Nig. J. Biotech. Vol. 38 (1) : 137-145 (June 2021)
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and www.biotechsocietynigeria.org
DOI: https://dx.doi.org/10.4314/njb.v38i1.16

Mushroom-mediated delignification of agricultural wastes for bio-ethanol production

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Abstract
Biological pretreatment is a cost-effective method of delignifying lignocellulosic biomass, making it less recalcitrant to hydrolysis into fermentable sugars. In this study, selected agricultural wastes were pretreated with mushrooms (*Lentinus squarrosulus* and *Pleurotus ostreatus*) to delignify them for bioethanol production. The substrates were supplemented with 0.2 % $CaCO_3$, inoculated with 12 % (w/w) *L. squarrosulus* and *Pleurotus ostreatus* spawns and incubated at 25 °C for 21 days. The highest lignin removal and highest bioethanol yield of 77.45 % and 13.98 % were obtained from bean husks pretreated with *L. squarrosulus*. Similarly, 64.29 % and 60.92 % lignin were removed from the *Pleurotus ostreatus*-pretreated banana leaves and sawdust, respectively, while 12.08 % and 13.05 % bio-ethanol yields were recorded, respectively. These findings demonstrate that affordable and straightforward mushroom delignification of abundant and cheap biomass can improve hydrolysis outcomes, thus easing bioethanol production.

Keywords: Agricultural waste, bioethanol, biological pretreatment, delignification, mushrooms.

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Introduction

The global energy demand is increasing due to the rapid development of new technologies, industries and transport (Hawrot-Paw et al., 2020). Fossil fuels that belong to non-renewable sources of energy are currently being used to meet these needs. In addition, the global population is predicted to reach 10 billion by 2050 (Goujon, 2019), increasing the demand for fuel (Rempel et al., 2019). Thus, it is estimated that fossil fuel supplies will be exhausted by the middle of this century (Shokrkar et al., 2017), which will necessitate the need for an alternative source of fuel (Wood and Roelich, 2019). One such potential alternative is bioethanol (Chamnipa et al., 2017), offering several advantages, including its renewable nature and low carbon emissions (Qureshi et al., 2015). Bioethanol is the most important and commonly used liquid biofuel, with a global market amounting to $6.8 billion in 2019, estimated to grow to $7.2 billion in 2024 (McWilliams, 2020). World production of bioethanol is believed to rise to about 137 billion litres in 2026, with a continual increase in production in the United States of America and Brazil (Dev et al., 2019). Furthermore, it is anticipated that the next generation of clean energy in Africa will come from renewable energy, with biofuels playing a pivotal role (Adewuyi, 2020).

First-generation (1-G) bioethanol is produced from crops with a high sucrose content (such as sugarcane, sugar beet and sweet sorghum) and high starch content (corn, wheat, rice, potato etc.) (Malik et al., 2020). Production of 1-G

bioethanol poses ethical challenges, leading to the utilisation of inedible crops, agricultural residues, food wastes and other lignocellulosic materials as a sustainable alternative feedstock for the production of second-generation (2-G) bioethanol (Lee et al., 2020). Lignocellulosic feedstocks are widely distributed and abundant in nature, attracting increasing attention (Bhatia et al., 2017). However, the three polymeric components of lignocellulosic biomass: cellulose, hemicellulose and lignin, are bound together to form a rigid matrix that is difficult to disrupt (Chen *et al*., 2017; Abdullah et al.,2020; Malik et al.,2020).

There are four steps in the conversion of lignocellulosic biomass to ethanol. The first is the pretreatment step that disrupts the cell wall of plants, i.e. destroys the matrix and makes the plant's structure more susceptible to enzymatic hydrolysis (Zabed et al., 2017). Pretreatment also removes lignin, the aromatic polymer that glues the other two carbohydrate polymers together. Pretreatment could be physical (milling or grinding), chemical (dilute acid hydrolysis), or biological (microbial degradation of lignin by white- and brown-rot fungi) (Solarte-Toro et al., 2019). Pretreatment is usually performed in combination with a typical process involving some form of fungal delignification and mild chemical treatment (Balan et al., 2008; Ma et al., 2010). Saccharification is the hydrolysis of the residual carbohydrates to fermentable sugars using either acids or commercial enzymes (Silverstein et al., 2007), a process made more efficient by pretreatment. The sugars obtained are then converted into bioethanol by employing ethanol-producing microorganisms (Malik et al., 2020) in a fermentation process. Finally, the ethanol is collected via product separation.

Biological pretreatment is the most efficient, eco-friendly and least severe pretreatment option (Taufikurahman et al., 2020). Biological pretreatment utilises microorganisms with the ability to produce ligninolytic enzymes that break down lignin. Common examples include the white-rot fungi *Trametes versicolor* and *Phanerochaete chrysosporium*, the brown-rot fungi *Coniophora puteana*, *Postia placenta* and *Aspergillus niger* (Ray et al., 2010; Ahmed El-Imam et al., 2020). In addition, this pretreatment method is sustainable, cost-efficient and has high energy efficiency (Taufikurahman et al., 2020).

Nigeria is a largely agrarian country, and its annual biomass potential is estimated at 144 million tonnes (Shaaban and Petinrin, 2014). However, much of this wastes accumulate in the environment or are fed to animals as low-value feed (Ahmed El-Imam et al., 2019). Consequently, this research investigated the effect of using ligninolytic fungi in the biological removal of lignin in the common lignocellulosic waste materials banana leaves, beans husks, and sawdust and the impact of this delignification on bioethanol production.

Materials and methods

Substrate collection and processing

The substrates (sawdust, banana leaves and cowpea bean husks) were obtained from different locations in Ilorin, Nigeria. The substrates were sorted to remove dirt and ground into a fine powder using a laboratory blender (Philips, China).

Microorganism and culture maintenance

Fresh spawns of *Lentinus squarrosulus* and *Pleurotus ostreatus* were obtained from a seller in Osogbo, Nigeria. Six different in-house *Saccharomyces cerevisiae* strains (Ahmed El-Imam et al., unpublished work) were maintained on PDA slants at 4 ^{o}C. The organisms were subcultured regularly to maintain viability.

Pretreatment conditions

Initial experiments were performed to investigate the ability of the substrates to support the growth of the various mushrooms. Twenty-five grams of each substrate was weighed into wide-mouthed one-litre bottles, wetted to a moisture content of 75 % (w/w), and then sterilised at 121 ^{o}C for 30 min. The substrates were allowed to cool and supplemented with 1 ml of sterile 0.2 % $CaCO_3$. Next, pairs of bottles were inoculated with 12 % (w/w) of mushroom spawn, then incubated at 25 ^{o}C for 21 d. Controls comprised the same biomasses that were not inoculated but were subjected to the same treatments. The substrate-mushroom combinations that showed the most florid growth were then selected for analyses and further experiments.

Lignin estimation

The lignin compositions of untreated and the treated samples with the most growth for each

substrate were estimated (Ramamoorthy and Sahadevan, 2019). Exactly 1 g of each sample was treated with 0.5 M NaOH solution, and the volume made up to 150 ml. It was maintained at 80 ^{o}C for 3½ hours. The substrate was then washed and dried at 40 ^{o}C to a constant weight. The hemicellulose content was the difference in the weight before and after the process.

For lignin estimation, 0.5 g of hemicellulose-removed biomass was treated with 15 ml of 98 % H_2SO_4 and incubated for 2 hours at 30 ^{o}C. Deionised water was used to reduce the acid strength of the mixture to 4 % H_2SO_4. The mixture was then autoclaved at 121 ^{o}C and 15 psi for one hour. 10 % $BaCl_2$ solution was used to remove sulfate ions from the remaining biomass. It was then dried to a constant weight at room temperature, and this gave the lignin content.

Percentage delignification was calculated by using the equation (Irfan et al., 2011):

$$\text{Delignification (\%)} = \frac{L_u - L_t}{L_u} \times 100$$

Where L_u = Lignin (untreated substrate) and L_t = Lignin (treated substrate).

Dilute acid hydrolysis

Dilute acid hydrolysis was carried out using nitric acid at a 3 % (v/v) concentration. From the mushroom-treated and untreated samples, 45 g were weighed into separate 250 ml Erlenmeyer flasks containing 180 ml solution of 3 % HNO_3 (20 % solid-loading ratio). The mixture was autoclaved at 121 ^{o}C for 30 m. The cooled slurry was filtered using Whatman No.1 filter paper. The pH of the hydrolysate was adjusted to 5.5 using NaOH pellets or 0.1 M HNO_3 solution.

Reducing sugars estimation

The reducing sugars in the hydrolysate were estimated using the DNS method (Sana et al., 2017). Reducing sugar content (%) was calculated from a glucose standard curve.

Yeast screening/Spot plate test

Spot plate screening was performed, with the hydrolysates serving as the only carbon source (Ahmed El-Imam et al., 2019). Hydrolysates of treated and untreated substrates were solidified using 1.5 % bacteriological agar. The media were sterilised at 121 ^{o}C for 30 min and poured into sterile Petri dishes upon cooling.

A cell suspension of each *S. cerevisiae* strain with OD_{600} of 1.0 was diluted in a ten-fold series. A 5 µL aliquot of each strain from 10^{-1} to 10^{-4} dilutions was spotted onto a matrix of dots (6 x 4); that is, four dilutions of six yeast strains were spotted per plate of the hydrolysate agar. Spotting was performed in duplicate plates, which were left undisturbed until the spots were dry. They were then incubated at 30 ^{o}C for 72 h (Ahmed El-Imam et al., 2019). After incubation, the strain with visible colonies was selected for the fermentation process.

Fermentation

The selected strain from the screening experiment was used to ferment the hydrolysates. Fermentation experiments were carried out using 25 ml of sterile hydrolysate at pH 5.5 in 250 ml conical flasks. Yeast cells were pitched at 1 x 10^7 cells/ml (Ahmed El-Imam et al., 2019), the flasks were plugged with sterile cotton wool and incubated at 30 ^{o}C for 5 d. Samples were withdrawn at 24-hour intervals to determine reducing sugars consumption and ethanol yield.

Ethanol estimation

Quantitative ethanol estimation was carried out using the potassium dichromate method reported by Koshy et al. (2014). First, 1.5 ml of the hydrolysate was withdrawn and made up to 25 ml with distilled water. Next, 0.1 ml and 5 µl of 2 N NaOH solution and potassium dichromate solution were added to the sample, respectively. Finally, the solution was incubated in a water bath at 50 ^{o}C for 30 min. The absorbance was read at 600 nm, and the ethanol content was estimated from a standard curve.

Results and Discussion

Effect of biological pretreatment on biomass lignin content

Three under-utilised lignocellulosic biomass (banana leaves, beans husks and sawdust) in Nigeria were investigated for their potential use in bioethanol production. First, the substrates were subjected to particle size reduction, then biologically pretreated using *Lentinus*

squarrosulus and *Pleurotus ostreatus* to disrupt the biomass' structure. Table 1 shows the growth pattern of the mushrooms on the various substrates. Spawn run was most dense in the beans husk substrate inoculated with *L. squarrosulus*, and least was sawdust.

Table 1: Growth pattern of the two mushrooms on the biomass substrates after 21 days

Biomass	**Mushroom**	
	Lentinus squarrosulus	***Pleurotus ostreatus***
Beans husks	**++++**	**+++**
Banana leaves	**++**	**+++**
Sawdust	**++**	**+++**

++ : Moderate growth; +++: Heavy growth; ++++: very heavy growth

From Table 1, it is evident that beans husk was the most amenable substrate as it supported the growth of both mushrooms best, while sawdust was the least ideal. Beans husks contain 55.1 % carbohydrates, 11.2 % protein and 6.1 % lignin (Amadioha and Nwazuo, 2019; Okechukwu et al., 2019), which makes it an adequate substrate, and the mushrooms can proliferate quickly. Conversely, sawdust contains about 59.7 % carbohydrates (Stoffel et al., 2017), and its protein content is neither routinely determined nor available in the literature. The absence of information on its protein content may suggest that it is negligible, explaining the poorer performance as a substrate.

Table 2 shows the lignin contents of the untreated substrates and the mushroom-substrate treatment showing the most mushroom growth (Table 1). The amount of lignin removed after 21 days is expressed as a percentage of the content in the untreated biomass.

Table 2: Lignin content of treated and untreated substrates

Substrates + Pretreatment fungus*	***Lignin content***		***Delignification (%)***
	Untreated biomass (%)	***Mushroom-treated biomass (%)***	
Beans husks (+ L. squarrosulus)	***20.4***	***4.6***	***77.45***
Banana leaves (+ P. ostreatus)	***18.2***	***6.5***	***64.29***
Sawdust (+ P. ostreatus)	***17.4***	***6.8***	***60.92***

* Substrates were treated with the two mushroom species, but the lignin (and subsequent) analyses were only performed on the substrate-mushroom combinations that showed the most mushroom growth after 21 days

The highest delignification level of 77.5 % was observed in *L. squarrosulus* fermentation of bean husks. This level of delignification is significantly higher than the reports of Li et al. (2018), who

observed lignin degradation of up to 52% in switchgrass samples treated with *Pleurotus ostreatus* for 80 days at 75 % MC and 5 ml inoculum. The findings are also higher than the reports by Waghmare et al. (2018) of 26 % lignin removal from sorghum husk pretreated with *Phanerochaete chrysosporium*. Yasid et al. (2019) delignified shredded oil palm empty fruit bunch using the mycelial culture of *Ganoderma lucidum*. They reported a lignin content of 12.69 % in the untreated sample, 10.05 % lignin after four weeks of incubation, 8.58 % lignin after eight weeks of incubation and 7.49 % lignin after 12 weeks of incubation.

This outstanding delignification level not only affirms mushroom treatment as an efficient pretreatment method, it also demonstrates that the specific fungi-biomass combination impacts the delignification outcomes.

Spot plate screening

Spot plate screening was performed to determine the yeast strains' abilities to utilise the sugars in the various hydrolysates. The results revealed that all the *S. cerevisiae* strains tested could grow on and tolerate the different hydrolysates. *Saccharomyces cerevisiae* OR6 grew most robustly among the tested strains (Table 3). As was to be expected, the colony sizes of the strains decreased with increasing dilution (results not shown).

Table 3: Spot plate screening of six *Saccharomyces cerevisiae* strains on dilute acid biomass hydrolysates solidified with 1.5 % agar

Hydrolysate medium	***OR1***	***OR2***	***OR3***	***OR4***	***OR5***	***OR6***
Beans husk	***+***	***+***	***+***	***+***	***+***	***+++***
Sawdust	***+***	***+***	***+***	***+***	***+***	***++***
Banana peel	***+***	***+***	***+***	***+***	***+***	***++***
Coconut pith	***+***	***+***	***+***	***+***	***+***	***+***
Potato peel	***+***	***+***	***+***	***+***	***+***	***++***

+: Negligible growth; ++ : Moderate growth; +++: Heavy growth

This result compares to the findings reported in Ahmed El-Imam et al. (2019) of substantial growth with yeast strains on sorghum bran hydrolysate-based media.

Fermentation

OR6 was then employed in a five-day fermentation using treated and control substrates to compare bioethanol production levels, thereby verifying the efficacy of the pretreatment method. The results are shown in Figure 1. *Lentinus squarrosulus*-treated beans husks (BHLS), with the most considerable lignin degradation of 77.45 %, also produced the highest ethanol concentration of 13.98 ± 0.23 % at day 5 (Fig. 1a), which was higher than yields of 12.33 ± 0.63 % from the untreated sample. This observation compares to the findings of Li et al. (2018), who reported an ethanol yield of 31 % from switchgrass with the highest lignin degradation of 52 %. Similarly, *P. ostreatus*-treated banana leaves (BLPO) yielded 12.08 ± 0.07 % ethanol compared to the untreated sample, with 9.89 ± 0.63 % (Fig. 1b). Higher yields of bio-ethanol were also recorded in treated sawdust on the fifth day with a concentration of 13.05 ± 0.23 % compared to the untreated sample, which yielded a 9.64 ± 0.57 concentration of ethanol (Fig. 1c). The trend indicates that the biologically-treated substrates produced higher ethanol yields than the control substrates.

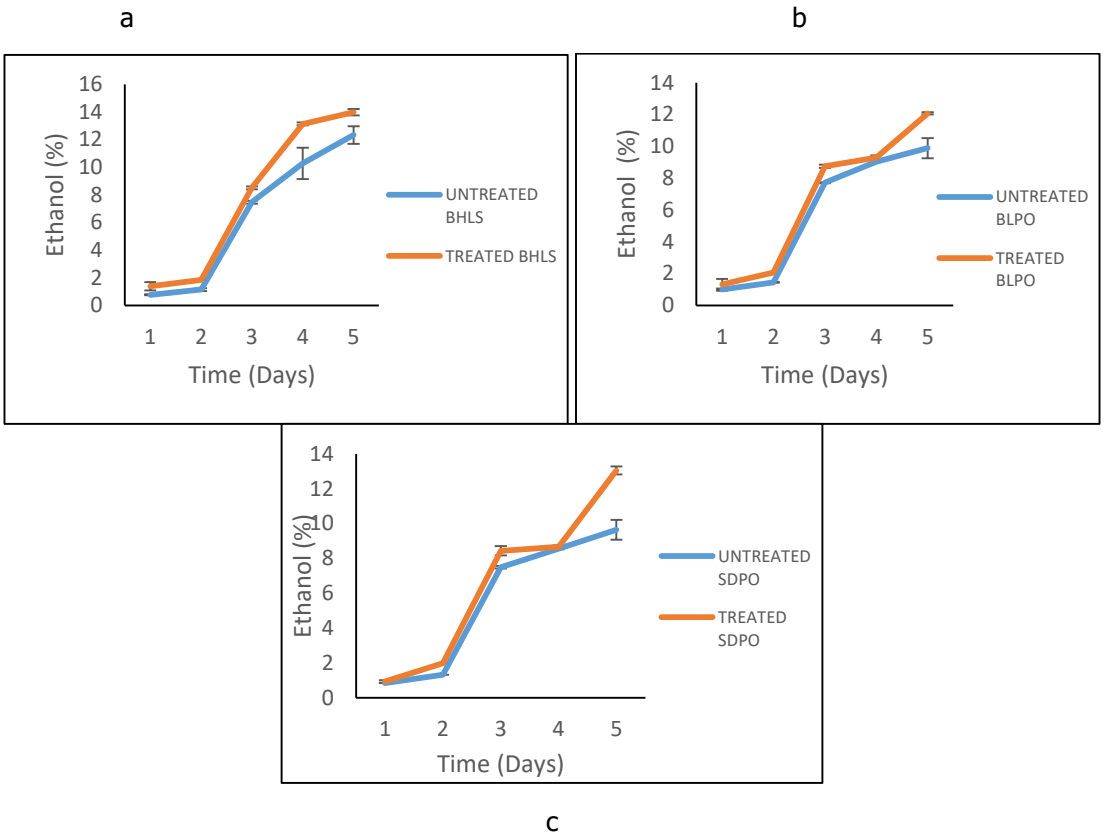

Fig.1: Trends in ethanol yields from different biomass (a): Beans husks + *Lentinus squarrosulus* (BHLS) (b): Banana leaves + *P. ostreatus* (BLPO) (c): Sawdust + *P. ostreatus* (SDPO).

The significant increase in yields observed in this report is similar to reports available in the literature. Nazarpour et al. (2013) also reported a 53 % bioethanol yield from rubberwood pretreated with *Ceriporiopsis subvermispora* compared to the untreated counterpart. Similarly, Ramamoorthy and Sahadevan (2019) reported high ethanol yields of 51.24 g/L from the fermentation of the biologically pretreated substrate (novel mixture of surgical waste cotton and cardboard). Megersa (2020) also pretreated sawdust samples with wood rot wild mushrooms and obtained high bio-ethanol yields compared to the non- pretreated sample. The substrate treated with *Ganoderma applanatum* yielded the highest amount of bioethanol of 1.77 g/L. This research demonstrates that biological pretreatment of biomass substrates is an effective, uncomplicated and sustainable method in the bioethanol production process.

Conclusion

The present study investigated the mushroom-mediated delignification of the agricultural wastes bean husks, banana leaves, and sawdust for bioethanol production. The biological pretreatment significantly improved bioethanol yield compared to the untreated substrates. In addition, the biological pretreatment eliminated the need for complex conventional delignification processes, which may result in secondary pollution problems. If adopted on a large scale, this efficient and straightforward process could significantly expand the biofuel industry in Nigeria and globally.

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