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Production, characterization and evaluation of bacterial protease as a potential additive to enhance detergency of endod

By

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Table of contents page

ACKNOWLEDGMENT i
List of abbreviations vi
Lists of tables vi
List of figures vii
Abstract viii
1. INTRODUCTION 1
2. Objectives of the study 3
2.1. General objective 3
2.2. Specific objectives 3
3. Literature review 4
3.1. History of enzyme supplementation on detergents 4
3.1.1. Protease 4
3.1.2. Occurrence 5
3.1.3. Plants 5
3.1.4. Animals 5
3.1.5. Microorganism 5
3.2. Types of protease 6
3.2.1. Cysteine proteases 6
3.2.2. Serine proteases 6
3.2.3. Threonine proteases 8
3.2.4. Aspartic proteases 8
3.2.5. Metalloprotease 9
4. Applications of alkaline proteases 9
4.2. Detergent industry 9
4.3. Food and feed industry 10
4.4. Peptide synthesis 10
4.5. Leather industry 10
4.6. Management of industrial and household waste 11
4.7. Photographic industry 11
4.8. Medical usage 11
4.9. Silk degumming 12

5. MATERIALS AND METHODS 12
5.1. Media and culture conditions for isolation, screening and enzyme production 12
5.2. Enzyme assay 12
5.3. Morphological and biochemical characterization of isolate aau 2106 13
5.4. Characterization of the enzyme 13
5.4.1. Effect of pH on the activity and stability of proteases 13
5.4.2. Effect of temperature on the activity and stability of proteases 13
5.5. Enzyme production through solid state fermentation 14
5.5.1. Effect of incubation time on protease 14
5.5.2. Effect of moisture on the production of enzyme 14
5.5.3. Effect of nitrogen on the production of enzyme 14
5.6. Concentration of enzyme 14
5.7. "Endod" or green detergent formulation as detergent 14
5.7.1. Effect of pH on the detergency of endod 15
5.8. Stability of the enzyme with different detergents and endod 15
5.9. Effect of enzyme concentration in stain removal activity 15
5.10. Wash performance 15
5.11. Data analysis 15
6. Results 16
6.1. Isolation and screening of alkaline protease producing bacteria 16
6.2. Characterization and identification of isolate aau 2106 17
6.3. Characterization of protease enzyme 17
6.3.1. Effect of pH on the activity of proteases 17
6.3.2. Effect of temperature on the activity of protease 18
6.3.3. Effect of temperature on stability of protease 19
6.4. Enzyme production through solid state fermentation 19
6.4.1. Effect of nitrogen source on the production of protease 19
6.4.2. Effect of moisture on the enzyme production 20
6.4.3. Effect of incubation time on enzyme production 21
6.5. Effect of pH on the detergency of endod 21
6.6. Enzyme stability in the presence of endod and commercial detergents 21
6.7. Effect of enzyme concentration on stain removal efficiency 22

6.8. Stain removal activity of alkaline protease 23
7. Discussion 25
8. Conclusion and recommendation 27
9. References 28

List of abbreviation

aau	Addis Ababa University
EC	Enzyme commission
Fig	Figure
h	Hour
SSF	Solid state fermentation
TEV	Tobacco etch virus

Lists of tables

Table 1. List of isolates and their activity on the casein agar plate 16
Table 2 Morphological identification and biochemical test of isolate no aau 2106 17
Table 3. Effect of enzyme concentration on stain removal efficiency 22

List of figure

Fig.1: Protease catalysis of peptide bonds. 1
Fig.2: Isolate aau 2106 grown on a casein agar plate and the clear zone show that presence of proteolytic activity. 16
Fig. 3: Effect of pH on the activity of protease 18
Fig. 4: Effect of temperature on the activity of protease 18
Fig.5: Effect of temperature stability on protease. 19
Fig.6: Effect of nitrogen on the crude protease aau 2106 20
Fig.7: Effect of moisture on the enzyme activity 20
Fig.8: Effect of time on the production of protease 21
Fig.9: The stability of protease with commercial detergents and endod 22
Fig.10: Stain removal activity of endod and comercial deteragents. 23
Figure 11 Wash performance checked on egg yolk and blood stained clothes using commercial detergent and endod with and without enzyme. 24

Abstract

At present alkaline proteases are widely used in the detergent, leather tanning, pharmaceutical, food, and feed processing industries. Although proteases are found in all living organisms (plant, animal, and microorganisms), the bulk of commercially important enzymes are from microorganisms. The aim of this study was to isolate protease producing bacteria, characterize the enzyme, and evaluate potential application as detergent additive to enhance the washing performance of endod berries. Different bacterial strains were isolated from soil and screened using an alkaline casein agar media. One isolate designated as aau 2106 was selected for further study because of its high proteoliytic activity, level of enzyme production and, ability to grow under solid state fermentation (SSF). Cultivation condition for the production of the enzyme under SSF, such as, moisture content, nitrogen content, and incubation period were optimized. The enzyme was active in the pH range of 6 to 10.5, temperature of 40 to 75^{O}C. And optimum in; 96h incubation period, 1:1 ratio of moisture content and, casein in the production of enzyme under SSF and the enzyme was stable in the presence of endod and commercial detergents. The potential of the enzyme as a detergent additive was tested by adding the protease (57 U/g) in detergent formulations and used to clean pieces of cloth stained with blood and egg. Addition of the enzyme improved the cleaning efficiency of both endod and commercial detergents. This indicates that enzyme supplementation of endod could result in the development of an ecofriendly detergent.

Keywords: Detergent, Endod, Protease

1. INTRODUCTION

Proteases are enzymes that hydrolyse proteins by breaking down the peptide bonds via the addition of water resulting in the formation of peptide fragments (Fig. 1) (Sookkheo *et al.*, 2000).

Proteases derived from microorganisms, animals, and plants have been commercially used for different applications. But because of their rapid growth rate, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for the various applications, currently microorganisms are the major sources of commercially important proteases (Kocher and Mishra, 2009).

Proteases exhibit different properties in terms of substrate specificity, active site configuration and catalytic mechanisms, response to pH and temperature for activity and stability. These differences offer great potential for a variety of biotechnological applications (Gupta *et al*., 2002). Today proteases find important application in the detergent, leather tanning, pharmaceutical, and food and feed processing industries (Amare Gessesse 1997).

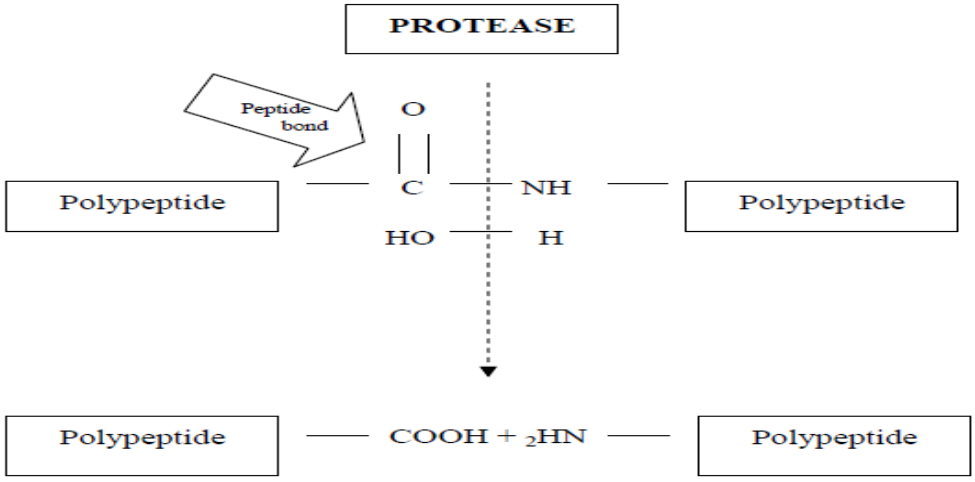

Fig.1: Protease catalysis of peptide bonds or proteolysis (Sookkheo *et al.*, 2000).

Proteases are classified into different groups based on their composition of amino acids in their active site or their pH optimum for activity. Based on their active centers proteases are classified

as serine (EC. 3.4.21), cysteine (thiol) (EC 3.4.22), aspartic (EC3.4.23) and metallo-protease (EC 3.4.24) (Chu, 2007). Based on optimum pH, proteases are divided as acidic, neutral, and alkaline. Due to their relatively high activity and stability at high pH alkaline proteases are the most commonly used industrial enzymes (Guleria *et al*., 2016), their major use being as components of house hold laundry detergents (Nehra *et al*., 2002). This is because the pH of most laundry detergents is generally in the range of pH 9.0 – 12.0.

Proteolytic enzymes added into detergents must have characteristics such as, good stability and activity at relatively high temperature, broader specificity to be able to hydrolyze different proteins, active and stable at alkaline pH values, and stable in the presence of other detergent components like perfumes, surfactants, and bleaches. In addition stability during storage and washing are also of desirable properties (Horikoshi, 1990).

Before commercial detergents were available, most people in the highlands of Ethiopia used endod berry as a traditional detergent to wash cloths. Endod (*Phytolacca dodecandra*) is a plant, commonly known as the African soapberry plant. With the introduction of commercial detergents the use of endod for laundry application is gradually forgotten. If the washing efficiency of endod is improved, there is still a possibility to use it as a green laundry detergent. One way to improve the washing efficiency of detergents is addition of proteolytic enzymes. At present, more than 90% of the commercial detergents contain enzymes as washing supplements. Therefore, identifying proteolytic enzymes that are active and stable in the presence of endod, the washing to temperature and pH could lead to the development of an effective and ecofriendly endod based detergent. Alkaline proteases are highly used in detergent industry because they are used as cleaning additives in detergents to facilitate the release of proteins within a short period of time but large number of alkaline proteases currently used for detergent applications have some limitations such as low activity and stability in the presence of chelating agents, surfactants, and bleaches (Gupta *et al.,* 2002) and also the components of detergent including surfactant and enzyme are imported from other countries. So, in this study, we focused on producing a stable alkaline protease enzyme and supplement it on endod as “green detergent” for use as a laundry detergent.

2. Objectives of the study

2.1. General objective

The main objective of this study is to identify a microbial protease and evaluate its potential as an additive to enhance detergency of endod.

2.2. Specific objectives:

- To isolate and screen alkaline protease producing microorganisms and characterize the enzymes.
- To optimize cultivation conditions for protease production under solid state fermentation.
- Evaluate the enzyme as an additive to endod and formulate a green detergent.
- Examine the wash performance of commercial detergents in presence and absence of enzyme.

3. Literature review

3.1. History of enzyme supplementation on detergents

Detergent supplemented with enzyme was started in 1914, by German scientists Rohm and Haas; they used pancreatic protease and sodium carbonate in washing detergents. The name of the product is Burnus, after the white Arab cloak. In 1956 the first detergent with bacterial enzyme was introduced into the market under the trade name Bio-40. In 1963 an alkaline protease, alcalase, was effectively incorporated in to detergent powder and was marketed by Novo Industry, Denmark under the trade name Biotex. Unfortunately, in the early 1970s, detergent proteases faced a setback because some workers developed an allergic reaction due to the enzyme dust and this problem was solved by the introduction of dust-free encapsulated products (Gupta *et al*., 2002).

The major market share (>55%) of the detergent enzyme was held by Gist-Brocades in the Netherlands, Genencor International in the United States, Solvay in Belgium and Showa-Kenko in Japan in the 1980s and early 1990s. These suppliers sell a full range of enzymes for powder and liquid detergents (Beg *et al*., 2002).

Starting from 1995 up to 95% of the global market of proteases is supplied by Novo Nordisk and Genencor. Today, the market of detergent enzyme accounts for 89% of the total protease sales in the world and it is captured by bacteria for example subtilisins and/or alkaline proteases from many *Bacillus* species and streptomycin species of actinomyctes. The detergent enzyme market has grown nearly 10-fold during the past 20 years (Gupta *et al*., 2002).

3.1.1. Protease

Microorganisms account about 70% of commercial protease production in the world. Proteases are important to all forms of life on the earth that we can find it from plants, animals, fungi, virus and prokaryotes. They can be produced in large quantities within a short period of time using fermentation to produce an abundant and regular supply of enzyme (Kumar and Takagi, 1999).

Protease is a hydrolytic enzyme that can degrade proteinous substance for different applications in food, detergent, and leather industry. Protease is applied in large amount for the use of

laundry detergent and nowadays it's being profitable, where a total global market size was about 0.6 billion USD in 2000 (Fan *et al*., 2001).

3.1.2. Occurrence

Protease enzyme is found from all organisms starting from prokaryotes to eukaryotes to viruses. These enzymes are used for digestion of protein to highly regulated cascades (e.g., apoptosis pathways, the blood-clotting and the invertebrate prophenoloxidase-activating cascade). There protein degradation can either be limited which breaks specific peptide bonds, or unlimited which breaks down a complete peptide (Zelisko and Jackowski, 2004).

3.1.3. Plants

The genome of plant encodes over hundreds of protease enzyme with are used for the regulation of plant development; they are also used in photosynthesis regulation (Zelisko and Jackowski, 2004).

3.1.4. Animals

Protease enzymes are used in organisms for many metabolic processes; for example, acid proteases secreted into the stomach (such as pepsin) and serine proteases present in duodenum (trypsin and chymotrypsin) enable animals to digest the protein in food. It also plays important role in blood-clotting, as well as lysis of the clots, and the correct action of the immune system. Some snake venoms are also proteases, such as pit viper haemotoxin and interfere with the victim's blood clotting cascade (Kumar and Takagi, 1999).

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3.1.5. Microorganisms

Plenty of microorganisms present in soil with thousands of species from this many of them are involved in protein degradation and they are also used to control the quality of protein such as protostomes has a protease enzyme which degrades misfolded or unfolded proteins. The other use of the secreted bacterial protease is they can act as an exotoxin, and be an example of virulence factor in bacterial pathogenesis (such as, exfoliative) and they also destroy extracellular structures. Also some viruses such as polio, norovirus, and Tobaco etch virus (TEV) use a protease to degrade massive polyprotiens into functional units (Sims *et al*., 2002).

3.2. Types of protease

Proteases are divided into various groups; based on their active sites. Those are metallo- (EC.3.4.24), aspartic- (EC.3.4.23), cysteine- or sulphydryl- (EC.3.4.22), or serine-type (EC.3.4.21) and depending on their pH; acidic, neutral and alkaline. Alkaline proteases are enzymes that are active in a neutral to alkaline pH range. They either have metallo-type (metalloprotease) or are of a serine center (serine protease); and the alkaline serine proteases are the most important group of enzymes used for different markets (Gupta *et al*., 2002).

3.2.1. Cysteine proteases

Cysteine proteases are also called **thiol proteases** because in their catalytic mechanism they involve a nucleophilic cysteine thiol. Cysteine proteases are found in many plant families mostly they are found in fruits such as pineapple, papaya, fig and kiwi fruits. The amount of protease will be higher when the fruit is unripe. Cysteine proteases are mostly applied for tenderizing meat because of their high capacity to hydrolyze proteins (Domsalla *et al*., 2008).

Catalytic mechanism

Deprotonation of thiol in the active site of enzyme next to amino acid mostly a histidine residue is the first step of cysteine protease for the hydrolysis of peptide bond. Then, anionic sulfur will attack on carbonyl carbon substrate. An amine terminus will be released with the substrate and the histidine residue in the protease enzyme is restored to its deprotonated form, and a thioester intermediate linking the new carboxyl-terminus of the substrate to the cysteine thiol will be formed. Finally, thioester bond is hydrolyzed in order to form new enzyme and carboxylic acid (Domsalla *et al*., 2008).

Biological importance

Cysteine protease is used for physiology and development. They are also involved in programmed cell death meaning apoptosis and in signaling pathways and in the response to stresses which are biotic and abiotic. In humans they are used for bone development by remodeling extracellular matrix and they are also important in MHC class II immune responses and prohormone processing (Grudkowska *et al.*, 2004).

3.2.2. Serine proteases

Serine proteases are enzymes which are also called serine endopeptidase because they hydrolyze peptide bonds in protein (Hedstrom, 2002). They are found almost everywhere

such as in eukaryotes and prokaryotes. Serine protease are divided into two these are chymotrypsin-like (trypsin-like) or subtilisin-like structures (Madala *et al*., 2010).

Biological importance

They are important for coordinating different physiological functions like reproduction, digestion, blood coagulation and immune response (Hedstrom, 2002).

Catalytic mechanism

Catalytic tried is the main player in the catalytic mechanism in the serine proteases. It is in the active site of the enzyme in which catalytic process occurs. The triad is a structure consisting of three amino acids: His 57, Asp 102 and Ser 195 as there name indicates ‘’serine protease” and they are very important amino acids in protease enzyme to make it degrade proteins (Iván *et al*., 2009).

The task of each amino acid in the triad performs: First serine has an -OH group that can act as a nucleophile which attack carbonyl carbon of the scissile peptide bond of substrate then on the histidine nitrogen there are pairs of electrons that can accept hydrogen from serine-OH group by this process it coordinate the attack of peptide bond and the carboxyl group on the aspartic acid in turn hydrogen bonds with the histidine to make nitrogen atom more electronegative.

The whole reaction can be summarized as follows, first on the serine enzyme a polypeptide substrate binds then serine –OH attacks the carbonyl carbon next the nitrogen of histidine accepts the hydrogen from the serine –OH after this two electrons from double bond of carboxyl oxygen moves to the oxygen to form a tetrahedral intermediate so the bond joining the nitrogen. And the carbon in the peptide bone is broken to generate acyl-enzyme intermediate, the electrons that before moved from carbonyl oxygen double bond move back from the negative oxygen to recreate the bond so water is formed in to the reaction this water replaces the N-terminus of the hydrolyzed peptide to attack carbonyl carbon. So the electrons form double bond and move to oxygen to make it negative this is done by nitrogen of histidine which accepts a proton from the water so another tetrahedral intermediate generated. Finally the bond formed in the first step between serine and carbonyl carbon go and attack the hydrogen in which histidine just acquired

then electron-deficient carbonyl carbon re-forms the double bond with the oxygen. Thus, the C-terminus of the peptide will be ejected (Iván *et al*., 2009).

3.2.3. Threonine proteases

Threonine proteases are enzymes that can hydrolyze protein containing threonine (Thr) residue in its active site. The prototype members of this class of enzymes are the catalytic subunits of the proteasome; however, the acyltransferases convergently evolved the same active site triad geometry and enzyme mechanism (Ekici *et al.,* 2008).

Catalytic mechanism

Threonine proteases use its N- terminal threonine as a nucleophile to perform catalysis (Cheng and Grishin, 2005). The threonine must be N-terminal because the terminal amine of the same residue acts as a general base by polarizing water that deprotonates alcohol in order to increase its activity as a nucleophile (Ekici *et al*., 2008).

Catalysis takes place in two steps: first a nucleophile attacks the substrate for the formation of covalent acyl-enzyme intermediate so that the first product is released and finally water hydrolyzes the intermediate to form the free enzyme and release the second product acyltransferase (Ekici *et al.,* 2008).

3.2.4. Aspartic proteases

Aspartic proteases are enzymes that have highly conserved aspartates in their active site, are active in acidic pH, use an activated water molecule bound to one or more aspartate residues for catalysis of their peptide substrates, and all aspartic protease are inhibited by pepstatin (Szecsi, 1992).

Catalytic mechanism

Aspartic proteases are proteases having an ability to digest dipeptide bonds which have beta-methylene group and hydrophobic residues. One aspartate activates the water by removing a proton; this enables water to perform a nucleophilic attack on the carbonyl carbon of the substrate scissile bond, generating a tetrahedron oxyanion reaction intermediate. Rearrangement of this intermediate can lead to protonation of the scissile amide which results in the splitting of the substrate peptide into two product peptides (Brik and Wong, 2003).

3.2.5. Metalloprotease

Metalloprotease is (also known as metalloproteinase) any protease enzyme whose catalytic mechanism involves a metal. For example, meltrin which plays a very important role in the fusion of muscle cells during the development of embryo. This process is called myogenesis. Metallo-proteases require zinc and some use cobalt. The metal ion is related to the protein via three ligands. The ligands coordinating the metal ion can vary with histidine, glutamate, aspartate, lysine and arginine (Suguna *et al.*, 1987).

4. Applications of alkaline proteases

Alkaline proteases are enzymes which are very important for many applications and are used in every country (Godfrey and West, 1996). Especially alkaline proteases which are found from bacteria are used for different applications in various industries.

Pronod 153L is cleaner containing protease enzyme which is used to clean surgical instruments fouled by blood proteins and also subtilopeptidase A is an enzyme based optical cleaner which is used in Indians market (Kumar *et al*., 1998). Alkaline proteases are also used for cleaning contact lens (Nakagawa, 1994), degumming of silk (Kanehisa, 2000), isolation of nucleic acid in molecular biology (Puri, 2001), pest control (Kim *et al*., 1999), and selective delignification of hemp (Dorado *et al*., 2001), but this application of alkaline protease are not being used widely. The major uses of alkaline proteases in industrial sectors are described as follows.

4.2. Detergent industry

Detergent industries are interested in supplementing enzyme to make their product best in removing stains that are proteinous. Application of detergent proteases is highly used in household laundry detergent formulations. The need of detergent industries on the enzyme technology is because of the addition of completely new performance benefits, consumer-recognizable cleaning benefits, fabric restoration and an increased performance/cost ratio. In addition, enzyme producers and detergent suppliers are involved in developing new enzyme in that can fulfill the consumer's interest such as, improved cleaning, fabric care and antimicrobial benefits. However, apart from their use in laundry detergents, household dish washing detergents and both industrial and institutional cleaning detergents are also popular (Godfrey and West, 1996; Showell, 1999).

4.3. Food and feed industry

Microbial proteases have been used in the food industries in many ways like in preparation of protein hydrolysates which are used in food formulations, blood pressure regulation, and specific therapeutic dietary products and to make fruit juices and soft drinks stronger (Neklyudov *et al*. 2000). The alkaline proteases are known to produce hydrolysate from different natural protein substrates such as soy protein (Proup; Novo Nordisk, Bagsvaerd, Denmark), whey (Lacprodan; MD Foods) and casein (Miprodan; MD Foods, Viby, Germany). Fujimaki *et al*. (1970), produced soy protein hydrolysates from alkaline protease. Dalev (1994), studied the hydrolysis of feather keratin, to obtain a protein concentrate for fodder production from alkaline proteases (B72 from B. *subtilis* and B. *licheniformis* PWD-1).

4.4. Peptide synthesis

The protease enzyme has been used for peptide synthesis since the 1937 by using the reverse-enzymatic reaction of hydrolysis (Morihara 1987; Kise *et al*., 1990; Clapes *et al*., 1997). The use of enzyme for peptide synthesis increase solubility of non-polar substrates, the reactions can be performed stereospecifically and reactants do not require side-chain protection, or shifting thermodynamic equilibria to favor synthesis over hydrolysis and decreases the use of hazardous chemicals, organic solvents, expensive protecting-groups (Morihara, 1987). Now day's proteases have been widely applied for the synthesis of dipeptides (Barros *et al*, 1999) and tripeptide (So *et al*., 2000), and sugar esterification (Riva *et al*., 1988). An industrial protease, neutrase which are co-deposited with sorbitol onto polyamide was used for the synthesis of several N-protected dipeptide derivatives in acetonitrile (Clapes *et al*., 1997). The proteinase from an extremophile, Thermus Rt41A, immobilized on controlled-pore glass beads, was used for peptide synthesis, using the synthesis of Bz-Ala- Tyr-NH2 as a model system (Wilson *et al*., 1994).

4.5. Leather industry

Leather processing involves the use of chemicals like hydrogen sulfide which causes environmental pollution and safety hazards. In order to solve this problem, the use of protease enzyme is preferable and easy to control, thus being ecofriendly (Andersen, 1998). Alkaline proteases are used in leather industry in the soaking, bating and dehairing stages of skin preparation within a short period of time because the alkaline conditions enable the swelling of hair roots; and the subsequent attack of protease on the hair follicle protein allows easy removal

of the hair. Hameed *et al*., (1999), used *Bacillus subtilis* K2 alkaline protease in bating and leather processing.

4.6. Management of industrial and household waste

Proteases are used in management of industrial and household wastes by lowering the biological oxygen demand of aquatic systems which resulted in the ability of protease solubilizing proteinaceous waste.

The application of alkaline protease in the management of waste from various food-processing industries and household activities is from microorganisms especially from *B. subtilis* (Dalev, 1994).

A formulation containing protease enzyme from *B. subtilis*, *B. amyloliquefaciens* and *Streptomyces* sp. and a disulfide reducing agent (thioglycolate) which enhances hair degradation and cleans pipes which are closed with hair-containing deposits were prepared and patented by Genex (Jacobson *et al*., 1985).

4.7. Photographic industry

Alkaline proteases are used for the use of silver recovery in the bioprocessing of photographic films or X-ray. The film contains gelatin layer containing 1.5–2.0% silver by weight which can be used as a good source of silver for a variety of purposes. Thus silver can be extracted from the protein layer or gelatin by proteolytic treatments and this also helps for recycling polyester film base. Alkaline protease of *Bacillus* sp. B21-2 for the enzymatic hydrolysis of gelatin layers of X-ray films to release silver particles (Ishikawa *et al*., 1993). Before this technology silver was recovered by burning the films causing undesirable environmental pollution.

4.8. Medical usage

Medical products are also produced from alkaline protease for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kudrya and Simonenko (1994) reported the elastolytic activity of B. *subtilis* 316M for the preparation of elastoterase. Kim *et al*., (1999), found the use of alkaline protease from *Bacillus sp*. CK 11-4 as a thrombolytic agent having fibrinolytic activity.

4.9. Silk degumming

Only a few patents described the use of protease for the degumming of silk by using protease (Kanehisa, 2000). Sericin is a protein that surrounds fibers that becomes silk. This sericin was conventionally removed by an expensive method by conducting twist-setting and shrink-proofing for the silk yarns, using starch (Kanehisa, 2000).

RGR-14 was studied and results were analyzed gravimetrically (fiber weight reduction) and by scanning electron microscopy 26 of treated silk fiber. After 5 h of incubation of silk fiber with protease from Bacillus sp., the weight loss of silk fiber was 7.5% (Puri, 2001).

5. MATERIALS AND METHODS

This study was carried out from November 2016 to July 2017 in the Department of Microbial, Cellular and Molecular Biology, College of Natural Sciences, Microbial Biotechnology Laboratory, Addis Ababa University, Addis Ababa. All chemicals used in the study were purchased from the local market and were of analytical grade.

5.1. Media and culture conditions for isolation, screening and enzyme production

In this study, all media preparations were accomplished by following the procedures developed by Amare Gessesse and Birhanu Abegaz Gashe (1997). Soil samples were collected from 5-10 cm depth into plastic bags from Addis Ababa University and Mojo tannery from decomposing hair and used for the isolation of different microorganisms. Isolates were purified through repeated streaking and 56 isolates was used for screening for enzyme production.

The media used for isolation, screening and enzyme production was composed of g/l: casein 5; peptone 5; yeast extract 2; NaCl 5; $MgSO_4.7H_2O$ 0.2; $CaCl_2$ 0.1; K_2HPO_4 1; and pH at 10. Sodium carbonate was separately autoclaved and added to the rest of the medium after cooling. A loopful of sample was streaked on casein-agar plates and incubated at 32°C for 48 h. Enzyme production was carried out in 500 ml flasks containing 100 ml medium. After 48 h incubation at 32°C with rotary shaking the culture was harvested and cells separated by centrifugation and the supernatant was used as the enzyme source for the assay procedure.

5.2. Enzyme assay

The reaction mixture in a total volume of 500 µl was composed of 1% casein in 50 mM glycine NaOH buffer, pH 10 and 50 µl of crude enzyme. After 30 min incubation at 60°C the reaction

was terminated by adding 450 µl of 10% trichloro acetic acid. After centrifugation at 10,000 rpm for 5 min, 150 µl supernatant was taken and mixed with 750 µl of 0.5 M sodium carbonate followed by addition of 150 µl 1 N Folin-Ciocalteu's phenol reagent. The reaction mixture was allowed to stand for 30 min at room temperature and absorbance measured at 660 nm. One unit of protease activity was defined as the amount of enzyme that released 1 µg tyrosine per min under the conditions described above (Shimogaki *et al*., 1991).

5.3. Morphological and biochemical characterization of isolate aau 2106

Colony characterization such as configuration, margin, pigment, arrangement and shape was investigated microscopically and direct observation of the 24h old colony on the agar plate. Gram test was carried using general procedure of gram test (Duncan, 2005) and 3% KOH. Biochemical characteristics such as catalase, casein, gelatin, and starch hydrolysis test were undertaken as follows. Catalase test was investigated using 3% H_2O_2 by dropping on the slide with 48 h at 32^{o}C grown fresh colony and observing whether air bubble was detected (Muhammed, 2011). Casein hydrolysis test was examined on casein agar plate (Amare Gessesse and Birhanu Abegaz Gashe, 1997), starch hydrolysis test was examined on a starch medium, and gelatin hydrolysis test was examined in 12% gelatin and a loop full sample of isolate aau 2106 was incubated for 48h and placed in refrigerator for 20 min and see if the gelatin medium is hydrolyzed (Leboffe *et al*., 2010).

5.4. Characterization of the enzyme

5.4.1. Effect of pH on the activity and stability of protease:

The activity of protease was measured at different pH values from 6.5 to 10.5. The pH was adjusted using one of the following buffers (100mM): phosphate (pH 6.0–8.0), Tris–HCl (pH 7.5-9), glycine–NaOH (pH 8.5–10.5) with a final concentration of 50mM in a reaction mixtures. After incubation at 60°C the activity of protease was measured (Amare Gessesse and Birhanu Abegaz Gashe, 1997).

5.4.2. Effect of temperature on the activity and stability of protease

The activity of protease was measured at different temperature values from 40 up to 75^{o}C and also effect of calcium was measured by adding 5mM $CaCl_2$ into substrate 1% casein and the activity of protease was measured. The stability of protease was measured by incubating the

enzyme at different time from 0 min up to 60 min at 55 and 60^{o}C then the activity of protease was measured (Amare Gessesse and Birhanu Abegaz Gashe, 1997).

5.5. Enzyme production through solid state fermentation

5.5.1. Effect of incubation time on protease

To investigate the effect of incubation time on protease production flasks containing 10g wheat bran with mineral salts (NaCl 0.5g, $CaCl_2.H_2O$ 0.01g, $MgSO_4.7H_2O$ 0.02g, K_2HPO_4 1g) having 1:1.5 ratio of moisture content were incubated at 32^{o}C from 24 to 120 h. Enzyme extraction and assay was carried out following the standard procedure.

5.5.2. Effect of moisture on the production of enzyme

The effect of moisture for the production of protease was determined by incubating the culture media containing 10g of wheat bran and mineral salts with in the ratio of 1: 0.5 to 1:3 at 32^{o}C for 96 h followed by enzyme extraction by following the standard procedure. To optimize the extraction process the residue was re-extracted up to three times and each extract was tested for enzyme activity separately. Those extracts' having significant activity was pooled together and the activity measured from the pool.

5.5.3. Effect of nitrogen on the production of enzyme

The activity of protease was measured by adding 0.5% (w/v) nitrogen sources: ammonium sulphate, sodium nitrate, yeast extract, peptone, and casein in a media containing 10g wheat bran with mineral salts at the ratio of 1:1 for 96 h incubation period. Enzyme extraction and assay was carried out following the standard procedure.

5.6. Concentration of enzyme

Enzyme was purified using 96% ethanol with enzyme in the ratio of 1:3 and it was precipitated at - 4^{o}C for 1 h. Then, the supernatant was discarded and the pellet was centrifuged at 10,000 rpm and again the supernatant was discarded and the pellet was diluted with buffer.

5.7. "Endod" formulation as detergent

Endod berry was harvested from Addis Ababa University College of Natural Science around chemistry department and sun dried for 2 days followed by grinding using an electric grinder. Weighed amount of 60g powdered endod was then added to equal volume of 60ml water and set aside for 8 days. The endod was then extracted using 300ml methanol followed by removal of

the solvent methanol through evaporation then formulated a detergent with other chemicals in g: Na_2HPO_4 0.62; Citric acid 0.124; Na_2CO_3 0.04; $NaHCO_3$ 0.39; Endod 0.21.

5.7.1. Effect of pH on the detergency of endod

The effect of pH on the detergency of endod was investigated by observing the formation of foam and the time that the foam stayed by adjusting the pH of the formulated detergent using buffers (Tris-Hcl and Glycine) with pH values of 7.5, 8, 8.5,9, 9.5, and10.

5.8. Stability of the enzyme with different detergents and endod

Detergent solutions (arial and omo) at a concentration of 7mg/ml were prepared separately in distilled water. The solutions were boiled for 30min at 100^{o}C to destroy any protease that already present in the detergents and cooled. Equal enzyme concentration was added to each detergent and endod solution and the mixture was incubated at 50°C for different time intervals from 0-120 min. Then stability of the enzyme was then measured.

5.9. Effect of enzyme concentration in stain removal

Different concentrations of enzyme was supplemented in endod and commercial detergent with different concentration amounts in U/g of 9.5, 19, 28.5, 38, 47.5, and 57 and wash a 24 h stained clothes by egg and blood from Kara slaughter house. And this was evaluated by 5 students to rank as 1- 4 depending up on the cleanness of the clothes in ascending order meaning the more clean cloth will get the highest rank by comparing with the control (a cloth only washed by water).

5.10. Wash performance

White clean cotton cloth piece (4 cm × 4 cm) stained with 200µl blood and 400µl egg and dried. Then after 24 h all the stained clothes were subjected to wash treatments with only endod, commercial detergents (arial and omo), and also supplemented with 57 U/g enzyme, commercial detergents without enzyme (by boiling 7mg/ml detergent at 100^{o}C for 30 min and cooled) and also with only tap water and was taken as a control then the clothes were dried and visually examined by 5 students and took the average then converted it into percentage.

5.11. Data analysis

All data presented here in this study were the average of two measurements and all graphical and numerical data values generated by using Microsoft Excel 2010 and Sigma Plot version 10.

6. Results

6.1. Isolation and Screening of Alkaline Protease Producing Bacteria

Based on proteolytic activity, out of a total of 56 isolates tested, one isolate, designated as aau 2106, was selected for further study. The isolate showed high casein degradation within 3 days of incubation at 32^{o}C (Fig. 2).

Table 1. List of isolates and their activity on the casein agar plate

No activity	Moderate activity	Good activity	Very good activity
aau 1803, aau 1804, aau 1805	aau 1802, aau 1809	aau 1801, aau 1806	aau 1908
aau 1904, aau 1905, aau 2001, aau 2006, aau 2009	aau 1901, aau 1907, aau 1909, aau 2002	aau 1808, aau 1902	aau 2003
aau01, aau 02, aau 05, aau 07,	aau 2004, aau 2005, aau 2007	aau 1905, aau 1906	aau 2008
aau 08, aau 09, aau 10, aau 12	aau 2202, aau 2107, aau 2109	aau 1903, aau 1807	aau 2104
aau 2201, aau 2203	aau 04, aau 06	aau 2101, aau 2108	aau 2105
aau 2207, aau 11	aau 2204, aau 2205, aau 2206	aau 2103, aau 2102	aau 2106, aau 03

aau, Addis Ababa University

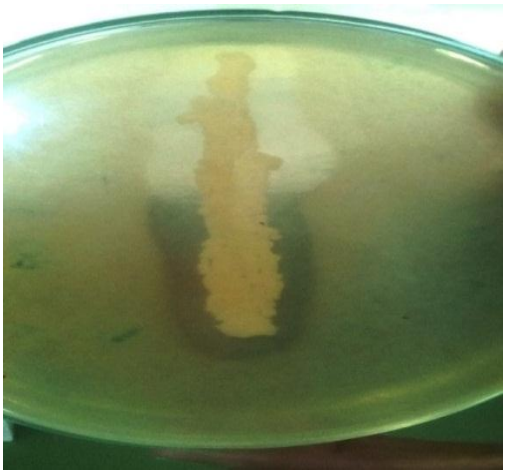

Fig.2: Isolate aau 2106 grown on a casein agar plate and the clear zone shows the presence of proteolytic activity.

6.2. Characterization and identification of isolate aau 2106

Table 2 shows results of morphological and biochemical tests of isolate aau 2106. Based on its morphological and physiological characteristics the isolate was characterized as gram positive and catalase positive. The colonies were characterized as chalky, circular configuration, hard, and white. It is rod shaped cell when observed under light microscope with 1000X magnification power. And it has the ability to hydrolyze casein and cannot hydrolyze starch and gelatin.

Table 2 Morphological identification and biochemical test of isolate no aau 2106

Test on colony morphology	**aau 2106**
Configuration	Circular
Margin	Hard
Pigment	Chalky
Cell shape	Rod
Cell arrangement	Pairs
Gram test	Positive
Catalase test	Positive
Casein hydrolysis	Positive
Gelatin hydrolysis	Negative
Starch hydrolysis	Negative

6.3. Characterization of protease enzyme

6.3.1. Effect of pH on the activity of protease

The effect of pH on activity of the enzyme was determined from pH 6.0-10.5. The enzyme was active in a broad pH range with an optimum at pH 9.5, and retained greater than 50% of its maximum activity in the pH range of 6.5-10.5 (Fig. 3). The enzyme lost 63.2% of its maximum activity under slightly acidic conditions (below pH 6.5).

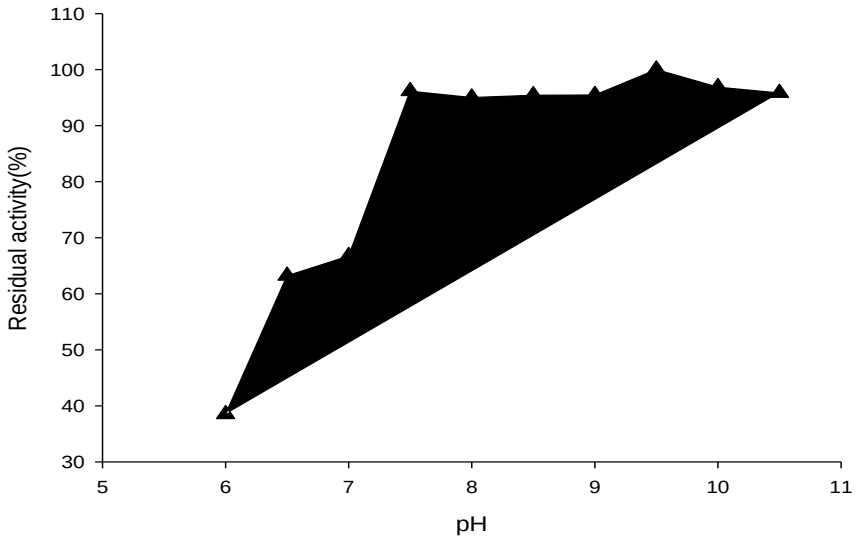

Fig. 3: Effect of pH on the activity of protease

6.3.2. Effect of temperature on the activity of protease

The effect of temperature on proteolytic activity of protease extracted from aau 2106 was determined in the temperature range of 40 – 75^{o}C. The enzyme showed higher activity in temperature range of 55-65^{o}C with an optimum at 60^{o}C (Fig. 4) both in presence and absence of calcium.

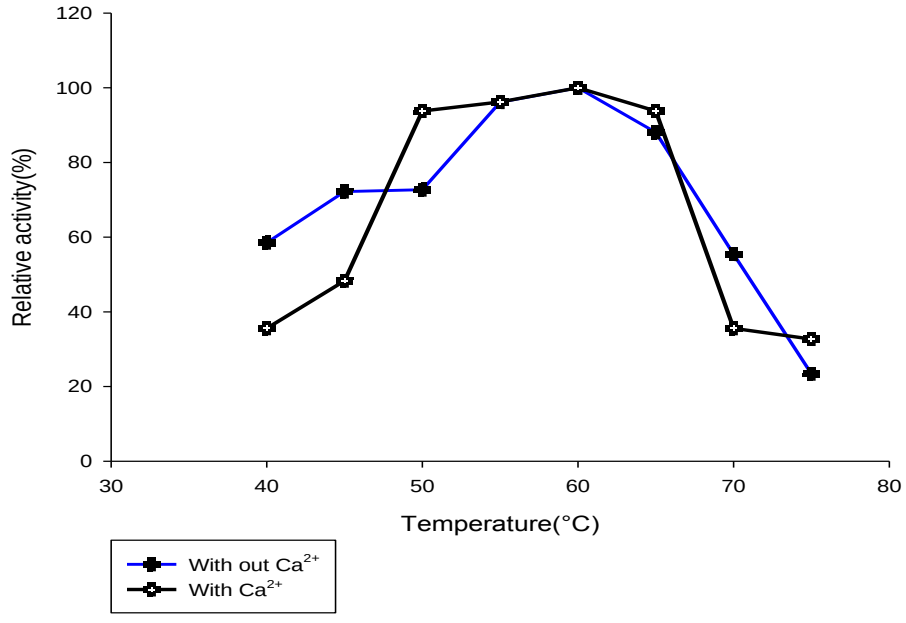

Fig. 4: Effect of temperature on the activity of protease

6.3.3. Effect of temperature on stability of protease

The enzyme of aau 2106 retained more than 50% of its maximum activity after 10min incubation in both temperature values (55 and 60^{o}C) (Fig.5).

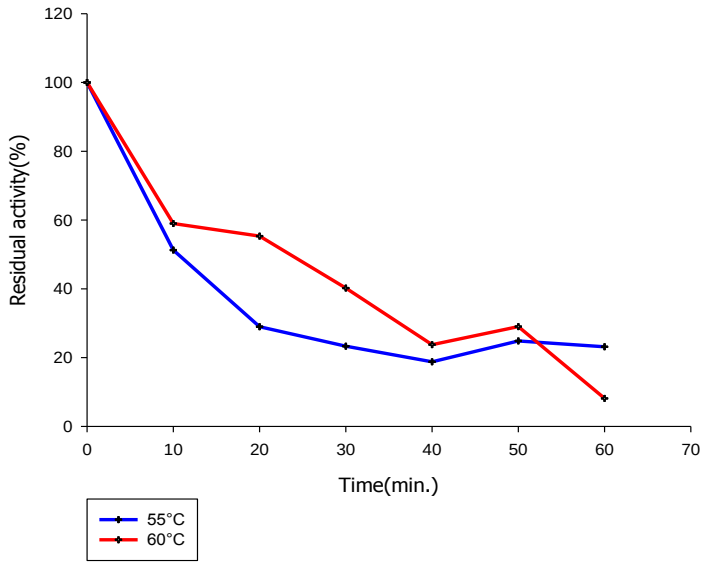

Fig.5: Effect of temperature stability on protease.

6.4. Enzyme production through solid state fermentation

6.4.1. Effect of nitrogen source on the production of protease

When enzyme production was tested in the presence of casein, peptone, yeast extract, ammonium sulphate, high activity was recorded in the presence of casein followed by peptone, and yeast extract (Fig. 6).

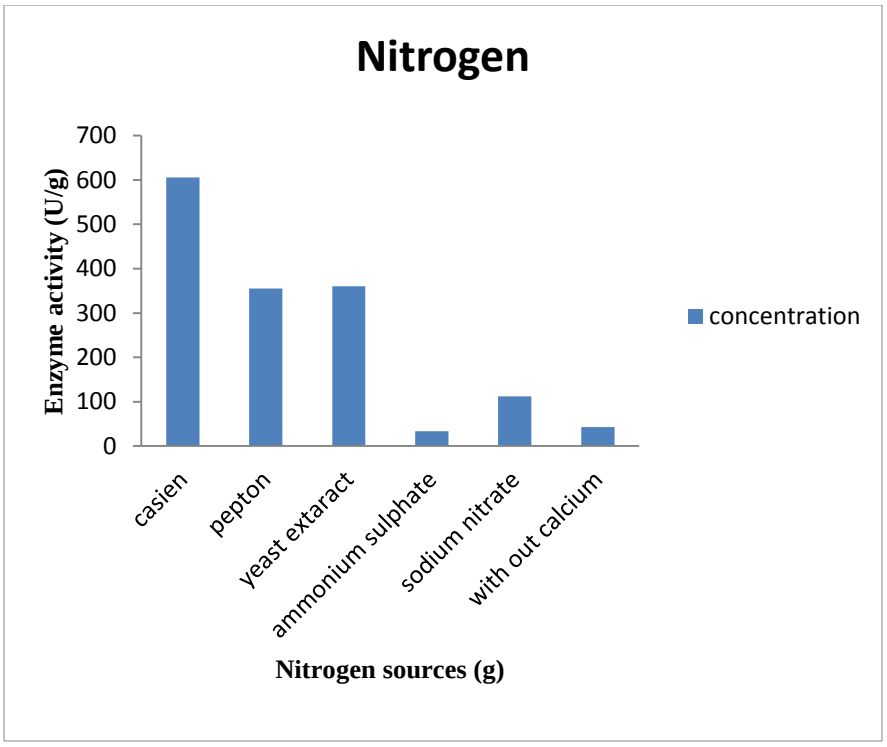

Fig.6: Effect of nitrogen on the crude protease aau 2106

6.4.2. Effect of moisture on the enzyme production

When the organism is grown under solid state fermentation the highest protease activity was observed at moisture to solid ratio of 1:1. At moisture content above the optimum resulted in gradual decrease of enzyme activity (Fig. 7).

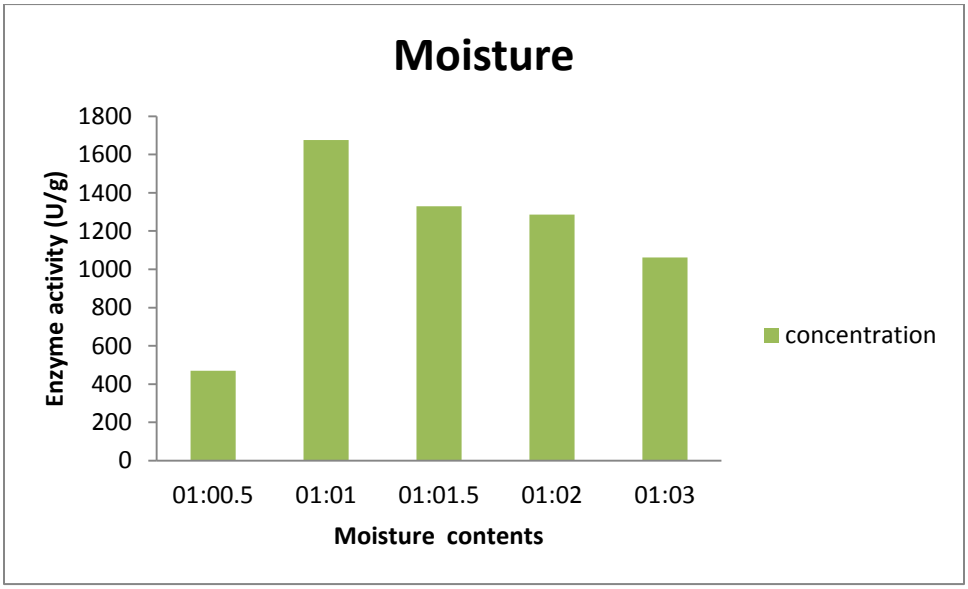

Fig.7: Effect of moisture on the enzyme activity

6.4.3. Effect of incubation time on enzyme production

The time course of protease production using solid-state fermentation was measured from 24-120h. Less enzyme production was observed from 24-48 h. Then the production started to increase progressively from 72 up to 96 h and started to decrease at 120 h (Fig. 8).

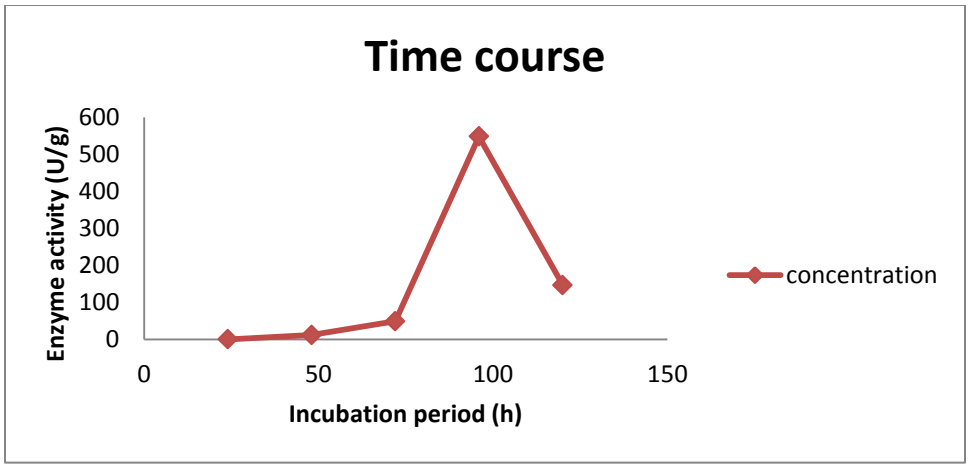

Fig.8: Effect of time on the production of protease

6.5. Effect of pH on the detergency of endod

The effect of pH on the detergency of endod was investigated and formation of foam was high in pH range from 7.5 up to 9 and at pH 8 the highest foam formation was observed. And the time that the foam stayed was over 24 h. The detergency decreased in pH values of 9.5 and 10 and it stayed only for about 30 min.

6.6. Enzyme stability in the presence of endod and commercial detergents

The stability of protease with commercial detergents (Omo, Arial) and endod was measured and the enzyme was stable in all detergents including endod and the enzyme showed higher stability on endod (Fig 9).

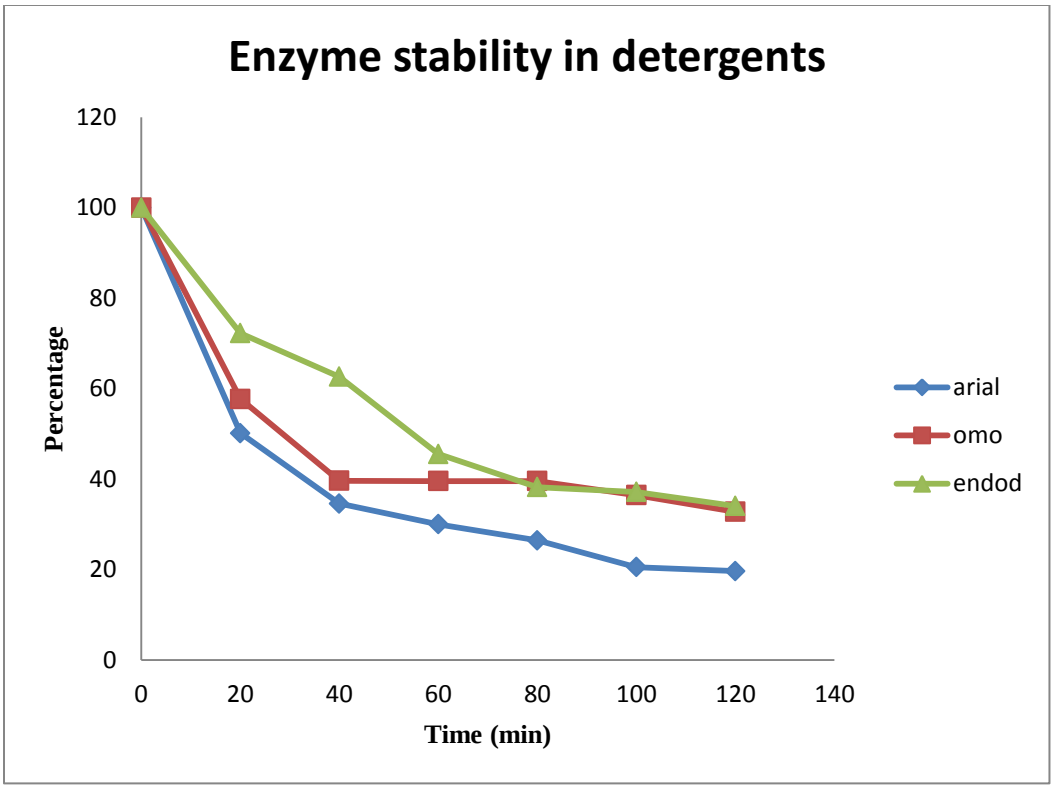

Fig.9: The stability of protease with commercial detergents and endod

6.7. Effect of enzyme concentration on stain removal efficiency

The concentration of enzyme has also an effect on the stain removal efficiency. . More than 50% stain removal efficiency was observed for enzyme concentrations ranging from 28.5-57U/g with the highest stain removal activity at 57U/g.

Table 3. Effect of enzyme concentration on stain removal efficiency

Enzyme concentration (U/g)	Percentage (%)
9.5	25
19	45
28.5	60
38	70
47.5	85
57	100

U/g, units per gram

6.8. Stain removal activity of alkaline protease

Stain removal activity of protease was measured using cotton cloth that was stained with egg and blood. A sample containing protease showed high removal of stain. For example in a cloth which is stained by egg have high stain removal activity on Arial supplemented with protease enzyme and in a cloth which is stained by blood showed high activity on endod supplemented with protease.

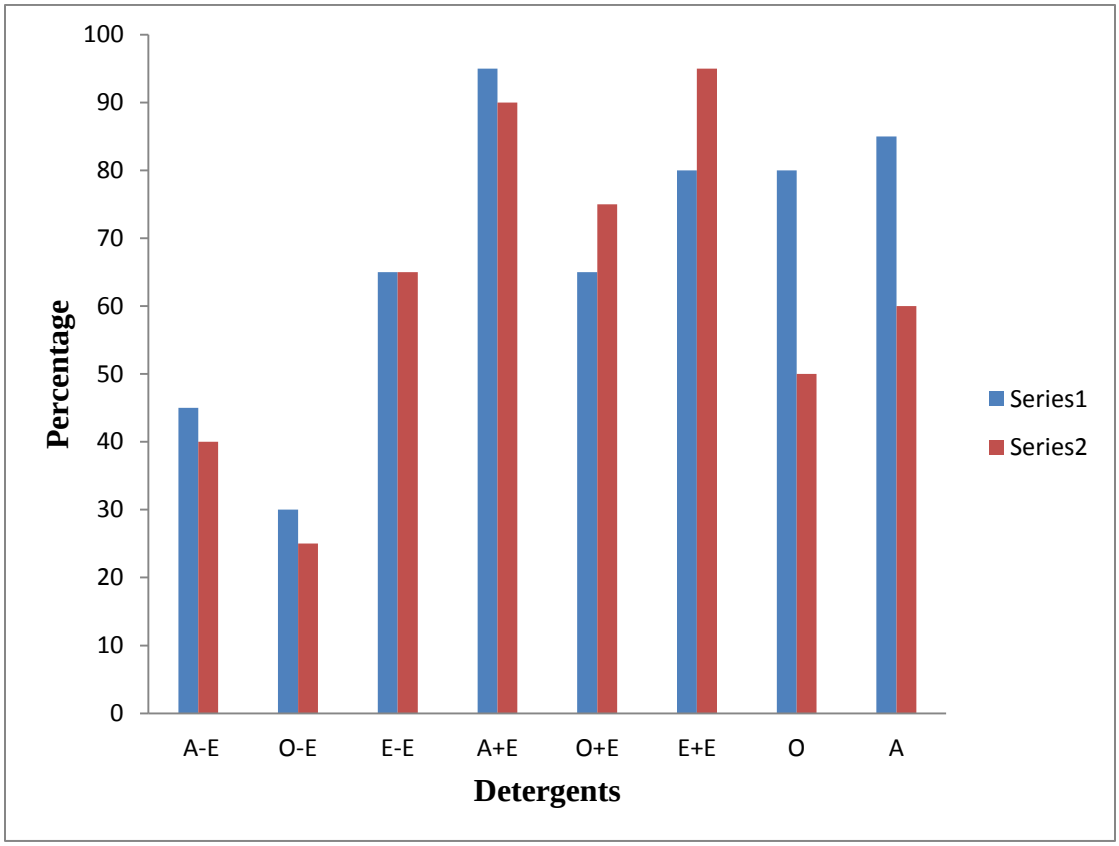

Fig.10: Stain removal activity of A-E- Arial without enzyme, O-E- Omo without enzyme, E-E- Endod without enzyme, A+ E - Arial with enzyme, O+E- Omo with enzyme, E+E- Endod with enzyme, O- Omo, A- arial, and on a cloth stained by egg (series 1) and blood (series 2).

High stain removal activity was observed when the commercial detergent Arial supplemented with enzyme from isolate aau 2106 was used for the washing of egg stained cloths. And endod plus enzyme from aau 2106 have high stain removal activity and Arial plus enzyme from aau 2106 and Omo without any enzyme showed low activity, Endod and the commercial detergents Arial and Omo showed relative result which is good.

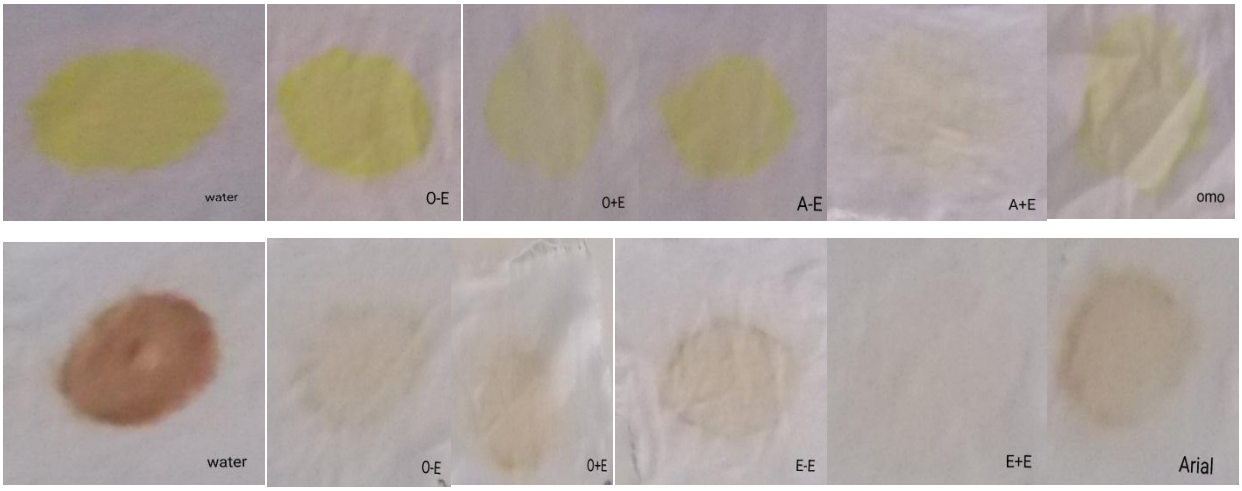

Figure 11 Wash performance checked on Egg yolk and blood stained clothes using commercial detergent and endod with and without enzyme; C (O-E)- Omo without enzyme, H (O)- Omo, F (O+E)- Omo with enzyme, and E (A+ E) - Arial with enzyme; A-E- arial without enzyme, E-E- endod without enzyme, E+E- endod with enzyme, A- Arial.

7. Discussion

Enzyme from isolate aau 2106 showed higher activity in the alkaline pH range 7-10.5 with an optimum pH at 9.5-10. The fact that it is optimally active in the alkaline range makes it an important enzyme for use in detergent formulation (Kalisz, 1988). This is because the pH of laundry detergents is generally in the range of 9.0–12.0.

Activity and stability in the normal laundry washing temperature range is considered important. Microbial alkaline proteases with optimum activity between 37-70°C have been reported by different researchers (El-Sawah and El- Din 2000; Eftekhar *et al* 2003). The protease reported in this study was active in the temperature range of 40-75^{o}C with maximum activity observed at 60^{o}C. Two of the most important alkaline proteases of the detergent industry, subtilisin carberg produced by *B. licheniformis* and subtilisin Novo or bacterial protease Nagese (BPN), produced by *B. amyloliquefaciens* also showed higher activity at 60^{o}C (Horikoshi, 1990).

Calcium is needed for the stability of enzyme and proteases which are used as detergent additives are also using calcium to increase the enzyme stability (Amare Gessesse and Birhanu abegaz Gashe, 1997). The activity of the enzyme reported in this study increased in the presence of calcium. Similarly the activity of alkaline protease from *Aspergillus oryxea* and *Canidiobolus coronatus* was increased in the presence of calcium (Bhosale *et al*., 1995).

Two researchers reported addition of soya bean, casein, gelatin, peptone, yeast extract, tryptone, etc. in the growth medium as nitrogen sources result in high alkaline protease production (Banerjee *et al.,* 1999; Hameed *et al.,* 1999). In the current study casein resulted high alkaline protease production. Similarly, Thomas *et al.* (2007), showed maximum protease production with casein from *Virgibacillus pantothenticus* and reported soya bean meal to be the highest for the optimal production of alkaline protease using *Bacillus sp* RJ14.

At a solid to moisture ratio of 1:1 higher enzymatic production was observed (Fig 7). Below and above the optimum enzyme production was reduced. For microbial growth under solid state fermentation the moisture content is known to have huge impact on enzyme production. With increasing moisture content free water molecules occupy the inter-particle space of replacing air.

This thus leads to a decrease in the porosity of the medium, interfering with gas exchange. On the other hand low amount of moisture decreases diffusion of nutrients and metabolites (Ellaiah *et al.,* 2002).

Under solid-state fermentation maximum enzyme production by isolate aau 2106 was reached after 96 h. Different organisms differ in the time course of their enzyme production. For example, isolates belonging to the genus *Bacillus* that produced high enzyme activity in the time range of 18 h to 96 h were reported (Banerjee *et al*., 1999; Singh *et al*., 2001). Ideally production in a short period could be preferred because it has huge cost implication and also reduce the chance for spoilage by contaminants. But from large scale production point of view the time isolate aau 2106 took is not that short.

The use of protease as supplement of endod and commercial detergents showed that the enzyme is stable in the detergent formulations indicating that it is resistant to surfactants, bleaching agent, and oxidizing agent that are present in each formula. This is very important property because bleach-stable enzymes are not generally available except for a few reports (Tsuchiya *et al.,* 1992). Oberoi *et al*., (2001), reported enzymes which are supplemented into detergent formulations must be active and stable at high pH range (9-12) and high temperatures (40-50^{o}C).

As shown in Fig 11, the enzyme which is supplemented in to Arial, Omo and endod removed the stain (blood and egg); higher removal of stain was observed at endod plus enzyme from isolate aau 2106.

The stain removal was done mechanically without any incubation with a minimum enzyme concentration 57 U/g. Kumar and Bhalla (2004), reported the protease that was used to remove egg yolk stain from test fabric needs 2 h for complete egg stain removal even at 40^{o}C. The removal of stains even at room temperature with a minimum concentration of enzyme implies that this study was highly cost-effective and needs less amount of enzyme supplementation during detergent formulation.

8. Conclusion and recommendation

The alkaline protease produced by isolate aau 2106 has a very good potential to be used as detergent additive supplementing endod. When used with endod it was very efficient for stain removal. The enzyme was also stable in the presence of endod. This shows enzyme supplementation of endod could allow the development of an ecofriendly detergent formulation.

The organism that produces the enzyme was able to grow under solid state fermentation using wheat bran as substrate and this help to reduce the production cost of the enzyme which makes the process highly economical.

However, detailed biochemical and biophysical characterization of the enzyme is not yet carried out. Therefore, it is recommended that:

1. The enzyme is purified and characterized in detail to have a better understanding of its biochemical and biophysical properties
2. The gene coding for the enzyme is cloned and some of the properties be modified to through protein engineering.

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