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BIOCONVERSION OF LIGNOCELLULOSIC AGRICULTURAL BY-PRODUCTS BY MICROORGANISMS INTO HIGH MYCOPROTEIN FEEDS

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ABSTRACT

This research study was conducted to produce high mycoprotein feeds (HMPF) from lignocellulosic agricultural by-products through solid-substrate-fermentation. Six species of fungi were used as fermenting organisms and four agricultural wastes as substrates. The nutrient composition of the fermented wastes was determined through proximate analysis. Results of the study showed that the three macrofungi *Pleurotus* spp., *Ganoderma* spp., and *Psilocybe* spp. and the three microfungi *Aspergillus* spp1, *Trichoderma* spp., and *Aspergillus* spp2 were capable of producing HMPF through solid-substrate-fermentation of lignocellulosic agricultural by-products. Further, banana leaves, rice straw, corn cob and sugarcane bagasse are candidate substrates for the production of HMPF. Proximate analysis revealed that the crude protein (CP) of the fermented banana leaves obtained a four- to five-fold increase (24.41%-28.16%) in CP while an eight to nine-fold increase (19.66%-22.63%) in corn cobs after fermentation. The fermented sugarcane bagasse attained 11- to 13-fold (21.37%-25.83%) rise in the CP content while the fermented rice straw obtained two to five-fold increase (18.88%-29.51%) in the CP content. The ash contents (ACs) of the fermented products likewise increased while the crude fiber (CFr) and crude fat (CF) of the lignocellulosic agricultural by-products decreased after fermentation. The present results demonstrate the feasibility of utilizing lignocellulosic agricultural-wastes as substrates of fungal organisms to produce high protein feeds for animals.

Key words: protein biomass, high mycoprotein feeds (HMPF), lignocellulosic, solid-substrate-fermentation, macro and microfungi

INTRODUCTION

Agricultural wastes comprise a major proportion of agricultural production. These crop residues are made up mostly of lignocellulosic materials. These lignocellulosic crop residues are rich in dietary fibers (Valéro *et al*., 2012) which are great potential energy resources for livestock and poultry. However, they are characterized with very poor inherent

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feeding value due to low digestible dry matter and protein content (Nasehi *et al*., 2017). Likewise, these fiber-rich wastes have an enormous potential to be exploited for the production and recovery of several products and ingredients to improve animal nutrition and the worldwide supply of protein and calories for animal production in lesser environmental footprints. By using appropriate technologies, the economic value of lignocellulosic crop residues could be increased through nutrient enrichment and production of products that are safe not only for animal use but also for human feeding.

Animal production in the Philippines is a lucrative viable enterprise and progressively developing. However, the high cost of animal feeds due to the importation of expensive feed ingredients is a major problem that limits the sustainability of animal production. Aside from this, the extensive use of synthetic growth promoters and additives is now a worldwide concern because of its impact on consumers' health and disputes on multidrug resistance. While feed comprises the bulk of the total cost of production, efforts in the production of safe and cost-effective alternative feed ingredients could lead into a cost-efficient and successful animal production by not compromising the quality of feeds, nutrition of animals and health of consumers.

Microbial degradation of lignocellulosic materials brings a variety of changes in their bio-physicochemical properties. Microbial treatment can enhance the digestibility of various agricultural residues. Filamentous fungi are potential candidates that can improve the nutritional quality of lignocellulosic residues by degrading lignin with the use of the complex extracellular cellulolytic enzymes and converting these complex polysaccharides into simple sugars (Singh and Kumar, 2015). The use of fungal fermentation to cycle and recycle these residues will not only result in the reduction of pollution but also help to produce low cost and high-quality protein biomass. Further, production of protein biomass from fibrous wastes is an eco-friendly approach to recovering renewable energy resources from agri-crop production into valuable low-cost products. This research was conducted to produce high mycoprotein feeds from lignocellulosic agricultural by-products through solid-substrate-fermentation. Specifically, it aimed to identify species of filamentous macro and microfungi that could produce mycoprotein feeds and determine the proximate composition of the mycoprotein feeds produced from different agricultural by-products.

MATERIALS AND METHODS

Four independent studies were conducted utilizing four agricultural by-products as substrates and six fungal organisms. The agricultural by-products were sugarcane bagasse, rice straw, corn cobs and banana leaves. Each of the study was set-up with five replications to produce high mycoprotein feeds.

A 39 g potato dextrose agar (PDA) basal medium (TM Media) was weighed using a toploading analytical balance (National LCS-3000, 1200gx0.1g, Nagata Scale Co., Ltd, Taiwan). It was placed in a beaker and dissolved in 1 Li of distilled water. The beaker was placed on top of a hot plate (All American™ Corning™ PC-420 Fisher Scientific, USA) stirred continuously until the PDA was completely dissolved. A 20 ml mixture was dispensed in sterilized flat bottles. The bottles were covered immediately with cotton wool and aluminum foil to reduce contamination. Then, the bottles were sterilized for 15 min using an autoclave (All American™ Stove Top, Wisconsin Aluminum Foundry, USA) maintaining a pressure of 15 psi. After sterilization, the bottles were slanted and allowed the

basal medium to cool down and solidify.

The three macrofungi (*Pleurotus* spp., *Ganoderma* spp., *Psilocybe* spp.) were kindly given by Mr. Benjie L. Garcia of the Central Luzon State University and the three microfungi (*Aspergillus* spp1, *Trichoderma* spp., *Aspergillus* spp2) were obtained from the Department of Agriculture-Philippine Center for Postharvest Development and Mechanization (DA-PhilMech).

Sub-cultures of the six fungal organisms were prepared from pure cultures. Before inoculation, the glass chamber was surface sterilized to minimize contamination. Each pure culture of the fungal organisms was sub-cultured one at a time. The inocula of the pure culture of macro and microfungi were aseptically transferred to flat bottles containing the basal medium using an inoculating loop. After inoculation, the flat bottles containing the inoculants were properly labeled and arranged on the shelves and incubated at room temperature for 5 to 7 days until full mycelial ramification of the fungal mycelium is evident on the surface of the basal medium.

A standard nutrient solution (SNS) based on that used by Pham *et al*. (1992) with some modifications by Demo-os *et al*. (2013) were prepared. Urea, ammonium sulfate, ammonium phosphate, vinegar and sugar were dissolved and mixed in water.

The sugarcane bagasse was obtained from a sugarcane producer and processor in Sta. Ignacia, Tarlac while the banana leaves were obtained in San Jose, Tarlac. The corn cob, on the other hand, was obtained from Pindangan 2nd, Camiling, Tarlac and the rice straw was obtained in Sta. Ignacia, Tarlac. The fibrous agricultural by-products were shredded using a shredding machine, sundried and used as substrates. In the preparation of the substrates, the shredded agricultural by-products were weighed, and the SNS was mixed with the shredded agricultural by-products. For the corn cob, a ratio of 60 SNS:40 corn cob substrate was used while a ratio of 50 SNS:50 substrates was used for sugarcane bagasse, rice hay and banana leaves. The SNS was gradually added to the fibrous sources individually and mixed thoroughly until a uniform mixture was attained. The substrates were packed in an equal quantity of 1 kg in polypropylene bags fitted with polyvinyl chloride (PVC) necks. The mouth of the PVC was covered with cotton wool and used paper and tightly sealed with a rubber band to avoid contamination. The bagged substrates were sterilized in an autoclave for 30 minutes maintaining a pressure of 15 psi. The sterile substrates were placed in a tray to cool down and were arranged on the shelves before inoculation.

The substrates were inoculated using the previously prepared sub-cultures of the six fungal organisms. Each of the cultures was aseptically inoculated in the substrates using an inoculating loop. After inoculation, the substrates containing the inoculum were properly labeled, placed in fermenting shelves and incubated for 21 days at room temperature. Every week, the inoculated substrates were kneaded to mix the fungal colonies in the substrate for efficient colonization and fermentation.

After 21 days of incubation, the resulting products of fermentation were kneaded and were removed individually from the propylene bags, placed in trays and sun-dried to about 90% dry matter.

After sun-drying, a 250 g fermented product was compositely sampled from the five replicates of each substrate, placed in ziplock plastics and labeled for proximate analysis. The finished products were brought to the Department of Agriculture-Regional Feed Chemical Analysis Laboratory in San Fernando City, Pampanga for the analysis of the nutrient composition in terms of moisture content (MC), crude protein (CP), crude fat (CF),

ash content (AC), crude fiber (CFr) and dry matter (DM). Data were gathered from the average of two runs of proximate analyses of the same analytical condition.

RESULTS AND DISCUSSION

The results of the proximate analysis of banana leaves before and after fermentation by macrofungi is shown in Figure 1. It shows that unfermented banana leaves contain 4.07% CP, 1.87% CF, 41.43% CFr, 10.00% AC and 91.00% DM. The fermentation of *Pleurotus* spp. brought a four-fold increase in the crude protein. After fermentation, the CF decreased by 0.70% which is an indication of the use of fats by *Pleurotus* spp. as a source of energy. Moreover, a 0.55% rise in AC indicating an increase in the inorganic components of the fermented banana leaves. A lower %MC brought the high %DM. Similar results were observed in *Ganoderma* spp. and *Psilocybe* spp. when used as fermenting organisms. A remarkable four-fold increase in the protein content was obtained after 21 days of fermentation. The CF decreased by 0.57% and 0.59% for both fungal organisms, respectively. The ACs of the fermented products improved by 0.33% and 0.73%, respectively. DM content was 84.70% and 86.10%, respectively. Proximate analysis of the banana leaves fermented by microfungal species is presented in Figure 2. Fermentation of banana leaves by *Trichoderma* spp. and *Aspergillus* spp2 brought a four-fold increase in the CP of fermented banana leaves while a five-fold CP was attained when *Aspergillus* spp1 was used in fermenting the banana leaves. A slight decline in the %CF

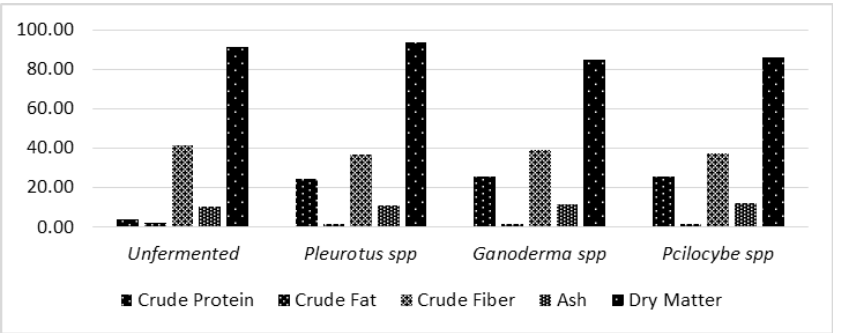

Figure 1. Proximate analysis of banana leaves before and after fermentation by macrofungi for 21 days at room temperature.

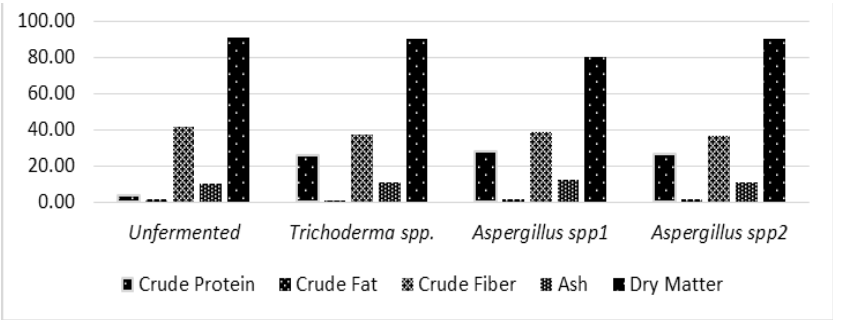

Figure 2. Proximate analysis of banana leaves before and after fermentation of microfungi for 21 days at room temperature.

of banana leaves was observed after fermentation by the three microfungal organisms. The CFr of fermented banana leaves decreased by 3.83% for *Trichoderma* spp., 2.76% for *Aspergillus* spp1 and 5.09% for *Aspergillus* spp2 when compared to unfermented banana leaves having a CFr of 41.43%. The AC increased by 1.23% in *Trichoderma* spp.-fermented banana leaves while 2.02% and 1.15% in the two *Aspergillus*-fermented banana leaves. There was a slight decline in the DM of the banana leaves fermented by *Trichoderma* spp. and *Aspergillus* spp2 while an 11.10% DM was attained in banana leaves fermented by *Aspergillus* spp1.

The results of the proximate analysis of corn cobs before and after fermentation of macrofungi is shown in Figure 3. It shows that unfermented corn cobs contain 2.09% CP, 0.8% CF, 41.90% CFr, 1.87% AC and 90.70% DM. The high organic matter, particularly the CFr, is an indication of high degradability and can be used as substrates for microbial fermentation converting complex carbohydrates into protein by several species of microorganisms. A nine-fold increase in the CP content was attained from the fermented corn cobs after 21 days of fermentation by *Pleurotus* spp. at 21.11%, *Ganoderma* spp. at 21.69% and *Psilocybe* spp. at 19.66%. The CF decreased by 0.63%, 0.49% and 0.35%, respectively, which indicates that the macrofungi utilized CF during the process of fermentation.

Moreover, a 0.5% rise in AC indicates an increase in the inorganic components of the fermented banana leaves. A lower %MC brought the high DM. Similar results were observed in *Ganoderma* spp. and *Psilocybe* spp. when used as fermenting organisms. A remarkable four-fold increase in the protein content was obtained after 21 days of fermentation. The CF of the corn cob decreased by 3.13%, 2.16% and 4.43% after fungal fermentation by *Pleurotus* spp., *Ganoderma* spp. and *Psilocybe* spp., respectively. There was also an improvement in AC of fermented corn cobs at 1.71%, 1.77% and 1.60% respectively, as compared to the unfermented corn cobs. The DM of *Pleurotus* spp. was 81%, 77% for *Ganoderma* spp. and 95% for *Psilocybe* spp.

On the other hand, Figure 4 shows that fermented corn cob by microfungal organisms had resulted in eight- to nine-fold rise in the %CP content of the fermented corn cobs. The CF content decreased by 0.43% when *Trichoderma* spp. was used in fermenting the corn cob while 0.64% and 0.76% decrease in CF when corn cob was fermented by *Aspergillus* spp1 and *Aspergillus* spp2, respectively. The CFr of fermented corn cob decreased by 2.51% for *Trichoderma* spp., 1.90% for *Aspergillus* spp1 and 3.79% for *Aspergillus* spp2 when compared to unfermented corn cob having a CFr of 41.90%. There was also a rise is the AC of the fermented corn cobs by the three fungal organisms compared with the unfermented substrates. The DM of the fermented corn cobs were 86.60% for *Trichoderma* spp., 83% for *Aspergillus* spp1 and 86.60% for *Aspergillus* spp2.

The results of the proximate analysis of unfermented and fermented sugarcane bagasse are shown in Figures 5 and 6. It shows that the unfermented sugarcane bagasse has very low CP, CF and AC but a very high DM constituting about 91.10% DM. After fermentation, there was a 12- to 13-fold increase in the CP content of the fermented sugarcane bagasse. The AC was likewise escalated from 2.41% unfermented sugarcane bagasse to 5.87%, 6.40% and 7.67% after fermentation by *Psilocybe* spp., *Ganoderma* spp., and *Pleurotus* spp., respectively. The CF of the fermented sugarcane bagasse, on the other hand, decreased when compared to the unfermented one whereas similar results were attained in the CFr of the unfermented sugarcane bagasse after fermentation. The fermentation of sugarcane bagasse by the microfungal organisms resulted to an 11- to 13-fold significant

increase in the CP and a 1.5 to 1.8-fold rise in the AC with a percent increase of 3.28% for *Trichoderma* spp., 4.16% for *Aspergillus* spp1 and 3.49% for *Aspergillus* spp2. The CF and the CFr of the fermented sugarcane bagasse also decreased.

Figures 7 and 8 show a snapshot of the proximate analysis of unfermented and fermented rice hay by several species of fungal organisms. The figures reveal that the unfermented rice straw has 5.60% CP, 0.99% CF, 37.14% CFr, 15.16% AC and 91% DM. After 21 days of fermentation, there was a two- to five-fold increase in the CP content of fermented products. Among the macrofungal isolates, *Psilocybe* spp. had produced the highest CP content of the fermented rice straw at 29.51%. This was followed by *Ganoderma* spp. with 22.09% CP and *Pleurotus* spp. with 19.83% CP. The CFr of rice straw fermented by *Pleurotus* spp. was 37.16%, *Ganoderma* spp. was 34.41% and *Psilocybe* spp. was 32.40%. The ACs of the fermented rice straw was 16.28%, 17.84% and 16.09%, respectively, slightly higher when compared to 15.16% of the unfermented rice hay. The DM of the fermented rice straw was 95.60% for *Pleurotus* spp., 86.90% for *Ganoderma* spp. and 93.20% for *Psilocybe* spp. Figure 8 further discloses that the fermentation of the three microfungal isolates resulted in a 2.37 to a 2.73-fold increase in the CP content of the fermented product. The CP of rice straw fermented by *Trichoderma* spp. was 20.19%. *Aspergillus* spp1 fermentation resulted in 18.88% CP and 19.65% CP by *Aspergillus* spp2. There was also an increase in the ACs of the fermented products that ranged from 0.93% to 2.68% by the three isolates. The CFr of rice straw fermented by *Trichoderma* spp. was 34.66% which was lower by 2.46% compared

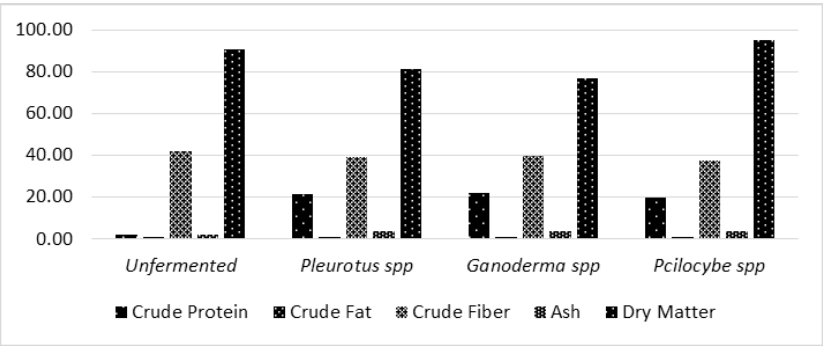

Figure 3. Proximate analysis of corn cob before and after fermentation of macrofungi for 21 days at room temperature.

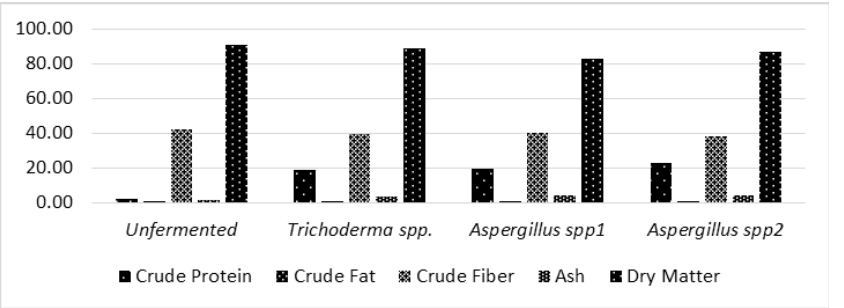

Figure 4. Proximate analysis of corn cob before and after fermentation of microfungi for 21 days at room temperature.

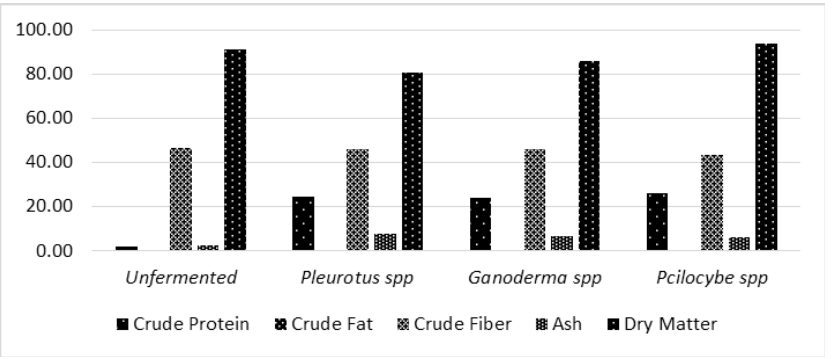

Figure 5. Proximate analysis of sugarcane bagasse before and after fermentation of macrofungi for 21 days at room temperature.

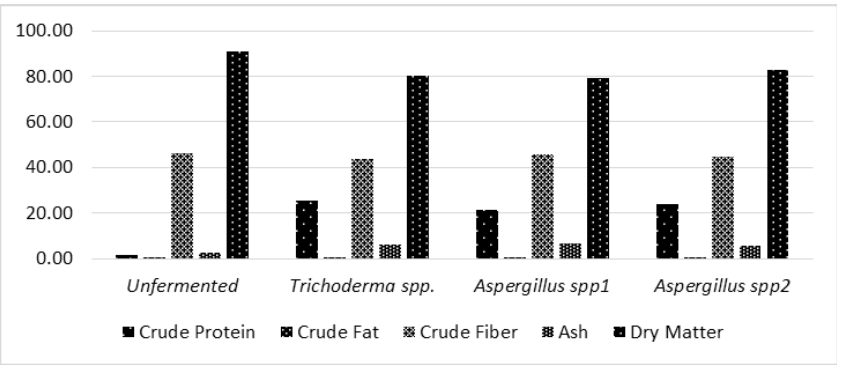

Figure 6. Proximate analysis of sugarcane bagasse before and after fermentation of microfungi for 21 days at room temperature.

to the unfermented one. The CFr obtained from *Aspergillus* spp1 fermented rice straw was 36.29% while rice straw fermented by *Aspergillus* spp2 attained 36.99% CFr.

Production of inexpensive high protein biomass from agricultural crop residues plays a substantial role in improving not only the economic utilization of agricultural by-products but also a tangible solution in replacing imported feed ingredients for animal production. Agricultural by-products like rice straw, sugarcane bagasse, banana leaves and corn cobs contain biologically important nutrients that require microbial bioconversion through biotechnological approaches for the efficient utilization of animals. Most of the agricultural crop residues used in the present study are generally made up of complex carbohydrates like cellulose, hemicellulose and lignin that are difficult to degrade. The physicochemical composition of crops residues is dependent on the types. In the case of rice straw, it is composed mainly of cellulose, hemicellulose, lignin, ash and other extractives. It contains on average between 30% to 45% cellulose, 20% to 25% hemicellulose, 15% to 20% lignin, as well as some minor organic compounds. Rice straw is poor in nitrogen, but relatively high in inorganic compounds, often referred to as ash (Boschma and Kwant, 2013). On the other hand, sugarcane bagasse, a by-product after extracting the juice for sugar production has similar chemical constituents to rice straw. However, it varied in percent composition. The cellulose, hemicellulose and lignin constitute 90% of the dry weight of the fiber (Rezende *et al*., 2011). The ash content is low which implies that the non-fiber extractive compounds

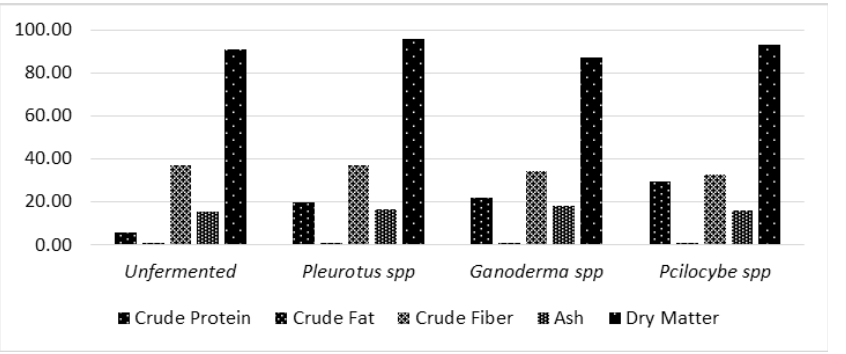

Figure 7. Proximate analysis of rice straw before and after fermentation of macrofungi for 21 days at room temperature.

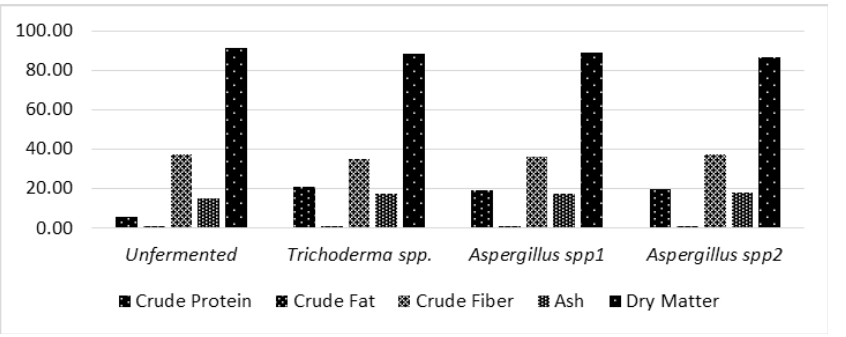

Figure 8. Proximate analysis of rice straw before and after fermentation of microfungi for 21 days at room temperature.

represent most of the dry weight of the sugarcane bagasse.

Furthermore, the polymeric fiber composition of corn cobs is made up mainly of monomeric molecules. Cellulose is made up of C6 sugars while hemicellulose is mainly made-up of the C5 sugars xylose and arabinose. Lignin consists of phenolic macromolecules (Pointner *et al*., 2014). Reports on the proximate composition of banana leaves revealed that it also contains a high amount of lignocellulosic materials mainly cellulose, lignin and hemicellulose. Banana leaves contain approximately 12% ash with very high organic matter of around 83% and around 5% carbon and very low nitrogen at 0.2%. However, the protein contents of these crop residues were found low. The high organic matter of the agricultural crop residues implies their high degradation potentials through enzymatic hydrolysis by several species of microorganisms. However, Mosier *et al*. (2004) and Menon and Rao (2012) revealed that the cellulose, hemicellulose and lignin that are embedded in a complex matrix are very resistant to enzymatic degradation. Screening and selection of several species of fungal organisms that are highly efficient in the biodegradation and conversion of the complex carbohydrates into protein are essential. Filamentous microfungi produce fibrous materials that can be easily converted to textured food products with protein content as high as 30% to 50%. The biomass produced by filamentous fungi can be used as food for animals without any further processing because it provides carbohydrates, lipids, minerals, vitamins and proteins. Also, nucleic acid contents of fungal protein are lower than that of yeast and bacteria (Chahal, 1982). White rot macrofungi, on the other hand, are efficient

lignocellulolytic decomposers capable of metabolizing plant cell constituents particularly cellulose, hemicellulose and lignin by their enzymes (Eriksson *et al*., 1990). Many species are capable of degrading lignin and can improve the nutrient composition of fodder for ruminant nutrition (Howard *et al*., 2003).

The intricate enzyme systems bring the ability of fungal organisms to degrade complex carbohydrates. According to Dashtban *et al*. (2009), the bioconversion of lignocellulosic residues could be done by microorganisms like fungi and bacteria that are capable of degrading lignocellulolytic materials through enzymatic hydrolysis. The conversion of cellulosic biomass to fermentable sugars requires the synergistic action of three cellulolytic enzymes namely 1,4 endoglucanase, -1,4 exoglucanase and -1,4 glucosidase. The most extensively studied cellulases are those produced by efficient lignocellulose-degrading fungi, particularly *Trichoderma* (Narsimha *et al*., 2006) and *Aspergillus* spp. (Baig, 2005). Such innate characteristic is an opportunity in the utilization of these organisms to produce protein biomass. Sibtain *et al*. (2017) revealed that using *Trichoderma harzianum* to produce fungal biomass protein from rice polishing resulted to a maximum of 49.50% crude protein, 32.00% true protein, 19.45% crude fiber, 9.62% ash, 11.50% cellulose content and 0.325% RNA content. The profile of amino acids of the final fungal protein biomass (FPB) exhibited that all essential amino acids were present in great quantities. The *T. harzianum* produces FPB with high nutritional value suitable as supplement for poultry animals.

It was reported by Patyshakuliyeva *et al*. (2016) that fungi such as *Trichoderma reesei* and *Aspergillus niger* produce enormous amounts of extracellular cellulolytic enzymes which is found to have synergistic interaction in degrading cellulose like the endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases. Meanwhile, similar enzymes called cellulosome associated with the cell wall are also secreted by bacteria and strains of fungal anaerobes capable of degrading cellulose. In basidiomycetes, lignocellulose degradation could be attributed to having unique oxidative systems and cellulolytic and hemicellulolytic activities (Dashtban *et al*., 2009).

Aspergillus niger and *Aspergillus oryzae* are also the most commonly used industrial *Aspergillus* species for the production of pharmaceuticals, food ingredients and enzymes (Berka *et al*., 1992; Pandey *et al*., 1999). *A. niger* and *A. oryzae* produce a broad range of enzymes related to the degradation of plant polysaccharides, such as cellulose, xylan, xyloglucan, galactomannan and pectin (de Vries and Visser, 2001). These enzymes are essential in converting natural carbon sources into smaller molecules easily permeable to cells. *A. oryzae* also produces some enzymes like proteases which rapidly reduce the viscosity of gelatin solutions and cause rapid digestion of the lower molecular weight components of gelatin have been detected in the culture filtrate. Esterase, phosphatase, amylase, sucrase and catalase were also liberated from the mycelium in sufficient quantity for convenient estimation. Enzymes catalyzing the liberation of inorganic phosphate from phytic acid and lecithin, various dipeptidases, lactase, maltase, salinase, nuclease and urease were also detected. White rot fungi is also an alternative and safe source of biologically active extracellular cellulolytic enzymes. It was reported that *Ganoderma* spp. possesses three major families of fungal lignin-modifying enzymes (LMEs) (Thurston, 1994). These include laccases, manganese-dependent peroxidases (MnPs) and lignin peroxidases (LiPs) (Youn *et al*., 1995). It is believed that these enzymes can degrade lignin that can be used in degrading lignocellulosic material as a renewable resource for the production of paper products, feeds, chemicals and fuels. These LMEs can oxidize phenolic compounds thereby

creating phenoxy radicals, while non-phenolic compounds are oxidized via cation radicals. LiP and MnP oxidize non-phenolic aromatic compounds with high oxidation-reduction potentials, the major components of the lignin polymer. Laccase oxidizes non-phenolic aromatic compounds with relatively low oxidation-reduction potentials (Kirk *et al*., 1987).

Reports also revealed that several species of *Pleurotus* spp. are among the most efficient in utilizing lignocellulosics (Zhang *et al*., 2002; Salmones *et al*., 2005; Albores *et al*., 2006). Similar studies on three *Pleurotus* spp., namely, *P. florida*, *P. ostreatus*, and *P. sajor-caju* for cellulolytic enzymes production showed that *P. florida*, produced the highest levels of enzyme activity, indicating high production of extracellular cellulases under optimum cultural and nutritional parameters using submerged fermentation conditions (Goyal and Soni, 2011).

Based from the results of the study, the three macrofungi *Pleurotus* spp., *Ganoderma* spp., and *Psilocybe* spp. and the three microfungi *Aspergillus* spp1, *Trichoderma* spp., and *Aspergillus* spp2 are capable of producing mycoprotein feeds from agricultural wastes of banana leaves, rice straw, corn cobs and sugarcane bagasse with an increase in CP content through solid-state fermentation. Further study could be conducted by varying moisture content of the substrates, inoculum size, and duration of fermentation, C/N sources concentration, qualitative protein and lipid content analysis, enzymatic analysis and feeding trials in livestock and poultry animals.

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