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Extraction and Partial Purification of Protease from Fermented Beans Using *Aspergillus niger*

Mgbede Timothy[1], Roy Yohanna Emochone[2], Isaac John Umaru[3]
Federal University Wukari, Taraba State, Nigeria
umaruisaac@gmail.com

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Abstract

Proteases are enzymes that are found everywhere and play important roles in both the creation and breakdown of substances in living organisms. The utilization of microbial proteases in food fermentation is a long-standing practice that has now expanded to various industries due to advancements in the 'omics' era and genetic and protein engineering techniques. The protease enzyme was derived from *Aspergillus niger*, which was isolated from fermented cooked discarded beans. The enzyme was partially purified and described utilizing an affordable and environmentally friendly modified technique. The impact of temperature, pH, and manufacturing time on the enzyme was assessed. The optimal conditions for protease generation were a temperature of 40°C, a pH of 7.0, and duration of 6 days. The kinetic parameter values for Km and Vmax were determined to be 0.9181 mg/mL and 1.08914 µmol/min respectively. The protein determination experiment showed that the highest protein production occurred on day 6, following 5 days of optimal protein production. The research has shown that *Aspergillus niger* can efficiently generate protease at a low cost, making it suitable for various industrial applications including pharmaceuticals, food production, detergents, and cosmetics.

Keywords: Protease, Fermented Beans, *Aspergillus Niger,* Enzyme, Fungus

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INTRODUCTION

Proteases represent a broad group of enzymes which break down or hydrolyze proteins or peptides (Preeti *et al.,* 2021). The proteases act on the peptide bonds joining the adjacent amino acid residues in a protein molecule and cleave them leading to formation of shorter peptides and amino acids (Razzaq *et al.,* 2019). These hydrolytic enzymes are ubiquitous in nature and have been found in all living forms encompassing the eukaryotes like plants, animals, fungi, protists as well as the prokaryotic domains of bacteria and archaea. Even several viruses are also known to encode their own proteases (Bernardo *et al.,* 2018).

As per the Enzyme Commission classification, proteases are placed in the class 3 (hydrolases), sub-class 4 with each proteolytic enzyme assigned unique number as EC 3.4.x.x (Contesini *et al.,* 2018). These enzymes have been categorized on the basis of various parameters like the site of action, the type of substrate, active pH range, mechanism of action involving particular amino acid present in the active site (Guleria *et al.,* 2016a, b). Based on their site of action at the N or C terminus, they are classified as amino and carboxypeptidases (Naveed *et al.,* 2021). Aminopeptidases (EC 3.4.14.x) act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. And the carboxypeptidases act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine peptidases (EC 3.4.16.x), metallopeptidases (EC 3.4.17.x), and cysteine peptidases (EC 3.4.18.x), based on the nature of the amino acid residues at the active site of the enzymes. Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain. The endopeptidases are divided into four subgroups based on their catalytic mechanism, serine proteases (EC 3.4.21.x), cysteine proteases (EC 3.4.22.x), aspartic proteases (EC 3.4.23.x), metalloproteases (EC 3.4.24.x) (Rao *et al.,* 1998; Contesini *et al.,* 2017). The enzymes are categorized primarily on the basis of their phylogenetic relationships and mechanism of action in this database (Rawlings *et al.,* 2018). Similarly, the proteolytic enzymes have also been sorted according to the pH range which they have a higher activity: acidic (pH 2.0 to 6.0), neutral (pH 6.0 to 8.0) and alkaline (pH 8.0 to 13.0) (Gupta *et al.,* 2002; Tavano *et al.,* 2018).

Proteases from microbial sources have dominated applications in industrial sectors. Fungal proteases are used for hydrolyzing protein (Paula *et al.,* 2015). Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively.

Proteases from microorganisms have attracted a great deal of attention in the last decade because of their biotechnology potential in various industrial processes such as detergent, textile, leather, dairy and pharmaceutical preparations (Saran *et al.,* 2007). Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively. This group represents one of the largest groups of industrial enzymes and accounts for approximately 60% of the total enzyme sales in the world (Zambare *et al.,* 2011). Fungal proteases have attracted the attention of environmental biotechnologists because fungi can grow on low cost substrates and secrete large scale of enzymes into culture medium which could ease downstream processing (Anitha *et al*., 2013). The increase in demand for proteases and relative rise in price triggers this research work which aim at producing protease from fungi (isolated from wasted beans) which could offer some inherent advantages like rapid production rates, cheaper investment and ease downstream process. To satisfy the outstanding want of economic society as well as proffer a recycling means of a percentage organic waste.

MATERIALS AND METHODS

Materials

Spectrophotometer, beaker, test tubes, spatula, syringes, petri dish, Whatman filter paper, measuring cylinder, weighing balance, candle, wasted boiled beans, micro pipette, Erlenmeyer flask, fungi isolate, rice bran, cotton wool, incubator, autoclave, pH meter, microscope, slide, cover slip, water bath, centrifuge, thermometer.

Reagent

Distilled water, Casein, Follin reagent, TCA (trichloroacetic acid), KH_2PO_4 (buffer pH= 7.0), KNO_3, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, NaOH, HCl, PDA, chloramphenol, lactophenol, HCl, folin-ciocalteu, NaK tartrate, $CuSO_4$, Na_2CO_3, Tris-HCl buffer, sodium acetate buffer, A. 2% Na_2CO_3 in 0.1N of NaOH, 1% NaK tertrate in H_2O., 0.5% $CuSO_4$ in H_2O, Reagent I. 48ml of A, 1.0 mL of B and 1.0 mL of, Reagent II. 1part Folin-Ciocalteau (2N), 1part distilled water.

Source of Sample Collection

Beans was bought opposite Federal University Wukari, boiled and allow to spoil after some days. Fungi was isolated from it.

Preparation of Sample

The beans sample was boiled and transferred into a bowl in which it was stored in a cool and moist area, with the bowl left open to allow fungal growth. The beans stayed for 3 days and the fungus produced was collected.

Isolation of Protease Producing Fungi

Fungi were isolated from decayed beans sample, using a tenfold serial dilution-plating technique on potato dextrose agar (PDA) plates into which 30 µg of chloramphenicol was added. This was incubated at room temperature, that is, 28 ± 2°C Mohammed (2013). The culture was observed daily and fungal growth was subcultured onto fresh plates of PDA until pure isolates were obtained.

Identification of Fungal Isolates

The alkaline protease producing fungi were identified based on morphological characteristics and microscopic observation of fungal spores using lactophenol cotton blue staining. For morphological characterization, the fungal isolates were cultivated on dextrose agar medium. The shape, size, arrangement and development of conidiophores, phialides and conidiospores were studied using the taxonomic tools of Hoog *et al.* (2000).

Screening of the Fungal Pure Culture Isolate

The screening method used by Suganthi *et al.,* (2013), was used. Proteolytic production of the fungi strains was screened on agar plates supplemented with 5% NaCl and 1% casein (MNA). The plates were incubated o vernight at 37 °C. The protease producing strains were selected based on the zone of clearance. That is by the formation of a clear zone around colonies after precipitation with 1 M HCl solution (Sethi and Gupta 2015).

Fermentation

Proteases are generally produced using submerged fermentation due to its apparent advantages in consistent enzyme production characteristics with defined medium and process conditions and advantages in downstream in spite of the cost-intensiveness for medium components Sharma *et al.*, (2017). Submerged fermentation involves inoculation of the microbial culture into the liquid medium for production of the desired product.

Fermentation was carried out in 250 mL Erlenmeyer flask containing 50 ml of sterilized molasses. The medium was cooled to room temperature and was inoculate with 2 mL of

fungal spore suspension. The flasks after inoculation were placed in the orbital shaker rotating at 150 rpm and at 32^0C for 7 days. Later, the contents of the flasks were filtered using Whatman No. filter paper and the filtrate was used for the assay of protease enzyme by modified Anson's method Radha *et al.*, (2011).

Protease Activity

Protease activity was assayed by the modified Anson's method, (1938) as described in Thangam and Rajkumar, (2002) and Park *et al.,* (2013) using casein as the substrate. 1.0 mL of the culture broth was taken in a 100 mL flask and 1.0 mL of pH 7.0 phosphate buffer added to it. One mL of the substrate (2% casein) was added to the buffer enzyme solution and incubated at 37°C for 10 minutes in a water bath. At the end of 10 minutes, 6.0 ml of 5% TCA (trichloroacetic acid) was added to stop the reaction. The precipitated casein was then filtered off and 1.0 mL of the filtrate were taken into each test tube. To this, 3.0 mL of 0.5% NaOH solution and then 1.0 mL of the folin-ciocalteu reagent each was added. Final readings were taken in a spectrophotometer at 280 nm. Blanks of the samples were prepared by adding the TCA into buffer solution before the addition of substrate. One unit of protease activity was defined as the amount of enzyme required to liberate one microgram (1 μg) of tyrosine from casein per minute at 40°C under the assay conditions described above. The effect of various factors like inoculums size, carbon source, nitrogen sources, pH and temperature on the production of protease was studied.

Protein Determination

The protein content of the enzyme was measured according to the Lowry method of determining protein concentrations which lies in the reactivity of the peptide nitrogen with the copper (II) ions under alkaline conditions and the subsequent reduction of the Folin Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids (Lowry *et al.,* 1951)

1.0 mL of the crude was pipetted into each test tube and the other is make up to 1.0 mL using distilled water. The test tube with 1.0 mL distilled water serve as blank. Add 4.5 mL of Reagent I and incubate for 10 minutes. After incubation add 0.5 ml of reagent II and incubate for 30 minutes. Measure the absorbance at 660 nm

Purification of Protease

Ammonium Sulphate Precipitation

The principle of ammonium sulfate precipitation lies in "salting out" proteins from the solution. The proteins are prevented from forming hydrogen bonds with water and the salt facilitates their interaction with each other forming aggregates that afterward precipitate out of solution Grodzki and Berenstein (2010).

Ammonium sulphate precipitation profile was carried out to determine the concentration of ammonium sulphate suitable for protease production. This was done at different ammonium sulphate saturation ranging from 20 – 100% sulphate at intervals of 10% in each test tube containing 10 mL of crude enzyme. These were allowed to stand at cold temperature of about 4°C for 30hours. The test tubes were centrifuged at 3500 rpm for 30mins and pellets redissolved in equal volumes of phosphate buffer pH 7.0. Protease activity of the precipitates was assayed to determine the percentage ammonium sulphate saturation that has the highest activity.

Characterization of Protease

Effect of pH Change on Protease Activity

The effect of pH on enzyme activity was determined using sodium acetate buffer (pH range, 5.0–6.0), phosphate buffer (pH range, 6.5–7.5) and Tris-HCl buffer (pH range, 8.0–9.0) Yogesh *et al.* (2009). Each pH buffer was use for the enzyme assay according to the modified Anson's method, as described in Thangam and Rajkumar, (2002) to obtain optimum pH for the enzyme.

Effect of Temperature Change on Protease Activity

The optimum temperature for protease activity was obtained by changing temperature of incubating the enzyme at the interval of 5unit ranging from (25-55)°C for 1hour and at pH 7.0. The activity was assayed according to the modified Anson's method, method (Thangam and Rajkumar, 2002)

Substrate concentration was varied at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 respectively. To each test tube used 1.0 ml of phosphate buffer and 1.0 ml of enzyme was added to the varied substrate concentration and incubated at 40°C for 10 minutes. 6ml of trichloroacetic acid was added to terminate the reaction and the mixture was filtered using Whatman filter paper, then 3ml of NaOH and 1ml of Folin Ciocateau reagent were added to 1.0mL filtrate,

allowed to stand for 10 minutes and assayed at 280nm using a spectrometer. (Thangam and Rajkumar, 2002).

RESULTS



Figure 1: Plate for fungi screening and isolation

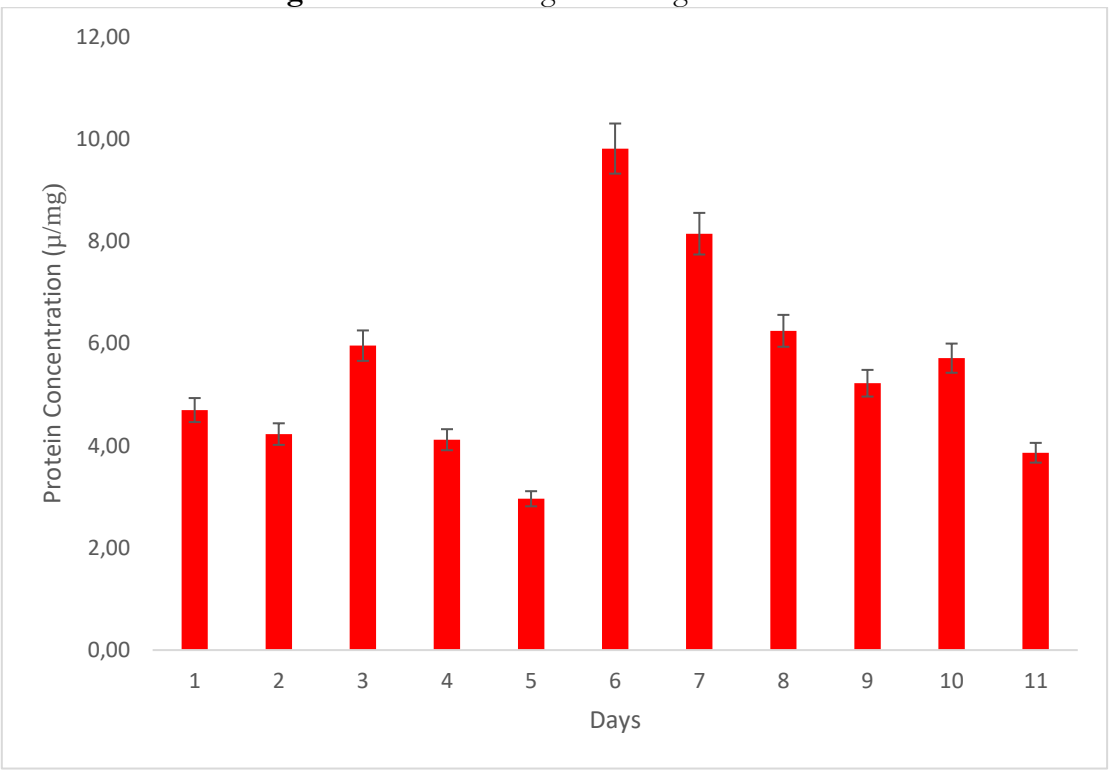


Figure 2: Effect of time on protein determination

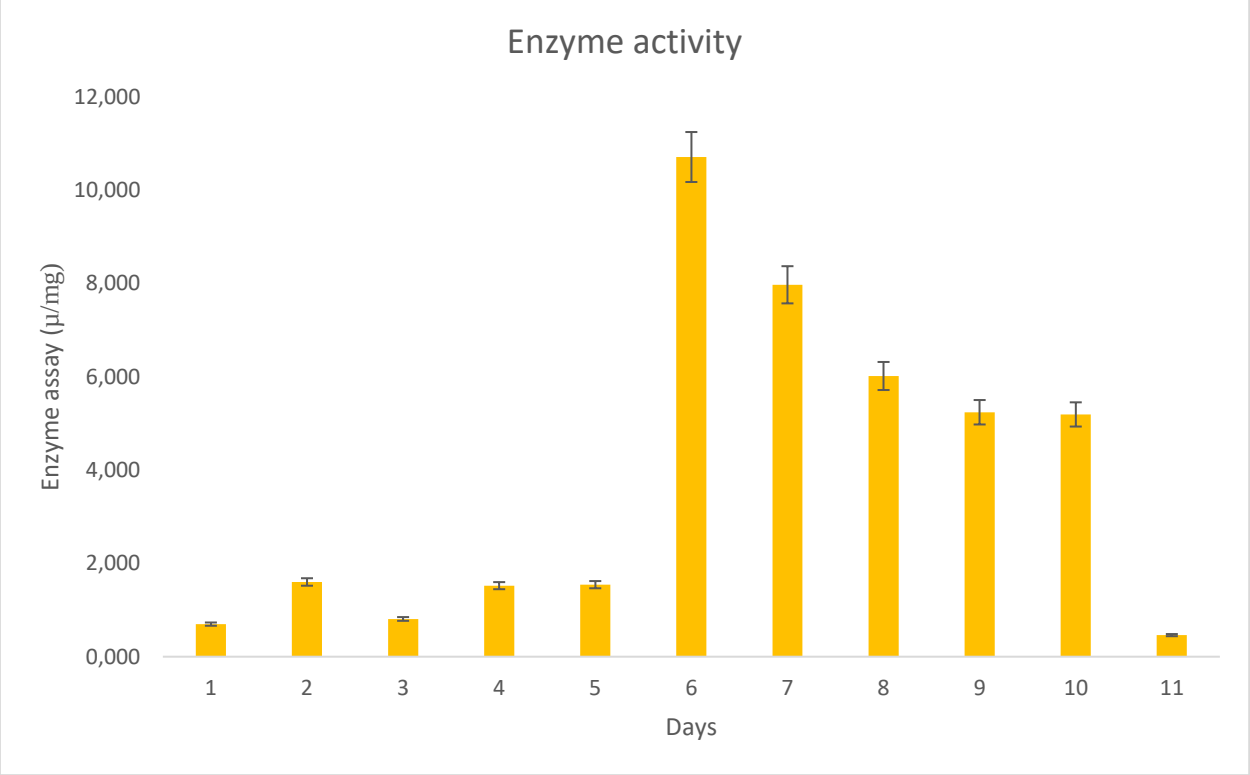


Figure 3: Effect of time of fermentation on enzyme assay

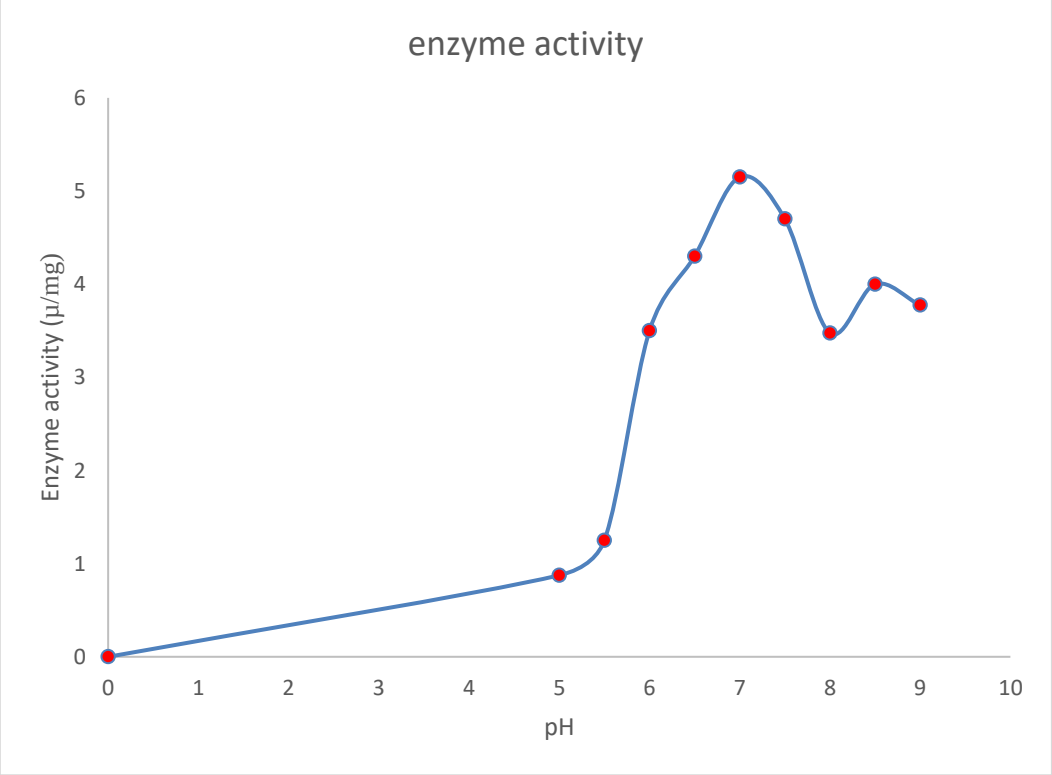


Figure 4: Effect of pH on enzyme activity

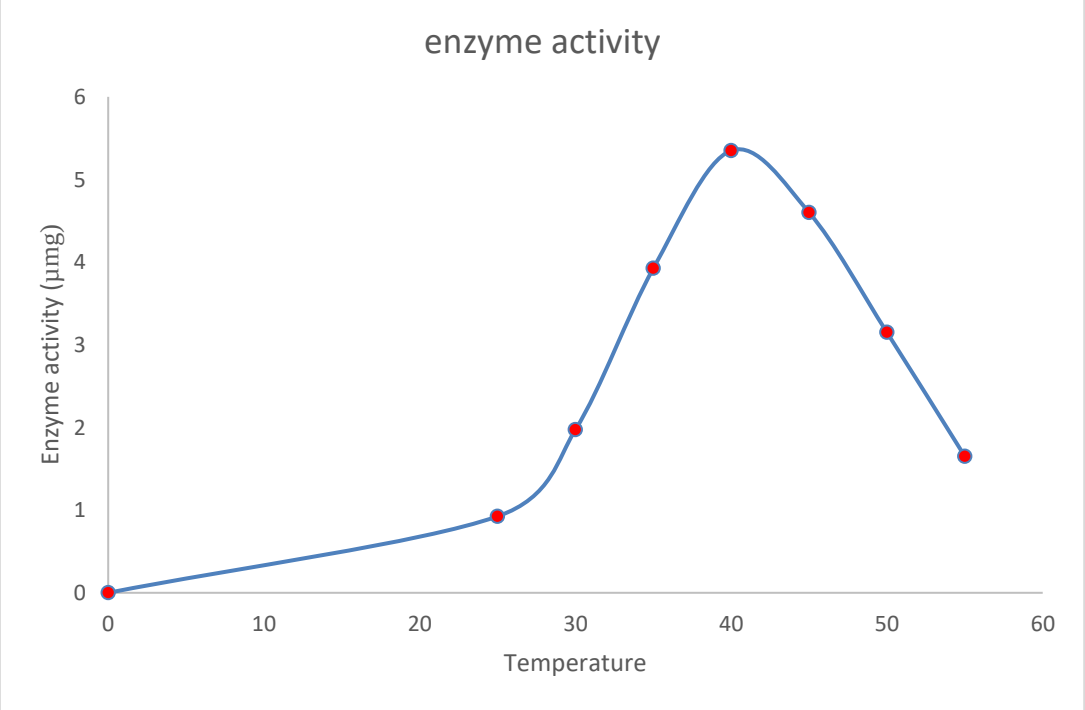


Figure 5: Effect of temperature on enzymes activity

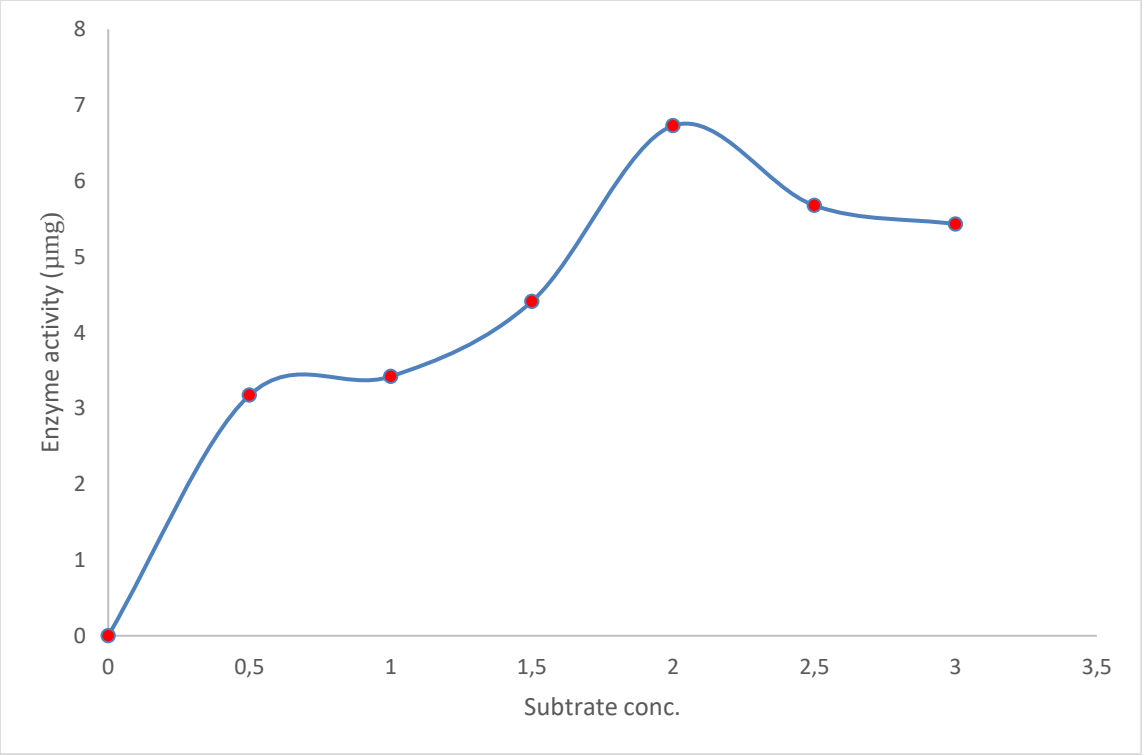


Figure 6: Effect of substrate concentration on enzyme activity

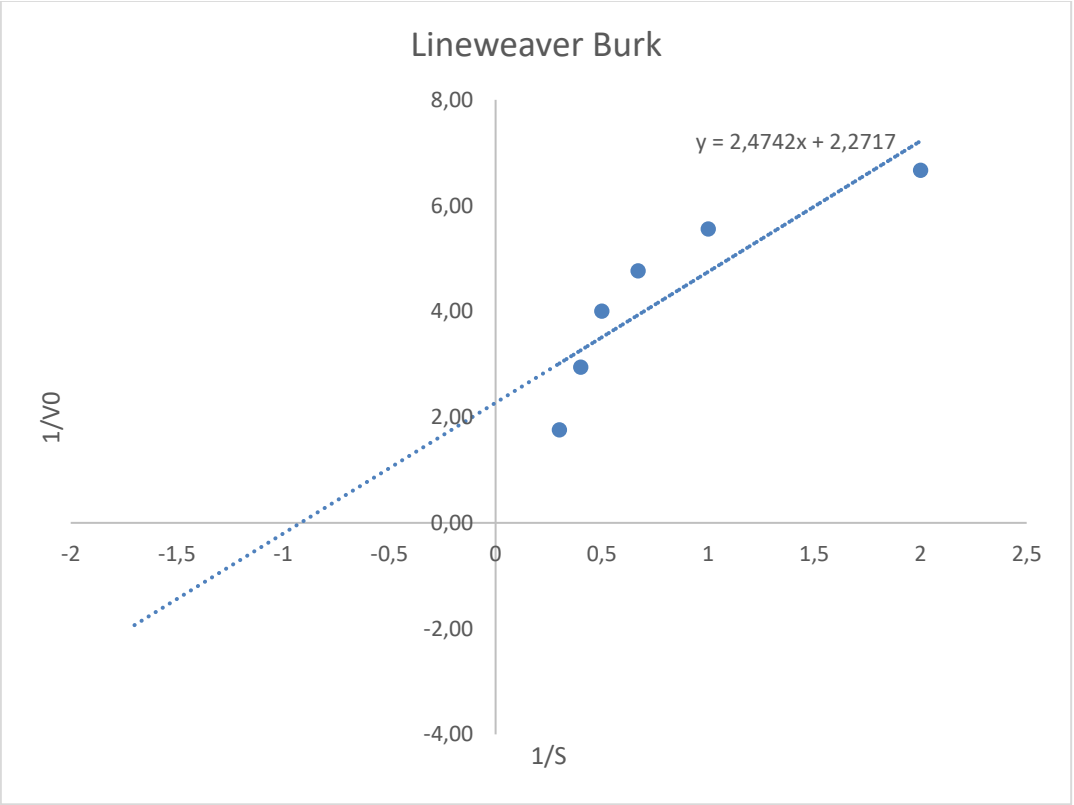


Figure 7: Line weaver bulk plot of the enzyme activity and substrate concentration

DISCUSSION

Proteases are found everywhere in animals, plants, and microorganisms. Microbes are highly abundant in proteases and are considered the preferred source of enzymes due to their fast growth, ability to thrive in limited space, and susceptibility to genetic modifications. Decades ago, several industries such as pharmaceutical, food, dairy, and detergent, utilized protease enzymes derived from diverse types of microbes. The study utilized *Aspergillus niger* to isolate and partially purify the protease enzyme from fermented beans. The enzyme reached its peak activity after 5 days of fermentation (Figure 3), coinciding with the end of the lag phase of fungal growth. This finding aligns with the findings of Morimura *et al.* (1994), who observed that *Aspergillus flavus* reached its peak production of protease within 10 days of incubation. Furthermore, the impact of the number of incubation days on protein levels and protein determination produced a noteworthy outcome, as depicted in figure 3. On the sixth day, the protein determination was the greatest, and it was followed by the seventh day. Based on the figure, it can be inferred that there was no substantial protein determination until after 5 days. Therefore, on the sixth day, protein production reached its maximum level.

The ideal temperature for the partly purified protease was 40°C. The findings indicated that the protease maintained a substantial level of enzymatic activity at low temperatures. This suggests that the protease exhibited significant stability at low temperatures. The optimal temperature for the protease is 50°C, as seen in the protease of *Aspergillus terreus* (Bushra *et al*., 2010) and *Aspergillus oryzae* (Sumantha *et al.,* 2005 and Ao *et al*., 2018). The investigation of the enzyme's thermostability at a temperature of 40°C indicates promising prospects for its utilization in several culinary industries, including baking and brewing.

pH refers to the measure of acidity or alkalinity in a solution, and even a slight alteration in pH can impact the activity of an enzyme. The partly purified protease had a distinct pH profile, as documented. The enzyme exhibited a wide spectrum of activity across acidic, basic, and neutral pH values. The highest level of activity was observed at pH 7.0, while it also showed significant activity at pH 6.5 and 7.5. The data aligns with previous research findings, specifically indicating that fungi have the ability to produce proteases with acidic, neutral, and alkaline properties (Siala *et al*., 2009; and Sumantha *et al*., 2005). In their study, Ao *et al.* (2018) analyzed the properties of a neutral protease derived from Aspergillus oryzae Y1, which was obtained from organically fermented broad beans. They found that this protease is a protein with a molecular weight of 45 kD and has an optimal pH of 7.0. De Oliveira *et al.* (2020) documented the synthesis of a protease with high heat resistance by the fungus *Moorella speciosa*, with an optimal pH of 6.5. However, when immobilized on MAT540 microspheres, the pH that yielded the best results shifted to 5.74. The protease studied in this research has a broad activity range (pH 5.0 to 9.5), which is significantly different from the limited pH range (3.0 to 4.0) identified for cathepsin D (CtsD) from *Aspergillus niger* ATCC 26933 (Vickers et al., 2007). The protease derived from *Aspergillus niger*, after undergoing partial purification, exhibited remarkable stability at pH 7.0, suggesting that the enzyme is a neutral protease.

The kinetic parameters (Vmax and Km) were determined to be 1.08914 μmol/min and 0.44019 mg/mL-1, respectively. These results demonstrate that the partly purified protease exhibits a high rate of degradation and a strong affinity for its substrate. The study conducted by Shankar *et al.* in 2011 revealed the presence of a protease enzyme in Beauveria species, which had a Km value of 5.1 mgml-1 when casein was used as the

substrate. The study conducted by Devi *et al.* in 2008 reported a reduced Km value of 0.8 mgml-1 while utilizing casein.

CONCLUSION

This work describes the extraction and partial purification of protease as well as characterization of *Aspergillus Niger* using a reformed method from different approaches. The result from the research has shown that protease can be produced from *Aspergillus Niger* isolated from beans, at rapid and cause effective rate. From the studies, it was revealed that, the enzyme could be of good use for industrial purposes, such as food, pharmaceutical, cosmetic, *etc.*

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