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Isolation, Partial Purification and Characterization of Proteases from *Aspergillus niger* under Solid-State Fermentation

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Proteases are enzymes with highly specialized proteolytic functions. They are ubiquitous, being found in all living organisms, they are essential for cell growth and differentiation. Besides their physiological functions and roles in living organisms, they also show great importance in various industries. The shortage of plant and animal proteases to meet the present world demand of industrial enzymes has directed increased interest in microbial proteases. Several researchers have reported on protease production from various sources. However, little is known about protease production using *A. niger* under solid-state fermentation. This present investigation was carried out to isolate and screen fungi from soil samples for the production, optimization, and characterization of protease. A*. niger* was identified morphologically and screened for protease production. Soli-state fermentation was carried out and crude protease was harvested. The effect of pH on protease activity was assayed, and different temperatures were used to test for protease activity. Also, the kinetic parameters (K_m and V_{max}) of the crude enzyme were also determined. The results of this investigation revealed that the optimal pH and temperature of the enzyme were 8.0 and 40°C, respectively. The enzyme was found to be more stable at alkaline pH than acidic pH. It also retained 80% of its activity at 50°C for 60 minutes. Protease activity was revealed to be highest at substrate concentration 1.0 mM. All these data suggest that the selected strain of *A. niger* can significantly produce protease enzyme under solid-state fermentation.

Keywords: Proteases; catalytic activity; substrate; fermentation; Aspergillus niger.

1. INTRODUCTION

"Proteases constitute a large and complex group of enzymes that play both nutritional and regulatory roles in nature. They are ubiquitous and found in a wide diversity of sources such as plants, animals and microorganisms, but they are mainly produced by bacteria and fungi. The molecular weight of proteases ranges from 18–90 kDa" [1]. "Proteases, also known as proteinases or proteolytic enzymes, belong to a group of hydrolases. They act as catalyst in the hydrolysis of proteins to polypeptides and oligopeptides to amino acids" [2]. "They can be classified as acidic, neutral and alkaline with regards to their pH working range" [3]. "Proteases are generally used in detergents and food industries, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds" [4]. They also have medical and pharmaceutical applications.

"Fungi such as *Aspergillus niger, Fusarium culmorum*, and *Penicillium* can excrete significant amount of enzymes and metabolites through solid-state fermentation (SSF) as this technique can stimulate their natural habitat" [5]. "There are approximately 60% of commercially available enzymes produced from fungi. Yeasts are also suitable for SSF. Filamentous fungi and yeasts are able to grow in an environment with low water activity. Some species of bacteria, such as *Bacillus thuringiensis, Pseudomonas sp.,* and *Bacillus subtilis,* have also been employed to produce enzymes through SSF" [6]. "Microbes are commonly used to produce proteases due to their feasibility in genetic manipulation, rapid growth rate, and wide biochemical diversity. Fungal species like *Aspergillus, Penicillium,* and *Rhizopus* are also generally used for proteases production as they are considered safe" [7]. "*Aspergillus niger* is one of the main sources of fungal proteases" [8,9]. "Some microorganisms produce heat stable proteases which are active at higher temperatures. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperature" [10].

"Solid-state fermentation has been reported to have more significant advantages in enzyme production as it requires lower energy consumption and produces lesser wastewater. Enzyme production through SSF is less subjected to the effects of the pH of media and temperature and inhibition by the substrate" [11,12]. "Economically, SSF offers many advantages including superior volumetric productivity, use of simpler machinery, and use of inexpensive substrates, simpler downstream processing, and lower energy requirements when compared with submerged fermentation" [4]. Solid-state fermentation has been reported by researchers to be of greater productivity than submerged fermentation in the production of

enzymes. Little is known about protease production using *A. niger* under solid-state fermentation. Hence, this present investigation was carried out to isolate, partially partially and characterize proteases using *A. niger* under solid-state fermentation [13].

2. MATERIALS AND METHODS

2.1 Study Area

The present study was carried out in Federal University Wukari Central Research Laboratory, Wukari, Taraba state, Nigeria, from the period of December 2022 to April 2022.

2.2 Sample Collection and Preparation

A total of 5 soil samples (5 g each; 5–10 cm below the surface) were collected aseptically from three regions in a dairy farm located at Federal university Wukari. Soil samples were sieved (3–4 mm mesh), homogenized, and stored at 4°C at the Central Research Laboratory, Wukari, Taraba state for further analysis.

2.3 Media Preparation

About 5g of potato dextrose agar (PDA) was measured using weighing balance and 200mg of chloramphenicol was added to inhibit the growth of bacteria. It was transferred into 250ml Erlenmeyer flask and 150 ml of distilled water was added and mix thoroughly. The flask was tightly stock with cotton wool and wrapped with aluminium foil. The media was sterilized in an autoclave at 121°C for 30 minutes and was removed from the autoclave and allowed to cool. The media was poured into a petri plate and allowed to gel after which the fungi isolate was inoculated into the media for culturing.

2.4 Isolation of Fungi

Fungal strains were isolated from soil of dairy farm located at Federal university Wukari, by serial dilution plate method. Fungus was isolated from 10^{-2}- 10^{-4} dilutions by plating into Potato Dextrose Agar (PDA) medium containing 0.05g chloramphenicol. Isolated fungal cultures were screened for protease enzyme production. The organisms were identified using lacto phenol cotton blue mounting method. The plates were incubated at 30°C for 5–7 days to isolate distinctive colonies; the isolated culture (*Aspergillus niger*) was partially purified by routine sub-culturing and stored at 4°C for further use.

2.5 Screening for Protease-producing Fungi

The method of Sanna et al. [14] was used to carry out primary screening for protease production. "Protease production was tested using the skim milk agar medium for the production of the clear zone. The detection medium was prepared using 20 g of skim milk dissolved in 200 ml distilled water, and 600 ml of 0.2 M phosphate buffer (K_2HPO_4 pH 6.8). All the three media components were autoclave separately to avoid coagulation and charring of milk due to the presence of buffer salts and later mixed under sterile conditions. The plates were then subsequently inoculated with mycelia from 5-day-old culture and incubated at 30°C for 2 days. The plates were examined for the formation of the clearing zone by flooding them with a solution of 10% Trichloroacetic acid (TCA)" [14].

2.6 Solid-state Fermentation

Solid-state fermentation was carried out in 250ml conical flask contains 10g of substrates with 10 ml of salt solution (g/l). KNO_3 2.0, $MgSO_4$ $.7H_2O$ 0.5, K_2HPO_4 1.0, $ZnSO_4$ $.7H_2O$ 0.437, $FeSO_4$ $.7H_2O$ 1.116, $MnSO_4$ $.7H_2O$ 0.203, pH 7.0 and it was autoclaved at 121°C for 20 min. After sterilization, the flasks were inoculated with 1.0 ml of spore solution (106 spores/ ml) and incubated at 30°C for seven days in an incubator. At the end of fermentation, cultures were extracted with 100ml of distilled water by shaking for 2 hours. The filtrate obtained was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was used as crude enzyme extract and little quantity was taken for enzyme activity and determination of protein content.

2.7 Enzyme Extraction

The enzyme of interest was extracted according to the method described by Tunga [15]. The fermented substrates were dispersed in 100 ml of distilled water at room temperature for 40 min and filtered using a cotton cloth. The filtrate was then centrifuge at 10,000 rpm for 10 min. The supernatant was used as a crude enzyme.

2.8 Protease Assay

Protease activity was determined according to the modified method of Vishwanath [16] as described by Bigelow and Wyman [17] using

Table 1. Protease assay protocol

	Test 1	Test 2	Test3	Blank
Casein solution	1ml	1ml	1ml	1ml
Phosphate buffer	1ml	1ml	1ml	1ml
Crude enzyme	1ml	1ml	1ml	-
Incubation temperatute	40°C	40°C	40°C	40°C
Trichloroacetic acid (TCA)	6ml	6ml	6ml	6ml
Filtrate Na_2SO_4 Solution	1ml	1ml	1ml	-
Na_2SO_4 Solution	3ml	3ml	3ml	3ml
Folin-Ciocalteau reagent	1ml	1ml	1ml	1ml
Time	20 mins	20 mins	20 mins	20 mins
Absorbance at 520nm	0.227	0.231	0.260	0.195

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 pH 7.0) 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 5.0 ml of the filtrate were taken into 3 test tubes. To this 10.0 ml of 0.5% NaOH solution and then 3.0 ml of the folin-ciocalteu reagent was added. Final readings were taken in a spectrophotometer at 280 nm. Blanks of the samples were prepared by adding the TCA 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".

2.9 Effect of Incubation Temperature on Protease Activity

The fungal spores inoculated into the SSF medium in 250 mL Erlenmeyer flasks were incubated at temperatures of 30°C, 35°C, 40°C, 45°C and 50°C for 120 hour to determine the optimum temperature for protease activity. Five test tubes were used to determine the effect of temperature on protease activity in which 1ml of culture supernatant was added to the varied temperature and the mixture was incubated for 10 minute in a beaker containing water and the temperature was controlled using a thermometer.

2.10 Effect pH on Protease Activity

The effect of pH on protease activity was determi ned using the method described by Yu and Xu [18]. 2g of casein (substrate) was dissolved in 100 ml of phosphate buffer and 20ml of the phosphate buffer solution was transferred into five beakers and the pH was varied at pH 5,6,7,8 and 9. 1ml each of NaOH and HCL acid was used to adjust the pH and1ml of culture supernatant was added to the varied pH respectively. The mixture was incubated for 10 minutes at 40°C in a water bath in which 6 ml trichloroacetic acid (TCA) was added to terminate the reaction and the mixture was filtered using Whatman filter paper and placed under ice water. Then 3ml of Na_2SO_4 solution and 1ml of Folin-ciocalteau reagent was added to the filtrate and allowed to stand for 10 minutes to develop a colour. Finally, it was assayed for enzymes activity, using spectrophotometer set at 520 nm.

2.11 Effect of Substrate Concentration on Protease Activity

Substrate concentration was varied at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM respectively. Six test tubes were used in which 1ml of phosphate buffer and 1ml 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 under ice using Whatman filter paper, then 3ml of Na_2SO_4 and 1ml of Folin Ciocateau reagent were added and allowed to stand for 10 minutes the absorbance was taken at 520 nm using a spectrometer.

3. RESULTS

3.1 Effect of pH on Protease Activity

Protease activity was maximum at pH 8.0. Varying the pH level showed effect on protease activity as shown in Fig 1.

Table 2. Effect of pH on protease activity

pH	Enzyme activity (U/ml)
5.0	0.300
6.0	0.321
7.0	0.300
8.0	0.406
9.0	0.329

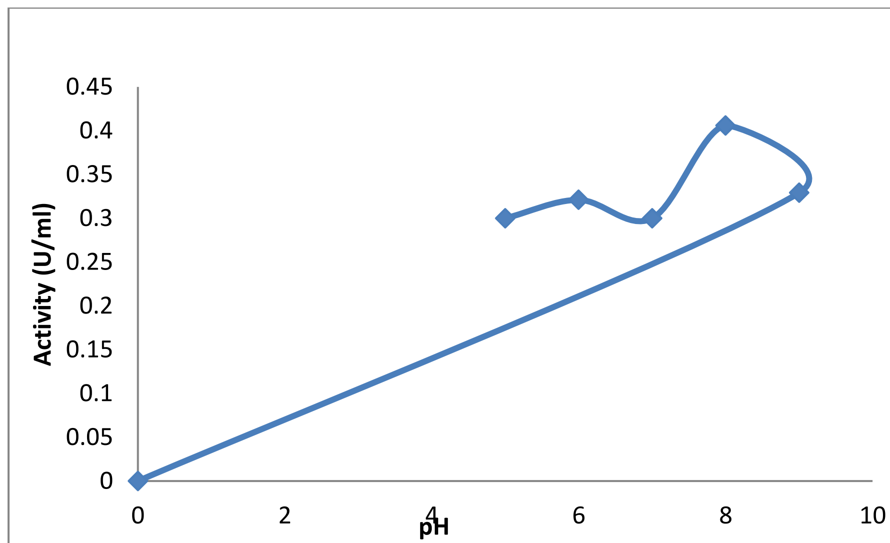

Fig. 1. Effect of pH on protease activity

Table 3. Effect of temperature on protease activity

Temperature (° C)	Protease activity (U/ml)
30	0.307
35	0.446
40	0.70
45	0.664
50	0.549

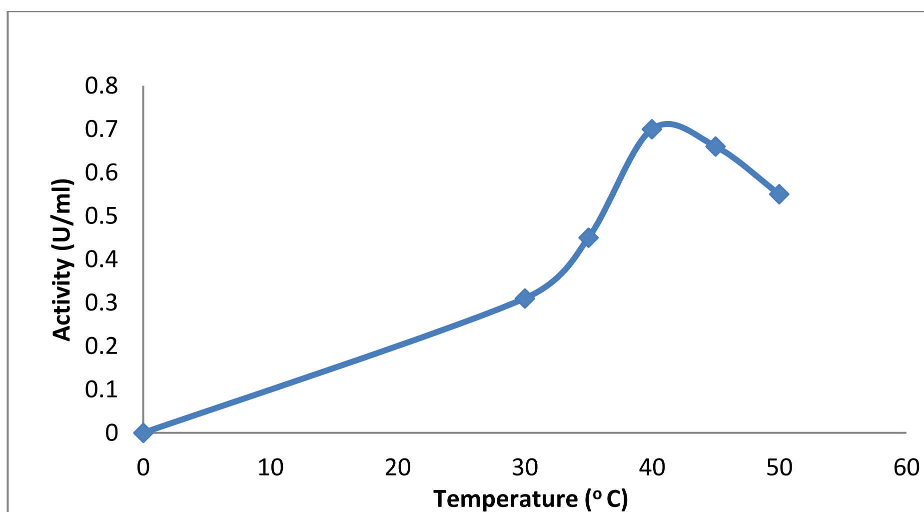

Fig. 2. Effect of temperature on protease activity

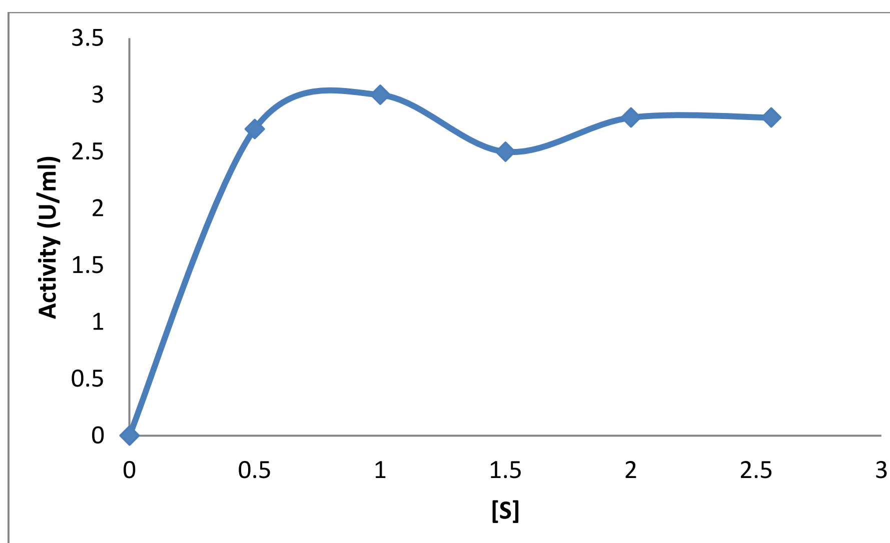

Fig. 3. Effect of substrate concentration on protease activity

3.2 Effect of Temperature on Protease Activity

Change in temperature showed effect on protease activity. The optimal temperature for protease activity was found to be 40ºC as shown in Fig. 2.

3.3 Effect of Substrate Concentration on Protease Activity

Enzyme activity changed as substrate concentration was varied. Protease activity was revealed to be highest at substrate concentration 1.0 mM as presented in Fig. 3.

4. DISCUSSION

"Enzyme production by micro-organisms is greatly influenced by media components, especially carbon and nitrogen sources, and also physical factors such as temperature, pH, incubation time inoculum density among others. It is important to produce enzymes in inexpensive and optimized media on a large scale for the process to be commercially viable" [17].

In the present study, enzyme production was gradually increased with the passage of time and highest enzyme activity was obtained on 7th day of incubation. It was also observed that prolonged incubation decreased the enzyme activity. However, the growth of the microorganism was not significantly affected.

Protease activity slightly increased at pH 6.0 and was highest at pH 8.0 showing that the enzyme is more active in an alkaline medium. (Fig. 1). This result is contrary to the report by Yu and Xu [18] that showed "maximum enzyme activity at pH 5.0, and De Vries and Visser [19]. that reported maximum activity at pH 6.5, both acidic media". This may be as a result of change in shape and charge properties of the enzyme and substrate. pH change causes alteration of the ionization state of amino acids residue in a protein which leads to alteration of the ionic bonds which determine the tertiary structure and charge properties of the protein. This ultimately results in enzyme inactivation or altered substrate recognition.

Temperature is known to have great effect on protease activity as it may reduce, give stable activity or express maximum activity of the enzyme. The effect of temperature on protease activity in this study was assayed by subjecting the enzyme to different temperature values. According to the result presented in Fig 2, protease activity at temperature 40⁰C was maximm. However, there was a sharp decrease in protease activity at 50⁰C. This may be as a result of enzyme denaturation. This result is contrary to the finding of Yandri et al. [20] who reported that the optimum temperature of protease isolated from *Enterococcus faecalis* was 30⁰C. In a different study carried out by Akel et al. [21], *Streptococcus lactis* and *lactococus lactis* both produced proteases which had maximum activity at 28⁰C and reduced activity after 30⁰C. The protease in this study was

completely inactivated at 30^{0}C A similar result reported by Lowry et al. [22] showed that “protease of *xenorhabdus nematophila* displayed sharp decrease in enzyme activity at temperature above 30^{0}C”. “Enzymes are known to be sensitive to temperature changes, and the change in activity of any particular enzyme to such conditions is a distinguishing characteristic of such enzyme” [23].

Protease showed maximum activity at 1.0mM substrate concentration and began to decrease at 1.5 substrate concentration (Fig. 3). This result is not in tandem with the findings of Gupta et al. [24] who reported that protease from *Lactobacillus acidophilus* had maximum activity at 2 casein concentration. Matta et al. [25] obtained “a slightly lesser value of 1.2 for protease produced by *Bacillus subtilis* in their study on the effect of casein concentration on protease activity by the bacterium”.

5. CONCLUSION

In this study, Protease was found to be more stable at alkaline pH than acidic pH. It also retained 80% of its activity at 50°C for 60 minutes. All these data suggest that the selected strain of *A. niger* can significantly produce protease enzyme under solid-state fermentation. The results obtained in this research can guide producers of protease on what parameters to vary for maximum utilization of this enzyme in industrial fields.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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