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Production and Characterization of Extracellular Protease Enzyme from Aspergillus Niger Using Different Agro-Industrial Residues

Anas Muazu
Department of Biomathematics, National Mathematical Center, P. M. B. 118 Abuja, Nigeria

ABSTRACT

Protease enzymes have many applications and are used in many industries including textile, detergent and pharmaceuticals. This work was aimed to produced extracellular protease enzyme from *Aspergillus niger* using different agro-industrial wastes, determine the effect of incubation periods, substrate concentration, pH and temperature on the enzyme activity. *Aspergillus niger* was isolated from local soil and used to produced extracellular protease enzyme under solid state fermentation conditions using wheat bran, rice husk, maize chaff and potato peel. Wheat bran proved to be the best substrate for protease production with highest yield of enzyme (0.94µg/ml) while potato peel had the least yield (0.46µg/ml). Fermentation carried at 96 hours produced the highest enzyme yield (0.95µg/ml) while least enzyme production (0.32µg/ml) was obtained at 144 hours. High concentration of casein (16 mg/ml) as substrate resulted in highest protease activity of 0.44µg/ml. The optimum pH for protease activity was 9 with maximum activity of 0.66µg/ml while the optimum temperature for protease activity was 45 ^{0}C with maximum activity of 0.86µg/ml. The highest protease activity obtained at high pH and temperature showed that the enzyme produced by the *Aspergillus niger* is an alkaline protease and thermostable.

Keywords: *Aspergillus Niger*, Protease, Casein, Solid State Fermentation

1. INTRODUCTION

Protease is an enzyme that breaks the peptide bonds of protein (Mitchel e*t al.,* 2007) to produce amino acids and other simpler peptides. It can be obtained from a variety of sources such as plants, animals and microbia (fungi and bacteria). Proteases are among the three largest groups of industrial enzymes accounting for about 60% of the total worldwide sale of enzymes from biological sources (Adinarayana *et al*., 2003). Although, proteases are found ubiquitously in plants, animals and microorganisms, the microorganisms are the preferred sources of proteases and dominated the commercial application (Nisha, and Divakaran 2014). 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 (Burhan *et al.,* 2003).

Microorganisms vary greatly from one another in their ability to produce enzymes. Commercial microbial enzyme production utilizes mainly various fungi, bacteria and yeasts (Geethanjali and Reshma 2014). Fungal enzymes are gaining importance in agriculture and industry as they are often stable at high temperature and extreme pH than the enzymes derived from plants and animals (Waiter and Morgan 2007). Protease is the single class of protein degrading enzymes, which occupy pivotal position with respect to their applications in both physiological and commercial fields (Geethanjali and Reshma 2014) . Protease represent one of the largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation process (Walsh, 2002).

Agro-industrial residues are generally considered the best substrate for the solid state fermentation (SSF) processes because they are usually simpler and can use wastes such as defatted soybean cake, gram bran, wheat bran, rice bran, banana waste, etc. for enzyme production (Germano *et al.,* 2003; Kashyap *et al.,* 2003).

This present work aims to exploit the locally available, inexpensive agro-substrates, wheat bran, rice husk, maiz chaff and potatoe peel for protease enzyme production under solid state fermentation conditions, determine the effect of culture conditions, substrate concentrations, pH and temperature on protease production.

2. MATERIALS AND METHODS

2.1. Preparation of Agro industrial waste (Substrates)

The Agricultural wastes (Wheat bran, Potato peel, rice husk and maize chaff) were dried at 50 ^{0}C and pounded to smaller particles. Five grams of each substrate was transferred to 50 ml of the basal medium in a conical flask and the whole content was sterilized by autoclaving at 121 ^{0}C for 15 minutes and was allowed to cool to room temperature.

2.2. Screening of Protease producing fungi

Soil sample was collected at 10 - 20 cm depth of soil using a hand trowel and transported to the laboratory in a clean polythene bag. The preparation of inoculum was carried out using the spread plate method described by Aneja (2003) with slight modification. Samples were serially diluted up to 10^{-6} dilutions and inoculated on Potatoe Dextrose Agar (PDA) to isolate protease producing fungi.

2.3. Identification and Maintenance of Isolates

Macroscopic and microscopic identification of fungal isolates was carried out based on morphological appearances such as color, texture, spores and hyphae using lactophenol cotton blue stain and with the aid of mycological atlas. Isolated *Apergillus niger* were stored on PDA slants at 40c for further use.

2.4. Preparation of Inoculum

The inoculum was prepared by dispersing the spores from a week-old fungal slant culture in 10 ml of distilled water with a sterile inoculation loop.

2.5. Solid State Fermentation

Five grams of each of the substrate was separately poured into 250 ml of Erlenmeyer flask, moisten with salt solution containing g/l KH_2PO_4 1.4, NH_4NO_3 10, KCl 0.5, $MgSO_4.7H_2O$ 0.1, $FeSO_4.7H_2O$ 0.1, soluble starch 20, sterilized at 121.5°C for 15 min, cooled, inoculated with 1 ml of fungal spore suspension (10^6 spores/ml), incubated at 30 ^{0}C for 144 hours on a rotary shaker at a speed of 150 rpm (Devi et al., 2008).

2.6. Extraction of Crude Enzyme

After 144 hours of incubation, each of the fermented substrate containing the growing fungus was centrifuged at 8000 x g 5 °C for 10 min and the supernatant obtained was used as crude enzyme for protease assy.

2.7. Enzyme Assay

Protease enzyme assay was conducted using the method of Kocher et al., (2003). 1 ml of crude enzyme was added into a 15 ml test tube containing 1 ml of casein solution (1%, w/v casein solution prepared in 50 mM glycine–NaOH buffer, pH 7.5) and incubated in a water bath at 50 ^{0}C for 30 min. After 30 min, the reaction was stopped by adding 3 ml of 10% trichloroacetic acid and allowed to act for 1 hour. After 1 hour, 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 by spectrophotometer at 540 nm. Protease activity was calculated using tyrosine standard curve. One unit protease activity was defined as 1 microgram (µg) of tyrosine liberated per mill (ml^{-1}) under the assay conditions.

2.8. Effect of Different Agro-industrial Residues on Protease Production

One (1) ml of the crude enzyme form the different agro-industrial substrate was inoculated into 50 ml test tube containing 1 ml of casein solution prepared in 50 mM glycine–NaOH buffer, pH 7.5) and incubated in a water bath at 30 ^{0}C for 30 min. The absorbance for the enzyme activity was read by spectrophotometer at 540 nm and the values obtained were recorded. Wheat bran which secreted highest protease activity was selected for further protease assays.

2.9. Effect of Incubation Period on Protease Production

The effect of incubation period on protease was determined by incubating 1 ml of the crude enzyme in the production medium at different incubation periods ranging from 24, 48, 72, 96, 120 to 144 hours.

2.10. Effect of Casein as Substrate on Protease activity

To determine the effect of substrate concentration on protease activity, 1ml of the crude enzyme was added into 50 ml test tubes containing various concentrations of casein ranging from 4, 8, 12, 16, 20, to 24 mg/ml and incubated at 30 ^{0}C for 24 hours.

2.11. Effect of pH on protease activity

The effect of pH on protease enzyme activity was studied by incubating 1 ml of the crude enzyme in 50 ml test tubes containing 1 ml of casein at various pH concentrations starting from 6, 7, 8, 9, and 10 to 11. The reaction mixture was incubated at 30 ^{0}C for 24 hours.

2.12. Effect of Temperature on protease activity

The effect of temperature on protease enzyme activity was studied by incubating 1 ml of the crude enzyme in 50 ml test tubes containing 1 ml of casein at various temperatures ranging from 30, 35, 40, 45, and 50 to 55. The reaction mixture was incubated for 24 hours. All enzyme activity assays were done spectrophotometrically at 450 nm.

3. RESULTS AND DISCUSSION

3.1. Effects of Agro-industrial Residues

Protease enzyme produced by *Aspergillus niger* form the different agricultural residues presented various result. Maximum enzyme activity of 0.94 µg/ml was obtained following fermentation with wheat bran while lowest enzyme activity of 0.46 µg/ml was obtained after fermentation with potato peel (Figure1). Rice husk and maize chaff have moderate activity having 0.71 and 0.58 µg/ml respectively. The highest enzyme activity obtained in this study was in line with the study of Nisha and Divakaran (2014) who reported a highest protease activity of 490.12 U/ml on wheat bran by *Bacillus subtilis*. However, the high enzyme activity by *A. niger* on wheat bran obtained in this work varied with the work of Geethanjali and Reshma (2014) who reported less enzyme activity of 1.1 U/ml by *A. niger* and highest enzyme activity of 2.01 U/ml by *Rhizopus* spp using wheat bran.

3.2. Effect of Incubation Time

The effect of different incubation time on protease production by *A. niger* was studied. Protease production was found to be increasing following the first 24 hours of incubation up to 96 hours where highest activity of 0.95 µg/ml was obtained (Figure 2). Beyond 96 hours, protease production started decreasing resulting in lowest activity of 0.32 µg/ml after 144

hours. This can be attributed to the growth phase of the fungus probably reaching their decline phase leading to decrease in secretion of the extracellular enzyme. The high protease production obtained at 96 hours in this work was in agreement with the work of Nisha and Divakaran (2014) who found that Rhizopus spp secreted highest protease production at 4th day of incubation (96 hours) and also found that protease production decreases beyond 4th day of incubation. A work by Oyeleke et al., (2010) also reported increase in protease activity by *A.niger* from 3rd day to 5th day (120 hours) of incubation. However, the increase in protease activity with increase incubation time by *A. niger* obtained in his work was on the contrary to the work of Devi et al., (2008) who reported highest enzyme production of 100 U/ml following 30 hours of incubation and a gradual decrease in enzyme production after 72 hours of incubation. A gradual decrease in enzyme units with increasing incubation periods clearly suggests the enzyme's role as a primary metabolite, being produced in the log phase of the growth of the fungus for utilization of nutrients (proteins) present in the solid substrate (Sumantha et al., 2006).

3.3. Effect of Casein as Substrate

The effect of various concentrations of casein as substrate was studied and different protease activities were obtained. Highest protease activity of 0.44 µg/ml was obtained at a concentration of 16 mg/ml (Figure 3). Increase in protease activity was found to seize beyond the concentration 16 mg/ml, remained relatively constant and later decrease to 0.39 mg/ml at a concentration of 24 mg/ml (figure 3). The increase in enzyme activity with increase in casein concentration in this work was in agreement with the work of Sumantha et al., (2006), who found that protease activity by *Rhizopus microspores* increase when the substrate concentration was increased from 5 to 10 mg/ml and remained constant all through form 10 to 25 mg/ml.

3.4. Effect of pH on Protease activity

The effect of pH on protease activity was determined by incubating the reaction mixture containing 1 ml of the crude enzyme for 24 hours at different pH values ranging from 6 to 11. Protease activity was found to be increasing with increase in pH values to certain level. Highest protease activity of 0.66 µg/ml was obtained at pH value of 9. The activity of the enzyme diminished beyound pH value of 9 resulting in low enzyme activity of 0.35 µg/ml at pH value of 11 (Figure 4). On the contrary to this work, a study by Bankole et al., (2014) reported lowest protease activity of 0.15 µg/ml at pH value of 9 by *A. niger*, with highest protease activity of 0.50 at pH value of 7. However, work by Devi et al., (2008) reported optimum protease activity of 8.5 U/ml at optimum pH of 8.5 which is close to pH value of 9 employed in this work. Nisha and Divakaran (2014) reported optimum protease activity of 397 U/ml at pH value of 9 by Bacilus subtilis.

3.5. Effect of Temperature on Protease activity

Protease activity was found to be increasing with initial temperature until an optimum temperature was reached where protease activity started declining. The optimum temperature for protease activity was found to be 45 ^{0}C with optimum activity of 0.86 µg/ml while the lowest protease activity was recorded at 30 ^{0}C (figure 5). The optimum protease activity at 45 ^{0}C obtained in this work was in accordance with the work of Devi et al., (2008) who reported optimum protease activity of 85 U/ml at 45 ^{0}C from *A. niger*. The lowest protease activity obtained in this work at 30 ^{0}C was however in contrast to the work of Geethanjali and Resham (2014) who reported highest protease activity of 1.90 U/ml at 30 ^{0}C by Rhizopus species but also reported high protease activity by Aspergillus species at 40- 45 ^{0}C. Although protease activity in this work seized to increase beyound 45 ^{0}C (50 and 55 ^{0}C), the activity of the enzyme was still higher than the initial temperatures. A review by Daniel et al., (2010) 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. Temperature is a critical factor for maximum enzyme activity and it is a prerequisite for industrial enzymes to be active and stable at higher temperature.

4. CONCLUSION

From the results of this study it can be concluded that agricultural wastes can be good substrates for production of commercially industrial protease enzymes under solid state fermentation chief among them being wheat bran. The assays for protease activity in this work have shown that high incubation time (96 hours), amount of casein (16 mg/ml) resulted in high protease production. The optimum pH of 9 and temperature of 45 ^{0}C have proved best the enzyme activity and that showed that the protease enzyme produced by *A. niger* in this work is an alkaline and thermostable.

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Figures

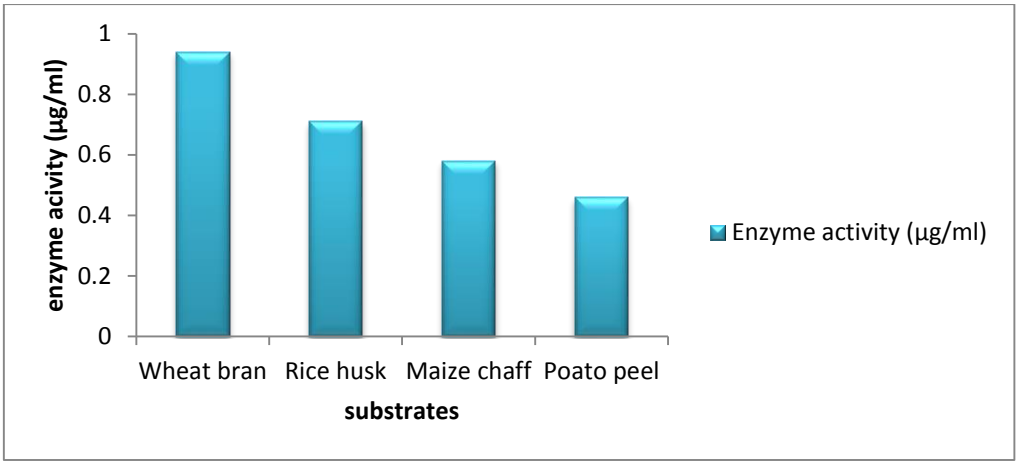

Figure 1: Effect of Different Agro-industrial Residues on Protease Production

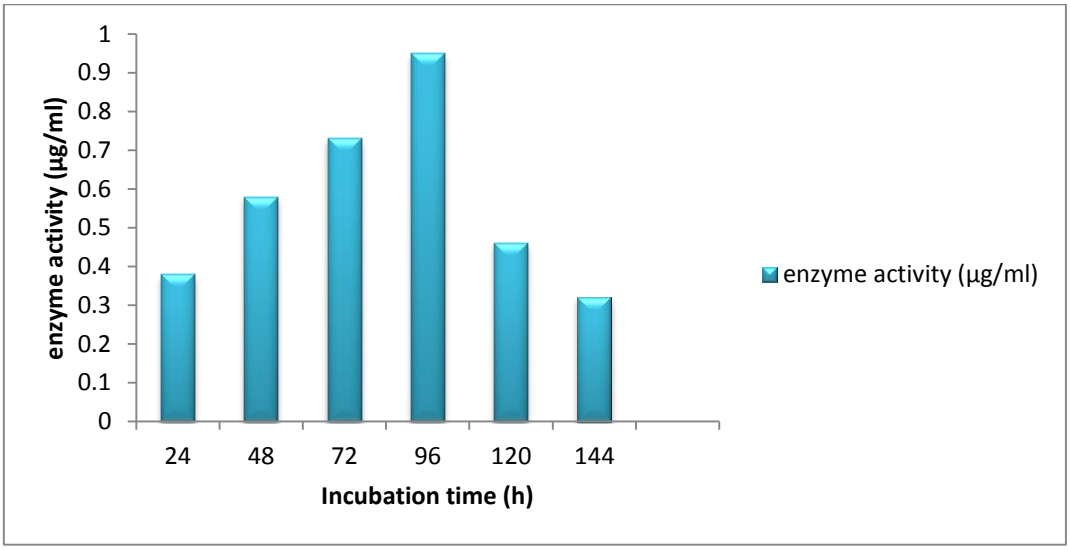

Figure 2: Effect of Incubation Time on Protease Production

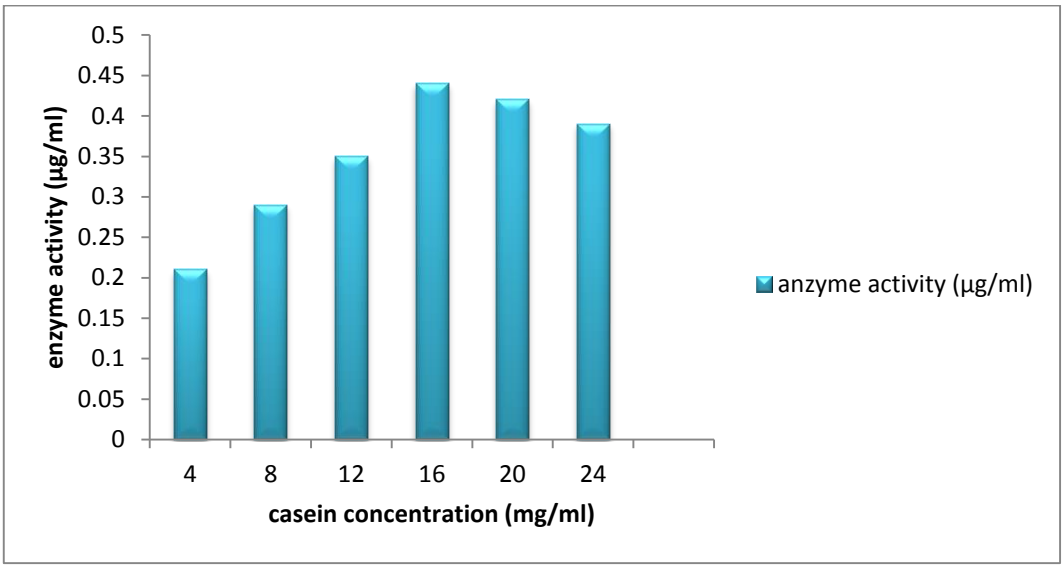

Figure 3: Effect of Different Casein Concentrations (as substrate) on Protease activity

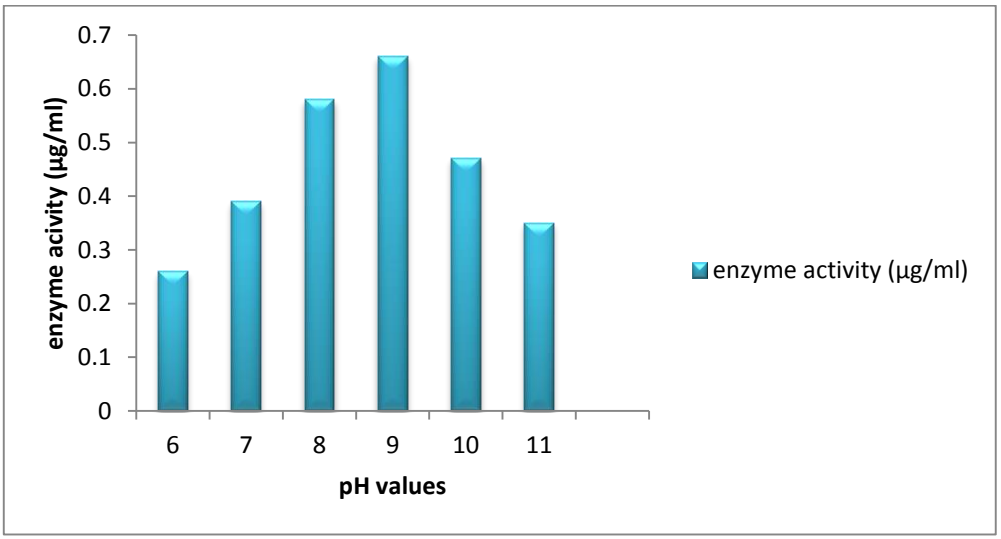

Figure 4: Effect of Different pH Values on Protease activity

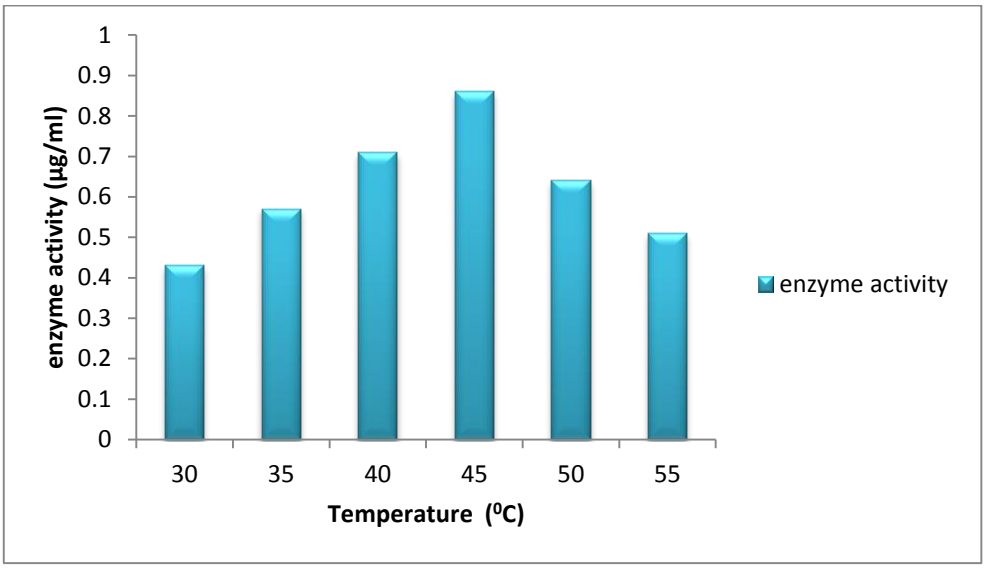

Figure 5: Effect of Different Temperatures on Protease activity

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