Biodegradation characteristics of pine lignin by *Raoultella ornithinolytica* MP-132

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ABSTRACT

The high cellulose content of pine sawdust causes it to be highly valued as an excellent source for biorefining. However, the complicated interaction between lignin and cellulose presents an obstacle to the successful utilization of pine sawdust. The process of breaking down lignin has become essential for effectively using pine sawdust. The objective of the investigation was to explore the lignocellulolytic enzymes activity and its effect on lignin degradation by strain MP-132. A culture medium with pine sawdust as the solo carbon source was investigated. The performance of lignocellulose breakdown was evaluated through determining the activity of the lignocellulolytic enzymes. Fourier-transform infrared spectroscopy and thermogravimetric analyzer were used to investigate the changes in the structure and appearance of the degraded pine lignin. The maximum activities of lactase, lignin peroxidase, cellulase, and xylanase secreted by strain MP-132 reached 55.97 U/L, 142.6 U/L, 2.92 U/mL, and 2.08 U/mL, respectively. On day 15th, the degradation rates of cellulose, hemicellulose, and lignin were 28.78%, 32.9% and 30%, respectively. After treatment with strain MP-132, significant changes in the lignin structure have been observed in pine sawdust. These findings indicate that *Raoultella ornithinolytica* MP-132 exhibits potential as an alternative candidate for the lignocellulosic bioenergy industry.

Keywords: Lignin, *Raoultella ornithinolytica*, biodegradation, depolymerization, laccase, lignin peroxidase, cellulase, xylanse

1. INTRODUCTION

Wood is a crucial and extensively utilized raw material in several fields such as building, furnishings, and paper manufacturing in modern society. However, there is a significant inefficiency in the utilization of resources during the wood processing procedure. For example, the byproduct generated from wood processing is also a valuable resource, however, the majority of this byproduct is typically regarded as waste and is either directly disposed of or burned. This process not only contaminates the environment, but also wasted valuable wood fiber resources that could be reused. Pine sawdust is highly regarded as an exceptional source for biorefining due to its elevated cellulose, hemicellulose, and lignin content. Nevertheless, the intricate interplay between lignin and cellulose poses a challenge to the effective exploitation of pine sawdust. The process of breaking down lignin is crucial for the effective use of pine sawdust.

Lignocellulose can be converted into energy through bio-refining approaches. With converting it into biofuels, bio-based chemicals, and bio-based polymers, mankind might reduce the dependence on fossil fuels[1]. The process of transforming cellulose and hemicellulose into chemical compounds including ethanol and butanol typically includes three primary steps: lignin depolymerization, enzymatic hydrolysis cellulose and hemicellulose, and monosaccharide fermentation[2]. Lignin depolymerization is a crucial barrier to the transition from cellulose to bio-chemicals. As lignin is completely embedded in cellulose fiber, it provides an inherent defense against enzymatic and physical destruction. However, in the natural environment, numerous biological systems possess sophisticated and diverse enzyme systems that may effectively tackle this obstacle[3]. Study findings has demonstrated that lytic polysaccharide monooxygenases may function as an association of enzymes that degrade polymeric lignin structure, leading to the breakdown of β-O-4 and C-C bonds[4]. *R. ornithinolytica* MP-132 is a symbiotic bacterium found in the gut of termite that can break down cellulose and hemicellulose. The whole genomes of *R. ornithinolytica* have been investigated, revealing a diverse array of genes responsible for production of enzymes, including auxiliary enzymes, lignin-degrading enzymes, and cellulolytic enzyme.

Therefore, in this study, the biodegradation rates of cellulose, hemicellulose, and lignin by *R. ornithinolytica* MP-132 during the bio-treatment of pine were investigated. Simultaneously, the enzyme types and activities that causes the decomposition of pine were determined. In addition, the study investigated the changes in the chemical structure of the

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pine lignin. In general, the purpose of this study is to improve the techniques used for researching the decomposition and transformation of lignin through investigating the structural changes in lignin during the bio-treatment pine.

2. MATERIALS AND METHODS

2.1 Culture medium

Luria-Bertani (LB) medium: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl.

The fermentation medium was comprised of 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $FeSO_4$, 0.1 g/L $CaCl_2$, 2 g/L $(NH_4)_2SO_4$, 0.02 g/L $MnSO_4$, 0.001 g/L $ZnSO_4$, 0.001 g/L $CuSO_4$, 0.05 g/L tryptone and 10 g/L pine sawdust.

2.2 Biodegradation of pine sawdust

Raoultella ornithinolytica MP-132 is inoculated into an LB medium for culture and incubated at 30°C with agitation at 150 r/min. The culture is allowed to grow until the optical density at 600 nm (OD600) reaches approximately 1.0. The inoculum of strain MP-132 are then transferred to a fermentation culture medium and incubated at 30°C with agitation at 180 r/min in a shaker for 15 days. Samples are taken at regular intervals for subsequent analysis experiments. The content of cellulose, hemicellulose, and lignin in pine were determined following the methodology described in earlier publications[5].

2.3 Measurements of lignocellulolytic enzyme activity

2 mL samples were extracted from fermentation cultures every 24 hours to conduct experiments measuring the activity of lignocellulolytic enzymes. The enzymatic activity of laccase, lignin peroxidase, cellulase and xylanase were measured using a UV-Vis spectrophotometer, following the approach described in previous publications[6].

2.4 Milled wood lignin (MWL) extraction

Following the procedure described in the literature for extracting milled wood lignin from pine[7], the exact procedures are as follows: the powder of raw pine sawdust or pine residues that have been destroyed by bacteria was dissolved in a solution of 90% dioxane, and then it was placed in a lightless environment, continuously stirred at a typical temperature for a duration of 12 hours, and undergoes extraction three times in series. The filtrate was collected and place them into a rotary evaporator set at a temperature of 40°C. As the solution evaporates, it produces raw milled wood lignin (MWL). The samples of raw MWL were dissolved in a 90% acetate water solution (with a solvent volume of 25 mL per gram of lignin), and then a certain quantity of water was added to the mixture to precipitate it. The obtained solids were MWL extracted from pine. The MWL samples were dried in a freeze drier. The dehydrated MWL samples are mixed with a 2:1 (V/V) of dichloroethane and ethanol solution, and the insoluble portion is then removed. Anhydrous ether was added into the filter, resulting in the formation of lignin sediment, and then was placed in the frozen dryer for drying. The dry lignin samples are being used for further experimental analysis.

2.5 Characterization of pine lignin

The functional groups included in the lignin samples were analyzed using Nicolet nexus 470 Fourier transform infrared spectroscopy (FTIR, Thermo, USA), following the methods described by Li et al.[8]. Furthermore, a thermogravimetric analysis of lignin was conducted using a TGA4000 thermogravimetric analyzer (Perkin-Elmer, USA) following the approach suggested by Li et al.[8].

3. RESULTS AND DISCUSSION

3.1 Determination of cellulose, hemicellulose, and lignin content in raw and treated pine sawdust

In order to find out about *R. ornithinolytica* MP-132's degradation capabilities in pine sawdust, the changes in the composition of cellulose, hemicellulose, and lignin in pine sawdust by strain MP-132 can be examined. The result has been displayed in Table 1. The content of cellulose, hemicellulose, and lignin in pine residual were 32.8%, 17.8%, and 22.5% after 7 days of bacterial treatment. The degradation rates were 18.6%, 23.9%, and 18.47%, respectively. After 15 days of processing, the degradation rates of cellulose, hemicellulose, and lignin were 28.78%, 32.9% and 30%, respectively. *R. ornithinolytica* MP-132 is renowned for its exceptional capacity to break down hemicellulose,

particularly after it has been exposed to the ball grinding process. As a result, it exhibits an excellent level of hydrolysis efficiency.

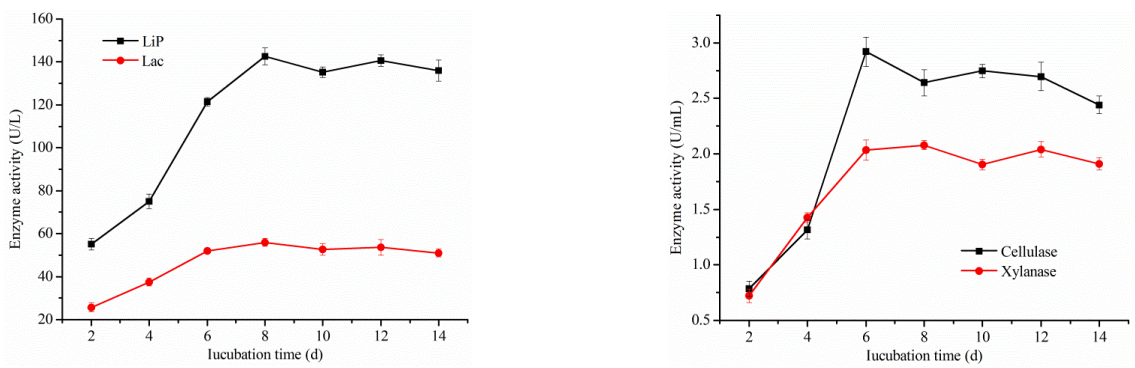
Table 1. The content of cellulose, hemicellulose, and lignin in pine sawdust treated by *R. ornithinolytica* MP-132.

Samples	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated	40.3±0.2	23.4±0.3	27.6±0.3
Treated 7 days	32.8±0.2	17.8±0.3	22.5±0.1
Treated 15 days	28.7±0.3	15. 7±0.2	19.3±0.2

3.2 Analysis of lignocellulolytic enzymes activities

The degradation of lignocellulose by microorganisms takes place primarily through the secretion of lignocellulolytic enzymes. This study identified the four main enzymes (laccase, lignin peroxidase, cellulase and xylanase) that are required for the degradation of pine sawdust by *R. ornithinolytica* MP-132. The results have been shown in Figure 1. During the initially four days of the process of fermentation, the enzyme activity of cellulase, xylanase, laccase (Lac), and lignin peroxidase (Lip) were relatively low, measuring at 1.31 U/mL, 1.43 U/mL, 37.5 U/L, and 75.1 U/L, respectively. As the fermentation time was prolonged by eight days, the activity of the lignin peroxidase reached its peak at 142.6 U/L; however, for the sixth day, the growth rate had significantly slowed; a slight decline was observed during the last 10 to 14 days of fermentation, indicating a dynamic change in trend.

Figure 1b illustrates that cellulase and xylanase exhibited patterns of growth six days before fermentation. The activity of cellulase initially followed the same growth trend as xylanase for the first four days. However, following the fourth day, the growth rate of cellulase activity rose dramatically. On day 6, the enzyme activity of cellulase reached a peak of 2.92 U/mL. As the fermentation time increased, the enzyme activity started to decrease. The dynamics of xylanase were comparable to those of cellulase, with their enzymatic activity peaking at 2.08 U/mL on the 8th day. When the last phase of fermentation, the enzyme activity of cellulase and xylanase declines. This decrease may be attributed to dead bacterial cells and lower vitality in the late stage, or it could be caused by the appearance of aromatic compounds in the fermenting liquids as a result of lignin degradation. These aromatic compounds inhibit the metabolic processes of the bacteria.



(a) (b)

Figure 1. Time course of laccase and lignin peroxidase activities (a), cellulase and xylanase activities (b) during the degradation of pine sawdust by R. ornithinolytica MP-132.

3.3 FTIR analysis

An FTIR analysis is carried out on untreated and treated pine lignin samples in order to obtain more knowledge of the decomposition of pine lignin. As shown in Figure 2, after bacterial treatment, pine lignin retains the basic structure of the bone structure, but its strength shifts dramatically.

The peaks observed between 2937 cm^{-1} and 2852 cm^{-1} represents the asymmetric and symmetrical vibrations of the C-H bonds in the methyl and methylene groups, which are in the side chain of lignin[9]. The relative intensity of these peaks decreased after bacterial treatment, which was associated with changes in the structure of lignin. The characteristic peak

at 1710 cm^{-1} indicates the presence of ketone or aldehyde functional groups in lignin, while the reduced absorption peak intensity at 1375 cm^{-1} (symmetric deformation vibration of –CH3), indicating that lignin side chain oxidation has resulted in the cleavage of ester bonds[10]. The absorption peaks at 1465 cm^{-1}, 1420 cm^{-1}, 1269 cm^{-1}, and 1219 cm^{-1} correspond to the lignin skeletal structure[11]. The decrease in peak intensity indicates the degradation of some lignin structures. The intensity of absorption peaks at 1142 cm^{-1} (deformation of the C-H ring) and 1033 cm^{-1} (C-O-C, vibration of C-O, and C=O vibration in the C-H ring) decreased revealing a reduction in the ether bonds of the lignin structure[12]. The above-mentioned findings demonstrate that *R. ornithinolytica* MP-132 is highly efficient in breaking down the lignin structure of pine.

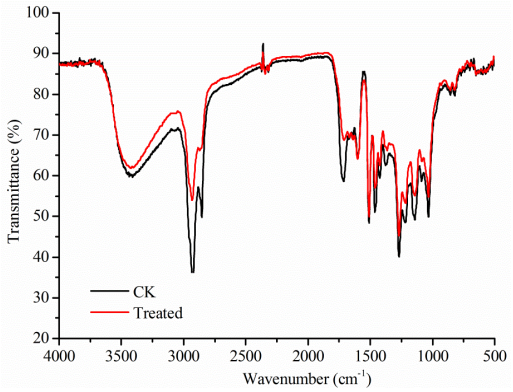
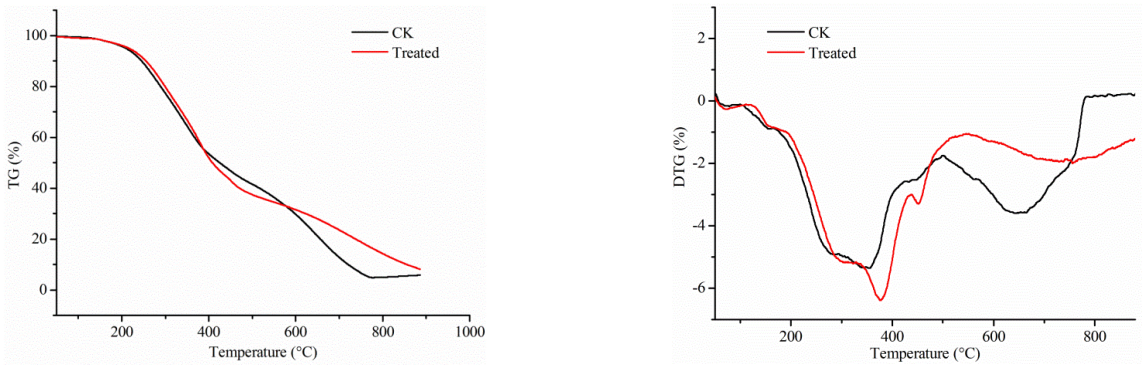


Figure 2. FTIR spectrum analysis of milled wood lignin (MWL). (CK) MWL of untreated pine sawdust and (Treated) MWL of pine sawdust treated with *R. ornithinolytica* MP-132.

3.4 Thermal analysis

Lignin is mostly composed of phenylpropane units, which are linked to other molecules through various chemical bonds. The pyrolysis of lignin is directly impacted by the types of chemical bonds and functional groups present in it[13]. Figure 3 displays the thermogravimetric (TG) and differential thermogravimetric (DTG) curves of both the control sample and MWL samples. The curve of TG and DTG illustrates the difference in thermal stability between both kinds of ground wood. This difference is mainly noticeable in the initial and final temperatures of the heat degradation reaction, as well as the rate and peak of loss of weight. As shown in Figure 3a, the simultaneous rate of degradation of the two MWL samples occurred within the temperature range of 50 to 190°C. This process is mostly caused by the removal of water and other volatile molecules present in the lignin samples[14]. Within the temperature range of 200 to 385°C, the degradation rate of the control samples is faster compared to the treated samples. This stage mainly involves the breakdown of the side chain and certain bonds of chemicals with low energy[15]. This indicates that the lignin samples had an increase in side chains and chemical bonds after bacterial treatment. Between 385 and 580°C, the rate of degradation of the treated MWL sample was faster than that of the control sample. It was primarily caused by the decomposition of methoxy group, leading to fewer methoxy groups in the bacterial-treated sample[16]. From 580°C to the end of the heating phase, the control sample degrades faster than the treated samples; this stage mainly involves the C-C bonds of the lignin skeleton structure, indicating that the bacterial treated samples contain more C-C bonds than the control samples[17]. Furthermore, at a temperature of 775°C, the control sample's loss of weight ended decreasing to a final residue weight of 4.8%, whereas the loss weight of treated samples decreased until the heating finish, with a final residual weight of 8.13%.



(a) (b)

Figure 3. TG and DTG curves of untreated and MWL treated by *R. ornithinolytica* MP-132.

4. CONCLUSION

Strain MP-132 exhibits the capability to utilize pine sawdust as the sole carbon source. It's possible to both qualitatively and quantitatively determine its secreted lignocellulolytic enzymes. Furthermore, the structure and thermolysis characteristics of pine lignin exhibit changes following bacterial bio-treatment. The maximum activities of lactase, lignin peroxidase, cellulase, and xylanase secreted by the strain MP-132 reached 55.97 U/L, 142.6 U/L, 2.92 U/mL, and 2.08 U/mL, respectively. On day 15th, the degradation rates of cellulose, hemicellulose, and lignin were 28.78%, 32.9% and 30%, respectively. These findings demonstrate that the MP-132 strain possesses an effective ability to deconstruct lignocellulose and provides an invaluable resource for lignin valorization.

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