

Extraction, purification and characterization of protease from *Aspergillus Niger* isolated from yam peels

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Abstract: Protease was obtained from *Aspergillus niger* isolated from yam peels; a food waste, purified and characterized. Purification was achieved using ion exchange DEAE column and gel filtration (Sephadex G-200) chromatography. Effects of temperature; pH and production time on protease production were investigated. Also, physicochemical characteristics of the purified enzyme were investigated. The optimum production of protease was at temperature, pH and time of 37°C, 7.0 and 42 hrs respectively. The results showed that purified protease had more specific enzymatic activity than crude samples from *Aspergillus Niger*. whereas the specific activity of crude enzyme was 0.51 (U/mg), while the purified enzyme had an improved specific activity of 8.51 (U/mg). Optimum temperature and pH values of the purified protease were found to be 50°C and 10.0, respectively. pH stability of the enzyme ranged from 3.0- 12.0. At 3.0 and 10.0 it retained 70% and 60% of its activity after 5 hrs of incubation. Temperature stability ranged between 30°C and 90°C but most stable at 50°C retaining 94% of its activity after 1 h of incubation. The enzyme exhibited maximum activity on casein, among other protein substrates. EDTA, Cu²⁺, Fe²⁺, Mg²⁺, and Ca²⁺ inhibited its activity while Na⁺ enhanced it. The enzyme was purified 16.60-fold, had a yield of 10.96 and the apparent molecular weight was 46.90 kDa. The study revealed that protease from *A. niger* can be exploited for protein conversion biotechnologies.

Keywords: Protease, *Aspergillus Niger*, Yam Peels, Fermentation, Purification, Characterisation

1. Introduction

Waste is defined by (Oresanya, 1998) as any unwanted material intentionally thrown away for disposal. However, certain wastes may eventually become resources valuable to others once they are removed from the waste stream (Wiebe, 2003). Waste products arise from our ways of life and they are generated at every stage of process of production and development. Solid waste is used to describe non-liquid waste material arising from domestic, trade, commercial and public services. One of the most critical problems of developing countries is improper management of vast amount of wastes generated by various anthropogenic activities. More challenging is the unsafe disposal of these wastes into the ambient environment. Water bodies especially freshwater reservoirs are the most affected. This has often rendered these natural resources unsuitable for both primary

and/or secondary usage (Fakayode, 2005). Wastes entering water bodies are both in solid and liquid forms. They are mostly derived from industrial, agricultural and domestic activities. The resultant effects of this on public health and the environment are usually great in magnitude (Osibanjo *et al.*, 2011).

Edible foods are also wasted when cultural or individual preferences say that food is undesirable Both edible and inedible foods may be considered garbage and therefore wasted. Edible foods are considered inedible when their quality deteriorates until they become unhealthy or noxious. Deterioration of food occurs from microbial contamination or from rotting due to overproduction, storage problems, or improper preparation (Osibanjo *et al.*, 2011).

Protease finds applications in various industries like

detergent, leather, pharmaceutical, food and waste water treatment. Biotechnological importance of these enzymes has been realized by the leather industries in bating hides (Bhosale, 1995). Detergent industries tapped its potential in stain removal. In food industries they are used in crude preparations. In pharmaceutical industries they are useful as ingredients in preparing medicines (Rao *et al.*, 1998).

This research justify the need to explore native fungal isolate, capable of producing proteases and at the same time relatively stable under prevailing operating conditions. The use of cheap agricultural residue (organic substrate) has enormous potential in reducing enzyme production cost. With this in mind, the objectives of the research were to: isolate and purify protease from fungi grown on yam peels, screen the isolates for protease, investigate and characterize the properties of the extracellular enzymes (protease) and investigate if the proteases obtained are relatively stable at operating condition.

2. Materials and Methods

Sources of Material: The material (yam peels) used in this study were sourced from a local fried yam seller in Akure metropolis, Ondo state, Nigeria. The yam peels were collected in clean cellophane bag, allowed to undergo natural bio- deterioration and transferred to the laboratory. All the reagent and chemicals used in the analysis were of analytical grade.

2.1. Isolation, Inocula and Fermentation Medium for Protease Production

Potato dextrose agar (PDA) with nutrients compositions of Potato Extract (Solid) 4g, Dextrose 20g, Agar 15g and pH 5.6 used for isolation was prepared according to the manufactures' specifications. Serial dilution was carried out while Potato dextrose agar (PDA) supplemented with 0.2g/L chloramphenicol was used in the mycological analysis of *Aspergillus niger*. One milliliter of dilution $1:10^3$ was transferred with a pipette into sterile plates. Molten agar cooled at 45°C was poured over the samples in each plate and then incubated at room temperature for 72 h. The spores on the potato dextrose agar plates were counted using a digital illuminated colony counter model no.KA00-74A (B. Bran Scientific & Instrument Co. England). Each distinct spore of fungi was transferred to freshly prepared potato dextrose agar plates for purification and incubated at room temperature for 72-168 h. The colonies forming clear zones around them were picked up and streaked on potato dextrose agar plates to get pure culture, and finally transferred to potato dextrose agar slants and maintained at 4°C.

Determination of optimum growth conditions: The protease production medium was modified according to the method of (Keay *et al.*, 1972) to contain sucrose 250mg, casein 500 mg, locust beans 50mg, di-potassium hydrogen phosphate 1gm, magnesium sulphate 200mg per liter at pH 7 at 28°C in an incubator (200 rpm). The flask containing 100 ml of sterile fermentation medium was inoculated at 28°C for

72 h in a rotary shaker regulated at 180 rpm. The culture medium was centrifuged at 3200 g for 1 h in a refrigerated centrifuge at 4°C to remove the fungal mycelia and medium debris. The supernatant was used as crude enzyme solution and was precipitated gradually up to 80% ammonium sulphate saturation. The precipitate after centrifugation at 3200 g for 30 min dissolved in minimal volume of 0.1% Tris-HCl buffer (pH 9) and was dialysed against 0.1mM potassium phosphate buffer for 48 h at 4°C. The supernatant was collected and subjected to estimate the protease activity and protein content.

Assay of protease activity: Extracellular proteolytic activity was determined according to the modified method of Anson, (1938) as described in Thangam and Rajkumar (2002) using casein as the substrate. The reaction mixture contained 1ml 1% (w/v) casein in 0.1M phosphate buffer (pH 7.0) and 1 ml of culture supernatant. The mixture was incubated at 40°C for 30 min. The enzyme reaction was terminated by addition of 6 ml 5% (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand for 10 min and filtered through Whatman filter paper. To 1 ml of filtrate, 3 ml of 0.5 M Na₂CO₃ solution and 1 ml of 3-fold-diluted Folin-Ciocalteu reagent (BDH Chemicals Ltd, Poole England) were added and mixed thoroughly. The colour developed after 30 min of incubation at 30°C and was measured in a Unico UV-2102 PC spectrophotometer at 660 nm. 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.

Ion exchange chromatography (DEAE Sephadex A-50): Further purification of protease enzyme was carried out using DEAE (Di-ethyl amino ethyl) cellulose anion exchange chromatography. The dialysed sample was applied to a DEAE column. The column was washed with 50mM, Tris buffer pH 8, and eluted with serially increasing concentration of NaCl (0.1M, 0.2). The eluted fractions were monitored by UV absorption spectrophotometer at 280nm.

Gel Filtration Chromatography (using Sephadex G-200): Preparation of the gel column and the fractionation procedures was determined as mentioned by Ammar (1975). Sephadex G-200 (Pharmacia, Upsalla, Sweden) was used, 0.1M phosphate buffer of pH 7.0 was added and the slurry was allowed to swell for 3 days at room temperature. Sodium azide (0.02%) was added to prevent any microbial growth. The enzyme solution was collected and dissolved in phosphate buffer 0.1M; pH 7.0 and fractionated through the Sephadex G-200 column (2.6 x 7.0cm) previously equilibrated with the same buffer. Seven (7) ml of the enzyme preparation sample was applied carefully to the top of the gel and allowed to pass into the gel by running the column. Buffer was added without disturbing the gel surface and to the reservoir. Elution was carried out with the respective buffer at a flow rate of 20ml/h. Fifty fractions (5ml each) were collected and absorbance read at 280nm using spectrophotometer (Jenway, 6305). Protein and protease activity in the fractions were estimated. The eluted enzymatically active fractions were pooled and used as the

purified enzyme.

SDS-Polyacrylamide gel electrophoresis: Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) at 12% was carried out to determine the purity and molecular weight of the enzyme, as described by (Laemmli, 1970). The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers consisting of bovine serum albumin (66 kDa), egg white ovalbumin (45 kDa), glyceraldehyde-3-P dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and bovine -lactalbumin (14.2 kDa). Protein bands were visualized after staining with Coomassie brilliant blue R-250.

Effect of pH on the activity of the enzyme: The optimum pH of the protease was investigated by the method of Yang *et al.*, (1996) at 40°C and pH values ranging from 3-12 with 100mM of (citrate-phosphate buffer) pH 3-7, Tris-HCL buffer pH 8-9 and glycine-NaOH buffer (10-12). The effect of pH on protease activity was performed at 40°C in the different buffers at 100mM for 30 min.

Effect of temperature on the activity of the enzyme: The optimum temperature was determined using the method of Rehman *et al.*, (2011) by estimating the protease activity at pH 7 and temperature ranging from 30-90°C. It was carried out by incubating the enzyme at temperatures ranging from 30-90°C for 30 min and the residual activity was measured at 40°C, pH 7 and expressed as percentage residual activity.

Stability of the purified enzyme toward pH: The enzyme stability of purified enzyme was examined by measuring the residual activity of the enzyme being incubated for 5 h at pH 3-12 and temperature based on the method applied by Yang *et al.*, (1996) and Rehman *et al.*, (2011). The effect of pH on the activity of protease was measured by incubating 0.5 ml of the diluted enzyme and 0.5 ml of phosphate buffer ranging pH from 3 to 12, containing 1% casein for 30 min at 40°C.

Stability of the purified enzyme The effect of temperature on the enzyme activity was determined by performing the standard assay procedure for 30 min at 40°C, pH 7.0 between a temperature ranges of 30 to 90°C, after which the residual enzyme activity was assayed for according to the method described earlier.

Determination of kinetic parameters: The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the protease for casein was determined at different substrate concentrations. They were evaluated by plotting the data on a Lineweaver-Burke double-reciprocal graph ($1/V_o$) versus ($1/[S]$) (Lineweaver and Burk, 1934).

2.2. Effect of Metals ions and Enzyme Inhibitor on Protease Activity

The effect of various metal concentrations on enzyme activity was investigated using $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$, NaCl and EDTA. The purified enzyme was pre-incubated with ions for 30 min at 40°C and then the remaining enzyme activity was estimated using casein as a substrate. The activity of the enzyme without metals was taken as the control.

2.3. Substrate Specificity of the Purified Protease

The ability of the purified protease to degrade various native proteins was evaluated with the following substrates (1% w/v). Casein, yeast extract, malt extract, beef extract, urea and peptone. Protease activity was determined as described earlier.

3. Results

Growth Culture of *Aspergillus niger*: The pattern of growth is illustrated in Figure 1.

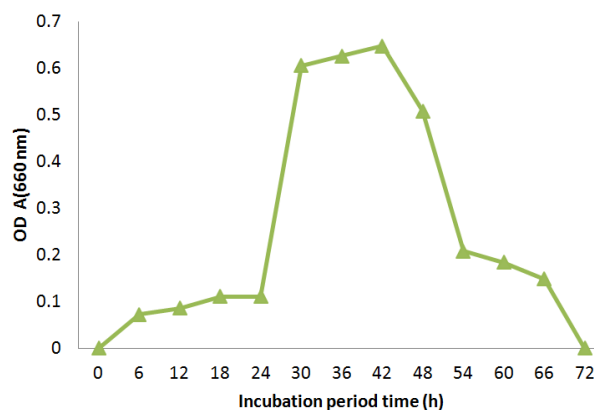


Figure 1. Fermentation profile of *Aspergillus niger* showing the growth and activity against time.

The exponential phase is observed immediately at 24 h; the rate of growth was between 24 h to 48 h reaching peak growth at 42 h of incubation. The growth started declining at 48 h and the culture entered into the death phase. It was observed that there was a prolonged lag phase from 6 h to 24 h and sharp growth phase from 24 h to 42 h after which there was a gradual death phase from 48 to 72 h.

Purification profile: Elution profile on ion exchange is shown in

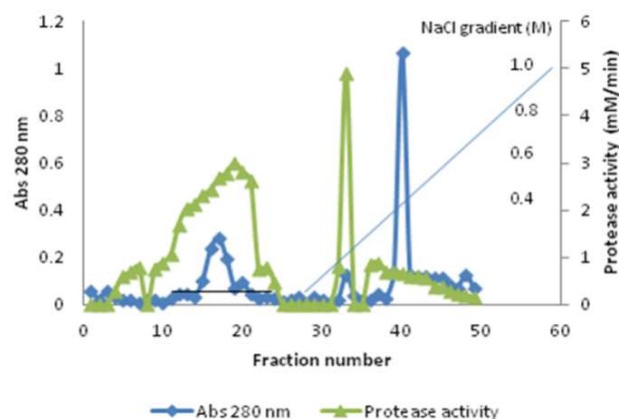


Figure 2. Elution profile of protease from *Aspergillus Niger* by an ion exchange A-50 column.

Figure 2 while Gel filtration chromatogram is shown on

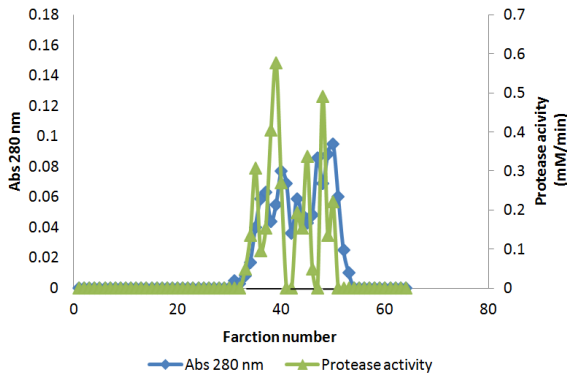


Figure 3. Purification elution profile of protease by gel filtration chromatography on Sephadex G-200. A further purification step.

Table 1. Purification of extracellular protease from *Aspergillus niger*

Steps	PC	EA	TEA	TP	SA	FOLD	YIELD (%)
Crude enzyme	0.318	0.163	40.75	79.5	0.513	1	100
(NH ₄) ₂ SO ₄ precipitation and dialysis	0.297	0.200	25.0	37.13	0.673	1.31	61.35
DEAE- cellulose	0.110	0.564	4.5	0.88	5.11	9.96	11.04
Sephadex G-200	0.070	0.596	4.47	0.525	8.51	16.60	10.96

PC: Protein concentration (mg/ml)
 EA: enzyme activity (mm/min)
 TEA: total enzyme activity (mm/min)
 TP: Total protein
 SA: Specific activity

SDS-PAGE Electrophoresis: The electrophoretogram of purified protease (PP) is shown in

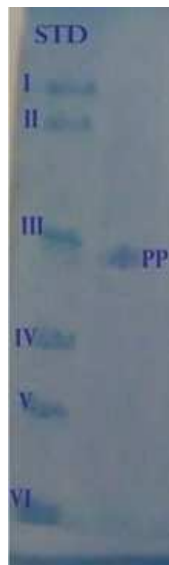


Figure 4. Electrophoretogram of purified protease (PP) by a combination of ion exchange chromatography and gel filtration chromatography on Sephadex.

Figure 4. Purified protein was analyzed on 10% Bis –Tris gel. Standard protein marker consisted of (I) Phosphorylase b, 103.14 kDa; (II) Bovine serum albumin, 81.35 kDa; (III) Ovalbumin, 47.05 kDa; (IV) Carbonic anhydrase, 34.17 kDa; (V) Soybean trypsin inhibitor, 27.26 kDa and (VI) Lysozyme, 17.67 kDa. The protein band occurred as a single band and it was spotted between protein standard iii and iv. The

Figure 3.

A summary of the results from the purification of protease is presented in Table 1 where it showed that specific activities for crude extract, ammonium sulphate precipitation, ion exchange chromatography, and gel filtration were 0.51, 0.67, 5.11, and 8.51 (U/mg) respectively. Purification (fold) for crude extract, ammonium sulphate precipitation, ion exchange, and gel filtration were 1, 1.31, 9.96, and 16.60 respectively. Yield for gel filtration was 10.96, an indication that purification increased with each purification step while percentage enzyme yield reduced with each purification step. The Lineweaver-Burk plot of the protease activity indicates that the protease enzyme has apparent K_m and V_{max} values for casein hydrolysis of 40.13 mg/ml and 7.8 U respectively.

estimated molecular weight for purified protease was 46.90 kDa.

Effect of pH on the Activity and Stability of Protease: The influence of pH on activity of the protease is shown in

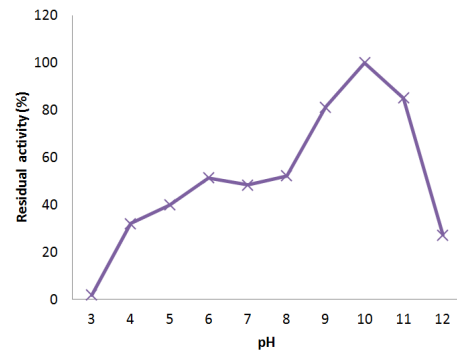


Figure 5. Effect of pH on the residual activity of protease

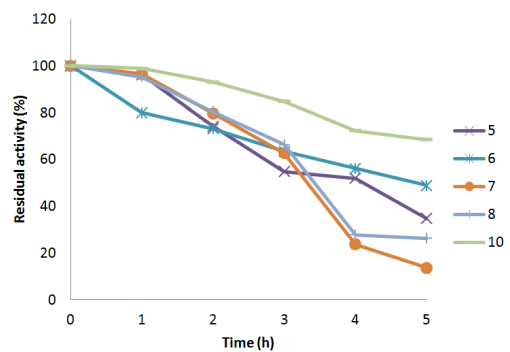


Figure 6. Effect of pH on the stability of protease activity

It revealed optimum pH at pH 10. At pH 11 and 12 the

enzyme was relatively stable and at lower pH it was observed that activity was low at pH 3-7. At pH 8 there was a sharp and sudden increase in the activity which makes the enzyme more stable at alkaline pH.

The pH stability is shown on Figure 6 depicting residual activity against time. At pH 10- 12 over a time of 5 h the enzyme was relatively stable and had residual activity of 70-55 %. At pH lower than 10 the enzyme was not stable with residual activity ranging from 55- 20%. Also from the plot, the enzyme was relatively stable at pH 9 to 12 than at pH 3 to 8 for a time of 5 h.

Effect of Temperature on the Activity and Stability of Protease.

The effect of temperature on the enzyme is shown on

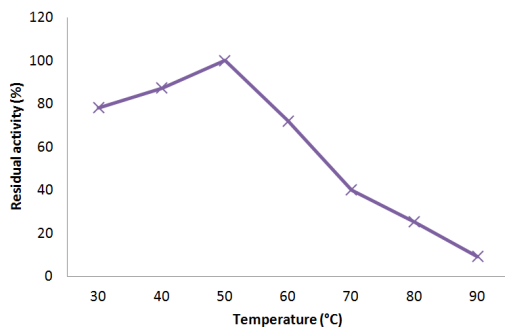


Figure 7. Effect of temperature on the activity of protease from Aspergillus Niger

Figure 7. At temperature ranging between 30-50°C, the activity was high, while the optimum temperature was at 50°C. There was a sharp and steady decrease in activity from 60-90°C. It was observed that, the higher the temperature the lower the activity. The thermostability of protease at various temperatures is shown in

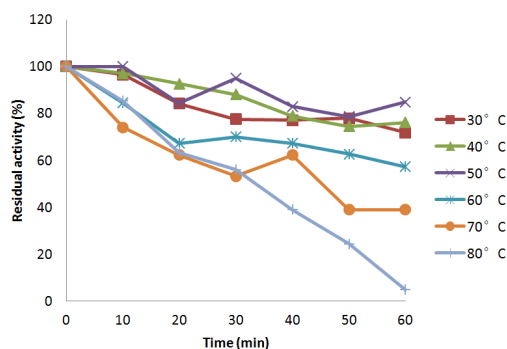


Figure 8. Temperature stability of the activity of protease from Aspergillus Niger

Figure 8, where it was revealed that protease was more stable from 30-60°C, after an hour of incubation. At higher temperature, there was a decline in residual activity. At incubation time of 20 min it was observed that residual activity was high at temperatures between 30-50°C and at temperatures between 60- 90°C the residual activity was moderate.

Effect of metal salts and inhibitor: The effect of various metals ions in

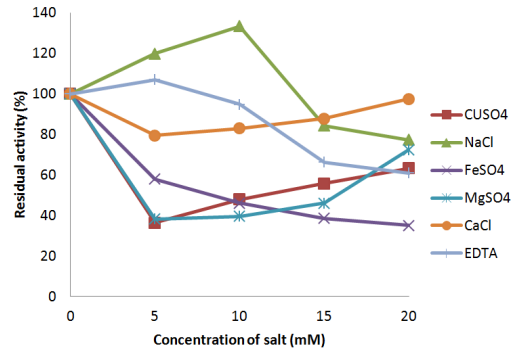


Figure 9. Effect of metallic ions on the activity of protease from Aspergillus Niger

Figure 9 shows that FeSO₄, MgSO₄ and CuSO₄ inhibited protease activity while EDTA, NaCl₂ and CaCl₂ induced the protease activity respectively.

Effect of different protein substrate on protease:

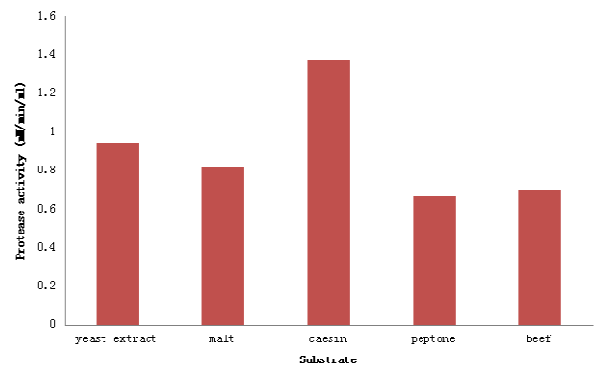


Figure 10. Effect of different protein substrates on protease activity.

Figure 10 shows the substrate specificity of protease. Different protein substrates at 1% concentration were used and it was found that maximum protease activity was observed in casein followed by yeast extract. The least activity was observed in peptone followed by beef extract and urea.

4. Discussion

Incubation time shows the result for optimal incubation period. This indicated that protease yield increased gradually and attained high titer of enzyme activity (280 U/g) at 42 h of incubation after which there was declination and the growth was eventually terminated at 72 h, further incubation reduced the yield. (Chakraborty *et al.*, 1995) isolated a new fungal isolate identified as *Aspergillus Niger* with the capacity to produce enormous amounts of a highly acidic extracellular protease on solid substrate. The fungus can be grown on inexpensive substrate and is capable of producing high yield of protease and relatively stable at the operating condition (Mulimani *et al.*, 2002).

The optimum temperature of the purified protease was 50°C. The result revealed that the protease retained a considerable amount of its activity at low temperatures. The

protease retained up to 94% of its original activity at 50°C. It reduced to about 28% of the original activity at 80°C, respectively. This indicates that the protease was considerably stable at low temperatures. The optimum temperature of the protease is consistent with the protease of *A. terreus* 50°C (Bushra *et al.*, 2010); and *A. oryzae* 50°C (Sumantha *et al.*, 2005).

Thermostability study of this enzyme at 50°C shows potential for applications in food industries such as baking, brewing etc. The enzyme of *Rhizopus oryzae* acted optimally at 60°C and was stable in the temperature range of 30–45°C. The acid protease of *Mucor rouxii* showed temperature optima of 50°C and was inactivated at 70°C. (Tremacoldi *et al.*, 2004) reported temperature optima of 40°C by acid protease of *Aspergillus clavatus*. The result was also different from the protease of *A. Niger* (32°C) (Aalbaek *et al.*, 2002). In addition, unlike other fungal proteases, *A. Niger* protease exhibited unique wide range thermostability even at low temperatures for 30 min incubation. The enzyme retained about 55% of its original activity at 90°C. The purified protease was completely stable at 50°C and is consistent with some previous reports (Sumantha *et al.*, 2005; Wang *et al.*, 2005). The temperature and stability profile of the purified protease suggested that the enzyme was thermostable. However, this enzyme could find potential commercial application as a food processing agent since most food industries such as brewing, baking, etc. that utilize proteases require their processes to be carried out at temperatures around 50 and 60°C.

The purified protease exhibited a very unique pH profile. The enzyme displayed broad range of activity at acidic, basic and neutral levels. The optimum activity was at pH 10.0, but equally exhibited remarkable activity at pH 7.0 and 11.0. The data is similar to literature reports particularly that fungi may secrete acidic, neutral and alkaline proteases (Siala *et al.*, 2009; and Sumantha *et al.*, 2005). The wide activity range (pH 3.0 to 12.0) of the protease from this research work contrasts markedly with the narrow pH range (3.0 to 4.0) observed for CtsD from *A. Niger* ATCC 26933 (Vickers *et al.*, 2007). The purified protease from *A. Niger* was very stable at pH 11.0. However, the result of this study differs from *A. clavatus* protease. This indicates that the protease exhibited broad activity at acidic, basic and alkaline range.

In enzyme action, metallic cofactors are important because their presence or absence regulates enzyme activity. The presence of specific metallic ions along with peels from food can inhibit or enhance protease activity. Inhibitors and metal chelators can reduce the hydrolysis of substrate by proteolytic enzymes. Due to their specificity, inhibitors and chelators can also aid in characterization of novel proteases by examining hydrolysis rates of protease in their presence. The inhibition studies of the purified protease indicated that specific metallo-protease inhibitors, a chelator's agent, EDTA significantly stimulated the protease suggesting that the protease is not a metallo-protease. The high activity of the enzyme in the presence of EDTA is very useful for application as detergent additive. These observations indicate

that the *A. niger* protease is a thiol (cysteine) protease. To this end, the protease could be of great potential in brewing industries and in meat tenderization where it is used to remove protein hazes.

The strong activation of the protease in the presence of Mg²⁺ suggests that the protease could be protected from thermal denaturation due to their probable presence in the catalytic domain of the enzyme. The involvement of Mg²⁺ and Ca²⁺ in the protection of protease thermal denaturation has been reported (Kumar and Tagagi, 1999). Na⁺ was slightly inhibitory to the protease.

The important feature of proteases is their ability to discriminate among competing substrates and utility of these enzymes often depends on their substrate specificity (Shankar *et al.*, 2011). The purified protease was able to degrade natural substrates like yeast extract, malt extract, peptone, urea, casein and beef extract to varying degrees. It was more active against casein compared to yeast extract, malt extract, peptone, urea and beef extract. The ability to degrade casein by this enzyme is consistent with the report of (Shankar *et al.*, 2011). The substrate specificity profile of the purified protease indicates that the enzyme has a wide range of hydrolytic activity on various protein substrates which is a great potential in biotechnological applications. The result confirms that sources of substrate significantly affected both affinity and digestibility of the substrates by the protease.

The kinetic parameter (V_{max} and K_m) values 7.8 and 40.13 respectively confirmed that the purified protease possessed high affinity and degradability. (Shankar *et al.*, 2011) has reported protease with K_m value of 5.1 mgml⁻¹ with casein as substrate from *Beauveria* sp. Report from (Devi *et al.*, 2008) gave a lower K_m values of 0.8 mgml⁻¹ using casein. When assayed with native protein as substrates, the protease showed high level of hydrolytic activity with all the substrates tested with highest specificity for casein (130 %), indicating broad substrate specificity of this enzyme (Wang *et al.*, 2005).

With an approximate 17-fold purification and overall yield of 10.96%, during passage through the ion-exchange column, the enzyme of interest eluted as an unbound fraction. Gel filtration chromatography depicted only one activity peak. In a Brilliant Blue G-Colloidal Concentrate stained SDS-PAGE gel, the protease migrated as a single band with an estimated molecular weight of 46.90 kDa. These observations indicate that the enzyme is homogeneous and probably monomeric. However, bands of unknown contaminant proteins were observed. Zymogram analysis revealed that only a single protein band possessed proteolytic activity which corresponded with the molecular weight of 46.90 kDa on SDS-PAGE. This confirms that the other minor bands were contaminant proteins. The molecular weight of the protease is at variance with other literature reports on fungi. The molecular weight of *A. Niger* protease is higher than that reported for *Beauveria* sp 29 kDa (Shankar *et al.*, 2011); *A. terreus* 35 kDa (Bushra *et al.*, 2010). The value was comparable with 75 and 74 kDa reported for *Pleurotus ostreatus* protease (Palmieri *et al.*, 2004).

5. Conclusion

This work describes the purification and characterization of *Aspergillus niger* strain from protease. The enzyme was purified from the culture supernatant by two steps with a 16.60-fold increase in specific activity and 10.96% recovery. The result from the study has shown that protease can be produced from *Aspergillus niger* isolated from yam peels which is a food waste. From the physicochemical 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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