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Production of Amylase and Protease Enzymes by *Aspergillus niger* and *Penicillium frequestans* Isolated from Abattoir Effluent

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ABSTRACT

The production of enzyme by *Aspergillus niger* and *Penicillium frequestans* isolated from abattoir effluent was carried out. The enzyme amylase was found to be thermostable, while protease had its optimum activity at 40°C and was active at a wide range of pH 3-8. The amount of amylase and protease liberated by each fungus was quantified by estimating the amount of reducing sugars and amino acid produced, when specified quantity of starch and casein were hydrolyzed after incubation at 28 and 40°C, respectively, with known concentration of enzyme solution. Results showed that *A. niger* exhibited the highest enzyme production after 4 days with 4.48×10^{4} mg mL^{-1} sec^{-1} and *P. freuqestans* 3.28×10^{-4} mg mL^{-1} sec^{-1}. Optimum temperature for the activity of amylase produced by *A. niger* and *F. frequestans* was 70 and 60°C, respectively and 40°C in the case of protease for both fungi. Optimum pH for activity of amylase produced by both *A. niger* and *P. frequestans* was pH 5 whereas, that of protease were 5 and 7, respectively. The result suggests that both isolates were good producers of amylase and protease which can be of industrial importance.

Key words: Amylase, protease, *Aspergillus niger*, *Penicillium frequestans*

INTRODUCTION

Abattoir effluents are waste water of animal slaughter house, usually contaminated with animal excrement and blood. Some of the consequences of man-made pollution are transmission of diseases by water-borne pathogen, eutrophication of natural water bodies, accumulation of toxic or recalcitrant chemicals in the soil, destabilization of ecological balance and negative effects on human health (Bridges *et al*., 2000; Amisu *et al*., 2003).

Abattoir effluent and in fact other industrial effluents can be converted to products that are of commercial interest such as enzymes, ethanol, antibiotics and single cell protein (Oyeleke *et al*., 2010). Another major approach to the improvement in the utilization of abattoir effluent is microbial enzyme production. There are three major sources of enzymes, that is derived from a variety of plants, e.g., pappain, animal enzymes-derived from animal glands, e.g., trypsin, pepsin and microbial enzymes-derived from micro organisms (fungal and bacterial) through the process of fermentation, e.g., amylase and protease. Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable (Oyeleke and Oduwole, 2009; Oyeleke *et al*., 2010).

Only few microorganisms including *Aspergillus* species have been reported to possess the ability to produce raw starch degrading amylase (Oyeleke *et al.*, 2010). The production of protease and amylase from microorganisms isolated from abattoir effluent will certainly help in conserving foreign exchange and in reducing environmental pollution caused by these waste waters. Therefore, the objective of this study is to produce amylase and protease enzymes using *Aspergillus niger* and *Penicillium frequestans* isolated from abattoir wastewater.

MATERIALS AND METHODS

Collection of sample: Abattoir effluent was aseptically collected from gutter at the abattoir in Minna, Niger State, Nigeria and transported to the microbiology laboratory of Federal University of Technology, Minna, Niger State, Nigeria. Ten milliliter of the wastewater was diluted in 90 mL of sterile distilled water, followed by serial dilution. Then, the serial diluent was aseptically inoculated onto different plates of sterile Potato Dextrose Agar (PDA). Sub-culturing was carried out until pure cultures of *A. niger* and *P. frequestans* were obtained. The isolates were characterized based on the colour of aerial and substrate hyphae, type of hyphae, shape and kind of asexual spores, sporangiophore and conidiophores and the characteristics of spore head.

A small portion of the mycelia growth was carefully picked with the aid of a pair of sterile inoculating needles and placed in a drop of lactophenol cotton blue on a microscope slide and covered with a cover slip. The slide was examined under the microscope, first with (x10) and then with (x40) objective lens for morphological examination as described by Cheesbrough (2003) and Oyeleke and Manga (2008). The isolates were identified by comparing their characteristics with those of known taxa using the schemes of Domsch and Gams (1970).

Screening for amylolytic activity: The amylolitic activity of the test isolates was determined by using the starch agar plate method as described by Bertrand *et al.* (2004), by inoculating the test organism individually into Potatoes Dextrose Agar medium which was supplemented with 1 g of starch. The agar plates were then incubated at 30°C for 5 days. After the incubation period, Lugol's iodine solution was added to the culture plate to identify the zones around the cultures. The diameter formed after the addition of iodine solution was measured to represent the amylolytic activity.

Medium for amylase production: The medium was prepared as described by Ali *et al.* (1998) by weighing the following medium composition in grams per litre; KH_2PO-1.4 g, NH_4NO_3-10 g, KCL-0.5 g, $MgSO_4.7H_20$-0.1 g, $FeSO_4.7H_2O$-0.01 g, Substrate-20 g. The medium composition were dissolved in 1000 mL of distilled water after which 100 mL of the medium was measured into conical flask (250 mL capacity each) heated on hot plate to homogenize and then sterilized in an autoclave at 121°C for 15 min after which they were removed and allowed to cool.

Extraction of amylase enzyme: Extraction of the crude enzyme was done by centrifugation of the fermented media at 2000 rpm (revolution per minute) for 5 min, supernatant collected and filtered off using Whatman No.1 filter paper. The filtrate was used as crude enzyme extract (Ali *et al.*, 1998; Oyeleke *et al.*, 2010).

Demonstration of enzyme activity: Amylase activity was assayed as described by Ramakrishna *et al.* (1982) by pipetting 0.5 mL of culture extract enzyme into test tubes and 1 mL of 1% soluble starch in citrate phosphate buffer having a pH of 6.4. The reducing sugars liberated were estimated by the 3, 5-dinitrosalicyclic acid (DNSA) method (Bertrand *et al.*, 2004). The

reaction mixture was incubated in a water bath at 40°C for 30 min. A blank consisting of 1 mL of soluble starch in citrate-phosophate buffer (pH 6.4) was also incubated in a water bath at the same temperature and time with the other test tubes. The reaction was terminated by adding 1 mL of DNSA reagent in each test tube and then immersing the tubes in a boiling water bath for 5 min after which they were allowed to cool and 5 mL of distilled water was added. The absorbance for all the test tubes was measured at 540 nm with spectrophotometer (JENWAY, 6305).

Determination of the reducing sugar: The reducing sugar content, following hydrolysis of starch by the enzyme extract, was determined using the method of Bertrand *et al.* (2004). The DNSA reagent was prepared by adding 1.8 g of 3.5 DNSA to 20 mL of 1.0 N NaOH and 60 mL of distilled water. Potassium sodium Tata rate (60 g) was added and the mixture was diluted to 200 mL with distilled water. The reducing sugar content from hydrolyzed starch by amylase enzyme was assayed by adding 2 mL of 3.5 DNSA reagents to I mL of the sample. The mixture was heated in boiling water for 5 mm and then cooled under running tap water. The absorbance at 540 nm of the resulting coloured solution (slight brown) was read in a spectrophotometer against a blank, prepared by substituting the hydrolyzed sample with distilled water. The reducing sugar content was subsequently determined by making reference to a standard curve of known glucose concentrations.

Effect of pH on amylase activity: Effect of pH was determined by using 0.IM acetate buffer for pH range 3.0-5.0 while 0.1 M phosphate buffer was used for pH range 6.0-8.0 to study the effect of pH on both amylase and protease activities (Bertrand *et al.*, 2004).

Effect of temperature on amylase activity: The optimum temperature was estimated by incubation of reaction mixtures at the various temperatures viz., 30, 40, 50, 60 and 70°C (Ali *et al.*, 1998).

Medium for protease production: The medium used for protease production was composed of (g/l): $CaCl_2$ $7.H_2O$ 0.4, KH_2PO_4 7.0, Na_2HPO_4 2.5, $MgSO_4.7H_2O$ 0.5, $ZnCl_2$ 0.1, NaC1 0.3, Casein 2.0; pH 6.0. Media were autoclaved at 120°C for 20 min.

Extraction of protease enzyme: Cultures were inoculated with 10^7 spores mL^{-1} and incubated in a rotary shaker at 150 rpm for 72 h at 30°C, in 250 mL Erlenmeyer flasks with a working volume of 50 mL. The cultures were centrifuged at 10,000 rpm for 10 mm to remove fungi mycelia and supernatants were used as the enzyme extract as described by Ali *et al.* (1998).

Assay for protease activity: Protease activity was determined as per Shimogaki *et al.* (1991), with slight modification using casein as the substrate. The reaction mixture comprise of crude enzyme extract (1.0 mL) and 1.0 mL of substrate solution (1.0% casein in 0.1 M Sodium phosphate buffer, pH 7.4). The mixture was incubated at 37°C for 60 mm. The reaction was terminated by the addition of 3.0 mL of 5% Trichioroacetic acid, and the mixture was kept at room temperature for 10 min followed by filtration through Whatman filter paper No. 1. The absorbance of the filtrate was measured at 280 nm against the blank. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 mg of tyrosine/mi/min under assay conditions.

Effect of pH and temperatures on the protease activity: The effect of the pH on the activity of protease were determined in the pH range of 3 to 8 whereas optimum temperature was estimated

by incubation of reaction mixtures at the various temperatures viz., 30, 40, 50, 60 and 70°C (Ali *et al*., 1998).

RESULTS

Effect of pH on amylase activity: Figure 1 shows the effect pf pH on amylase activity by *A. niger* and *P. frequestans*. Amylase was active at pH 4.0 to 7.0, with pH 5.0 having the highest activity for both *A. niger* and *P. frequestans* (4.48×10^3 and 1.99×10^3 mg mL^{-1} sec^{-1}, respectively).

Effect of temperature on amylase activity: Figure 2 shows the effect of temperature on amylase activity by *A. niger* and *P. frequestans*. Amylase produced was thermostable, the

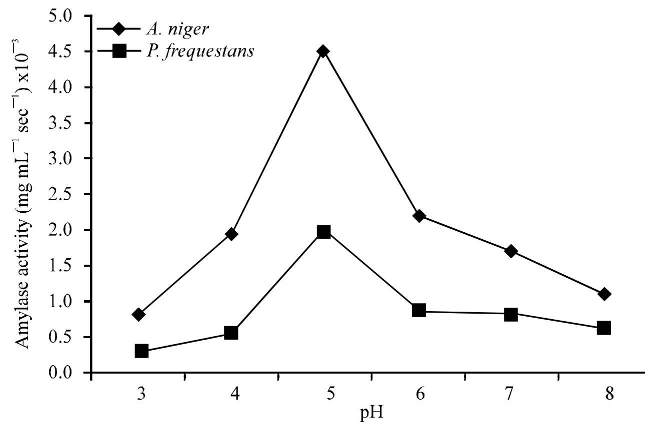

Fig. 1: Effect of pH on amylase activity

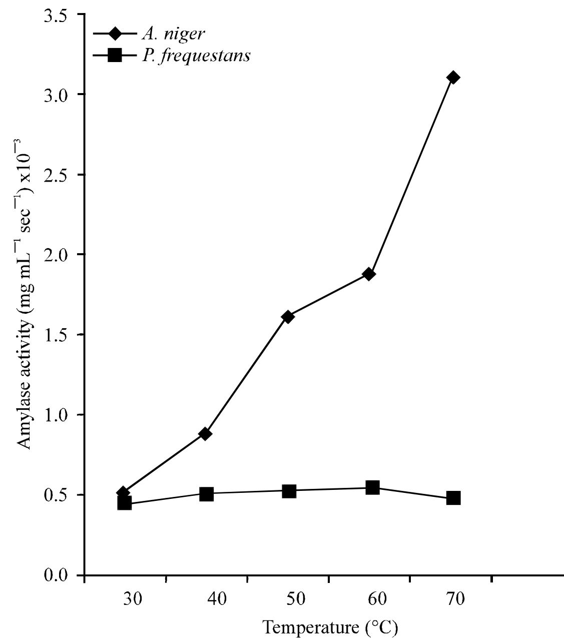

Fig. 2: Effect of temperature on amylase activity

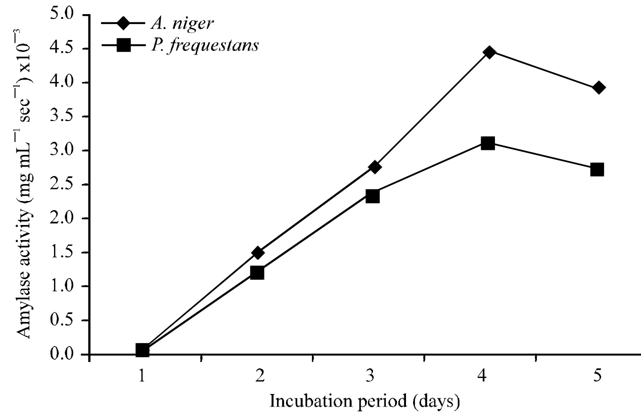

Fig. 3: Effect of incubation period on amylase activity

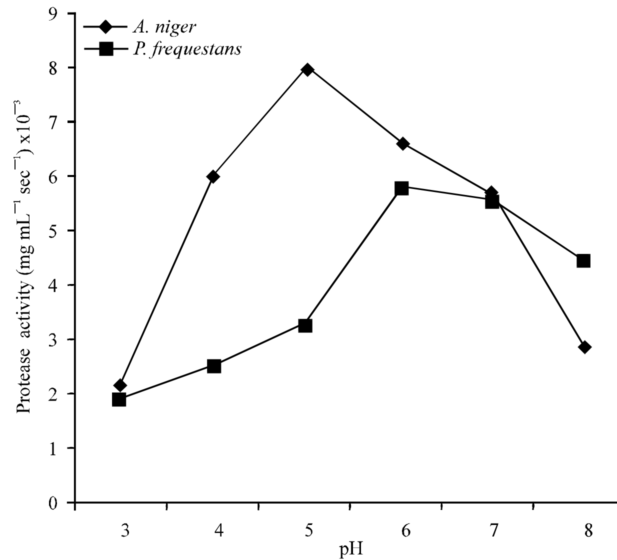

Fig. 4: Effect of pH on protease activity

optimum temperature for amylase produced by *A. niger* was 70°C with amylase activity of 3.11×10^{-3} mg mL^{-1} sec^{-1} and *P. frequestans* with amylase activity of 1.2×10^{-3} mg mL^{-1} sec^{-1} with a decrease at 70°C.

Effect of incubation period on amylase activity: Figure 3 Shows the effect of incubation period on amylase activity by *A. niger* and *P. frequestans*. High yields of amylase by *A. niger* and *P. frequestans* were noticed after 4 days with amylase activities of 4.48×10^{-3} mg mL^{-1} sec^{-1} for *A. niger* and 3.2×10^{-3} mg mL^{-1} sec^{-1}, the production of amylase by both fungi increase with time.

Effect of pH on protease activity: Figure 4 shows the effect of pH on protease activity yield by *A. niger* and *P. frequestans*. pH increased the production of protease also increased until the 3 ptimurn pH for both *A. niger* and *P. frequestans* at 5.0 and 7.0 (with protease activity of 8.16×10^{-4} and 5.84×10^{-4} mg mL^{-1} sec^{-1}, respectively). Enzyme yield then decreased till pH 8.0.

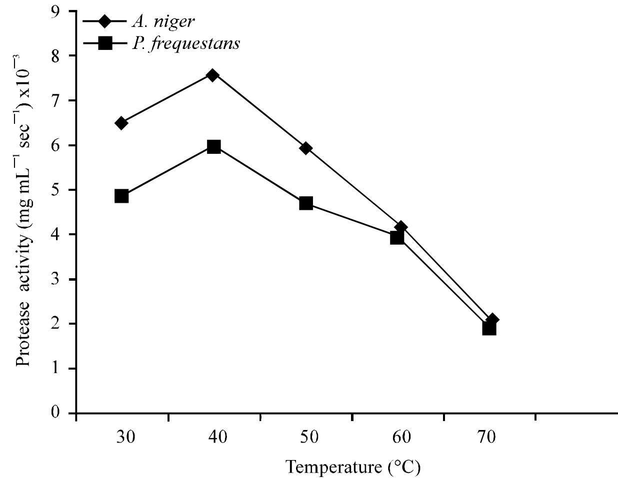

Fig. 5: Effect of temperature on protease activity

Effect of temperature on protease activity: The optimum temperature for proteases produced by both *A. niger* and *P. frequestans* were 40°C (with protease activities of 7.6×10^{-4} mg mL^{-1} sec^{-1} and 6.5×10^{-4} mg mL^{-1} sec^{-1}, respectively) as shown in Fig. 5. Temperature beyond 40°C led to decrease in protease.

DISCUSSION

The production of amylase by *A. niger* and *P. frequestans* isolated from abattoir effluent as summarized in Fig. 1 shows enhanced amylase production by *A. niger* (54%) than *P. frequestans* (46%). Protease production was found to be enhanced by 49% in the *A. niger* culture compared to that of *P. frequestans* (44%) The differences in enzyme yield by both fungi could be due to the difference in genetic make-up as reported by Oyeleke *et al.* (2010). It may also be due to the degree of susceptibility of substrate to chemical modification. Media composition has been reported by Ueda and Saha (1983) to significantly influence enzyme production and activity.

The optimum pH for amylase activity is 5.0 for both *A. niger* and *P. frequesans* whereas, for protease activity, 5.0 and 6.0 were optimum for *A. niger* and *P. frequesans*, respectively. Ali *et al.* (1998) recorded similar optimum pH of 5.0 for fungal amylases production. Okolo *et al.* (2000) also reported similar pH of progression of 4.0 to 7.0 for raw starch digesting enzyme using 0.1M acetate buffer of pH 3.0 to 70 when *A. niger* was grown on native starch. The optimum pH observed with the crude enzyme suggests the presence of amylolytic and proteolytic activities in the preparation.

The temperature study on amylase activity showed gradual increase in amylase from 30 to 70°C for *A. niger* while for *P. frequestans*, there was an increase from 30 to 60°C and a gradual decrease at 70°C. This implied that the enzymes are thermostable as reported by Goyal *et al.* (2005). A similar study was carried out for protease activity which revealed that the optimum protease activity occurred at 40°C, lower enzyme activities observed might be due to enzyme inactivation or thermal denaturation of enzyme protein. This is variance with the report of Oyeleke *et al.* (2010) who reported an optimum temperature of 30°C for protease activity when screening for *A. flavus* and *A. fumigatus* strains. Oyeleke *et al.* (2010) also reported that increase in temperature led to a decrease in protease activity.

The result shows that amylase and protease production by *A. niger* was enhanced an greater than *P. frequestans* by 49 and 45%, respectively. It was observed that the pH range 4.0-6.0 favours highest amylase activity and protease activity. Furthermore, temperature studies over the range of 30 to 70°C indicates that amylase activity has optimum activity at 70°C, on the other hand, the result reveals that protease activity was optimum at 40°C. However, industrial production of amylase and protease could be encouraged using solid state technique.

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