EXTRACTION AND ACTIVITIES OF CRUDE PROTEASES FROM SOLID STATE FERMENTATION OF *Aspergillus nigeR* GROWN ON VARIOUS AGRO WASTE

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ABSTRACT

Agro-industrial residues are generally considered the best substrates for the solid-state fermentation process. Food processing wastes such as shells of nuts and eggs in Nigeria are usually disposed in dumpsites or are incinerated, causing environmental pollution. This study extracted, estimated and compared enzyme activities from *Aspergillus niger* under solid-state fermentation (SSF) by utilizing Ground nut shell (GS), Bambara nut shell(BS), Guinea fowl egg shell(GFS) and Layer egg shells (LES) powder as solid substrates. The Agro wastes were dried under the sunlight for four days, ground using a laboratory pestle and mortar separately and sieved to obtain fine powder. The powder was sterilized, supplemented with sodium acetate buffer and pure casein, inoculated with the fungi then left to ferment for six days. The crude extract of the substrates were obtained by filtration and centrifugation. The extracts were assayed for enzyme activity using Sigma Aldrich's method of assay. The result obtained from the assay demonstrated that proteases were active in all the extracts. However, there was significant difference ($P < 0.05$) in protease activities in the samples studied. The result revealed that BS and LES showed the highest enzymatic activities (0.797± 0.035 µ/ml), (0.788 ± 0.028 µ/ml), (0.574 ± 0.032 µ/ml), (0.590± 0.040 µ/ml) at 0.2, 0.4, 0.6 and 0.8ml of the crude enzyme extract volume concentrations respectively followed by GS which had the enzymatic activities (0 .456 ± 0.001 µ/ml), (0.391 ± 0.003 µ/ml), (0.335 ± 0.003 µ/ml), (0.205± 0.032 µ/ml) at the volume extract volume concentrations of 0.2, 0.4, 0.6, 0.8ml respectively, meanwhile guinea fowl egg shell had the least enzymatic activities (0.401± 0.028 µ/ml), (0 .336 ± 0.047 µ/ml), (0.250 ± 0.018 µ/ml), (0.255 ± 0.010 µ/ml) at the volume of crude enzyme extract of 0.2, 0.4, 0.6, 0.8ml respectively. In all the shells the highest proteases activities were recorded at 0.2ml of the crude extract volume concentration and the least proteases activities were recorded at 0.8ml of the crude extract volume concentration. This study has proven that proteases were extracted and active from solid state fermentation of *Aspergillus niger* grown on various agro waste, Therefore proper utilization of these agro-wastes in industrial and scientific settings will not only serve as means of polluted environmental remediation but also as an iualternative to commercially available substrates for microbial growth.

Keywords: Proteases, Substrates, Fermentation, Enzyme

INTRODUCTION

Enzymes are biological molecules which speed up the rate of chemical reactions but remain unchanged at the end of the reaction (Oyeleke and Oduwole, 2009). They are usually found in trace levels in the cells of living animals. Proteases are examples of such molecules which are also known as proteinases or proteolytic enzymes. They belong to the hydrolase class and have the catalytic function of hydrolyzing protein peptide links (Theron, 2014). These enzymes

are degradative enzymes that can cleave protein meals and release peptides and amino acids that the body requires.

Enzymes have the ability to speed up chemical reactions linked with life processes while remaining unaffected by the reaction; they increase the rate of the reaction without necessarily initiating it. Proteolytic enzymes are ubiquitous because they are required for the survival of all living creatures and are produced by all prokaryotes and eukaryotes. These enzymes are degradative enzymes that can cleave protein meals and release peptides and amino acids that the body requires. However, Burhan *et al.,* (2003) established that microbial enzymes are preferred over those derived from plants and animals because they are less expensive to produce and have more predictable, controllable and reliable enzyme contents. Specificity in action is one of the essential characteristics of enzymes thereby gaining international recognition for their physiological and biotechnological applications (Sawant and Saraswathy, 2014).

Biotechnology is fast gaining traction because to the numerous advantages it provides over traditional chemical methods, particularly in terms of the environment and economics. Demand for enzymes has been boosted by new emergent applications, and the industry is responding with a steady supply of new products (Chander and Kaur, 2015). Technical enzymes, food enzymes, and animal feed enzymes are the three application areas of the industrial enzyme industry, and these proteases are employed in the food, dairy, detergent, leather, and pharmaceutical industries, among others (Rao *et al.,* 1998). Alkaline proteases account for approximately 52% of technical enzymes used in detergent, pulp, and paper manufacture (Chander and Arora, 2014). There has been an interest towards less chemical intensive processes. Enzyme has been used to replace toxic chemicals because of its eco-friendly nature, for example, leather treatment process has been accompanied with alkaline proteases which have successfully contributed to the reduction of pollutants and water usage while also enhancing the quality of leather. This therefore, increased the awareness about reducing industry's environmental impact which may be hazardous. Kumar *et al*., (2010) reported the use proteases in conversion of milk to curd when stored in bags made up of calf stomach. It was discovered that, Rennet an enzyme whose main component is chymosine, is a protease involved in the transformation of milk into curd (Kumar *et al*., 2010). Oyeleke and Oduwole, (2009) noted that enzymes mainly produced by microorganisms such as fungi and bacteria are often not readily available in sufficient quantities for food applications or industrial use though, they are naturally produced. Proteases are produced either by microorganisms or through chemical synthesis (Haq *et al.,* 2006). However, commercial quantities can be obtained through fermentation medium, either by solid state fermentation (SSF) or submerged fermentation by isolating microbial strains that produce the desired enzyme and optimizing the conditions for growth. Many researches have revealed that solid state fermentation is the best, with fungus as the fermenter organism because of their ability to create thermo-stable enzymes with high economic value (Kumar *et al*., 2010; Haq *et al.,* 2004; Pedersen *et al.,* 2015). Enzymes produced by microbes are cheaper and safer. Haq *et al.,* (2004) postulated that fungi exhibit a wide range of proteolytic enzyme compared to bacteria, because of their ability to utilize large range of substrates as nutrients and as well develop under different environmental conditions such as temperature, pH and time.

Aspergillus niger is known to be one of the most exploited microbes in different

fermentation methods for years, because of its several promising characteristics. *A. niger* is one of the most important industrial and medical fungi (Schuster *et al*., 2002). It is a ubiquitous soil fungus that can be found in a wide range of geographical regions, ecosystems, and inhabitants, as well as many types of soil and other substrates. The increase in demand for proteolytic enzymes by detergent and leather industries and also in research and medicines necessitate the use of strains of microorganisms that secrete a diverse range of highly active enzymes (Pedersen *et al.,* 2015).

In Nigeria, agro-wastes and industrial-wastes are usually disposed carelessly in dumpsites or are incinerated instead of using them as raw materials for production of useful products. Interestingly, study by Jayasree *et al*, (2009) revealed that agro-industrial residues such as eggs and nuts shells are best substrates for solid state fermentation process which in turn support production of important enzymes proteases. Therefore, this study determined the activities of crude proteases extracted from solid state of fermentation (SSF) of *Aspergillus niger* grown on Ground Nut Shell (GS), Bambara Nut Shell (BS), Guinea Fowl (GFS) and Layer Egg Shells (LES) instead of commercially available substrates for microbial growth.

MATERIALS AND METHODS

Study Site

Kaduna is located in the North West geopolitical zone of Nigeria and between latitude $10^{0}20^{0}$ and 10.33^{0}N and 7.75^{0}E. The vegetation is attributing of guinea savannah type with distinct wet and dry seasons. The state shares border with Kano, katsina and Zamfara states to the North, plateau and Bauchi state to the East, Nasarawa state and Federal Capital Territory to the south and Niger state to the west. Kaduna state occupies 46.53 square kilometers and has a population of more than 5 million according to 2006 census (Mohammad *et al*., 2019).

Sample Collection and Preparation

Groundnut shells (*Arachis hypogea*) and Bambara nut shells (*Vigna subterranea*) were obtained from central market, Kaduna State. Guinea fowl (*Numididae*) egg shells and Layer egg shells (*Gallus gallus domesticus*) were obtained from households, bakeries and market, the fungus *Aspergillus Niger* were obtained from a stock culture in the Biology Department of Kaduna State University. The research was carried out in the Biotechnology laboratory of the department of Biological science, Kaduna state university. The shells were collected individually, Groundnut and Bambara nut shells were achieved by separating the seeds from the shells manually,

Chicken (layers) and guinea fowl egg shells were also separated manually from the egg white and egg yolk by breaking off the shells from the egg content, all the shells were individually dried under sunlight for four days, after which they were ground into powdered form using mortar and pestle before sieving to get a powdered form. They were each weighed to 240g and put separately into air tight plastic container for further use. The shells powders were applied as the substrate. *A.niger* stock was plated onto agar slopes in 5ml bottles and then stored at 4^{0}C for further use.

Microorganism and Inoculum Preparation

The fungus *Aspergillus niger* used in this study was isolated from a stock culture in the Biological Department of Kaduna State University. Exactly 2.8g of nutrient medium (Himedia M001-500G) was weighed and dissolved in 100ml of distilled water into a beaker. It was heat up to 100^{0}C, then sterilized by autoclaving at 121^{0}C for 15minutes and allowed to cool at room temperature (25^{0}C). A long handled glass rod

was used to pick up some *A.niger* into the prepared media and incubated.

Experimental Design

The design of this study was carried out in duplicate with their controls. The experiment was composed of three treatments i.e. treatments 1, 2 and treatment 3, each containing four beakers that contained the substrates (groundnut, bambara nut, layer chicken egg and guinea fowl egg shells) which were treated with *A. niger*. Their controls were not treated with *A. niger* as summarized in Table 1.

Table 1: the summary of research experimental design.

	Substrate (g)	Casein (g)	SB (ml)	DW (ml)	*A.n* (ml)
T**1**	60	20	30	100	1.5
T**2**	60	20	30	100	1.5
T**3**	60	20	30	100	-

KEY: SB = Sodium buffer, DW = Distilled water, *A. n = Aspergillus niger.*

Treatment (T) 1: GFS= Guinea Fowl Shell 1, BS = Bambara nut Shell 1, GS= Groundnut Shell 1, LES = Layer Egg Shell 1.

Treatment (T) 2: GFS= Guinea Fowl Shell 2, BS = Bambara nut Shell 2, GS= Groundnut Shell 2, LES = Layer Egg Shell 2.

Treatment (T) 3: GFS= Guinea Fowl Shell Control, BS= Bambara nut Shell Control, GS= Groundnut Shell Control, LES = Layer Egg Shell Control.

Solid State Fermentation (SSF)

Solid- state fermentation was carried out in twelve (12) beakers of 250ml; the beakers contain 60g of groundnut shell (GS) powder each. Similar measurements were done for Bambara nut shell (BS) guinea fowl eggs (GFS) and layer egg shell (LES). Four among the eight beakers (one for each shell substrate) served as their controls while the other four served as a replicate; to all the twelve (12) beakers containing the substrates, 20g of pure casein, 100ml of distilled water and 30ml of sodium acetate buffer at pH 4.5 were evenly measured into the beakers; 1.5ml of *Aspergillus niger* obtained from the stock culture was inoculated into the replicated beakers containing substrates. The controls were not inoculated with *A. niger*. The content of the beakers was thoroughly mixed and covered with aluminum foil paper and sealed with masking tape. The beakers containing the inoculum was labeled as GS 1, BS 1, GFS 1, and LES 1, the replicate were labeled GS 2, BNS 2, GFS 2, and LES 2 While the controls were labeled as GS C, BNS C, GFS C, and LES C. All the beakers were left to stand for six days at room temperature (25^0C) for solid state fermentation (SSF) to take place.

Crude Enzyme Extraction

After the inoculation period, extraction of the crude enzyme was done by centrifuging the fermented substrates of the shells of groundnut, Bambara nut, eggs of guinea fowl and chicken at 15000rpm for about 10min. Supernatant of each of these substrates were collected individually and filtered using whatman's number 1 filter paper. The filtrates containing the crude enzyme were preserved in labeled vials at -20^0C in a refrigerator.

Assay for Enzyme Activities

The assay for enzyme activity was determined according to the method of Sigma Aldrich's Assay (2006). Suitable vials that hold up to 15ml was used for this assay, one vial among the five vial used, served as blank and the four others were used to determine the assay activity of proteases. To each of the five vials

5ml of 1% casein solutions were added and allowed to equilibrate in a water bath at 37^0C for about 5 minutes, after which varying volume of enzyme solutions of 0.2, 0.4, 0.6, and 0.8 to be tested were added to four of the suitable vials that can hold up to 15ml but not the blank, and then the mixture was gently swirled and incubated at 37^0C for exactly 10minutes, and 5ml of tri-chloroacetic acid (TCA) was added to each tube to stop the reaction. Then appropriate volume of the enzyme solutions of 1.8, 1.6, 1.4 and 1.2 ml were added to each tube containing the initial enzyme solution of 0.2, 0.4, 0.6 and 0.8 respectively including the blank so that the final volume of enzyme solution in each tube was brought up to 2ml, the tubes were then incubated at 37^0C for 30 minutes. During incubation, tyrosine standard dilutions were set up, which was done using four-dram vials that can easily hold up to 8ml. To the four drams vials 1.1Mm tyrosine standard stock solutions were added with the following volumes in ml: 0.20, 0.40, 0.60, and 0.80 but tyrosine standard was not added to the blank. Appropriate volume of 1.8, 1.6, 1.4, 1.2ml purified water was added respectively to each standard to bring the volume to 2ml, and were incubated for 30 minutes at the temperature of 37^0C. To all the vials containing the standards and standard blank, 5ml of sodium carbonate was added, and also 1ml of folin's reagent was added immediately afterwards for the best result, the dram vial was mixed by swirling and was also incubated at 37^0C for 60 minutes. After the incubation, 2ml of the solutions was filtered using a 0.45 µm polyethersulfone syringe filter into suitable cuvettes, the absorbance was read with a spectrophotometer at 660nm.

Statistical Analysis

Analyses were performed in triplicate. The data obtained from the study was subjected to analysis of variance (ANOVA) to determine the significant differences between the substrates, where $p > 0.05$ levels was considered significant. Duncan's Multiple Range Test was used to separate the mean ± standard deviation. Results were presented in tables and graphs.

RESULTS AND DISCUSSION

Crude Enzyme Extract

Table 2 shows the activities of crude enzyme on the relative concentrations of samples studied. The result revealed that different concentration (0.2, 0.4, 0.6 and 0.8 ml) of each sample brought about significant difference ($p < 0.05$) in the enzyme activities on Groundnut Shell (GS), Bambara Nut Shell (BNS), Guinea Fowl Egg Shell (GFS) and Layer Chicken Egg Shells (LES). The activities of the enzyme were dependent on the amount of sample extract as increase in concentration of each sample decreased the enzyme activities. The relative activities of the enzyme were found higher at 0.2 ml more than other concentrations (0.4, 0.6 and 0.8 ml) of each sample studied. However, the relative activities of the enzyme was higher in $BS_{0.2}$ and $LES_{0.2}$ with concentration of 100% (0.858 ± 0.027 and 0.797 ± 0.035µ/ml respectively) while GFS had the least crude enzyme activities at 0.2, 0.4, 0.6 and 0.8ml concentrations compared to those of BS, GS and LES.

Table 2: Crude enzyme extract and relative activities of Groundnut Shell (GS), Bambara Nut Shell (BNS), Guinea Fowl Egg Shell (GFS) and Layer Chicken Egg Shells (LES)

Sample/Conc. (ml)	Extract (ml)	R.A (%)	M ± S.D (µ/ml)
$GS_{0.2}$	50	100	0.456± 0.001
$GS_{0.4}$		85.5	0.391± 0.003
$GS_{0.6}$		73.5	0.335 ± 0.003
$GS_{0.8}$		46.9	0.205± 0.032
$BS_{0.2}$	50	100	0.797 ± 0.035
$BS_{0.4}$		74.9	0 .788 ± 0.028
$BS_{0.6}$		64.8	0.574± 0.032
$BS_{0.8}$		26.2	0.522 ± 0.053
$GFS_{0.2}$	45	100	0.401± 0.028
$GFS_{0.4}$		83.4	0.336 ± 0.047
$GFS_{0.6}$		61.8	0.250 ± 0.018
$GFS_{0.8}$		13.4	0.255 ± 0.010
$LES_{0.2}$	50	100	0.797 ± 0.035
$LES_{0.4}$		98.9	0 .788± 0.028
$LES_{0.6}$		72.0	0.574± 0.032
$LES_{0.8}$		65.5	0.522 ± 0.053

Key: R.A= Relative activity, M= Mean, S.D= Standard diviation, GS = Groundnut shell, GFS = Guinea Fowl Shell, LES = Layer chicken egg shell, BNS= Bambara-nut shell.

DISCUSSION

Waste management has been one of the greatest applications of biotechnology, where wastes have been utilized in the production of novel compounds such as enzymes. Chutmanop *et al*, (2008) carried out a work to demonstrate that cheaper agro wastes can be used to produce commercially valuable enzymes such as protease, this is in line with the current study which pays attention by focusing on protease production by *Aspergillus niger* through bioconversion of agro residues, serving as cheaper solid substrates through solid state fermentation. From the results obtained in this study, proteases have been successfully extracted from *A.niger* in solid state fermentation (SSF) by using agro wastes Groundnut Shells (GS), chicken egg Shells (LES), Guinea fowl egg shells (GFS) and Bambara nut shell (BS) as substrates. This was confirmed by Thakur *et al.*, (2015) who also successfully extracted proteases from agro waste (Soybean meal) through solid state fermentation (SSF) using *Aspergillus Oryzae* is very useful for microbial enzyme production as well as for solving pollution problems.

Table 2 shows the crude enzyme extraction and relative enzyme activities from GS, BS, GFS and LES, The variation volumes of their crude enzyme extract shown could be as a result of the different moisture absorption capabilities of the different substrates during the Solid State Fermentation process. The highest enzyme activities of all the substrates were recorded at 0.2ml and the least enzyme activities was recorded at 0.8ml. GFS had the least enzyme activities compared to BS, GS and LES while BS and LES had the highest enzyme activities. enzyme activities is higher at 0.2ml enzymes solution concentration than at 0.4, 0.6 and 0.8ml which means the activity of the enzymes decreased as the assay volume increased, this may have been due to molecular crowding. Molecular crowding is an effect in which a large number of macromolecules in a solution alter the equilibrium constant of enzyme reactions. This is in line with the study carried out by Minton, (2001) who studied the influence of macromolecular crowding and macromolecular confinement on Biochemical reactions in physiological media. The samples used crude enzymes, that is to say, other enzymes and macromolecules were present in the solution apart from the proteases. The combined amount of the other enzymes (background molecules) in the extract might be higher than the amounts of proteases; therefore, the molecular crowding may have had an inhibitory effect on the activity of the enzymes. This is because the overall activities of an enzyme also depend on the amount of free energy and the total

volume fraction of macromolecules in its environment.

Figure 1 shows that there is significant difference in the enzyme activities among GS, GFS, LES, BS compared to Tyrosine standard solution. No significant difference observed in the enzymatic activities between GS and GFS. Meanwhile, LES and BNS showed considerable increase in the enzyme activities compared to other samples at 0.2ml of the sample concentration. Similarly, at 0.4ml concentration, significant increase in the enzyme activities was recorded between LES and BNS followed by GS whereas the least activity was observed in GFS comparable to that of Tyrosine. However, at 0.6ml and 0.8ml of the test samples concentrations, significant difference was not observed between the enzyme activities in GS and GFS but they differ across other samples. Meanwhile, the results revealed that the enzyme activity is inversely proportional to the concentration (which means that increase in the concentration, decreases the enzyme activities down the group) which is contrary to the Tyrosine except for 0.4ml of the test samples concentration.

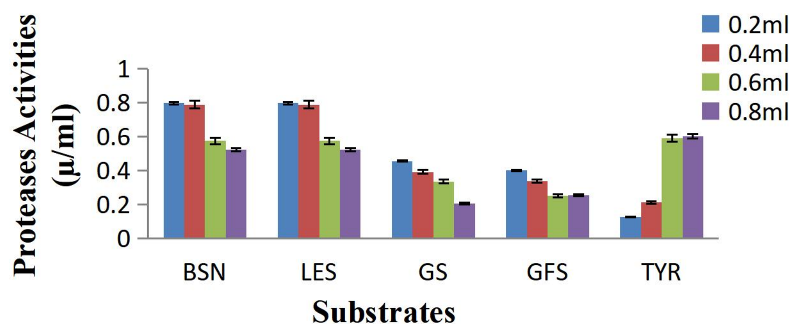

Figure 1: Enzyme activities of GFS, GS, LES, BNS and TRY standard at the sample concentrations of 0.2, 0.4, 0.6, and 0.8ml.

KEY: GS = Groundnut shell, GFS = Guinea Fowl Shell, LES = Layer chicken egg shell, BNS= Bambara-nut shell. TYR= Tyrosine

CONCLUSION

This study shows that, enzyme was successfully extracted from *A.niger* grown on agro-wastes of the shells of Groundnut, Bambara nut, eggs of Guinea fowl and chicken via Solid State Fermentation. Bambara nut shell showed the highest enzyme activities alongside with Layer egg shells at 0.2ml of the crude extract volume concentration followed by Groundnut shells then guinea fowl egg shells. This research demonstrates the potential of various agro-industrial leftovers as microbial production substrates. Therefore, proper utilization of these agro-wastes in industrial and scientific settings should be encouraged as a tool for environmental remediation and but also as an alternative to commercially available substrates for microbial growth.

We recommend that further studies on the importance of proteases should be carried out to stop economic loss and perhaps the useful application of the enzyme and to also

determine the suitable substrate for microbial production of proteases.

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