

Production, Characterization and Evaluation of Bacterial Protease as a Potential Additive to Enhance Detergency of Endod

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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 proteolytic 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°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 (Figure 1) [1].

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 [2].

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 [3]. Today proteases find important application in the detergent, leather tanning, pharmaceutical, and food and feed processing industries [4].

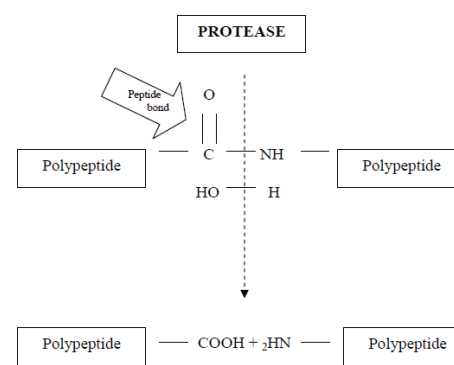


Figure 1. Protease catalysis of peptide bonds or proteolysis [1].

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 center proteases are classified as serine (EC. 3.4.21), cysteine (thiol) (EC 3.4.22), aspartic (EC3.4.23) and metalloprotease (EC 3.4.24) [5]. 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 [6], their major use being as components of household laundry detergents [7]. 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 [8].

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 the 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 a 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 [3] 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. Materials and Methods

All chemicals used in the study were purchased from the local market and were of analytical grade.

2.1. Media and Culture Conditions for Isolation, Screening and Enzyme Production

In this study, all media preparations were accomplished by following the procedures developed by [9]. 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 were 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; MgSO₄·7H₂O 0.2; CaCl₂ 0.1; K₂HPO₄ 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.

2.2. 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% trichloroacetic 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 the enzyme that released 1 µg tyrosine per min under the conditions described above.

2.3. Morphological and Biochemical Characterization of Isolate aau 2106

Colony characterization such as configuration, margin, pigment, arrangement, and shape were investigated microscopically and direct observation of the 24h old colony on the agar plate. Gram test was carried using the general procedure of gram test and 3% KOH. Biochemical characteristics such as catalase, casein, gelatin, and starch hydrolysis test were undertaken as follows. Catalase tests was investigated using 3% H₂O₂ by dropping on the slide with 48 h at 32°C grown fresh colony and observing whether an air bubble was detected [11]. Casein hydrolysis test was examined on casein agar plate [9], 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 the refrigerator for 20 min and see if the gelatin medium is hydrolyzed [12].

3. Characterization of the Enzyme

3.1. 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 mixture. After incubation at 60°C the activity of protease was measured [9].

3.2. 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°C and also the 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°C then the activity of protease was measured [9].

4. Enzyme production Through Solid State Fermentation

4.1. 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 0.01g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02g, K_2HPO_4 1g) having 1:1.5 ratio of moisture content were incubated at 32°C from 24 to 120 h. Enzyme extraction and the assay was carried out following the standard procedure.

4.2. 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 within the ratio of 1: 0.5 to 1:3 at 32°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 were pooled together and the activity measured from the pool.

4.3. 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 were carried out following the standard procedure.

4.4. Concentration of Enzyme

The enzyme was purified using 96% ethanol with in the ratio of 1:3 and it was precipitated at - 4°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.

4.5. “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. A weighed amount of 60g powdered endod was then added to an 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.

4.6. 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, and 10.

4.7. Stability of the Enzyme with Different Detergents and Endod

Detergent solutions (Ariel and Omo) at a concentration of 7mg/ml were prepared separately in distilled water. The solutions were boiled for 30min at 100°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.

4.8. Effect of Enzyme Concentration in Stain Removal

Different concentration 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 slaughterhouse. And this was evaluated by 5 students to rank as 1- 4 depending upon the cleanness of the clothes in ascending order meaning the cleaner cloth will get the highest rank by comparing with the control (a cloth only washed by water).

4.9. 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 the only endod, commercial detergents (Ariel and Omo), and also supplemented with 57 U/g enzyme, commercial detergents without enzyme (by boiling 7mg/ml detergent at 100°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 a percentage.

4.10. Data Analysis

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

5. Results

5.1. 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°C (Figure 2).



Figure 2. isolate aau 2106 grown on a casein agar plate and the clear zone shows the presence of proteolytic activity.

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, baau 1805	aau 1802, aau 1809	aau 1801, aau 1806	aau 1908
aau 1904, aau 1905,	aau 1901, aau 1907,	aau 1808, aau 1902	aau 2003
aau 2001, aau 2006, aau 2009	aau 1909, aau 2002		
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

5.2. 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. Characterization of Protease Enzyme

6.1. 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 (Figure 3). The enzyme lost 63.2% of its maximum activity under slightly acidic conditions (below pH 6.5).

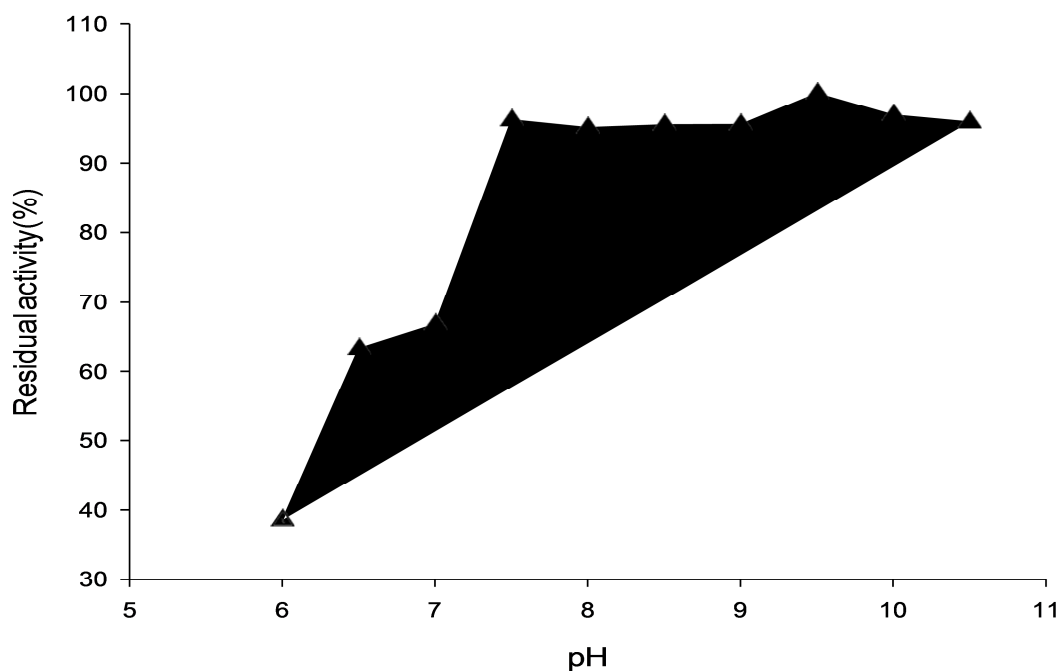


Figure 3. Effect of pH on the activity of protease.

6.2. 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°C. The enzyme showed higher activity in temperature range of 55-65°C with an optimum at 60°C (Figure 4) both in presence and absence of calcium.

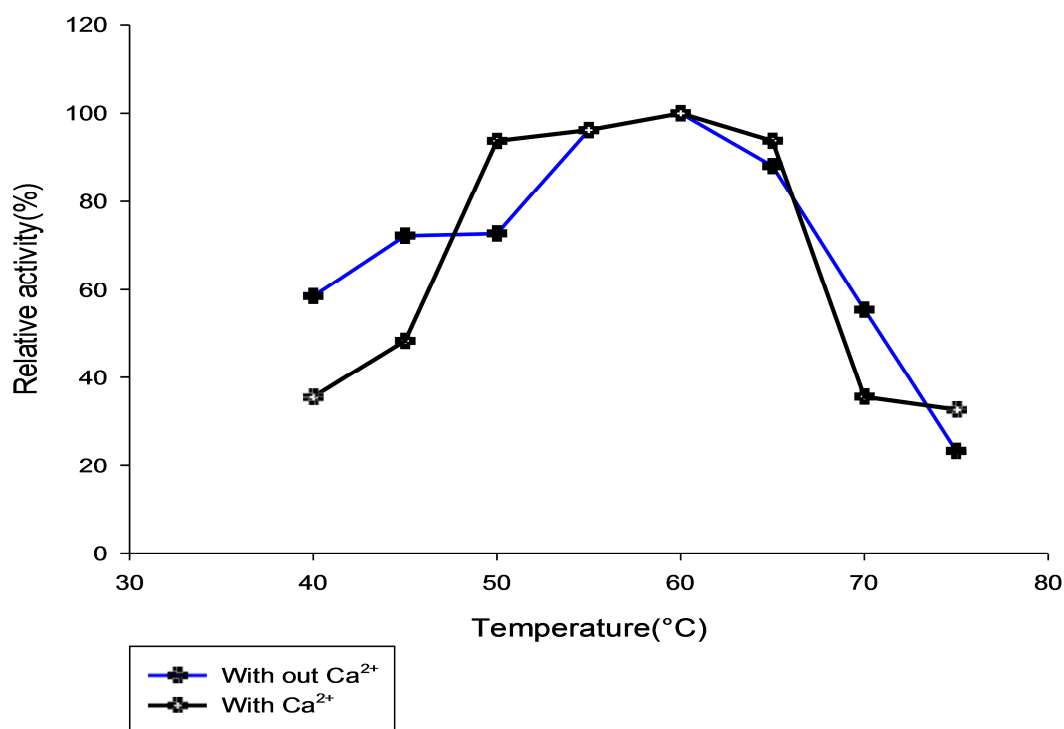


Figure 4. Effect of temperature on the activity of protease.

6.3. 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°C) (Figure 5).

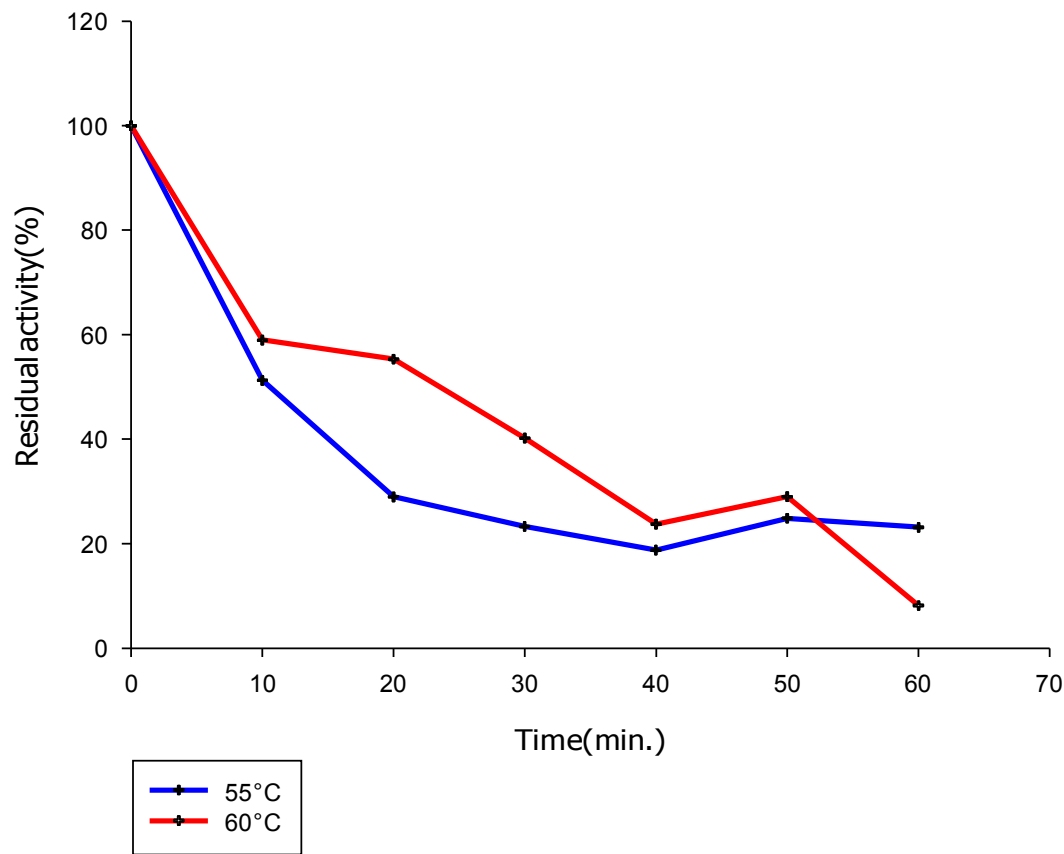


Figure 5. Effect of temperature stability on protease.

6.4. Enzyme Production Through Solid State Fermentation

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 (Figure 6).

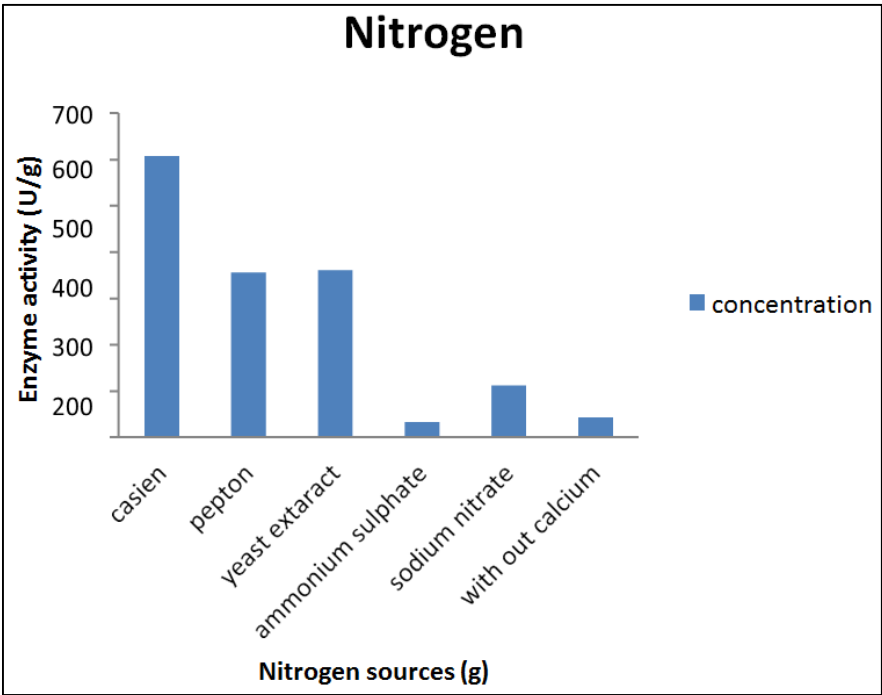


Figure 6. Effect of nitrogen on the crude protease aaU 2106.

6.5. 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. A moisture content above the optimum resulted in gradual decrease of enzyme activity (Figure 7).

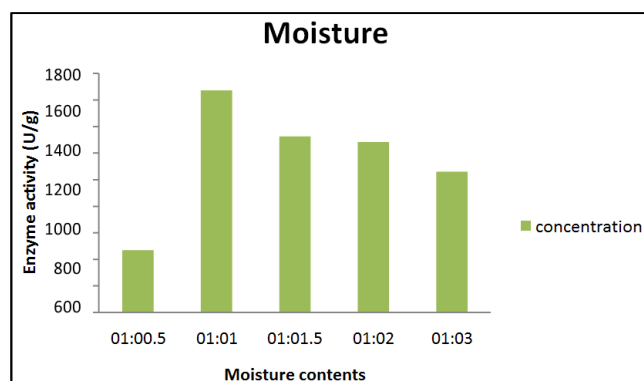


Figure 7. Effect of moisture on the enzyme activity.

6.6. 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 (Figure 8).

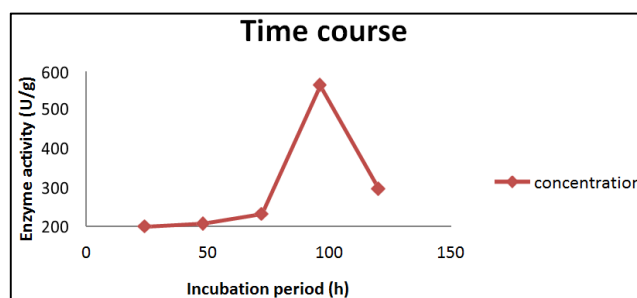


Figure 8. Effect of Time on the Production of Protease.

6.7. 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.

6.8. Enzyme Stability in the Presence of Endod and Commercial Detergents

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

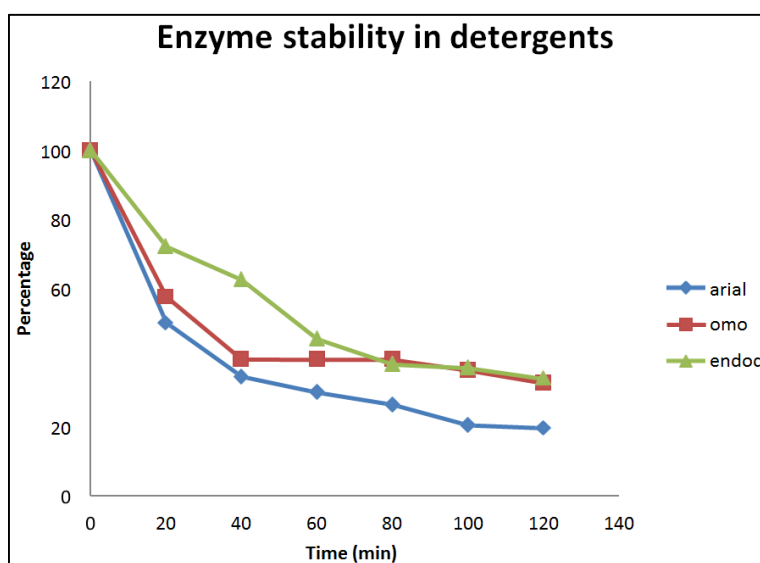


Figure 9. The stability of protease with commercial detergents and endod.

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.

6.9. 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.

6.10. 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 Ariel supplemented with protease enzyme and in a cloth, which is stained by blood showed high activity on endod supplemented with protease.

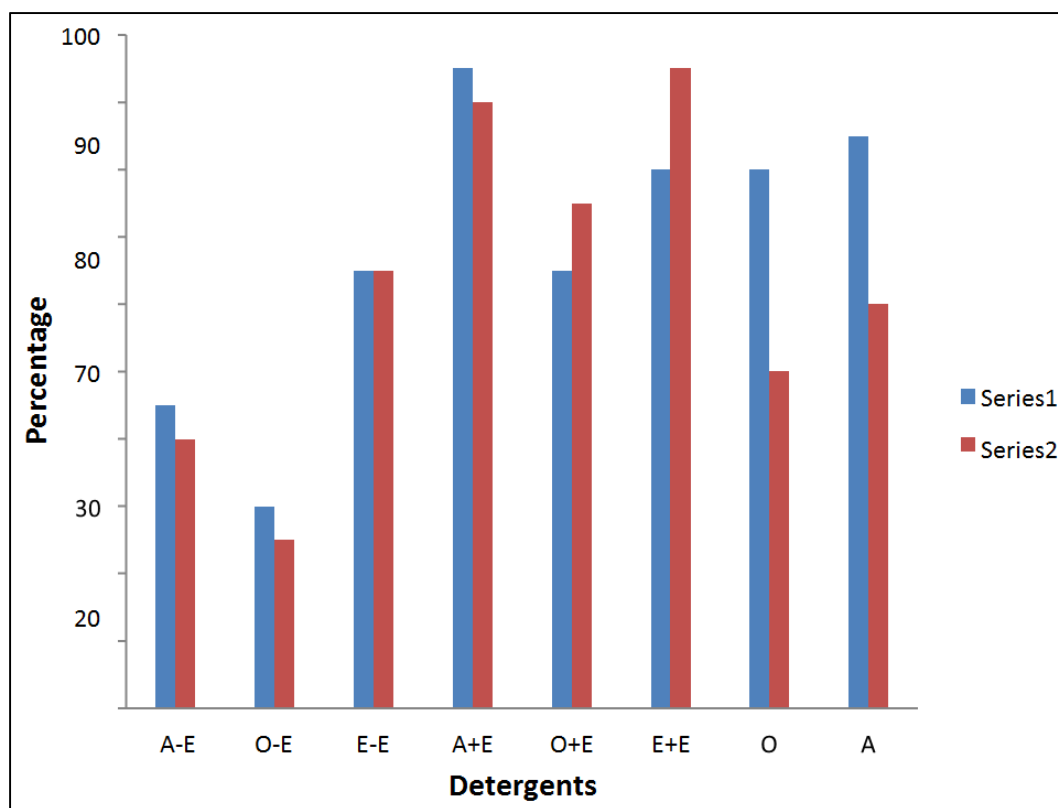


Figure 10. Stain removal activity of A-E- Ariel without enzyme, O-E- Omo without enzyme, E-E- Endod without enzyme, A+ E - Ariel with enzyme, O+E- Omo with enzyme, E+E- Endod with enzyme, O- Omo, A- arial, and on a cloth stained by egg (series 1) and blood (series 2).

High stain removal activity was observed when the commercial detergent Ariel supplemented with enzyme from isolate aa2106 was used for the washing of egg stained cloths. And endod plus enzyme from aa2106 have high

stain removal activity and Ariel plus enzyme from aa2106 and Omo without any enzyme showed low activity, Endod and the commercial detergents Ariel and Omo showed relative result which is good.

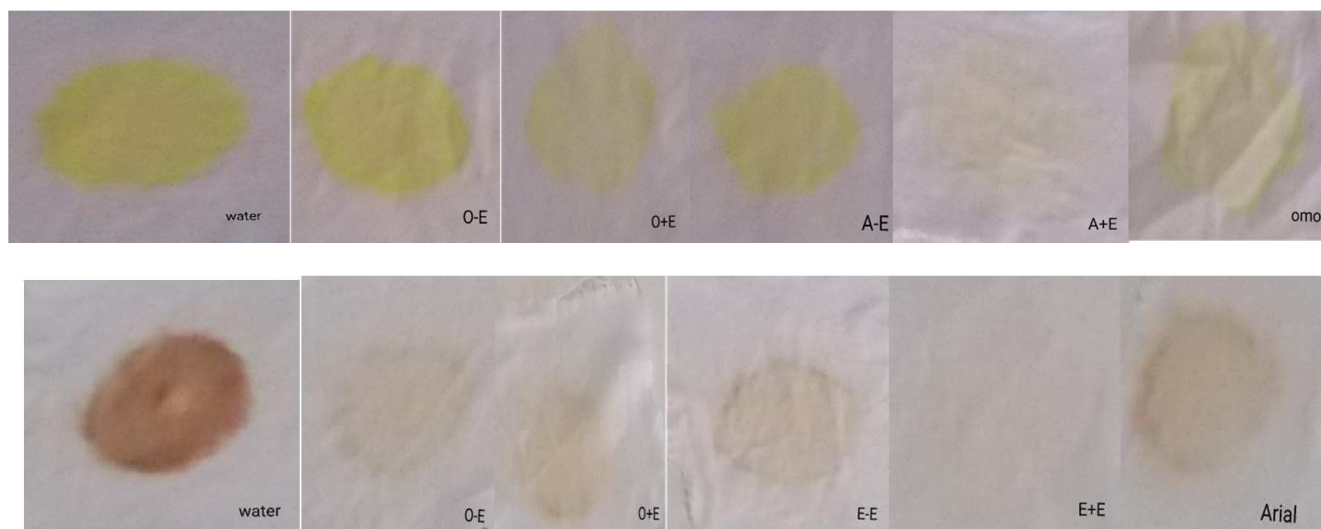


Figure 11. Wash performance checked on Egg yolk and blood stained clothes using commercial detergent and endod with and without enzyme; C (O-E)- Omo without enzyme, H (O)- Omo, F (O+E)- Omo with enzyme, and E (A+ E) - Ariel with enzyme; A-E- arial without enzyme, E-E- endod without enzyme, E+E- endod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

7. Discussion

The 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 the detergent formulation [13]. 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 are considered important. Microbial alkaline proteases with optimum activity between 37-70°C have been reported by different researchers [14, 15]. The protease reported in this study was active in the temperature range of 40-75°C with maximum activity observed at 60°C. Two of the most important alkaline proteases of the detergent industry, subtilisin carberg produced by *B. licheniformis* and subtilisin Novo or bacterial protease Nagease (BPN), produced by *B. amyloliquefaciens* also showed higher activity at 60°C [8].

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 [9]. The activity of the enzyme reported in this study increased in the presence of calcium. Similarly, the activity of alkaline protease from *Aspergillus oryzae* and *Canidiobolus coronatus* was increased in the presence of calcium [16].

Two researchers reported an addition of soya bean, casein, gelatin, peptone, yeast extract, tryptone, etc. in the growth medium as nitrogen sources results in high alkaline protease production [17, 18]. In the current study, casein resulted high alkaline protease production. Similarly, Thomas *et al.* (2007), showed maximum protease production with casein from *Virgibacillus pantothenicus* 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 (Figure 7). Below and above the optimum enzyme production was reduced. For microbial growth under solid state fermentation the moisture content is known to have a 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 a low amount of moisture decreases the diffusion of nutrients and metabolites [19].

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 [17, 20]. Ideally, production in a short period could be preferred because it has a 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 a 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 is present in each formula. This is very important property because bleach-stable enzymes are not generally available except for a few reports [21]. [22], reported enzymes which are supplemented into detergent formulations must be active and stable at high pH range (9-12) and high temperatures (40-50°C). As shown in Figure 11, the enzyme which is supplemented in to Ariel, 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. [23], 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°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 a 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.

List of Abbreviation

AAU: Addis Ababa University

H: Hour

SSF: Solid state fermentation

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