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Research Paper

Isolation and Molecular Detection of Aspergillus Niger from Groundnuts for the Fermentation of Agro-Allied Wastes for Improvement of Nutrients of Fish Feeds

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ABSTRACT
Agro-allied wastes are by-products or the remains of crops and animals after the main products have been utilized. They include manures, plant stalks, bedding, hulls, leaves and vegetable matter. These wastes are often discarded after consuming the edible portions, if properly utilized could be used in the production of biofuels, enzymes, vitamins, antioxidants, animal feed, antibiotics and other useful products through fermentation techniques (Pardeepet al., 2018). The present study was aimed at evaluating the effect of fermentation of selected agro-allied waste using Aspergillus niger on the nutritional quality and composition of fish feeds. The method described by (Egwim, 2014) was employed for the isolation of the organism. The organism was further identified molecularly using PCR, and Agarose Gel electrophoresis.Solid state fermentation of agro waste using the isolatewas performed by adopting the method by (Egwim, 2014). The targeted ITS 4 and ITS 5 region for the isolate was determined using 100bp Bioline Hyper Ladder.The levels of cellulose in the feed after the period of fermentation (144 hours) decrease considerably.The decrease of cellulose ranged between 68 to 87%. There was an increase in sugar levels in the substrates at 48, 96 and 144 hours of fermentation. A progressive increase in protein level shows the highest percentage increase in soya bean (45%) after 144 hours of fermentation.

***Key Words:** Microbial, Fermentation, Agro-allied, Waste.*

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I. Introduction

Agro-allied wastes are by-products or the remains of crops and animals after the main products have been utilized. They include manures, plant stalks, bedding, hulls, leaves and vegetable matter. These wastes are often discarded after consuming the edible portions, if properly utilized could be used in the production of biofuels, enzymes, vitamins, antioxidants, animal feed, antibiotics and other useful products through fermentation techniques (Pardeep*et al.*, 2018).

The extent of food wastes in Nigeria is a cause for serious concern. It is estimated that Nigeria generates more than 32 million tons of solid wastes annually, out of which only 20-30% is collected (Wale, 2019). Agricultural wastes are a renewable resource of great biotechnological potential. The value-added utilization of agricultural and agro-wastes or by-products not only offers opportunities for income generation in rural/urban areas, but also reduces the impact of environmental pollution (Adejo, 2016). Cellulose, present in most agro-wastes is a potentially valuable source of fibre, fuel and feeds (Egwim and Evans, 2018). In nature, much of the cellulose exists as waste matter from-agro-industry in the form of peels, husk, stalks and stems.

Description of Clariasgariepinus

The genus *Clarias*has four species; *Clariasmossambicus, C. lazera, C. anguaris and C.gariepinus,* and all havestriking resemblance. The African catfish *Clariasgariepinus* is a species of the order siluriformes and of the family clariidae (that is the air breathing catfish). Clariasgariepinus is a large, eel like fish characterized with a sharp tooth, dark gray or black coloration which extends dorsally as it fades to its white belly.The adult *Clariasgariepinus* has an average length of 1- 1.5m (Froese *et al.,* 2014). Also, it can develop to a maximum length of 1.7m and weigh up to 60kg (13016).

Distribution of *Clariasgariepinus*

The species *Clariasgariepinus* is mostly found in Africa and the Middle East. Its habitat is freshwater such as rivers, swamps, lakes, and also human-constructed habitat like ponds as well as cities' sewage systems. In the early 1980, the African catfish was introduced throughout the world for the purpose of aquaculture. Therefore, this species can be found in countries like Indonesia, Brazil, India and Vietnam which are far outside its original habitat.

Experimental Conditions for Feeding Habits of *Clariasgariepinus*

Like many catfish, *Clariasgariepinus* is a nocturnal fish. It may be classified as an omnivore, and still has high tendency of feeding carnivorously. Due to its wide mouth, the clariid can feed entirely on anything it comes across which is why it is often described as opportunistic feeder. It is able to consume a wide variety of food such as a whole large prey (water birds) moorhen, small fishes, insect larvae, snails, crustaceans and decomposing organic matter (Bakare, 2006). Clarias has the ability to adapt to all kinds of conditions to escape drying pools, it has the ability to crawl on dry ground, and between rainy seasons, it also has the ability to adapt to shallow mud for a long period of time.

Rearing suitability of *Clariasgariepinus* for aquaculture

The rearing of *Clariasgariepinus* began in the early 1970s in central and western Africa, as the species was discovered to be suitable for aquaculture because of the qualities it possesses. These qualities include; the ability to grow fast and feed on a wide variety of agricultural by products, its ability to tolerate poor water quality and difficult conditions, its ability to relatively reproduce in captivity and matures easily; it can withstand low oxygen and pH levels (Everest, 2017).It can be raised in high densities resulting in high net yields, high fecundity and palatability; its resistance to diseases as well as being higher in price than tilapia because it can be sold live at the market in most countries (Froese *et. al.,* 2014). The possession of these qualities by *Clariasgariepinus* makes it suitable for this research study.

Biology of *Aspergillus niger*

Classification of Aspergilli

Aspergilli are a large genus of about 300 species of mould in the family Trichocomaceae. They can be found in various habitats of the world and their growth rate is dependent by the temperature range in the environment in which they grow and the availability of moisture. Aspergilli are often regarded as conidial fungi and majority of them reproduce by asexual means of sporulation, though some have been shown to exhibit sexual reproduction. They are largely found in the soil or land (i.e. they are terricolous).

Aspergilli have become extremely important in agriculture, biotechnology, and in human health since their discovery in the 1720's (Feng wang, 2014).

Characteristics of Aspergilli

Aspergilli are found everywhere in nature with abundance of spores which predominate the air. They are saprophytes like other fungi which obtain their food from feeding on dead and decaying organic matter, though some species are capable of causing diseases to man and animals as well as causing damages to plants.

Aspergilli lack chlorophyll, and so are unable to produce their own food, but depend on other materials in their environments for feeding. They cannot absorb organic matter in their surroundings on their own, but secrete various enzymes like amylase which help to degrade the materials into simpler compounds so that they can be absorbed via the vegetative hyphae. The high amount of enzymes so released helps in the complete decomposition of organic matter in their surroundings and the availability of different sources of food which is necessary for their growth and reproduction.

Distribution of *Aspergillus niger*

Aspergillus niger are common and widely distributed in many geographic areas. They are however known to be prevalent especially in areas with extreme temperatures.

Studies have shown that the black spores of *Aspergillus niger* protect them from ultraviolet radiation of the sum which makes them adapt to warmer regions.

Reproduction in *Aspergillus niger*

Aspergillus niger belongs to the phylum Ascomycota (ascomycetous fungi), commonly referred to as sac fungi because their spores are produced within the sacs asexually (Asci)

The colonies of *Aspergillusniger* like *Aspergillus nidulans* when exposed to air as a favorable condition for growth tend to form reproductive and vegetative hyphae. The conidia (spores) are produced at the tip of the

reproductive hyphae that looks like a vesicle while the vegetative hyphae absorb nutrients from feeding on dead and decaying organic matter.

II. Materials and Method

Isolation of *Aspergillus niger*

*Aspergillus niger*was isolated from groundnuts. The isolate was sub cultured on Selective Dextrose Agar (SDA) in a Petri dish and acidified with Chloramphenicol to prevent contamination by bacteria. The sub cultured isolate was incubated at a range at normal room temperature and observe for growth (Egwim, 2014).About 65g of Saboraud Dextrose Agar (SDA) was introduced with (1 L) of distilled water into a conical flask according to the manufacturer's instruction. The mixture was autoclaved at 120℃ for 15 minutes. Six (6) test tubes containing (9mL) distilled water was also autoclaved. After autoclaving, the mixture was allowed to cool and then acidified with chloramphenicol to prevent bacterial growth.

Isolation

The sample (Groundnuts) was purchased from Sabo market, Kaduna. The sample was grinded to fine powder using a mortar. One gram (1g) of the sample was weighed using a digital weighing balance. Serial dilution was performed from the stock solution to 5 test tubes labeled10^{-1},10^{-2},10^{-3}, 10^{-4}and10^{-5}.

Morphological Characterization of *Aspergillus niger*

Aspergillus niger was identified according to colony morphology and microscopic examination. The isolate was transferred onto SabauroudDextrose Agar (SDA) Slant for species identification and was carried out in the Department of Biological Science, Kaduna State University (Isitua and Ibeh, 2010).

Molecular Identification of *Aspergillus niger*

Molecular identification of the isolate was carried out using the following techniques: DNA Extraction, DNA Amplification using PCR,Gel Electrophoresis,Purification of PCR Products,DNA Sequencing and BLAST.The DNA extraction of cells grown on Sabauroud dextrose broth at 30°C for 48 h was carried out according to the procedure described by Lachance*et al*. (2006).

The primer sequences for fungi used for the analysis were ITS4: TCCTCCGCTTATTGATATGS and ITS5: GGAAGTAAAAGTCGTAACAAGG. The size of the amplicon used was 650bp while the DNA ladder used was 50bp from NEB.The polymerase chain reaction (PCR) was performed in a cocktail mix consisting of 2.5ul of 10x PCR buffer, 1ul of 25Mm MgCl2, 1ul each of forward and reverse primer, 1ul of DMSO, 2ul of 2.5mMDNTPs, 0.1ul of 5u/ulTaq DNA polymerase and 3ul of 10ng/ul DNA. The total reaction volume was made up to 25ul using 13.4ul nuclease free water.

Agarose gel electrophoresis was performed to resolve the amplified using a standard molecular weight marker 50 bp DNA ladder from NEB as DNA marker. The amplified fragments were visualized on safe view stained 1.5% agarose electrophoresis gels. The gel was stained with ethidium bromide, visualized under UV light. The purified PCR products were sequenced using ABI PrismTM Big DyeTM Terminator Cycle sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) following the manufacturer's instructions. Sequencing was done with the same primers as used in the PCR reaction.

Solid State Fermentation

Sixty grams of dried peel was moistened with 30mL distilled water in 250mL Erlenmeyer flasks and autoclaved at 121^0C for 40minutes. one flask containing each sample was inoculated with 1mL spore suspension (109spores per mL) and incubated at 30^0C for 144hours. During the process, the samples were withdrawn at regular intervals (48,96 and 144hours) to determine cellulose, glucose and protein levels. Control was set up for each sample (Egwim, 2014).

III. Results

Molecular Identification of *Aspergillus niger*

The molecular result as presented in figure 4.1 showed that *Aspergillus niger*was subjected to molecular identification using universal primers ITS1 and ITS2 respectively. The amplified DNA of the isolate showed that *Aspergillus niger*had an amplicon size of 650bps. The blast showed that the isolate *Aspergillus niger*had 99% homologous identity.

Effect of Solid State Fermentation on Cellulosic Degradation

The levels of cellulose in the feed after the period of fermentation (144 hours) as presented in figure 2 decreased considerably. The decrease of cellulose levels was paramount in the treated wastes as compared to the control group. The decrease of cellulose ranged between 68 to 87%.

Effect of Solid State Fermentation on Sugar Yield

Figure 3 shows the increase in sugar levels in the substrates at 48, 96 and 144 hours of fermentation. The highest yield was observed in cassava as a result of the fermentation of substance by the isolate. The sugars increased were also influenced by the degradation of cellulose due to the activities of cellulosic enzymes to simple sugars.

Effect of Solid State Fermentation on Protein Yield

The progressive increase in protein level as presented in figure 4:4 shows the highest percentage increase in soya bean (45%) after 144 hours of fermentation. The isolate, *Aspergillusniger* correlated with the crude protein content as well as the biomass produced in the substrates to bring about increased protein level. The bioconversion of sugar into proteins influenced the increase level of protein in the substrates.

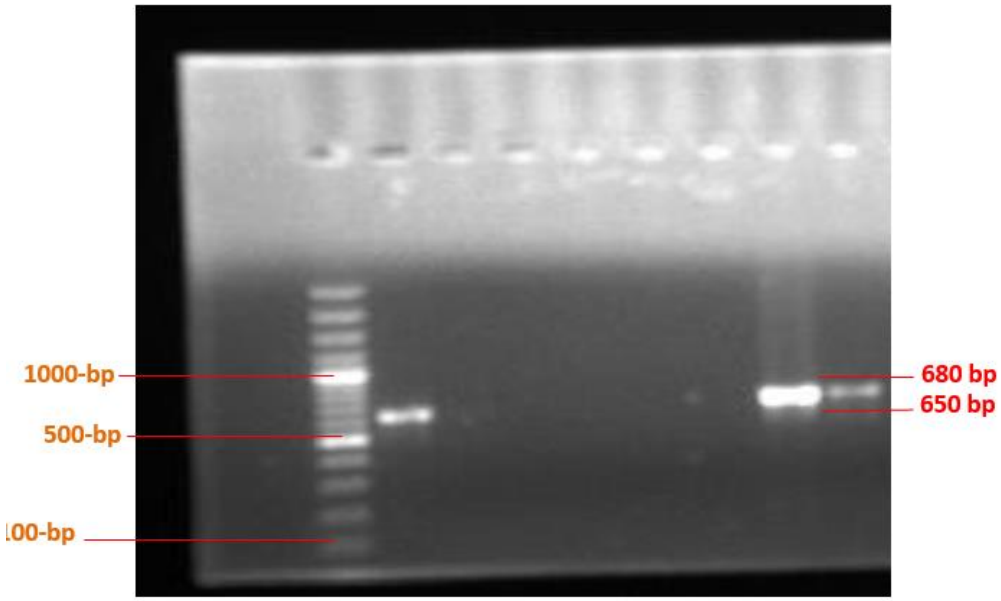

Plate 1: Agarose Gel electrophoresis of targeted ITS 4 and ITS 5 region for the isolate (*Aspergillus niger* D_2) using 100 bpBioline Hyper Ladder

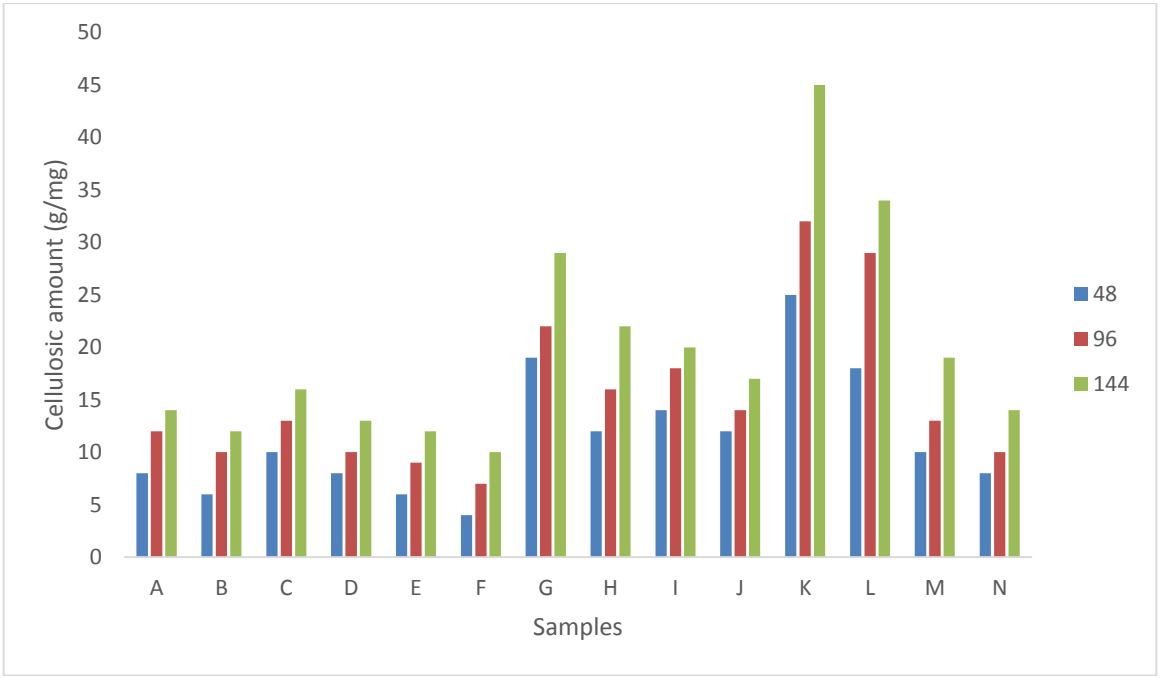

Figure 1: Effects of solid state fermentation on cellulose degradation of fermented agro wastes

Key:

A: fermented banana; B: Banana control; C: fermented pineapple; D: pineapple control; E: fermented mango; F: mango control; G: fermented tigernut; H: tigernut control; I: fermented cassava; J: cassava control; K: fermented soyabean; L: soyabean control; M: fermented plantain; N: plantain control.

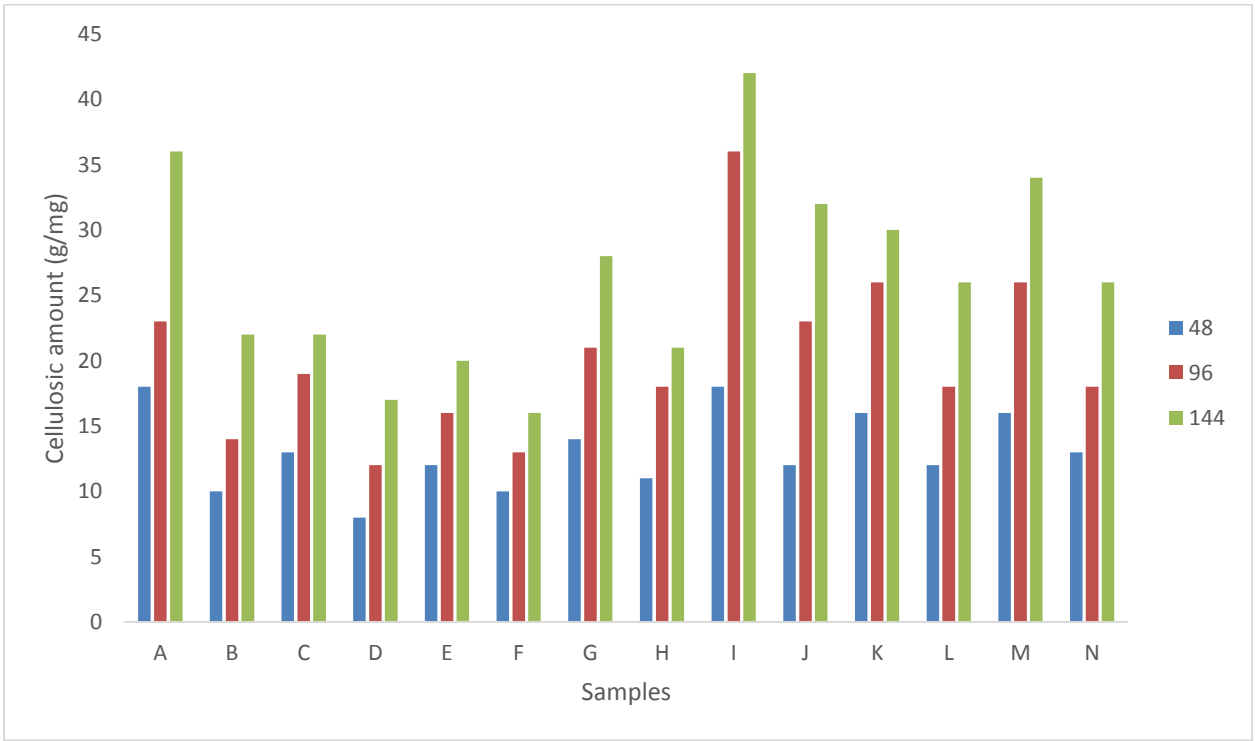

Figure 2: Effects of solid state fermentation on sugar yield of fermented agro wastes

Key:

A: fermented banana; B: Banana control; C: fermented pineapple; D: pineapple control; E: fermented mango; F: mango control; G: fermented tigernut; H: tigernut control; I: fermented cassava; J: cassava control; K: fermented soyabean; L: soyabean control; M: fermented plantain; N: plantain control

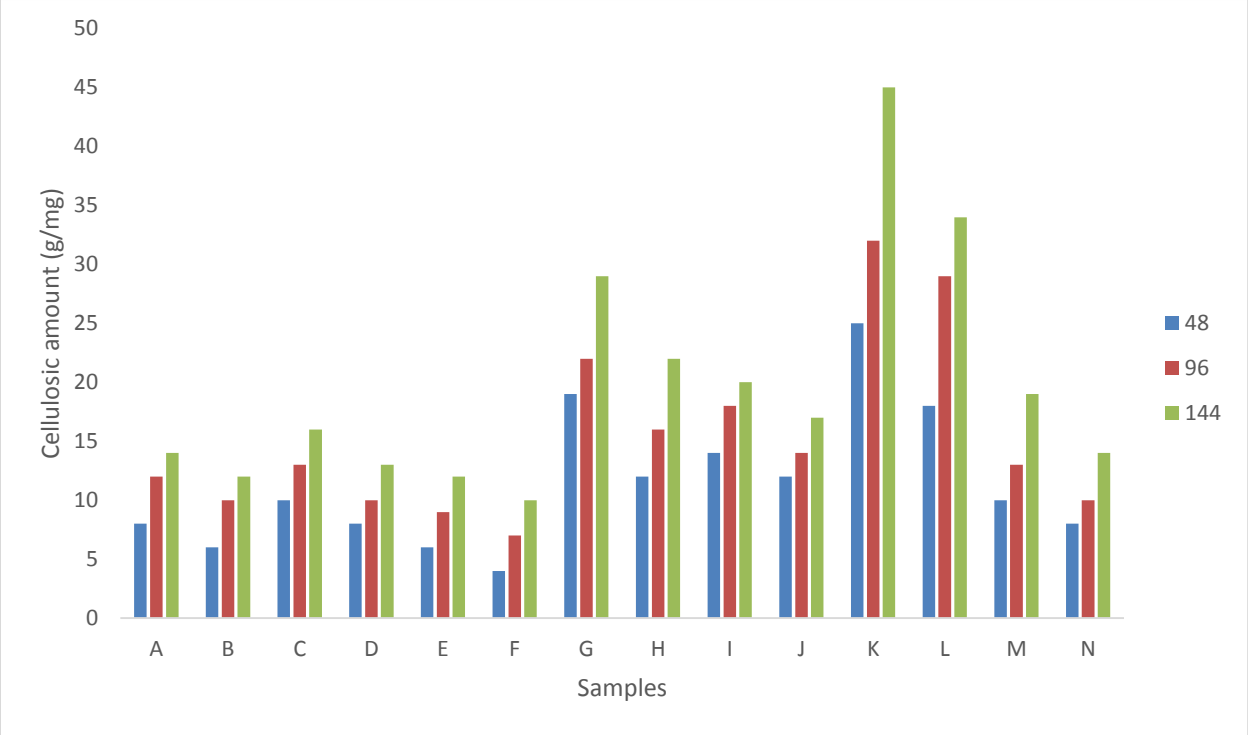

Figure 3: Effects of solid state fermentation on protein yield of fermented agro wastes

Key:
A: fermented banana; B: Banana control; C: fermented pineapple; D: pineapple control; E: fermented mango; F: mango control; G: fermented tigernut; H: tigernut control; I: fermented cassava; J: cassava control; K: fermented soyabean; L: soyabean control; M: fermented plantain; N: plantain control

IV. Discussion

Cellulose levels in the feed decreased in the substrates after 144 hours of fermentation and the decrease in cellulose ranged between 68 to 87%.The decrease in the levels of cellulose was significantly higher in the treated wastes than the untreated wastes and this is in agreement with reports byOfuya and Nwanjiuba (1990), Iyayi and Aderolu (2003), Agboro and Akinyele, (2007)and Egwim, (2014). The authors reported that as a result of solid state fermentation, over 35% of the original content of cellulose was lost in the substrates which were attributed to the ability of fungi to secrete enzymes that degrade cellulose as they multiplyon the substrates.

The enzymes so secreted also have the ability to degrade other complex carbohydrates to simple sugars within a period of 148 hours of fermentation. The optimum time required for the degradation of non-starchy polysaccharides in agro-allied wastes using *Aspergillus niger*within 2 weeks which is in agreement with findings by Iyayi and Aderolu (2003) and Ayayi and Losel (2001) for agro-industrial by – products like corn bran, rice bran, wheat offal, maize offal, brewer's dried grainsand palm kernel meal, as against 7 days by Egwim, (2014).The result of changes in sugar level ranged from 12 to (42%) with cassava observed to obtain the highest yield as a result of fermentation by the isolate. The sugar production was influenced by the decrease in cellulose due to the activities of cellulosic enzymes.

There was a progress in the increase in protein level.After 144 hours of fermentation with*Aspergillus niger*, the highest percentage increase in protein level of 45% was observed in soyabean. Akinyele and Agbro (2007) recorded (34%) highest percentage increase in protein level in Unripe and Ripe plantain peels after a period of seven (7) days fermentation using *Aspergillus niger*. Parani and Eyini (2012) reported an increase in the levels of protein by 15.8 to 22.5% with mono cultures of coffee pulp fermented with fungi.

V. Conclusion

The fungus, *Aspergillus niger* was isolated, and molecularly detected from this study. The organisms showed high degradability of complex carbohydrates to simple sugars within some days of fermentation, with increased in sugar (12-42%) as well as increase in protein level (45%) respectively.

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