



Optimization Studies on Extracellular Protease Production by *Aspergillus niger* and *Aspergillus terreus* Using Skim Milk Casein as Substrate

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Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed to the study conception and design. Authors AIO and FEE designed the study and wrote the protocol. Authors CTO, USAO and JMM managed the literature review. Author FEE wrote the first draft of the manuscript. Authors AIO, FEE and IAO performed the statistical analysis. Authors AIO and JMM managed the analysis and editing of the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Proteases are proteolytic enzymes that have a wide range of applications in the industrial sectors. This study investigated the production and optimization of protease by protease-producing fungi, (*Aspergillus niger* and *A. terreus*) using skim milk casein as substrate.

Materials and Methods: *Aspergillus terreus* and *A. niger* obtained from the Department of Plant Science and Biotechnology, University of Jos, Nigeria were screened for extracellular protease on skim milk agar medium. Optimization studies across different parameters: temperatures (30-80°C), pH (3-10), substrate Concentration (0.25-2 %), and incubation period (6 days) using submerged fermentation were done for maximum protease activity.

Results: The maximum temperature of protease activity was 50°C (116.5 IU/ml) for *Aspergillus niger* and 40°C (121.86 IU/ml) for *Aspergillus terreus*. The Optimum pH for protease activity was

pH 9 (106.85 IU/ml) for *Aspergillus niger* and pH 8 (107.90 IU/ml) for *Aspergillus terreus*. Optimum protease activity (29.36 IU/ml) at an incubation period of 48 hours was recorded for *Aspergillus niger* and 72 hours (182.2 IU/ml) for *Aspergillus terreus*. At 2% substrate concentration a maximum activity of 87.21 IU/ml and 76.5 IU/ml was recorded for *Aspergillus niger* and *A. terreus*, respectively.

Conclusion: The test fungi *Aspergillus niger* and *Aspergillus terreus* exhibited maximum hydrolytic potential for protease production which has a vast application in different industries.

Keywords: Hydrolytic; enzyme; *Aspergillus niger*; *Aspergillus terreus*; protease assay; skim milk.

1. INTRODUCTION

Enzymes are macromolecular biological catalysts, most of which are proteins in nature, although a few are catalytic RNA molecules. Enzymes catalyze or accelerate chemical reactions. At the start of a chemical reaction, substrates are converted by enzymes into products [1]. More than hundreds of enzymes are used industrially, more than half of which are from fungi and yeast, and over a third are from bacteria, while the rest are divided between animal (8%) and plants (4%) sources. Enzymes are widely spread virtually in all plants, animals, and microorganisms. Microbial enzymes are preferred to plants and animal because of the following reasons: low cost of production, predictable and controllable enzyme contents, and also because unlike those of plant and animal tissues contain less harmful materials [1]. "Fungal enzymes are more suited for industrial applications as fungi are easily cultivated and are fast growing. Fungal proteases are used in the food, dairy, detergent, leather, and pharmaceutical industries. They are used for bioremediation and production of therapeutic peptides. Furthermore, fungal proteases are more diverse and exhibit wider substrate specificity" [2].

Proteases are a large and complex group of enzymes that catalyze the hydrolysis of protein molecules into peptides and amino acids [3, 4]. "They are also called proteolytic enzymes or proteinases. Proteases are classified into three groups, that are, neutral, acidic, and alkaline proteases, based on their acid-base nature. Fungi are the main source of acid proteases" [5]. "Proteases form a large group of enzymes belonging to the class of hydrolases. Serine protease (EC 3.4.21), cysteine (thiol) protease (EC 3.4.22), aspartate proteases (EC 3.4.23), and metallo-protease (EC 3.4.24) constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market" [1,6,7]. Traditionally the proteinases have been regarded as degradative

enzymes which are capable of cleaving protein foods. They liberate small peptides and amino acids needed by the body. Proteolytic enzymes have the ability to carry out selective modification of inactive precursor proteins or zymogens by limited cleavage of one or more peptide bonds, thereby activating the zymogenic forms of proteolytic enzymes such as trypsinogen. This is also used in other crucial biological processes including metabolism, enzyme modification, apoptosis pathways, prophenol oxidase activating cascade, blood clotting, hormone production and fibrinolysis [8]. Furthermore, "a study of proteolytic enzymes is valuable because of their use in various forms of medical therapies important as reagents in laboratory and industrial processes" [9]. Proteases play an important role in many processes in human's body such as fertilization, digestion, growth, maturation, aging, and even death of the organism. They regulate many physiological processes by controlling the activation of the synthesis and degradation of proteins.

The demand for newer and novel natural products with improved qualities to enhance man's existence in life has led researchers to conduct a series of researches over the years. Among these natural products are the biological enzymes. Among all enzymes, extracellular proteases have a renewed interest due to their role in the cellular metabolic processes and their importance in industries. Their application is very broad and they have been used in many fields for years mainly in industries such as the detergent, food, pharmaceutical, leather, and diagnostics industries, waste management, and silver recovery [10]. Currently, "the largest share of the enzyme market has been held by detergent proteases, being active and stable at alkaline pH" [11]. Fungi of the genera *Aspergillus*, *Penicillium*, and *Rhizopus* are especially useful for producing proteases. This study aims to investigate the extracellular proteases production of *Aspergillus niger* and *A. terreus* using skim milk casein as a substrate.

2. MATERIALS AND METHODS

2.1 Collection and Isolation of Protease Producing Fungi

The test fungi (*Aspergillus niger* and *Aspergillus terreus*) were obtained from preserved samples at the Department of Plant Science and Biotechnology, University of Jos. The samples were inoculated on starch agar and kept in an incubator at $25\pm 2^{\circ}\text{C}$ for 5 days. Macroscopic and microscopic examinations were carried out to confirm the identities of the test fungi. Identification of species was done using a fungal atlas and the identification schemes of [12,13]. Pure fungal colonies of *Aspergillus niger* and *A. terreus* were stored at 4°C until used [14].

2.2 Screening of the Test Fungi for Proteolytic Activity Using Plate Assay

Skim milk agar plates were prepared using selective medium containing [g/l: KH_2PO_4 (0.7) NH_4NO_3 (5), KCl, 0.2; $\text{MgSO}_4\cdot\text{H}_2\text{O}$, 0.2; $\text{FeSO}_4\cdot\text{H}_2\text{O}$, 0.5; distilled H_2O , 1000L; Agar, 10; 1% skim milk casein] and inoculated with 5mm mycelia discs from the edge of actively growing 4-day old cultures of the test fungi. After 5 days of incubation on skim milk agar at $25\pm 2^{\circ}\text{C}$, the plates were observed for growth.

2.2.1 Effects of incubation period on fungal growth and crude protease production using submerged fermentation (SmF)

The effects of incubation period on crude protease production were determined using modified method of Asha et al. [15]. Submerged Fermentation was performed by inoculating disc of pure cultures of actively growing *Aspergillus niger* and *A. terreus* into the modified protease production medium composed of [g/l: KH_2PO_4 , 1.5; NaCl, 0.5; K_2HPO_4 , 1.0; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.2; skim milk substrate, 10g) and then incubated for 6 days at room temperature ($27\pm 2^{\circ}\text{C}$) without agitation [15]. Aliquots were withdrawn at 24 hours' intervals centrifuged at 8000rpm and supernatant of crude enzyme was used for protease assay. The protease activity was assayed according to the method of Cupp Enyard [16] by using skim milk casein as substrate. The test tubes, containing the reaction mixtures, composed of 1ml crude enzyme, 1ml phosphate buffer; (pH 7.5), and 1 ml of 1% skim milk casein and blank containing 1 ml of the enzyme solution only, were incubated in water

bath, at temperature of 37°C for 30 mins to allow for an enzymatic reaction. The reaction was terminated by the addition of 5ml of Trichloroacetic acid (TCA). The tubes were then allowed to stand for 15 min. at room temperature. Precipitated proteins from both test tubes were then filtered by centrifugation at 8000rpm for 10 minutes. Two ml of filtrates mixed with 5 ml of NaCO_3 was then added to both test tubes turning the solution cloudy. This was followed by the addition of 1ml Folin ciocalteus phenol reagent which reacted primarily with free tyrosine liberated in the mixture. The resultant solution was mixed properly by swirling and incubated at 37°C for 30 minutes for the formation of blue color. Volumes of 2 ml of each of the solution were then placed into suitable cuvettes and absorbance was measured at 650nm against a reagent blank using tyrosine standard. One protease unit was defined as the amount of enzyme that released $1\mu\text{g}$ of tyrosine per ml, per minute, under standard conditions [5].

2.3 Optimization Studies on the Enzyme Using Different Parameters

2.3.1 Effects of temperatures of incubation on protease production

The effects of different incubation temperatures on enzyme activity of *Aspergillus niger* and *A. terreus* were studied using modified method of Asha et al. [15]. The optimum temperature for protease activity was determined by performing the enzyme reaction at incubation temperatures: 30°C , 40°C , 50°C , 60°C , 70°C , and 80°C . Standard protease assay method was performed, using the phosphate buffer at pH 7.5.

2.3.2 Effects of pH on protease activity

The effects of different pH on the protease activity of *Aspergillus niger* and *A. terreus* were determined using 1% skim milk casein as substrates. The phosphate buffer varied pH of 3, 4, 5, 6, 7, 8, 9 and 10, using 2M Hydrochloric acid (HCl) and 1M of Sodium hydroxide (NaOH). The pH was measured using a pH meter and then the standard protease assay was performed. The values obtained from protease assay were recorded in triplicates as reported by Asha et al. [15].

2.3.3 Substrate concentration dependence on protease production

Standard protease assay at pH 7.5 (phosphate buffer) was performed to determine the effects of

substrate concentration on protease activity. Varying concentrations (0.25%, 0.5%, 1.0%, 1.5%, and 2.0%) of skim milk casein (substrate) were used for the assay. The values obtained from protease assay were recorded in triplicates as reported by Asha et al. [15].

3. RESULTS

Identification of test fungi (*Aspergillus niger* and *Aspergillus terreus*) using their distinct characteristics was confirmed. The screening for proteolytic activity, done using plate assay, showed that *Aspergillus niger* and *A. terreus* initiated growth on the skim milk media used. It was observed that by the end of the incubation period the test fungi have covered the entire plates, indicating that were both proteolytic. The details of the fungal morphology are shown in Fig. 1 and Fig. 2 respectively.

The effects of the incubation period of 6 days on fungal growth and crude protease production are shown in Fig. 3. Both test fungi had growth with progression from the 1st day. *Aspergillus niger* had the highest enzyme activity on day 2 (48 hours) liberating 29.36 IU/ml. *Aspergillus terreus* had a peak of enzyme activity on day 3 (72 hours) of incubation, liberating 182.21 IU/ml.

The effects of incubation temperature on enzyme activity indicate that the organisms had enzyme activity at all incubation temperatures but the optimum temperature for enzyme activity was 50°C (116.5 IU/ml) for *Aspergillus niger* and 40°C (121.86 IU/ml) for *Aspergillus terreus*. Enzyme activity then declined up to 49 IU/ml and 47.57 IU/ml at 80°C for *Aspergillus niger* and *A. terreus*, respectively. The details of the effects of incubation temperature on enzyme activity are shown in Fig. 4.

The results of the effects of varying the pH range from 3-10 on enzyme activity for both test fungi are presented in Fig. 5. The optimum protease activity for *Aspergillus niger* was recorded at pH 9 (106.85 IU/ml) and optimum protease activity for *Aspergillus terreus* was recorded at pH 8 (107.9 IU/ml). The decline on enzyme activity was observed at pH 10 with *Aspergillus niger* and *A. terreus* recording 78 IU/ml and 90 IU/ml respectively.

The different substrate concentrations (0.25%, 0.5%, 1.0%, 1.5%, and 2.0%) of skim milk showed that protease activity gradually increased with increase in concentration (Fig. 6). Enzyme activity was highest at 2% concentration for both *Aspergillus niger* and *A. terreus* yielding 87.21 IU/ml and 76.5 IU/ml, respectively.



Fig. 1. (a) Culture of *Aspergillus niger* (b) *Aspergillus niger*



Fig. 2. (a) Pure Culture of *Aspergillus terreus* (b) *Aspergillus terreus*

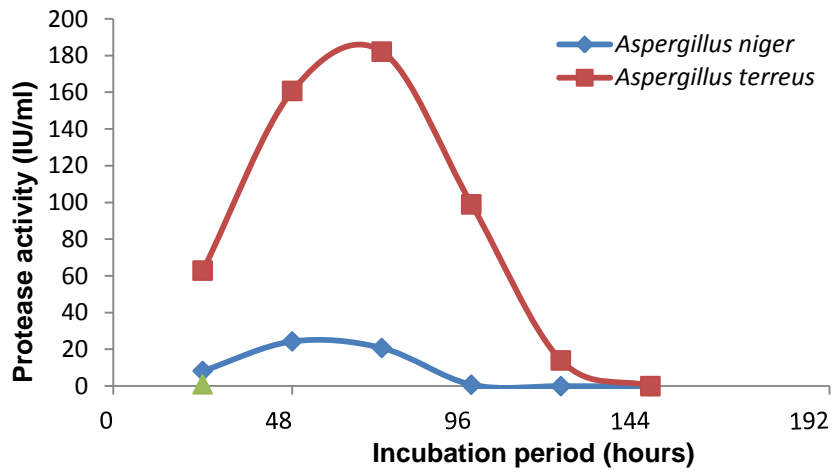


Fig. 3. Effects of incubation period on protease production

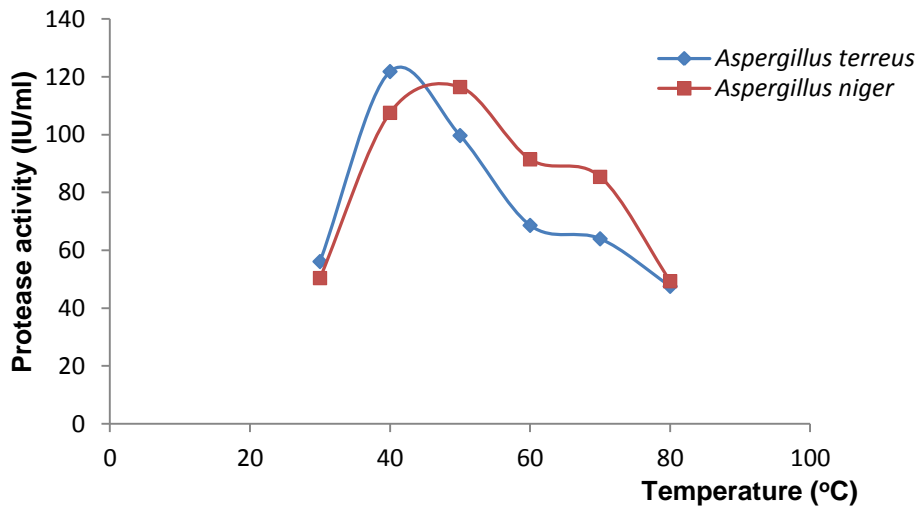


Fig. 4. Effects of different incubation temperatures on protease production

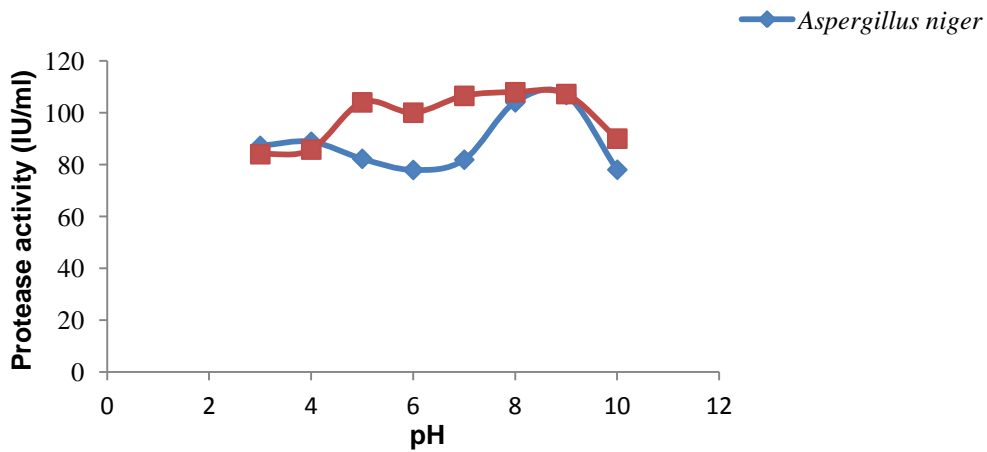


Fig. 5. Effects of Different pH on Protease Production

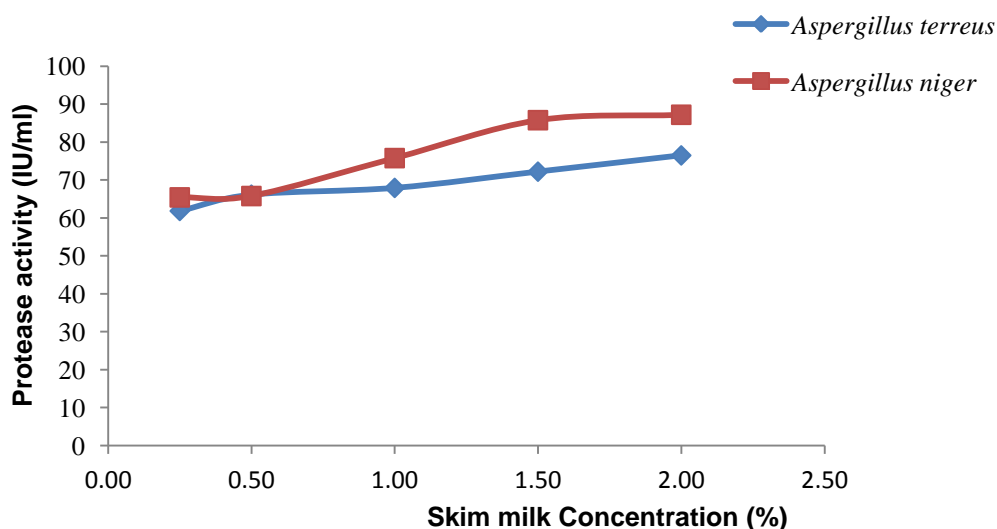


Fig. 6. Substrate concentration dependence on protease production

4. DISCUSSION

The proteolytic activity of *Aspergillus niger* and *A. terreus* was studied using skim milk casein as a substrate. Incubation period was an important parameter for enzyme production for the investigated fungi *Aspergillus niger* and *A. terreus*. *Aspergillus niger* showed a maximum protease activity on the second day (48 hours) of its incubation after that there was a decline. The maximum protease activity (Fig. 3) for *Aspergillus terreus* was observed on day 3 (72 hours) after which there was a decline. It was observed that *A. niger* has significantly lower protease activity than *A. terreus*. *Aspergillus terreus* has been reported to be a better protease producer with higher protease activity than *A. niger*. El-Shora and Metwally [17], in their work on 'Production, purification and characterization of proteases from whey by some fungi' reported that "the specific activity of the purified proteases from *A. niger* and *A. terreus* were 179.0 and 294.7 U mg⁻¹ protein respectively which was accompanied with higher fold of purification of the protease (81.9) from *A. terreus* compared to 28.9 fold for the enzyme from *A. niger*". In a similar result, Budak et al. [18] reported that *A. terreus* had higher amount of proteases identified in wheat bran (WB) than in other *Aspergillus* species studied including *A. niger*. The decline in protease activity could be due to the exhaustion of nutrients as reported by [1,14,19]. The findings of this research work are in line with the work of Chellapandi [20]. The decrease in the enzyme units could probably also be due to inactivation of the enzyme by other constituent proteases [21].

Environmental conditions play important roles in the microbial growth and in suppression or initiation of the enzymes by specific compounds [22]. Temperature is a critical parameter in microbial growth and usually varies from one organism to another [23]. The optimal temperature for fungal proteases ranged between 35°C and 50°C with few exceptions [24]. For *Aspergillus niger* (Fig. 4), there was a gradual increase in protease activity from 30°C up to 50°C (116.5 IU/ml) and then decline was observed from 60°C to 80°C. Similar result was reported by Siala et al [25]. It was deduced that *Aspergillus niger* had maximum protease activity at high temperature of 50°C probably due of its melanin pigmentation which confers protection against adverse environmental conditions. Esbelin et al. [26] reported that spores of the dark *A. niger* N402 and the fawn-color mutant were resistant to moist heat at 56°C while spores of the white-color mutant were highly sensitive indicating that melanin protects pigmented spores of *A. niger* from pulse light. Other researchers also reported that melanin pigmentation in the spores of *A. niger* have a beneficial role in its survival under extreme conditions [27,28]. For *Aspergillus terreus* (Fig. 4), the results showed that there was a gradual increase in protease activity up to 40°C (121.86 IU/ml) and then successive decrease. The optimum temperature (40°C) as observed in this study is in line with the previous works by other researchers [29,30]. "Fungal proteases are usually thermo labile and show reduced activities at high temperatures. Higher temperature is found to have adverse effects on metabolic

activities of microorganisms and cause inhibition to the growth of fungi. The enzyme is denatured by losing its catalytic properties at high temperatures due to stretching and breaking of weak hydrogen bonds within enzyme structure” [31].

Secretion of enzyme by microbial strains is strongly influenced by the extracellular pH. Changes in pH can make and break inter- and intramolecular bonds leading to changes in the structure of the enzyme and its effectiveness [1]. The transport of nutrients and enzymatic processes across the cell membrane are strongly influenced by the culture pH [15]. From the result obtained (Fig. 5) *Aspergillus niger* showed an optimum activity at pH 9.0. According to reports recorded by Devi [32] and Dubey et al. [33], *Aspergillus niger* produced proteases at varied pH in the range 4.4- 9.0. *Aspergillus terreus* optimum activity was observed at pH 8.0. Gopulkumar et al. [34] reported a pH optimum for *Aspergillus terreus* also at 8.0. *Aspergillus terreus* and *A. niger* have been reported to produce alkaline protease. Ahmed [35] in his research work reported protease production in *A. niger* within the pH range of 7-9 with maximum protease production at pH 8. This result agrees with the findings of our work. Similar results were reported by other researchers [36,37,38].

Substrate concentration critically affects the enzyme activity. Enzyme production is influenced by nitrogen source; and skim milk (casein) has been reported to be a major nitrogen source for many microorganisms for maximum protease production [39]. From the result in Fig. 6, *Aspergillus niger* and *A. terreus* showed a maximum protease activity at 2.0% substrate concentration. Kezia et al. [39] reported protease activity at the highest concentration (1.6%) of the substrate.

5. CONCLUSION

Optimization studies were carried out on different parameters (Incubation temperature, Temperature, pH and substrate concentration) for maximum protease production by *Aspergillus niger* and *A. terreus* using skim milk casein as substrate under submerged fermentation. The test fungi exhibited maximum hydrolytic potentials for protease production. Proteases could have vast application in different industries including detergent, textile, food processing, photography and drug manufacturing industries.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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