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Sugarcane bagasse degradation and characterization of three white-rot fungi

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HIGHLIGHTS

- *Lentinula edodes* LE16 was the best fungus for selective delignification.
- Polyphenol oxidase and MnP activities are reported in *L. edodes* on sugarcane bagasse firstly.
- PPO as one of the primary ligninolytic enzymes is the first report.
- Role of esterase in degrading the hemicellulose–lignin matrix was confirmed.
- Lignin selective removing could be reached by controlling biodegradation process.

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ABSTRACT

In order to investigate the details of lignin biodegradation, the characteristics and process of sugarcane bagasse (SCB) degradation by three lignin degrading fungi, *Phanerochaete chrysosporium* PC2, *Lentinula edode* LE16 and *Pleurotus ostreatus* PO45, were studied. We found that the ligninolytic enzymes polyphenol oxidase (PPO) and manganese peroxidase (MnP) were produced first, and that the cellulolytic enzyme CMCase was produced subsequently. These three fungi were more efficient to degrade lignin (85–93%) than hemicelluloses (64–88%) and cellulose (15–67%) in 12 weeks, in which *P. chrysosporium* PC2 was the most efficient strain to degrade all the ingredients. Results of the FTIR and CP/MAS ^{13}C NMR revealed that the three fungi preferentially degraded syringyl units. The PPO and MnP as the main ligninolytic enzymes, especially the presence of PPO, were new findings in this study, which improved our knowledge of biopretreatment of SCB and evidenced these strains as valuable resource for SCB biotransformation.

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1. Introduction

Sugarcane bagasse (SCB) is one of the most abundant by-products of agroindustry in the world, creating 540 million tons of residue per year (Satyanarayana et al., 2008). SCB typically contains (on a washed and dried basis) approx. 40% cellulose, 24% hemicelluloses, and 25% lignin (Saha, 2003), as well as small proportions of ash and waxes. For the large-scale biological production of fuel ethanol, SCB is considered to be an attractive feedstock because of its availability in large quantities at low cost. However, the primary limit of SCB usage is its high degree of complexity due to its mixed composition of cellulose, hemicelluloses and lignin in extremely inhomogeneous fibers, in which the lignin is the compounds most resistant to degradation. Therefore, removal of the lignin from the complex by some kind pretreatment is the most important step in bioethanol production from SCB (Cardona et al., 2010). Compared to the numerous physical and chemical pretreatments, biological methods to remove lignin from SCB are superior because their mild condition, less consume of energy and being environmentally friendly (Camassola and Dillon, 2009). However, the biological processes to remove lignin are generally slow and difficult to control, which has limited their application at the industrial level.

To improve the biological removal of lignin, selection of effective microorganisms and enhancing the activities of lignin degrading enzymes are essential. For this purpose, many white-rot fungi, such as *Phanerochaete chrysosporium*, *Cyathus stercoreus*, *Phlebia* sp., *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, and *Irpex lactues* have been tested in microbial pretreatments for corn stover, wheat straw, rice straw, cotton stalks and sugarcane bagasse (Singh et al., 2010; Sharma and Arora, 2010; Yang et al., 2010a,b; Camassola and Dillon, 2009). According to the previous studies, the fungi mentioned were the most efficient microorganisms for

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biomass deconstruction and delignification (Dinis et al., 2009), and they secrete a unique set of extracellular oxidases and peroxidases that enable them to effectively degrade lignin (Elisashvili et al., 2008). Lignin peroxidase (LiP), manganese peroxidase (MnP) and copper-containing enzymes are common ligninolytic enzymes found in these fungi. One copper-containing enzyme is polyphenoloxidase, which possess three different but connected activities: (i) catechol oxidase; (ii) laccase and (iii) monophenol monooxygenase (Sheptovitsky and Brudwig, 1996). This enzyme catalyzes the oxidation of polyphenolic compounds in the presence of molecular oxygen. Researchers have noticed the laccase activities of polyphenoloxidase in delignification, but no attention has been paid to the catechol oxidase activity in the lignin degradation.

In several studies, some white-rot fungi were found to simultaneously produce cellulolytic, hemicellulolytic and ligninolytic enzymes (Isikhuemhen and Mikiashvilli, 2009), which are necessary for the degradation of complex materials such as SCB. In addition, white-rot fungi also produce a group of accessory enzymes such as esterase when they degrade a substrate. In general, researchers have primarily emphasized the efficiency of lignin degradation, the types and activities of the ligninolytic enzymes and the characteristics of degradation products, but little attention has been paid to the accessory enzymes in lignin degradation. According to our knowledge, there is no systematic investigation about the kinetic of the cellulolytic, hemicellulolytic and ligninolytic enzymes, as well as their cooperation with the esterase. In addition, no information is available about the suitable period of pretreatment, although different lignin degrading fungi have been reported.

To better understand the SCB degradation process, we investigated the lignocelluloses degradation characteristics and degradation products of three white-rot fungi strains, *P. chrysosporium* PC2, *Lentinula edodes* LE16 and *P. ostreatus* PO45. The results provided useful information for establishing an industrial procedure of SCB biopretreatment and offered additional critical insights for understanding the complicated lignocelluloses degradation mechanism of white-rot fungi in natural environment.

2. Methods

2.1. Material

Raw sugarcane bagasse samples were obtained from Liujiang Sugar Factory (Guangxi Province, China). They was dried under sunlight, cut into small pieces, oven-dried at 80 °C to constant weight, ground and sieved through a 20 mesh size screen, and stored in a sealed container with dehydrated $CaCl_2$ for further experiments.

2.2. Fungal strains and inoculation

Three white-rot fungi, *P. chrysosporium* PC2, *L. edodes* LE16 and *P. ostreatus* PO45, previously isolated and verified as lignin degraders (unpublished data) were used in this study. Generally, *L. edodes* grows in suitable woody materials, and *P. ostreatus* prefers to grow on herbaceous plant. These strains were preserved on potato dextrose agar (PDA) plates at 4 °C. To prepare the inocula for the solid state fermentation of SCB, mycelium agar plugs with 10 mm in diameter, were cut along the edge of the actively growing colonies, which had been cultivated on PDA plates for 7 days at 25 °C.

2.3. Solid state fermentation (SSF) and quantification of SCB degradation

In 150-mL conical flasks, 5 g of dry SCB was added and moistened with 20 ml liquid Czapek culture medium. After autoclaving 1 h at 121 °C, the flasks were inoculated with two mycelium agar plugs each prepared from the three fungi strains and incubated at 25 °C in dark (Isikhuemhen and Mikiashvilli, 2009). In all cases, SCB samples treated identically but without fungal inoculation were used as the control. All experiments were performed in triplicate.

The fermentation flasks were sampled every 7 days. The fermented SCB was weighed and transferred into a rotary shaker, soaked with deionized water in a ratio of 1:10 (SCB : water) by incubating with shaking (150 rpm) in an ice-water bath for 0.5 h, and then filtered through Whatman No.1 paper. The liquid fraction was assayed for enzyme activity (Dinis et al., 2009), and the solid SCB residues were oven-dried at 80 °C to constant weight for the subsequent determination of weight loss and change in chemical composition.

2.4. Changes of pH during the degradation of SCB

At the same time, prior to determine the weight loss mentioned above, 1 g of the fermented SCB was mixed with 1 ml of deionized water according to Haddadin et al. (2009), with the modification that the mixture was blended with a rotary shaker (150 rpm) for 0.5 h, and the pH was measured using a Compact Twin pH Meter (Horiba Ltd., Japan).

2.5. Determination of SCB degradation

After incubation, the mycelia were removed from the samples by immersing the SCB residues in acetic acid: nitric acid (8:1, v/v) for 1 h and then washed with deionized water until the washed water was neutral. The mycelium free SCB residues were dried at 80 °C and 0.3 g of dry residues were used for analyzing the lignocelluloses composition. The cellulose content in the SCB was determined using a colorimetric method with anthrone reagent (Ververis et al., 2004). For quantification of lignin, the SCB samples were hydrolyzed by H_2SO_4 (72% w/w) and the acid insoluble lignin content on ash basis was weighed (Ballesteros et al., 2004). The holocellulose content in the SCB residues was measured according to the method of Klaudiz (1957). The decrease in the total weight was calculated from the weight difference between the uninoculated controls and the treated dried samples (Locci et al., 2008). The lignin degrading ratio was calculated using the following formula: $Ri\ (\%) = 100 \times (m_0 - m_i)/m_0$, where Ri is the degrading ratio for the ith week sampling; m_0 is the initial content of lignin; m_i is the ith week sampling content of lignin. The same method was applied to the total organic mass, cellulose and hemicelluloses degradation rates.

2.6. Enzyme assays

In each of the enzyme assays, 0.5 ml of the filtrates obtained in the section 2.3 was used as the enzyme sample.

2.6.1. Ligninase assays

Lip activity (EC 1.11.1.14) was assayed by determining the oxidation of veratryl alcohol using H_2O_2 (Tanaka et al., 2009). MnP activity (E.C. 1.11.1.13) was assayed by monitoring the oxidation of DMP (2,6-dimethoxyphenol) using H_2O_2, at presence of $MnSO_4$ (Heinfling et al., 1998). Laccase activity (E.C. 1.10.3.1) was determined by examining the oxidation of ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate)] (Pickard et al., 1999). One unit activity is defined as the amount of enzyme that transformed 1 μmol of substrate per min. PPO (E.C. 1.10.3.1) was assayed as reported by Simsek and Yemenicioglu (2007), with the modification that it was assayed at 30 °C using catechol (1.67 mM) as substrate

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