

## Comparative Study of Diverse Pretreatment Approaches to Degrade Lignin from *Bambusa balcooa*

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Bamboo biomass is a potential source of monomeric sugars containing a high cellulose content with a low amount of lignin. However, for efficient hydrolysis, an effective biomass pretreatment technique is required to minimize the lignin content and other barrier components. In the present study, bamboo biomass was treated with different physical, chemical, biological, and combined treatments to reduce the lignin content. Among all the pretreatments, the maximum lignin removal amount (14.5%) was obtained with the combined chemical and biological treatment under 2% NaOH + 1% H<sub>2</sub>O<sub>2</sub> + WDP2 fungal culture (5 plugs) conditions. In addition, the ligninolytic fungus and NaOH pretreatment was primarily effective in removing lignins, whereas the H<sub>2</sub>O<sub>2</sub> pretreatment efficiently minimized cellulose crystallinity. Scanning electron microscopy and Fourier-transform infrared spectroscopy was utilized to analyze the structural changes of the raw and treated biomass. The structural analysis indicated that all the treatments caused disruption in the biomass structure and reduced the compactness of the biomass, which facilitated the biomass conversion during the hydrolysis process. The findings of the present study indicated effective pretreatment methods in overcoming the recalcitrancy of potential lignocellulosic biomass for maximum hydrolysis.

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### INTRODUCTION

The increased worldwide energy demand has a noticeable effect on the economic sustainability and stability of the world (Moe *et al.* 2012). Many researchers in the biotechnology field have centered on the biological alteration of lignocellulosic biomass into simple sugars *via* the enzymatic hydrolysis of plant cell wall polyoses (Den *et al.* 2018). This can be applied to create a variety of downstream fuels and chemicals that meet global energy demands, as well as make an effort to decrease the reliance on conventional petroleum resources. Currently, most of the biorefinery approaches profoundly rely on either the biological alteration of lignocellulosic biomass or thermo-chemical progressions to produce transportation fuels (Foston and Ragauskas 2012). For the expansion of the plant based eco-friendly fuel industry, the chosen biomass resource should be present on a sustainable basis and also be affordable, with project sustainability as the concern. Bamboo has many special features, *e.g.*, a very fast growth rate, short reconstruction, easy propagation, and is rich in cellulose and hemicellulose and low in lignin; as such, it promises to be a potential feedstock for bioethanol or other biofuels production (Kuttiraja *et al.* 2013). There are approximately 1,500 bamboo species in the world and more than 20

million hectares of bamboo woodlands and plantations (Nirala *et al.* 2017). India is the second primary reserve of bamboo in the world and 25% of the bamboo species are found in India, which are broadly dispersed in nearly all states. They are predominantly plentiful in the Western Ghats and the “Sister States “of North-east India (Biswas 1988). The extent of the Indian bamboo manufacturing was assessed to be approximately Rs. 6505 crores, which increased in 2015 to Rs. 26000 crores and continues to rapidly increase (Sulaimani 2009).

Bamboo is known as one of the fastest growing plant species, with a harvestable time of 3 years to 5 years versus 10 years to 20 years for most of the softwood species in the ecosphere. It has auspicious features for gasification, diesel, and gasoline synthesis. The heating value of bamboo is higher compared to agricultural remains, grasses, and straw (Singh *et al.* 2017). In addition, bamboo has a high biomass productivity, is self-regenerating, and bamboo can contest with the most effective wood species in terms of sequestration of carbon. It acts as a carbon sink, thus helping in CO<sub>2</sub> mitigation. Its CO<sub>2</sub> storage rate per unit area of plantation is 4 times that of hard wood, with a higher release of oxygen (approximately 35%) into the atmosphere (Southern Metropolis Daily Mark 2012).

Biomass resistance built by the chemical composition of lignocelluloses and their interactions has developed a restrictive factor for inexpensively releasing sugars from the plant cell wall for ethanol invention. Consequently, lignocellulosic biomass frequently should be pretreated to overcome the biomass resistance and increase the cellulose digestibility. These pretreatments increase the hydrolysis of cellulose by using enzymes to remove the hemicellulose and modify the lignin structure to diminish the particle size and to raise the substrate porosity (Wu *et al.* 2016). Autoclaving and hot water treatments are generally used as a physical treatment, causing thermolysis by solubilizing the hemicellulose and slightly removing lignin polymers from the biomass (He *et al.* 2016). However, for proficient hemicellulose and lignin removal, the physical approaches are not considered an effective technology and also cause the release of various fermentation inhibitors (Madadi *et al.* 2017). Chemical pretreatments are purely dependent on chemical utilization, which is an effective way to disrupt the lignocellulosic biomass structure. Alkaline (NaOH, KOH, and Na<sub>2</sub>CO<sub>3</sub>) and oxidative (H<sub>2</sub>O<sub>2</sub>) treatments, as a chemical approach, involve the dissolution of the lignin-carbohydrate bonds, thus promoting the breakdown of the lignin structure. However, the use of chemicals in a biorefinery results in a harsh methodology that causes chemical pollution.

In addition to the methods mentioned above, the biological approach has received renewed attention as a greener technology that depends on the microbial enzymatic activity (wood rotting fungi) to hydrolyze the lignin polymers (Alvira *et al.* 2010). Numerous pretreatment methods have been established in the recent decades for furthering the enzymatic hydrolysis of numerous woody and non-woody biomass. However, most of the previous studies utilized energy extensive strategies or higher chemical concentrations to achieve better delignification of agricultural lignocellulosic biomass (Sun and Cheng 2002). In addition, limited detailed information has been found for bamboo biomass utilization for biofuel production. To improve the energy-saving methods for the pretreatment and bioconversion of biomass, reducing the high input of chemicals and/or energy is a crucial step. For the successful conversion of agroforestry biomass, therefore, new, and more effective process technologies are needed among the several alternative pretreatment techniques being investigated in this study.

The present study investigated the impact of different physical, chemical, and biological methods, as well as their combination, on bamboo biomass to select the best environmentally friendly delignification methodology. A comparison of different pretreatment methods has not been thoroughly investigated yet, especially the chemical and biological pretreatment methods. Therefore, an innovative methodology is required that enhances the cellulosic fiber content for easy hydrolysis while simultaneously decreasing the lignin cementing. This paper described a new ligninolytic fungal culture that showed maximum delignification of the bamboo biomass along with an alkaline-oxidative treatment and also clearly illustrates the effect of different methods in terms of the removal of recalcitrant lignin polymers.

## EXPERIMENTAL

### Preparation of Bamboo Biomass

Two species of bamboo, *B. balcooa* and *B. nutans*, were used in this study. Green woody samples of 1-year-old bamboo plants of the two species were collected from the agroforestry research center (AFRC), G.B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. All the selected fresh stem samples were chopped into small pieces and dried in an oven at a temperature of  $60\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$  for 2 weeks until a constant weight was attained. The dried pieces were then ground using a Wiley mill into a fine powder. The powder was then sieved through a 2-mm-diameter sieve and stored in an air tight container at a temperature of  $4\text{ }^{\circ}\text{C}$  until used later during the study. The moisture content in the samples was determined using the formula shown in Eq. 1,

$$\text{Moisture Content} = \frac{\text{Fresh weight of wood} - \text{Dry weight of wood}}{\text{Fresh weight of wood}} \times 100 \quad (1)$$

### Chemical Analysis of Bamboo Biomass

To select one of the two potential bamboo species for further saccharification processing, the powdered wood biomass from the two bamboo species was checked for its cellulose, ash, moisture, and lignin contents. The cellulose content in the bamboo biomass was estimated by the method outlined by Zhang *et al.* (2012). The analysis of the ash, moisture, and lignin contents were performed as per ASTM standard, respectively, as outlined in Sluiter *et al.* (2008a,b). The prepared powder (0.5 g) was mixed with a 1.25% solution of  $\text{H}_2\text{SO}_4$  (150 mL) and boiled for 30 min, followed by washing 3 times with hot distilled water. The washed biomass was further boiled for 30 min in a 1.25% solution of KOH (150 mL) and again washed 3 times with hot distilled water, followed by washing once with cold distilled water and then 3 times with acetone. It was then dried in an oven for 1 h at a temperature of  $60\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$  and weighed again. The biomass was further heated in muffle furnace at a temperature of  $540\text{ }^{\circ}\text{C}$  for 5 h. The resulting ash was cooled down and the weight was measured. The cellulose content was determined using Eq. 2,

$$\text{Cellulose Content (\%)} = \frac{F_1 - F_2}{F_0} \times 100 \quad (2)$$

where  $F_0$  is the weight of the fresh biomass (0.5 g),  $F_1$  is the weight of the dried biomass, and  $F_2$  is the weight of the ash.

Next, oven dried bamboo wood powder (1.0 g) was mixed with an acid detergent solution. Cetyl trimethyl ammonium bromide (CTAB, 20.0 g) was dissolved in 1.0 N sulphuric acid (1.0 L) (100 mL) and boiled for 15 min continuously and further refluxed for 60 min on a heating mantle. The contents were then filtered using filter paper and washed 3 to 4 times with hot distilled water until foam generation stopped. The biomass was then washed thoroughly with acetone until the filtrate was colorless. After the acetone washing, the biomass was dried in an oven at a temperature of  $60\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$  until a constant weight was reached. The dried acid detergent fibres (ADF) content was determined using Eq. 3:

$$\text{ADF (\%)} = \frac{\text{Weight of acid detergent fibres}}{\text{Weight of wood powder}} \times 100 \quad (3)$$

The dried acid detergent fibre was mixed with a 72%  $\text{H}_2\text{SO}_4$  solution (30 mL) and allowed to sit for 3 h with intermittent stirring. The solution was then diluted with distilled water (100 mL) to reduce the consistency of the fibres. The content was then filtered and washed with distilled water until a clear filtrate was obtained, which was then dried in an oven at a temperature of  $60\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$  until a constant weight was reached. The acid detergent lignin (ADL) content was determined using Eq. 4:

$$\text{ADL (\%)} = \frac{\text{Weight of acid detergent lignin}}{\text{Weight of wood}} \times 100 \quad (4)$$

The acid detergent lignin (the residue left after the acid treatment) was transferred to a muffle furnace and heated at a temperature of  $575\text{ }^{\circ}\text{C}$  for 3 h. The remaining ash content was calculated using the Eq. 5,

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of wood}} \times 100 \quad (5)$$

Finally, the lignin content (%) in the bamboo wood powder was calculated using Eq. 6,

$$\text{Lignin (\%)} = \frac{\text{Acid detergent lignin} - \text{Ash}}{\text{Weight of wood}} \times 100 \quad (6)$$

### Biomass Pretreatment: Delignification Process

On the basis of the moisture content, cellulose content, lignin content, and biomass yield, the *B. balcooa* variety of bamboo was selected for further pretreatment studies. The processed dry bamboo (*B. balcooa*) powder was subjected to various physical, chemical, physico-chemical, biological and physico-chemical+biological pre-treatments, as described in Table 1. For the biological treatment of the biomass, a laccase producing *Lenzites elegans* WDP2 fungal culture, isolated from decaying wood samples, either as a whole cell catalyst or its laccase enzyme, was used and provided with NCBI accession number MF289192. For the production of the lignin degrading (laccase) enzymes, the fungal culture was initially grown in laccase production medium (glucose -3.0 L<sup>-1</sup> w/v;  $\text{KH}_2\text{PO}_4$  - 5.0 L<sup>-1</sup> w/v;  $\text{NH}_4\text{NO}_3$  - 12.5 Mm;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  - L<sup>-1</sup> w/v 1.0; Tween 20 - 0.2 L<sup>-1</sup> w/v; KCl - 0.5 L<sup>-1</sup> w/v;  $\text{FeSO}_4$  - 0.001 L<sup>-1</sup> w/v; veratryl alcohol - 1 mM; trace metal solution - 0.1 %, and a pH of 5.0) for 12 d at a temperature of  $28\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$  (Pandey *et al.* 2018).

After the incubation period, the culture was centrifuged at 8000 rpm (at a temperature of 4 °C) for 10 min, and the supernatant was used as a source of crude enzyme. The laccase activity was estimated in the collected supernatant according to the method outlined in Kalra *et al.* (2013).

Briefly, a reaction mix was initially prepared containing 1.0 mL of crude enzyme and 3.0 mL of sodium acetate buffer (10 mM and a pH of 5.5) into which 1.0 mL of guaiacol substrate (2 mM) was added. The above solution was thoroughly mixed and incubated at a temperature of 30 °C for 15 min in a hot water bath. After the incubation, absorbance was measured at 450 nm against a blank containing water (1 mL) instead of the enzyme source.

One unit of laccase enzyme was defined as “the amount of enzyme required to oxidize 1 μmol of guaiacol in 1 min”. The laccase activity (in U/mL) was calculated using Eq. 7,

$$\text{Enzyme activity (EA)(U/mL)} = \frac{A \times V}{t \times e \times v} \quad (7)$$

where EA is the enzyme activity,  $V$  is the total mixture volume (mL),  $A$  is the absorbance,  $v$  is the enzyme volume (mL),  $t$  is the incubation time (min), and  $e$  is the extinction coefficient of guaiacol ( $0.674 \mu\text{M} \cdot \text{cm}^{-1}$ ).

The structural changes occurring in the bamboo wood powder after the different pretreatments were determined using Fourier-transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM).

### Fourier-transform Infrared (FTIR) Spectroscopy

The selected dried wood samples after certain treatments, as mentioned in Table 1, were also analyzed *via* FTIR spectroscopy to determine any changes in the molecular bonding. The FTIR peaks of the treated samples were also compared with the FTIR peaks of the untreated samples. The spectroscopic analysis was performed with a Thermo-Nicolet 670 FTIR spectroscopy. The changes in the peaks were recorded at  $4 \text{ cm}^{-1}$  in the region of 400 to  $4000 \text{ cm}^{-1}$ .

### Scanning Electron Microscopy (SEM)

The bamboo samples after the selected pretreatment process (alkaline, oxidative, alkaline-oxidative, WDP2 whole cell fungal catalyst, and alkaline-oxidative + WDP2 whole cell fungal catalyst) that showed the highest exposed cellulose content were analyzed for structural changes in the surface *via* SEM imaging, which were compared with the untreated control samples. The samples were initially completely dried in an oven at a temperature of  $60 \text{ °C} \pm 2 \text{ °C}$  to remove the moisture content and were further processed for SEM imaging. The SEM images were captured at different magnifications using a LEO 435 VP.

### Statistical Analysis

All tests were conducted in triplicate and accessible as the mean  $\pm$  standard deviation (SD). The data from the pretreatments study was compared *via* two-way analysis of variance using SPSS and the Dunnett’s test.

**Table 1.** Summary of the Various Physical, Chemical and Biological Pretreatments Used for Delignification of the Woody Biomass from the Selected Bamboo Sp. (*Bambusa Balcooa*)

Sample No.	*Physical	*Chemical	Biological	Physico-chemical	Physico-biological	Chemical + Biological	Physical + Chemical + Biological
1.	<sup>a</sup> Autoclaving	NaOH	* <i>Lenzites elegans</i> WDP2 whole cell catalyst	NaOH + autoclaving + H <sub>2</sub> O <sub>2</sub>	Autoclaving + <i>Lenzites elegans</i> WDP2 Culture	H <sub>2</sub> O <sub>2</sub> + NaOH + <i>Lenzites elegans</i> WDP2 culture	H <sub>2</sub> O <sub>2</sub> + NaOH + autoclaving + <i>Lenzites elegans</i> WDP2 culture
2.	<sup>b</sup> Hot water method	H <sub>2</sub> O <sub>2</sub>	<i>Lenzites elegans</i> WDP2 laccase enzyme	–	Autoclaving + <i>Lenzites elegans</i> WDP2 enzyme	H <sub>2</sub> O <sub>2</sub> + NaOH + <i>Lenzites elegans</i> WDP2 enzyme	H <sub>2</sub> O <sub>2</sub> + NaOH + autoclaving + <i>Lenzites elegans</i> WDP2 enzyme
3.	-	TiO <sub>2</sub> + NaOH	-	-	-	NaOH + <i>Lenzites elegans</i> WDP2 culture	-
4.	-	TiO <sub>2</sub> + NaOH + H <sub>2</sub> O <sub>2</sub>	-	-	-	NaOH + <i>Lenzites elegans</i> WDP2 enzyme	-
5.	-	H <sub>2</sub> O <sub>2</sub> + Fe shavings	-	-	-	-	-
6.	-	H <sub>2</sub> O <sub>2</sub> + NaOH	-	-	-	-	-
7.	-	H <sub>2</sub> O <sub>2</sub> + Fe Shavings + NaOH	-	-	-	-	-

\* Physical treatments: a) wet powdery biomass (2 g) was heated in an autoclave for 1 h at a temperature of 121 °C (15 lb psi); b) wet powdery biomass was subjected to a hot water treatment in a water bath at temperature of 100 °C for 30 min

\* Chemical treatments: the dry powdery biomass adjusted to a moisture content of 1 to 15 (solid to liquid ratio) was treated with any one of the chemical agents at a time as per details given: (i) NaOH (2%), (ii) H<sub>2</sub>O<sub>2</sub> (1%), (iii) Fe shavings (0.5 gm) or (iv) TiO<sub>2</sub>(0.2%) at 80 °C for 1 h in hot air oven

\*Biological treatment: the sterilized wet biomass (2 gm) was treated with WDP2 fungal culture as biological catalyst (5 plugs) or 5 mL of crude laccase enzyme solution (28.44 U mL<sup>-1</sup>) from the same fungal culture using laccase medium adjusted with (1:7) solid: liquid ratio. Here TiO<sub>2</sub> photo-catalyst nano-powder was used.

## RESULTS AND DISCUSSION

### Selection of Potential Bamboo Species

To select an appropriate biomass for efficient bioconversion into fermentable sugars, the biomass should have a high biomass yield and cellulose with low lignin, ash, and moisture contents. In this study, the biomass yield, and cellulose, lignin, ash, and moisture contents were estimated for the two bamboo species in order to select the potential bamboo species for further pretreatment studies.

### Biomass Yield and Moisture Content

The results indicated that a higher biomass yield was obtained in the *B. balcooa* species (14.7 kg/culm) compared to *B. nutans* (8.52 kg/culm), as described in Table 2. Furthermore, both the samples were dried in an oven to check the moisture content. The moisture content was found to be quite similar in *B. balcooa* (25.2%) and *B. nutans* (22.5%). A plant biomass with a low moisture content and high biomass yield is desirable for the industrial production of bioethanol. The total available amount of organic biomass was higher in the *balcooa* sp. in contrast to *nutans* sp. and had the low moisture content generally required for bioenergy production. The presence of a higher moisture content in the biomass is also not desirable from a microbial contamination point of view since it can affect the storage stability of the biomass (Bassam 1998). In previous studies, lower moisture contents, at the levels of 14.3%, have also been reported in *B. beecheyana* species (Truong and Le 2014).

**Table 2.** Lignocellulosic, Ash, and Moisture Contents of the Green Woody Biomass from 1-Year-Old Plants of Two *Bambusa* sp.

Biomass sp.	Cellulose (%)	Lignin (%)	Biomass (kg)	Ash (%)	Moisture Content (%)
<i>Bambusa balcooa</i>	44.2 ± 2.15	28.9 ± 2.26	14.7 ± 1.49	0.002 ± 0.001	25.2 ± 2.73
<i>Bambusa nutans</i>	28.2 ± 2.45	27.8 ± 2.39	8.52 ± 1.40	0.002 ± 0.001	22.5 ± 0.86
Sem ± CD (5%)	1.33 5.20	1.34 5.24	0.834 3.26	0.0004 0.0018	1.16 4.57

\*Sem: standard error of mean, CD: critical difference at 5% ( $p$ -value less than 0.05), a P-level less than 0.05 indicates that the values are significant

### Chemical Composition of Biomass

The dried fine powdered biomass from the two bamboo species was further analyzed for the cellulose, ash, and lignin contents. In terms of the cellulose content, a much higher level was obtained in *B. balcooa* (44.2%) compared to *B. nutans* (28.2%). The lignin and ash contents between the two species were found to be 28.9% and 0.002% in *B. balcooa* and 27.8% and 0.002% in *B. nutans*, respectively. A higher cellulose content with a low lignin concentration is desirable, which positively correlates with the glucose release during the hydrolysis process that is appropriate for biorefinery industries. However, the chemical components in plant biomasses greatly differ and depend on ecological influences, growth circumstances, and plant maturity (Michelin *et al.* 2014). In a few studies, the cellulose and lignin contents of several bamboo species were described and varied from 29.3% to 49.1% and 23.8% to 26.1%, respectively (Higuchi 1957). Celluloses are approximately 70% of the total biomass and are strongly connected to lignin by

covalent and hydrogen bonds, which make the structure extremely resilient and hydrolysis-resistant. This extremely recalcitrant behavior is due to the lignins, degree of cellulose crystallinity and polymerization of polysaccharides, surface area availability, and the moisture content (Michelin *et al.* 2014).

### **Pretreatment of *B. balcooa* Biomass: Delignification Process**

The selected *B. balcooa* biomass underwent further various individual and combination pretreatments to determine the best method that would result in a maximum decrease in lignocellulose recalcitrance by decreasing the lignin levels and simultaneously increasing the cellulose release.

### **Physical Treatment**

#### *(a) Autoclaving*

In the first instance, the biomass was subjected to autoclave treatment by exposing it to high pressure under wet conditions for a prolonged time period to delignify the biomass. The autoclaving process resulted in a slight increase in the cellulose content (51.6%), which was slightly different in comparison to the control (44.2%), along with a slight decrease in the lignin content (21.0%) compared to the control (28.9%). The results showed a lower level of delignification in the *balcooa* sp.; this might be due to the utilization of only water as a solvent in the autoclaving step. Furthermore, the efficiency of this process is dependent on the pressure, temperature, and reaction time, along with the biomass type (Kumar and Sharma 2017). During the present study, the pretreatment conditions were kept at a neutral level to diminish the energy requirement for the delignification, which was also responsible for a lower amount of lignin removal from the biomass. In addition, individual autoclaving treatments do not seem to significantly enhance the delignification of the biomass, which has also been reported by earlier studies (Debiagi *et al.* 2020).

#### *(b) Liquid hot water treatment*

In this study, a liquid hot water pretreatment resulted in a significant increase in the cellulosic content in *B. balcooa* (62.2%) compared to the untreated sample (44.2%). Similarly, the lignin content was also decreased in the treated sample (19.7%) compared to the control (28.9%). The liquid hot water treatment primes the lignin and hemicellulose hydrolysis and cellulose accessibility (Yang and Wyman 2004)

In conjunction with autoclaving, the hot water treatment resulted in a higher removal of lignin, which directly increased the cellulosic fiber exposure in *balcooa* sp. The reason for lignin degradation was that the liquid hot water (LHW) process operated at a temperature of 100 °C for 60 min, where water acts as a solvent and catalyst, which disrupts the ester and ether bonds and is accompanied by a release of organic acids from the biomass which ultimately aid in cell wall matrix disruption and the releasing of ferulic acid, lignin, and carbohydrate residues (Zhuang *et al.* 2016). Here, a cost-effective approach using a boiling water bath without any external pressure was utilized. Under such conditions, a significant lignin reduction was achieved that allowed increased cellulose accessibility. However, due to the energy and water requirement in large amounts involved in downstream processing, the technique has not been very useful in industrial applications. In addition, most of the studies using this method have been carried out at a higher temperature/pressure range (greater than 100 °C), which makes it unsafe and unfit in the bio-refinery field.

In this approach, both autoclaving and liquid hot water methods were employed to remove the lignin polymer from the *balcoa* sp. Under wet conditions during autoclaving, the biomass was exposed to high pressure for prolonged incubation to delignify the biomass. The steam explosion method was first introduced by Mason (1926) as a biomass pretreatment method. This process causes an explosive effect on lignin residues by hydrolyzing and transforming the lignin component and depends mainly on the temperature and pressure (Kumar and Sharma 2017). In the present investigation, the bamboo powder was treated under steam explosion conditions in distilled water, which helped with the liberation of small cellulosic fibers by breaking the complex lignin fibers. The steam enters the fibers of the biomass under high temperature and pressure conditions and causes the expansion of the cell wall fibers, which liberates the cellulosic fibers by partial hydrolysis of the lignin and hemicellulose network. However, the use of water alone is not sufficient in breaching the phenolic polymers of lignins; hence it is unable to interrupt the composite ether bonding of cellulose and lignins. In earlier reports by Debiagi *et al.* (2020), an increase in the cellulose content with a slight decrease in the lignin content when compared to the control was reported when the biomass from wheat bran and oat hulls was exposed to autoclaving.

## Chemical Pre-treatment

### (a) Alkali treatment

Usually lime (calcium hydroxide) or sodium hydroxide is used in an alkaline treatment study. Here, the biomass treatment with NaOH (2% v/v) did not show a significant increase in the cellulosic content (50.6%) nor an effective decrease in the lignin content (24.5%). A slight difference was observed in comparison to the untreated biomass. Similarly, Zhao *et al.* (2008) have reported the low impact of a mild alkali treatment of NaOH on different biomass containing less than 26% lignins, *e.g.*, wheat straw, switchgrass, hardwoods, and softwoods. A somewhat higher lignin removal amount (72.3%) with the same concentration of NaOH (2%) from lignocellulosic biomass was reported in previous studies; however, this was achieved at a much higher temperature (Zhang *et al.* 2012). A slight difference was observed in comparison to the untreated biomass.

### (b) Oxidative treatment

A pretreatment with H<sub>2</sub>O<sub>2</sub> resulted in an increase of the cellulosic content (52.6%) in *B. balcoa* compared to the control (44.2%), along with a decrease in the lignin content (22.1%) compared to the control (28.2%). The oxidative pretreatment was conducted in three other different combinations, *i.e.*, with NaOH, Fe shavings, and Fe shavings + NaOH. The maximum cellulose recovery was observed in the combined treatment of NaOH + H<sub>2</sub>O<sub>2</sub>, which showed an increased cellulosic content of 64.8% with a reduced lignin content of 18.7%. In the combined case, H<sub>2</sub>O<sub>2</sub> leads to the oxidation and cleavage of lignin through various chemical reactions, *e.g.*, oxidative cleavage of ether linkages in an aromatic ring of a lignin polymer, which displaces the polysaccharides and lignin bonding. The displaced weak lignin polymer gets affected by the presence of the NaOH compound, which further disrupts the lignin bonds by separating the lignin linkages (Zhang *et al.* 2012). Thus, these reactions assist in the release of a higher cellulose content from the biomass. Similar results were observed from the combined effect of NaOH + H<sub>2</sub>O<sub>2</sub> + Fe shavings, with a recovery of cellulosic content of 60.4% and a lignin content of 19.5%, while the treatment with H<sub>2</sub>O<sub>2</sub> + Fe shavings showed a cellulosic content of 54.8% and a lignin content of 21.6%. These

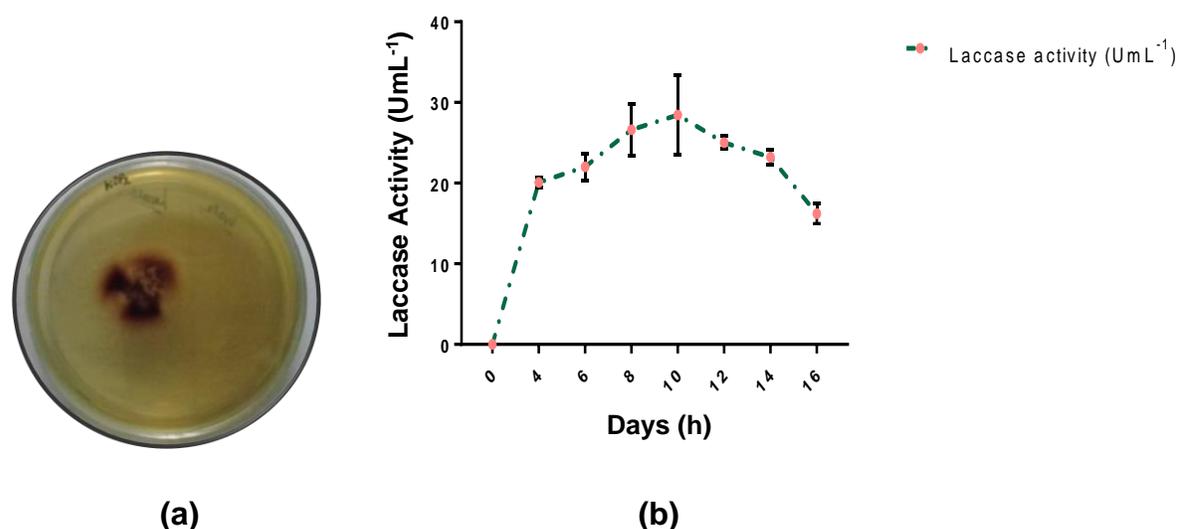
treatment conditions are similar to a Fenton oxidation reaction, whereby the metal catalysts in presence of H<sub>2</sub>O<sub>2</sub>, according to their catalytic activity generate oxidizing agents, which increases the overall conversion of lignin into degraded products and prevents the accumulation of inhibitory products. All combinations of the H<sub>2</sub>O<sub>2</sub> treatment showed good effect on the bamboo biomass treatment in contrast to the H<sub>2</sub>O<sub>2</sub> only treatment in terms of enhancing the cellulose fiber exposure with lignin removal. However, similar to the study performed by the authors, a non-significant increase in the cellulose release was achieved with the H<sub>2</sub>O<sub>2</sub> treatment alone, compared to its combination with other treatments, *e.g.*, metal catalysts (Fe shavings); in addition, NaOH has been observed to show promising results (Cao *et al.* 2012).

(c) *Nano-catalyst with alkali-peroxide treatment (titanium dioxide)*

Two combinations of TiO<sub>2</sub>, *i.e.*, with NaOH and H<sub>2</sub>O<sub>2</sub> + NaOH, were tested. The maximum cellulose recovery (60.8%) and lignin removal (19.2%) was observed in the TiO<sub>2</sub> + NaOH + H<sub>2</sub>O<sub>2</sub> pretreatment when compared to the TiO<sub>2</sub> + NaOH combination, which yielded a lower lignin (20.3%) removal and cellulose release (56.8%) was observed. The results were in accordance with the earlier results, where NaOH and H<sub>2</sub>O<sub>2</sub> was shown to result in a significant improvement in the cellulosic fiber recovery, up to 77.25%, and in the presence of a TiO<sub>2</sub> nano-powder catalyst, further carried out the efficient catalytic degradation of lignins or related compounds (Kansal *et al.* 2008; Diaz *et al.* 2013). However, few attempts have been made to study the impact of nanoparticle supplementation in the pretreatment of lignocellulosic biomass. However, the present attempt also shows that a TiO<sub>2</sub> supplementation in conjunction with certain chemical treatments (NaOH in this case) could enhance lignin degradation and increase the availability of cellulosic fibers compared to NaOH alone.

### Biological Pre-treatment

The selected bamboo biomass was separately treated with the white rot fungal isolate *Lenzites elegans* WDP2 as a whole cell catalyst as well as using the laccase enzyme from *Lenzites elegans* WDP2. It was observed that treatment with the whole cell fungal culture resulted in a significant increase in the cellulose content (68.4%) along with a lower lignin content (16.6%) in contrast to the laccase enzyme treatment (a cellulose and lignin content of 60.4% and 18.0%, respectively). The fungal culture was grown with up to 2 weeks of incubation, and the laccase activity was estimated every 2 days of incubation. The highest enzyme activity (28.4 U·mL<sup>-1</sup>) from the fungal culture (as shown in Fig. 1) was achieved on the 10<sup>th</sup> day of culture incubation during the 12 d of the pretreatment study. The supernatant was collected as a crude enzymatic source and subjected to delignification of the *balcooa* powder. Here, the enzymatic treatment was found to be not as effective as the cell catalyst treatment, which could be because of the use of a crude enzyme with low activity, in addition to the laccases (lignin degrading enzymes), and other enzymes, *e.g.*, peroxidases also assisting in the increased degradation of the lignins by the white rot fungi (Vats *et al.* 2013). In the case of the whole cell pretreatment, the fungal delignification was processed under solid surface fermentation conditions, where the fungal hyphae completely grew over the *balcooa* powder and effectively hydrolyzed the lignin polymer from the substrate. In employing the fungal culture (*L. elegans* WDP2), it also caused the release of other enzymes, *e.g.*, peroxidase, which simultaneously enhances the depolymerization of lignin complexes (data not shown).



**Fig. 1.** Laccase activity of the fungal culture *Lenzites elegans* WDP2 during the biological pretreatment: (a) brown zone of guaiacol oxidation through laccase catalysis; and (b) laccase activity ( $28.44 \text{ U mL}^{-1}$ ) quantification

**Table 3.** Influence of Different Pretreatment Methods on the Lignin and Cellulosic Contents of the Woody Biomass of 1-Year-Old *Bambusa balcooa*

Sample No.	Treatments	Exposed Cellulose Content (% w/w)	Lignin Content (% w/w)
I.	<b>Physical Pre-treatments</b>		
i	Autoclaving	$51.6 \pm 1.49^{****}$	$21.0 \pm 0.63^{****}$
ii	Hot water method	$62.2 \pm 2.11^{****}$	$19.7 \pm 0.62^{****}$
II.	<b>Chemical Treatments</b>		
i	NaOH	$50.6 \pm 1.35^{****}$	$24.5 \pm 0.47^{**}$
ii	H <sub>2</sub> O <sub>2</sub>	$52.6 \pm 1.14^{****}$	$22.1 \pm 0.23^{****}$
iii	TiO <sub>2</sub> + NaOH	$56.8 \pm 0.71^{****}$	$20.3 \pm 1.10^{****}$
iv	TiO <sub>2</sub> + NaOH + H <sub>2</sub> O <sub>2</sub>	$60.8 \pm 0.96^{****}$	$19.2 \pm 0.55^{****}$
v	H <sub>2</sub> O <sub>2</sub> + Fe Shavings	$54.8 \pm 2.38^{****}$	$21.6 \pm 0.42^{****}$
vi	H <sub>2</sub> O <sub>2</sub> + NaOH	$64.8 \pm 1.35^{****}$	$18.7 \pm 0.22^{****}$
vii	H <sub>2</sub> O <sub>2</sub> + Fe Shavings+ NaOH	$60.6 \pm 2.82^{****}$	$19.5 \pm 0.30^{****}$
III.	<b>Biological Treatments</b>		
i	<i>Lenzites elegans</i> WDP2 culture	$68.4 \pm 1.16^{****}$	$16.6 \pm 1.65^{****}$
ii	<i>Lenzites elegans</i> WDP2 enzyme	$60.4 \pm 1.03^{****}$	$18.03 \pm 1.44^{****}$
IV.	<b>Control (untreated)</b>		
		$44.2 \pm 2.15$	$28.9 \pm 2.26$
	Sem $\pm$ CD (5%)	0.823 2.40	0.573 1.67

Note: \* Statistical analysis was performed by Dunnett's multiple comparison test; \*\*\*\* signifies the probability level ( $p$ -value less than or equal to 0.0001) and \*\* for  $p$ -value less than or equal to 0.01; Sem: standard error or mean; CD: critical difference at 5%

Biological pretreatment methods have an advantage over other physical and thermochemical processes in terms of the equipment needed and the energy consumed. Currently, research has been conducted on wood rot fungi, which tend to be more potent

because of their special oxido-reductive enzyme systems, *e.g.*, laccase, in terms of a biomass pretreatment process (Pandey *et al.* 2018). Bari *et al.* (2016) also reported a high level of lignin degradation (57.4%) in beech wood after 120 d of pretreatment time using *Trametes versicolor*. Similarly, another study reported 24% delignification of bamboo culms by *Echinodontium taxodii* 2538 and *Trametes versicolor* spp. after 28 d of treatment (Tsegaye *et al.* 2019). However, in this study, the pretreatment could achieve a significant delignification (43%) within the first 12 d of incubation with the fungal culture, *L. elegans*. The effects of all the different pretreatment methods on the cellulose and lignin content of the bamboo biomass are listed in Table 3.

It is apparent that between the different physical, chemical, and biological pretreatment approaches, the best results were obtained *via* the biological treatment of the biomass with the whole cell fungal catalyst. Alternatively, chemical treatment with alkaline-peroxide was also found to give satisfactory results, although the effect was not as considerable as the biological treatment. It is hard for a single process to split the lignin and hemicellulose structures and release cellulose (Huang *et al.* 2017; Bhowmick *et al.* 2018). Hence, the employment of two or more different pretreatments as a combined method is necessary to achieve increased lignin degradation and hemicellulose solubilization compared to what a single method can do alone.

## Combined Pretreatment

### *Physical + chemical treatments*

During individual treatment experimentation, the alkali-peroxide method resulted in considerable deformation of the complex phenolic polymer from the bamboo biomass, which in turn enhanced the cellulosic part of the substrate. For the efficient treatment of the biomass, the alkali-peroxide treatment was combined with the autoclaving method to further increase the intensity of delignification. Therefore, in the present study, the biomass was subjected to a physical treatment (autoclaving) followed by an alkaline peroxide (NaOH + H<sub>2</sub>O<sub>2</sub>) treatment. An increased cellulose content of 55.6% was noted along with a decreased lignin content of 21.1% as compared to control (as described in Table 4). Significant results were not found, which contrasted with the alkaline and peroxide treatment. Diminutive changes were observed in the terms of the lignin degradation, but the cellulose content of the biomass was slightly increased in contrast to the individual treatments. The drawback of the steam related methods is the generation of repressive composites that become toxic for further processes (Baruah *et al.* 2018). The reason behind this was not clear, but it might be that the inhibitory compounds released during the autoclaving method interfered with the alkali-peroxide treatment and hence became less suitable for decreasing the lignin complexity of the biomass.

### *Physical + biological treatments*

As in the previous combination, the lignin degradation was not effective, so the bamboo biomass was subjected to physico-biological methods using two different processes *i.e.*, autoclaving with *L. elegans* as a whole cell culture and autoclaving with the laccase enzyme separately. After pretreatment, the cellulose content was found to be 68.5% and 65.0% with lignin contents of 20.7% and 20.8% for both the combined whole cell and enzymatic pretreatment method (as listed in Table 4), respectively. It was observed that the cellulose content was enhanced, and the lignin content was reduced compared to the autoclaving method alone. However, compared to both biological methods, no differences were found in the cellulose content with a lower lignin degradation amount. Wang *et al.*

(2012) reported that a combined biological pretreatment along with liquid hot water pretreatment for the enzymatic saccharification processing of *Populus tomentosa* resulted in a two-fold rise in the glucose yield over the pretreatment carried out using liquid hot water individually. Similarly, in this research, the combined effect was found to be significant in compared to autoclaving alone. It was reported that the autoclaving method released undesirable lignocellulose-derived inhibitors from the lignocellulosic substrate, which affects the other processes of plant-based biomass hydrolysis (Baruah *et al.* 2018). It was concluded that the combined treatment of autoclaving with chemical and biological methods was less suitable for breaking lignin polymers from the bamboo feedstock.

#### *Chemical + biological treatments*

In this case, the biomass was pretreated with an alkaline-peroxide (NaOH + H<sub>2</sub>O<sub>2</sub>) mixture under similar conditions used in the alkaline-peroxide treatment alone. The chemically treated biomass was washed to remove the chemical residues and dried to remove the water content. Then, the biomass was treated with active *L. elegans* WDP2 culture and laccase enzyme, separately. After both the treatments, the cellulose content increased to 76.6% and 64.8%, and the lignin content decreased to 14.5% and 20.5%, respectively (as shown in Table 4). The combined effect of the alkaline-peroxide treatment followed by the *L. elegans* WDP2 treatment showed considerable influence in terms of decreasing the recalcitrant-cementing lignin polymer. The results of this study were in accordance with those of Yu *et al.* (2009), who observed the effect of a chemical and biological treatment on the substrate, rice husk, as well as the removal of lignin. In case of the alkali-peroxide treatment combined with a biological enzymatic treatment, the results were not significant in terms of decreasing the amount of lignin. The presence of any residual chemicals in the biomass caused the ineffectiveness of the enzyme. The other reason for this was the use of unpurified crude enzyme. In the case of the biological pretreatments, the whole cell culture method was found to be more effective compared to the enzymatic method and showed satisfactory results in combination with the alkali-peroxide treatment as the best delignification method. The chemical pretreatment conditions were also maintained at a low chemical concentration to decrease the effect of the harsh chemicals on the biological processing of the ligninolytic fungal system to maximize the cellulosic fibers release from the complex lignin cementing. From the above results, alkaline oxidative as the chemical treatment and *L. elegans* WDP2 as the biological treatment was found to be the best treatment under their combined conditions.

Another approach was also utilized in the combined delignification of bamboo biomass, in which the substrate was subjected to alkali (NaOH) treatment and further treated with either a *L. elegans* WDP2 whole cell culture or laccase enzyme, separately. After the combined treatment, the cellulose content of the biomass increased to 70.2% with a decreased lignin content of 20.1%. In the case of combining the enzymatic and NaOH pretreatment, the biomass showed considerable results in terms of exposing the cellulose fibers with a total cellulose content of 74.8% and a decreased total lignin content of 20% (as described in Table 4). The combined effect of the alkali and biological method was also found to be an efficient treatment compared to the alkaline and biological treatment methods separately. Previous researchers have also reported that a combination of several methods with a biological process is more suitable compared to a single pretreatment method (Biswas 1988).

### Physical + Chemical + Biological Treatments

To maximize the delignification of the biomass together with an increase in the exposed cellulose content, a combination of a physical, chemical, and biological pretreatment was performed. Two different methods were employed in which the biomass was first autoclaved, then treated with an alkaline-peroxide solution. After these two steps, the pre-treated biomass was further treated with a biological pretreatment. In the biological method, both the whole cell treatment and laccase enzyme treatment were used separately. A higher cellulose release was observed in the case of the physical-chemical and biological combined treatment using a whole cell culture, yielding a 72.6% cellulose content and a 19.8% lignin content. However, in the case of the enzymatic treatment, only a 70.0% cellulose content and a lignin content of 19.9% was estimated (as described in Table 4).

**Table 4.** Combined Influence of a Physical, Chemical, and Biological Pretreatment on the Lignin and Cellulosic Contents of the Woody Biomass of *Bambusa balcooa*

Sample No.	Treatments	Cellulose Content (% w/w)	Lignin Content (% w/w)
I.	<b>Physical + Chemical Treatments</b>		
i	Autoclaving + NaOH + H <sub>2</sub> O <sub>2</sub>	55.6 ± 2.57****	21.1 ± 0.55****
ii	Physical + biological treatments		
II.	Autoclaving+ <i>Lenzites elegans</i> WDP2 culture	68.5 ± 0.09****	20.7 ± 0.39****
i	Autoclaving + laccase enzyme	65.0 ± 0.16****	20.8 ± 0.40****
ii	Chemical + biological treatments		
iii	H <sub>2</sub> O <sub>2</sub> +NaOH + <i>Lenzites elegans</i> WDP2 whole cell culture	76.6 ± 1.00****	14.5 ± 0.06****
iv	H <sub>2</sub> O <sub>2</sub> + NaOH + laccase enzyme	64.8 ± 0.02****	20.5 ± 0.33****
v	NaOH + <i>Lenzites elegans</i> WDP2 whole cell culture	70.2 ± 0.30****	20.1 ± 0.71****
vi	NaOH + laccase enzyme	74.8 ± 1.41****	20.0 ± 0.01****
vii	<b>Physical + Chemical + Biological Treatments</b>		
III.	H <sub>2</sub> O <sub>2</sub> + NaOH + Autoclaving + <i>Lenzites elegans</i> WDP2 whole cell culture	72.6 ± 0.31****	19.8 ± 1.40****
i	H <sub>2</sub> O <sub>2</sub> + NaOH + Autoclaving + laccase enzyme	70.0 ± 0.23****	19.9 ± 0.79****
V.	<b>Control (untreated)</b>		
		44.2 ± 2.15	28.9 ± 2.26
	Sem ± CD (5%)	0.556 1.65	0.318 0.945
Note: * Statistical analysis was performed by Dunnett's multiple comparison test; **** signifies the probability level ( <i>p</i> -value less than or equal to 0.0001) and ** for <i>p</i> -value less than or equal to 0.01; Sem: standard error or mean; CD: critical difference at 5%			

The overall summary of the combined pretreatments showed that the different methods yielded improved effects in contrast to single operated treatments. In all the cases, the cellulose content increased and the lignin content decreased. Among all methods, the best method was an alkaline-peroxide treatment along with a fungal cell treatment using *Lenzites elegans* WDP2, which showed maximum cellulose exposure with the maximum lignin decrease. The percent increase in the cellulose content and the percent decrease in the lignin content of the *B. balcooa* biomass varied during different pretreatment studies. The combined pretreatment approach showed agreeable results in terms of enhancing the

accessibility of the cellobiose polymer (cellulose) and overall phenolic polymer (lignin) degradation from *B. balcooa* biomass.

### Monitoring of the Structural Changes in the Pretreated Bamboo Biomass

Analytical techniques, *i.e.*, FTIR analysis and SEM, were used to visualize the structural changes caused by the pretreatment in the bamboo biomass.

#### Fourier-transform Infrared Spectroscopy (FTIR)

In the present study, 6 pre-treated bamboo samples were selected for FTIR analysis, *i.e.*, the H<sub>2</sub>O<sub>2</sub> treated, NaOH treated, NaOH + H<sub>2</sub>O<sub>2</sub> treated, fungal culture treated, NaOH + H<sub>2</sub>O<sub>2</sub> with fungal culture, and untreated biomass as the control. The alkali-peroxide and whole fungal cell treatment and alkali-peroxide + whole fungal cell (*L. elegans* WDP2) were found to be the best methods for the degradation of lignin polymers with maximum cellulose recovery. The H<sub>2</sub>O<sub>2</sub> or NaOH treated biomass showed changes in the wave numbers in the region of 500 to 3000 cm<sup>-1</sup>, which depicts O-H stretching and C-H stretching in the lignin component of the biomass (as shown in Fig. 2b and 2c). In the untreated biomass, a peak at 2269.8 cm<sup>-1</sup> was observed, but it was not present in either of the NaOH or H<sub>2</sub>O<sub>2</sub> treated samples, which indicated the breaking of the bands, which lead to lignin degradation in the bamboo samples (Fig. 2a). Slight changes were observed at 1630 to 1500 cm<sup>-1</sup> in both the cases, which could be related to C=O stretching (unconjugated) and aromatic ring vibration in the lignin component of the biomass. The reduction of bands was found at a range of 700 to 1000 cm<sup>-1</sup>, which was attributed to glycosidic linkages and C-O vibration in the sample (Figs. 2b and 2c). The FTIR spectra of the samples subjected to the alkaline-peroxide pretreatment showed an emergence of new peak at 897.6 cm<sup>-1</sup>, which is assigned to the β-glycosidic bond in the cellulose polymer of the biomass, which agreed with the chemical analysis data (Zhang *et al.* 2019). A very minute change was observed at 1100 cm<sup>-1</sup> wave number which illustrated C-O-C asymmetrical extending in the hemicellulose and cellulose components of the plant biomass. A new peak was observed at 1500 cm<sup>-1</sup>, which showed an aromatic ring vibration in the lignin component (Fig. 2d).

The results, according to the chemical methods, show that the greatest cellulose content was retained in the alkali-per-oxide pretreated biomass with maximum degradation of the lignin aromatic bonding. The biomass was also treated biologically using the fungal culture *Lenzites elegans* WDP2 and by a combined treatment (alkaline-peroxide treatment + biological treatment). The FTIR spectra of the biologically treated biomass revealed a new peak at 1731 cm<sup>-1</sup>, which indicated ketone/aldehyde C-O stretching in the hemicellulose or carboxylic groups in the lignins.

The broadening of the peak located at 3100 cm<sup>-1</sup> was clearly revealed in the case of the combined pretreatment when compared to the untreated or other chemical treated samples; this related to the C-H and O-H bonds stretching. Differences in the biologically treated and chemical + biologically treated samples were also observed at the region of 1600 to 1650 cm<sup>-1</sup> which depicts aromatic ring vibration and C-O stretching of the lignin components of the wood (as shown in Fig 3e and 3f). The FTIR spectrum of the combined chemical + biological treatment revealed maximum degradation in the lignin component of the biomass, which was directly related to the pretreated data. Fourier-transform infrared spectroscopy has been broadly useful in plant cell wall examination for comparison among different samples of different ages that undergo developmental and compositional changes in the cell wall.

The pretreatment results were indicated by the C-O bond vibration movement, C-O-C stretching, aromatic ring vibration in the cellulose, hemicellulose components, and lignin component of the biomass. Zheng *et al.* (2018) described the FTIR spectrum of pretreated wheat straw and showed that the intensities of the absorption peaks were stronger within the range of 1450 to 1630  $\text{cm}^{-1}$  of the spectrum involved in the lignin aromatic skeleton stretch.

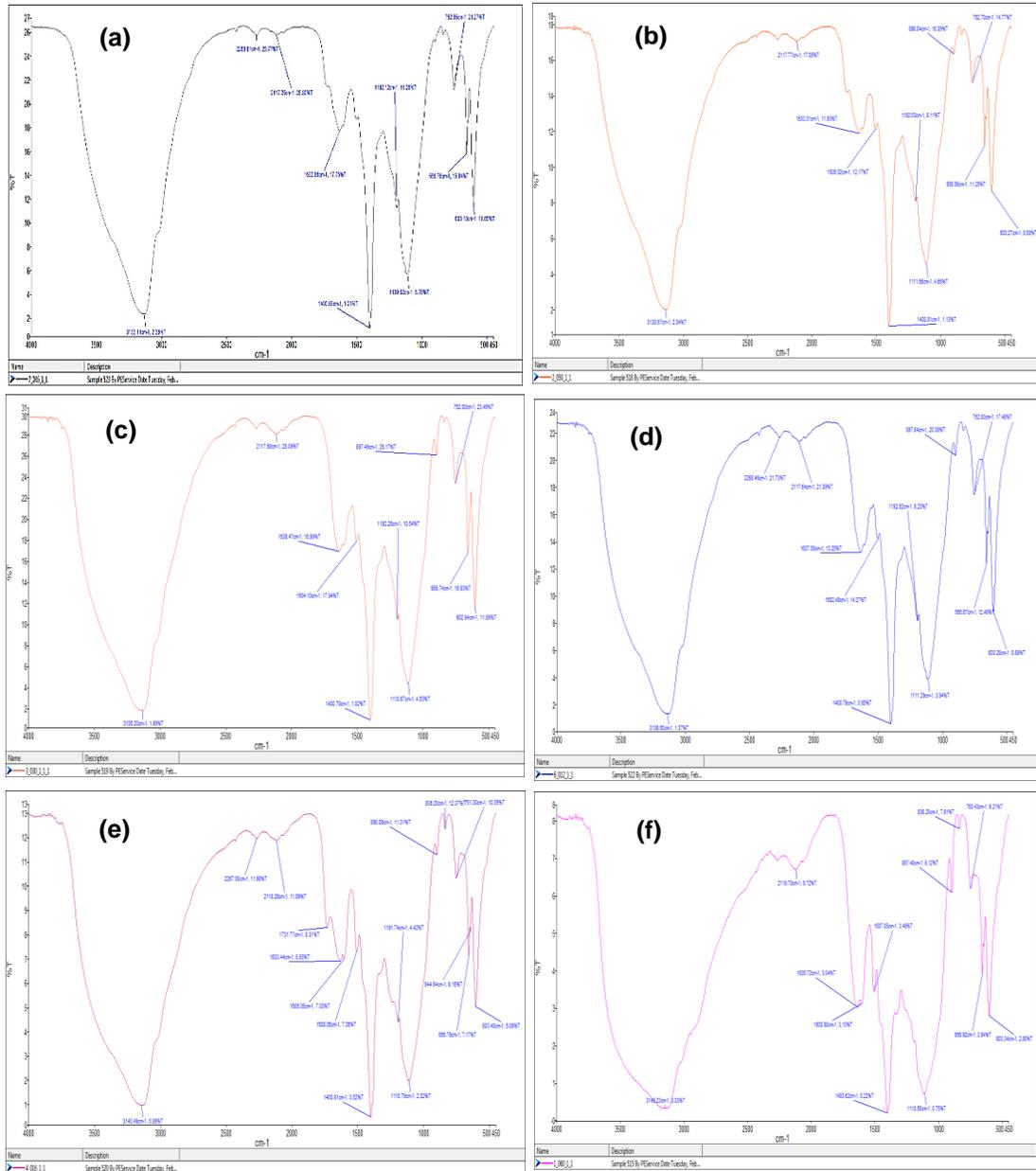
The biological treated biomass showed a peak at a range of 1700 to 1750  $\text{cm}^{-1}$ , which was absent in the untreated biomass. This suggested the presence of carboxylic and ester bonds stretching in the lignin and hemicellulose from bamboo substrate. The extensive band at a range of 3000 to 3500  $\text{cm}^{-1}$  was assigned to the stretching of the O-H bond (Phitsuwan *et al.* 2016). The intensities of these peaks indicated the cellulose content in the pretreated bamboo samples, which was significantly higher in the chemical and biological treatment samples compared to the other treatment methods.

The results of the chemical analysis also suggested that the cellulose content in the combined treatment was higher in comparison to the other methods. Another array of 500 to 1770  $\text{cm}^{-1}$  was measured as the lignin impression area, and it was observed that maximum ring vibration and C-O bond stretching occurred at these wave numbers (Xu *et al.* 2013). A reference FTIR spectrum with absorbance bands indicating hydrolysis in the biomass was used to describe the changes in the treated and untreated bamboo wood biomass (Kline *et al.* 2010).

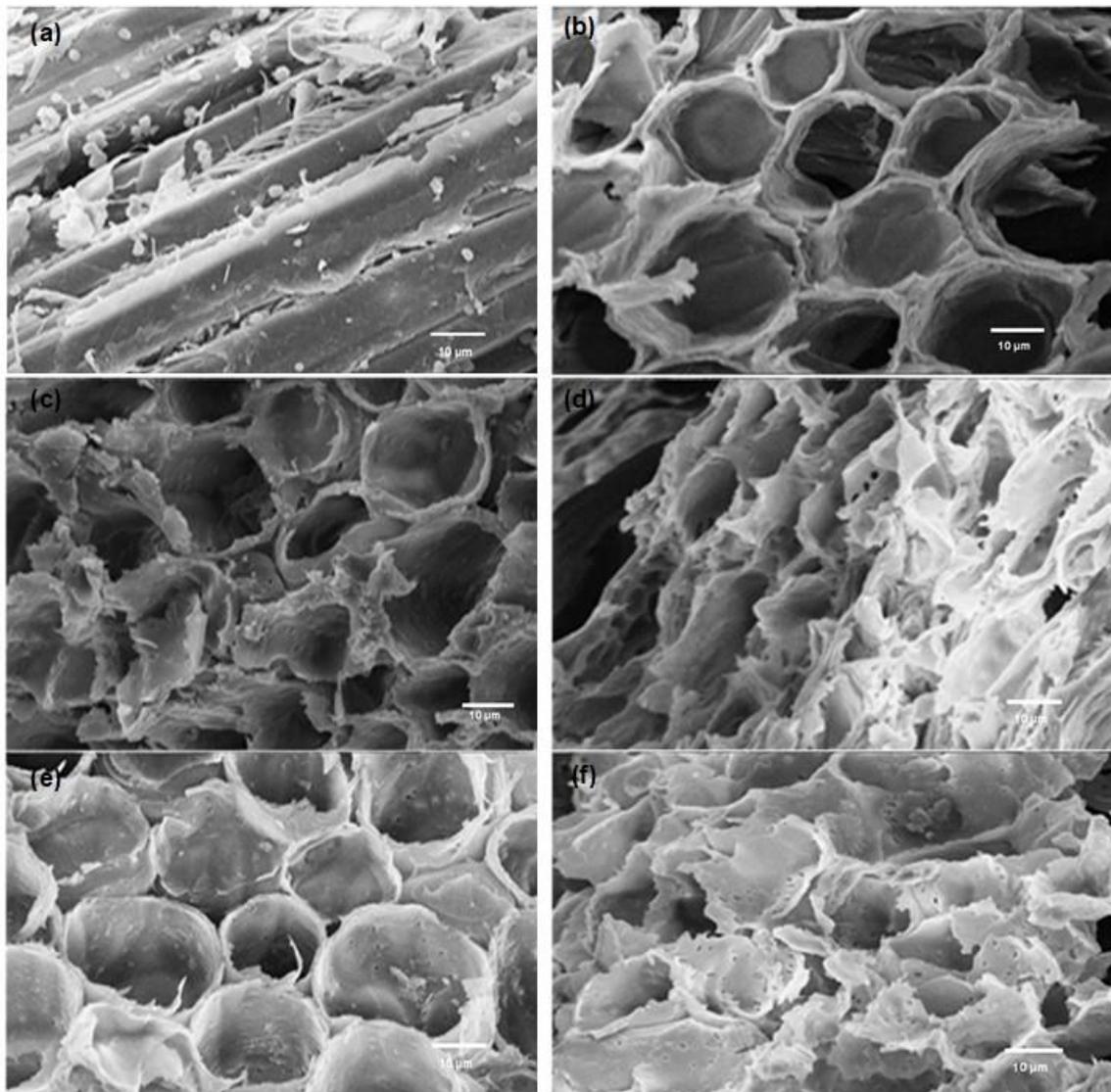
### Scanning Electron Microscopy (SEM) Analysis of the Biomass

In this study, the morphological alterations were observed in the pre-treated bamboo powder subjected to various treatments. The untreated bamboo samples revealed stiff and well-ordered stack line fibrils, with nonporous surfaces, which hindered the availability of the cellulases to cellulose. However, the SEM analysis of the alkaline (NaOH), peroxide ( $\text{H}_2\text{O}_2$ ), alkaline peroxide, biological (fungal isolate WDP2), and combined pretreatment using alkaline-oxidative along with the biological method revealed that the biomass lost its rigidity and compactness (as shown in Fig. 3a through 3f). The photomicrographs of all the treated bamboo samples showed the appearance of heterogeneous cracks that were scattered throughout the samples. Similar disruption in the lignocellulosic biomass after various treatments has also been well documented in previous studies (Shafiei *et al.* 2014).

Scanning electron microscopy analysis has been called the most important technique to study the surface of lignocelluloses (Amiri and Karimi 2015). It was clearly observed that the entire pretreated biomass surface had lost its compressed structure, which led to the formation of pits and cracks. The formation of cracks and pits are due to the breakdown and exclusion of huge part of lignins and hemicelluloses, and a reduction in crystallinity of the cellulose after the pretreatment (Kumar *et al.* 2009). The combined treatment showed a greater disruption and hydrolysis of the biomass using the biological treatment and chemical treatment method and was found to be a more effective method for causing deformation with the disruption of the hemicellulose fibres. The combined effect of both the methods resulted in the disconnection of the fibers with the appearance of the pores on the substrate surface, which could diminish the structural barrier and assist enzyme accessibility *via* the cumulative surface area. Scanning electron microscopy analysis is reliable with the FTIR spectra results. The surface characterization through SEM revealed the deformation of the biomass, which directly related to the breaking of the lignin cementing and hemicellulose mesh over the cellulose fibers.



**Fig. 2.** Fourier-transform infra-red (FTIR) spectra of the pretreated *Bambusa balcooa* samples: (a) untreated; (b) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) treatment; (c) alkali (NaOH) treatment; (d) alkaline-oxidative (NaOH-H<sub>2</sub>O<sub>2</sub>) pretreatment; (e) biological treatment (*L. elegans* WDP2); and (f) alkaline-oxidative + biological treatment



**Fig. 3.** Scanning electron photomicrographs of the chemically treated *Bambusa balcooa* wood powder showing the hydrolytic breakdowns and topological changes at 1000X: (a) untreated biomass; (b) H<sub>2</sub>O<sub>2</sub> treated biomass; (c) NaOH treated biomass; (d) alkaline-oxidative treated biomass; (e) biologically treated biomass; and (f) biologically + chemically treated biomass

## CONCLUSIONS

1. The present study attempted to investigate the effect of different available pretreatments (physical, chemical, and biological), either individually or their combination, on bamboo biomass delignification. All the tested pretreatments, whether individual or combination, showed a significant increase in the cellulose content and a decrease in the lignin content.
2. At the individual level, the biological treatment using a *L. elegans* WDP2 culture was found to be the superior method compared to the physical or chemical treatments and resulted in a higher delignification of phenolic polymers. However, a satisfactory

outcome could only be achieved by using combination treatments. Overall, the combined effect of a chemical and biological (alkali-peroxide with *L. elegans* WDP2) pretreatment method could produce the best results for the delignification of *B. balcooa* biomass.

3. This research presents an efficient and ecofriendly method that employs lesser chemical treatments and uses bamboo as the biomass, which could be an effective future source of bioethanol as India houses the second largest bamboo plantation.

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## REFERENCES CITED

- Alvira, P., Tomás-Pejó, E. Ballesteros, M., and Negro, M. J. (2010). "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review," *Bioresource Technology* 101(13) 4851-4861. DOI: 10.1016/j.biortech.2009.11.093
- Amiri, H., and Karimi, K. (2015). "Improvement of acetone, butanol, and ethanol production from woody biomass by using organosolv pretreatment," *Bioprocess and Biosystems Engineering* 38, 1959-1972. DOI: 10.1007/s00449-015-1437-0
- Bari, E., Taghiyari, H. R., Naji, H. R., Schmidt, O., Ohno, K. M., Clausen, C. A., and Suhaimi, E. (2016). "Assessing the destructive behaviors of two white-rot fungi on beech wood," *International Biodeterioration & Biodegradation* 114, 129-140. DOI: 10.1016/j.ibiod.2016.06.010
- Baruah, J., Nath, B. K., Sharma, R., Kumar, S., Deka, R.C., Baruah, D. C., and Kalita, E. (2018). "Recent trends in the pretreatment of lignocellulosic biomass for value-added products," *Frontiers in Energy Research* 6, 1-19. DOI: 10.3389/fenrg.2018.00141
- Bassam, N. E. (1998). *Energy Plant Species: Their Use and Impact on Environment and Development*, James & James, London, United Kingdom.
- Bhowmick, G. D., Sarmah, A. K., and Sen, R. (2018). "Lignocellulosic biorefinery as a model for sustainable development of biofuels and value added products," *Bioresource Technology* 247, 1144-1154. DOI: 10.1016/j.biortech.2017.09.163
- Biswas, S. A. S. (1988). "Studies on bamboo distribution in North-eastern region of India," *The Indian Forester* 114(9), 514-531. DOI: 10.36808/if/1988/v114i9/9259
- Cao, W., Sun, C., Liu, R., Yin, R., and Wu, X. (2012). "Comparison of the effects of five pretreatment methods on enhancing the enzymatic digestibility and ethanol production from sweet sorghum bagasse," *Bioresource Technology* 111, 215-221. DOI: 10.1016/j.biortech.2012.02.034
- Debiagi, F., Madeira, T. B., Nixdorf, S. L., and Mali, S. (2020). "Pretreatment efficiency using autoclave high-pressure steam and ultrasonication in sugar production from liquid hydrolysates and access to the residual solid fractions of wheat bran and oat hulls," *Appl. Biochem. Biotechnol.* 190, 166-181. DOI: 10.1007/s12010-019-03092-0

- Den, W., Sharma, V. K., Lee, M., Nadadur, G., and Varma, R. S. (2018). "Lignocellulosic biomass transformations via greener oxidative pretreatment processes: Access to energy and value-added chemicals," *Frontiers in Chemistry* 6, 1-23. DOI: 10.3389/fchem.2018.00141
- Diaz, A., Toullec, J. L., Blandino, A., Ory, I. d., and Caro, I. (2013). "Pretreatment of rice hulls with alkaline peroxide to enhance enzyme hydrolysis for ethanol production," *Chemical Engineering Transactions* 32, 949-954. DOI: 10.3303/CET1332159
- Foston, M., and Ragauskas, A. J. (2012). "Biomass characterization: Recent progress in understanding biomass recalcitrance," *Industrial Biotechnology* 8(4), 191-208. DOI: 10.1089/ind.2012.0015
- He, L., Huang, H., Zhang, Z., and Lei, Z. (2015). "A review of hydrothermal pretreatment of lignocellulosic biomass for enhanced biogas production," *Current Organic Chemistry* 19(5), 437-446. DOI: 10.2174/1385272819666150119223454
- Higuchi, H. (1957). "Biochemical studies of lignin formation, III," *Physiologia Plantarum* 10(4), 633-648. DOI: 10.1111/j.1399-3054.1957.tb06971.x
- Huang, W., Wang, E., Chang, J., Wang, P., Yin, Q., Liu, C., Zhu, Q., and Lu, F. (2017). "Effect of physicochemical pretreatments and enzymatic hydrolysis on corn straw degradation and reducing sugar yield," *BioResources* 12(4), 7002-7015. DOI: 10.15376/biores.12.4.7002-7015
- Kalra, K., Chauhan, R., Shavez, M., and Sachdeva, S. (2013). "Isolation of laccase producing *Trichoderma* spp. and effect of pH and temperature on its activity," *International Journal of ChemTech Research* 5(5), 2229-2235.
- Kansal, S. K., Singh, M., and Sud, D. (2008). "Studies on TiO<sub>2</sub>/ZnO photocatalysed degradation of lignin," *Journal of Hazardous Materials* 153(1-2), 412-417. DOI: 10.1016/j.jhazmat.2007.08.091
- Kline, L. M., Hayes, D. G., Womac, A. R., and Labbe, N. (2010). "Simplified determination of lignin content in hard and soft woods via UV-spectrophotometric analysis of biomass dissolved in ionic liquids," *BioResources* 5(3), 1366-1383. DOI: 10.15376/biores.5.3.1366-1383.
- Kumar, A. K., and Sharma, S. (2017). "Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review," *Bioresource and Bioprocessing* 4, 1-19. DOI: 10.1186/s40643-017-0137-9
- Kumar, P., Barrett, D. M., Delwiche, M. J., and Stroeve, P. (2009). "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production," *Industrial & Engineering Chemistry Research* 48(8), 3713-3729.
- Kuttiraja, M., Sindhu, R., Varghese P. E., Sandhya, S. V., Binod, P., Vani, S., Pandey, A., and Sukumaran, R. K. (2013). "Bioethanol production from bamboo (*Dendrocalamus* sp.) process waste," *Biomass and Bioenergy* 59, 142-150. DOI: 10.1016/j.biombioe.2013.10.015
- Madadi, M., Tu, Y., and Abbas A. (2017). "Pretreatment of lignocellulosic biomass based on improving enzymatic hydrolysis," *International Journal of Applied Sciences and Biotechnology* 5(1), 1-11. DOI: 10.3126/ijasbt.v5i1.17018
- Mason, W. H. (1926). "Process and apparatus for disintegration of wood and the like," US Patent No. 1578609A.
- Michelin, M., Ruiz, H. A., Silva, D. P., Ruzene, D. S., Teixeira, J. A., and Polizeli, M. L. T. M. (2014). "Cellulose from lignocellulosic waste," in: *Polysaccharides*, K. G. Ramawat and J-M. Mérillon (eds.), Springer, New York, NY, pp. 1-33.
- Moe, S.T., Janga, K. K., Hertzberg, T., Hägg, M.-B., Øyaas, K., and Dyrset, N. (2012).

- “Saccharification of lignocellulosic biomass for biofuel and biorefinery applications - A renaissance for the concentrated acid hydrolysis?,” *Energy Procedia* 20, 50-58. DOI: 10.1016/j.egypro.2012.03.007
- Nirala, D. P., Ambasta, N., and Kumari, P. (2017). “A review on distribution of bamboos,” *Life Sciences Leaflets* 92, 70-78.
- Pandey, R. K., Chand, K., and Tewari, L. (2018). “Solid state fermentation and crude cellulase based bioconversion of potential bamboo biomass to reducing sugar for bioenergy production,” *Journal of the Science of Food and Agriculture* 98(12), 4411-4419. DOI: 10.1002/jsfa.8963
- Pandey, R. K., Tewari, S., and Tewari, L. (2018). “Lignolytic mushroom *Lenzites elegans* WDP2: Laccase production, characterization, and bioremediation of synthetic dyes,” *Ecotoxicology Environ. Safety* 158, 50-58. DOI: 10.1016/j.ecoenv.2018.04.003
- Phitsuwan, P., Sakka, K., and Ratanakhanokchai, K. (2016). “Structural changes and enzymatic response of Napier grass (*Pennisetum purpureum*) stem induced by alkaline pretreatment,” *Bioresource Technology* 218, 247-256. DOI: 10.1016/j.biortech.2016.06.089
- Shafiei, M., Karimi, K., Zilouei, H., and Taherzadeh, M. J. (2014). “Enhanced ethanol and biogas production from pinewood by NMMO pretreatment and detailed biomass analysis,” *BioMed Research International* 2014, 1-10. DOI: 10.1155/2014/469378
- Singh, S., Adak, A., Saritha, M., Sharma, S., Tiwari, R., Rana, S., Arora, A., and Nain, L. (2017). “Bioethanol production scenario in India: Potential and policy perspective,” in: *Sustainable Biofuels Development in India*, A. K. Chandel and R. K. Sukumara (eds.), Springer International Publishing, Cham, Switzerland, pp. 21-37.
- Sluiter, A., Hames, B., Ruiz, R.O., Scarlata, C., Sluiter, J., and Templeton, D. (2008). *Determination of Ash in Biomass* (NREL/TP-510-42622), National Renewable Energy Laboratory, Golden, CO, USA.
- Sluiter, A., Ruiz, R. O., Scarlata, C., Sluiter, J., and Templeton, D. (2008). *Determination of Extractives in Biomass* (NREL/TP-510-42619), National Renewable Energy Laboratory, Golden, CO, USA.
- Southern Metropolis Daily Mark. (2012). “Bamboo: An ultimate green material,” Jan. 8, <http://gcontent.oeeee.com/8/18/818f4654ed39a1c1/Blog/df2/752aa2.html>.
- Sulaimani, K. (2009). “Kheda-farmer-shows-the-way-in-bamboo farming,” (<https://indianexpress.com>), accessed 06 March 2021.
- Sun, Y., and Cheng, J. (2002). “Hydrolysis of lignocellulosic materials for ethanol production: A review,” *Bioresource Technology* 83(1), 1-11. DOI: 10.1016/S0960-8524(01)00212-7
- Truong, A. H., and Le, T. M. A. (2014). “Overview of bamboo biomass for energy production,” *HAL Open Science* 1-25.
- Tsegaye, B., Balomajumder, C., and Roy, P. (2019). “Microbial delignification and hydrolysis of lignocellulosic biomass to enhance biofuel production: An overview and future prospect,” *Bulletin of the National Research Centre* 43, 1-16. DOI: 10.1186/s42269-019-0094-x
- Vats, S., Maurya, D. P., Shaimoon, M., Agarwal, A., and Negi, S. (2013). “Development of a microbial consortium for the production of blend enzymes for the hydrolysis of agricultural waste into sugars,” *J. Sci. Indust. Res.* 72(9-10), 585-590.
- Wang, W., Yuan, T., Wang, K., Cui, B., and Dai, Y. (2012). “Combination of biological pretreatment with liquid hot water pretreatment to enhance enzymatic hydrolysis of *Populus tomentosa*,” *Bioresource Technology* 107, 282-286. DOI:

- 10.1016/j.biortech.2011.12.116
- Wu, R., Zhao, X., and Liu, D. (2016). "Structural features of formaline pretreated sugar cane baggase and their impact on the enzymatic hydrolysis of cellulose," *ACS Sustainable Chemistry & Engineering* 4(3), 1255-1261. DOI: 10.1021/acssuschemeng.5b01298
- Xu, F., Yu, J., Tesso, T., Dowell, F., and Wang, D. (2013). "Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: A mini-review," *Applied Energy* 104, 801-809. DOI: 10.1016/j.apenergy.2012.12.019
- Yang, B., and Wyman, C. E. (2004). "Effect of xylan and lignin removal by batch and flow through pretreatment on enzymatic digestibility of corn stover cellulose," *Biotechnology and Bioengineering* 86(1), 88-95. DOI: 10.1002/bit.20043
- Yu, J., Zhang, J., He, J., Liu, Z., and Yu, Z. (2009). "Combinations of mild physical or chemical pretreatment with biological pretreatment for enzymatic hydrolysis of rice hull," *Bioresource Technology* 100(2), 903-908. DOI: 10.1016/j.biortech.2008.07.025
- Zhang, M., Song, X., Sun, X., Wang, Z., Li, Z., Ji, H., and Li, J. (2012). "The relationship between cellulose content and the contents of sugars and minerals during fiber development in colored cotton cultivars," *Cellulose* 19, 2003-2014. DOI: 10.1007/s10570-012-9776-3
- Zhang, H., Huang, S., Wei, W., Zhang, J., and Xie, J. (2019). "Investigation of alkaline hydrogen peroxide pretreatment and Tween 80 to enhance enzymatic hydrolysis of sugarcane bagasse," *Biotechnology for Biofuels* 12, 1-9. DOI: 10.1186/s13068-019-1454-3
- Zhao, Y., Wang, Y., Zhu, J. Y., Ragauskas, A., and Deng, Y. (2008). "Enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature," *Biotechnology and Bioengineering* 99(6), 1320-1328. DOI: 10.1002/bit.21712
- Zheng, Q., Zhou, T., Wang, Y., Cao, X., Wu, S., Zhao, M., Wang, H., Xu, M., Zheng, B., Zheng, J., *et al.* (2018). "Pretreatment of wheat straw leads to structural changes and improved enzymatic hydrolysis," *Scientific Reports* 8, 1-9. DOI: 10.1038/s41598-018-19517-5
- Zhuang, X., Wang, W., Yu, Q., Qi, W., Wang, Q., Tan, X., Zhou, G., and Yuan, Z. (2016). "Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products," *Bioresource Technology* 199, 68-75. DOI: 10.1016/j.biortech.2015.08.051

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