Production of value-added chemicals from wheat straw lignin by bio-refinery process

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Abstract. Lignocellulosic-based bio-refineries are essential for a secure energy in future. Lignin degradation is required for carbon recycling and production of renewable chemicals. Lignin due to the high level of color and low biodegradability, is categorized as a serious pollutant particularly in the aquatic ecosystem. In this study, biodegradation of organosolv lignin by a white rot fungi, Bjerkandera adusta, was verified. The FTIR spectra of lignin, before and after treatment with fungi displayed modification by changing in the structure of lignin. Some of the functional groups have disappeared and some new bands appeared in the spectra. The SDS-PAGE chromatographs also showed that the fungi protein has a molecular weight of 44 kDa. This molecular weight increased to 46 kDa after the treatment of lignin by fungi. The heavier molecular weight occurred due to the bonding of fungi protein with lignin. The results from surface morphology also showed the attachments of lignin molecules to fungi mycelia. As a result, the extracted lignin after treatment with B. adusta showed significant changes in its physical and chemical structure which makes it a noble candidate for carrying out value added products and refinery of lignin waste in pulp and paper industries.

1. Introduction
Finding new ways to produce chemicals and fuels from renewable sources, particularly from plant biomass has increased due to the necessity of reduction in greenhouse gas emissions [1]. Lignin is a by-product of pulp and paper industry which is produced in a large scale [2]. Each year 60-100 ktoms lignin, 1 Mtonne lignosulfonates, 5-10 ktomes sulphur-free soda lignin and 3 ktomes Organosolv lignin are produced by Kraft, Sulfite, Soda and Organosolv processes respectively [3]. The lignin produced in Kraft process is generally very condensed and has high amounts of reduced $\beta$-O-4 content. Furthermore, the sodium sulphide (Na$_2$S) consumed in Kraft process causes thiol groups generation. In Organosolv treatment, a greater amount of $\beta$-O-4 is preserved due to a milder biomass treatment process [4]. Lignin, the only naturally synthesised polymer, is a cross-linked macromolecule with an aromatic backbone. It is obtained from oxidative coupling of monolignols via radicals made by oxidases. The most common links in lignin structure are $\beta$-1, $\beta$-5, $\beta$-O-4, $\beta$-$\beta$, 5-5 and 5-O-4 [5]. The existence of different organic and inorganic impurities, distinctive chemical reactivity and non-uniform structure of the lignin macromolecule are the restrictions in the way of converting lignocellulosic biomasses into profitable products. Therefore, the main goal of modern bio-refineries is to overcome these obstacles. For example, by controlling the cleavage of C-O and C-C bonds in a selective depolymerisation method, new categories of aromatic and monomeric species can be obtained [6]. Furthermore, lignin is the most resilient component among lignocellulosic materials. The chemical and physical methods for removing lignin from wastewater have many impediments. One of which is the high price of chemicals applied for this process. The use of absorbents for lignin decoloring, have some other setbacks.
such as saturation of absorbent sites due to high Total Suspended Solids (TSS) and Total Dissolves Solids (TDS) [7]. Lignin degradation by microorganisms is a more effective substitute [8]. There are a few anaerobic bacteria of gastrointestinal flora and some species of fungi such as basidiomycetes, brown-rot and white-rot fungi which are capable of producing lignocellulolytic enzymes. Wood rot basidiomycetes decompose all components of plant cell wall to CO$_2$ and H$_2$O by two key mechanisms: brown-rot and white-rot decay [8]. Degradation of lignin throughout wood decay process is carried out by basidiomycetes white-rot fungi in nature. White rot fungi, on the other hand, are the only microorganism in nature which are capable of lignin mineralization. Therefore, their ability to degrade lignin can be evaluated in vitro by assessing CO$_2$ discharged when lignin decomposes thoroughly [9]. White-rot fungi, due to their ability to yield great amounts of extracellular lignocellulolytic enzymes are attractive microorganism for bio-degradation of biomasses. Bjerkandera species are intersecting microorganism in biotechnology due to their ability in degrading aromatic xenobiotics in addition to lignin [10]. B. adusta is one of the white rot fungi which belongs to basidiomycetes. This fungus produces white rot on trees or wood by penetrating its mycelium in to the cell holes while diffusing ligninolytic enzymes. This fungus breaks down the lignin in the plants cell walls for accessing to the cellulose. Culture medium composition, inoculum concentration, pH, temperature, duration and speed of incubator have major effect on the mycelium growth of B. adusta. Ligninolytic enzymes produced by white-rot fungi can oxidise and degrade lignin. Furthermore, they increase the accessibility of the lignin structure. Enzymatic reactions, catalytic oxidation, catalytic reduction and hydrolysis reactions are the key approaches for depolymerisation of lignin [11]. Four enzymes mainly interfere in lignin degradation; manganese peroxidases (MnP), versatile peroxidases (VPs), lignin peroxidases (LiPs) and laccases (Lac). Due to large size of lignocellulolytic enzymes, their permeability into undecomposed wood cell walls is reduced. Hence, for native lignin decomposition, reactive oxygen species might play an important role. Fungal MnP, LiP and VP are all from the class II peroxidases in the superfamily of heme peroxidases. Lip and MnP are glycoprotein with 38-46 kDa and 40-50 kDa molecular weights respectively [12].

In this study, the biodegradation of the Organosolv lignin to yield value-added chemicals was investigated. For this purpose, white rot fungi, Bjerkandera adusta was applied and changes in the structure of lignin were verified.

2. Materials and methods

2.1. Lignin sample

In previous study, the lignin was extracted by applying ethanol organosolv treatment from wheat straw. First, the extraction conditions were optimized in a 600-ml Parr reactor. The maximum amount of yield (>90%) and the least amount of carbohydrates impurities and ash was isolated in 200 °C, 400 psi and 120 min. The optimum experiment’s conditions in which the highest amount of lignin was yielded, were selected for further extraction. Subsequently, for large-scale production, the lignin extraction was carried out in a 20L reactor [13].

2.2. Fungi

Bjerkandera adusta (UAMH 8258) was purchased from University of Alberta Microfungus Collection and Herbarium, Alberta, Canada (UAMH) and was cultivated on a commercial Potato Dextrose Agar (PDA) from HiMedia Laboratories at 26°C. To monitor the alterations in the lignin structure and investigate the enzymes production, the experiment was carried out in a liquid medium. To prepare the inoculum, 10 mm$^3$ of B. adusta agar plugs was suspended in a 5-ml medium. Then the suspension was transferred into a 2000 ml Erlenmeyer flasks containing 1000 ml of Potato Dextrose Broth (PDB) and 2 g of the extracted lignin. The pH was adjusted to 5.5 before autoclaving. All the culture media were sterilized by autoclaving at 121°C for 15 min. The flasks were incubated in a shaker (120 rpm). Two controls were designed for the experiment. One flask was contained PDB and 2g lignin and the other one PDB with B. adusta suspension. The cultures were harvested at the 5th and 7th days of incubation to verify the changes in lignin structure and production of enzymes from the fungi.
2.3. **Lignin Extraction and Fungi Cultivation**

2.3.1. **Fungus Modified Lignin Analysis**

Each sample was verified for lignin structure, surface morphology and proteins. For this purpose, each culture medium was taken under the biological fume hood and 500 ml of media were collected in the beakers. The contents of culture flasks were filtered with n°1 Whatman filter paper under vacuum. The filtered parts were kept in the freezer. 100 ml NaOH (10N) was added to the solid parts and the mixture were stored overnight in the beaker to dissolve all the lignin inside the mycelia. After 24 hours, the dissolved lignins in NaOH were centrifuged for 20 mins and 10000 rpm. The supernatants were kept in the freezer and the pellets were dried in the oven at 60°C [14]. The samples were named as follow:

Sample #1: *B. adusta* + 2 g lignin in PDB medium after 5 days  
Sample #2: *B. adusta* + 2 g lignin in PDB medium after 7 days  
Sample #3: *B. adusta* in PDB medium after 5 days (control)  
Sample #4: *B. adusta* in PDB medium after 7 days (control)  
Sample #5: 2 g lignin in PDB medium (control)

2.3.1.1. **Scanning electron microscopy (SEM)**

SEM was carried out by a JOEL machine (Hitachi S-2500, Tokyo, Japan), model 6610LV with a usual SEI Scintillator detector and BSEI diode detectors. Two different magnifications (800X and 6000X) were applied for SEM images. The mycelia of the sample which contained lignin treated by *B. adusta* and the control which contained only fungi were applied for the SEM imaging. The samples were cut carefully to maintain the structure of inner and outer surface of fungi mycelia. The surface was gold coated to bypass electrostatic charge during the study.

2.3.1.2. **Fourier transform infrared spectroscopy (FTIR) analysis of lignin sample before and after treatment by fungi**

Chemical changes in functional groups and polymer structure in culture media contained sample 1, 2, 3, 4, 5 were analyzed by FTIR machine. The dried samples were mixed with KBr. The samples structures were documented in the range of 4000-400 cm\(^{-1}\). A Bruker Tensor 27, Model Vertex 70 (Germany) with a temperature controller –I0977 detected the FTIR spectra. The spectra were noted with a resolution of 4 cm\(^{-1}\) and scan rate of 32 scans per minute.

2.3.1.3. **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of extracted protein**

SDS-polyacrylamide gel electrophoresis with marker protein (Bio-Rad) was applied to separate the extracellular proteins. The gel gradient concentration was 4% to 12%. To denature the proteins, 2-mercaptoethanol were applied as reducing agent. A Bis-Tris buffer system at 150 V was used for electrophoresis and Coomassie Brilliant was used for staining.

3. **Results and Discussions**

3.1. **Decolorization of lignin upon the fungi growth in the broth medium**

To increase the ability of fungi to degrade lignin, the organosolv lignin with high purity was applied. Figure 1. shows the change in the color of Organosolv lignin culture media after fungal growth. Figure 1. a. shows the culture media before the inoculations. In Figure 1. b. the flask which contained the fungi and lignin (middle) shows the changing in the color of medium. In Figure 1.c. the absorption of the black color particles inside the fungal mycelium, is observed. This could be explained by the decolorizing ability of *B. adusta*. Later by cutting the mycelium and observing them under the SEM the existence of lignin molecules inside the mycelia was confirmed.

Whit-rot fungi, among the potential microbes, due to having a robust lignin degrading enzymatic system are good candidate for lignin degradation and decolorization of pulp and paper waste [15].
3.2. Surface morphology

Figure 2. shows the fracture surfaces of the B. adusta observed by SEM after 7 days of incubation with lignin. These images clearly show the bonding of extracted lignin particles inside B. adusta mycelia. These spheres could not be observed on the control sample (without lignin). The SEM image of B. adusta from the media with Organosolv lignin shows the lignin granules with the size of ~4 μm which is compatible with data collected in the previous studies [16].

3.3. Analysis by fourier transform infrared spectroscopy

The FTIR analyses were performed on samples obtained from the 5th (#1) and 7th (#2) days of culture media contains B. adusta, lignin and PDB (Figure 3). The FTIR analyses also were performed on controls consist of B. adusta in PDB culture medium form day 5th (#3), day 7th (#4) and the one with lignin and PDB (#5). FTIR analysis of treated samples from the 5th and 7th days showed certain changes in the spectra as compared with the controls. The FTIR spectra of Organosolv lignin biodegraded by B. adusta displayed significant variations from 500 to 4000 cm⁻¹. The control sample which consists of only lignin and PDB shows strong peaks in FTIR spectrum and the results were consistent during the time due to the absence of fungi in medium. When the fungi continued the growth in the flasks consist
of fungi and lignin from day 5\textsuperscript{th} and day 7\textsuperscript{th}, some peaks showed only a weak peak in the lignin molecule and some of the peaks disappeared.

![FTIR spectra of treated lignin samples](image)

Figure 3. FTIR spectra of treated lignin samples.

The FTIR spectrum of control sample (#5) which contained only lignin and PDB is displayed. The sample showed a peak at 1267 cm\(^{-1}\) which is assigned to C-O in guaiacyl ring. This band disappeared in FTIR spectra of sample #1 and #2, which represents the conversion of guaiacyl to other bands. Besides, in sample #5 the band at 1452 cm\(^{-1}\) signifies the existence of C-H bend in aromatic ring, this band also disappeared in sample #1 and #2. The band at 3413 cm\(^{-1}\) which is associated with oscillation of hydroxyl group is disappeared in the treated samples. Furthermore, the absorption band at 1597 cm\(^{-1}\) in sample #5 corresponds to aromatic ring vibration and C=O stretching, this band also could not be detected in the sample #1 and #2. In FTIR spectra of sample #1, the appearance of new C-H bond around 1462 cm\(^{-1}\) is a direct result of deformation in methyl and methylene groups. Additionally, new aromatic bands at 1400 cm\(^{-1}\) and 2855 cm\(^{-1}\) of sample #1 spectra are corresponded to C-N stretch of amides and O=C-H:C-H stretch of aldehydes. After treatment of lignin with \textit{B. adusta}, a band at 1654 cm\(^{-1}\) originating from conjugated carbonyl in lignin is appeared, which proposed the formation of new carbonyl. By comparing sample #1 with #2, in sample #2, new band at 708 cm\(^{-1}\) was observed which was not presented in sample #1. This could be the result of changes in lignin structure during the growth of fungi. By comparing the FTIR spectra in different samples, biodegradation of lignin in sample #1 and #2 could be observed. By comparing the results with sample #3 and sample #4, it can be concluded that fungi growth alone on the PDB has a limited effect on the overall results. Finally, band absorption between 458 and 600 cm\(^{-1}\) corresponded to C-Br stretch, which could be detected in all the samples [15], [16].

Studies showed, lignin degradation microorganisms, breakdown guaiacyl unit to vanillic acid as an intermediate [17]. The structure of lignin can be modified in existence of enzymes. For instance, laccases can modify the lignin structure by making dissimilar mesomeric forms that might couple in many potential forming inter-unit bonds. The mesomeric effect can be detected in \(\beta\)-5, \(\beta\)-O-4 and 5-O-4 bonds of lignin molecules [18]. Peroxidases, on the other hand, can oxidase 5-O-4 nonphenolic and phenolic structures in lignin. They also enhance the reactivity of lignin by rising its phenolic content. The biodegradation Organosolv lignin by \textit{B. adusta} can be valuable for a variety of applications such as flocculants, adhesive, sizing agents and corrosion inhibitor [19].
3.4. Gel Electrophoresis

The fungi proteins of lignin treated sample by B. adusta (day 7) and the control which contained only fungi (day 7) detached by gel electrophoresis. Figure 4 shows the Gel electrophoresis of lignin treated by fungi and the control which only had fungi.

![Figure 4. Gel electrophoresis of (M) marker, (1) lignin treated by fungi and (2) control (with only fungi protein).](image)

Although the treated and non-treated samples showed similar protein profiles, but the bands from lignin treated media by fungi expressed more. It also exhibited higher protein concentration. The band positions of treated sample also were slightly different from the control. The molecular mass of proteins produced by treated lignin was ~46kDa and non-treated lignin was ~44kDa which can be defined due to the bonding between protein and polymer. The gel electrophoresis of culture contained lignin without fungi did not show any visible proteins bands. This confirmed that the origin of the protein detected in treated sample and control were from B. adusta activity, not from the lignin itself. Furthermore, the molecular weights of MnP and LiP are 44-45 and 41-43kDa respectively [20], which are closed to what has been shown in our results. The results from gel electrophoresis suggested that the nature of B. adusta proteins, is capable of lignin biodegradation.

The lignin modifying enzymes (LMEs) secrete extracellularly to facilitate decomposing process. These extracellular enzymes degrade lignin effectively but not selectively [9]. B. adusta also produces manganese peroxidase- lignin peroxidase hybrid enzyme. These enzymes can oxidize numerous phenolic and nonphenolic precursor such as guaiacol, 2,2’-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and veratyl alcohol, 2,6-dimethoxyphenol in the lack of Mn(II). MnP and Lip are glycosylated heme-containing enzymes. Mnp, on the other hand, is capable of cleaving and oxidizing lignin by consuming H2O2 for its activity [20].

4. Conclusion

Although the total energy demand has grown 24% in worldwide from 2005 to 2015, the bioenergy portion in overall global primary energy utilization has stayed comparatively stable (about 10%). The annual production of lignin in large quantities is more than 70 million tons but only 2% of lignin is applied for industrial purposes and 95% is burnt to produce energy. This is while 80% of world’s primary energy supply is still from fossil fuels [21], [22]. The United States and European Union established goals to develop lignin valorization process so it can replace fossil fuels. One of the investment priorities of European Union to achieve a sustainable development within 2020 is lignocellulosic biomass alteration into biofuel which is considered a promising choice to substitute fossil fuels. Lignocellulosic-based bio-refineries are essential for a secure energy in future. Lignin has a critical effect on increasing the amount of liquid transportation fuel accessible from biomass over the carbohydrate particles [23]. Ultimately, lignin is a green biofuel and has a countless market in sustainable energy and bio-polymeric materials [1].
According the results obtained from surface morphology, bonds between lignin particles and mycelia were observed, which appeared essential for lignin degradation. Also, the changes in FTIR spectra of different samples, confirmed the lignin modification during the fungi growth. The results suggest the proposed fungi, *B. adusta*, as a suitable choice for decolourization and removal of lignin wastes. Ultimately, lignin degradation resulted in production of aromatic chemicals which can be applied for further applications.

5. References

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