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Enzyme Activity of Microorganisms Associated with Fermented Husk and Testa of *Cola acuminata*

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ABSTRACT

The availability of microbial enzymes in addition to its low cost, large production, environmental protection, plasticity and chemical stability, makes them widely used for industrial processes. Agricultural and forestry waste which can serve as substrate in producing biologically important secondary metabolites such as cellular proteins, organic acids, prebiotic, enzymes are economically advantageous due to its low cost and availability. The study investigated and compared the potential of same micorganisms isolated from *Cola acuminata* husk and testa waste to produce hydrolytic, proteolytic and lipolytic enzymes. The screened enzymes included α-amylase, β-amylase, cellulase, protease and lipases using appropriate procedures, with their activity measured in μmol min^{-1} mL^{-1}. Microorganisms were isolated using standard microbiological techniques from *Cola acuminata* husk and testa subjected to liquid state fermentation for 10 days. Thirteen microorgainsms were isolated in all and examined for potentials to produce the named enzymes. The bacterial isolates included; *Bacillus subtilis*, *Bacillus sphaericus*, *Bacillus cereus*, *Bacillus laterosporus*, *Bacillus licheniformis*, *Bacillus firmus*, *Micrococcus luteus* and *Lactobacillus fermentum*. *Trichoderma viridiae*, *Articulospora inflata*, *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger* were the fungal species isolated. The *α*-amylase, β-amylase and cellulase activity of the bacterial isolates ranged from 0.093 ± 0.006^{a}-0.383 ± 0.015^{f} μmol min^{-1} mL^{-1}. Protease activity ranged from 1.723 ± 0.147^{a}-3.300 ± 0.170^{g} μmol min^{-1} mL^{-1} while the lipase from 1.000 ± 0.160^{a}-1.500 ± 0.200^{f} μmol min^{-1} mL^{-1}. The activity of the fungal isolates on the other hand ranged from 0.013 ± 0.005^{a}-0.430 ± 0.001^{e}, 2.416 ± 0.313^{a}-10.137 ± 0.083^{i} and 1.000 ± 0.050^{a}-2.267 ± 0.289^{f} μmol min^{-1} mL^{-1} for the hydrolytic, proteolytic and lipolytic enzymes respectively. Protease and lipase had highest activity. Bacterial and fungal isolates from the testa showed higher enzymatic activity as compared to same isolates from the husk. Kolanut husk and testa can thus serve as an alternative substrate for microorganisms for the production of the screened enzymes.

Key words: Enzyme, *Cola acuminata,* microorganisms, substrate, enzyme, agricultural waste, kolanut

INTRODUCTION

The concern aimed at reducing environmental pollution has led to search for clean up technologies. This clean up technology can be used in the production of important commodities to serve the energy, chemical and food industries. The technology makes use of materials that

requires less energy and reduces pollutants in industrial effluents, in addition to being more economically advantageous due to its cost reduction. In view of these, the use of residues from agricultural industries and forestry in bioprocesses has aroused the interest of scientists lately (Sanchez, 2009). Kolanut husk and testa is an example of agricultural waste that can be used as substrate for the production of microbial enzymes.

Kolanut is classified into the family Malvaceae and subfamily Sterculiaceae (Sonibare *et al*., 2009). The seeds of kolanut has been used in the beverage, food, pharmaceutical and textile industries (Esther *et al*., 2010). Asogwa *et al.* (2006) reported that kolanut husk is used for the production of activated carbon, candles, soap, replaces up to 60% of maize in poultry feed formulations and the testa can be used as ingredient in the formulation of fertilizer because of the high potassium content. Locally, the husk can be used as traditional medicine to reduce labour pains, treating swellings and fresh wounds. The root of the tree can be used as chewing stick for cleaning the teeth and for carving (Asogwa *et al*., 2006).

The use of agricultural and forestry waste as substrate in bioprocesses produces biologically important secondary metabolites such as enzymes, organic acids, cellular proteins and prebiotic. Enzymes decompose complex molecules into smaller units such as carbohydrates into sugars, which are natural substances involved in all biochemical processes. Each substratum has a corresponding enzyme due to the enzymes' specificity (Deb *et al*., 2013). Microbial enzymes are more advantageous than those from animal or plant sources. The advantages comprises of possibility of large-scale production in industrial fermentors, lower production cost, use of non-burdensome methods and rapid culture development, wide range of physical and chemical characteristics, possibility of genetic manipulation and there are no seasonal effects. The mentioned characteristics make microbial enzymes suitable biocatalysts for different industrial applications (Hasan *et al*., 2006).

Microbial enzymes are more useful than enzymes derived from animal or plant, because of variety of catalytic activities, possibility of higher yield, regular supply due to the absence of seasonal fluctuations and rapid growth of microorganisms and ability by scientist to manipulate the genetic makeup of the organisms (Iftikhar *et al*., 2010). Valuable array of enzymes are yielded by fungi and bacteria when appropriate fermentation substrates and fermentation methods are used. In the case of bacterial enzymes, various enzymes such as amylase, cellulase and xylanase to mention a few has been produced (Subramaniyam and Vimala, 2012). The α-amylase is a microbial enzyme used in many industries such as brewing, food, paper and pharmaceutical industries. Because of the wide use of these enzymes, there is a need to select potent strains of microorganisms that can produce it in very large quantities. Strains such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been described to have this ability (Haq *et al*., 2010). Alpha and beta-amylases share similar catalytic domain conformation, although the catalytic mechanisms differ from each other (Liu *et al*., 2003).

When glucosidic bonds of cellulose microfibrils are broken, oligosaccharides, cellobiose and glucose are released. The breakage of the bond is usually caused by cellulases which is an hydrolytic enzyme used in food, cosmetics, detergent, drugs textiles, paper industries, in waste management and in the medical-pharmaceutical industry (Soares *et al*., 2012). Cellulase is an important component of all plants and it's the world's most abundant resources replenished by photosynthesis and accounts for half of the 18-20 Mt of organic carbon annually fixed by photosynthesis (Lynd *et al*., 2002). Cellulase has the ability to catalyze the hydrolysis of cellulose and cellooligosaccharide derivatives (Chinedu *et al*., 2008). They play an important role in natural biodegradation processes in plant lignocellulosic materials (Chinedu *et al*., 2010). Proteases on the

other hand are hydrolytic enzymes with ability to catalyze cleavage of peptide bonds in other proteins into smaller peptide fragments. They are ubiquitous in nature, occurring in all plants, animals and microorganisms which account for about 40-60% of the world's total enzyme in the market (Radha *et al*., 2012). Proteases from microorganisms are the most important industrial enzymes and are predominantly extracellular which can be concentrated in fermentation medium (Ikram-Ul-Haq *et al*., 2006).

Lipases are known to catalyze the hydrolysis of long chain acyl glycerol. Lipases are serine hydrolases of physiological significance and industrial potential which can catalyze a number of reactions such as esterification, alcoholysis, aminolysis and hydrolysis (Prasad and Manjunath, 2011). The natural substrate of lipolytic enzymes are the triacylglycerols which has low solubility in water, lipases catalyses the hydrolysis of ester bonds under natural conditions and can convert triglycerides into diglycerides, monoglycerides, glycerol and fatty acids (Bisht *et al*., 2012). The metabolic activities of microorganisms are sensitive to pH changes which has marked effect on the type and amount of enzyme production. Another effect pH change might or can have is the denaturation of the enzyme, thus resulting in the loss of catalytic activity (Ikram-Ul-Haq *et al*., 2006).

The identification and dissemination of new microbial sources, mainly those which are non-toxic to humans are of high strategic interest, besides guaranteeing enzyme supply to different industrial processes and the development of new enzymatic systems which cannot be obtained from plants or animals. This study is thus aimed at determining the enzyme production of microorganisms isolated from fermented kolanut husk and testa of *Cola acuminata*.

MATERIALS AND METHODS

Sample collection: *Cola accuminata* waste was obtained from Owena-Ijesha in Osun, Osun state, Nigeria and transported to the laboratory of Microbiology Department, Federal University of Technology, Akure Ondo State for analysis. The specie was authenticated at Crop Science and Production Department, Federal University of Technology, Akure Ondo State, Nigeria.

Isolation, identification and enzyme screening of the associated microorganisms: Microorganisms were isolated and identified according to microbiological standards. Broth cultures of bacteria and fungi served as crude enzyme. The organisms were prepared in broth and incubated for 24-72 h, respectively for bacteria and fungi. The broths were after wards filtered, refrigerated and screened for α-amylase, β-amylase, protease, lipase and cellulase.

Alpha amylase: One percent soluble starch was prepared in 0.02 M sodium phosphate of pH 6.9 containing 0.006 M NaCl. The 0.5 mL of crude enzyme solution was added to 0.5 mL of substrate and incubated at 25°C for 3 min. One millilitre of DNSA was added and the mixture heated in a water bath (100°C) for 5 min. After heating, mixture was cooled and 10 mL of distilled water was added and mixed properly. The mixture was read in a spectrophotometer (Gulfex Medical and Scientific England, Spectrumlab 23A, model number 23A08215) at an absorbance of 540 nm against a blank containing buffer without enzyme. A calibration curve was made with maltose to convert the reading to unit of activity (Bernfeld, 1951).

Beta amylase: One percent soluble starch was prepared in 0.016 M sodium acetate of pH 4.8. The 0.5 mL of enzyme solution was added to 0.5 mL of substrate and incubated at 25°C for 3 min. One millilitre of DNSA was added. The mixture was then heated in water bath (100°C) for 5 min. After

which, the mixture was cooled and 10 mL of distilled water was added, then read in a spectrophotometer (Gulfex Medical and Scientific England, Spectrumlab 23A, model number 23A08215) at 540 nm against a blank containing buffer without enzyme. A calibration curve was made with maltose to convert the reading to unit of activity (Bernfeld, 1951).

Protease assay: One percent casein was prepared in 0.05 M citrate buffer of pH 7.5, the solution was heat-denatured at 100°C for 15 min in a water bath. One millilitre of 1% casein solution was pipette into test tubes. It was incubated at 37°C for 15 min, 0.2 mL of enzyme solution was added and allowed for 1 h inside the water bath. Three millilitre of 10% trichloroacetic acid (TCA) was added to terminate the reaction. The tubes were centrifuged at 300 rpm and the supernatant was read at 280 nm using UV spectrophotometer (model number 80-2102-14, serial number 58591) (Ladd and Butler, 1972).

Cellulase assay: One percent Carboxyl Methyl Cellulose (CMC) was prepared in sodium acetate buffer of pH 5.5 (0.5 M). The 0.25 mL of enzyme solution was added to 0.25 mL of the substrate solution and incubated at 37°C for 30 min. The 0.5 mL DNSA was added to the solution and heated for 5 min in a boiling water bath. The solution was allowed to cool and 10 mL of distilled water was added. The same procedure was carried out on the substrate without the addition of the enzyme solution instead of distilled water. The absorbance was read in a spectrophotometer (Gulfex Medical and Scientific England, Spectrumlab 23A, model number 23A08215) at 540 nm (Miller, 1959). The absorbance at the 540 nm obtained was extrapolated from the glucose standard curve to obtain the amount of glucose liberated.

Lipase assay: Lipase activity was assayed by modifying the method of Yong and Wood (1977). Olive oil was used as substrate. The 0.2 mL of the crude enzyme was dispensed into the test tube as well as a 0.2 mL of olive oil was added. The mixture was incubated at 40°C for 1 h. The reaction was terminated by adding absolute ethanol. The mixture was titrated with 0.1 M NaOH with phenolphthalein as an indicator. A change is observed when the mixture turns to pink. The values were read and recorded.

RESULTS

Isolates from liquid state fermented kolanut husk and testa were screened for production of α-amylase, β-amylase, protease, cellulase and lipase as shown in the Fig. 1-2. The fungal isolates included; *Trichoderma viridiae* (1 and 2), *Articulospora inflata* (3 and 4), *Aspergillus fumigatus* (5 and 6), *Aspergillus flavus* (7 and 8) and *Aspergillus niger* (3 and 4), while the bacterial isolates included; *Bacillus cereus* (A), *Micrococcus luteus* (B) *Bacillus firmus* (C), *Bacillus licheniformis* (D), *Lactobacillus fermentum* (E) *Bacillus subtilis*, (F) *Bacillus laterosporus* (G) and *Bacillus sphaericus* (H). The activities of the enzymes were measured in μmol min^{-1} mL^{-1}. The alphabet and numbers are used to represent the isolates on the figures. Odd numbers represent isolates from kolanut husk samples, while the even represents isolates form kolanut testa for the fungal isolates.

The enzyme activity of fungi isolates from kolanut husk and testa is presented in Fig. 1. It was observed that isolates 4 and 8 had the highest activity for α-amylase with 0.133 ± 0.005^{c} and 0.130 ± 0.020^{c} μmol min^{-1} mL^{-1}, respectively, while the least activity was recorded for isolate 6 with activity of 0.020 ± 0.000^{a} μmol min^{-1} mL^{-1}. Isolate 4 and 5 had the highest β-amylase activity with a value of 0.430 ± 0.000^{e} and 0.160 ± 0.020^{d} μmol min^{-1} mL^{-1}, respectively. The lowest activity was

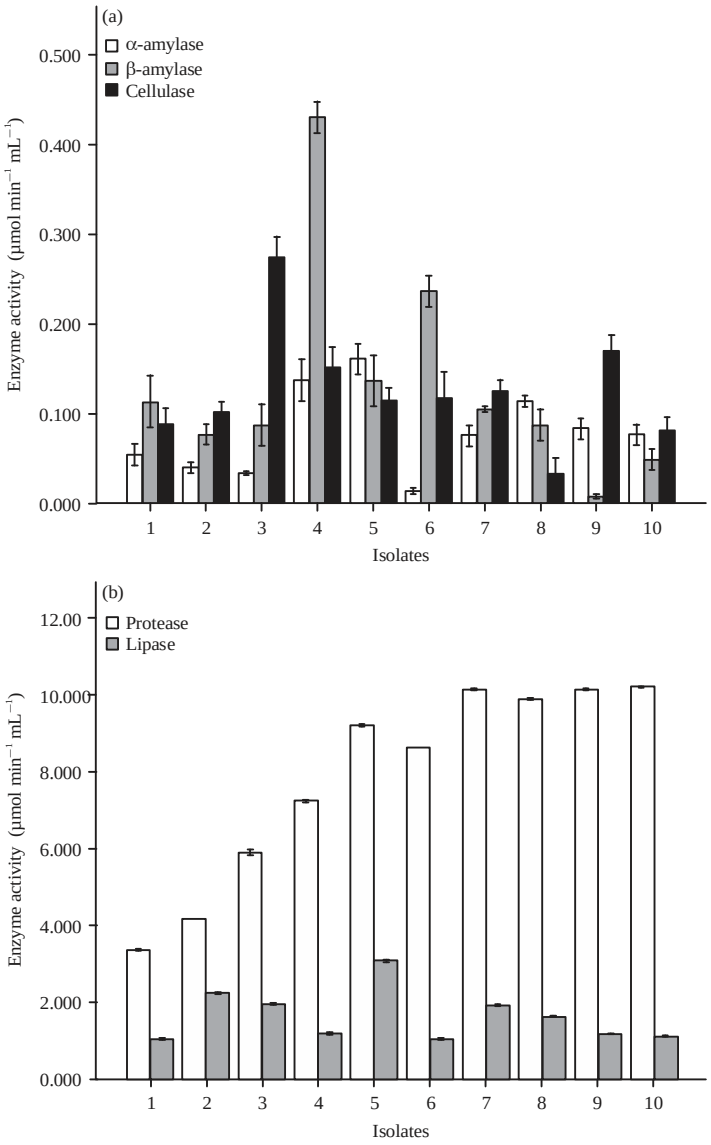

Fig. 1(a-b): Enzyme activity of fungi isolated during fermentation of kolanut husk and testa, (a) α-amulase, β-amylase and cellulase and (b) Protease and lipase. The odd numbers on the horizontal line represents isolates from the husk samples while the even numbers represents the testa, 1 and 2: *Trichoderma viridae*, 3 and 4: *Articulosporum inflata*, 5 and 6: *Aspergillus fumigatus*, 7 and 8: *Aspergillus flavus*, 9 and 10: *Aspergillus niger*

observed for isolate 9 reading 0.013±0.005^{a} µmol min^{-1} mL^{-1}. The activity of cellulase was observed to be prominent and least for isolates 3 and 2 with values 0.2560±0.015^{d} and 0.083±0.015^{a} µmol min^{-1} mL^{-1} individually. In comparing the activity of the enzymes, it was observed that β-amylase had highest activity followed by cellulase.

Figure 1b shows the activity of protease and lipase of the fungal isolates. There was no significant difference between the protease activity of isolates 7, 8, 9 and 10, all of which had

a high activity as compared to isolates 1 and 2. The values of isolates 7, 8, 9 and 10 ranged from 9.010±0.089^{g}-10.137±0.085^{i} while the minimum values ranged from 02.416±0.313^{a}, 3.336±0.289^{b} μmol min^{-1} mL^{-1}. For the lipase activity, isolates 5 and 2 had high activity of 2.267±0.289^{f} and 2.000±0.250^{e} μmol min^{-1} mL^{-1}, respectively. Whereas, isolates 1, 4, 6, 9 and 10 had minimum activity which ranged from 1.000±0.050^{a}-1.166±0.297^{b} μmol min^{-1} mL^{-1}.

The isolated bacterial enzyme activity is presented on Fig. 2. Isolates D and H had a maximum activity of 0.333±0.032^{f} μmol min^{-1} mL^{-1} for α-amylase while the minimum was recorded for isolates C and B with 0.140±0.017^{a} and 0.150±0.020^{b} μmol min^{-1} mL^{-1}, respectively. Isolate H had highest β-amylase activity while isolate F had the least activity of 0.093±0.006^{a} μmol min^{-1} mL^{-1}. Cellulase activity of isolate A and F were observed to be at upper limit of 0.383±0.015^{f} and 0.300±0.010^{e} μmol min^{-1} mL^{-1} correspondingly, while isolate D recorded the least activity of 0.053±0.006^{a} μmol min^{-1} mL^{-1}.

Figure 2b shows the protease and lipase activity of bacterial isolates form fermented husk and testa. Almost all the isolates had high activity for protease with no significant difference, which was also the case for lipase. In comparing the activity of protease and lipase by the bacterial isolates, protease was observed to have higher activity by all the isolates. An overview of the screened enzymes showed both bacterial and fungal isolates to have maximum activity for protease and lipase as compared to activity of α-amylase, β-amylase and cellulase.

DISCUSSION

Enzymes are referred to as nature's catalysts and are produced by the fermentation of biobased materials. Microbial enzymes have a greater benefit when compared to their plant and animal counterpart. Approximately 2% of the world's microorganisms have been tested to have enzyme sources (Hasan *et al*., 2006). The same microorganisms isolated from different sources, in this case husk and testa of *Cola acuminata* have varying enzymatic activity which is evident from the study.

Amylases are enzymes that break down starch or glycogen. *Bacillus* Species isolated in this study showed highest activity for α and β-amylase as compared to activity of the enzymes by the isolated fungi. This shows that *Bacillus* has the ability to break down the starch present in the samples than the fungi isolates. Although, amylases of fungi origin has been found to be more stable than bacterial enzymes on commercial scale but their production is low. If cultural condition and strain of fungi can be optimized, they will produce more (Abu *et al*., 2005). Production of amylase by the isolated organisms confirms the ability of a variety of microorganisms and other living organisms to produce the named enzymes (Pandey *et al*., 2000).

Nagamani *et al.* (2012) reported that fermentation time, temperature, inoculum age, pH and initial moisture content can have effect on the production of protease. In this research, either of afore mentioned parameters could have had effect on the enzyme activity by the organisms. Protease production by *Bacillus* species is dependent on cell growth (Olajuyigbe and Ajele, 2008). Incubation period, temperature, different carbon sources and nitrogen sources have been reported to often affect cellulase productivity by *Bacillus subtilis* (Shabeb *et al*., 2010; Xu *et al*., 2011). High lignin has been reported to make cellulose inaccessible for action by cellulase as lignin provides compressive strength, stiffens the cell wall and protects the carbohydrate from chemical and physical damages (IIyas *et al*., 2012). Cellulases have been studied to be produced by a diversity of fungus population, such as the genera *Trichoderma*, *Chaetomium*, *Penicillium*, *Aspergillus*, *Fusarium* and *Phoma*; aerobic bacteria, such as *Bacillus*, Pseudomonas, *Staphylococcus*, *Acidothermus*, *Celvibrio*, *Streptomyces* and *Xanthomonas* (Moreira and Siqueira, 2006). Anaerobic

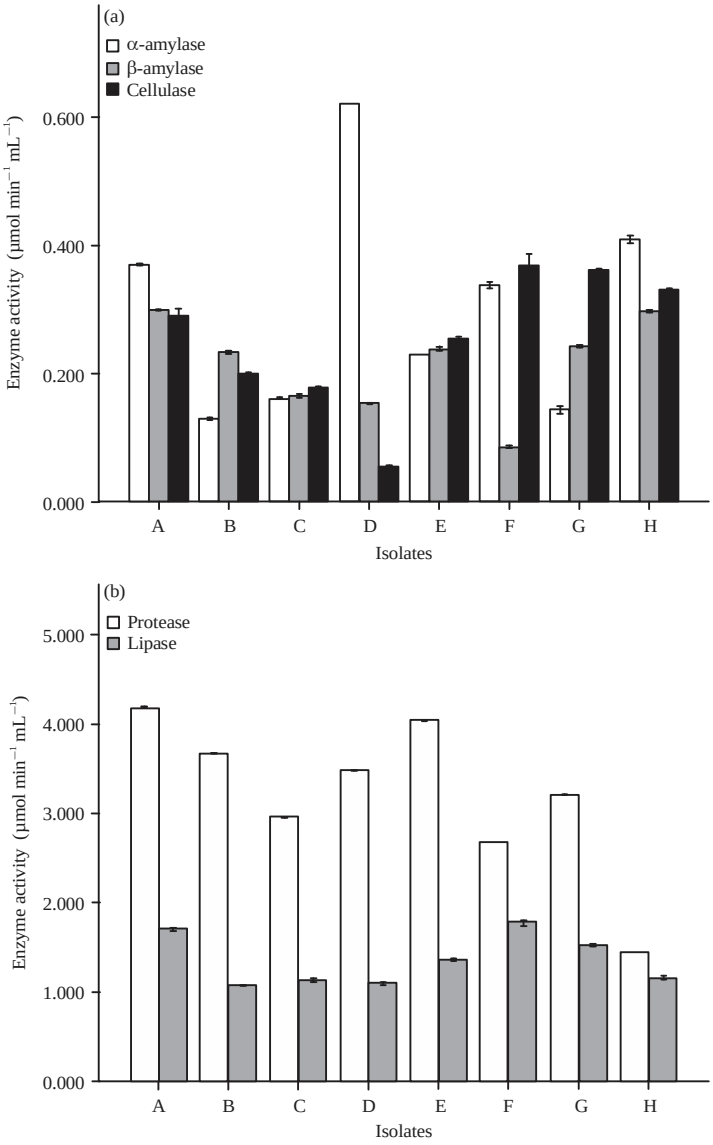

Fig. 2(a-b): Enzyme activity of bacteria isolated during fermentation of kolanut husk and testa, (a) α-amulase, β-amylase and cellulase and (b) Protease and lipase, Alphabets A, C, E, G and B, D, F, H on the horizontal line represents isolates from the husk and testa samples, respectively; A: *Bacillus cereus*, B: *Micrococcus luteus*, C: *Bacillus firmus*, D: *Bacillus licheniformis*, E: *Lactobacillus fermentum*, F: *Bacillus subtilis*, G: *Bacillus laterosporus*, H: *Bacillus spaericus*, WS: Testa sample, HK: Husk sample, β-beta, α-alpha

bacteria, such as *Clostridium*, *Eubacterium*, *Acetovibrio*, *Bacteroides*, *Butyrivibrio*, *Caldocellum*, P*seudonocardia*, *Ruminococcus* and *Thermoanaerobacter* (Zhang and Lynd, 2006) have been implicated in cellulase production, although none of these bacterial species was isolated in this study. Difference in the quantity of enzymes produced by the microorganisms may be due to the nature of cellulose or hemicelluloses, presence of some components (activators or inhibitors) in the sample (Mabrouk and El Ahwany, 2008).

Lipases and esterases are an important group of lipolytic enzymes associated with the metabolism of lipid degradation. The industrial demand for new lipase sources with different enzymatic characteristics and production at a lower cost has led to the isolation and selection of new lipolytic microorganisms from a variety of substrate and modifying the production process (Colen *et al*., 2006). For lipase production, different lipid based carbon sources have been reported as inducer as well for the production of this enzyme in large quantity (Bisht *et al*., 2012).

Generally, enzyme production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources and physical factors such as temperature, pH, incubation time and inoculum size (Muthulakshmi *et al*., 2011). Ajiboye *et al*. (2013) also reported that magnesium has been found to be an activator of many enzyme systems. Enzyme production by bacteria had being believed to be best produced using submerged fermentation but over time, solid state fermentation has been observed to be more effective which is attributed to metabolic differences and accumulation of a variety of intermediate metabolites amounting to a lowered enzyme activity and production efficiency (Subramaniyam and Vimala, 2012).

CONCLUSION

In this study, all the bacteria and fungi isolates elaborated protease and lipase with bacteria producing more than the fungi. The ability of the microbial isolates to produce protease and lipase is a clear indication that the husk and testa of the kolanut have protease and lipid as part of their composition. Therefore, fermented husk and testa of *Cola acuminata* are good sources of microorganisms that are capable of producing enzymes of biotechnological applications.

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