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Full Length Research Paper

Screening of *Aspergillus flavus* and *Aspergillus fumigatus* strains for extracellular protease enzyme production

Oyeleke, S. B.[1]*, Egwim, E. C.[2] and Auta, S. H.[1]

[1]Department of Microbiology, Federal University of Technology, Minna- Nigeria.
[2]Department of Biochemistry, Federal University of Technology, Minna- Nigeria.

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The fungal isolates identified as *Aspergillus fumigatus* and *Aspergillus flavus* isolated from a local rice husk dumpsite were screened for protease enzyme production. High yields of protease enzyme were obtained by both fungi after 144 h with concentrations of 0.96 µg/ml/min for *A. flavus* and 0.84 µg/ml/min for *A. fumigatus.* Optimum temperature for the activity of protease produced by *A. flavus* and *A. fumigatus* was at 30°C with protease activities of 0.46 and 0.43 µg/ml/min, respectively. Optimum pH for the activity of protease produced by *A. flavus* and *A. fumigatus* were pH 8 and 5, respectively. The result showed that both isolates were good producers of extracellular protease enzymes which can be useful in industries.

Key words: Protease, rice husk, optimization, *Aspergillus fumigatus, Aspergillus flavus.*

INTRODUCTION

Protease is an enzyme that breaks the peptide bonds of proteins (Mitchell et al., 2007). Protease breaks down peptide bonds to produce amino acids and other smaller peptides. It can be isolated from a variety of sources such as plants, animals and microbia (fungi and bacteria). Its application is very broad and has been used in many fields for years, and is mainly used in food and detergent industries (Yandri et al., 2008). Proteases work best in acidic conditions except alkaline proteases which has its optimal activity shown in alkaline (basic) pH (Mitchell et al., 2007). Proteases are one of the most important classes of industrial enzymes and accounts for about 60% of commercial enzymes in the world (Barrette and Rawlings, 2003). They find application in a number of biotechnological processes, viz. in food processing and pharmaceuticals, leather industry, detergent industry, etc. (Nascimento and Martins, 2004; Beg and Gupta, 2003). Two third (2/3) of the industrial produced proteases are from microbial sources (Ellaiah and Adinarayana, 2002). A variety of microorganisms such as bacteria, fungi, yeast and *Actinomycetes* are known to produce these enzymes (Madan et al., 2002; Devi et al., 2008). Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Devi et al., 2008). *Aspergillus clavatus* ES1 has been recently identified as a producer of an extracellular bleaching stable alkaline protease (Hajji et al., 2008).

Protease is the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields (Pastor et al., 2001; Ward, 1985). Therefore, the objectives of the study were to isolate and identify protease producing fungi from local yam peel waste dumpsites, to perform partial characterization of the enzyme production and its properties with regard to the effect of temperature and pH and to maximize the reserve of huge foreign exchange spent on the importation of protease enzyme.

MATERIALS AND METHODS

Isolation and characterization of organism

The fungi species used in this research were isolated from clayey

*Corresponding author. E-mail: droyeleke@yahoo.com.

soil by serial dilution method. The soil sample was aseptically collected by sweeping off yam peel debris from the top soil of a yam peel waste dumpsite along Minna/Bida road in Niger State, and a hand trowel was used to collect about 10 g each of the top soil into a plastic bag. 1 g of the soil sample was suspended in 9 ml of sterile distilled water and was properly mixed. 1ml of the soil suspension was pipetted from each of the above and transferred into another 9 ml of sterile distilled water. The soil suspension was further diluted in seven more 9ml of sterile distilled water blanks. About 0.1 ml from the 10^{-5} dilution was spread on potato dextrose agar plates using a glass spreader, sterilized by dipping in 95% ethanol and flaming. The plates were incubated at room temperature for 5 days. The growth of fungal colonies was observed after incubation. The individual colonies were then subcultured. Identification was based on cell and colony morphology characteristics (morphological and microscopic features). Among the characteristics used were colonial characteristics such as size, surface appearance, texture and colour of the colonies. In addition, microscopy revealed vegetative mycelium including presence or absence of cross walls, diameter of hyphae and types of sexual and asexual reproductive structures. Appropriate references were then made using mycological identification keys and taxonomic description. Based on this, two common fungal species were isolated namely *Aspergillus flavus* - which exhibited a yellowish grey colouration and *Aspergillus fumigatus* - which exhibited a greenish colouration. The young colonies of the *Aspergilli* were aseptically picked up and transferred into PDA slants and incubated at 27°C for 5 days for maximum growth.

Cultivation was carried out in the Czapek Dox medium with the following composition (g/l):

K_2HPO4, 1; $MgSO4 \cdot 7H2O$, 0.5; KCl, 0.5; $FeSO4 \cdot 7H2O$, 0.01; sucrose, 30; casein, 1% (w/v)

Crude extract enzyme production

A. flavus and *A. fumigatus* were cultured in 250 ml Erlenmeyer flasks for five days at 37°C on a rotary shaker set at 250 revolutions per min. The cultures were centrifuged at 10,000 x g for 15 min and the supernatants thus obtained were used as crude enzyme extract as described by Ali et al. (1989).

Enzyme assay

The protease activity was assayed by the method of Lovrien et al. (1985). Three ml of reaction mixture containing 0.5% casein in 2.95 ml of 0.1 M Tris-HCl buffer, pH 8.0 and 0.1 ml of each enzyme was incubated at 40°C. After 30 min, the reaction was stopped by adding 3 ml of cold 10% trichloroacetic acid. After 1 h, each of the culture filtrate was centrifuged at 8,000 rpm for 5 min to remove the precipitate and absorbance of the supernatants was read spectrophotometrically at 540 nm. The amount of amino acids released was calculated from a standard curve plotted against a range of known concentrations of tyrosine. One unit of enzyme (µ/ml/min) was defined as the amount of enzyme that liberated 1.5 µg tyrosine per ml per minute under assay conditions.

Effect of pH on protease production

The effect of pH on activity of protease produced by *A. flavus and A. fumigatus* was carried out using different pH ranges like 3, 4, 5, 6, 7, 8, 9 and 10. Adjustments of the pH were done by addition of hydrochloric acid (0.1 N) and 0.1 N sodium hydroxide to achieve acidity and alkalinity respectively. The optimization media with the above pH were inoculated with the test sample and the protease assay was done after 24 h. The best pH was concluded by reading the absorbance at 540 nm.

Effect of temperature on protease production

The effect of temperature on activity of protease produced by *A. flavus and A. fumigatus* was studied by taking various temperatures ranges like 30, 40, 50, 60, 70, 80, 90 and 100°C. The optimization media was inoculated with the test samples at different temperatures and the protease assay was done after 24 h.

Protein assay

The total protein content of the samples was determined using the method of Biurette as described by Jayaraman (1981). 4 ml of each culture filtrate were taken and 6ml of Biurette's reagent was added to each test tube. The contents were mixed well and the tubes were kept at 37°C for 10 min during which a purple colour developed. The optical density of each tube was measured at 540 nm using the reagent blank. The concentrations of protein in the enzyme samples were determined with reference to Standard Bovine Serum Albumin (BSA).

RESULTS

The effect of incubation period on protease production by *A. flavus* and *A. fumigatus* is shown in Figure 1. High yields of proteases by *A. flavus* and *A. fumigatus* were noticed after 144 h with protease activities of 0.96 µg/ml for *A. flavus* and 0.84 µg/ml for *A. fumigatus*. The production of proteases by both fungi increased with passage of time.

The optimum temperature for proteases produced by *A. flavus* was 30°C (with protease activity of 0.46 µg/ml) and *A. fumigatus* (with protease activity of 0.43 µg/ml, as shown in Figure 2. Temperatures beyond 30°C led to decrease in protease yield.

The effect of pH on protease yield by both fungi is shown in Figure 3. From the figure, it can be seen that as pH increased, the production of protease also increased until optimum pH for protease production by *A. flavus* (pH 8) with protease activity of 0.74 µg/ml/min and by *A. fumigatus* (pH 5) with protease activity of 0.70 µg/ml, was reached. Then the production of enzyme decreased till pH 10.

DISCUSSION

The production of proteases by both fungi increased with increase in time. *A. flavus* recorded a higher protease yield (0.9 µg/ml/min) than *A. fumigatus* (0.84 µg/ml/min).

The differences in protease yield by both fungi could be due to the differences in their genetic makeup. Similar observations were also recorded by Ali (1992) who worked on the production of proteases by *A. fumigatus* and *Penicillium* sp.

At temperatures beyond 30°C, both fungi produced

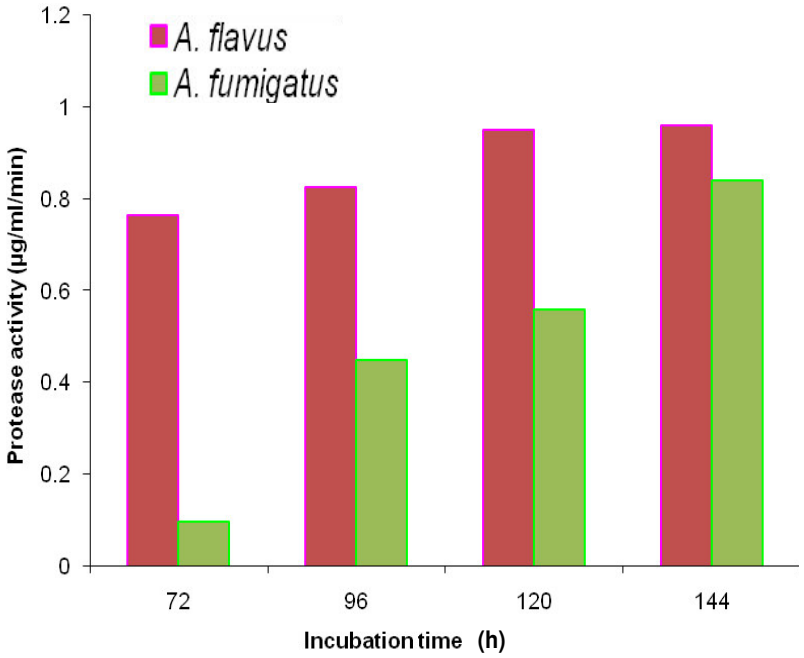

Figure 1. Effect of incubation period on protease yield by *A. flavus* and *A. fumigatus.*

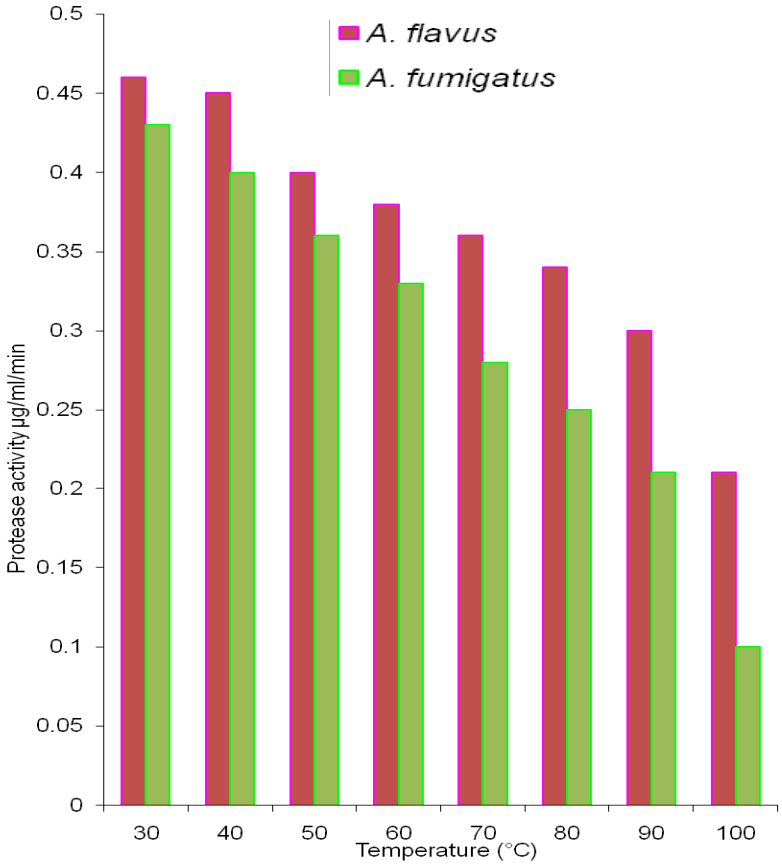

Figure 2. Effect of temperature on protease produced by *A. flavus* and *A. fumigatus.*

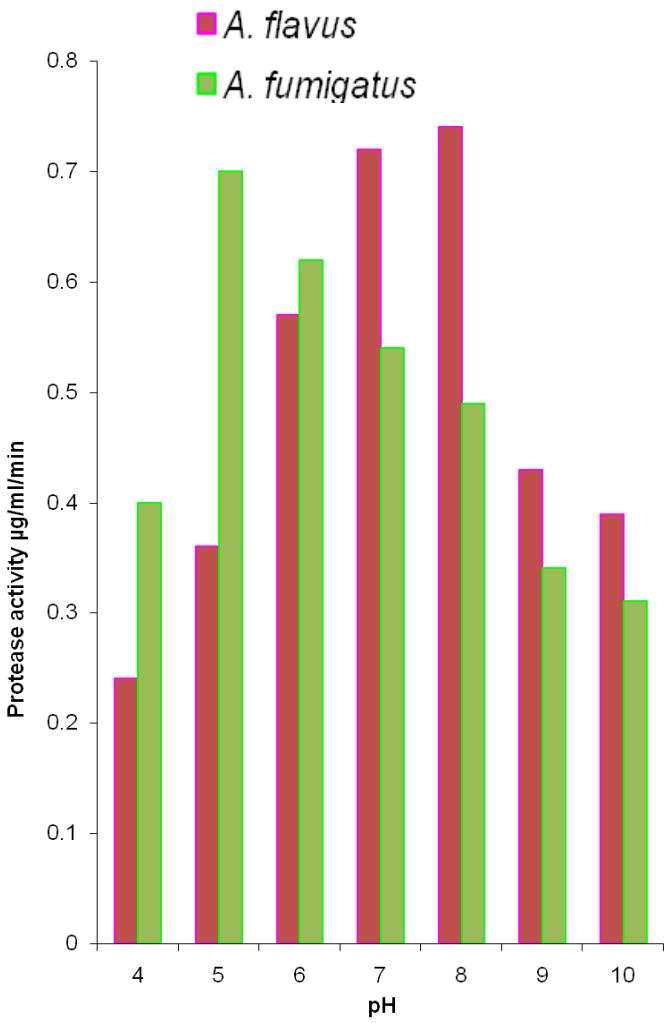

Figure 3. Effect of pH on protease produced by *A. flavus* and *A. fumigatus*.

protease but in lesser yields than that produced at optimal temperature. These temperatures might not have been suitable for protease production. This is in accordance with the review of Daniel et al. (2010) who stated that increase in temperature led to increase in activity but that there was limit to the increase in activity because higher temperatures led to a sharp decrease in activity. This could be due to the denaturing of protein structure. Ali (1992) also recorded optimum temperature of 30°C for protease produced by *A. fumigatus*. These results are at variance with those recorded by Hossain et al. (2006) and Devi et al. (2008) who reported optimum temperatures of 45°C for *Aspergillus* spp. and 45°C for *A. flavus*, respectively.

The optimum pH recorded for protease production by *A. flavus* and *A. fumigatus* were pH 8 and 5, respectively. The results showed that the protease produced by *A. flavus* was an alkaline protease demontrating the alkalinophilic nature of the fungus, while that produced by *A. fumigatus* is an acidic protease. Hossain et al. (2006) also recorded optimum pH of 8 for proteases production by *A. flavus*. Devi et al. (2008) recorded optimum pH of 8.5 for protease production by *Aspergillus niger.* These results are at variance with the study of Coral et al. (2003) and Siala et al. (2009) who recorded optimum temperature of 60 and 90°C and optimum pH of 3.0 and 9.0 for proteases produced by *A. niger*.

Alkaline and acidic proteases produced from locally isolated fungi, *A. flavus* and *A. fumigatus*, have been characterized. Their desirable characters such as stability at high and low pH and at fairly high temperatures are significant characteristics of any enzyme for industrial application.

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