 http://dx.doi.org/10.4314/bajopas.v5i1.33

Bayero Journal of Pure and Applied Sciences, 5(1): 187 – 190
Received: October 2011
Accepted: April 2012
ISSN 2006 – 6996

SCREENING OF *PLEUROTUS OSTREATUS* AND *GLEOPHYLUM SEPIARIUM* STRAINS FOR EXTRACELLULAR PROTEASE ENZYME PRODUCTION

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ABSTRACT
The fungal isolates identified as Pleurotus ostreatus and Gleophylum sepiarium isolated from sawdust dump-site were screened for protease enzyme production. High yields of protease enzyme were obtained by both fungi after 96h with concentrations of 1.6ug/ml/min for P ostreatus and 1.5ug/ml/min for G.sepiarium. Optimum temperature for the activity of protease produced by P.ostreatus and G.sepiarium was at 70^{0}C with activity of 060ug/ml/min and 0.55ug/ml/min respectively. Optimum pH for the activity of protease produced by P.ostreatus and G.sepiarium was pH 7 with activity of 0.55ug/ml/min and 0.60ug/ml/min. The result showed that both isolates were good producers of extracellular protease enzyme which may be useful in industries.
Key words: Protease, sawdust, optimization, Pleurotus ostreatus, Gleophylum sepiarium.

INTRODUCTION

Protease is an enzyme that breaks the peptide bonds of protein to produce amino acids and other smaller peptides (Mitchell,*et al*., 2007). It can be isolated from a variety of sources such as plants, animals and microbes (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) . Protease work best in acidic conditions except alkaline proteases which has its optimal activity shown in alkaline pH (Michell *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 pharmaceutical, leather industry, detergent industry, etc (Beg and Gupta, 2003) Nascimento and Martins, 2004) Two third (2/3) of the industrial produced proteases are form 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 ciavatus* ESI has been recently identified as a producer of extracellular bleching stable alkaline protease (Haiji *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*., 2002;). Therefore, the objectives of the study were to isolate and identify protease producing fungi from sawdust dumpsites to partially purify and characterize the enzyme.

MATERIALS AND METHODS

Isolation and characterization of organism

The fungi species used in this research were isolated from sawdust by serial dilution method. The sample was aseptically collected from sawdust dump-site at Maitumbi in Minna, Niger State and a hand trowel was used to collect about 10g each of the sample into a plastic bag. Exactly1g of the sample was suspended in 9ml of sterile distilled water and was properly mixed. Sample suspension (1ml) was pipetted from each of the above and transferred into another 9ml of sterile distilled water. The suspension was further diluted in seven more 9ml of sterile distilled water (Lovrien *et al*). About 0.1ml from 10^{-5} dilution was spread on potato dextrose agar (PDA) plates using a glass spreader, sterilized by dipping in 95% ethanol and flaming. The plates were incubated at room temperature for 4days. The growth of fungal colonies was observed after incubation and individual colony 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 type 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: *Pleurotus ostreatus* which exhibited a black colouration and *Gleophylum sepiarium* which exhibited a white colouration. The young colonies of the fungi were aseptically picked up and transferred into PDA slants and inoculated at 27^{0}C for 4days for maximum growth (Oyeleke *et al.,* 2010).

Cultivation was carried out in the Czapek Dox medium with the following composition.
K_2HPO_4,1g; MgSO4. $7H_2O$, KCl, 0.5g; $FeSO4.7H_2O$, 0.01g; sucrose, 30g; casein, 1% (w/v)

Crude extract enzyme preparation
P.ostretus and *G.sepiarium* were cultured in 250ml Erlenmeyer flasks for five days at 37^0C on a rotary shaker set at 250 revolutions per min. The cultures were centrifuged at 10,000 x g for 15 min and 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 Lovrieri et al. (1985). The reaction mixture (3ml) containing 0.5% casein in 2.95ml of Tris HCl buffer, pH 8.0 and 0.1ml of each enzyme was incubated at 40^0C. After 30min, the reaction was stopped by adding 3ml of cold 10% trichloroacetic acid. After 1h, each of the culture filtrate was centrifuged at 8,000rpm for5min to remove the precipitate and absorbance of the supernatants was read spectrophotometrically at 540nm. 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 (u/ml/min) was defined as the amount of enzyme that liberated 1.5ug tyrosine per minute under assay condition.

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

Effects of temperature on protease production
The effect of temperature on activity of protease produced by *P.ostreatus* and *G. sepiarium* was studied by taking various temperatures like 30, 40, 50, 60, 70, 80, 90, and 100C. The optimization media was inoculated with the test samples at different temperatures and the protease assay was done after 24hrs.

Protein assay
The total protein content of the sample was determined using the Biurette method as described by Jayaraman (1981). Each culture filtrate (4ml) 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 37^0Cfor 10min during which a purple colour developed. The optical density of each tube was measured at 540nm using the reagent blank. The concentrations of protein in the enzyme samples were determined with reference to Standard Bovine Serum Albumin (BSA) concentration.

RESULTS
The results of the research were presented in figures below. Maximum production of proteases by P.ostreatus and G.sepiarium were 1.6ug/ml/min and 1.5ug/ml/min respectively. The maximum time for the two enzyme production was at 96hours of incubation. Further increment in time of incubation leads to denature of the enzyme.

Optimum temperature for proteases activity produced by *P.ostreatus* and *G.sepiarium* were recorded at 70^0c with activity of 0.60mg/ml and 0.58mg/ml. However, temperatures below or above 70^0c caused a sharp decrease in protease activity as compared to the optimum temperature.
Maximum protease activity for the two fungal isolates were (0.55mg/ml) and (0.60mg/ml) respectively at pH of 7. This shows that the protease produced by the two fungal isolates is neutral protease. As the pH increased, the activity of the protease enzyme decreased

The effect of incubation period on protease production by P.ostreatus and G.sepiarium is shown in fig. 1. High yield of proteases by *P.ostreatus* and *G.sepiarium* were noticed after 96h with protease activities of 1.6ug/ml and 1.5ug/ml respectively. The production of proteases by both fungi increased with passage of time.

The optimum temperature for proteases produced by *P.ostreatus* was 70^0C with protease activity of 0.60ug/ml) and *G.*sepiarium (with protease activity of 0.55ug/ml) as shown in fig.2. Temperatures beyond 70^0C led to decrease in protease yield.

The effect of pH on protease yield by both fungi is shown in fig.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 *P.*ostreatus and *G.*sepiarium (pH 7) with protease activity of 0.55ug/ml/min and 0.60ug/ml/min was reached. Then the production of enzyme decrease till pH 12

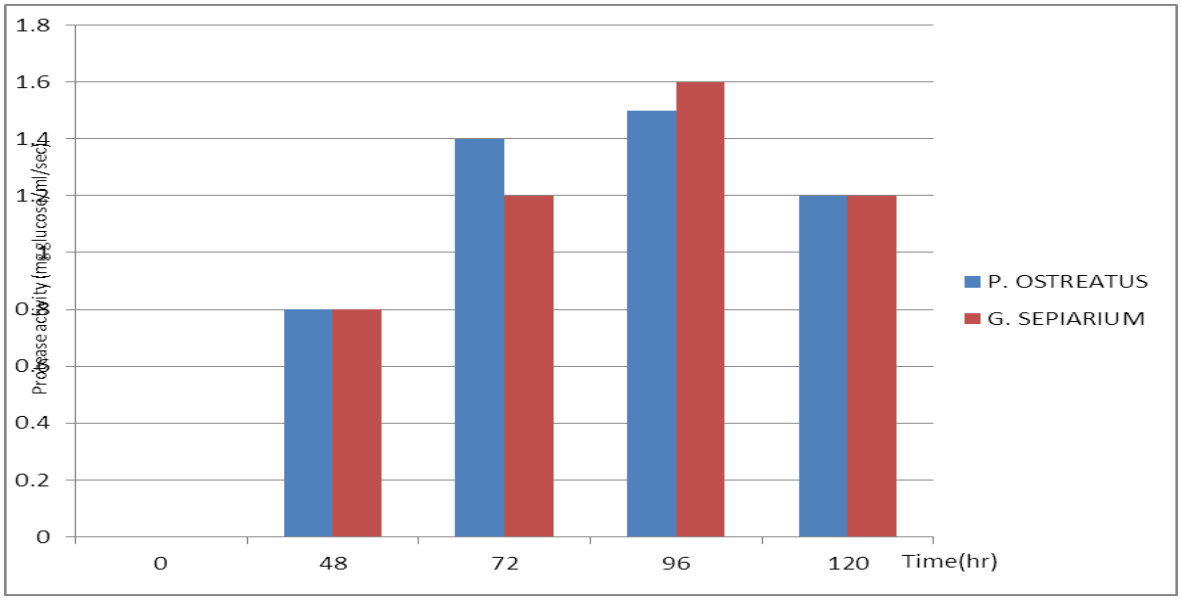

Figure 1: Incubation profile of protease from P.ostreatus and G.sepiarium

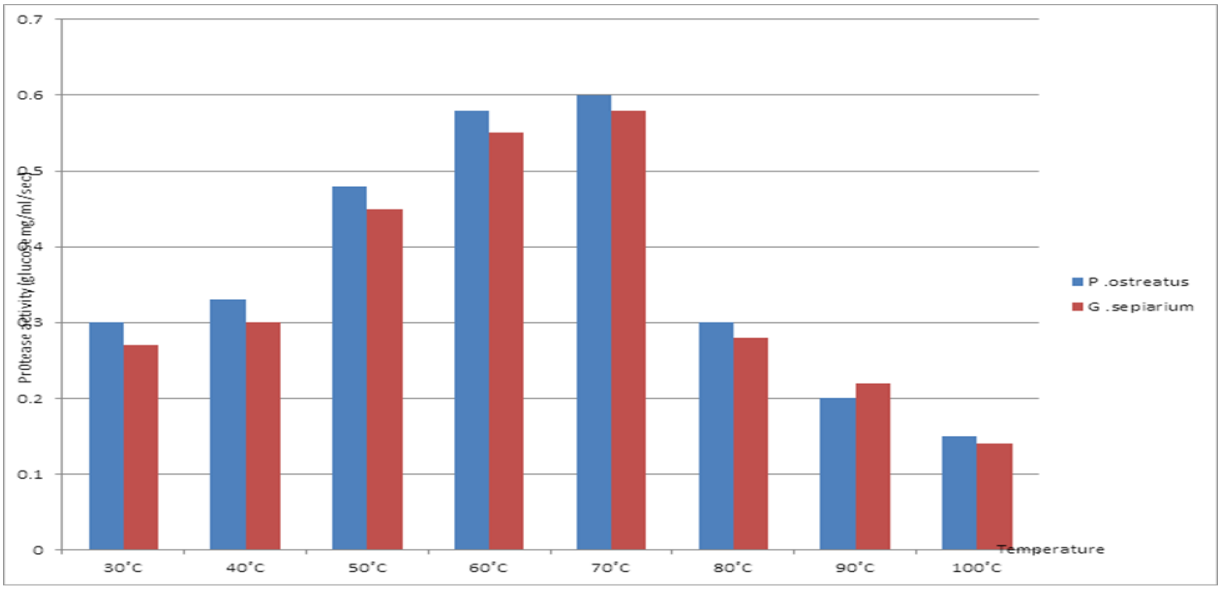

Figure 2: Temperature activity profile of protease from *P.ostreatus* and *G.sepiarium*

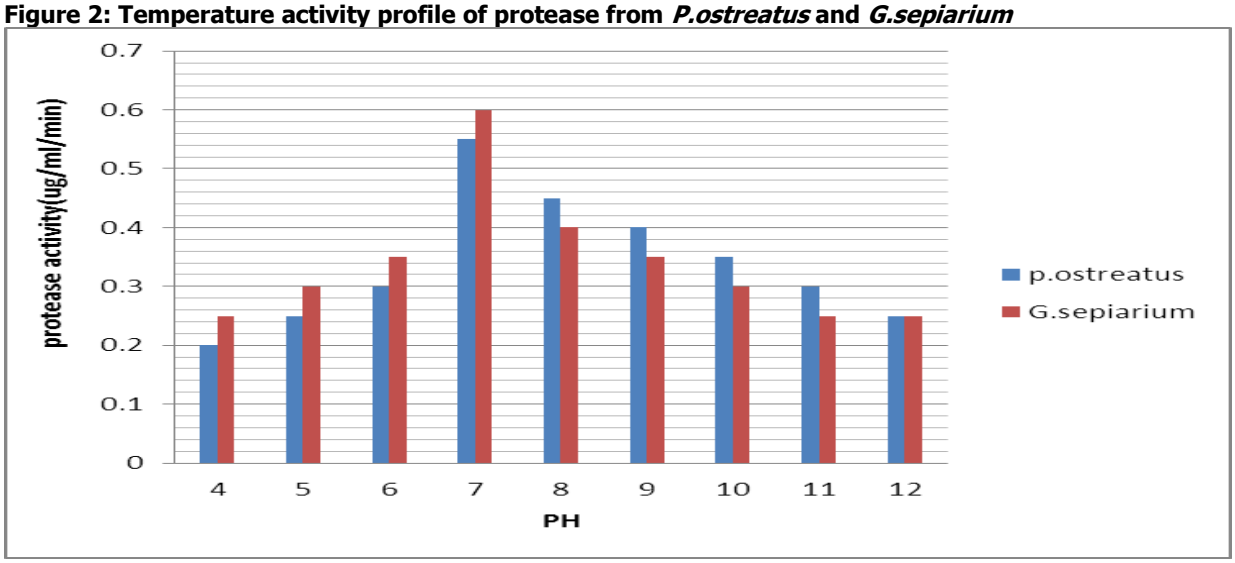

Figure 3: pH activity profile of protease from *P.ostreatus* and *G. sepiarium*

DISCUSSION

The production of protease by both fungi increased with increase in time. *P. ostreatus* recorded a higher protease yield (1.6ug/ml/min) than G.*sepiarium* (1.5ug/ml/min). The differences in protease yield by both fungi could be due to the differences in their genetic makeup. Similar observation was made by Oyeleke *et al.*, (2010) who worked on production of proteases by *A.flavus* and *A.fumigatus*. It is also in accordance with observation made by Ali (1992) who worked on production of proteases by *A.fumigatus* and *Penicillium* sp.

At temperatures beyond 70^0C, both fungi produced 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 increase in activity because higher temperatures led to a sharp decrease in activity. The result is also in agreement with observation made by Devi *at al.*,(2008) who reported optimum temperatures of 45^0C for *Aspergillus* spp .

The optimum pH recorded for protease production by *P. ostreatus* and *G.sepiarium* was pH 7. The results showed that the proteases produced by P.ostreatus and G.sepiarium were a neutral protease demonstrating the neutraphilic nature of the fungi. Hossain et al. (2006) also recorded optimum pH of 8 for proteases production by *A.flavus.* and by *A.niger*. These results are at variance with the study of Coral *et al*. (2003) who recorded optimum temperature of 80^0C and 90^0C and optimum pH of 3.0 and 9.0 for protease production by *A. niger* Devi *et al.* (2008) recorded optimum pH of 7.5 for proteases produced by *A.niger*.

CONCLUSION

The study revealed the potential of agricultural wastes capability to produce protease by *P, ostreatus* and *G. sepiarium*.It can be concluded that these isolate can be industrially exploited for the synthesis of protease enzyme.

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