Enhancing lignin degradation by Mn^{2+} ion supplementation to assist enzymatic hydrolysis of cellulose in a two-stage pretreatment of bamboo

Liu Jian*, Gan Lihui, Long Minnan

(*College of Energy, Xiamen University, Xiamen 361005, China*)

Abstract: It is of great significance to reveal a key agent to improve the efficiency of conversion of lignocellulosic biomass to fermentable sugars for the production of bioethanol. This study investigated a two-stage pretreatment of bamboo comprising a microbial treatment using *Ceriporiopsis* sp. followed by a hydrothermal treatment to facilitate the process with Mn^{2+} as an accelerator. The effects of Mn^{2+} nutritional addition on ligninolytic activity, lignin degradation, total weight loss, pulp yield, sugar yield and sugars in the soluble fraction were examined. The results showed that the time required for the incubations supplemented with Mn^{2+} to achieve sufficient lignin degradation and sugar yield from both the pulp and soluble fractions was significantly shortened, whereas all three ligninolytic activities were significantly decreased. The enzyme activity varied according to the presence of Mn^{2+}, although the amount and species of the expressed protein are similar. Considering the cost, microbial treatment with a one-time fed-batch supply of metal nutrition ($MnSO_4$) was the most preferable contribution to hydrothermal pretreatment, resulting in 19.7% lignin degradation, 66.7% pulp yield and 26.1% sugar yield over a period of 21 d. It was proven that microbial treatment by solid state incubation with Mn^{2+} nutrition has the potential to be a low-cost, environmentally friendly alternative to chemical approaches.

Keywords: hydrolysis, fungi, Mn^{2+} ion, pretreatment, bamboo, *Ceriporiopsis* sp.

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1 Introduction

The lignocellulosic biomass has the potential to provide an economical application because of its sustainable production, widespread availability and low starting cost. Bamboo is a potential feedstock that can be transformed into biofuels and biochemicals[1]. The conversion of lignocellulosics into biofuel employs the following three major steps: pretreatments to reduce the crystallinity of cellulose and breakdown the molecular structure of lignin; hydrolysis of cellulose with cellulose to obtain glucose; and bioconversion of glucose to butanol or ethanol[2,3]. Among the challenges found in all of the steps, pretreatment is the prerequisite to obtain fermentable sugars for an industrially acceptable application due to the robust structure of lignocellulose[4].

The existing pretreatment process have mainly been developed based on physical and chemical technologies such as autoclave, dilute acid, alkali, ionizing radiation, microwave, steam explosion and oxidation or varied combinations[5-7]. Nevertheless, typical pretreatments require high-energy (electricity or steam), corrosion-resistant equipment and high-pressure reactors, thereby increasing the cost. In addition, chemicals in pretreatment are detrimental to the subsequent enzymatic hydrolysis and microbial process. Accordingly, hydrothermal technology has been shown to be an efficient tool for pretreatment and is widely accepted as a

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Biographies: Gan Lihui, Engineer, research interest: biomass chemical engineering, Email: ganlihui@xmu.edu.cn; **Long Minnan,** Professor, research interest: biochemistry, Email: Longmn@xmu.edu.cn.

***Corresponding author: Liu Jian,** PhD, Senior Engineer, research interest: biomass chemical engineering. B313, College of Energy, Xiamen University, Xiang'an, Xiamen, Fujian, China. Tel/Fax: +86-592-5952787/2188053; Email: jianliu@xmu.edu.cn.

controllable mild processing method[8]. Hot water is helpful as an effective solvent for hemicellulose extraction and decrystallization, but other reports have indicated its poor selectivity in delignification[9,10]. This indicates the importance of microbial treatment by white rot fungi associating with hydrothermal pretreatment for the delignification of lignocellulose.

The unique ability of white rot fungi to degrade lignin specifically has attracted considerable interest. Microbial treatment employs white rot fungi and their enzyme systems to break down the rigid structure of lignin in cellulosic biomass[11,12]. It is an environmentally friendly approach that has received increased attention recently and has potential advantages over the popular technologies of physicochemical pretreatment due to its lower energy and cost, simplified process and use of sustainable catalysts. A series of fungi such as *Ceriporiopsis subvermispora, Phanerochaete chrysosporium* and *Pleurotus ostreatus* can metabolize lignin efficiently in various natural lignocellulosic biomass[13,14]. The physiology of oxidative enzymes that degrade lignin has been investigated extensively for their unique potential in delignification[15]. Reducing the content of lignin in biomass helps expose the highly ordered crystalline structure of lignocellulose and facilitates the access of hydrolytic enzymes to substrates[16]. White rot fungi can decompose lignin efficiently by excreting special extra-cellular enzymes, especially laccase and peroxidases (MnP), as secondary metabolites under metabolic control[17]. The efficiency of delignification and minimal consumption of holocellulose relative to other white rot fungi make *Ceriporiopsis* sp. a suitable candidate for microbial treatment and biopulping[18].

It has been reported that the addition of some types of nutrient salts, especially Mn^{2+} ions, can enhance the performance of white rot fungi in pure artificial substrates by stimulating the secretion of lignin-degrading enzymes responsible for delignification processes[19]. However, few studies have been carried out on the influence that alternative Mn^{2+} ions have on the microbial treatment of lignocelluloses. The substrate characteristics, moisture content, nutrient supplements, culture duration and aeration are important variables for the performance of white rot fungi and impact both growth and metabolite formation, but those processing parameters were fully explored in former studies excluding Mn^{2+} ions[20].

In the present studies, microbial treatment by white rot fungi was focused on strengthening the efficiency in the hydrothermal pretreatment of bamboo. This work examined the microbial treatment of bamboo powder using oxidative enzymes, especially manganese-dependent peroxidases, as a key factor that affected the lignin-degrading performance of *Ceriporiopsis* sp. We propose that conventional delignification using white rot fungi can be improved by the nutritional addition of Mn^{2+}, requiring less time for processing. Therefore, to better understand the potential of this study, the effects of the nutrition content ($MnSO_4$, an inorganic salt providing Mn^{2+}) in the substrate and culture time (0-40 d) on ligninolytic activity, lignin degradation, total weight loss, pulp yield, sugar yield and the composition of sugars in the soluble fraction were investigated to evaluate the effectiveness of the process.

2 Materials and methods

2.1 Strain and inoculation

The fungal strain *Ceriporiopsis* sp. was obtained from dead wood and was identified according to the Bergey's Manual of Determinative Bacteriology. Propagation of the organism was performed on potato dextrose agar (PDA) plates for 2 weeks at 25°C.

2.2 Materials

The bamboo used in this study was moso bamboo grown in Fujian, China. The full-sized culm aged approximately six months to one year was harvested and then dried naturally to approximately 10% moisture content. The feedstock was ground to a 1 mm particle size by a mill and stored in air-tight containers. The average main components (w/w) of bamboo as determined by three replicate analyses were 49.4% cellulose, 14.2% hemicellulose and 28.3% lignin. The standard deviations were less than 1.5%.

2.3 Microbial treatment

The process flowchart of the experiment was list in Figure 1. The solid cultures were grown in flasks capped with cotton filters containing 50.0 g of bamboo powder, 5.0 g of wheat bran, 0-0.1 g of $MnSO_4$ and a

certain amount of distilled water. Approximately 1.0 cm^2 of precultured agar was incubated in each flask. The control without the addition of $MnSO_4$ was CASE0, and the sample with initial $MnSO_4$ was named CASE1. For the culture of the fed-batch, 0.02 g of $MnSO_4$ was supplied as initial nutrition, and then 0.5 mL of sterilized solution containing 0.02 g of $MnSO_4$ was fed on day 7 for one time (CASE2) or on both day 7 and day 14 for two times (CASE3). Samples were autoclaved for 20 min (121°C) and then cooled prior to incubation. Pretreatments were carried out in an air convection incubator at 28°C, 75% initial moisture, 70% air moisture and destructively sampled at predetermined time intervals (0-40 d). All of the treatment combinations were completed in triplicate.

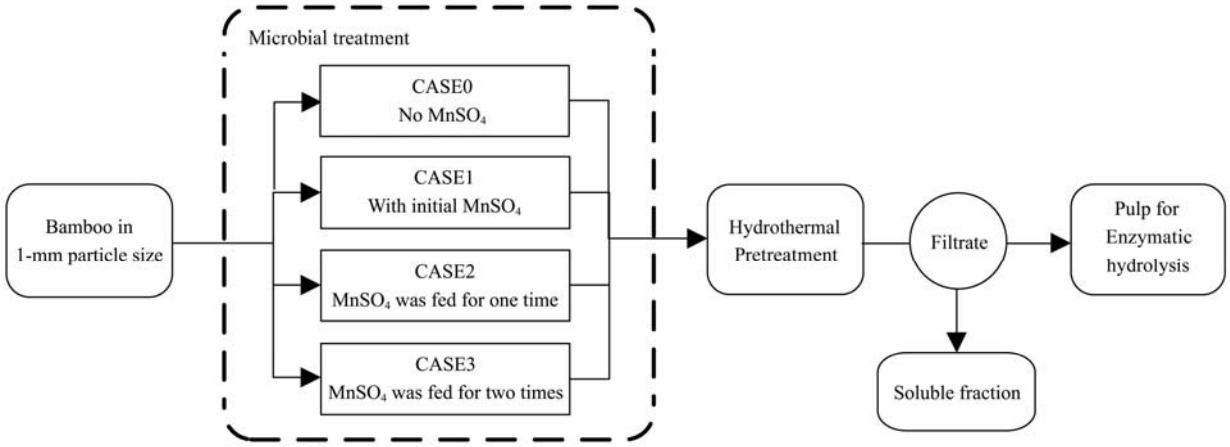


Figure 1 Process flowchart of the experiments

2.4 Hydrothermal pretreatment

An autoclave was used for the hydrothermal pretreatment followed by microbial treatment. The autoclave was a batch type equipped with a stirrer, pressure sensor and thermocouple. The power was controlled automatically by monitoring the inner temperature of the reactor. In all of the experiments, distilled water was used as the solvent. A liquid to solid ratio of 20:1 (w/w) was used to ensure that the bamboo powder was fully submerged in the liquid. The temperature was 200°C for 10 min of pretreatment time (not including the heating time of 3 min and cooling time of approximately 15 min). The hydrothermal pretreated bamboo was filtered through filter paper and separated into pulp and soluble fractions. The filtrates consisting basically of a mixture of pentoses and hexoses may be converted into ethanol by selected microorganisms[21,22].

2.5 Analysis methods

The pulp yield was calculated as a percentage of the total solids recovered after pretreatment based on the initial sample (dry weight). Sugar yield was estimated as a percentage (w/w) of the total glucan and xylan in the solids recovered. The holocellulose and lignin contents were determined according to the standard of Technical Association of the Pulp and Paper Industry (TAPPI). The neutral sugar composition was analyzed after hydrolysis with H_2SO_4 for bamboo and pulp according to a previously reported method[23]. The effectiveness of a pretreatment method can be reflected by the percentage of lignin degradation with simultaneous conservation of cellulose polymers, which can be figured by a parameter, the ratio of lignin degradation to total weight loss (change in both the lignin and glucan contents after pretreatment), implying increased delignification with the preservation of cellulose[24]. The reducing sugar content was determined according to the DNS method[25].

The enzymes from incubated fungi cultures were collected by soaking in sodium acetate buffer (pH 4.8) at every time node. The supernatant of each sample was collected and transferred to a new tube after centrifugation at 4000×g for 3 min. Three types of ligninolytic activities were monitored during the incubation. Manganese peroxidase (MnP), manganese-independent peroxidase (MIP) and laccase activities were determined as described by Vyas et al.[26]

For SDS-PAGE, the supernatants were boiled for 5 min and cooled in ice before loading onto the gel. A 12.0% resolving gel was prepared containing 2.0 mol/L Tris-HCl (pH 8.8), 12% potassium persulphate, 20% SDS and TEMED. The stacking gel (5.0%) was made using 0.5 mol/L Tris-HCl (pH 6.8), 12% potassium persulphate, 20% SDS and TEMED. Electrophoresis was performed at a constant voltage of 160 V for 4 h using a DYCZ-24F Standard Vertical Electrophoresis Unit (Liuyi Crop., Beijing) with running buffer consisting of 25 mmol/L Tris base, 200 mmol/L glycine and 3 mmol/L SDS. Prestained protein markers were used. The gel was stained with 0.6% Coomassie Brilliant Blue R-250 in 48% (v/v) methanol, 8% (v/v) acetic acid for 1 h and destained for 10 h in 18% (v/v) methanol and 8% (v/v) acetic acid. Finally, the gel was viewed and photographed using an imager.

FTIR spectroscopy is a powerful tool to study the physicochemical and conformational properties of lignin and polysaccharide. The relative absorbance of different bands was determined via the baseline correction method to compare the spectra.

2.6 Enzymatic hydrolysis

The wet pulp fraction was hydrolyzed with a commercial cellulose from *Trichoderma viride*. Cellulase loading was performed using 8 Filter Paper Units (FPU)/g substrate. Enzymatic hydrolysis was performed in 0.05 M sodium citrate buffer (pH 4.5) at 45°C on a rotary shaker at 140 r/min for 48 h. The saccharification ratio per pulp was calculated according to the NREL LAP-009 procedure[27]. The sugar yield was based on the weight percentage of reducing sugars to the original biomass. All of the enzymatic hydrolysis experiments were performed in duplicate.

3 Results and discussion

3.1 Effects of Mn^{2+} supplementation on ligninolytic activities and delignification

The results showed that Mn^{2+} provided a negative stimulating effect on ligninolytic activity during the first 21 d of the incubation that was incompatible with the highly significant nutrition interaction for lignin degradation (Table 1). The initial purpose of supplementing Mn^{2+} was to improve the ligninolytic activity, thereby increasing lignin degradation. It was anticipated that inorganic nutritional Mn^{2+} supplements would improve ligninolytic activity, resulting in better selective lignin degradation. Nevertheless, by contrast, the results indicated that supplementing Mn^{2+} ions inhibits all three ligninases but enhanced lignin degradation significantly in 21 d of bamboo incubation.

Another interesting phenomenon was the higher selectivity of lignin degradation associated with Mn^{2+} addition compared with that of the control during the first 21 d of the incubation (Table 1). Moreover, the addition of Mn^{2+} in this study improved both the efficiency of delignification and preservation of cellulose. This may be related to the composition of the biomass and their deficiency in providing insufficient nutrients to meet the requirements of fungi.

Although the main effects of Mn^{2+} addition are significant for short-term microbial treatments (no longer than 28 d), Mn^{2+} addition did not enhance lignin degradation in bamboo powder by *Ceriporiopsis* sp. after 35 d of incubation. As shown in Table 1, incubation with a different initial amount of $MnSO_4$ resulted in a laccase activity level of 1.3-2.4 IU/g, an MIP activity level of 0.3-0.7 IU/g and an MnP activity level of 4.1-5.7 IU/g on day 35, findings that were not significantly different from those following incubation without Mn^{2+} (laccase: 3.0 IU/g; MIP: 1.1 IU/g; MnP: 8.2 IU/g). Incubation with supplemental $MnSO_4$ degraded 20.1%-22.0% lignin compared with the control (19.4%) at the end of 35 d, indicating almost identical activity levels. A statistical analysis of lignin degradation was performed based on $MnSO_4$ and incubation time, and p-value $_M$ and p-value $_I$ are found both higher than 0.05, suggesting no significant difference. There was a long initial lag period before 21 d in the control compared with Mn^{2+}-added samples, most likely because the fungi needed to produce a combination of manganese- dependent enzymes to facilitate degradation and use the bamboo powder as a carbon source[28].

Table 1 Major effects and interactions between the $MnSO_4 \cdot 5H_2O$ level (0-0.1 g) and time factors (day 21, day 28, day 35) on ligninolytic activity, lignin degradation, total weight loss, pulp yield and sugar yield following incubation at 75% initial moisture content and 28°C

Incubation time /d	$MnSO_4 \cdot 5H_2O$ /g	Lac/IU·g^{-1}	MIP/IU·g^{-1}	MnP/IU·g^{-1}	Lignin degradation/%	Lignin degradation/ Total weight loss	Pulp yield /%	Sugar yield /%
21	0	4.1	1.2	18.2	7.6	0.8	71.8	14.1
21	0.01	1.7	0.8	14.9	10.3	1.5	68.4	22.3
21	0.02	1.9	1.5	10.2	18.4	1.7	67.7	25.0
21	0.05	6.0	1.0	6.9	17.0	1.5	70.5	17.3
21	0.1	3.3	1.2	5.2	18.9	1.6	71.1	20.5
28	0	2.6	2.1	9.1	12.7	1.1	69.7	16.6
28	0.01	2.0	0.5	5.6	15.6	1.3	68.4	22.5
28	0.02	1.8	0.6	4.5	19.0	1.2	67.0	23.9
28	0.05	1.6	0.6	3.2	19.3	1.2	65.3	23.4
28	0.1	1.7	0.8	5.0	20.4	1.3	64.9	22.8
35	0	3.0	1.1	8.2	19.4	1.2	66.2	19.1
35	0.01	2.4	0.7	5.4	20.3	1.3	66.1	19.2
35	0.02	1.3	0.6	4.1	21.2	1.2	65.7	19.7
35	0.05	2.0	0.3	5.7	20.1	1.2	65.4	21.6
35	0.1	2.1	0.5	4.2	22.0	1.3	64.5	22.7
0	0	0	0	0	0	-	79.6	5.4
p-value $_M$*						0.3313	0.3330	0.0884
p-value $_I$**						0.1162	0.0059	0.4507

Note: * *p*-value $_M$ means that *p*-value is computed based on $MnSO_4 \cdot 5H_2O$, g; ** *p*-value $_I$ means that *p*-value is computed based on Incubation time, day.

3.2 Effects of Mn^{2+} supplementation on pulp yield and sugar yield

Delignification is a critical process during pretreatment for enzymatic hydrolysis, and higher cellulose content in the recovered solids eventually provides a higher sugar yield for ethanol fermentation. Microbial treatment that results in a higher pulp yield is expected to provide more sugar during the hydrolysis step. Therefore, aside from delignification, pulp yield and sugar yield are important criteria for evaluating performance. The effects of Mn^{2+} on pulp yield and sugar yield for the 21 d, 28 d, and 35 d culture periods are presented in Table 1. The results showed that Mn^{2+} had a significant impact on pulp yield and sugar yield across all quantity levels before day 28. Supplementation with 0.1 g of $MnSO_4$, followed by supplementation with 0.05 g (70.5%), 0.01 g (68.4%), and 0.02 g, (67.7%) at day 21, resulted in a high pulp yield (71.1%) that was lower than that of the control (71.8%). It was assumed that higher unselective enzyme activities typically improve the ability of the fungus to degrade lignin, but it may also lead to unexpected cellulose consumption and more total weight loss, lowering the pulp yield and overall sugar yield. Nevertheless, supplementation with Mn^{2+} produced a significantly higher selectivity ratio (1.5-1.7) and sugar yield (17.3%-25.0%) than that of the control (0.8% and 14.1%, respectively) on day 21. However, after long-term incubation, pretreatment with Mn^{2+} showed similar performance to that without Mn^{2+} across all concentrations on day 35, and no significant difference was observed across the 4 nutrition levels. The observed results for the 0.02 g, 0.05 g and 0.1 g $MnSO_4$ supplementation were similar, indicating that the extent of lignin degradation in bamboo powder does not require excessive Mn^{2+} supplementation. A statistical analysis of pulp yield and sugar yield was performed based on $MnSO_4$ and incubation time, and only *p*-value $_I$ of pulp yield are found lower than 0.05, suggesting a significant influence of incubation time on pulp yield. The results showed that well-optimized Mn^{2+} provided the best sugar yield (0.02 g of the supplement, 25.0%, day 21) much earlier than the control (19.1%, day 35), saving the incubation time significantly.

3.3 Effects of Mn^{2+} supplementation on sugar availability from the soluble fraction

The resulting substrates were highly digestible by cellulolytic enzymes at relatively low enzyme loadings and had a strong susceptibility to a high total sugar yield.

Table 2 shows the composition of the filtrates obtained, referred to as 100 g of raw biomass. The sugar compositions are expressed as the monomer content after the heating step. Mn^{2+} nutritional addition contributed to the selection for lignin removal over the relatively minimal removal of xylan. It was assumed that the total sugar in filtrates decreased as the incubation time increased for the fungi metabolic requirement. However, a maximum concentration of 8.7% of sugars was determined in the filtrates obtained at 28 d with Mn^{2+}. The contents of galactose and arabinose were stable concomitantly with the incubation time and Mn^{2+} supplementation, except for the contents on day 28, probably due to structural change in subsequent delignification reactions. A significant difference was observed such that recovery sugars ranging from 5.1% to 5.8% were obtained in incubations performed without nutritional supplementation, whereas it increased to 7.0%-8.7% on the supplementation of Mn^{2+} (Table 2). Xylose is proven to be the most abundant hemicellulose-derived sugar in filtrates due to the high hemicellulose content in the raw material, which will be released to the liquid after steam pretreatment. Considering that the content of xylose presented in the extractive fraction of raw material was 5.1% of the total raw biomass, it is obvious that more xylose recovery was available than any other sugars, indicating more effective Mn^{2+}-assisted selectivity of the xylan fraction.

Table 2 Major effects and interactions between the $MnSO_4 \cdot 5H_2O$ level (0-0.02 g) and time factors (day 21, day 28, day 35) following incubation at 75% initial moisture content and 28°C on the compositions of the filtrates obtained after steam pretreatment, referred to as 100 g of raw bamboo

Incubation time/d	$MnSO_4 \cdot 5H_2O$/g	Glucose/g	Xylose/g	Galactose/g	Arabinose/g	Mannose/g	Total/g
21	0	1.1	2.2	0.4	0.8	0.6	5.1
21	0.02	1.7	3.4	0.5	0.8	1.2	7.6
28	0	1.2	2.5	0.5	0.8	0.8	5.8
28	0.02	1.8	3.6	0.8	1.4	1.1	8.7
35	0	1.2	2.3	0.5	0.9	0.7	5.6
35	0.02	1.5	2.9	0.5	1.0	1.1	7.0

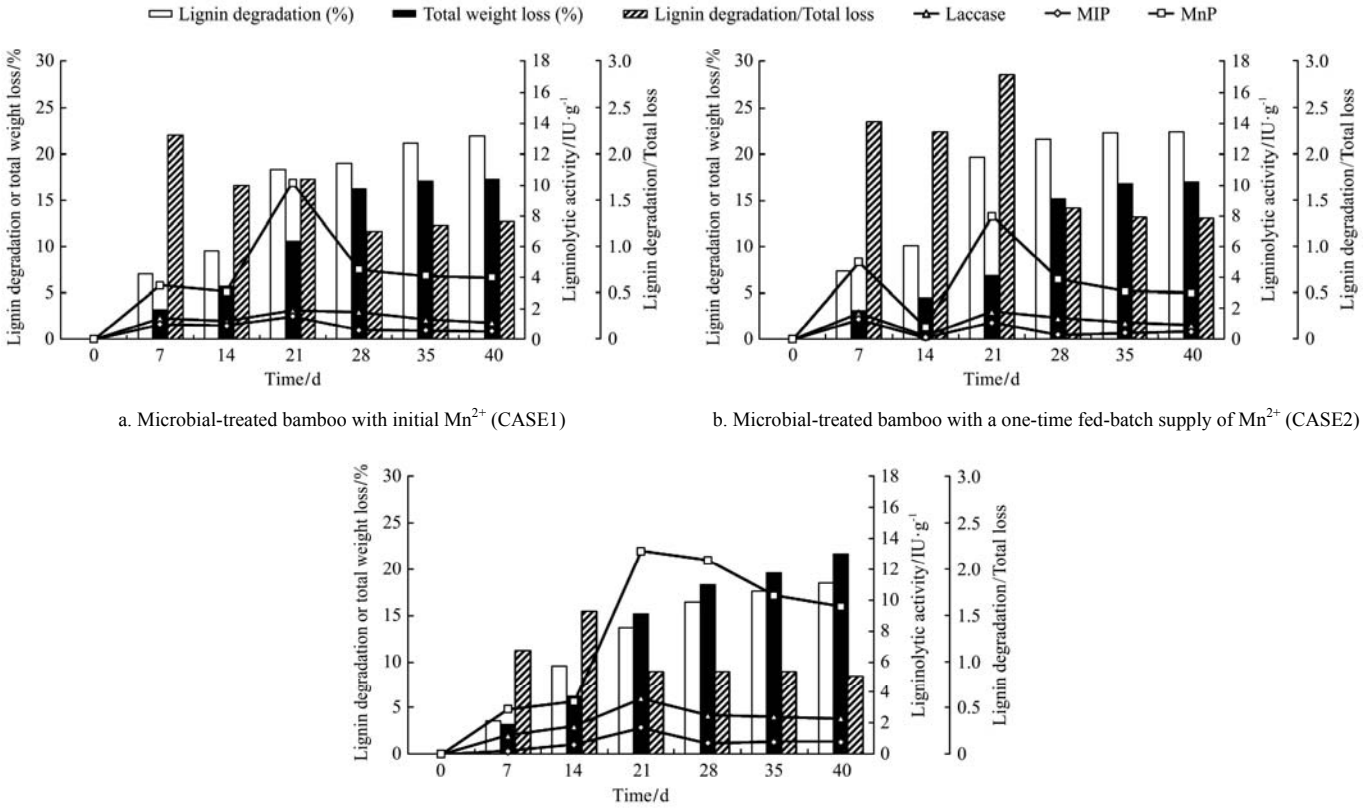
3.4 Effects of the Fed-batch supply of Mn^{2+} on ligninolytic activities and delignification

In addition to supplementing the initial nutrients, the fed-batch supply is another key factor that affects fungal growth and metabolic activities, especially in cases where the microorganism requires certain nutrients crucial to support metabolism[29]. The effects of Fed-batch/non-fed-batch supply on delignification over time are presented in Figure 2. A lag period (7 d or 14 d) before significant lignin degradation was observed in all incubations. Between 14 d and 21 d, lignin was degraded significantly at a rate of 5.0%-10.0% for all incubations. For the no fed-batch supply (CASE1), the lignin degradation rates slowed down after 28 d, and the final lignin degradation ratio over 40 d was 22.0%, on average (Figure 2a). Incubation with 1 and 2 times the fed-batch supply (CASE1 and CASE2) showed similar trends within 14 d, but higher lignin reduction occurred after 14 d of incubation than with CASE3 (Figures 2a, 2b and 2c). For all three processes, a longer incubation time resulted in significantly higher biomass degradation. A noticeable difference was noted in the ratio of lignin degradation to total weight loss. All of the CASEs continued to degrade lignin selectively at the beginning, whereas the total biomass was degraded together in CASE3 without significant selectivity after 14 d. The lignin degradation observed in the CASE2 was significantly higher than that in CASE1 and CASE3 within 21 d of incubation.

CASE3, on average, produced the highest levels of ligninolytic activity of the other cases at day 21 with a low ratio of lignin degradation to total weight loss (Figure 2c), indicating that more cellulose was accessible to the fungus through decomposition of the lignin structure in the excess Mn^{2+} system. Although this level of Mn^{2+} content supported active fungal growth and expression, it may not fully support the metabolic functions of *Ceriporiopsis* sp. This limitation in the available Mn^{2+} could be one possible explanation for the hampered ligninolytic activity in CASE1. In contrast, an

interesting phenomenon was that the ratio of lignin degradation to total weight loss was lower in CASE3 than in the other cases. Table 3 and Figure 2 show that CASE1 and CASE2 demonstrated, respectively, 18.4% and 19.7% lignin degradation, 67.7% and 66.7% pulp yield, and 25.0 % and 26.1% sugar yield after 21 d, better than CASE3, indicating that the Mn^{2+} levels were adequate for lignin degradation. It is possible to increase the Mn^{2+} content to improve performance, but the accumulation of metal ions limits metabolic activity, often inhibiting microbial cultures and increasing susceptibility to limit metabolism[28]. Thus, there is a parabolic relationship between ligninolytic activity and Mn^{2+}, with CASE2, the only one-time fed-batch supply as the optimum level.

Therefore, it was assumed that, in this microbial treatment process, lignin degradation relies on fungal growth and ligninase formation mainly during the 21 d incubation on bamboo powder. For all of the incubations, additional culture time may have been beneficial to overall lignin degradation, but the selectivity of degradation decreased after 21 d, i.e. the total weight loss also increased significantly. However, the pulp yield and sugar yield would have been reduced. Within each condition, a significant difference in selectivity was observed. This indicates that the compatible Mn^{2+} fed-batch supply with a short incubation time may be helpful in preserving cellulose and sugar. This approach achieved a high enzymatic sugar yield rapidly from bamboo using substantial Mn^{2+} loadings.



a. Microbial-treated bamboo with initial Mn^{2+} (CASE1)

b. Microbial-treated bamboo with a one-time fed-batch supply of Mn^{2+} (CASE2)

c. Microbial-treated bamboo with a two-time fed-batch supply of Mn^{2+} (CASE3). Laccase

Note: △: MIP; ◇: MnP; □: Lignin degradation. Blank: Total weight loss; Black: Ratio of lignin degradation to total weight loss. The incubations were carried out at 28°C and 70% air moisture for 40 d. The cultures contained 50.0 g of air-dried bamboo powder, 5.0 g of wheat bran, 0.02 g of $MnSO_4 \cdot 5H_2O$ and 75% water.

Figure 2 Ligninolytic activity, lignin degradation and total weight loss after 40 d of incubation.

3.5 Analysis of the protein profile by SDS-PAGE

Protein profiling of the fungi is important to understand the enzyme gene expression occurring during the microbial process which can, in turn, lead to improved delignification. Figure 3 showed that little visual difference in the protein profiles between the CASEs was obtained among the variations in the number of bands, major band patterns, band patterns and band intensity, and no induced-stress proteins were observed. The high similarity in the protein profiles suggested that the secretion of proteins was not sensitive to Mn^{2+}. Although the amount and species of expressed protein are

similar, the enzyme activity may vary in accordance with Mn^{2+} even under the same cultural media and conditions. No noticeable additional protein was expressed, demonstrating the suitability of the culture conditions with Mn^{2+} to the strain causing the same amount and type but different activities of protein expression.

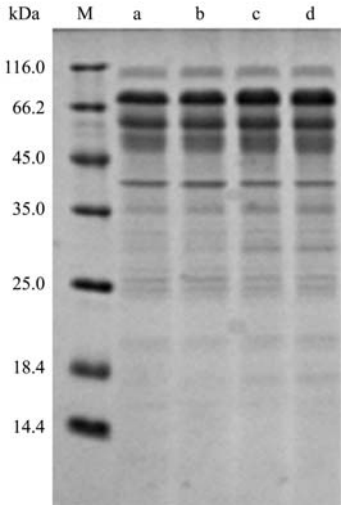


Figure 3 SDS-PAGE analysis of the protein extracted from (a) microbial-treated bamboo without Mn^{2+} (CASE0), (b) microbial-treated bamboo with initial Mn^{2+} (CASE1), (c) microbial-treated bamboo with a fed-batch supply of Mn^{2+} (CASE2), and (d) microbial treated bamboo with a two-time fed-batch supply of Mn^{2+} (CASE3)

3.6 Infrared spectroscopy

The IR spectra of lignocellulosic materials were influenced by three main biopolymers, namely lignin, hemicellulose and cellulose. Figure 4 provides the infrared spectroscopy for the microbial-treated lignocellulosic materials and their control, in which 4 samples were included: (a) raw material; (b) microbial-treated bamboo without Mn^{2+}(CASE0); (c) microbial-treated bamboo with initial Mn^{2+} (CASE1); (d) microbial-treated bamboo with a fed-batch supply of Mn^{2+} (CASE2). All of the samples presented two main absorbance regions: the first at low wavelengths in the range of 800-1900 cm^{-1}, and the second one at higher wavelengths corresponding to the range of 2600-3600 cm^{-1}. Specific absorptions peaks could be identified for each particular component.

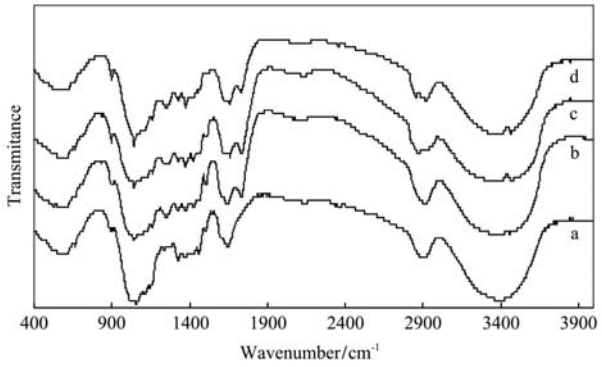


Figure 4 FTIR spectroscopy for (a) raw material, (b) microbial-treated bamboo without Mn^{2+} (CASE0), (c) microbial-treated bamboo with initial Mn^{2+} (CASE1), and (d) microbial-treated bamboo with a fed-batch supply of Mn^{2+} (CASE2)

The band at approximately 900 cm^{-1} is attributed to the asymmetric out-of-plane ring stretching in cellulose due to the β-linkage and also to the amorphous form in cellulose. Its absorbance intensity increased from (a) to (b), (c) and (d) due to the microbial selectivity on lignocellulosic materials. The peaks at 1045 cm^{-1} indicated the stretching vibration of C-O ether in β-O-4 bonds, and these peaks increased in relative intensity upon Mn^{2+} supplementation for its contribution to selectivity. The band near 1125-1160 cm^{-1} assigned to C-O-C asymmetric valence vibration was decreased in the absorbance intensity ratio after microbial treatment, especially for (c) and (d). This observation could be due to the depolymerization of lignin following microbial treatment. The band at 1375 cm^{-1} representing symmetric C-H bending from the methoxyl group exhibited a slight increase in the relative intensity due to its rigid bio-degradability. The relative intensities of the bands related to -CH, $-CH_2$ vibrations and aromatic ring modes (1410 cm^{-1} and 1460 cm^{-1}) decreased slightly after microbial treatment, indicating an increase in the oxidation degree. The mean value of the relative absorbency of the different bands of aromatic rings at 930 cm^{-1}, 1520 cm^{-1} and 1600 cm^{-1} were lower for microbial-treated than for untreated raw materials. Moreover, lignin presenting characteristic peaks in the range of 1500-1600 cm^{-1}, corresponding to aromatic skeletal vibration, decreased in the relative intensity for Mn^{2+} supplementation. The absorption band at 1640 cm^{-1}, which could be attributed to the variation of the -C=O stretch of conjugated p-substituted aryl ketones, decreased marginally in the relative intensity due to the biodegradation of lignin. Bands in the range of 2820-2880 cm^{-1}, which are very characteristic of $-OCH_3$ groups, were shifted to a higher relative intensity after

microbial treatment. The band at 3420-3460 cm^{-1} is attributed to the hydrogen-bonded O-H stretching vibration, and its relative absorbance intensity decreased upon microbial treatment. This was due to the biodegradation of bonds between the carbohydrate and lignin and the biodegradation of the hydrogen bond between the cellulosic chains during the microbial treatment process.

4 Conclusions

Mn^{2+} significantly affected lignin degradation, providing better results than controls and a shorter incubation time (no more than 21 d). The results suggested that Mn^{2+} supplementation is an important consideration for microbial treatment because it significantly improved process performance during a limited incubation time (no more than 21 d). FT-IR analysis also confirmed the depolymerization of lignin following microbial treatment, especially for the significant influence by Mn^{2+} supplementation. This work demonstrated that this two-stage pretreatment process is well suited for converting lignocellulose to fermentable sugars. Microbial treatment by the solid-state incubation of *Ceriporiopsis* sp. could degrade lignin and has the potential to be an energy-saving, low-cost, simple and environment friendly approach that can extensively reduce the severity of chemical pretreatments. Further work is required to identify the most economical configuration, including process designs, techno-economic analysis and investigation of processing strategies to economize Mn^{2+} usage.

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