Protease Production From the Pod of African *Parkia biglobosa* Using Resident Fungi in Solid State Fermentation

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Abstract: Protease production from the pod of naturally fermenting African *Parkia biglobosa* by bioprocess is a good value added to agro industrial residues. The production of enzyme protease by fungiwas carried out under solid state fermentation (SSF). The effects ofincubation period, pH of the medium and addition of supplements onprotease production (enzyme activity) by *Aspergillus niger* and *Rhizopus stolonifer* were determined. The optimal values of factors influencing the production were determined during theincubation period of 5 days. The enzyme activity increased until day three before it began to decline in both supplemented and non-supplemented fermented substrate at 28 ± 2^0C. The pH range for enzyme production was 6.0 – 7.0. Protease production was 1% enhanced when supplemented with nitrogen source(1% ammonium nitrate) but was not enhanced when supplemented with carbon source(1% maltose). In conclusion, production of protease under SSF using *Parkia biglobosa* pod as substrate is effective and could be encouraged in industrial processes like food processing, baking and other related processes.

Keywords: protease, *Parkia biglobosa*, enzyme activity, fungi, solid state fermentation (SSF)

Introduction

Enzymes are the catalytic corner stones of metabolic activities of living things and catalyse most of the reactions in living organisms. They are biological molecules that are responsible for thousands of metabolic processes (Girsham *et al.*, 1999). Enzymes commercially available now are not economically comparable to the chemical process. Hence any substantial reduction in the cost of production of enzymes will be a positive stimulus for the commercialization of enzymatic depilation. Extracellular enzymes are usually capable of digesting insoluble nutrient material such as proteins, cellulose and starch (Aniflousa *et al.*, 2002).

Protease refers to group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of protein (Rawlings and Barrett 1993). Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme in market (Woods *et al*., 2001). Proteasesare produced commercially from plants, animals and microbial sources (Shafee *et al*., 2005). Microorganisms offer an attractive source of protease enzyme because they can be cultured in large quantities in a short period using established fermentation techniques (Rao *et al.*, 1998). Proteases are used in pharmaceutical, food processing, meat tenderization, dairy, taming, brewing, baking, animal feed, detergent, textile and other industries (Godfrey and West, 1996). Extracellular proteases are naturally produced by microorganism mainly to degrade large polypeptide inthe medium into peptides and amino acids before cellular uptake (Shafee *et al*., 2005).

Solid state fermentation for protease production with the use of low cost substrate and cost effective method allows possible production and application of crude enzyme for industrial processes. For the production of enzymes for industrial use, isolation and characterization of new promising strain is a continuous process (Sumantha *et al*., 2005). Solid state fermentation involve microbial modification of a solid, undissolved substrate in which microbial cultures are grown on moist solid with little or no free water, although capillary water may be present (Pandey, 1999). It not only provides a natural substrate for fungal growth and fermentation, they also result in improved value of these agro-industrial residues (Pandey, 1999). The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of microbial population. The most important among them are medium pH, incubation period and incubation temperature (Pandey, 1999).

Parkia biglobosa (Jacq) Benthis perennial plant belonging to thefamily Mimosaceae of the order Leguminisae. *P. biglobosa* is found commonly everywhere in the Savannah and it grows up to about 20m high. In Yoruba,it is called "igbaOdo", "dorowa", in Hausa, and"origiliokpi" in lgbo. The fermented seeds of *P. biglobosa* are use in all parts of Nigeria and indeed the West Coast of Africa as condiments for seasoning traditional soups. Pods are usually pink brown to dark brown when mature, about 45 cm long and 2 cm wide (Orwa *et al*., 2009). In some communities, whole pods serves as fodder and are eaten by domestic stock, including cattle, while in some others pods and roots are used as sponges and as strings for musical instruments. Most often, pods are found discarded as wastes during the time of harvest hence

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becoming a source of aesthetic pollution to the environment. Presently, pods are not of any industrial application, the use of it in protease production will be an effective means of pollution control and most importantly, conversion of waste to wealth. Hence, this research aimed at producing protease from the pods as a means of adding value to waste and controlling environmental pollution.

Materials and Methods

Substrate Collection

The substrate (*Parkia biglobosa* pod*)* was collected from Budo-Egba along Ilorin-Ogbomosoexpress way, Kwara State and authenticated at Plant Biology Department of the University of Ilorin with number UIH001/948.

Preparation of sample

The dried substrate (*Parkia biglobosa* pod) was crushed with mortar and pestle, grinded with Panasonic AC-311 electric blender and sieved to finely powdered form with a plastic sieve of 125mm-20µm round hole.The powdered substrate was stored in a clean airtight container for further use.

Determination of water holding capacity

The water holding capacity was determined because the quantity of water to be added should be an amount that the substrate (*Parkia biglobosa* pod) can take in without being water logged to maintain SSF condition. The water holding capacity was determined according to the method described by Zazueta-murale *et al.*(2006). Five gram of substratewas moistened with 10ml of water.

Isolation and Identification of organisms

The organisms were isolated from the substrate (*Parkia biglobosa* pod). Twenty gram of the dried substratewas place into a container and 40ml of water was added according to the water holding capacity of the substrate. It was then covered and left until visible fungal growth was observe after 3 days. Distinct fungal colonies were carefully picked and sub cultured severally until pure cultures were obtained. The organisms were maintained on PDA slant at 4 ^{0}C for further research. Identification of fungal isolates was base on cultural and microscopic characteristics. Microscopy was done on wet mount and after staining with cotton blue in Lactophenol. Characters obtained were compared with information in literature to identify the organisms (Singh *et al*., 1991; Samson *etal*., 2004).

Screening of isolates for protease production

The isolates were screened by using the skimmed milk medium according to Sharmer *et al.*(2006).Hundred grams of casein was mixed with 250ml of PDA and 5 g of peptone water containing streptomycin(2ml) and poured into the Petri dishes, after which the isolates for screening were inoculated on the different labelled plates and incubated at 28±2 ^{0}C for48 hours.

Fermentation for enzyme production

Five grams of the substrate(*Parkia biglobosa* pod)was placedintoeach often conical flasks. Ten millilitres of sterile distilled water was added to the substrate and plugged with cotton wool and aluminium foil. It was autoclaved at 121^{0}C for 15 minutes. Inoculation was done by adding 10ml of sterile distilled water into a fully sporulated PDA slant of each screened organism that showed positive reaction for protease production and shake vigorously to dislodge the spores. One milliliters of spore suspension containing about 2×10^4 spores/ml was dispensed into the appropriate flasks and labelled. The flask were incubated for 120 hours at 28± 2^{0}C (Kayode and Sanni, 2008).

Effect of incubation periodon protease production

The ten flasks inoculated with spore suspension of the organisms were incubated for 24, 48, 72, 96 and 120 hours. Samples were analyzed for protease production every day (Chinnasamy *et al* ., 2011).

Effect of nitrogen sourceon protease production

The previously used procedure for incubation period was repeated but there was the addition of 1% ammonium nitrate into 20g of the substrate in a conical flask, sterilized in the autoclave at 121^{0}C for 15minutes. The flasks were inoculated and incubated at 28± 2^{0}C for 120 hours (Monod *et al.,* 1991).

Addition of carbon supplements

The previously used procedures for incubation period was repeated but there was addition of 1% maltose as supplement into 20g of the substrate and incubated at 28±2^{0}C for 120 hours (Murthy and Lonsane, 1993).

Enzyme extraction

This was done after every 24 hours by weighing one gram of the fermented substrate into a conical flask, and 100ml of sterile distilled water was added and was placed on the rotary shaker at 200rpm for one hour. After shaking, it was filtered with Whatman No1 filter paper to obtain the crude enzyme (Ali, 1992).

Assaysfor protease

Protease assay was done by adding 2ml of 0.1M phosphate buffer of pH 7 containing 1% of casein into 2ml of the crude enzyme inside a test tube which was incubated at 37 ^{0}C for 30 minutes. After incubation, it was filtered again with Whatman No1 filter paper.Thereafter, 2ml of reagent C (containing mixture of reagent A:1g of anhydrous Na_2Co_3 in 100ml of 0.1M NaoH and reagent B: 0.25g of cupric sulphate in 50ml of 0.5g of sodium tartate) was added and left

for 10minutes followed by addition of 0.2ml of reagent D (1:3 of FolinClocalteau reagent and distilled water), left for 30 minutes. After 30minutes, the absorbance was measured using a spectrophotometer at wavelength 650nm (Tremacoldi*etal.*,2004).

Determination of pH of the crude enzyme

The pH was determined using a pHmetre (model:STARTER 2000) by dipping the electrode of the pH into the crude enzyme filtrate for 10 minutes before the pH of the crude enzyme was read (Sumantha *et al* ., 2005).

Protease activity

The values of absorbance obtained from the protease assay were used to calculate the amount of protease produced (protease activity). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 mg of tyrosine under assay conditions.

Results

Two fungi were isolated and characterized as *Aspergillus niger* and *Rhizopus stolonifer*

Table 1: Description of the fungal isolates

S/N	Isolates	Description	Suspected organism
1	A	It appeared on PDA in black, non-mucoid but powdry colonies and flat on the surface of PDA plate. It appeared under microscope as a conidia arising from a vegetative mycellium it has no protoplasm nor cross wall non-septate and it did not take up any colour but remained black.	*Aspergillus niger* .
2	B	It was very fast in its growth with whitish/greyish filaments with black spores. The filament appeared to be raised on the surface of PDA plate. It appeared under microscope as non-septate hyphae with unbranched sporangiospore and has a root like structure called rhizoid.	*Rhizopus stolonifer*

Effect of incubation period on enzyme activity.

The highest enzyme activity was obtained at 72 hours of incubation in both non-supplemented fermented substrate and supplemented fermented substrate by *A. niger* and *R. stolonifer* respectively as presented in Figures 1, 2 and 3.

Effect of pH on enzyme activity

The highest enzyme activity was at the lowest pH while the lowest enzyme activity was at the highest pH for both *A. niger* and *R. stolonifer* after 72 hours of incubation as shown in Figures 4 and 5.

Effect of pH on enzyme activity during fermentation with supplements

The highest enzyme activity was at the lowest pH as shown in Tables 1, 2,3 and 4 for both *A. niger* and *R. stolonifer*after 72 hours of incubation.

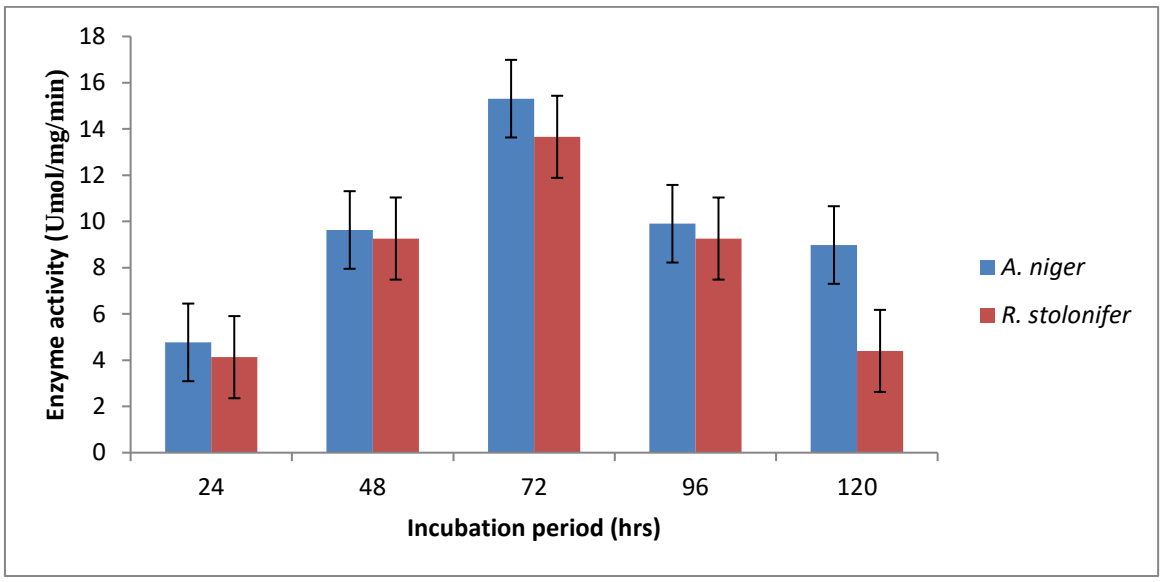


Figure 1-: Effect of incubation period on protease production at 28± 2^0C

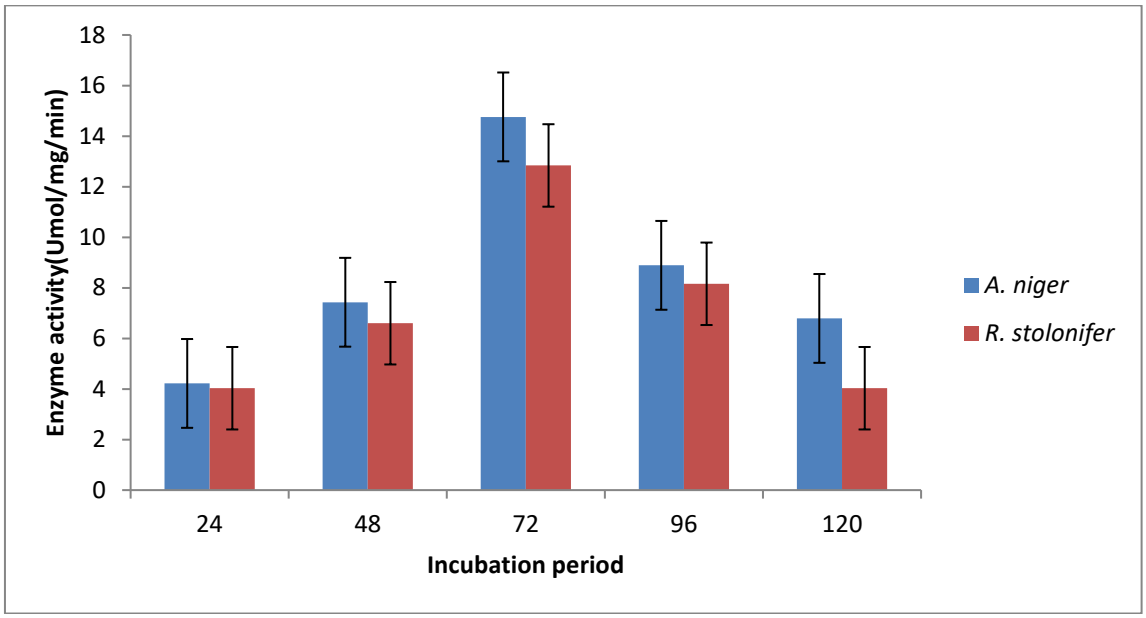


Figure 2-: Effect of maltose on protease production by *A.niger*and*R.stolonifer*28± 2^0C

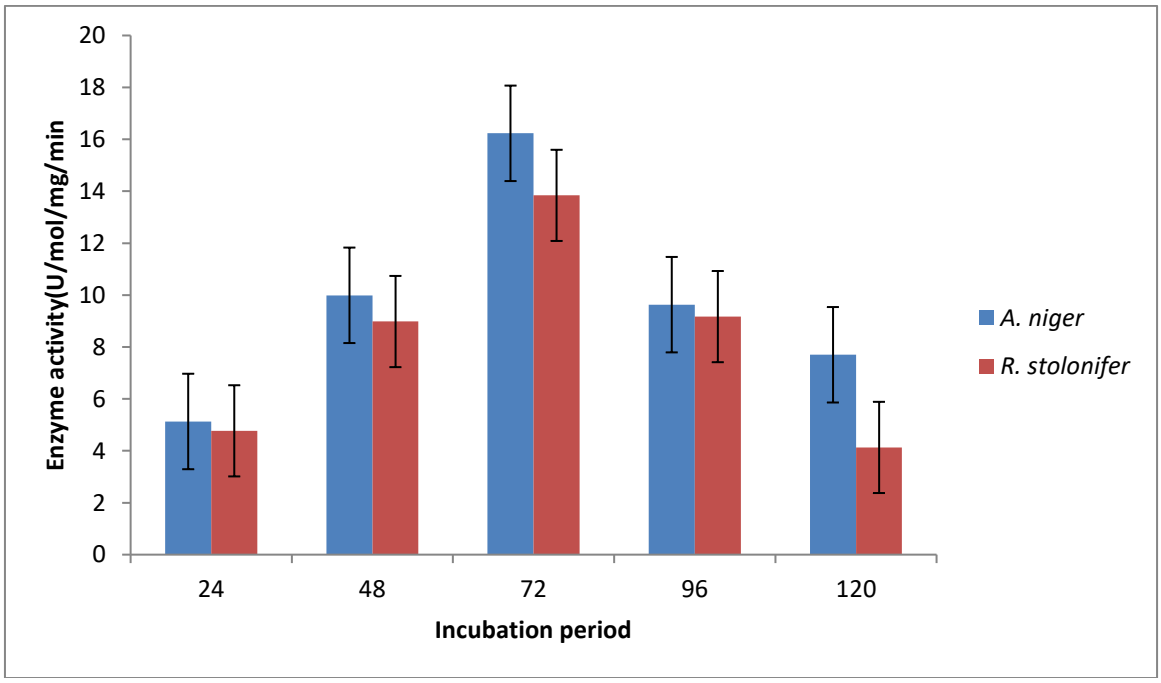


Figure 3-: Effect of ammonium nitrate on protease production by *A.niger*and*R.stolonifer* 28± 2^0C

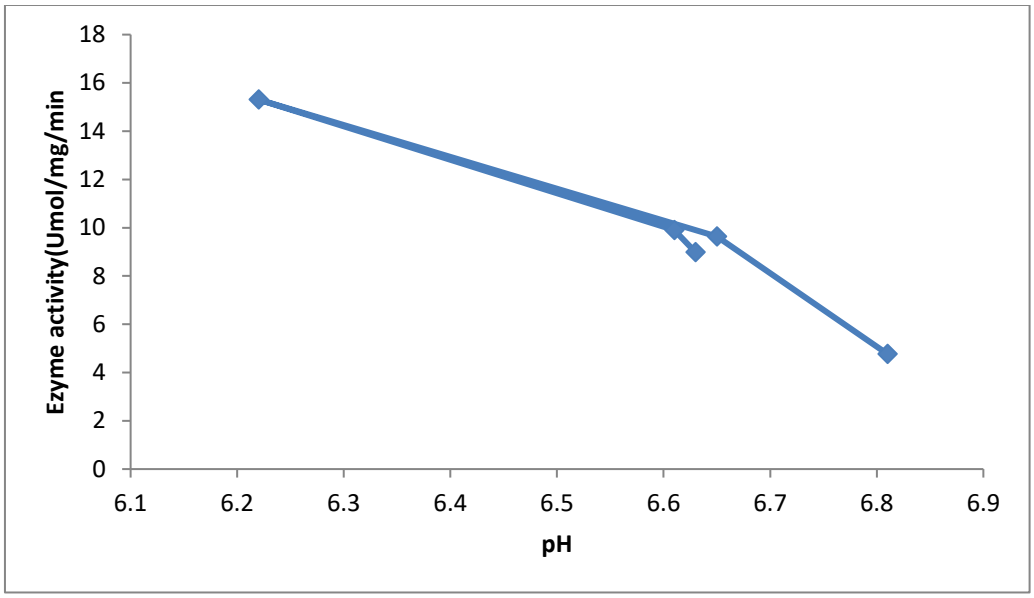


Figure 4:Changes in pH during fermentation by *A.niger* 28± 2^0C

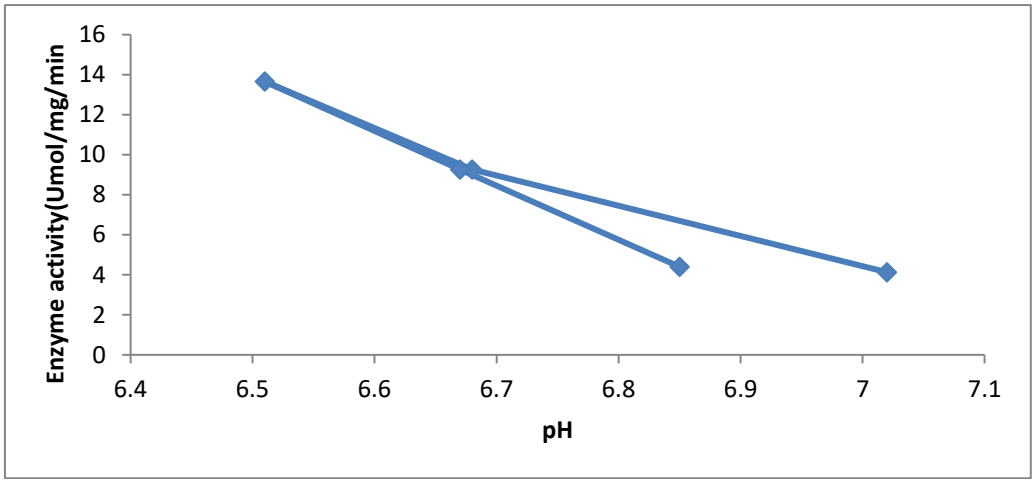


Figure 5:Changes in pH during fermentation by *R. stolonifer* 28± 2^0C

Table 2: Changes in pH during fermentation with maltose for protease production by *A.niger*

Incubation period (hrs)	pH	Enzyme activity(Umol/mg/min)
24	6.91	4.22 ± 0.00
48	6.61	7.43 ± 0.64
72	6.39	14.76 ± 0.13
96	6.63	8.89 ± 0.13
120	6.67	6.79 ± 0.26

Note: Values are mean of two replicates.

Table 3:Changes in pH during fermentation with maltose for protease production by *R.stolonifer*

Incubation period (hrs)	pH	Enzyme activity(Umol/mg/min)
24	6.89	4.03 ± 0.00
48	6.67	6.60 ± 0.00
72	6.59	12.84 ± 0.26
96	6.63	8.16± 0.13
120	6.88	4.03 ± 0.00

Note: Values are mean of two replicates.

Table 4:Changes in pH during fermentation with ammonium nitrate for protease production by *A.niger*

Incubation period (hrs)	pH	Enzyme activity(Umol/mg/min)
24	6.73	5.13 ± 0.00
48	6.42	9.99 ± 0.39
72	6.01	16.23 ± 0.13
96	6.61	9.63± 0.13
120	6.63	7.70 ± 0.25

Note: Values are mean of two replicates.

Table 5:Changes in pH during fermentation with ammonium nitrate for protease production by *R.stolonifer*

Incubation period (hrs)	pH	Enzyme activity(Umol/mg/min)
24	6.93	4.77 ± 0.26
48	6.63	8.98 ± 0.00
72	6.54	13.84 ± 0.13
96	6.65	9.17 ± 0.00
120	6.92	4.13 ± 0.13

Note: Values are mean of two replicates.

Discussion

The two fungi isolated and identified based on their macroscopic and microscopic characteristics; *A. niger* and *R. stolonifer* are wide spread in nature such as in naturally decomposing waste and also in the air. These two fungi gave positive result during screening indicating their ability to produce protease (Chakraborty *et al.*, 2000; Haq *et al.*, 2003).

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Protease production by microbial strains depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support cell growth and product production (Ellaiah, 2002).

The two fungi were able to produce proteaseunder the assay conditions. Enzyme production and activity tend to increase with incubation period until the 96th hour when it begins to reduce. Maximum enzyme activity was recorded at pH very close to neutral, above or below which there was a decline. This result is similar to the results reported by (Tuefel and Gotz, 1993; Mukhtar and Ul-Haq, 2009).In a similar study, Karuna and Ayyanna (1993) reported that maximum protease activity with *A. oryzae*was obtained after 72 hours of incubation. When incubation period was increase to 96 hours and beyond, enzyme activity decreased.

Enzymes, which are primary metabolites, are secreted into growth medium by fungi to enhance the breaking down of organic substrate for onward absorption for growth and metabolism. Production and release of enzyme increases as the organism moves progressively from lag to log or exponential phase of growth where maximum activity is display. As the organism progresses into stationary phase of growth, production of enzymes and other primary metabolites reduce. Incubation period has been reported to be directly proportional to enzyme activity and the peak usually correlates to late exponential phase of growth (Romero *et al.*, 1998; Mukhtar and Haq, 2009).
This can be attributed to reduction in utilizable substrate and accumulation of toxic wastes. Hence, growth and subsequently enzyme production reduces. The trend of the result obtained in this research follow this patern.

Supplementing the medium with maltose and nitrogen source in form of ammonium nitrate did not really increase enzyme activity. It might be suggested that 1 % maltose and ammonium nitrate did not meet the carbon and nitrogen requirement of the two organisms. It has been frequently described that in a defined medium, a protein and carbon source must be present for enzyme to be produced. Carbon is required for cell wall and biomass production while protein is needed for the synthesis of nucleic acid and all cellular components. Nitrogen is very important in metabolism of microorganism especially in the synthesis of enzyme and other proteins hence nitrogen has always been one of the important components of fermentation substrate/media (Chinnasamy *et al*., 2011).

However, it has also been noticed that the absence of a proper carbohydrate (carbon source) and protein in a medium results in a dramatic decrease in enzyme production (Gajju *et al.*, 1996).

Filamentous fungi have been recorded to have special ability for enzyme production in SSF (Tremacoldi *et al.*, 2004). *Aspergillus niger* recorded higher enzyme activity than *Rhizopus stolonifer* in this study. *A. niger* and other species of *Aspergillus*has been reported as a known organism with a special ability to produce various kinds of enzymes including protease from organic substrates under laboratory and industrial conditions. However, the ability of *R. stolonifer* to produce protease is also noteworthy. The two organisms have been employedpreviously to produce various types of enzymes using different organic substrates (Chakrabarty *et al.*, 2000; Haq *et al.*, 2003; Mukthar and Haq, 2009).

Conclusively, protease production can be achieved using *Parkia biglobosa* pod (agro-industrial waste) under solid state fermentation by fungi. The pod will be a low cost substrate/raw material for the production of protease through SSF. The utilization of this agro-industrial waste provides an alternative means of waste utilization and a sustainable environment for improving the quality of human life through wealth creation. *Asperigullusniger* and *Rhizopus stolonifer* both displayed a good quality and suitability for protease production under the conditions of assay.

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