Nig J. Biotech. Vol. 23 (2011) 28 - 34
ISSN: 0189 17131
Available online at www.biotechsocietynigeria.org.

NIGERIAN JOURNAL OF BIOTECHNOLOGY

Production of Microbial Protease from Selected Soil Fungal Isolates

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(Received 20.07.11, Accepted 09.11.11)

Abstract
This study was undertaken to monitor the production of protease enzyme from soil fungal isolates obtained from Omo natural forest in Ogun State of Nigeria. The study also sought to determine the kinetic parameters of the enzyme with the aim of establishing the industrial and biotechnological importance of this microbial enzyme. The harvested mycelia of the fungi were separately homogenized in buffered culture medium for five days using shaker incubator in which the protease activity was monitored. The results of the enzyme activity showed that the organisms; *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus and Penicillium italicum* produced the protease enzyme maximally between day three and day five of incubation while the effect of temperature and thermal stability on the enzyme production showed temperature optimal for enzyme production was between 30 and 60^{0}C and the thermal stability on the enzyme activity was between 30 and 50^{0}C. The optimal pH on the enzyme production was observed to be between pH 3.5 and 5.5 for the organisms.

Keywords: Soil microorganism, fungal isolate, incubation period, microbial enzyme

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Introduction

The soil is home to a large proportion of the world's genetic diversity. The linkages between soil organisms and soil functions are observed to be incredibly complex. The interconnectedness and complexity of this soil 'food web' means any appraisal of soil function must necessarily take into account interactions with the living communities that exist within the soil (Burges and Raw, 1967). We know that soil organisms break down organic matter, making nutrients available for uptake by plants and other organisms. The nutrients stored in the bodies of soil organisms prevent nutrient loss by leaching.

Microbial exudates act to maintain soil structure, and earthworms are important in bioturbation (Doran *et.al.,* 1994). The protease enzyme constitutes two thirds of total enzymes used in various industries and this dominance in the industrial market is expected to increase as year gone-by (Gupta *et al*., 2002). Of all proteases, alkaline proteases produced by *Bacillus* species are of great importance in detergent industry due to their high thermostability and pH stability. For production of enzyme for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process (Parekh *et al*., 2002).

Even though most commercial proteases originated from microorganisms belonging to the genus *Bacillus*, fungi exhibit a wider variety of proteases than bacteria. Furthermore, fungi are normally generally regarded as safe strains and they produce extracellular enzymes, which are easier to be recovered from fermentation broth (Sandhya *et al*., 2005). The microbial proteases of *Aspergillus* species, in particular, have been studied in detail since they are known for their capacity to secrete high levels of enzymes in their growth environment. Several of these secreted enzymes, produced in a large-scale submerged fermentation, have been widely used in the food and beverage industry for decades (Biesebeke *et al*., 2006).

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase activating cascade). Peptidases can break either specific peptide bonds (limited proteolysis), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (unlimited proteolysis). The activity can be a destructive change abolishing a protein's function or digesting it to its principal components, it can be an activation of a function or it can be a signal in a signaling pathway (Hooper, 2002).

Materials and Methods

Isolation of fungi: The fungi were isolated by the methods reported by Kader *et al.* (1999). In a typical assay, 1g of soil sample was mixed with 9ml of sterile distilled water in a test tube shaken vigorously. Series of dilutions were made until 1:1000 dilution. After preparing the appropriate dilution, 0.5mL was pipetted, poured and dispersed by swirling on potato dextrose agar (PDA) and incubated at 30^0C for five days. The various isolates were sub-cultured until single pure isolates were obtained.

Identification of fungi: Characterization method employed for the fungal isolates were made by both the inspection of colonial features, cellular characteristics at X100 and X40 microscopic magnification. Identification was done by employing the method of (Barnet and Hunter, 1972) and conventional techniques of isolating individual microorganisms and allowing them to grow and produce colonies.

Enzyme production medium: The composition of mineral salt medium for production of the fungi isolates enzyme was 1.0g K_2HPO_4; 0.5g $MgSO_4.7H_2O$; 2.0g $NaNO_3$; 0.001g $FeSO_4.7H_2O$; and 05g KCl. The culture medium was adjusted to pH 7.0 before sterilization by autoclaving at 121^0C for 25min., the carbon source used was 0.5% casein.

Preparation of Enzyme Solution: With the aid of a sterile cork borer, a 5mm disk from the advancing edge of a 4 day old fungal isolates were separately inoculated into the cultivation medium. Incubation was carried out at 30^oC for 5 days in a shaker incubator operated at 150rpm. Protease is an extracellular enzyme so its recovery is quite easy. After incubation the production medium was centrifuged at 12,000 rpm for 15 minutes to separate the cells. The supernatant was collected as it contained the crude enzyme and stored at 4^0C till further use.

Protease Assay: 0.5ml of 1% casein solution (1g of casein was prepared in 100mL of 0.05M citrate phosphate buffer pH 7.5 boiled for 15minutes) was pipetted into test tubes. It was incubated at 37^0C for 15 minutes. 0.2 ml of enzyme solution was added and allowed to stand for one hour inside the water bath with occasional shaking. 3.0 mL of 10% Trichloroacetic acid (TCA) was added to terminate the reaction. The tubes were allowed to stand for one hour in the cold chamber at 2^0C, the solutions were then centrifuged at 3000 rpm and the absorbance of the supernatant was read at 280nm for unprecipitated protein hydrolysate using UV/visible spectrophotometer (Ladd and Butler, 1972). The various days with highest activity were however used to determine the various effects on enzyme activity.

Effect of pH on Protease activity of the fungi: The effect of pH on the enzyme activity was determined by using the following buffers to prepare the substrate: 0.05M Sodium acetate buffer (pH 3.0 - 5.0), Citrate buffer (pH 5.0 – 7.0) and Tris-HCl buffer (pH 7.0 – 9.0) as described above.

Effect of Temperature on Protease Activity of the Fungi: 0.5ml of 1% casein solution was pipetted into test-tubes and put into water bath set at 37^0C and left for 15 minutes to equilibrate after which 0.2 ml of the crude enzyme with highest activity was added and the reaction mixture was incubated for 1hour. The reaction was terminated by adding 3mL of 10%

TCA and kept in the refrigerator at 2^0C for one hour. It was centrifuged at 3000 rpm and absorbance of supernatant solution was read at 280nm for unprecipitated protein hydrolysate using uv/visible spectrophotometer. This was repeated for 40^0C, 50^oC, 60^0C, 70^0C, 80^0C, 90^0C and 100^0C.

Assay of thermal stability on protease activity at various temperatures: 1.0ml of the crude enzyme solution from the various organisms with highest activity was pipetted into test tubes, incubated in water bath at various temperatures (30, 40, 60, 70 and 80) ^{0}C for 1hour. 0.2 ml each of the heat treated crude enzyme solution was added to 0.5ml of the 1% casein solution and incubated at 37^0C for 1hour. After this 3ml of cold 10% TCA was added to stop the reaction and it was kept in the refrigerator at 2^0C for 1hour. It was centrifuge and absorbance of the supernatant was read at 280nm.

Quantitative Assay for Protein Content of the enzyme using Biuret Method: 5mg/ml stock of bovine serum albumin (BSA) was prepared and serial dilutions were made into test tubes. Distilled water was added to make up each test-tube volume to 1ml, 0.2ml of crude enzyme was also made up to 1.0ml with distilled water, 3ml of Biuret was added and the solution was allowed to stand for 30minutes for incubation and color development. The absorbance was read at 540nm and the protein concentration of the crude extract was calculated from the standard calibration curve.

Results and Discussion

Protease Activity of Enzymes Extracted From Fungal Isolates During Five Days Incubation Period: Determination of protease activity from the fungal isolates showed that *Aspergillus flavus* had the highest activity at day 4 (7.5×10^{-4}) U/ml/min while it showed its lowest activity at Day 1 (3.2×10^{-4}) U/ml/min. *Aspergillus niger* has the highest activity at day 4 (4.30×10^{-3}) U/ml/min. while it showed its lowest activity at day 1 (1.8×10^{-4}) U/ml/min. *Aspergillus fumigatus* has the highest activity at day 4 (1.97×10^{-3}) U/ml/min. while it showed its lowest activity at day 1 (4.7×10^{-4}) U/ml/min. *Penicillum italicum* showed its highest activity at day 3 (1.87×10^{-3}) U/ml/min while it showed its lowest activity at day 5 (8.3×10^{-4}) U/ml/min.

Table 1: Protease Activity of Enzymes Extracted from Fungal Isolates During Five days Incubation Period.

Days	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus fumigatus*	*Penicillium italicum*
	U/ml/min	U/ml/min	U/ml/min	U/ml/min
1	3.2×10^{-4}	1.80×10^{-4}	4.70×10^{-4}	1.47×10^{-3}
2	6.3×10^{-4}	4.80×10^{-4}	1.45×10^{-3}	1.62×10^{-3}
3	7.0×10^{-4}	8.80×10^{-4}	1.63×10^{-3}	1.87×10^{-3}
4	7.5×10^{-4}	$4.30x\ 10^{-3}$	$1.\ 97x\ 10^{-3}$	1.18×10^{-3}
5	7.0×10^{-4}	1.83×10^{-4}	$1.\ 25 \times 10^{-3}$	8.30×10^{-4}

The protease production of *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus* and *Penicillium italicum* was observed for five days in liquid medium with 0.5% casein as a carbon source. The organisms were found to produce the enzyme maximally as they grow in the medium at different rates between two and four days and declined after fourth day was obvious as obtained in Table 1. These results showed that the protease enzyme production may be directly linked to the organism concerned and effectiveness and suitability of the culture medium, this observation is however similar to that obtained by (Pagare *et al.,* 2009). Though, Nascimento and Martins (1996); Johnvesly and Nailk (2001) and Ferrero *et al.*, 1996 have reported various other carbon sources to influence fungi protease production in culture medium, casein as used in

this work as carbon source for the fungi growth to produce protease has been used severally and have positively influenced the fungal protease production (Pagare *et al*., 2009).

Effect of pH on Protease Extracted from Four Fungal species: The range of pH (3-9) which were used in this study to check the optimum pH for the protease activity of the various fungi was observed with *Aspergillus flavus* having the highest activity at pH 5.5 (7.6 x 10^{-3}) U/ml/min.; *Asperlligus niger* with highest activity at pH 5.0 (11.4 x 10^{-3}) U/ml/min; *Aspergillus fumigatus* with highest activity at pH 5.5 (16.1 x 10^{-3}) and *Penicillum* with highest activity at pH 5.5 (8.3 x 10^{-3}) U/ml/min though the activity of all the organisms is relatively high between pH 3.5 and 9.0.

Table 2: Effect of pH on Protease Extracted from Four Fungal species

pH	*Aspergillus flavus italicum*	*Aspergillus niger*	*Aspergillus fumigatus*	*Penicillium*
	U/ml/min	U/ml/min	U/ml/min	U/ml/min
3.0	0.99 x 10^{-3}	5.95 x 10^{-3}	1.7 x 10^{-3}	2.6 x 10^{-3}
3.5	7.4 x 10^{-3}	10.3 x .10^{-3}	10.6 x 10^{-3}	2.8 x 10^{-3}
4.0	7.3 x 10^{-3}	10.7 x 10^{-3}	10.9 x 10^{-3}	7.7 x 10^{-3}
4.5	7.4 x 10^{-3}	11.1 x 10^{-3}	12.4 x 10^{-3}	7.8 x 10^{-3}
5.0	7.4 x 10^{-3}	11.4 x 10^{-3}	13.2 x 10^{-3}	8.1 x 10^{-3}
5.5	7.6 x 10^{-3}	11.1 x 10^{-3}	16.1 x 10^{-3}	8.3 x 10^{-3}
6.0	6.7 x10^{-3}	9.3 x 10^{-3}	12.9 x 10^{-3}	6.6 x 10^{-3}
6.5	6.4 x 10^{-3}	9.9 x 10^{-3}	12.8 x 10^{-3}	6.6 x 10^{-3}
7.0	6.6 x 10^{-3}	9.3 x 10^{-3}	12.3 x10^{-3}	6.7 x 10^{-3}
7.5	6.1 x 10^{-3}	9.4 x 10^{-3}	11.2 x 10^{-3}	6.1 x 10^{-3}
8.0	6.0 x 10^{-3}	8.8 x 10^{-3}	11.8 x 10^{-3}	6.2 x 10^{-3}
8.5	5.7 x 10^{-3}	8.3 x 10^{-3}	11.4 x 10^{-3}	7.2 x 10^{-3}
9.0	5.4 x 10^{-3}	7.4 x 10^{-3}	10.9 x 10^{-3}	6.9 x 10^{-3}

From the result obtained in Table 2, it was obvious that the organisms produced both acid and alkaline proteases as the effect of pH span between 3.5 and 9.0. It has been reported widely that protease production from microbial source can be acidic or alkaline proteases as reported by many researchers depending on the organisms and source of the isolation. (Pagare *et al*., 2009) obtained an optimum pH for protease activity of 8 for .but relatively high pH between 3.0 and 9.0 for extracellular protease from *Aspergillus niger* and *Bacillus subtilis*. Nascimento and Martins (1996) and Sookkheo et al., (2000) also reported the optimum pH for protease activity to be between 7.0 and 8.5. According to Borris (1987) alkaline protease production is found to be maximum at pH 9-13. Dutta and Banerjee (2006) reported that pH 7 is the maximum protease activity in *Pseudomonas*.

Effect of Temperature on Protease Extracted from Four Fungal species: The range of temperature between 30^{o}C and 100^{o}C was used in this study to check the optimum temperature for the protease activity of the various fungi and it was observed with *Aspergillus flavus* having the highest activity at 60^{o}C (6.1x 10^{-3}) U/ml/min.; *Asperlligus niger* with highest activity at 50 ^{o}C (6.8 x 10^{-3}) U/ml/min; *Aspergillus fumigatus* with highest activity at 50 (9.3 x 10^{-3}) and *Penicillum italicum* with highest activity at 40 ^{o}C (3.8 x 10^{-3}) as it was evidenced from the results all the organisms apart from *Penicillium italicum* completely lost their activity at 100^{o}C.

Table 3: Effect of *Tempe*rature on Protease Extracted from Four Fungal species

Temperature	*Aspergillus flavus* U/ml/min	*Aspergillus niger* U/ml/min	*Aspergillus fumigatus* U/ml/min	*Penicillium italicum* U/ml/min
30°C	9×10^{-4}	3.5×10^{-3}	1.1×10^{-3}	3.2×10^{-3}
40°C	8×10^{-4}	3.6×10^{-3}	9.0×10^{-3}	3.8 x 10
50°C	3×10^{-4}	6.8×10^{-3}	9.3×10^{-3}	2.1 x 10
60°C	$6.1x\ 10^{-3}$	4.2×10^{-3}	4.0×10^{-3}	9.5 x 10
70°C	7×10^{-4}	2.5×10^{-3}	2.1×10^{-3}	1.7 x 10
80°C	0.0	2.4×10^{-3}	2.5×10^{-3}	1.5 x 10
90°C	0.0	1.9×10^{-3}	5.5×10^{-4}	1.3 x 10
100°C	0.0	0.0	0.0	0.6 x 10

However, the optimum temperature range for all the organisms was between 40°C and 60°C (Table 3). The observations in this study fall in line with those reported previously by other Scientists that protease activity was increasing up to 60°C and a reduction in protease activity was also observed at above 60°C (Pagare *et al*., 2009). Horikoshi (1990) reported the optimum temperature of 60°C for protease activity.

*Effect of thermal stability on Protease Extracted from Four Fungal species:*The effect of thermal stability shows that *Aspergillus flavus* has the highest activity at 40°C (1.0×10^{-3}) U/ml/min, *Aspergillus niger* has the highest activity at 30°C (4.6×10^{-3}) U/ml/min, *Aspergillus fumigatus* has the highest activity at 50°C (6.2×10^{-3})mg/ml/min and *Penicillum* has the highest activity at 40°C (4.7×10^{-3}) U/ml/min.

Table 4: Effect of *thermal stability* on Protease Extracted from Four Fungal species

Temperature	*Aspergillus flavus* U/ml/min	*Aspergillus niger* U/ml/min	*Aspergillus fumigatus* U/ml/min	*Penicillium italicum* U/ml/min
30°C	0.2×10^{-3}	4.6×10^{-3}	1.7×10^{-3}	2.3×10^{-3}
40°C	1.0×10^{-3}	2.8×10^{-3}	1.4×10^{-3}	4.7×10^{-3}
50°C	0.2×10^{-3}	2.3×10^{-3}	6.2×10^{-3}	3.2×10^{-3}
60°C	0.3×10^{-3}	1.4×10^{-3}	4.1×10^{-3}	1.6×10^{-3}
70°C	0.9×10^{-4}	1.9×10^{-3}	2.3×10^{-3}	2.6×10^{-4}
80°C	0.3×10^{-4}	2.1×10^{-4}	0.8×10^{-3}	1.2×10^{-4}
90°C	0.8×10^{-4}	2.4×10^{-4}	0.3×10^{-3}	2.1×10^{-4}
100°C	0.0	0.2×10^{-4}	$0.05x10^{-3}$	0.2×10^{-4}

The thermal stability temperature range observed in this study was between 30°C and 100°C to check the temperature at which the enzyme activity of the various fungi would be affected by heat treatment and it was observed that *Aspergillus flavus* having the highest activity at 40°C ($1.0x\ 10^{-3}$) U/ml/min. but loss activity completely at 100 °C; while *Asperlligus niger* with highest activity at 30°C (4.6×10^{-3}) U/ml/min; *Aspergillus fumigatus* with highest activity at 50 °C (6.2×10^{-3}) and *Penicillum italicum* with highest activity at 40°C (4.7×10^{-3}) still retain slight activity even at 100°C.

Conclusion and Recommendation

In conclusion microorganisms especially fungi present in the soil like *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus* and *Penicillum italicum* produced hydrolytic enzymes of industrial and biotechnological importance. The protease enzyme from these fungi has ability to degrade the proteolytic materials present in the soil or otherwise for bioresource development

and environmental management based on the results obtained from this present work. Also further purification of this protease enzyme present in the forest and plantation soil will enhance its catalytic efficiency and use in the production of fertilizers.

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