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RESEARCH ARTICLE

Biological pretreatment of corn stover by solid state fermentation of *Phanerochaete chrysosporium*

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Abstract Biological pretreatment is a promising way to overcome the biorecalcitrance of cleaving the supermolecular structure of lignocellulose by lignin degrading enzymes from microorganisms. Solid state fermentation of corn stover with the white-rot fungus *Phanerochaete chrysosporium* was carried out and the efficiency of this pretreatment was evaluated. The enzymatic hydrolysis yield reached a maximum when the corn stover was biologically pretreated for nine days, and the hydrolysis yield decreased sharply if the solid state fermentation was carried out for more than nine days. A possible explanation for this sharp decrease is that not only the lignin degrading enzymes (LiP and MnP) were secreted, but also other metabolites, which were toxic or fatal to the hydrolysis enzymes resulting in the lower hydrolysis yield were generated during the prolonged period of biopretreatment. These results are useful to help determine the optimal timing and to understand the lignin structure and degradation mechanism in biological pretreatment processes.

Keywords biological pretreatment, *Phanerochaete chrysosporium*, solid state fermentation, biorecalcitrance, hydrolysis yield

1 Introduction

Biological pretreatment is the most promising way to overcome the biorecalcitrance of cleaving the supermolecular structure of lignocellulose through lignin degrading enzymes from microorganisms. The white-rot fungus *Phanerochaete chrysosporium* is the most intensively studied strain used for biological pretreatment. It secretes two major peroxidases, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP), both of which are capable of degrading the cross-linked aromatic polymers of lignin and improving enzyme accessibility [1–3]. Solid state fermentation of *P. chrysosporium* is generally used for the biological pretreatment of solid lignocellulosic materials [4,5]. The main barrier that hinders biological pretreatment from practical use is the low efficiency of breaking the lignocellulose structure. With solid state fermentation, it generally takes more than a week for *P. chrysosporium* to grow and secrete enough peroxidase and oxidase enzymes for lignin degradation. Therefore, a method of enhancing biological pretreatment so that it only requires a short period of time is needed before biological pretreatment can be practically used [6–8].

In this study, a submerged liquid fermentation of *P. chrysosporium* was first carried out and the effective production of the lignin degrading enzymes, LiP and MnP was confirmed. Then a biological pretreatment consisting of the solid state fermentation of corn stover with *P. chrysosporium* was carried out and the pretreatment efficiency was evaluated by a typical enzymatic saccharification method, that is, the hydrolysis of the solid state fermented corn stover into mono-saccharides, glucose and xylose. The hydrolysis yields using different culturing parameters were determined.

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2 Materials and methods

2.1 Strains

The white-rot fungus strain *P. chrysosporium* CGMCC 5.776 was purchased from China General Microbiological Culture Collection Center (CGMCC), Beijing, China. The strain was transferred and maintained on Potato Dextrose Agar (PDA) plates containing 20% potato juice, 20 g/L glucose, and 20 g/L agar. The fermentation medium contained 10 g of glucose, 2 mmol of ammonium tartrate, 2 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 mg of

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$CaCl_2 \cdot 2H_2O$, 1 mg of vitamin B_1, 3×10^{-3} mol of veratryl alcohol, 7 mL of trace element solution and 1 g of Tween 80 per liter of 10 mmol/L acetate buffer (pH 4.5). The trace element solution contained 7.8×10^{-3} mol of glycine, 1.2×10^{-2} mol of $MgSO_4 \cdot 7H_2O$, 2.9×10^{-3} mol of $MnSO_4 \cdot H_2O$, 0.017 mol of NaCl, 3.59×10^{-4} mol of $FeSO_4 \cdot 7H_2O$, 7.75×10^{-4} mol of $CoCl_2$, 9.0×10^{-4} mol of $CaCl_2 \cdot 2H_2O$, 3.48×10^{-4} mol of $ZnSO_4 \cdot 7H_2O$, 4×10^{-5} mol of $CuSO_4 \cdot 5H_2O$, 2.1×10^{-5} mol of $KAl(SO_4)_2 \cdot 12H_2O$, 1.6×10^{-4} mol of HBO_3 and 0.01 g of Na_2MoO_4 per liter of ionized water.

2.2 Chemicals and reagents

Veratryl alcohol and guaiacol were from Acros Organics (New Jersey, USA). The two industrial cellulase enzymes used were Spezyme CP from Genencor International (Rochester, NY, USA), and Novozyme 188 from Novo Industrial A/S (from Sigma-Aldrich, St Louis, MO, USA). All other chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.3 Inoculum and submerged liquid culture

P. chrysosporium CGMCC 5.776 was cultured on PDA slants for 7 d at 30°C and then transferred into sterilized water and dispersed with fine glass beads. The number of spores in the sterile water was counted with a hematimeter. The spores (8×10^4) were inoculated into 100 mL of fermentation medium in a 500-mL flask and cultured at 30°C and 150 r/mim for 13 d. The resulting liquid fermentation mixture was then analyzed for biomass and peorxidases or used as sedds cultures for the biological pretreatment of corn stover using solid state fermentation.

2.4 Assay of LiP and MnP

The LiP activity was measured by monitoring the hydrogen peroxide initiated conversion of veratryl alcohol to veratraldehyde. The conversion was measured by using an UV-Vis absorbance spectrometer DU800 (Beckman Coulter Inc., CA, USA) to monitor the absorbance of veratraldehyde at 310 nm. The assay system contained 50 $mmol \cdot L^{-1}$ sodium tartarate buffer at pH 2.5, 2 $mmol \cdot L^{-1}$ veratryl alcohol, 0.4 $mmol \cdot L^{-1}$ H_2O_2, and 550 μL of fermentation broth to make up a total volume of 1 mL [9]. One unit of lignin peroxidase activity is defined as the conversion of 1 μmol of veratryl alcohol substrate in 1 min at 30°C.

The activity of MnP was measured by monitoring the absorbance change at 465 nm when guaiacol (2-methoxyphenol) was oxidized to 4-methoxyphenol by hydrogen peroxide. The assay system contained 0.15 $mmol \cdot L^{-1}$ sodium acetate buffer at pH 4.5, 0.25 $mmol \cdot L^{-1}$ $MnSO_4$, 0.4 $mmol \cdot L^{-1}$ H_2O_2, and 500 μL of fermentation broth to make up a total volume of 1 mL [9]. One unit of MnP activity is defined as the conversion of 1 μmol of guaiacol substrate in 1 min at 30°C.

2.5 Solid state fermentation

The corn stover was harvested in the fall of 2007 from the north-east Jilin Province in China. The corn stover was milled into short pieces, washed with tap water and dried at ambient temperature. It was then dried to a constant weight at 105°C. Afterward the corn stover powder was pretreated with the following steps before the solid state fermentation: 1) fractionation though two sieves one with a pore size greater than 40 mesh/inch, and the other smaller than 20 mesh/inch; 2) sterilization by exposure to UV irradiation for 30 min; and 3) humidification using sterile water until the water content was 60% (dry weight basis). Then 2 g of corn stover powder (dry weight) was placed in a Petri dish. The *P. chrysosporium* submerged liquid culture broth was mixed with fresh medium in the ratio of 20% (broth): 80% (fresh) and was then inoculated onto the corn stover powder. The sample was then allowed to ferment at 30°C for 13 d. Thirteen Petri dishes and one control containing un-inoculated corn stover powder were prepared as one set for the solid state fermentation (biological pretreatment). Everyday one Petri dish was subjected to the hydrolysis assay. Each set was performed twice.

2.6 Enzymatic hydrolysis assay

The biologically pretreated corn stover was evaluated using two industrial cellulase enzymes, Spezyme CP and Novozyme 188. The assay was carried out on a well-mixed sample in a 100-mL shaking flask containing 20 mL of citrate buffer (50 $mmol \cdot L^{-1}$, pH 4.8) at an orbit shaking rate of 200 rpm. The pretreated corn stover solid loading was 5% (dry weight) and the enzymatic hydrolysis was performed at 50°C and pH 4.8 for 72 h. The Spezyme CP and Novozyme 188 enzymes were both added into the hydrolysis system at 7 FPU and 15 IU per gram of dried solid fraction, respectively.

The activity of Spezyme CP was assayed using the LAP-006 protocol of the National Renewable Energy Laboratory, USA [10]. The activity of the cellobiase Novozyme 188 was assayed in a reaction mixture containing 1.0 mL of diluted enzyme solution and 1.0 mL of 80 $mmol \cdot L^{-1}$ cellobiose solution in 50 $mmol \cdot L^{-1}$ citrate buffer at pH 4.8.

2.7 Analysis

The amounts of glucose, xylose, and lignocellulose degradation compounds were determined by high performance liquid chromatography (HPLC, LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-rad Aminex HPX-87H column at 65°C. The mobile

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phase was 0.005 $mol \cdot L^{-1}$ H_2SO_4 with a flow rate of 0.6 mL/min. Before analysis, all samples were centrifuged to remove the cell mass and other water insoluble substances, and then filtered through a 0.22-μm filter.

The water content of the corn stover was about 50% (total weight basis). The composition of the pretreated corn stover, analyzed according to the NREL two-step hydrolysis protocol (2005), was 32.6% cellulose, 26.4% hemicellulose and 8.1% lignin (dry weight basis) [11].

The hydrolysis yield of the corn stover was calculated according to NREL LAP-009 as follows [12].

$$\text{Hydrolysis yield} = \frac{[\text{glucose}] + 1.053 \times [\text{cellobiose}]}{1.111 \times [\text{fraction}] \times [\text{biomass}]} \times 100\%,$$

where [glucose] is the glucose concentration in the broth after enzymatic hydrolysis, [cellobiose] is the cellobiose concentration in the broth after enzymatic hydrolysis, [biomass] is the concentration of the dry biomass at the beginning of the enzymatic hydrolysis, [fraction] is the cellulose fraction of the dry biomass (g/g), 1.053 is the conversion factor to convert cellobiose to glucose and 1.111 is the conversion factor to convert cellulose to glucose.

3 Results and discussion

3.1 Submerged liquid culture of *P. chrysosporium*

After the liquid fermentation of the white-rot fungus strain *P. chrysosporium* CMGCC 5.776, the activities of the two lignin degrading enzymes in the submerged liquid culture were determined. Figure 1 shows the time courses of the cell growth, sugar consumption, and enzyme activity of *P. chrysosporium* during the 13 d of culturing at 30°C with vigorous shaking. Figure 1(a) shows that the cells grew rapidly in the first three days and stepped into the stationary phase from the 4th day until the culture ended. The glucose was steadily consumed even with the cell growth in the stationary phase. Figure 1(b) shows that the activities of lignin peroxidase (LiP) and manganese peroxidase (MnP) increased as the cell density increased and reached a maximum on the 8th day for LiP (1155 units/L), and on the 9th day for MnP (150 units/L). Then the activities of the both enzymes decreased and were completely lost by the end of the culture (the 12th day). As can be seen from the results, the amount of LiP produced is higher than the amount of MnP produced by the white-rot fungus strain *P. chrysosporium* CMGCC 5.776. These results are also in agreement with previous reports that LiP is the major lignin degrading enzyme whereas the secretion level of MnP is low for *P. chrysosporium* [2,6,8,13].

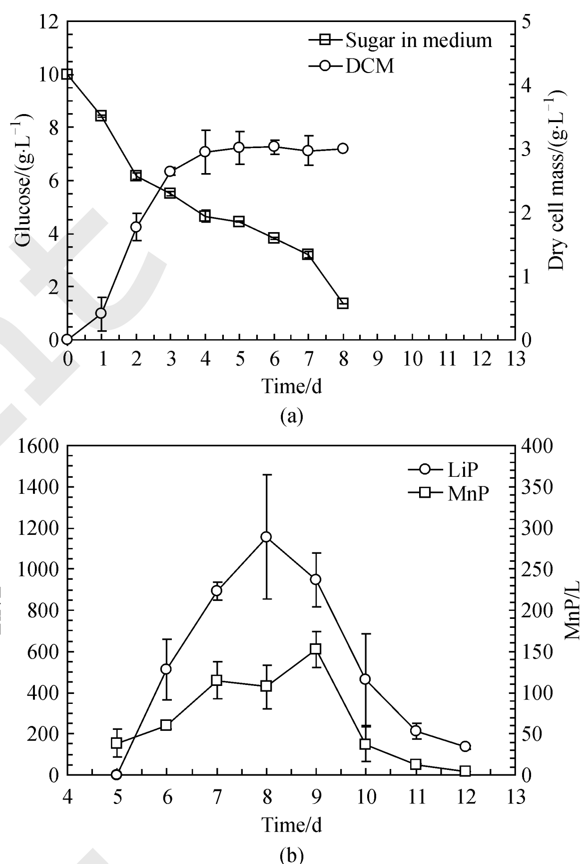

Fig. 1 Time courses in the submerged liquid culture of *P. chrysosporium*. (a) glucose and dry cell mass (DCM); (b) enzyme activity. Conditions: 30°C, 150 rpm, 100 mL of the nitrogen limited medium described in Material and Methods

3.2 Solid state fermentation of corn stover with *P. chrysosporium*

The activities and protein concentrations of LiP and MnP were assayed during the solid state fermentation of corn stover, but unfortunately, neither was detected. This may be because the assay method did not work for the cloudy solution which was formed during the washing of the corn stover, or because the secreted enzymes are bound to the lignin components of the solid substrate (CS) and are not easily washed into the liquid phase. Therefore, the efficiency of the biological pretreatment of the corn stover was assayed directly using enzymatic hydrolysis of the solid state fermented CS. Since the hydrolysis yield of the biologically pretreated CS within the first 6 days was very similar to that of the untreated CS, which was lower than 20% (Data was not shown), the hydrolysis yield of the corn stover was calculated from the sample pretreated for seven days. The daily water loss in the CS in the Petri dishes was approximately 10% of the total water in the corn stover. To maintain the growth of *P. chrysosporium*, water had to be added to the Petri dishes daily. The results of the solid state

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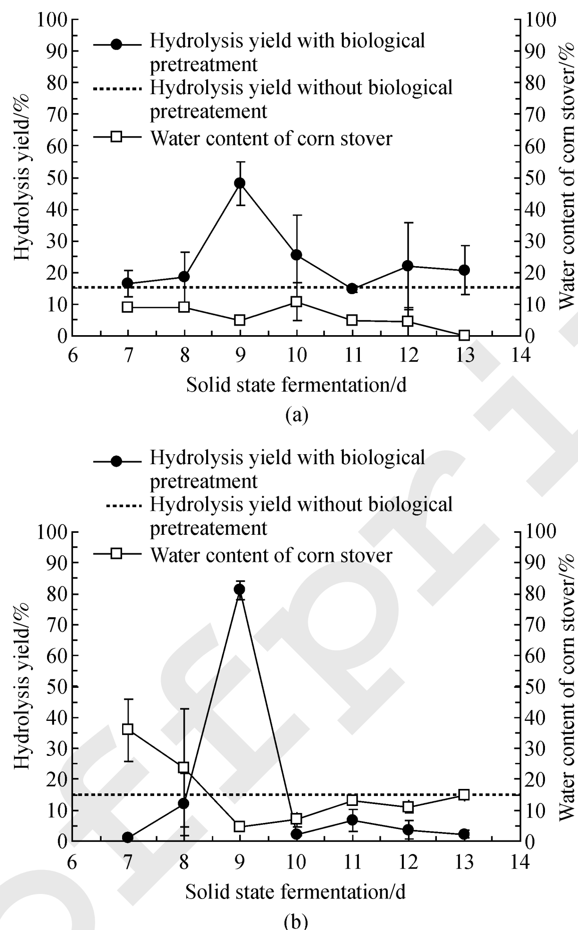

Fig. 2 Time courses of the hydrolysis yield and water content of corn stover during the solid state fermentation with *P. chrysosporium*. (a) without water supplement; (b) 10% of the total water supplemented daily in the whole process. 30°C, initial water content: 60% (w/w)

fermentation of corn stover with *P. chrysosporium* with and without a water supplement are shown in Fig. 2.

Figure 2(a) shows that for the case of no water supplement, the maximum sugar yield (50%) was threefold greater than the yield for the CS without biological pretreatment. Figure 2(b) shows that with a periodic water supplement (adding 10% of the total water every day for the first six days), the hydrolysis yield reached 80% of the theoretical yield which is almost 6-fold greater than that without biological pretreatment. These results indicate that the solid state biological pretreatment fermentation improved the hydrolysis yield significantly both with and without a water supplement; however the daily water supplement increased the maximum hydrolysis yield 60% compared to that without a waster supplement. Interestingly, in both cases, the hydrolysis yield increased from the 7th day to the 9th day where it reached a maximum. After that, the yield decreased sharply.

3.3 Analysis on the biological pretreatment of corn stover with *P. chrysosporium*

The present study shows that the biological pretreatment of CS with white-rot fungus improved its enzymatic digestibility. However, there has also been a study that reported that neither solid state nor submerged cultivation of *P. chrysosporium* had a positive effect on the cellulose hydrolysis of cotton stalks in spite of significant lignin degradation [14]. The difference between the negative report and the present experiment is that oil and fiber crop residues (cotton stalks) were used in that study and food crop residues (corn stover) were used in this study. The lower biological pretreatment efficiency may be due to the stronger recalcitrance of oil and fiber crop residues compared to food crop residues [15,16]. Our previous results on the pretreatment of different crop residues showed similar results: cotton and rapeseed stalks were harder than corn stover, rice straw and wheat straw [16].

The moisture content during the solid state fungal pretreatment of lignocellulose is another key factor for the pretreatment efficiency because of its effect on both the fungal growth and the secondary metabolism [17]. Since low moisture prevents fungal growth and inhibits lignin removal, it is necessary to add a water supplement water to compensate for the water loss during the solid state fermentation [18–21]. Usually, 70% of the initial moisture content is chosen as the optimum for fungal pretreatment [18–22]. But in this study, a moisture content of approximately 20% of the CS moisture content was maintained and a good hydrolysis yield was obtained. The reasons that this lower moisture content was acceptable may include effects of the substrate cultivar and particle size, the addition of nutrients, the white-rot fungi used, or synergistic actions among these factors [18,19,22].

Interestingly, the hydrolysis yield peaked at nine days of solid state fermenting, both with and without the water supplement. It is reasonable that the hydrolysis yield increased as the culture time increased and that it reached a maximum on the 9th day, because the secreted enzymes in the submerged liquid cultures also reached a maximum on the 8th or 9th day. The loss of enzyme activity with a prolonged culture time is also reasonable, especially for solid state fermentation. However, what is hard to understand is that on the 10th day, the hydrolysis yield decreased sharply to a yield that was equal to or only 1/3 of the corn stover yield without biological pretreatment (Fig. 2).

Although the catalysis mechanism is still not fully understood, the lignocellulose degradation by *P. chrysosporium* is thought to occur by the continuous cleavage of the supermolecular structure of lignin by the free radical ionic intermediates from the hydrogen peroxide and the lignin substrates [1,23,24]. In this way, the biorecalcitrance of the lignocellulose to hydrolytic enzymes and the subsequent microbial fermentation was partially overcome. Other reports have suggested that this fungus can

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also generate cellulase and xylanase enzymes during solid state or submerged liquid cultures, but concomitantly the hydrolyzed cellulose is consumed rapidly by the fungus [25,26]. This rapid consumption of the cellulose may be one reason for the sharp decrease in the hydrolysis yield after nine days of solid state culture.

Another possible explanation is a toxic effect of the fungus itself or its metabolites on the hydrolysis enzymes. Shi et al. reported that water or hot-water washed biologically pretreated cotton stalks showed a better hydrolysis performance but the performance was still lower than that of untreated stalk [14]. Many other studies on the biological pretreatment of lignocellulose have also used a water-wash or sterilization step before the next processing step [14,15,17–22]. The most likely reason for the sudden drop in yield, is that during prolonged pretreatment (more than nine days), the white-rot fungus not only secreted the lignin degrading enzymes (LiP and MnP), but also generated other metabolites, which were toxic or fatal to the hydrolysis enzymes resulting in the lower hydrolysis yield. Therefore, a washing or sterilization step before hydrolysis or SSF (Simultaneous saccharification and fermentation) is necessary. Unfortunately, this was not done in our study and a sharp decrease in the hydrolysis yield occurred after the peak value was reached. To fully prove this hypothesis will require further experimental studies which are now underway in our laboratory.

4 Conclusions

Solid state fermentation of corn stover with the white-rot fungus *P. chrysosporium* was carried out and the efficiency of this pretreatment was evaluated. The results show that enzymatic hydrolysis yield reached a maximum when the corn stover was biologically pretreated for nine days, and the hydrolysis yield decreased sharply with the prolonged biopretreatment period. A possible explanation for this sharp decrease is that not only the lignin degrading enzymes (LiP and MnP) were secreted, but also other metabolites, which were toxic or fatal to the hydrolysis enzymes resulting in the lower hydrolysis yield were generated. These results are helpful to determine the optimal timing and to understand the lignin structure and degradation mechanism in biological pretreatment processes.

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