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Comparative Study on Fungal Pretreatment and Hydrolysis of Cassava Peelings and Rice Husks for Second-Generation Bioethanol Production

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Abstract Fluctuating oil prices and its increasing environmental concerns have revived widespread interest in production of biofuel from renewable (lignocellulose) materials. Rice husk and cassava peelings (agro-wastes with little or negligible values to industries in Ghana) were evaluated as a substitute cost effective feed stock for bioethanol production. This project investigated second-generation bioethanol production by pretreating and hydrolysing agro-waste using *Pleurotus ostreatus*, *Aspergillus niger* and a combination of the two fungi. The various hydrolysates obtained were subsequently fermented to ethanol using *Saccharomyces cerevisiae*. The analysis of lignocellulose fractions was conducted using van Soest refractometer whilst fermentable sugars and bioethanol produced were analysed using gravimetric method. The combination of the fungi gave a better yield of fermentable sugars compared to the yield obtained from hydrolysis by either *P. ostreatus* or *A. niger*. Of the two fungi, *P. ostreatus* hydrolysis of rice husk and cassava peelings gave optimum fermentable sugar concentrations of 2.0 g/L and 34.11 g/L respectively, which were higher than 1.33 g/L and 28.64 g/L obtained from *A. niger* hydrolysis of rice husk and cassava peelings, respectively. The combination of the two fungi for hydrolysis gave the best results for fermentable sugar of 3.0 g/L and 36.51 g/L for rice husk and cassava peelings respectively, for equal weights of the two substrates. The fermentations results revealed that the maximum ethanol yields for cassava peelings and rice husk were 19.36% and 1.53% (w/w dry biomass), respectively. Hence, it can be concluded that cassava peelings can serve as a better feedstock for production of second-generation bioethanol.

Keywords Second-generation bioethanol, Lignocellulose, Fermentation, *Pleurotus ostreatus*, *Aspergillus niger* and *Saccharomyces cerevisiae*

1. Introduction

An alcohol made by fermentation, mostly from carbohydrates produced in sugar or starch crops is termed as bioethanol. Bioethanol is a product of microbial fermentation as opposed to synthetic ethanol produced from petrochemical sources. The fermentation of glucose or other sugars in biomass produces ethanol and carbon dioxide expressed as:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2CO_2 + \text{heat} \quad (1)$$

Ethanol is a colourless, clear liquid with a mild characteristic odour. It is volatile, miscible in both water and non-polar solvents at ordinary conditions with a density of 0.792 g/cm^3 at 15.5°C [1]. It has a boiling point of 78°C and freezes at -112°C. It is biodegradable, low in toxicity and creates hardly any environmental pollution if spilled.

Bioethanol has been used for many years for various purposes, like a starting material for the production of chemicals such as butanol, acetaldehyde, acetic acid, beverages, and also as an alternative fuel [2]. The forms in which bioethanol can be utilized as transportation fuels include: anhydrous ethanol (100% ethanol), hydrous ethanol (95% ethanol and 5% water), anhydrous ethanol-gasoline blend (10–20% ethanol in gasoline) [3]. Presently, in some advanced countries like USA, ethanol is blended with gasoline to serve as transportation fuel. The most common blend is 10% ethanol and 90% gasoline. Vehicle engines require no modifications to run on this blend. However only flexible fuel vehicles (FFV) can run on up to 85% ethanol and 15% gasoline blends [4].

There are three main types of raw materials that can be used for bioethanol production; (i) sucrose – containing raw materials, (ii) starch - containing raw materials and (iii) lignocellulose raw materials. The first two groups are known as “first-generation” and the last one is referred to as “second-generation”. Second-generation bioethanol refers

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to ethanol produced by biological fermentation of residual biomass [5]. It is expected that future demand for bioethanol is likely to increase. Given such a potentially enormous market in the coming years, prominence is now being placed on lignocellulose biomass as the main raw material for bioethanol production instead of sucrose and starch containing raw materials.

Lignocellulose biomass as the most abundant biopolymer on earth is a potential renewable resource for the production of bioethanol. Lignocellulose biomass is an attractive raw material for bioethanol production because of its advantages over first-generation feedstock. The first-generation bioethanol is obtained from main food sources (e.g. corn, rice, sorghum, sugar cane). Apart from competing with food production, bioethanol production from these feedstocks can cause high production prices that limit their industrial production [6]. Lignocellulose biomass lessens the potential conflict of feedstock for bioethanol production and food resource. It is also cheap, renewable and ubiquitous on earth. [7]. These factors make lignocellulose biomass preferably suitable for bioethanol production. The production of second-generation bioethanol typically consists of the following steps; pre-treatment, enzymatic hydrolysis, fermentation and downstream processing.

The main objective of this work is to assess the potential production of bioethanol from lignocellulose agricultural waste (cassava peelings and rice husk) materials in Ghana.

2. Materials and Method

2.1. Sources of Experimental Materials

The cassava peelings were obtained from cassava dumpsite in Kumasi, Ghana. The rice husks were obtained from a rice farm at Akuse in the Greater Accra Region of Ghana. Spawn of *P. ostreatus* k910 used for biological pretreatment/enzymatic hydrolysis was obtained from Robart Farms, Kumasi. *A. niger* Mena1E was also used for biological pretreatment and enzymatic hydrolysis and was obtained from the Pathology Laboratory of the Faculty of Agriculture, Kwame Nkrumah University of Science and Technology, Kumasi. Baker's yeast used for ethanol fermentation was acquired from Saf-instant, Wisconsin, USA.

2.2. Preparation of *A. niger* Spore Suspension for Hydrolysis

Under aseptic conditions, 20 mL of the sterile potato dextrose agar (PDA) acquired from Oxoid (CM1055) Fischer Scientific, Waltham MA, U.S.A was poured into sterilized petri dishes and inoculated with pure cultures of *A. niger*. The *A. niger* was subjected to lactophenol cotton blue staining, which confirmed the morphology and spore colouration characteristic of the species. A sterile inoculation pin was used to pick the spores from the pure culture unto the PDA in eight replicates. The inoculated Petri plates were incubated at room temperature for four days after which spores of the mold were collected by flooding the cultures with 20 mL sterile distilled water to dislodge the pores. The spore suspensions were then transferred into a sterile 250 mL Erlenmeyer flask and kept for further use. Serial dilutions of 1/10,000 mL were prepared. The diluted suspension was mixed thoroughly, a drop was sampled with a pipette, and the cell population was determined using a haemocytometer and light microscope.

2.3. Preparation of Stock Culture of *S. cerevisiae* for Ethanol Fermentation

Under aseptic condition, 2 g of dry baker's yeast was transferred into 10 mL of sterile nutrient broth in a McCartney bottle. The bottle was shaken rigorously for even distribution of the cells and incubated for 48 h for activation. Aseptically, an aliquot (0.1 mL) of the cell suspension was then used to inoculate yeast extract agar and the plates incubated at 30°C for 48 h. Pure colonies were subcultured on yeast extract agar and the pure culture was maintained on agar slants at 4°C [8]. Pure culture of a single colony of the yeast growing on the yeast extract agar plate was subsequently inoculated into 50 mL of sterile nutrient broth in a 100 mL Erlenmeyer flask and incubated at 30°C for 48 h in a shaking incubator at 100 rpm. This was used for the ethanol fermentation. The cell population was counted with the aid of a haemocytometer and light microscope. In addition, an aliquot of 1 mL of the stock culture was pipetted into 9 mL of the sterile peptone water in a McCartney tube. Serial dilutions up to fivefold were prepared and finally, 100 µL of each serial dilution was plated on malt extract agar and incubated at 30°C for 24 h. The viable cells were counted and colony-forming units determined.

2.4. Substrate Preparation

Dried cassava peelings and rice husks, which served as substrates for the hydrolysis, were ground using a blender and sieved with 2 mm sized mesh to obtain a uniform particle size. Quantities of 50 g of the various substrates were weighed into 250 mL conical flasks containing 50 mL of distilled water. With the aid of a spatula the contents were thoroughly mixed. This was done to obtain the optimum moisture content for the inoculum finally resulting in a weight of 100g. The flasks were then corked with absorbent cotton wool and covered with aluminium foil. These were done in triplicate for eight weeks. The conical flasks with their contents were then autoclaved at 121°C at 15 psi for 15 min.

2.4.1. Preliminary Study

In order to determine the sequence of hydrolysing the substrates with fungi in the double hydrolysis stage, a preliminary study was conducted. With the aid of a blender, 50 g of cassava peelings were milled into 2 mm particle size and mixed uniformly with 50 mL of distilled water. This was then transferred into a conical flask after the pH was

determined and the flask corked and autoclaved. After cooling, the substrate in each conical flask was inoculated with either *P. ostreatus* spawn, or *A. niger* spore suspension independently in triplicate and incubated for a one-week period at room temperature.

Growths were recorded in both fungi-inoculated substrates after the one-week incubation period. The *P. ostreatus* and the *A. niger* separately hydrolysed cassava peelings were autoclaved. The autoclaved *P. ostreatus* hydrolysed cassava peelings were then inoculated with the *A. niger* spore suspension. The autoclaved *A. niger* hydrolysed cassava peelings were also inoculated with *P. ostreatus* spawn and incubated at room temperature for another period of seven days. This was done in triplicate. The concentrations of sugar produced by the various hydrolysed substrates with the fungi were not determined at this stage. At this stage, the main purpose was to determine the sequence of inoculating the inoculum.

2.5. Determination of Moisture Content of Unhydrolysed Samples

Quantities of 2 g of the untreated but autoclaved samples were transferred into a cleaned crucible (weight known) in triplicate. The individual crucibles with their contents were put in an oven at a temperature of 110°C for 24 h. At the end of the 24 h, the crucibles were taken from the oven, allowed to cool in a desiccator at room temperature and reweighed. Moisture content was calculated by difference and expressed as a percentage of the initial weight of the samples [9].

2.6. Biological Pretreatment and Enzymatic Hydrolysis of the Substrates with *P. ostreatus*

After autoclaving, the substrates were allowed to cool at room temperature. Under aseptic conditions, the bottle containing the spawns of *P. ostreatus* was shaken to loosen the grains. Ten grams of the grains was weighed into a sterile container and subsequently evenly spread onto the prepared substrates, thus a ratio of 1:5 in each of the conical flasks and corked with cotton wool [10]. The experiments were done in triplicate for each substrate. In the negative experimental control set up, spawns of *P. ostreatus* were not placed onto the substrate. The inoculated substrates and the control were incubated at 28°C for a period of eight weeks with weekly interval terminations of cultures for each substrate. The termination of the culture was to stop the organism from further hydrolysing the substrate. This was done by flooding it with sterile distilled water to the extract released sugars. Aliquots of the hydrolysates of each substrate were then taken for further analysis.

2.7. Biological Pretreatment and Enzymatic Hydrolysis of the Substrates with *A. niger*

After autoclaving, the substrates were allowed to cool to room temperature. Under aseptic conditions, 10 mL of the spore suspension (with a concentration of 2.2×10^6 cells / mL) was poured unto the substrates in each flask and corked again. The above spore concentration was used because a minimum level of inoculum was required for effective colonisation and subsequent delignification. A further increase in the inoculum level had insignificant effect on fungal colonisation rate and subsequent growth [11]. The experiments were done in triplicate for each substrate. A negative control experiment was set up in which the substrates were not inoculated with the *A. niger* suspensions. The inoculated substrates and the control were incubated at 28°C for a period of eight weeks with weekly interval terminations of cultures. The termination of the culture was to stop the organism from further hydrolysing the substrate. This was done by flooding it with sterile distilled water to the extract released sugars. Aliquots of the hydrolysates of each substrate were then taken for further analysis.

2.8. Biological Pretreatment and Enzymatic Hydrolysis of *P. ostreatus* Hydrolysed Substrates with *A. niger*

The substrates were first hydrolysed with *P. ostreatus* and the hydrolysis process terminated weekly (for the eight weeks' period) by autoclaving. These served as substrates for the second stage hydrolysis using the *A. niger*. Under aseptic conditions, 10 mL of the *A. niger* spore suspension was poured onto the substrates in each flask and corked. The experiments were done in triplicates for each substrate. In the negative experimental control set up, spawns of *P. ostreatus* and suspensions of *A. niger* were not added on the substrate. These were subsequently incubated at 28 °C for five days. The incubation period was kept as a constant since it would be used for comparative analysis.

2.9. Extraction of Fermentable Sugars

At the end of each week, the hydrolysis process was terminated by flooding the cultures with sterile distilled water to immediately extract the sugars. The fermentable sugars from the hydrolysed substrates were extracted by pouring 300 ml of distilled sterile water at 28°C onto the content of the flasks. Afterwards, the flasks were fixed on a rotatory shaker at 200 rpm for 30 min. The mixtures were subsequently filtered using filter cloth and the filtrate collected into another sterile container. Distilled sterile water of 200 mL at 28°C was used to thoroughly rinse the conical flask, and the rinsed water was also filtered. The filtrate collected was centrifuged at 5,000 rpm for 30 min at 28°C and the supernatant stored in freezer at -20°C until needed for downstream application. The residue was oven-dried at 70°C for 72 h, milled to homogeneity and stored for chemical analysis.

2.9.1. Determination of Fermentable Sugars

Fermentable sugars were determined using a digital refractometer (HI96801). Distilled water was used to blank the refractometer and then 3 mL of supernatant was drawn and dropped on a groove on the refractometer. The total sugar values were read, converted from Brix to g/L and recorded.

2.10. Fermentation of Hydrolysates to Ethanol

Ethanol fermentation was conducted using a modified method of Zakpaa *et al.* [8]. Hydrolysates of 30 mL were dispensed into labelled 100 mL bottle and autoclaved at 121°C for 15 min and allowed to cool. The pH of the hydrolysates was adjusted to 5.5 (using sterile NaOH and HCl) and inoculated with 0.3 mL of the *S. cerevisiae* suspension (containing 2.9 x 10^6 cells/mL in sterile nutrient broth). In the control set-up however, the hydrolysates were not inoculated with *S. cerevisiae*. The bottles were tightly capped with polypropylene caps, sealed with the aid of an adhesive tape and incubated at 30°C at 100 rpm for 5 days in a shaking incubator for fermentation. The concentration of ethanol produced was determined at regular intervals of 12 h for the five days' fermentation duration using the gravimetric method. Thus, the difference in weight due to carbon dioxide production was determined every 12 h and this corresponds to mass of ethanol produced. The yields of ethanol produced were also determined using a relation;

1. Ethanol Yield (%W/W Sugar) = ((Mass of ethanol produced in reactor (g))/(mass of sugar in hydrolysate (g)) ×0.51)×100, where 0.51 is a constant conversion factor [3].
2. Ethanol Yield (%W/W Dry Biomass) = ((Mass of ethanol produced in reactor (g))/(mass of dry biomass (g))×0.51)×100, where 0.51 is a constant conversion factor [3].

2.11. Chemical Analysis

The chemical composition of the raw and the inoculated substrates was determined by proximate analysis using van Soest Method of Analysis [12] for the determination of the fibre components (neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL)) of the hydrolysed and un-hydrolysed substrates. These were used to estimate the cellulose, hemicellulose and lignin contents. Thus

1. NDF = Cellulose + Hemicellulose + Lignin
2. ADF = Cellulose + Lignin
3. Hemicellulose = NDF – ADF
4. Cellulose = NDF– Hemicellulose –Lignin
5. Lignin = ADF – Cellulose

The means ± SD for each of the three replicates in every experiment were determined. Using a significance level of $p < 0.05$, an analysis of variance (ANOVA) was carried out and graphs (with standard deviation errors bars) plotted using Graph Pad Prism software (Version 6.0).

3. Results

3.1. Preliminary Study Results

It was observed that *P. ostreatus* was unable to colonise and hydrolyse both the rice husk and cassava peelings substrates that were previously hydrolysed with the *A. niger*. However, *A. niger* was able to colonise and hydrolyse both substrates that were previously hydrolysed by *P. ostreatus* under the same conditions as the latter. This was used to determine the sequence of hydrolysis in the two-stage hydrolysis process.

3.2. Moisture Content of the Unhydrolysed Samples

The determination of the moisture content of unhydrolysed but autoclaved rice husks and cassava peelings samples gave moisture content results of 70.82% and 70.26%, respectively. These served as the optimum moisture content for the growth of the microorganism being used for the initial hydrolysis. During the moisture content determination, a desiccator was used to ensure no moisture was absorbed.

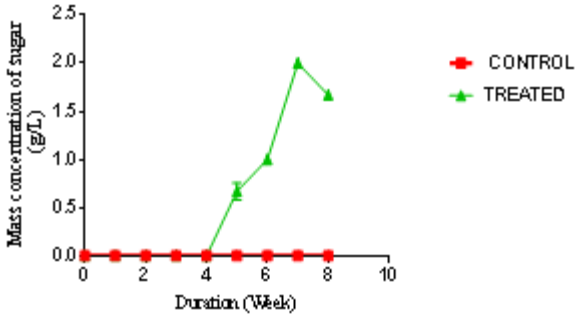

Figure 1. Concentration of sugar produced from *P. ostreatus* hydrolysis of rice husk. (Control = unhydrolysed rice husk hydrolysate, Treated = hydrolysed rice husk hydrolysate) using negative control

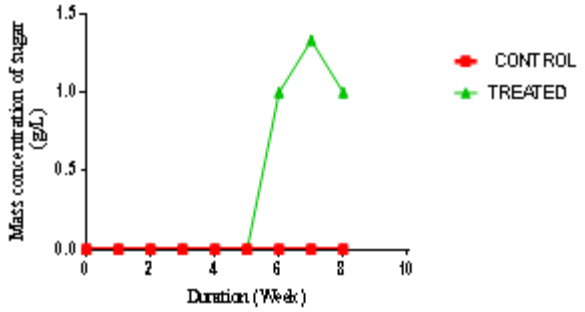

Figure 2. Concentration of sugar produced from *A. niger* hydrolysis of rice husk. (Control = unhydrolysed rice husk hydrolysate, Treated = hydrolysed rice husk hydrolysate) using negative control

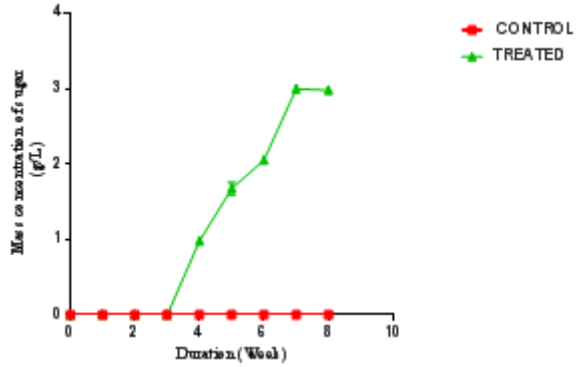

Figure 3. Concentration of sugar produced from *A. niger* hydrolysis of rice husk previously hydrolysed with *P. ostraetus*. (Control = unhydrolysed rice husk hydrolysate, Treated = hydrolysed rice husk hydrolysate) using negative control

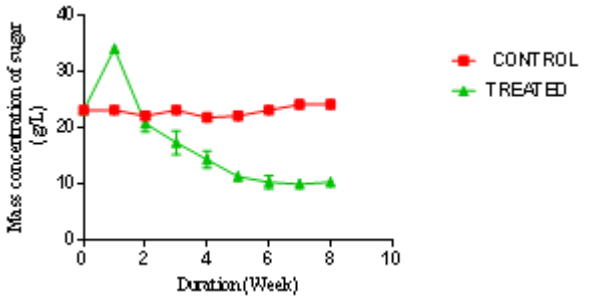

Figure 4. Concentration of sugar produced from *P. ostreatus* hydrolysis of cassava peelings. (Control = unhydrolysed cassava peelings hydrolysate, Treated = hydrolysed cassava peelings hydrolysate) using negative control

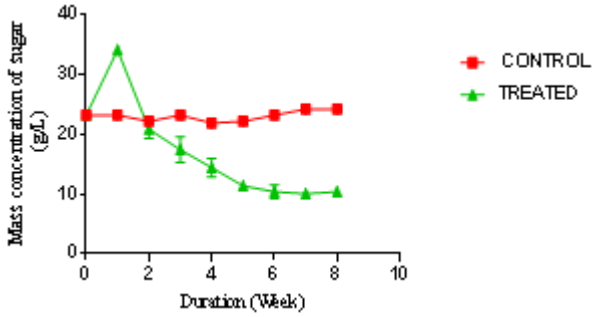

Figure 5. Concentration of sugar produced from *A. niger* hydrolysis of cassava peelings. (Control = unhydrolysed cassava peelings hydrolysate, Treated = hydrolysed cassava peelings hydrolysate) using negative control

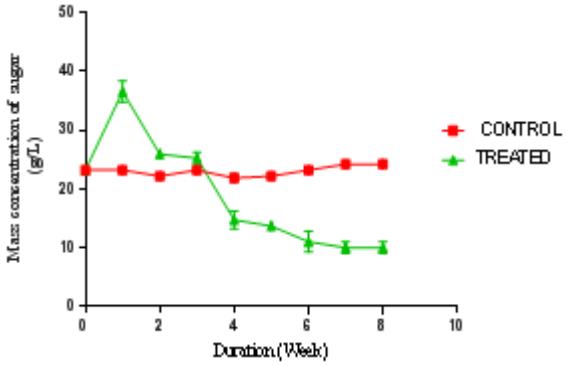

Figure 6. Concentration of sugar produced from *A. niger* hydrolysis on cassava peelings previously hydrolysed with *P. ostraetus*. (Control = unhydrolysed cassava peelings hydrolysate, Treated = hydrolysed cassava peelings hydrolysate) using negative control

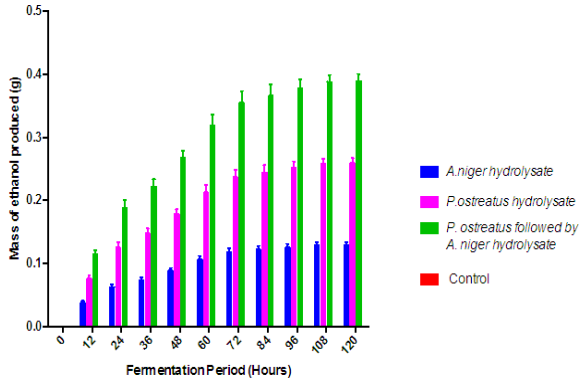

Figure 7. Ethanol production from the fermentation of the various rice husk hydrolysates with *S. cerevisiae* (Control = hydrolysate not inoculated with *S.cerevisiae*)

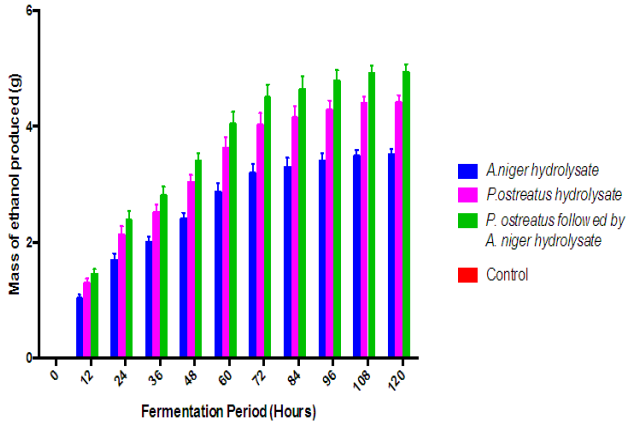

Figure 8. Effect of fermentation of the various cassava peelings hydrolysate with *S. cerevisiae* on ethanol production. (Control = hydrolysate not inoculated with *S.cerevisiae*)

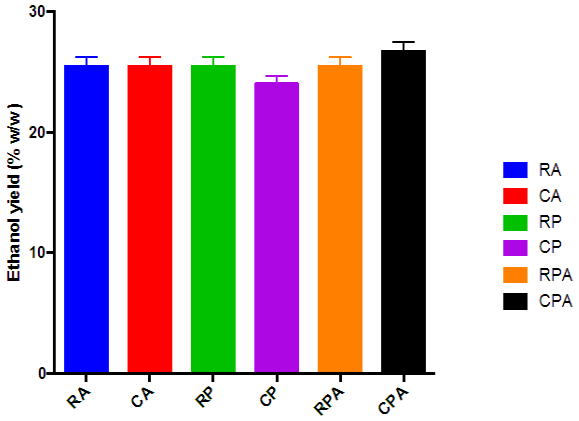

Figure 9. Percentage Ethanol yield (relative to sugar in the hydrolysate) of the various hydrolysates after 120 h fermentation period. (hydrolysate obtained from hydrolysis of cassava peelings with *P. ostreatus* followed by *A. niger* (CPA), hydrolysis of cassava peelings with *P. ostreatus*(CP), hydrolysis of rice husk with *A. niger* (RA), hydrolysis of cassava peelings with *A. niger* (CA), hydrolysis of rice husk with *P. ostreatus* (RP) and hydrolysis of rice husk with *P. ostreatus* followed by *A. niger* (RPA))

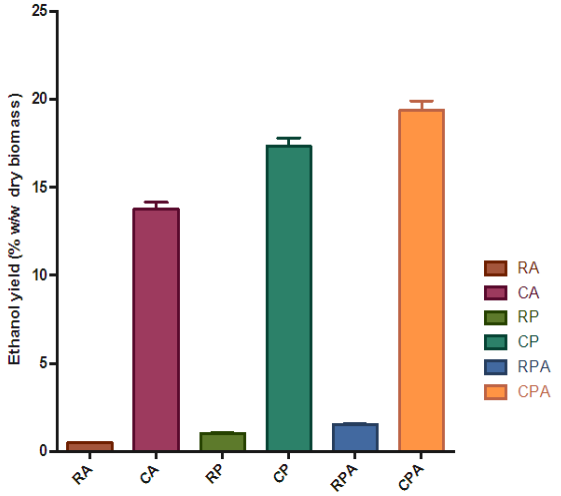

Figure 10. Effect of type of hydrolysis on percentage ethanol yield of dry biomass

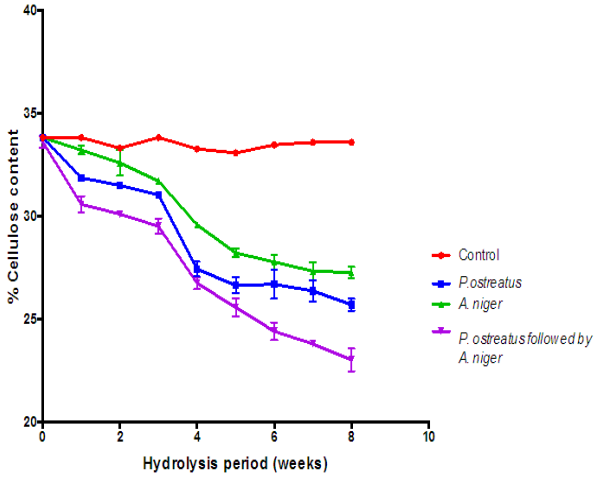

Figure 11. Comparison of the effect of hydrolysis of rice husk with the various fungi on cellulose content (Control= Rice husks not inoculated with fungi)

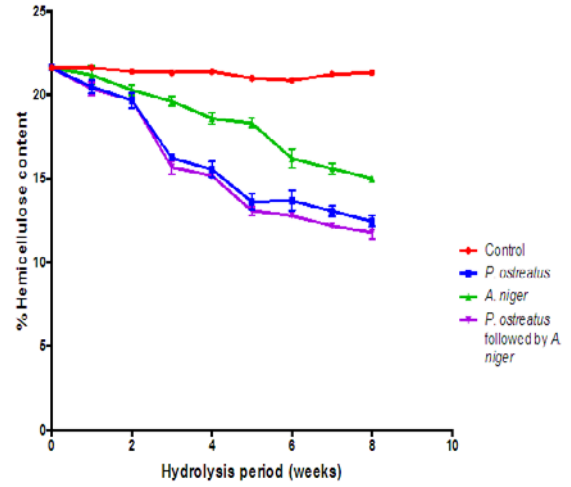

Figure 12. Compariosn of the effect of hydrolysis of rice husk with the various fungi on hemicellulose content (Control= Rice husks not inoculated with fungi)

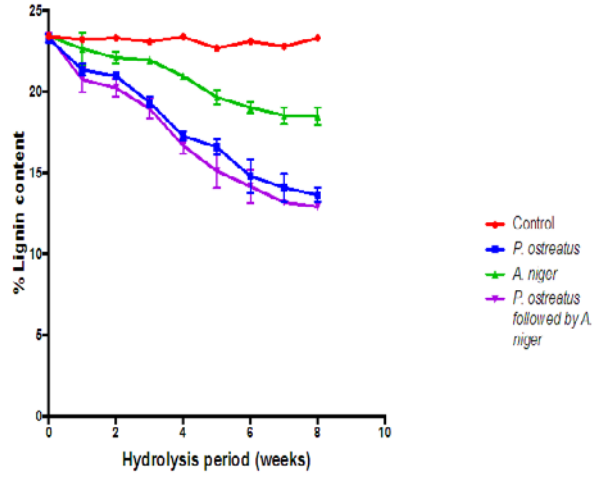

Figure 13. Comparison of the effect of hydrolysis of rice husk with the various fungi on lignin content (Control=Rice husk not inoculated with fungi)

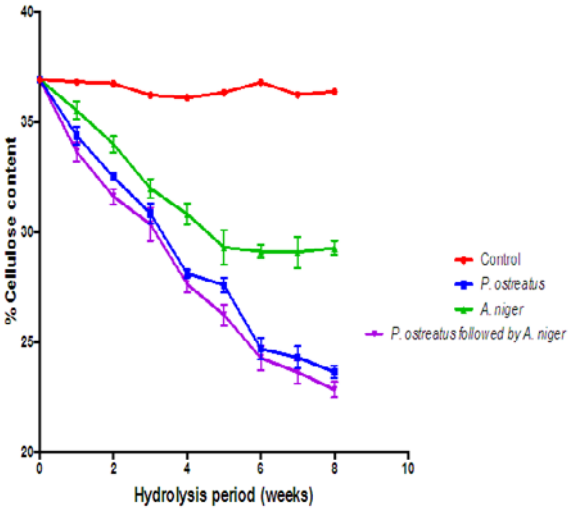

Figure 14. Comparison of the effect of *P. ostreatus* and *A. niger* hydrolysis of cellulose content of cassava peelings (Control=Cassava peelings not inoculated with fungi)

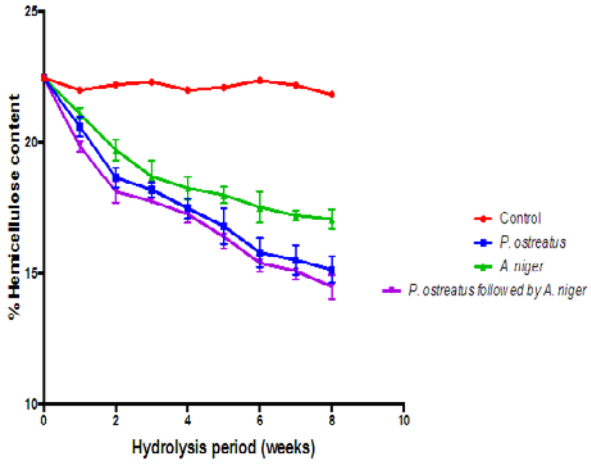

Figure 15. Comparison of the effect of *P. ostreatus* and *A. niger* hydrolysis of hemicellulose content of cassava peelings (Control=Cassava peelings not inoculated with fungi)

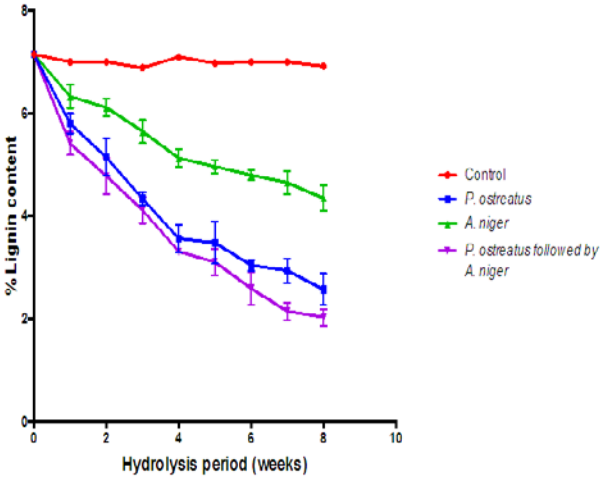

Figure 16. Comparison of the effect of *P. ostreatus* and *A. niger* hydrolysis of lignin content of cassava peelings (Control=Cassava peelings not inoculated with fungi)

4. Discussion

Preliminary studies were carried out to determine the sequence of hydrolysis in the two-stage fungal hydrolysis since growth of fungi could be inhibited by another fungus. The preliminary studies revealed that *P. ostreatus* was unable to colonise and hydrolyse both the rice husks and cassava peelings substrates that were previously hydrolysed with the *A. niger*. This could be a result of secondary metabolites such as aflatoxins, patulins and satratoxins produced by *A. niger* [13] which could be inhibitory to the colonisation of *P. ostreatus*. However, *A. niger* was able to colonise and hydrolyse both substrates that were previously hydrolysed by *P. ostreatus* under the same conditions as the latter, suggesting no inhibitory metabolites were produced by *P. ostreatus*. Bentil *et al*. [10], reported similar observations in their work. The preliminary studies results informed the decision to either hydrolyse the pretreated cassava peelings and the rice husks with *A. niger* alone or to first hydrolyse the pretreated cassava peelings and the rice husks with *P. ostreatus* and then followed by *A. niger*.

There was no fermentable sugar released immediately after autoclaving the rice husks. This could be due to the fact that rice husk is low in starch content, but has very high recalcitrant lignin, which strongly shields the cellulose and hemicellulose from degradation to monomer sugars units by autoclaving [14]. In contrast, although the initial fermentable sugar concentrations of the raw substrates were not determined before autoclaving, the sugar concentrations of the cassava peelings immediately after autoclaving were very high. This could be due to the fact that since cassava peelings have high starch content, subjecting it to autoclaving caused the release of monomer sugar units [15] which reflected in the graphs for the controls in Figures 4, 5 and 6. Thus autoclaving for sterilization of cassava peelings substrates before fungal hydrolysis had an impact on the substrates, resulting in the release of fermentable sugars. It is also possible that some amount of the cassava tuber content, which is rich in monosaccharaides was left attached to the cassava peels surface. This also, could have contributed to the high amount of fermentable sugars present in the cassava peelings hydrolysates after autoclaving.

The hydrolyses of cassava peelings and the rice husks with either *A. niger* or *P. ostreatus* alone, and the combination of first, with *P. ostreatus* and then with *A. niger* resulted in significant increase in fermentable sugars. The optimum fermentable sugars obtained from cassava peelings hydrolysates were significantly higher ($p < 0.05$) than those of rice husks hydrolysates. This may be due to the low lignin content but high starch content of cassava peelings as compared to rice husk (Figure 1 to 6). The celluloses (and starch) of cassava peelings are readily available and easily hydrolysed into sugar units by the extracellular enzymes produced by fungi as compared to those of rice husks. Cassava peelings treated with *P. ostreatus* produced highest concentration of 34.11 g/L of sugar as compared to 28.64 g/L obtained with the use of *A. niger* within one week of hydrolysis (Figure 4 and 15). This may be due to the synergistic nature of cellulases produced by the microorganisms and an indication that the enzymes from *P. ostreatus* may contain more of the components of the cellulase complex than *A. niger* [10]. *P. ostreatus* is also one of the fungi found to have an elaborate higher exoglucanase and endoglucanase activities [15].

There was a sharp increase in sugar concentrations from the time of inoculation (week 0) to the first week of hydrolysis of cassava peelings with the various fungi as compared to those of the rice husks (Figure 1 to 6). This implies that right after inoculating the cassava peelings, the fungi were able to colonise and secrete cellulases to break the celluloses. The microbes used the pre-existing sugars present to catalyse this reaction, and after which the fungi started using the available sugars for their growth. This could also be the reason for the accompanied decrease in the lignocellulose fractions. Hydrolysis of rice husks with the various individual fungi was characterised by low sugar concentrations (Figure 1 to 3). This is due to its higher lignin content; hence the fungi could not easily assess the cellulose to hydrolyse it to sugars.

The fermentable sugar concentration (36.51 g/L) obtained after hydrolysis of *P. ostreatus*-hydrolysed cassava peelings with *A. niger* for one week could be due to the fact that, the fungi used the substrates as carbon source and concurrently produced enzymes that broke cellulose accessible into fermentable sugar units [10]. Subsequently the continuous decrease in fermentable sugar yield of cassava peelings after first week by the combination of two fungi and individual fungi could be as result of the organisms feeding on the sugar produced. However, comparatively, the substrate treated with two fungi had relatively higher sugar concentrations than those treated with individual fungi. The highest fermentable sugar yield was observed in case of *P. ostreatus* and *A. niger* treated substrates. A similar trend was reported in a work done by Kumar *et al*. [16]. However, in their work, the substrates were rice straw, wheat straw and bagasse. This study proved that combining two fungi for hydrolysis improved sugar yield from rice husks. This is in agreement with the report of Olanbinwoninu and Odunfa [17] that combinations of pre-treatment boost the efficiency of sugar yield from cassava peelings.

Fermentation of the various hydrolysates by the yeast, *S. cerevisiae* yielded ethanol in accordance with the sugar concentration obtained. There was significant difference in the ethanol concentration over the fermentation period of each hydrolysate. However, from the third day, there was no significant difference ($p > 0.05$) in mean ethanol concentration, and this could be as result of other by-products in the broth that might have caused the inhibition of the yeast [18]. Additionally, the increase in ethanol concentrations of the fermentation cultures (Figure 8) could also be the reason for slowdown of the fermentation process after the third day [18]. It is also plausible that beyond the third day all the sugars that can be fermented by the *S. cerevisiae* would have been completely utilised [19].

Hydrolysates of cassava peelings with relatively high sugar concentrations gave the highest ethanol yield after fermentation with *S. cerevisiae* as compared to that of rice husks (Figure 7 and 8). This therefore implies the yield of ethanol is directly proportional to concentration of sugar in the fermenting broth. Theoretically, the maximum conversion efficiency of glucose to ethanol is 51% on a weight basis. In any case, since *S. cerevisiae* growth commences during the aerobic phase, some amount of sugar gets used up before the anaerobic stage, which is characterized by ethanol production. This could be the reason for the shortfall in the yields obtained in this study. Another reason for the short fall in the ethanol yields is that the yeast consumed sugar for growth and production of other metabolic products (example furfural, which can inhibit cells and affect the specific growth rate as well as cell-mass yield per ATP) [19]. Furthermore, the hydrolysates are not solutions of pure glucose (as used to estimate for the conversion efficiency) but contain other forms of sugars. This could be another reason for the shortfall in ethanol yields obtained in this study.

Considering the compositional analysis of the substrates, the unhydrolysed cassava peelings (control week 0) were found to contain 23.15% hemicelluloses, 37.57% cellulose and 7.56% lignin. These values were slightly different from the work of Aderemi and Nworgu, who reported 21.65% hemicelluloses and 6.98% lignin in the peels [20]. A possible explanation for the difference might be as results of varietal differences of the substrates. Also those of the rice husks (control week 0) samples were found to contain 33.75% cellulose, 21.47% hemicellulose and 23.16 % lignin.

The appearance of the fungal mycelia on the substrate after the first week was an indication that the degradation had commenced [20]. Generally, all the lignocellulose fractions of the substrates decreased significantly with respect to the hydrolysis time (Figure 2.0 to 2.5). On the other hand, no significant changes ($p > 0.05$) were observed in their respective controls. These point to the fact that, the *A. niger* and *P. ostreatus* did break down the various lignocellulose fractions as carbon source for their nourishment. Vijay *et al.* reported that most species of fungi (particularly white-rots) have the potential to degrade cellulose, hemicellulose and lignin [21]. According to Irfan *et al.*, degradation of lignocellulose biomass results in weight loss [22].

The progressive decrease in cellulose levels of all the hydrolysed substrates (Figure 11 and 14) are as result of some extracellular fungal hydrolases (cellulases) activities which breakdown the cellulosic substrates [23]. The extracellular enzymes (fungal hydrolases) released into the medium kept degrading the cellulose fractions to further break them into simple sugars for nourishment by the organisms. These processes continue until the enzymes are denatured. These results further confirmed those of Datta and Chakravarty [23], as well as that of Bentil *et al.* [10], who reported that; the breakdown of cellulose was a consequence of synergistic actions of some hydrolases (cellulases). *P. ostreatus* and *A. niger* have been reported to be sources of cellulases, hemicellulases and laccases which are hydrolytic in nature and therefore assist in the breakdown of complex carbohydrates into monomers [24].

Similarly, the hemicellulose content of all the substrates hydrolysed with the various fungi decreased significantly ($p < 0.05$) with time. Nevertheless, this was not true for the negative control, as this remained constant. The decrease in hemicellulose content of the various hydrolysed substrates could be due to hemicellulolytic enzymes (secreted by P. *ostreatus* and *A. niger*) activities on the substrates. This result agrees with previous works reported by Alemawor *et al*., [25] and Brimpong *et al*., [26]. They stated that there was 20-45% decrease in hemicellulose contents of some agro-wastes (lignocellulose materials) when hydrolysed with *P. ostreatus* over time. It has been established that *Pleuritic ostreatus* produces enzymes with the capability of degrading a variety of β (1,4)-linked glycan substrates as well as glycosides [27].

Though a decreasing trend of hemicellulose content was seen throughout the hydrolysis duration (Figure 12 and 15), no significant decrease ($p>0.05$) was observed after the sixth week of hydrolysis; this indicates that the optimum duration for hemicellulose degradation of cassava peelings hydrolysed with *P. ostreatus* is six weeks. By the end of six weeks, hemicellulose content of cassava peelings hydrolysed with *P. ostreatus* decreased by 39.99% after which there was no significant decrease. This could be as result of denaturation of secreted enzymes due to changes in pH or loss of moisture during the hydrolysis period. In congruence, [25] Brimpong *et al*., reported a 41% decrease in hemicellulose content of corncobs hydrolysed with *P. ostreatus* for a period of six weeks with no further decrease thereafter [26]. Melo *et al*. also reported that the enzyme activity dwindled with prolonged incubation [28].

Although the control displayed no significant changes ($p > 0.05$) in lignin content, the decrease in lignin content of the substrates hydrolysed with the various fungi was significant ($p < 0.05$) over the hydrolysis period. Lignin hinders the biological breakdown of hemicelluloses and cellulose as it covalently binds them to confer mechanical strength to cell walls of plants [29]. Hence the degree of lignin degradation observed gives a hint of hemicelluloses and celluloses available for fungi degradation and utilization. The degradation of lignin was due to activities of extracellular lignin-degrading enzymes. These enzymes include lignin peroxidases that oxidize aliphatic side chains as well as aromatic rings to produce compounds that are easily utilized by the fungi [30]. Other extracellular lignin degrading enzymes produced by fungi such as Mn-peroxidase and laccases were involved in the oxidative cleavage of non-phenolic lignin moieties [11]. They also catalyzed chemical reactions that oxidized numerous phenolic compounds [11]. Lignin degradation makes the cellulose more accessible [23]. Thus, *P. ostreatus* hydrolysis of the substrates for a period of at least 4 weeks will help enhance the availability of cellulose for further degradation to sugars. A similar work by Brimpong *et al*. reported a 42.3% decrease

in lignin content of corncobs hydrolysed with *P. ostreatus* for duration of six weeks with no further decrement thereafter [26]. This shows that lignin, one of the major constituents of rice husks and cassava peelings, limits the use of these substrates in the bioethanol industry.

The lignocellulose fractions of all the hydrolysed substrates with the fungi kept decreasing with time. However, the sugar concentrations of the respective substrates hydrolysed with the various fungi increased to an optimum level after which it began decreasing at a point. A possible explanation for this could be due to the fact that, since the enzymes for the degradation of the lignocellulose components are extracellular in nature; when released, they continued degrading the lignocellulose fractions with time till denatured [10]. However, these enzymes at a point further hydrolysed the celluloses available to monomer units of sugars (accounting for the increase in fermentable sugar), which the organism might have later utilised for its growth leading to the decrease in sugar concentration.

5. Conclusions

Bioethanol was successfully produced from rice husks and cassava peelings. *P. ostreatus*, *A. niger* and the combination of the two fungi were able to hydrolyse the substrates into fermentable sugars. The selected order of the two-stage biological pretreatment/ hydrolysis with *P. ostreatus* followed by *A. niger* significantly enhanced the concentration of fermentable sugars produced as compared to the individual fungus. The fermentable sugars produced were converted to bioethanol by *S. cerevisiae*. Evaluation of the various fermented hydrolysates with *S. cerevisiae* of the two substrates revealed that cassava peelings produced higher ethanol yield (19.36 % w/w dry biomass) than those of rice husks (1.53 % w/w dry biomass). These therefore confirmed cassava peelings as a useful and better raw material for second-generation bio-ethanol production.

REFERENCES

[1] Thomsen, M. H., & Thomsen, A. B. (2008). Pretreatment of Whole-Crop Harvested, Ensiled Maize for Ethanol Production. *Applied Biochemistry and Biotechnology*, *148*, 23–33.

[2] Chandrakant, P., & Bisaria, V. S. (1998). Simultaneous bioconversion of cellulose and hemicellulose to ethanol. *Critical Reviews in Biotechnology*, *18*(4), 295–331.

[3] Purwadi, R. (2006). *Continous ethanol production from dilute-acid hydrolyzates: detoxificacion and fermentation strategy*. Chalmers University of Technology, Gotebery, Sweden

[4] Graeme, M. W. (2010). *Bioethanol: Science and technology of fuel alcohol*. Dundee: Graeme M. Walker & Ventus Publishing ApS.

[5] Taylor, M. P., Eley, K. L., Martin, S., Tuffin, M. I., Burton, S. G., & Cowan, D. A. (2009). Thermophilic ethanologenesis: future prospects for second-generation bioethanol production. *Trends Biotechnology*, *27*(398-405).

[6] Alam, M., Zahangir, Kabbashi, A., N., Hussin, S., & I.S., N. (2009). Production of bioethanol by direct bioconversion of oil-palm industrial effluent in a stirred-tank bioreactor. *Journal of Industrial Microbiology and Biotechnology*, *36*(6), 801–808.

[7] Galbe, M., Hahn-Hagerdal, B. Gorwa-Grauslund, M. F., Liden, G., & Zacchi, G. (2006). Bio-ethanol - the fuel of tomorrow from the residues of today. *Trends in Biotechnology*, *24*(12), 549–556.

[8] Zakpaa, H. D., & Johnson, F. S. (2009). Production of bio-ethanol from corncobs using *Aspergillus niger* and *Saccharomyces cerevisiae* in simultaneous saccharification and fermentation. *Journal of Biotechnology*, *8*(13), 3018–3022.

[9] Association of Official Analytical Chemists (AOAC) (2002). Methods of Analysis, 15TH edition, Arlington, VA, Chapter 4, p. 54.

[10] Bentil, A. J., Dzogbefia, V. P., & Alemawor, F. (2015). Enhancement of the nutritive value of cocoa (*Theobroma cacao*) bean shells for use as feed for animals through a two-stage solid state fermentation with *Pleurotus ostreatus* and *Aspergillus niger*. *International Journal of Applied Microbiology and Biotechnology*, *3*, 20–30.

[11] Caixia, W., & Yebo, L. (2012). Fungal pretreatment of lignocellulosic biomass. *Biotechnology Advances*, *30*, 1447–1457.

[12] van Soest, P. J. (1995). Use of detergents in the analysis of fibrous feeds. *J. Assoc. Off. Anal. Chem*. *46*: 825-829.

[13] Praveena, Y. S. N., & Padmini Palem, P. C. (2011). Antibacterial activities of mycotoxins from newly isolated filamentous fungi. *International Journal of Plant, Animal and Evironmetal Science*, *1*(1), 8–13.

[14] Taniguchi, M., Suzuki, H. M., Watanabe, D., Sakai, K., Hoshino, K., & Tanaka, T. (2005). Evaluation of pretreatment with *P. ostreatus* for enzymatic hydrolysis of rice straw. *Bioscience and Bioengineering, 100*(6), 637–643.

[15] Chu, B. C. H., & Lee, H. (2007). Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation. *Biotechnology Advances*, *25*(5), 425–441.

[16] Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for pretreatment of lignocellulose biomass for efficient hydrolysis and biofuel production. *Industrial and Engineering Chemistry Research*, *48*(8), 3713–3729.

[17] Olanbiwoninu, A. A., & Odunfa, S. A. (2012). Enhancing the Production of Reducing Sugars from Cassava Peels by Pretreatment Methods. *International Journal of Science and Technology*, *2*(9), 650–657.

[18] Taherzadeh, M. J., & Karimi, K. (2007). Pretreatment of lignocellulose wastes to improve ethanol and biogas production: A review. *International Journal of Molecular Sciences*, *9*(9), 1621–1651.

[19] Hahn-Hagerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Liden, G., & Zacchi, G. (2006). Bio-ethanol - the fuel of

tomorrow from the residues of today. *Trends in Biotechnology*, *24*(12), 549–556.

[20] Aderemi, A. F., & Nworju, F. C. (2007). Nutritional status of cassava peels and root sieviate biodegraded with *Aspergillus niger*. *American-Eurasia. Journal of Agricultural &. Environmental Sciences*, *2*(3), 308–311.

[21] Vijay, P. M., Shyam, P. S., & Syed, A. A. (2007). Bioconversion of low quality lignocellulosic agricultural waste into edible protein by *Pleurotus sajor-caju* (Fr.) Singer. *Journal of Zhejiang University. Science B*, *8*(10), 745–751.

[22] Irfan, M., Gulsher, M., Abbas, S., Syed, Q., Nadeem, M., & Baig, S. (2011). Effect of various pretreatment conditions on enzymatic saccharification. *Songklanakarin Journal of Science and Technology*, *33*(4), 397–404.

[23] Datta, S., & Charkravarty, D. K. (2001). Comparative utilization of lignocellulose components of paddy straw by *Trichoderma lobyense* and *Volvariella volvacea*. *Indian Journal of Agricultur.*, *71*, 258–260.

[24] Iyayi, E. (2004). Changes in the cellulose, sugar and crude protein contents of agro-industrial by-products fermented with *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* sp. *Journal of Biotechnology*, *3*, 186–188.

[25] Alemawor, F., Dzogbefia, V. P., Oldham, J. H., & Oddoye, O. (2009). Effect of *Pleurotus ostreatus* fermentation on the composition of cocoa pod: Influence on time and Mn-ion supplementation. In *Proceedings of 2nd African Conference on Edible and Medicinal Mushrooms, Accra, Ghana, March 24-28* (Vol. 8, p. 60).

[26] Brimpong, B., Adamafio, N., & Obodai, M. (2009). Cultivation of oyster mushroom: Alteration in biopolymer profiles and cellulose digestibility of corncob substrate. In *Proceedings of 2nd African Conference on Edible and Medicinal Mushrooms, Accra, Ghana* (p. 57). Accra, Ghana.

[27] Highley, T. L. (1976). Hemicelluloses of white and brown rot fungi in relation to host preferences. *Mat. U Org.*, *11*, 33–46.

[28] Melo, I. R., Pimentel, M. F., Lopes, C. E., & Calazans, G. M. T. (2007). Application of fractional factorial design to levan production by *Zymomonas mobilis*. *Brazilian Journal of Microbiology*, *38*(1), 45–51.

[29] Chabannes, M., Ruel, K., Yoshinaga, A., Chabbert, B., Jauneau, A., Joseleau, J. P., & Boudet, A. M. (2001). In situ analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. *Plant Journal*, *28*(3), 271–282.

[30] Lo, S. C., Ho, Y. S., & Buswell, J. A. (2001). Effect of phenolic monomers on the production of laccases by the edible mushroom *Pleurotus sajor-caju*, and partial characterization of a major laccase component. *Mycologia*, *93*(3), 413–421.