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Optimization of Protease Production Using Agro Wastes by *Aspergillus spp.* In A Solid-State Fermentation

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ABSTRACT

Agricultural wastes are highly nutritious and facilitate microbial growth and metabolism. Most agro-wastes are lignocellulosic in nature, composed of lignin, hemicellulose and cellulose. Agricultural residues can be used for the production of various value-added products, such as industrially important enzymes. Extracellular proteases have high commercial value and multiple applications in detergents, food, dairy, pharmaceutical, leather, diagnostics, waste management and several recovery industries. Aspergillus oryzae was isolated from rice farmland, Rigasa, Kaduna. It was screened for protease production using skimmed milk agar assay with a wider clear zone of casein hydrolysis (halo zone). The main objectives of this study were to isolate, characterized, and optimize the production conditions by Aspergillus oryzae under solid state fermentation (SSF) using locally available agricultural wastes used as substrate. The results showed that the optimum conditions for maximum protease production are 84 hours of incubation; pH 6.0; temperature of 35°C; 3% inoculum size; peptone as Nitrogen source and rice husk as the best fermentation substrate. The highest protease activity of rice husk was 1350 Ug−1 of dry solids compared with an activity of corn cob 1005 Ug−1 respectively. Protease was produced from Aspergillus oryzae with broad substrate specificity, high pH and temperature stability.

Keywords: agro-waste; protease; characterization; *Aspergillus*

INTRODUCTION

The biological catalysts known as enzymes enable chemical reactions to take place in living things under normal environmental conditions. Proteases, also known as proteolytic enzymes, are among the families of enzymes that have undergone substantial research due to their vast range of applications in numerous sectors (Mohammed, 2015; Adebola, *et. al.,* 2018).

Although proteases are extensively present in both plants and animals, commercial proteases are only ever made from microorganisms. As a result of several species in these genera being generally regarded as safe, moulds of the genera *Aspergillus, Penicillium*, and *Rhizopus* are particularly helpful for manufacturing proteases (Ahmed *et al*., 2010). Due to their industrial applications and cost-efficient biotechnological procedures, microbial enzymes have drawn a lot of attention. According to Joo *et al*. (2002), a protease is an enzyme that carries out proteolysis, or protein catabolism, by hydrolyzing the peptide bonds that bind the amino acids together in the polypeptide chain that makes up the protein. Different peptide bonds can be hydrolyzed by different proteases. As a result, the specificity linked to each enzyme varies depending on the catalytic site (Mohammed, 2015; Ibrahim and Abdullahi, 2016; Ibrahim, *et. al.,* 2017).

According to Rathakrishnan and Nagarajan (2011), proteases are primarily categorized as acidic, neutral, and alkaline proteases based on how well they function in different pH ranges. Proteases break down long chains of protein into smaller pieces, break the peptide bonds connecting amino acids by adding water molecules across the peptide bonds, and catalyze the production of peptides in organic solvents with less water.

Proteases are spherical, relatively small molecules that hydrolyze the breaking of peptide bonds in proteins (Tanzadehpanah *et al*., 2012). The Extracellular (exoenzyme) has applications in the detergent, food, dairy, pharmaceutical, leather, diagnostic, and waste management sectors of the modern chemical and biochemical industries. Proteases are one of the most significant classes of industrial enzymes, accounting for more than 65% of all industrial applications, including the preparation of leather, the tenderization of meat, the synthesis of peptides, the food industry, the de-hairing process, and the pharmaceutical industry (Kirk *et al.*, 2002; Ibrahim, 2020a; Ibrahim, 2020b).

Proteases are thought to account for 40% of all enzyme sales globally (Castro and Sato, 2014). Due to their high production, wide biochemical variety, susceptibility to genetic modification, and ease of recovery from the fermentation medium, fungi are prospective sources of proteases (Vishwanatha *et al*., 2009; Ibrahim, *et. al.,* 2022a; Ibrahim, *et. al.,* 2022b; Ibrahim, *et. al.,* 2022c).

The U.S. Food and Drug Administration (FDA) classifies the filamentous fungus *Aspergillus oryzae* as a "Generally Recognised as Safe (GRAS)" organism, due to its high proteolytic activity, it has a long history of usage in the food industry for the manufacturing of conventional fermented foods (Matsushita-Morita *et al*., 2011). The molecular biology of this organism, according to Machida *et al.* (2015), reveals that it has the biggest expansion of hydrolytic genes (135 proteinase genes). This study focuses on the production of proteases by *Aspergillus oryzae* using rice husk and maize cob in solid-state fermentation.

Materials and Methods

Sample site

The research was conducted in the Microbiology Laboratory, Applied Biology Department, Kaduna Polytechnic and substrates were obtained from rice and corn processing company U/ Mu'azu, Kaduna.

Sample Collection

Soil sample was collected from Zango, Kabala West and Kurmin mashi abbatoir, kaduna state.

According to the method of Oyeleke *et al.,* (2011), soil samples were collected from the surface of the rice field soil and at a depth of about 15cm with the aid of a sterilized hand trowel. About 100g each of the top soil from 2 different places was taken. The hand trowel was applied perpendicular to the vertical surface of the profile, where all soil samples are collected in a sterile polythene bag and labeled accordingly. Exactly 50g each of the substrates was collected in sterile polythene and transported to Microbiology laboratory, Applied Biology Department, Kaduna Polytechnic for the isolation of microorganisms, processing and storage of samples for analysis.

Media Preparation

All other chemicals to be used in the present study were of analytical grade purchased from chemical suppliers, and prepared by the manufacturers guide. The wild strain of *Aspergillus* was isolated from soil, and grown on media slants at 37^{0}c for 5 days. (Oyeleke *et al.,* 2011; Ibrahim, 2019).

Pure culture of the isolates was obtained by culturing and sub-culturing of isolates on Potato dextrose agar (PDA) and maintained on PDA slant. The isolate was inoculated on appropriate media to differentiate species of *Aspergillus.*

Proximate Analysis of Samples

Chemical composition of samples collected was determined as the method described by the Association of Official Analytical Chemists (AOAC), 2004 for Moisture, Ash, Protein, Fat, Crude fibre and Total carbohydrate content of the waste samples (Aurand *et al., 1987;* Ibrahim*, et. al.,* 2022).

Inoculum Preparation

Spores were harvested by flooding the plates with sterile distilled water containing 0.05% Tween 80 as a wetting agent, after which the spores were scrapped from the surface of the colonies with a sterile spatula. The resulting suspension was shaken in a 100ml Erlenmeyer flask to break up the spores' chain. The concentration of spores was determined using a hemocytometer and the suspension was further diluted in a sterile Tween 80 solution to achieve the desired concentration (10^6 spores per ml).

Solid-State Fermentation

Fermentations were initiated from spores in 250mL Erlenmeyer flasks that contained 20g of rice husk as well as corn cob supplemented with 2g of wheat flour which serves as inducer. Effects of various combinations of rice husk and corn cob on fermentations were investigated.

Aspergillus flavus and *parasiticus* Agar (AFPA)

Exactly 22.75 g of dehydrated medium was suspended in 500 ml of distilled water, and heated to a boil with frequent agitation to dissolve the medium completely. Sterilized by autoclaving at 121°C for 15 minutes and cooled around 50°C. The freshly prepared medium was dispensed aseptically into sterile plates and stored at a temperature below 8°C.

Substrate

In this study, two agro-wastes (Rice husk and corn cob) were used as substrates/media for carrying out the Solid-state fermentation, which was collected in dried form and ground (Muthulakshmi *et al.,* 2011; Ibrahim and Abdullahi, 2019).

Isolation of Fungi from Soil

The serial dilution technique was employed for the isolation of fungi from the soil (Kutateladze *et al.,* 2016). About twenty-five grams of the soil samples were mixed with a solution containing 225 ml sterile water and 5.80 g peptone water (Choudhary and Jain, 2012). Five screw cap test tubes with 9ml distilled water were autoclaved and arranged in a test tube rack for further processing, and serial dilutions were made in 10^{-1}, 10^{-2}, 10^{-3}, $10^{-4,}$ and 10^{-5} dilutions respectively. The third and fifth dilution was used to obtain fungal colonies. The plates were incubated at room temperature for 5-7 days (Choudhary and Jain, 2012). The isolated culture of *Aspergillus oryzae* was purified by routine sub-culturing for a period of 5-7 days and stored at 4^{o}C for further use (Muthulakshmi *et al.,* 2011).

Differentiation on AFPA Selective Medium

The isolates were inoculated on AFPA selective/differential medium and incubated at 30°C for 48-72 hours to differentiate *A. flavus* and *A. parasiticus* based on reverse color. All the plates were incubated at 30°C with intermittent observation for every two days for 1 week to observe changes in the reverse color.

Identification of Isolated Fungi

The isolated fungus was subsequently identified using its morphological traits. As the mounting fluid, lactophenol cotton blue stain was employed. A little amount of fungal mycelium was extracted from the colony's edge using a sterilised needle and a drop of lactophenol solution. The fungal mycelium was spread onto a slide by using a second needle in order to tease out the fungal structures (Maitig *et al.,* 2018). The slides were observed under the microscope using low power objectives, the following morphological characteristics evaluated the colony growth (length, width, presence or absence of aerial mycelium, color, presence of wrinkles and furrows, and pigment production). The characteristics were compared with the standard description in Bergey's manual of determinative bacteriology (Holt *et al.,* 1994; Muhammad, *et. al.,* 2023).

Proximate Analysis of Samples

Chemical composition of the samples collected was determined as the method described by the Association of Official Analytical Chemist (AOAC), 2004 for moisture, ash, protein, fat, crude fibre, and total carbohydrate contents (Zubair *et al*., 2015).

Determination of Moisture Content

Rice husk and corn cob contain low amount of moisture. Moisture content of selected samples was determined according to the AOAC (2004) method. Moisture content is the amount of moisture present in a food product. This test is based on LOD (Loss on drying) at an oven temperature of 105^{o}C. Besides water, other volatile substances were lost at 105^{o}C.

A dried, cooled platinum dish was heated at 100 °C for 1 hour; the weight was measured and recorded as (W_1). 3g of the test sample was introduced into the dish and weighed accurately (W_2). The dish and its content were transferred into an oven at 105^{o}C to dry for about 3 hours and the dish was weighed and recorded as(W_3).

The test was performed repeatedly until two consecutive constant weights were attained (Zubair *et al*., 2015). The moisture content of the rice husk and corn cob was calculated using the formula below:

$$\% \text{ Moisture} = \frac{(W_2 - W_3)}{(W_2 - W_1)} \times 100$$

Where, W_1 = Weight of dish
W_2 = Weight of dish and sample
W_3 = Weight of dish and sample after drying in the oven.

Determination of Ash Content
Ash content of selected samples was determined according to the AOAC (2004) method. The organic component of the food was burnt off in the air; the residual ash consists of inorganic components in the form of oxides.

Weight of the clean, dry, and empty crucible was measured using an electrical balance, 3 grams of the sample was transferred into the crucible and the crucible with the samples was heated in a hot air oven at a temperature of 105 °C for 24 hours. After drying, the crucible was transferred into the muffle furnace and ignited at 600 °C for 5 hours, removed and cooled in a desiccator and the weight of the crucible with ash was measured (Zubair *et al*., 2015).

Ash content of the samples was calculated using the formula below:

$$\text{Ash content (g\%)} = \frac{A - B}{W} \times 100$$

Where, A= Final weight of the crucible and sample
B= Weight of empty crucible
W= Weight of sample (Zubair *et al*., 2015).

Determination of Fat Content
This was achieved according to the method described by Werner Schmid, which works based on the principle of heating with concentrated HCl which dissolves the fat and other materials. The fat was extracted with a suitable solvent (diethyl ether), and 2g of the sample was weighed into a boiling tube. Exactly 10ml of concentrated HCl was added into a boiling water bath until solid particles dissolved and the mixture turned brown. It was then cooled and transferred into a separating funnel. 10ml of ethanol and 30ml of diethyl ether were added and shaken to dissolve; it was then allowed to stand for some minutes to separate.

A clean dried conical flask (W_1) was weighed and the ether layer was transferred into the flask. The extraction was repeated twice with 25ml of diethyl ether and the extract was evaporated in a water bath. The fat was dried in an oven at 105^0C, cooled and recorded as (W_2).
The percentage of crude fat of the samples was calculated by the equation below:

$$\% \text{ Fat} = \frac{(W_2 - W_1)}{(W)} \times 100$$

Where, W = Weight of sample
W2 = Weight of conical flask
W3 = Weight of sample and conical flask
(Zubair *et al*., 2015).

Determination of Crude Fibre Content
Crude fibre content was determined according to the method described by AOAC (2004).

The percentage of crude fibre content obtained from the experiment was calculated using the formula below:

$$\% \text{ Crude fibre} = \frac{\text{Loss of weight}}{\text{Weight of sample}} \times 100$$

Determination of Protein Content
Protein content was determined according to the method described by AOAC (2004). Kjeldahl nitrogen method is one of the most widely recognized methods employed for crude protein determination. This involves three (3) stages: The digestion, distillation, and titration processes (Zubair *et al*., 2015).

Digestion
1g of the sample was weighed and transferred into a digestion flask. A spatula full of $CuSO_4$ salt and 25ml of concentrated H_2SO_4 solution were added. A significant amount of anti-bump was added and the digestion flask was connected to a glass tube (with a condenser neck-off) whose joint was rubbed with Vaseline. The whole digestion set-up was connected to the lower chamber of the Kjeldahl apparatus and the heat knob was on. The sample was heated until a clear solution was obtained (Zubair *et al*., 2015; Ibrahim and Abdulkadir, 2019).

Distillation
After complete digestion, 200ml of distilled water and 85ml of 50% NaOH solution were added to the digest respectively. The measuring cylinder used to measure the NaOH was rinsed with 50ml distilled water and the content was transferred to the digestion flask. Anti-bump was added and the distillation set-up was connected to the upper chamber of the apparatus. 50 ml of 2% H_3BO_3 was measured and transferred into a receiving flask. 3 drops of screened methyl red indicator were added. The receiving flask was placed in the middle chamber of the apparatus, and the delivery tube was immersed into the pinkish solution in the receiving flask. The heat knob of the upper chamber was turned on for distillation to begin, and about 200ml of the resulting bluish solution was collected for titration (Zubair *et al*., 2015).

Titration
After complete distillation, the bluish solution was titrated with 0.05M H_2SO_4 solution until a permanent pink color was developed, which indicated the endpoint (Zubair *et al*., 2015).

The percentage of protein content was determined using the formulae below:

$$\% \text{ Nitrogen (N)} = \frac{\text{TV} \times 0.0014}{\text{W}} \times 100$$

Where, TV = Titre value
W = Weight of sample taken.

$$\% \text{ Protein (P)} = \%N \times F$$

Where, F = Jones protein factor (5.70).

Determination of Total Carbohydrate Content
Total carbohydrate content of samples was calculated by subtracting the value of moisture, ash, protein, fibre, and fat from 100 as shown in the formula below:
% Carbohydrate = 100 – (% Moisture + % Ash + % Protein + % Crude fibre + % Fat).

Energy (kcal)
Energy of the samples was calculated using the formula below:
Energy (Kcal) = (total carbohydrate×4) + (protein×4) + (fat×9)

Inoculum Preparation
Spores were harvested by flooding the plates with sterile distilled water containing 0.05% Tween 80 as a wetting agent, after which the spores were scrapped from the surface of the colonies with a sterile spatula. The resulting suspension was shaken in a 100ml Erlenmeyer flask to break up the spores' chain. The concentration of spores was determined using a hemocytometer and the suspension is further diluted in a sterile Tween 80 solution to achieve the desired concentration (10^6spores per ml).

Solid-State Fermentation
Fermentations were initiated from spores in 250mL Erlenmeyer flasks that contained either 20 g of rice husk or corn cob supplemented with 2 g of wheat flour as inducer. The effects of various combinations of rice husk and corn cob on fermentations were investigated. The media was moistened to 50% (g per 100 g) moisture with tap water, autoclaved at 121 °C for 25 minutes, and cooled to room temperature before inoculation with 1 ml of fungal spore suspension (10^6 spores/ml). The flasks were incubated at 30 °C for 120 hours. A flask was harvested every 12 hours for various measurements. The contents of the harvested flasks were thoroughly mixed with a glass rod before sampling for various analyses (Chutmanop*et al.,* 2008).

Protease Assay
The moisture content of the freshly harvested fermented solid samples was determined in accordance with the AOAC (2004)method. The sample is dried at 103°C for 2 hours, cooled in a desiccator, and reweighted and the pH of the fermented solid was measured. The samples is then dried at 105 °C for 4 hours and cooled in a desiccator. 1 g sample of the dried solids was ground and soaked in 10mL of distilled water for 1 hour and the pH of the supernatant was measured (Chutmanop *et al*., 2008; Ibrahim and Adamu, 2020).

Determination of Enzyme Activity
To measure enzyme activity, 10mL of distilled water was added to a 1 g sample of fermented solids. The resulting slurry was agitated on a rotary shaker at 180 rpm for 30 minutes. The slurry was then centrifuged at 10000 × *g* for 10minutesheld at 4 °C. The supernatant was recovered and held at 4 °C for analysis. The storage period should not exceed 5 days.

Protease activity was determined as the method described by Agrawal *et al*., (2005). Exactly 1mL of the supernatant was mixed with 5mL solution of 2% (g per 100 mL) casein dissolved in 0.5mol L^{-1} carbonate buffer held at pH 10. The resulting solution was incubated at 40 °C on a rotary shaker at 300 rpm for 30 minutes. About 0.5 mL of the reaction mixture was taken and the reaction is quenched by adding 1.5mL of 10%pre-chilled trichloroacetic acid (TCA). The reaction tube was immersed in an ice bath for 5 minutes to completely precipitate the protein. The supernatant was recovered by centrifugation at 10000 × *g* for 10min; specific enzyme activity was expressed as units/mg of protein. Tyrosine liberated during casein hydrolysis was measured in the supernatant. (Chutmanop *et al*., 2008).

Calculation of enzyme activity
A unit of protease activity was defined as the amount of enzyme liberating 1 µg of tyrosine min^{-1} under standard assay conditions (Chutmanop *et al*., 2008).

The tyrosine concentration was calculated using the regression equation obtained from the standard curve of tyrosine.

$$\text{Tyrosine concentration } (\mu g/ml) = \frac{\text{Test} - \text{Control} + 0.285}{0.00926}$$

Enzyme activity (U/ml)
$$= \frac{\mu g \text{ of tyrosine} \times \text{dilution factor} \times \text{total volume}}{}$$

Or Reaction time

$$\text{Enzyme activity (U/g)} = \frac{A \times V1 \times V2 \times \eta}{m \times t}$$

Where;
A = Reading from tyrosine standard curve at given absorbance (660nm);
V1 = Total volume of diluted sample solution (ml);
V2 = Total volume of the reaction mixture (ml);
η = Dilution factor of the test sample;
m = weight of fermented substrate (g);
t = reaction time (minutes).

Protein Estimation
Protein was estimated as described by Lowry *et al*. (1951) using bovine serum albumin as standard protein. In this assay, three reaction solutions were prepared as a Lowry solution (SolA + SolB + SolC) with a ratio of 100:1:1 volume basis respectively. For SolA, about 2.86g of NaOH and 14.31g of Na_2CO_3 were dissolved in 100 ml of deionized water. SolB was prepared by dissolving 2.86g of $Cu_2SO_4.2(H_2O)$ in 100 ml of deionized water. SolC was freshly prepared by dissolving 2.86g of Tartaric acid. $2(H_2O)$ in 100ml of deionized water. Folin reagent was prepared for every assay by mixing 5ml of 2N Folin and Ciocalteu's phenol reagent with 6ml of deionized water. The assay was initiated by mixing 0.5ml sample with 0.7ml of Lowry solution. Then the sample mixture was immediately added to 0.1ml of Folin reagent and mixed vigorously. Subsequently, the mixture was incubated for 30 minutes at room temperature in dark conditions. The mixture was then vortex briefly and the absorbance was measured shortly. The protein content was estimated by measuring the absorbance at 750 nm using a spectrophotometer.

DISCUSSION
The *Aspergillus oryzae* was isolated from a soil sample which showed bright orange-yellow reverse colour on AFPA, this indicated that the strains were nonaflatoxigenic. This agrees with the work of Sooriyamoorthy (2013) who reported that the isolates produced bright orange-yellow reverse color with 48 hours of incubation at 30 °C. The color of the conidia can be a very useful starting point in identification, as well as metulae, phialides, shape, and size of vesicle. *A. oryzae* is closely related to *A. flavus* which produces colonies of similar or slightly smaller in size on the standard media. However, colonies of *A. flavus* remain green as they age, whereas those of *A. oryzae* are more floccose and turn olive-brown as they age.

Microbial proteases have a number of commercial applications in industries such as food, leather, detergent, and diary, pharmaceutical, diagnostic and waste management industries. Proteases are among the most important class of industrial enzymes which constitute more than 65% of the total industrial applications i.e., it helps in removing protein-based stains from cloth, leather preparation, meat tenderization, cheese making, peptide synthesis, de-hairing process, and pharmaceutical industries. It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions, the nature of proteases, and their catalytic potentiality under different conditions (Kirk et al., 2002).

Incubation time for the production of protease by *A.oryzae* was studied within 5 days. Researchers such as Chutmanop *et al.* (2008) reported that the maximum protease enzyme production occurred on 4th day of incubation. The incubation period shows a direct proportion to the production of enzymes and other metabolites which occurs when the organism is actively multiplying, after which the enzyme activity and the growth of the microorganism decreases; this could be attributed to the rate of nutrient limitation and the production of toxic metabolites (Chutmanop *et al.*, 2008). *Aspergillus oryzae* showed maximal protease production at pH 6. Identical observations were earlier recorded in *A. niger* and *A. nidulans* at pH 6.0 (Ahmed *et al.,* 2017). Protease production by microorganisms strongly depends on the extracellular pH which acts to influence many enzymatic processes and transport of various components across the cell membranes. This, in turn, supports cell growth and metabolite production.

The higher enzyme production was found to be at 35°C. Chutmanop *et al.* (2008) have also reported that maximum production of protease by *A. oryzae* was obtained at an incubation temperature of (34-35°C) and the enzyme production was reduced when the incubation temperature was increased above 35°C. Fungal proteases are usually heat-sensitive and show reduced activities at high temperatures. Higher temperature was found to have destructive effects on the metabolic activities of microorganisms and inhibit the growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperatures due to stretching and loss of normal conformation of the enzyme active site (Muthulakshmi *et al.*, 2011; Ibrahim and Falola, 2021).

At lower inoculum levels, the yield was very low. In this present study, the maximum protease synthesis was recorded with 3% inoculum size, whereas at higher concentrations, a decrease in enzyme activity was observed, this might be due to the clumping of cells to the substrates which could have reduced sugar and oxygen uptake rate as well as rate of enzyme released. Haq *et al.* (2003) reported that 3% of inoculum size gave maximum protease production by *A. oryzae*.

Various nitrogen sources namely, KNO_3, peptone, and yeast extract were used for optimization purposes, and peptone showed the maximum protease production at 3% concentration. This result corresponds to the work of Chutmanop *et al.* (2008) who reported that 3% inoculum concentration shows the highest enzyme productivity by *A. oryzae* under SSF. Certain nitrogenous salts tend to decrease the pH of the culture medium and have adverse effects on enzyme production, although they support the growth of the organism.

The highest protease activity on rice husk was 1350 Ug−1 dry solids compared with an activity of corn cob with 1005 Ug^{-1}. These results agree with the work of Elliah *et al.* (2002) who reported that protease production in the presence of different substrates and different carbon sources have different influences on enzyme production by different strains. It might be due to the fact that the increased level of substrates decreases the aeration and porosity of the medium, which were very essential for the proper growth of the organism.

Production of enzymes requires amino acids; molds can produce amino acids from inorganic nitrogen sources which can stimulate enzyme production. Although the protein content of the two substrates is not similar, rice husk proteins are more easily digested than the proteins in corn cob. This probably explains the better enzyme production recorded with rice husk (Chutmanop *et al.* (2008).

CONCLUSION

Protease was produced from a locally isolated fungus *Aspergillus oryzae*. It was shown to have broad substrate specificity, high pH, and temperature stability. These are significant characteristics of any industrially important enzymes. The conditions for protease production by *A. oryzae* in a solid-state fermentation medium were optimized. *A. oryzae* has a promising potential for protease production using inexpensive promising technology known as SSF. The optimum conditions for protease production in this study were observed at 3% inoculum size, pH 6.0, and peptone as a nitrogen source after 84 hours incubation period, with two agro-allied wastes (rice husk and corn cob) which have shown good potential for use as substrate for protease production by *A. oryzae* under solid state fermentation.

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