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Application of Fenton pretreatment on the degradation of rice straw by mixed culture of *Phanerochaete chrysosporium* and *Aspergillus niger*

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ABSTRACT

The rice straw (RS) is a kind of recalcitrant lignocellulosic material. The Fenton reaction has been widely used for the degradation of organic compounds and toxic chemicals. Therefore, applying it to pretreatment straw seems to have good potential. This study was to investigate the degradation of the pretreated RS by the mixed solid-state culture with *Phanerochaete chrysosporium* and *Aspergillus niger*t. The results showed that the degree of delignifition was 15.01% by using an optimized Fenton reagent (6 g/L of $FeSO_4{\cdot}7H_2O$ and 15% concentration of H_2O_2) at a solids loading of 5% (w/v) and a moderate temperature for 15 h. The analysis of the Fenton pretreated RS by Scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR) showed significant changes in physicochemical structure, favoring the subsequently susceptibility of ligninolytic enzymes. The volatile organic compounds collected from the mixed solid-state culture implied the degradation degree of the lignocellulose to some extent. In conclusion, Fenton pretreatment could effectively disrupt the recalcitrant structure of RS and accelerate its biodegradation, which has high potential application in the energy conversion and utilization of lignocellulose.

1. Introduction

The increasing demand for energy and the reduction of global fossil fuels, together with climate changes have attracted extensive attention. These frustrating challenges have aroused our interest in non-conventional energy sources (Sindhu et al., 2016). Rice straw (RS), as one of the most abundant agricultural wastes, there is around 200 million tons produced in China and a large number of them are simply disposed by burning in the fields (Ranjan and Moholkar, 2013). The utilization of RS in an economically feasible way would not only prevent environmental pollution but produce chemicals and biofuels.

RS is mainly composed of cellulose and hemicellulose surrounded by lignin sheath. The three main components formed a tightly packed, tough and complex, water-insoluble structure that is resistant to depolymerization by microbial and chemical attack (Chen et al., 2014; Kumar et al., 2008). Both cellulose and hemicellulose could be converted to fuels by enzymatic hydrolysis and fermentation (Blanch et al., 2011). Lignin with non-carbohydrates polymer structure is a complex aromatic derivative composed of phenylpropanoid units, which acts as a physical barrier to prevent the hydrolysis of lignocellulose (Ranjan and Moholkar, 2013). Chandler and Jewell showed that one percent lignin decreased organic matter digestion by about 3% (Chandler and Jewell, 1980). Therefore, the degradation of lignin is the key step to the lignocellulose transformation (Huang et al., 2010). In addition to the physical and chemical rigidity and recalcitrance, there are many other factors that restrict the digestibility of RS, such as lignin content, crystallinity of cellulose and particle size (Hendriks and Zeeman, 2009). Studies have shown that the highly recalcitrant nature of lignocellulose material limits the access of lignocellulolytic enzymes to penetrate its interior to carry out the hydrolysis of the glycosidic bonds (Allardyce et al., 2010).

Biological treatments are considered as more environmental friendly than physical and chemical methods in lignocellulose degradation due to its low energy consumption, mild reaction conditions and simple procedures and equipment (Ye and Cheng, 2002). Basidiomycete strains are the most efficient lignocellulose degraders among fungi (Sánchez, 2009). *Phanerochaete chrysosporium* (*P. chrysosporium*) is one of the typical basidiomycete white-rot fungi, which has

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prominent ability to degrade lignin (Chen et al., 2016; Yu et al., 2011; Zhang et al., 2014). *Aspergillus niger* (*A. niger*) is a well-known fungus for producing cellulases. Mixed culture of lignocellulolytic fungi can upregulate the secretion of ligninolytic enzymes and improve the degradation of lignin and cellulose (Chia et al., 2007; Gregorio et al., 2006). Therefore, the mixed culture of *P. chrysosporium* and *A. niger* was expected to achieve better degradation effect and reducing sugar yields. However, there are a few researches about improving the mixed culture by pretreatment of RS. To facilitate the hydrolysis of lignin degrading enzymes, pretreatment method is necessary in removing lignin and reducing crystallinity of cellulose as well as increasing material porosity. Various pretreatment methods have been investigated such as steam explosion, ammonia fiber explosion, alkali treatment, acid hydrolysis, hot water treatment, super-critical carbon dioxide explosion, extrusion pretreatment, and hydrodynamic cavitation (Al-Zuhair et al., 2013; Hilares et al., 2016). Although these pretreatment methods have been extensively studied, they still have some drawbacks such as high energy consumption and formation of toxic byproducts on enzymes and microorganisms (Younghoon et al., 2013). A more effective and economically feasible pretreatment method is still needed to make full use of potential lignocellulosic materials (Wyman et al., 2005). Fenton pretreatment has been widely developed and applied due to its simplicity, availability and low cost (Deng, 2007). In general, a classical Fenton process is that hydrogen peroxide produces high activity hydroxyl radical under the catalysis of ferrous ion. The Fenton reagent can increase the accessibility of the enzyme by oxidizing and disrupting the lignocellulosic structure (Neyens and Baeyens, 2003). The cellulose substrates was treated with 0.5 mM concentration of Fe^{2+} and 2% concentration of H_2O_2 and reacted for 48 h could provide the highest enzyme activity (Jain and Vigneshwaran, 2012). The combination of Fenton pretreatment and biological treatment by mixed fungi would be an acceptable way for RS degradation and reuse.

The present study is focused on the integrated process of Fenton pretreatment and biological treatment to determine the efficiency of the Fenton pretreatment for the delignification. Meanwhile, the subsequent digestibility of RS was evaluated by the ligninolytic enzymes in the mixed culture of *P. chrysosporium* and *A. niger*. The morphological structure changes of RS were investigated by SEM and FTIR. Meanwhile, the composition changes of VOCs from solid-state cultivation were analyzed to study the effect of Fenton pretreatment on the degradation of RS by mixed culture of *P. chrysosporium* and *A. niger*.

2. Materials and methods

2.1. Materials

The RS harvested from the suburb of Changsha (Hunan province, China). After washing and air-dried, the RS was grounded through a 60-mesh sieve, and then dried at 45 °C for 24 h to constant weight for subsequent experiments (Michalska et al., 2012). Ferrous sulfate ($FeSO_4{\cdot}7H_2O$) and hydrogen peroxide (H_2O_2, 30%) were obtained from Lingfeng chemical reagent company (Shanghai, China). All the chemicals were reagent grade and used as received without further purification.

2.2. Pretreatment by fenton reagent

The Fenton treatment was carried out in 500 mL Erlenmeyer flasks with 10 g RS prepared as described in Section 2. 1. $FeSO_4{\cdot}7H_2O$ was diluted to 3 g/L, 6 g/L, and 12 g/L. H_2O_2 (30%) was diluted to 5%, 10%, and 15%. The effects of different concentrations of $FeSO_4{\cdot}7H_2O$ and H_2O_2 on RS were investigated. The 100 mL of $FeSO_4{\cdot}7H_2O$ and 100 mL of H_2O_2 were respectively added into the shaking Erlenmeyer flasks at 25 °C, 200 rpm for2, 6, 12, 15, 18, and 24 h, respectively. The flask was covered in a layer of film to avoid quality loss and contamination. After the pretreatment, 20% concentration of NaOH was added to raise the pH value to above 10, which could precipitate iron (III) hydroxide. The pretreated RS slurry was washed by distilled water and separated by vacuum filtration several times until the pH of the filtrate was near neutral. Subsequently, the material was dried in an oven at 45 °C to constant weight for further experiment.

2.3. Lignin analysis

Acid soluble lignin (ASL) and acid insoluble lignin (AIL) in all samples were determined according to the Laboratory Analytical Procedure (LAP) published by the National Renewable Energy Laboratory (Sluiter Bh et al., 2010). This procedure uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. The ASL was measured by UV–vis spectroscopy, and the AIL was accounted for gravimetric analysis. All the analyses were conducted in triplicate.

2.4. FTIR analysis

The determination of the RS functional group was carried out through the FTIR apparatus (IRAffinity-1, Shimadzu, Japan) which was equipped with a 2-m gas cell and a DTGS KBr detector. The oven-dried RS was ground and sieved to desired mesh size (average diameter less than 0.2 mm) for analysis. The test samples were made by tableting the mixture of RS power and KBr (1 mg power/200 mg KBr) into a thin slice of 5 mm in diameter (Liu et al., 2008). The spectrum scope was recorded in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} and each spectrum was composed of 100 scans.

2.5. Mixed culture

After the Fenton pretreatment, the RS was subjected to enzymatic hydrolysis in mixed culture with *P. chrysosporium* and *A. niger*, which were purchased from China Center for Type cultivation Collection (CCTCC) and maintained on potato dextrose agar (PDA) at 4 °C. The inoculum was grown on PDA plates for 5 days at 30 °C, and then the spores from plates were diluted in sterile distilled water, then shake it and adjusted to a concentration of 1×10^7 CFU/ml by blood counting chamber. A 5 g of pretreated and untreated RS powers were added to the 9 cm (ϕ) sterile petri dishes, respectively, and then added 20 mL of substrate solution consisting of NaCl (0.5 g/L), KH_2PO_4 (1 g/L), $CaCl_2$ (0.3 g/L), $MgSO_4$ (0.5 g/L), $(NH_4)_2SO_4$ (3 g/L), $FeSO_4{\cdot}7H_2O$ (0.01 g/L), $MnSO_4{\cdot}7H_2O$ (0.015 g/L), $ZnSO_4{\cdot}7H_2O$ (0.015 g/L), and $CoCl_2$ (0.01 g/L) (Massadeh et al., 2001). The cultivation medium was sterilized at 121 °C for 20 min, aseptically inoculated with 1 mL of *P. chrysosporium* suspension and 1 mL suspension of *A. niger*, and cultured at 37 °C for 21 days. The samples taken on days 1, 3, 6, 10, 14, 18, and 21 were used for enzyme activity assays, FTIR, and GC–MS analysis. All assays were carried out in triplicate.

2.6. Extracellular enzymes activity assays

The effectiveness of Fenton pretreatment was evaluated by assessing the enzyme activities of LiP and MnP. Crude enzymes were extracted by suspending 0.5 g collected samples in 10 mL distilled water and incubating at 200 rpm in a rotary shaker at 25 °C for 1 h. Then the extracts were filtered by 0.45 μm membrane filters and stored at 4 °C. LiP activity was determined by using the reagent of veratryl alcohol (VA) (Tanaka et al., 2009). One enzyme unit (U) of LiP activity was defined as the amount of enzyme required to make 1 M veratryl alcohol completely oxidize to veratryl aldehyde per minute under assay conditions. MnP enzyme activity was determined by spectrophotometric method (Lopez et al., 2007). One enzyme unit (U) was defined as the amount of enzyme required to produce 1 M Mn^{3+} by oxidation of Mn^{2+} per minute under experimental conditions. The enzyme activity was expressed as U/g of dry residue. All assays were performed in triplicates