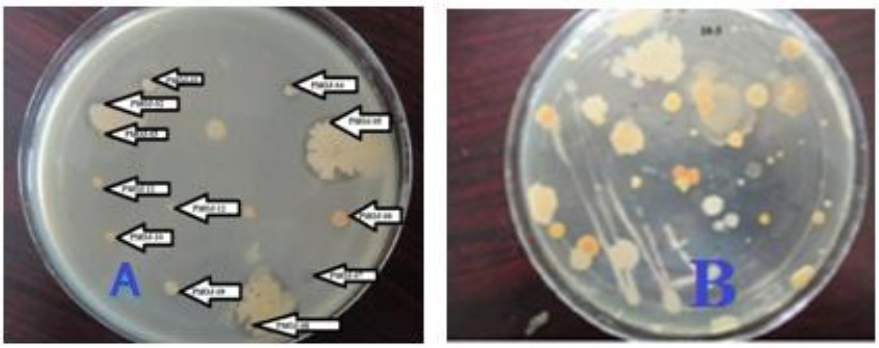

Figure 6: Clear zone formation by 12 potent bacteria over skim milk agar (A) and different bacterial isolates on nutrient agar (B)

After the primary screening bacteria only five bacteria were selected for secondary screening based on the size of clear zones (34, 21, 18, 13, and 27mm as shown in the Figure 7) which were positive for protease production in skim milk agar.

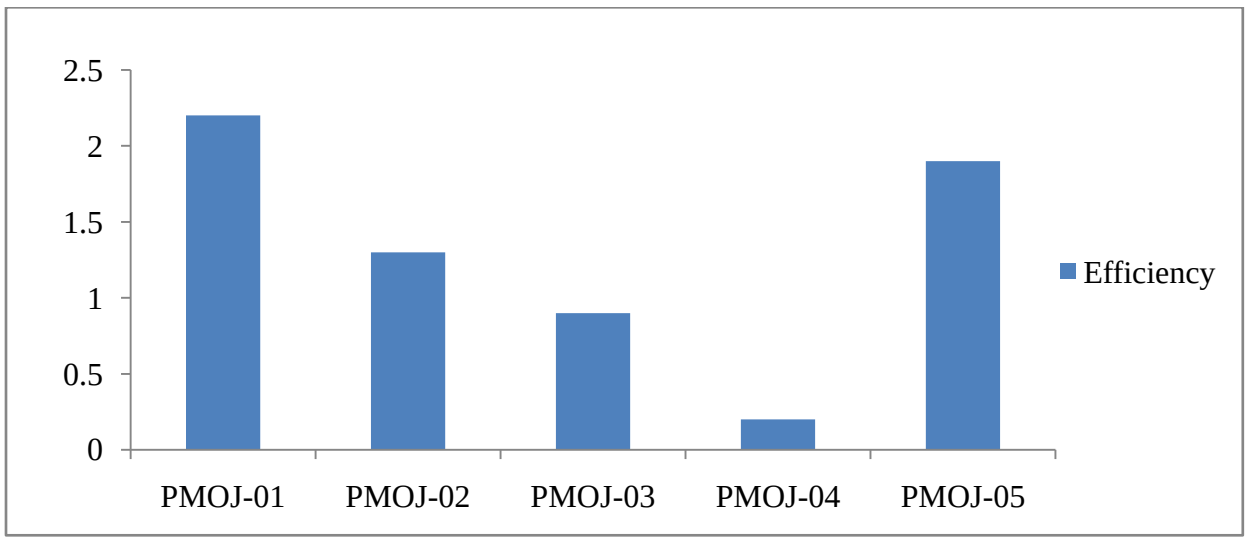

Figure 7: The efficiency of isolated bacteria on skim milk agar

The result of this study showed that all the five isolated bacterial groups were able to produce protease enzyme but isolate PMOJ-1 and PMOJ-5 recorded the highest production ability while isolates PMOJ-02, PMOJ-03 and PMOJ-04 had the least production ability as expressed by the diameter of clear zone observed for each isolate. Only two protease producing isolates were selected based on their incubation period, size of clearance zone (Figure 8) and efficiency (the ratio of diameter of zone of clearance to colony diameter) in 24 hrs.

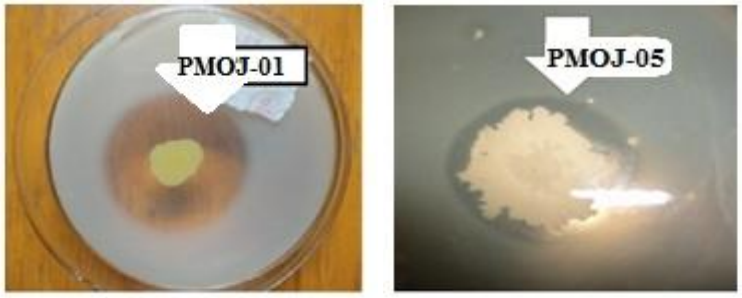

Figure 8: Skim milk agar hydrolysis by two best isolates: PMOJ-01 and PMOJ-05

4.2. Identification of Proteolytic Isolates

4.2.1. Morphological Identification

The first bacterial isolate (PMOJ-01) screened from sludge soil was found to be Gram positive and cocci shaped while the second bacterial isolate (PMOJ-05) was Gram positive and rod shaped (Annex 2 and 3). Morphologically PMOJ-01 was yellow pigmented, circular margin; concave shaped and smooth where as PMOJ-05 was white pigmented, irregular (partially circular), flat and pointed at middle of the colony (Figure 9).

Figure 9: Morphology of PMOJ-01 and PMOJ-05

4.2.2. Biochemical Identification

As indicated in table 6, PMOJ-01 was gram positive, cocci, facultative, non motile and gave positive results for Urease, Methyl red, Catalase and nitrate reductase (Table 6) tests and negative results for Citrate Utilization, H_2S, Indole, Vogues- Proskaeur (VP) tests. This means isolate PMOJ-01 can also produce Catalase, Urease and tryptophanase enzymes but not cystein desilferase enzyme while using Triple sugar iron agar as a carbon source.

Based on the results shown in Table 6, the strain PMOJ-05 was gram positive, rod shaped, spore forming, facultative, motile and gave positive results for Citrate Utilization, Methyl red, Indole, Vogues- Proskaeur (VP), Catalase and nitrate reductase (Table 6) tests and negative results for Urease, H_2S and Mannitol utilization. Therefore, it can produce enzymes such as catalase, formic hydrogenlyase, amylase and tryptophanase in addition to protease but not Urease and cystein desilferase. Isolate PMOJ-05 uses sodium citrate as a carbon source and ammonium salts as the nitrogen source.

Table 2: Biochemical Characterization of isolate PMOJ-01 and PMOJ-05

Culture	Isolate PMOJ-01	Isolate PMOJ-05
Colony pigment	yellow	white
Margin	Convex with an entire margin	Circular form and flat elevation
Gram reaction	+	+
Spore	-	+
Motility	-	+
Catalase	++	++
Oxygen requirement	facultative	facultative
Triple sugar iron test	+(very less)	+
MR	+	+
VP	-	+
Indole	-	+
Citrate	-	+
Mannitol utilization	-	-
Starch hydrolysis	-	+
Nitrate test	+(less)	+(less)
Growth in 0%NaCl	+	+
Growth in 2%NaCl	+	+
Growth in 5%NaCl	-	+
Growth at 0^0C	-	-
Growth at 20^0C	+	+
Growth at 37^0C	+	+
Growth at 65^0C	-	+(less)
Urease test	+	-
Identified as	*Micrococcus sp*	*Bacillus sp*

4.3. Characterization of bacterial isolates

4.3.1. Effect of Temperature on microbial density

Maximum cell density for *Micrococcus sp*. (PMOJ-01) and *Bacillus sp* (PMOJ-05) was observed when temperature was adjusted between 35 and 45^0C (Figure 10). The growth rate of both *Micrococcus sp* and *Bacillus sp* was high up to 45^0C with the highest Optical Density (OD) measurement of 0.429±0.001 and 0.488±0.004, respectively, but it declined thereafter. The least cell density was recorded at 4^0C with mean OD 0.142±0.002 and 0.127±0.007 for PMOJ-01 and PMOJ-05 respectively.

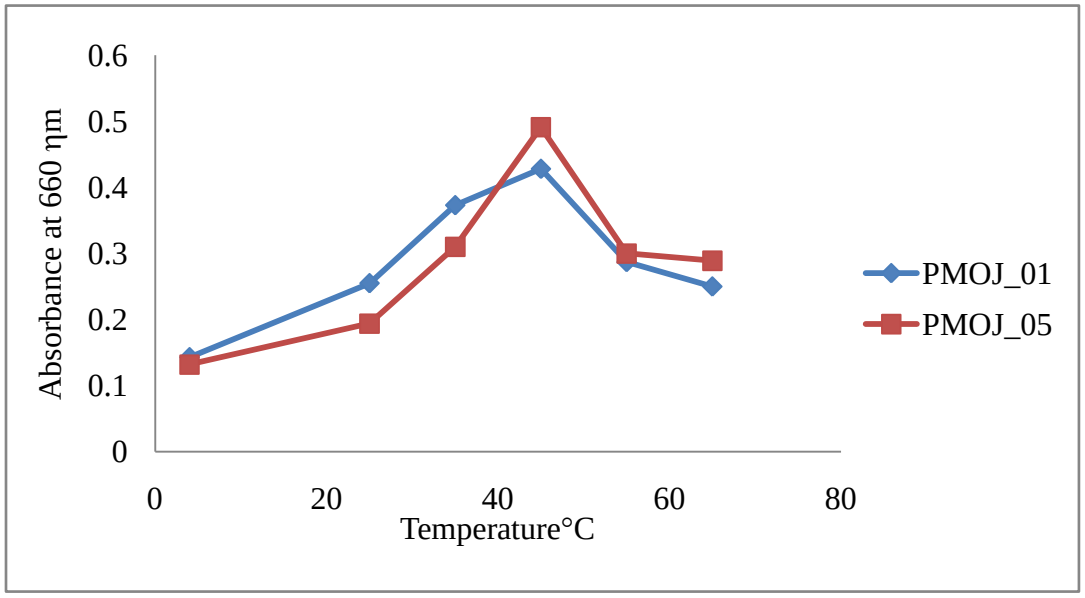

Figure 10: Effect of temperature on microbial growth

4.3.2. Effect of pH on Microbial Density

The two potent bacterial isolates, PMOJ-01 and PMOJ-05, were inoculated into nutrient broth of different pH values and the optical density of each culture was measured at 660 ηm absorbance. The least result of PMOJ-05 was observed at 10 with 0.096±0.005 OD, and the highest result was recorded at pH 7 with 0.899±0.013 OD where as isolate PMOJ-01 showed high growth at pH 8 with 0.454±0.001 OD and least growth at pH 10 with mean OD 0.137±0.001. Isolate PMOJ-01 grew over a broader pH ranges (pH 6-9) since the OD values recorded over the specified pH range were relatively the same unlike that of isolate PMOJ-05 (Figure 10).

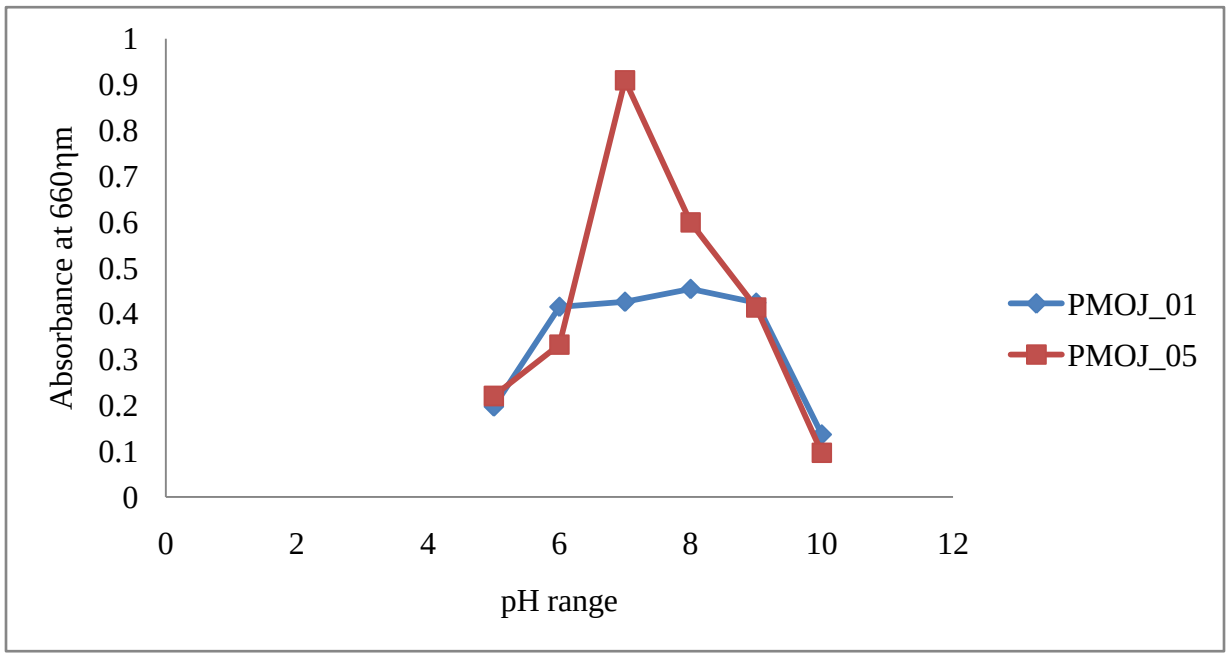

Figure 11**:** Effect of pH on growth pattern of isolates at 37^0C

4.3.3. Effect of NaCl concentration on microbial density

Effect of sodium ions on protease production and growth of PMOJ-01and PMOJ-05 were studied by amending the cultivation medium with different concentrations (w/v) of NaCl ranging from 0% to 7%. Maximum growth of PMOJ-01 was obtained at 1% NaCl with mean cell density (OD) of 0.71±0.035 but isolate PMOJ-05 with OD measurement of 1.31±0.035 was obtained at 2 % concentrations of NaCl. A decrease in growth was observed with further increase of NaCl concentration. Hence, 1% and 2 % concentration (w/v) was considered optimum for the growth of PMOJ-01and PMOJ-05, respectively (Figure 12).

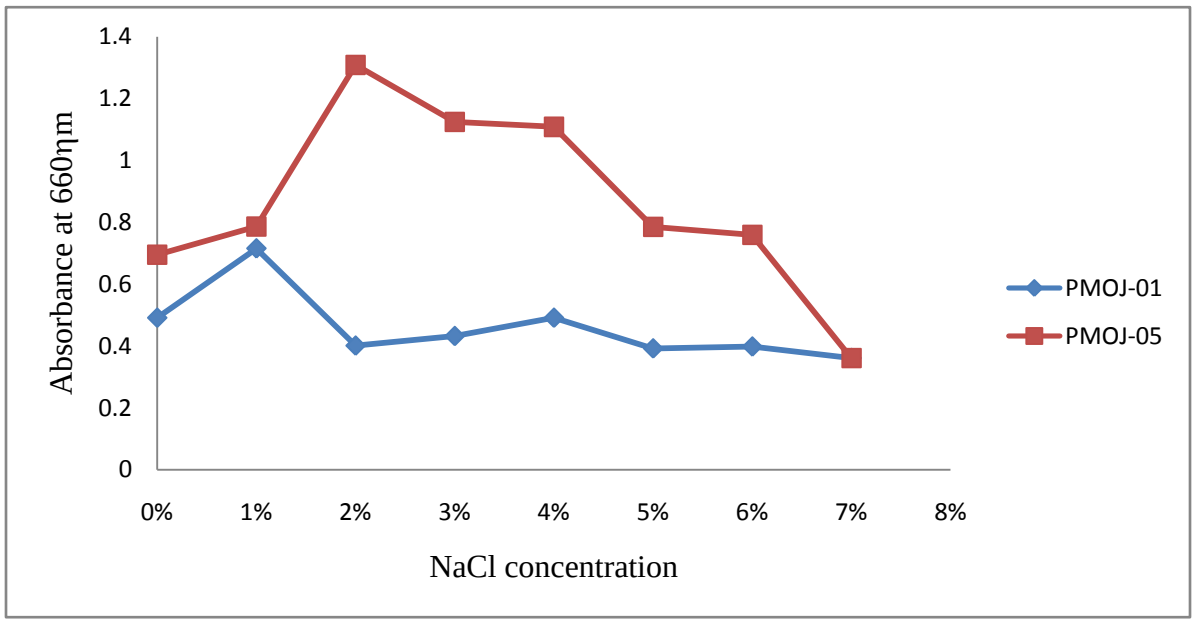

Figure 12: Growth pattern of the two potent isolates under different salt concentrations at 37°C

4.4. Protease production

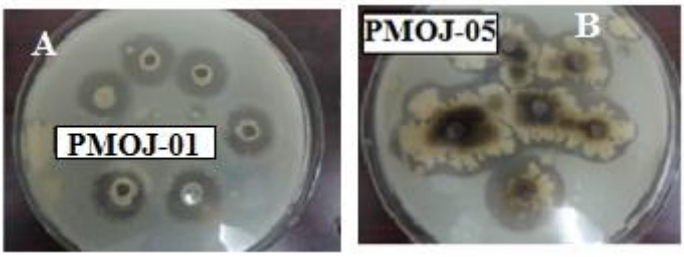

Figure 13: Clear zone formation as a result of hydrolysis of skim milk by protease (crude)

Figure 13 showed that the crude enzyme was obtained after appropriate temperature incubation of potent isolates of PMOJ-01 and PMOJ-05 in skim milk broth for 24 hrs (Figure 13). The effect of crude enzyme obtained from PMOJ-01 and PMOJ-05 (after centrifugation) was observed following hydrolysis of 1% skim milk agar after 24 hrs incubation at optimum condition.

4.5. Determination of Proteolytic Activity

The concentration for PMOJ-01 (*Micrococcus sp*) and PMOJ-05 (*Bacillus sp*) was 0.544 and 0.526 µmole and crude enzyme activity was calculated as 0.299 U/ml and 0.289U/ml, respectively (Table 7). Furthermore, enzyme activity of partially purified enzyme of PMOJ-01 and PMOJ-05 was 0.242 U/ml and 0.231 U/ml, respectively. The total solid protease sample of crude enzyme was 1.37 units/mg for PMOJ-01 (*Micrococcus sp.)* whereas 1.199 units/mg PMOJ-05 (*Bacillus sp)*. The total solid protease of partially purified enzyme of PMOJ-01 and PMOJ-05 was 2.05 U/mg and 11.55 U/mg respectively.

Table 3: Absorbance values of known concentrations of tyrosine at 660 ηm

Test tubes	volume of working standard	distilled water	Na_2CO_3	F.C	Concentration in µmole	OD of tyrosine standard solution
blank	0ml	2ml	5ml	1ml	0	0.201 ± 0.003^{f}
1	0.05ml	1.95ml	5ml	1ml	0.025	0.333 ± 0.001^{e}
2	0.10ml	1.90ml	5ml	1ml	0.05	0.410 ± 0.001^{d}
3	0.20ml	1.80ml	5ml	1ml	0.1	0.521 ± 0.014^{c}
4	0.40ml	1.60ml	5ml	1ml	0.2	0.923 ± 0.032^{b}
5	0.50ml	1.50ml	5ml	1ml	0.25	0.989 ± 0.002^{a}

4.6. Determination of protein content

The protein content in the crude enzyme of PMOJ-01(*Micrococcus sp)* and PMOJ-05(*Bacillus sp)* was 0.218 mg/ml and 0.241 mg/ml and partially purified enzyme was 0.118 mg/ml and 0.02 mg/ml respectively (Table 8).

Table 4: Absorbance values of known concentrations of Egg Albumin at 660 ηm

Test tube	volume of egg albumin	distilled water	Concentration (mg/ml)	Solution in ml	Lowery reagent D	F.C in ml	OD of protein standard solution
blank	0ml	5	0	0.5	5ml	0.5	0.206 ± 0.014^{f}
1	0.25ml	4.75	0.05	0.5	5ml	0.5	0.324 ± 0.031^{e}
2	0.5ml	4.5	0.1	0.5	5ml	0.5	0.340 ± 0.033^{e}
3	1ml	4	0.2	0.5	5ml	0.5	0.518 ± 0.405^{d}
4	2ml	3	0.4	0.5	5ml	0.5	0.615 ± 0.478^{c}
5	3ml	2	0.6	0.5	5ml	0.5	0.556 ± 0.433^{d}
6	4ml	1	0.8	0.5	5ml	0.5	0.926 ± 0.441^{b}
7	5ml	0	1	0.5	5ml	0.5	1.050 ± 0.441^{a}

4.7. Characterization on protease enzyme

4.7.1. Effect of pH on protease activity

The result showed that both organisms were affected by increment of pH as well as protease production varies with pH variation. Their maximum protease productions were at pH 7 and 8 with OD of 1.159±0.006 and 1.264±0.007 for *Bacillus* and *Micrococcus* respectively. The lowest protease activity in PMOJ 01 was at pH 10 (1.208±0.029) while PMOJ-05 was at pH 6 (0.969±0.035). The mean of optical densities was noted on the pH 6.0, 7.0, 8.0, 9.0, & 10.0 and thus confirmed the alkaline nature of PMOJ-05 protease enzyme and neutral protease enzyme of PMOJ-01.

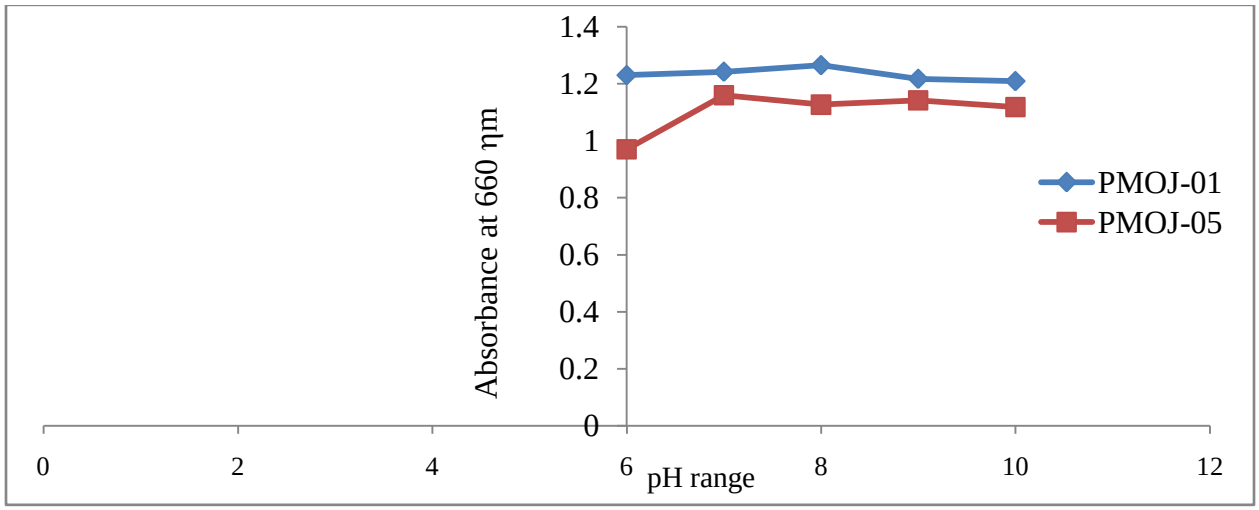

Figure 14: Effect of different pH on proteases activities produced by *Micrococcus* and *Bacillus*.

4.7.2. Effect of Temperature on protease activity

In this study temperature had effect on the production of protease in both organisms (Figure 17). There was an increase in protease production with increase in temperature up to 37^{o}C in *Micrococcus* (OD1.549±0.018). But, the protease production started decreasing thereafter. The same observation was made in *Bacillus* but the maximum protease production was obtained at 45^{0}C with maximum mean OD 1.512±0.068.

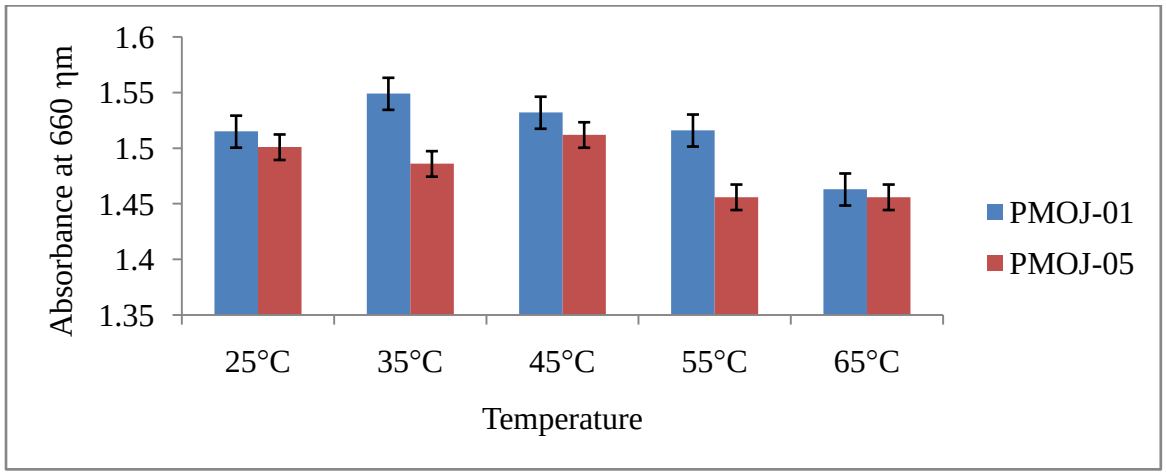

Figure 15: Effect of temperature on protease activities produced by *Micrococcus* and *Bacillus*

4.8. Partial Purification of protease

Ammonium sulphate precipitation was performed as the first step of protease purification of the enzyme (Figure 18). The results from the ammonium sulphate precipitated fraction of the selected proteases (PMOJ 01 and PMOJ-05) showed that the solid protease sample from PMOJ-01 had activity of 2.05 U/mg while PMOJ-05 had activity of 11.55 U/mg.

Table 5: Summary of results on extracted enzyme

isolate	**Crude enzyme**			
	Concentration in µmole	Enzyme activity (U/ml)	Protein content (mg/ml)	Solid enzyme (U/mg)
PMOJ-01	0.544±0.13	0.299 ±0.13	0.218±0.13	1.37±0.13
PMOJ-05	0.526±0.07	0.289±0.07	0.241±0.07	1.199±0.07
	Partially purified enzyme			
	Concentration in µmole	Enzyme activity	Protein content	Solid enzyme
PMOJ-01	0.44±0.036	0.242±0.036	0.118±0.036	2.05±0.036
PMOJ-05	0.42±0.086	0.231±0.086	0.020±0.086	11.55±0.086

4.9. Application of protease in industries

4.9.1. De-hairing effect

In the present de-hairing study, incubation of protease with sheep skin for 12 hrs showed removal of scum (Figure 19) and hair (Figure 20) very easily without affecting the skin quality as compared to skin treated with buffer only. However, the skin quality was damaged in chemically treated skin. The control skin that was incubated in distilled water, under identical conditions, showed no sign of Scum removal.

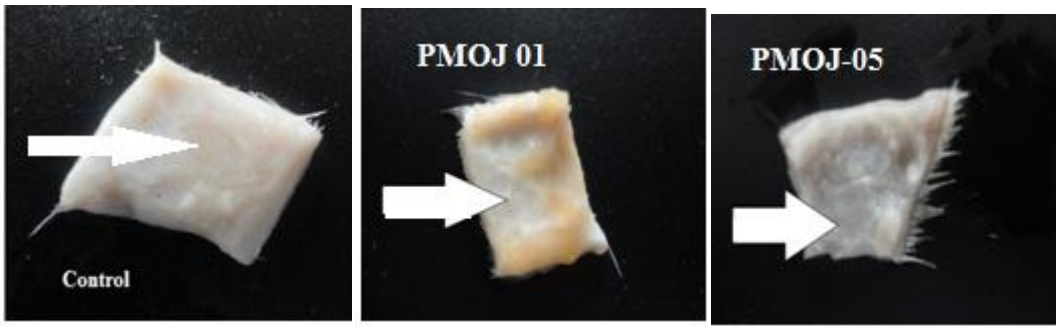

Figure 16: Scud removal effect of PMOJ-01 enzyme and PMOJ-05 enzyme after 12 hrs incubation

Figure 17: De-hairing effect PMOJ-01 enzyme and PMOJ-05 enzyme after 12 hours incubation

4.9.2. De-staining effect

In this finding the egg yolk which was spread and dried by oven was removed by enzymes of isolate PMOJ-01 and PMOJ-05 within 30 min, while the prepared control didn't show any type of de-colorization sign (Figure 21). Result of the blood removing study (Figure 22) revealed that protease from isolate PMOJ-05(*Bacillus)* is a promising additive for detergent industry.

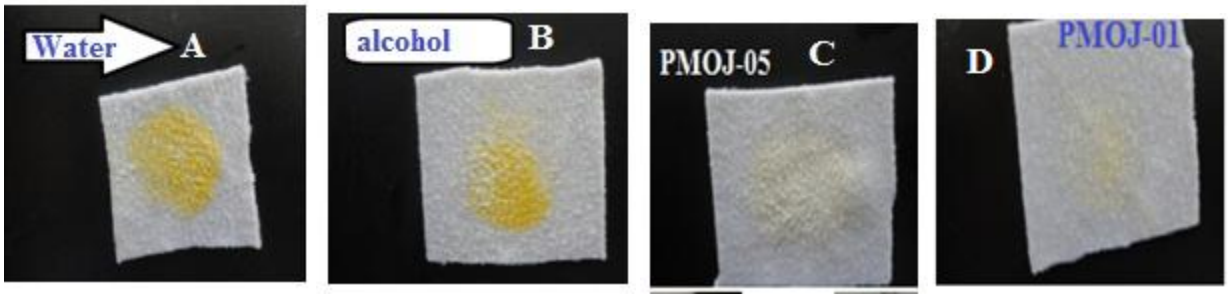

Figure 18: Effects distilled water (A), Alcohol (B), Enzyme of PMOJ-05 (C) and Enzyme of PMOJ-01(D) on prepared egg stained clothes after 30 minutes.

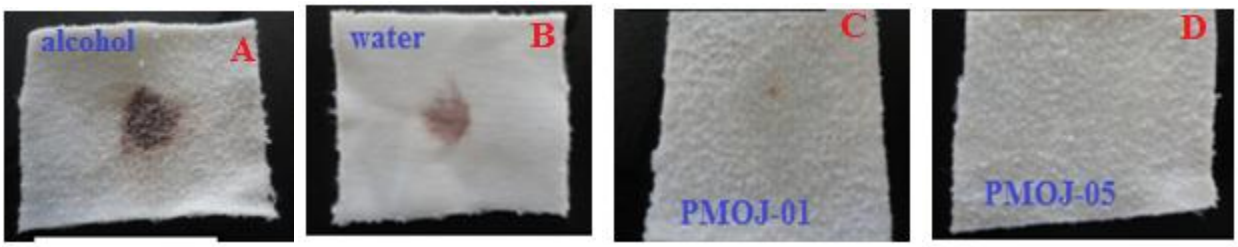

Figure 19: Effects alcohol (A), distilled water (B), enzyme of PMOJ-01(C) and enzyme of PMOJ-05 (D) on blood stained clothes after 30 minutes incubation.

5. DISCUSSION

A total of 20 different bacteria were isolated from the sludge samples collected from Modjo Tannery. Of these only twelve of them were potent in clear zone formation in 24 hrs on skim milk agar. According to Gupta *et al*., (2002), formation of clear zone on skim milk around the isolated colonies was considered as indication of good growth with the ability of extracellular protease production. Even if all these 12 isolates are proteolytic, they had different rate of protease production (clear zone diameter) in 24 hrs. The result revealed that five of the potent isolates, namely PMOJ-01, PMOJ-02, PMOJ-03, PMOJ-04 and PMOJ-05, formed clear zone diameter of more than 13 mm with in 24 hrs period. During screening, isolation and production of Protease from marine microbes, Guravaiah *et al*., (2010) reported that bacteria that form a large clear zone on skim milk agar were found highly proteolytic.

After evaluation of the entire morphological and biochemical test, the two most promising isolates in this study were identified as *Micrococcus* and *Bacillus*. Microbial proteases are produced from high yielding strains including species of *Bacillus* sp., *Alcaligenes faecalis, Pseudomonas fluorescens* and *Aeromonas hydrophilia* (Longo *et al*., 1999). Among these, *Bacillus* sp. is one of the most important groups of bacteria that are being used in the enzyme industry and this bacterium is also known to produce quite effectively proteolytic enzymes (Longo *et al*., 1999). As per the observation by Gopinath (2002), the predominant bacterial genera in tannery sludge were *Pseudomonas, Vibrio, Bacillus* and *Micrococcus*.

Most of the commercially important proteases are derived from *Bacillu*s. These bacteria are known for their abilities to secrete large amounts of proteases having significant proteolytic activity and stability at considerably high pH and temperatures (Pastor *et al.,* 2001). In another study concerned with the production of alkaline protease from soil samples, it was observed that 27 bacterial isolates out of 40 belonged to the genus *Bacillus* (Belma *et al*., 2002). In the current study, the predominant isolates were *Bacillus*, followed by *Micrococcus.* Amar (2001) obtained similar result, while studying the composition of microflora from tannery industries effluent.

It has been noted that microorganisms are dependent on the extra cellular temperature and pH for growth and enzyme production (Kumar and Takagi, 1999). Every organism possesses a minimal, optimum and maximum temperature for their growth and metabolism. Temperature below

optimum is bacteriostatic and above optimum is bactericidal. So, temperature is a critical parameter that has to be controlled in any fermentation. Proteases produced from *Bacillus* are known to be active over a wide range of temperature (Kumar and Takagi, 1999). In the present study evaluation of the effect of temperature on growth of the selected isolates (PMOJ-01 and PMOJ-05) showed that the maximum population was displayed at 45^0C, while the least cell density was recorded at 4^oC and 65^oC. While characterizing thermostable alkaline protease, Kaur *et al.,* (2001) observed that the optimum temperature for *Bacillus* species ranged from 30 to 80ºC. In addition, the enzyme from an obligatory alkaliphilic *Bacillus* P-2 showed an exceptionally high optimum temperature of 90ºC. The protease has also good thermostability at high temperatures, being thermostable at 90ºC for more than 1hour and retained 95 and 37% of its activity at 99ºC (boiling) and 121ºC (autoclaving temperature), respectively.

Majority of microorganisms which can synthesize protease favor alkaline conditions (Naidu and Devi, 2005). Guravaiah *et al*. (2010) stated that most of the *Bacillus* species grow in alkaline condition within the range of pH 8 to pH 11 even if there are other *Bacillus* species that prefers neutral conditions. Similar results were observed in the current study. The least result was recorded at pH 5 and 10 with 0.225±0.008 and 0.100±0.005 OD, respectively, while the highest result was at pH 7 with 0.899±0.013 OD for PMOJ-05 and pH 8 with 0.454±0.001 OD for PMOJ-01.

Salt concentration is another factor that affects microbial growth and enzyme production. In this study maximum growth of PMOJ-01 was obtained at 1% concentrations of NaCl with cell density of 0.71±0.035 but isolate PMOJ-05 with 1.31±0.035 cell density at 2 % concentrations of NaCl. Although the exact reason for the increasing protease production in the presence of sodium chloride is still unknown, it is well-known that sodium chloride at its optimum concentration level plays an important role in providing favorable osmotic environment for the growth of bacterial cells which might result in enhancement of protease production. Presence of sodium chloride enhanced the production of protease in *B. licheniformis* (Ferrero *et al.,* 1996). According to this report, maximum cell growth and enzyme activity was obtained at 0.05 % (w/v) of NaCl as compared to the control. The enhancing effect of sodium on bacterial protease production has been reported rarely. Chandrasekaran and Dhar (1983) observed the beneficial effect of sodium chloride on protease production by *Streptomyces moderatus* NRRL 3150.

Likewise, Shanmughapriya *et al.* (2007) reported the highest protease activity by a marine isolated *Roseobacter* sp at 3 % NaCl.

According to Quadar *et al*. (2009), the presence of crude protease enzyme can be indicated by forming clear zone on skim milk or casein agar after 24 hrs incubation. The partially purified protease enzymes are known for their significant proteolytic activity (Pastor *et al*., 2001). In the current study, the crude enzymes obtained from PMOJ-01 and PMOJ-05 after 24 hrs incubation and centrifugation were evaluated for its effectiveness in hydrolysis of 1% skim milk agar. The two cultures were found growing well in the medium by producing proteolytic enzyme. The result clearly demonstrated that the ability of PMOJ-01 and PMOJ-05 to produce protease enzyme and the proteolytic activity of its crude enzyme.

The enzyme activity of partially purified enzyme of PMOJ-01 and PMOJ-05 was 0.242 U/ml and 0.231 U/ml and the total solid protease of partially purified enzyme of PMOJ-01 and PMOJ-05 was 2.05 U/mg and 11.55 U/mg, respectively. According to Kumar (2002), the maximum enzyme activity at optimum temperature, pH and nutrient availability was 0.065 U/ml for *Bacillus pumilus* and 0.038 U/ml for *Staphylococcus auricularis*. Enzymes isolated from *Bacillus pumilus* and *Staphylococcus auricularis* showed efficient activity in leather processing and biofilm degradation. In relation to this, enzymes from the current isolates are much better in their activities.

The effect of pH on protease activities of our isolates was in agreement with related results reported by Gupta *et al*. (2002) on protease production, where the optimum temperature and pH of the partially purified protease of *Bacillus* species was found to be 40°C and pH 7. Likewise, the optimum pH for protease production in a *Flavobacterium* species was 7.4 (Morita *et al.*, 1998). On the contrary, protease production was best at pH 9 in *Bacillus polymyxa* (Madan *et al*., 2000). Optimum pH for protease production in various *Bacillus* species was in the range of 7 and 10 (Purva *et al*., 1998 and Kumar, 2002). Thangam and Rajkumar (2002) reported an optimum pH 8 for protease production by *Micrococcus.*

In this study, it was observed that temperature had effect on the production of protease in both isolates, with optimum temperature for protease activity of isolate PMOJ-05 being at 45^{o}C while that of isolate PMOJ-01 was at 37^{o}C. Such variation in temperature preference in the two

isolates, hence their enzyme activities, could make the two enzymes candidates for application under different setups. In related investigation, Odu and Akujobi (2012) also reported a similar trend in *Micrococcus luteus* and *Bacillus* species where the organism could produce protease in the range of 27-57^{o}C with production maximum at 37^{o}C. However, increase in temperature beyond 37^{o}C and 47^{o}C for *Micrococcus luteus* and *Bacillus* species, respectively, led to decline in protease production proving that temperature plays a major role in enzyme production. The actual mechanism by which temperature controls enzyme production is not well understood (Chaloupka, 1985). However, studies by Frankena *et al*. (1986) showed that a link existed between enzyme synthesis and energy metabolism in Bacilli, which was controlled by temperature and oxygen uptake.

Wide variation occurs in the optimum temperature of enzyme production from organism to organism. As reported by Hare *et al*. (1981), alkaline protease production in *Vibrio alginolyticus* was maximum at 30°C and its yields decreased above this temperature. In a *Flavobacterium* species optimum temperature was found to be as low as 10°C (Morita *et al*., 1998) while it was 30°C and 36°C for two *Serratia marcescens* strains (Longo *et al*., 1999; Romero *et al.,* 2001). For different *Bacillus* sp. the optimum temperature ranged from 30°C to 50°C (Romero *et al.,* 2001; Longo *et al*., 1999 and Kumar, 2002). However temperature was not a limiting factor for protease production by a dairy strain of *Aeromonas hydrophila* (Santos *et al.,* 1996).

Precipitation is the most commonly used method for the isolation and recovery of proteins from crude biological mixtures (Bell *et al*., 1983). It performs both purification and concentration steps. It is generally affected by the addition of reagents such as salt or organic solvent, which lowers the solubility of the desired proteins in the aqueous solution. In the present study, 80% saturation of ammonium sulphate has been used. Protease purification was monitored at various stages by enzymatic assay. A number of alkaline proteases have been purified and characterized by $(NH_4)_2SO_4$ precipitation from different sources including *Bacillus* species (Kumar *et al*., 1999); *Bacillus subtilis* (Laeur *et al*., 2000 ; Singh *et al*., 2001a) and *Bacillus pumilus* (Kumar, 2002). The results from the ammonium sulphate precipitated fraction of the selected proteases (PMOJ 01 and PMOJ-05) in the present study showed that the solid protease sample from PMOJ-01 had activity of 2.05 U/mg while PMOJ-05 had activity of 11.55 U/mg. Comparable results were reported for *Streptomyces* sp. G 15, where the specific activity after the first step of purification was 11 U/mg (Sampath *et al.,* 1997). In a related investigation, protease from

Aeromonas hydrophila was reported to have specific activity of 3 and 2 fold purification was obtained after ammonium sulphate precipitation (Pansare *et al*., 1986). Aderibigbe *et al* (1990) reported proteases from strains of *B. subtiIis*, which obtained 0.514 fold purification by ammonium sulphate precipitation. Another strain of *B. subtilis* had specific activity of 3.88U/mg and 4-fold purification after $(NH_4)_2SO_4$ precipitation (Yang *et al*., 2000). However, in *Aeromonas proteolytica* protease, the specific activity of the culture filtrate was 15 and that of ammonium sulphate precipitated fraction was 43 with 76% yield (Griffin and Prescott, 1970).

Leather processing industries generate a lot of toxic pollutants, such as sulphide and chromate, which are found to be detrimental to the environment. There are many reports of dehairing of goat/bovine skin by employing purified/semi purified Protease (Thangam *et al.,* 2001). The scum removal activity of protease created interest of researchers because of significant reduction in toxicity, in addition to improvement of leather quality compared to chemical methodology (Sunderaranjan *et al.,* 2011). In support of the above observations, the current study confirmed the scum removal activity of protease from the isolates with promising potential application in the leather industry. Furthermore, blood removing study revealed that protease from PMOJ-05 (*Bacillus)* was a promising additive for detergent industry in agreement with the well established practices reported by many authors including Nadeem *et al*. (2008). They studied the high capacity of blood stain removal by *Bacillus licheniformis* N-2 and *Bacillus subtilis.* Interestingly, our result indicated that the proteolytic enzyme produced by the isolates were even better than some of the commercially available enzymes. Proteases widely used in the detergent industry remove protein stains such as grease, blood, egg and human sweat which have a tendency to adhere strongly to textile fibers (Kumar, 2002).

The overall importance of this study was to reduce environmental pollution by substituting chemicals with protease enzyme that is secreted from sludge isolated bacteria and introducing enzyme technology against toxic chemicals in leather and detergent industries.

6. CONCLUSION

- The two bacterial strains (PMOJ-01 and PMOJ-05), isolated from Modjo tannery wastes, can produce extracellular protease.
- Based on their morphological and physiological properties, the two isolates were tentatively identified as *Bacillus* and *Micrococcus*.
- The optimum growth conditions for PMOJ-01was 45^0C in the presence of 1% NaCl while PMOJ-05 grow best at 45^0C at 2% NaCl concentration indicating that both isolates are mesophilic. Furthermore, the protease enzymes of these isolates were found active over a broad temperature range of 25-55ºC with the optimum temperature for protease activity of isolate PMOJ-05 being at 45ºC.
- pH profiles of PMOJ-01 and PMOJ-05 revealed that PMOJ-01 has a much broader tolerance to wider pH range than PMOJ-05 with optimum pH of 8 and 7 for PMOJ-01 and PMOJ-05, respectively. Enzymes extracted from the two isolates were found to be a typical neutral and alkaline protease, displaying their activity for casein predominantly in the region of pH 7.0-10.0.
- Protease was partially purified by employing standard protein purification procedures of ammonium sulphate followed by dialysis
- The bacterial strains PMOJ-01 and PMOJ-05 are a potent source for protease enzyme. Leather making with the help of protease enzyme could produce leather with maximum softness and eliminates the use of pollution causing chemicals such as sodium, lime and solvents. It is, therefore, reasonable to assume the promising nature of this enzyme for commercial applications in detergent industry since they are able to degrade animal blood and egg albumin in 30 minutes.

7. RECOMMENDATION

- Further evaluation need be made at species level as extracted DNAs of both PMOJ-01 and PMOJ-05 were stored in Addis Ababa Science and Technology University, Biological and Chemical Science Laboratory.
- The protease from PMOJ-01 and PMOJ-05 is significant for an industrial perspective because of its ability to function in broad pH and temperature ranges. The enzymatic properties of the protease also suggest its suitable application as additive in detergent formulations. Thus, further evaluation must be undertaken on its compatibility with various commercial detergents.
- Enzymes isolated from PMOJ-01 and PMOJ-05 showed efficient activity in dehairing process, discoloration of animal's blood and Egg Albumin. These isolates and purified proteases enzymes may further be exploited for various industrial applications.
- More parameters must be undertaken to optimize protease production including carbon sources, nitrogen sources, metal ions, surfactants and oxidants on stability of proteases.
- The partially purified protease needs further purification and characterization through SDS-PAGE and Gel electrophoresis for further research.
- Dehairing of hides is an important and unavoidable step involved in the processing of leather in the tanneries where implementation of new technology is necessary. The conventional lime sulfide method employed for this purpose must be replaced by protease and further thermostable protease to avoid chemical application.
- Leather and leather industry have been part of economy of many developed countries and many of the developing countries are earning foreign currency and creating job opportunities. Though it is part of economy of many countries, leather processing is one of the most important industrial processes that contribute to pollution as the conventional methods result in damage to the environment. Therefore, these industries need to practice the current approach or technology.

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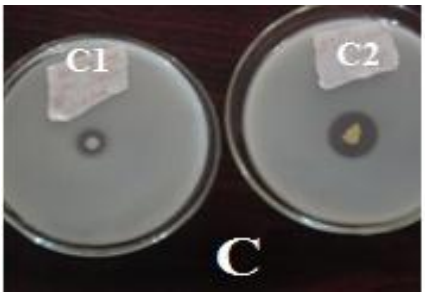
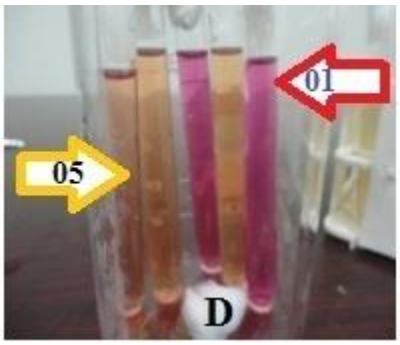
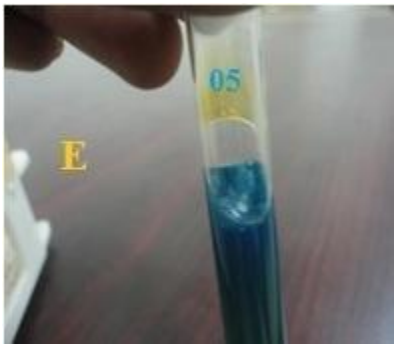
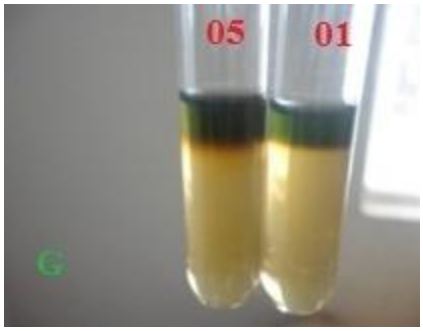
ANNEXES

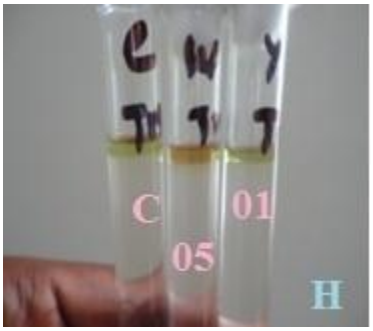
Annex 1: Images of morphological and biochemical tests of potent isolates of PMOJ-01 and PMOJ-05

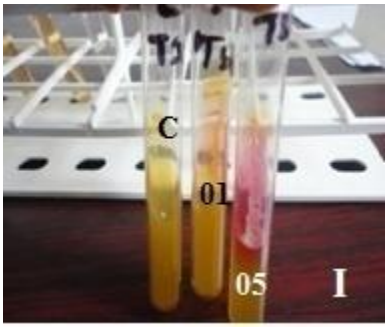


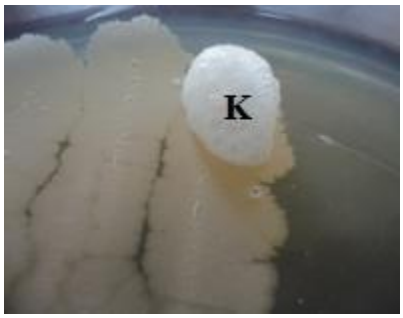


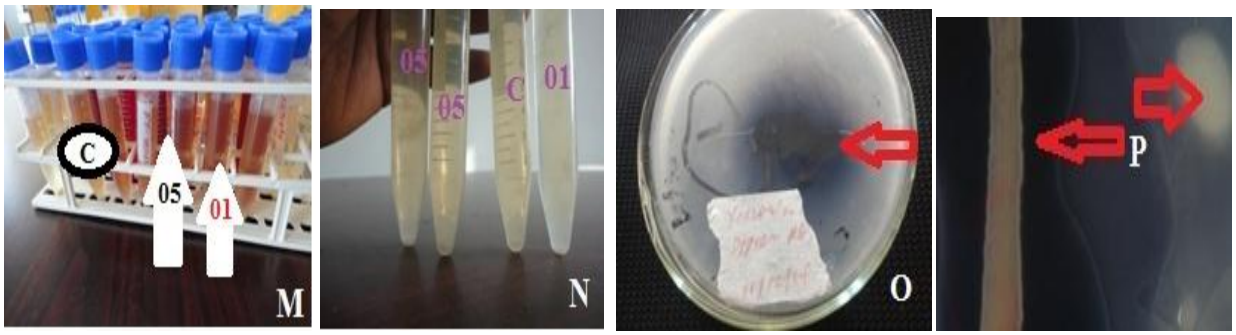












Where; C) skim milk agar hydrolysis by PMOJ-01(C_2) and PMOJ-05(C_1); D) Urease test of PMOJ-01(01) and PMOJ-05(05); E) Citrate utilization by PMOJ-05; F) Citrate utilization by PMOJ-01; G) Voges Proskauer test PMOJ-05 and PMOJ-01; H) Indole test of PMOJ-05 and PMOJ-01; I) Triple sugar iron test PMOJ-05 and PMOJ-01; J) Methyl red test of PMOJ-05 and PMOJ-01; K) catalase test of PMOJ-05; L) catalase test of PMOJ-01; M) Nitrate test of PMOJ-05 and PMOJ-01; N) Motility test of PMOJ-05 and PMOJ-01; O) Starch test of PMOJ-01; P) Starch test of PMOJ-05;Q) oxygen requirement.

Annex 2: Tables on medium preparation

Table 1: Composition (g/l) of the skim milk agar medium

No	Ingredient	Amount (g/ml)
1	Skim milk powder	10.0
2	Peptone	1.0
3	NaCl	5.0
4	Agar	15.0
5	Distilled water	1000

Table 2: Composition of nutrient broth for motility test

Ingredient	Amount (g/ml)
Beef extract	5.0
Peptone	5.0
Agar	3.0
NaCl	15
pH	7.5
Distilled water	1000

Table 3: Composition of starch agar

Ingredient	Amount (g/ml)
Peptone	5.0
Beef extract	5.0
Yeast extract	1.0
NaCl	15.0
Soluble starch	10.0
pH	7.5
Agar	15
Distilled water	1000

Annex 3: Tables on characterization of PMOJ-01 and PMOJ-05, efficiency of five potent proteolytic isolates on clear zone formation

Isolates	Diameter of clear zone in mm	Diameter of colony in mm	Efficiency
PMOJ-01	34	12	2.2
PMOJ-02	21	08	1.3
PMOJ-03	18	09	0.9
PMOJ-04	13	11	0.2
PMOJ-05	27	08	1.9

Annex 4; characterization of PMOJ-01 and PMOJ-05 through microbial density by different parameters

Effect of different temperatures on microbial growth

Temperature(°C)	**Mean of OD of microbial density**	
	PMOJ-01	PMOJ-05
4	0.142 ± 0.002^{h}	0.127 ± 0.007^{i}
25	0.256 ± 0.001^{f}	0.189 ± 0.006^{g}
35	0.374 ± 0.001^{c}	0.312 ± 0.003^{d}
45	0.429 ± 0.001^{b}	0.488 ± 0.004^{a}
55	0.289 ± 0.003^{e}	0.293 ± 0.009^{e}
65	0.253 ± 0.004^{f}	0.289 ± 0.001^{e}

Effect of different pH on cell density after 24 hrs incubation at 37^{o}C

pH	**Mean of OD of microbial density**	
	PMOJ-01	PMOJ-05
5	0.194±0.004^{g}	0.225±0.008^{f}
6	0.415±0.000^{d}	0.336±0.006^{e}
7	0.424±0.002^{d}	0.899±0.013^{a}
8	0.454±0.001^{c}	0.602±0.004^{b}
9	0.424±0.001^{d}	0.406±0.009^{e}
10	0.137±0.001^{h}	0.100±0.005^{i}

Effect of different NaCl concentration on cell density after 24 hrs incubation at 37^{o}C

Concentration of NaCl (%)	**OD of proteolytic isolates**	
	PMOJ-01	PMOJ-05
0	0.49±0.014^{e}	0.69±0.021^{d}
1	0.71±0.035^{c}	0.78±0.028^{c}
2	0.40±0.007^{e}	1.31±0.035^{a}
3	0.43±0.007^{e}	1.12±0.028^{b}
4	0.49±0.028^{e}	1.11±0.014^{b}
5	0.39±0.057^{f}	0.78±0.035^{c}
6	0.40±0.035^{e}	0.75±0.057^{b}
7	0.36±0.021^{f}	0.36±0.028^{f}

Annex 5: statistical data on protease characterization

Effect of different pH on protease after 24 hrs incubation at 45^{o}C

pH	**Mean of OD of protease activity**	
	PMOJ-01	PMOJ-05
6	1.229±0.035^{c}	0.969±0.035^{g}
7	1.241±0.004^{b}	1.159±0.006^{e}
8	1.264±0.007^{a}	1.126±0.009^{f}
9	1.216±0.009^{d}	1.141±0.070^{e}
10	1.208±0.029^{d}	1.117±0.029^{f}

Effect of different temperature on protease after 24 hrs incubation

Temperature (°C)	**Mean of OD of protease activity**	
	PMOJ-01	PMOJ-05
25	1.515±0.004^{c}	1.501±0.028^{c}
35	1.549±0.018^{a}	1.486±0.052^{d}
45	1.533±0.044^{b}	1.512±0.068^{c}
55	1.516±0.041^{c}	1.456±0.047^{e}
65	1.463±0.086^{f}	1.456±0.069^{e}

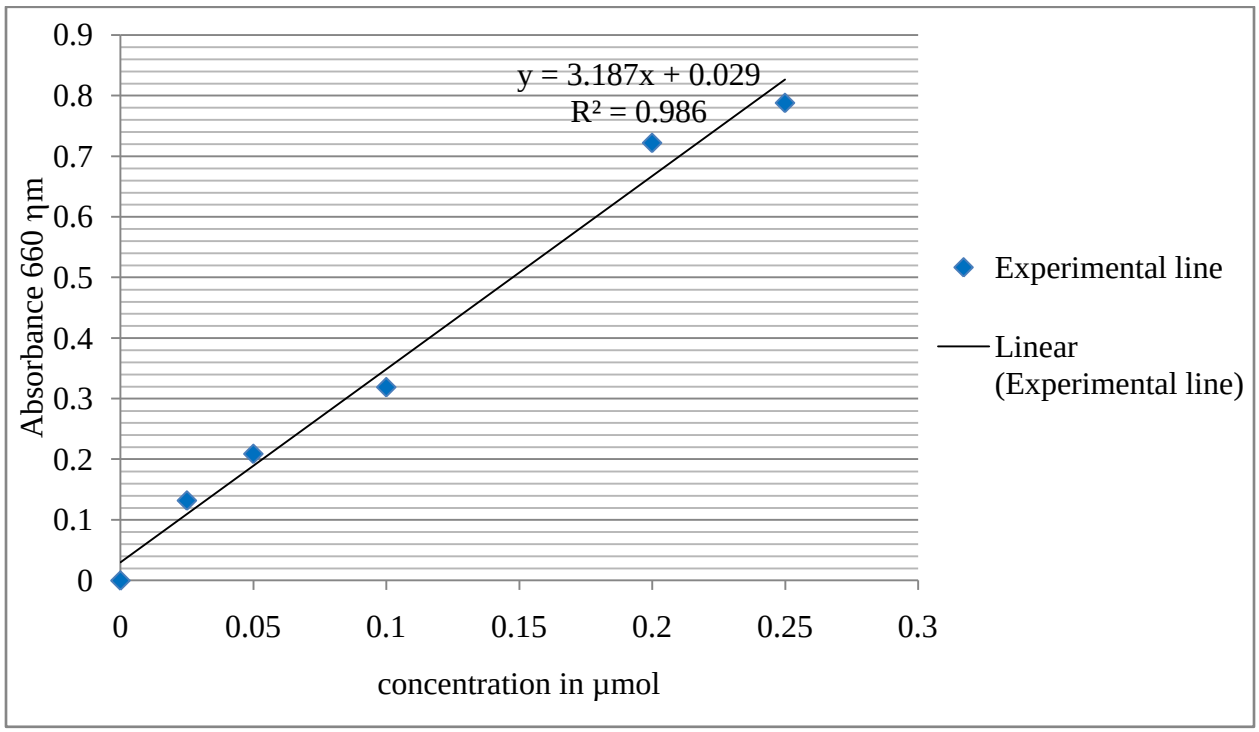
Annex 6: Protein content of partially purified protease of PMOJ-01 and PMOJ-05

Isolate	330ηm	600ηm	660ηm	net OD	Mean OD660ηm	concentration mg/ml
PMOJ-01 1	1.463	0.635	0.611	0.196		
PMOJ-01 2	1.37	0.589	0.568	0.153	0.152±0.044	0.118±0.044
PMOJ-01 3	1.043	0.374	0.523	0.108		
PMOJ-05 1	1.426	0.662	0.533	0.118		
PMOJ-05 2	1.184	0.495	0.462	0.047	0.078±0.036	0.02±0.036
PMOJ-05 3	1.172	0.486	0.486	0.071		
blank			0.415	0		

Annex 7: Enzyme activity of partially purified protease of PMOJ-01 and PMOJ-05

Isolate	OD at 660ηm	Net OD at 660ηm	Mean OD	Concentration mg/ml	Enzyme activity U/ml
PMOJ-01 1	1.802	1.387			
PMOJ-01 2	1.839	1.424	1.423±0.036	0.44±0.036	0.242±0.036
PMOJ-01 3	1.874	1.459			
PMOJ-05 1	1.797	1.382			
PMOJ-05 2	1.856	1.441	1.365±0.086	0.42±0.086	0.231±0.086
PMOJ-05 3	1.686	1.271			
blank	0.415	0			

Annex 8: Standard curve for tyrosine at 660 ηm



Annex 9: Standard curve for Egg Albumin at 660 ηm