Pretreatment of Natural Lignocellulose With Inorganic Salts Improves Ligninase Production Fermented By *Aspergillus Fumigatus*

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Abstract

This work screened out the optimal conditions for pretreatment of natural lignocellulose with inorganic salts and provided a simple, easy-to-operate, low-cost, clean and efficient pretreatment method for the efficient degradation of natural lignocellulose by strains. The results showed that the optimal pretreatment inorganic salt was $FeCl_2$ with a concentration of 11%, pretreatment at 60°C for 48 h, and the solid-liquid ratio was 1:11 (g/mL). According to the characterization results, after pretreatment of $FeCl_2$ solution, the smooth and dense structure of natural lignocellulose surface became rough and irregular, and surface fiber bundles showed spalling and fracture. Subsequently, the enzymes produced by solid-state fermentation of *Aspergillus fumigatus* were easier to enter the interior, which increased the contact area between materials and enzymes, and increased the amount of enzymatic loads, thereby improving the biodegradation effect.

Introduction

Lignocellulose is a rich and inexpensive renewable resource. Making ample use of it will help improve the current resource shortage and environmental pollution problems, and have important implications for the sustainable development of society. Typically, lignocellulosic biomass consists of cellulose, hemicellulose, lignin, as well as small amounts of extract, and widely exists in plants. However, lignin is an aromatic polymer having an amorphous, molecular structure containing structural units of oxyphenylpropanol or a derivative thereof [1]. In the process of degradation and utilization of lignocellulose, lignin and polysaccharide are cross-linked by ester bond and ether bond, resulting in high stability and stubbornness of lignocellulose, poor accessibility of ligninase, resulting in reducing the efficiency of enzymatic hydrolysis. The natural structure of lignocellulose determines its difficulty in resource utilization and harmless treatment, so effective pretreatment is required in its conversion [2]. Pretreatment can change the dense structure of lignocellulose, destroy the physical and chemical connection between the interior, reduce the crystallinity of cellulose, or remove lignin, and increase the porosity of the raw materials. Effectively promote contact between cellulase and cellulose, thereby greatly improving the efficiency of enzymatic hydrolysis [3].

At present, pretreatment methods mainly include physical methods (microwave treatment) [4], chemical methods (acid-base hydrolysis) [5], and biological methods (biological enzymatic method) [6]. Each pretreatment method gets its advantages and disadvantages. Mechanical comminution is a relatively simple physical pretreatment method, which destroys the complex structure of natural lignocellulose to a certain extent by shearing or grinding, and reduces the size of matrix particles, increases the joint point between exposed surface and lignin degrading enzyme, increases the reaction area. Some experiments have shown that the lignin enzyme digestion process and degradation efficiency could be improved after the matrix was pulverized [7]. However, the degradation of natural lignocellulose is directly related to the milling time and the degree of comminution. The smaller the particle size, the easier it is to react, but the more energy it needs to provide. Moreover, the amorphous state produced by physical pulverization is very unstable and easy to recrystallize, resulting in the limited application. Ultrasonic pretreatment is a

pretreatment method reported in recent years [8]. The physicochemical effect is generated by the action of energy, which destroys the intermolecular hydrogen bond and the crystalline structure, changes the size of the fiber crystallization zone, reduces the degree of polymerization, and thereby increases the rate of enzymatic hydrolysis. The Yu research group used the ultrasonic system to pretreat the rice husks and greatly improved the degradation rate of *Pleurotus ostreatus* on rice hulls. The increase in efficiency was attributed to the structural damage of the rice husks during ultrasonic pretreatment [9]. Despite the fact that microwave treatment has the advantages of high efficiency and pollution-free, it is sometimes difficult to obtain industrial applications due to its prohibitive cost. The acid treatment method is applied earlier, among which sulfuric acid is the most widely used. Various diluted inorganic acids, such as HCl, H_3PO_4 and HNO_3 have also been used to pretreat different lignocellulose materials. Acid pretreatment of lignocellulose can destroy the chemical bonds between lignin and hemicellulose to increase the accessibility of enzymes. At the same time, the average degree of polymerization of cellulose was reduced, which promoted the enzymatic hydrolysis of lignocellulose [10]. The Ali research group pretreated the remaining empty pods of Moringa with dilute H_2SO_4. It was found that the degradation of lignin was reduced and the recovery rate of xylan reached 24.7–50.2% [11]. Another team used maleic acid to pretreat lignin in the secondary wall of higher plant cells found that under the condition of low concentration of maleic acid pretreatment, some dense lignin was modified to become more looser, which increased the accessibility of ligninase [12]. Among the alkaline reagents, NaOH and $Ca(OH)_2$ are the most commonly used, and NaOH works well, but the cost of acid neutralization for subsequent treatment is higher. Currently, $Ca(OH)_2$ has been used to pretreat various lignocellulosic materials for further enzymatic hydrolysis [13]. Compared with NaOH aqueous solution pretreatment, $Ca(OH)_2$ pretreatment has lower cost and better environmental benefits because it can be easily recovered by reacting with CO_2 [14].

In recent years, with the development of science and technology, researchers at home and abroad have studied many methods of lignin treatment. Although there are many pretreatment methods that can quickly process lignin, researchers hope to use more environmentally friendly techniques to treat lignin to reduce costs [15], increase degradation rates, and reduce secondary pollution to the environment during recycling. The biological treatment method has mild conditions, low energy consumption, simple operation and no pollution, and has been proved to be the best way to treat lignocellulose. However, biodegradation has the disadvantages of a long treatment cycle and low degradation efficiency. Referring to previous pretreatment methods, the study used a completely new approach. The method uses an inorganic salt reagent to pretreat natural lignocellulose and then ferment with *Aspergillus fumigatus* G-13. It is intended to change the dense structure of lignocellulose by inorganic salt pretreatment, and improve the ability of strains to produce ligninase using natural lignocellulosic materials as substrates, thereby improving the degradation efficiency of lignin. For the subsequent release and utilization of cellulose, a method for removing lignin which is relatively clean, efficient, simple in operation and low in cost is provided. In this study, it is expected that different types of inorganic salts will be used to pretreat the Robinia, screen out the inorganic salts of the best pretreated Robinia, and the pretreatment conditions

are further optimized. Robinia is a typical broad-leaved natural lignocellulose. Robinia is mainly guaiacyl-syringyl lignin, which is composed of dehydrogenated polymers of coniferyl alcohol and erucyl alcohol.

Materials And Methods

2.1 Strain sources

Aspergillus fumigatus G-13 (*A. fumigatus* G-13), which could degrade lignin used in this work was isolated from the samples collected from soil near the sewage draining exit of a paper mill in Harbin.

2.2 Natural lignocellulosic sample

The Robinia from Songshan Forest Farm, Gongyi City, Henan Province.

2.3 Pretreatment processes

Take the natural lignocellulosic substrate sample Robinia, and the impurities were removed and passed through a 20-mesh sieve. 4 g of the Robinia under the sieve was weighed and placed in a 150 mL Erlenmeyer flask. $FeSO_4$, $Fe_2(SO_4)_3$, $FeCl_2$, $FeCl_3$, NaCl, $CaCl_2$, $ZnCl_2$ and $MgCl_2$ solutions were added to pretreat the samples respectively. After pretreatment, the samples were washed with distilled water until the pH value no longer changed. After filtration, the pH was adjusted with a citric acid-sodium citrate buffer solution (0.1 mol/L, pH 3) to 6. Dry to constant weight at 60°C.

2.4 Preparation of bacterial suspension

The oblique surface of *A. Fumigatus* PDA medium stored at 4°C was taken out, and the fungal spores were washed with sterile water to prepare a spore suspension of 10^6 cells/mL (measured by blood cell counting method). Keep it in a 4°C refrigerator. The sample was inoculated at 3.5 mL/vial.

2.5 Solid state fermentation culture

Used solid-state culture, 3 g of the pretreated natural lignocellulose sample was added to a 150 mL Erlenmeyer flask, and a large amount of elemental nutrient salt solution and a 0.1% trace element nutrient salt solution were added according to the solid-liquid volume ratio, and autoclaved at 120°C for 20 min. Afterwards, 3.5 mL of spore suspension was added and cultured at 30°C under constant temperature. The ligninase activity of the fermentation broth was determined by sampling on the 3rd, 6th, 9th, 12th, 15th, 18th and 21st, respectively, with 3 parallel samples in each group.

Among them, a large amount of elemental nutrient solution: 0.2150 g KH_2PO_4, 0.2160 g $(NH_4)_2SO_4$, 0.0073 g $MgSO_4$, 0.0150 g $CaCl_2$ dissolved constant volume to 100 mL, natural pH. The nutrient salt solution of trace elements: 3.9 g/L $CoCl_2 \cdot 6H_2O$, 1.4 g/L $ZnSO_4 \cdot 7H_2O$, 1.6 g/L $MnSO_4 \cdot H_2O$, 5 g/L $FeSO_4 \cdot 7H_2O$ dissolved constant volume to 1000 mL, natural pH.

2.6 Preparation of crude enzyme

After fermentation, the solid matrix was added to the centrifuge tube, and then 5 mL of acetic acid sodium acetate solution (pH value 4.5) was added to the 1 g substrate for oscillation extractionat at 30℃ and 100 r/min for 40 min. Crude enzyme was collected by centrifugation (20 min, 6000 r/min) at room temperature.

2.7 Ligninase activity assay

2.7.1 Manganese peroxidase activity assay

Add 3.4 mL of acetic acid-sodium acetate buffer solution (concentration: 200 mmol/L, pH 4.5) to the reaction system, and add 0.1 mL of 1.6 mmol/L $MnSO_4$ solution and 0.4 mL of crude enzyme solution. Finally, 0.1 ml of 1.6 mmol/L H_2O_2 solution was added to start the reaction, and the reaction was carried out at 37℃ for 3 min, and the absorbance at 240 nm was measured. One enzyme unit (U) is defined as the amount of enzyme required to oxidize 1 μmol Mn^{2+} per minute to Mn^{3+}. Each sample parallels operation three times and then averaged [16].

2.7.2 Lignin peroxidase activity assay

The reaction solution contained 1 mL of 15 mmol/L resveratrol solution, 1.5 mL of sodium tartrate buffer (concentration of 250 mmol/L, pH of 3) and 0.4 mL of crude enzyme solution. Finally, 0.1 mL of 20 mmol/L H_2O_2 solution was added to initiate the reaction, and the absorbance at 0, 1, 2 and 3 min was measured at 310 nm ultraviolet light. One enzyme unit (U) is defined as the amount of enzyme required to oxidize 1 μmol of resveratrol per minute to become veratraldehyde. Each sample was run in parallel three times and then averaged [17].

2.8 Fermentation substrate treatment

The centrifugal residue was washed with distilled water and suction filtered until the color of the filtrate did not change. Then, water was added to the residue, and the mixture was centrifuged at 5000 r/min for 10 minutes, and the precipitate was dried at 60°C to a constant weight. Subsequent characterization by scanning electron microscopy, infrared spectroscopy and X-ray diffraction.

2.8.1 Scanning electron microscope

The scanning electron microscope used a Japanese HITACHI S-3400M scanning electron microscope with an accelerating voltage of 5.00 kV and a working distance of 13.2 to 18.2 mm. During the observation, a conductive tape was attached to the scanning electron microscope sample stage and a 15 nm thick gold film was plated on the surface of the sample by an E-1010 (HITACHI) type ion sputter coater.

2.8.2 Infrared

Infrared spectroscopy was performed using NEXU type Fourier infrared Raman spectroscopy (FT-IR) from NICOLET, USA. Using KBr tableting method, the wave number range was 400-4000cm^{-1}, and the

resolution was higher than 0.09 cm^{-1}.

2.8.3 X-ray powder diffraction

X-ray powder diffraction (XRD) was performed by a multifunctional X-ray system diffractometer (Philips, Netherlands). The Cu/K α source has a wavelength of 1.5418, and the spectrum was recorded at a current of 40 mA·h and a voltage of 45 kV. The scanning range 2θ was 5°-40°, the step width was 0.03°, and the scanning rate was 10.0 s/step. Cellulose crystallinity (CrI) was calculated on the basis of the diffraction pattern according to the method proposed by Segal [18], See formula 1.

$$Q_{CrI} = (I_{002} - I_{am}) / I_{002} \times 100\% \quad (1)$$

Where: Q_{CrI} — Crystallinity; I_{002} — Maximum Diffraction Intensity of 002 Crystal Plane, 2θ = Maximum peak near 22°; I_{am} — Scattering Intensity of Non-crystalline Background Diffraction, 2θ = Absorption peak near 16.5°.

Results And Discussion

3.1 Effects of different kinds of inorganic salt pretreatment on ligninase activity produced by A. fumigatus G-13 fermented Robinia

Acid and alkali pretreatment are currently relatively mature pretreatment methods for the removal of lignin and hemicellulose from lignocellulosic materials. However, residual acid after pretreatment needs to be neutralized, which not only consumes additional reagents, increases costs, but also produces waste and causes pollution. If it is detoxified, the cost will need to be further increased [19]. In this experiment, the effects of pretreatment with eight inorganic salts of $FeSO_4$, $Fe_2(SO_4)_3$, $FeCl_2$, $FeCl_3$, NaCl, $CaCl_2$, $ZnCl_2$ and $MgCl_2$ on the ligninase produced by *A. fumigatus* fermented Robinia were researched. After pretreatment, the Robinia structure was destroyed, and the destruction of lignocellulosic structure by inorganic salts was used as an indicator of ligninase activity.

Maintain pretreatment temperature, time, solid-liquid ratio and inorganic salt concentration of 50°C, 48 h, 1:16 (g/mL) and 8%, respectively. The effects of inorganic salt pretreatment on the activity of lignin degradation enzymes of *A. fumigatus* G-13 fermented Robinia were investigated by changing different kinds of inorganic salts. The results were presented in Table 1, the data in the table were the highest values of ligninase activity produced within 21 days of fermentation of various inorganic salt pretreated fermentation substrates. Among them, Mnp and Lip enzyme activities reached a maximum around 15 d. It can be seen from the table that the pretreatment of $FeCl_2$ and $FeSO_4$ solution improved the enzyme activity significantly. After pretreatment with this two solutions, Mnp were all increased by 2.2 times compared with the control group, and Lip was increased by 1.8 times and 2.2 times compared with the

control group, respectively. The experimental screening, $FeCl_2$ and $FeSO_4$ were selected as the best pretreatment inorganic salts of *A. Fumigatus* G-13 fermented Robinia.

Table 1. Effect of different types of inorganic salt pretreatment on ligninase activities produced by *A. Fumigatus* G-13 on Robinia

Pretreatment Method	Pretreatment Conditions	Mnp (U/L)	Lip (U/L)
$FeCl_2$	Pretreatment temperature: 50°C; Time: 48 h; Solid-liquid ratio (g/mL): 1:16; Inorganic salt concentration 8%; (No inorganic salt was added to the control group)	1656	239.4
$FeCl_3$		949.1	155.3
$FeSO_4$		1570.5	299.9
$Fe_2(SO_4)_3$		985.9	175.6
NaCl		449.4	79
$MgCl_2$		557.7	67.2
$CaCl_2$		955.1	158.8
$ZnCl_2$		852.6	289
Control group		720.6	131.6

3.2 Effects of pretreatment temperature on ligninase activity produced by *A. fumigatus* G-13 fermented Robinia

Maintain pretreatment time, solid-liquid ratio and inorganic salt concentration of 48 h, 1:16 (g/mL) and 8%, respectively. The effects of $FeCl_2$ and $FeSO_4$ solution pretreatment on the activity of lignin degradation enzymes of *A. fumigatus* G-13 fermented Robinia were investigated by changing different pretreatment temperature. The results were presented in Fig. 1 and Fig. 2. The Mnp and Lip enzyme activities increased with increasing fermentation time and reached a maximum around 15 d. After continued fermentation, the enzyme activities of lignin-degrading enzymes (Mnp and Lip) showed a downward trend. With the increase of pretreatment temperature, the activity of lignin degrading enzyme produced by *Aspergillus fumigatus* fermented Robinia substrates showed an upward trend. When the pretreatment temperature was 60°C, the enzyme activity of the lignin degrading enzyme produced by fermentation of two inorganic salt pretreated Robinia substrates reaches a peak. Among them, after pretreatment of $FeCl_2$ solution, Mnp activity reached 1593.18 U/L, Lip activity was 257.33 U/L, and after $FeSO_4$ solution pretreatment, Mnp activity reached 1567.23 U/L, and Lip activity was 354.01 U/L. It was indicated that 60°C was the optimum temperature for pretreatment of Robinia by two inorganic salts.

3.3 Effects of pretreatment solid-liquid ratio on ligninase activity produced by *A. fumigatus* G-13 fermented Robinia

Maintain pretreatment temperature, time and inorganic salt concentration of 60°C, 48 h and 8%, respectively. The effects of $FeCl_2$ and $FeSO_4$ solution pretreatment on the activity of lignin degradation enzymes of *A. fumigatus* G-13 fermented Robinia were investigated by changing different solid-liquid ratio (g/mL). The results were shown in the figure below. Figure 3 and Fig. 4 showed the changes of lignin degradation enzyme activity after pretreatment of Robinia with different solid-liquid ratio $FeCl_2$ solution and $FeSO_4$ solution. As can be observed in Fig. 3 (a) and (b) and Fig. 4 (a) and (b), when the solid-liquid ratio was 1:6, the Mnp and Lip enzyme activities were lower. This solid-liquid ratio pretreatment cannot fully infiltrate the Robinia, resulting in poor pretreatment effect. For $FeCl_2$ solution, the volume of the pretreatment solution was increased until the ratio of solid to liquid was 1:16 and 1:21, the highest Mnp activity was reached 1560.9 U/L and 1641.6 U/L, respectively, and the highest Lip activity was reached 269.14 U/L and 265.78 U/L, respectively. When the ratio of solid to liquid was 1:16 and 1:21, the pretreatment effect was about the same as that of Mnp and Lip produced by 1:11 (Mnp was 1655.98 U/L, Lip was 302.4 U/L). When the solid-liquid ratio was 1:11, the highest enzyme activity appeared 3 days earlier than that of 1:16 and 1:21. Therefore, considering the pretreatment effect and saving raw materials, when the solid-liquid ratio was 1:11, it was the optimum solid-liquid ratio for the pretreatment Robinia with $FeCl_2$ solution.

When used $FeSO_4$ solution to treat Robinia, it can be seen from 4(a) that when the ratio of solid to liquid was 1:16, the activity of Mnp was most significantly increased, reaching a maximum of 1842.27 U/L. Under this solid-liquid ratio, the Lip activity was 200.46 U/L, which was lower than the 223.77 U/L when the solid-liquid ratio was 1:11, but the enzyme production trend was about the same. Comprehensive consideration, the solid-liquid ratio of 1:16 was chosen as the optimum solid-liquid ratio for the pretreatment Robinia with $FeSO_4$ solution.

3.4 Effects of pretreatment time on ligninase activity produced by *A. fumigatus* G-13 fermented Robinia

Maintain pretreatment temperature, solid-liquid ratio and inorganic salt concentration of 60°C, 1:11 ($FeCl_2$ solution) 1:16 ($FeSO_4$ solution) and 8%, respectively. The effects of $FeCl_2$ and $FeSO_4$ solution pretreatment on the activity of lignin degradation enzymes of *A. fumigatus* G-13 fermented Robinia were investigated by changing different pretreatment time. In Fig. 5, when the $FeCl_2$ solution was used, the pretreatment time was less than 48 h, the Mnp and Lip enzyme activities of *A. fumigatus* fermentation increased with the increase of the pretreatment time. When the pretreatment time was 48 h, the Mnp and Lip enzyme activities reached the maximum on the 15th day, which were 1200.90 U/L and 236.65 U/L respectively. Under other pretreatment time conditions, the trend of enzyme production was regular and stable. If the pretreatment time was extended, the activity of Lip enzyme decreased obviously, and the change of Mnp activity showed that the effect of pretreatment on 60 h and 48 h was equivalent. After

prolonging the pretreatment time to 72 h, the enzyme production declined slightly. The possible reason was that under this pretreatment condition, the cellulose content and xylan content in Robinia reduced with the increase of time, resulting in a slight increase in lignin content. Reduced accessibility of enzymes and substrates, resulting in decreased enzyme production efficiency.

As shown in Fig. 6, when the $FeSO_4$ solution was utilized to pretreat Robinia, the overall trend of enzyme production was approximately the same, reaching the maximum value of *A. fumigatus* enzyme production in the vicinity of 15 days. When the pretreatment time was 24 h, the Mnp activity achieved the maximum value of 1192.14 U/L, but the overall enzyme production tendency was not as good as the pretreatment for 48 h, the activity of Mnp was always maintained higher and stable. After 21 days of fermentation, when the pretreatment time was 60 h, the Lip enzyme activity reached the maximum value of 255.69 U/L on the 15th day. Compared with pretreatment for 36 h and 48 h, although the highest Lip enzyme activity was not as good as 60 h pretreatment, the highest enzyme activity difference was lower, and the overall enzyme production tendency was better. In summary, the optimum pretreatment time for pretreatment of Robinia with $FeCl_2$ and $FeSO_4$ solution was 48 h.

3.5 Effects of pretreatment with different inorganic salts concentrations on ligninase activity produced by A. fumigatus G-13 fermented Robinia

Figure 7 and Fig. 8 were the trend diagrams of enzyme activities after pretreatment of Robinia with different concentrations of $FeCl_2$ and $FeSO_4$ solutions. Maintain pretreatment temperature, time and solid-liquid ratio of 60°C, 48 h, 1:11 ($FeCl_2$ solution) 1:16 ($FeSO_4$ solution), respectively. Under different concentrations, the changes of lignin peroxidase and manganese peroxidase activities were examined. The results showed that the activities of the two lignin-degrading enzymes were all increased first and then decreased. The highest value of ligninase activity in the fermented substrate pretreated by $FeCl_2$ and $FeSO_4$ solution appeared near 15 d. As can be seen from the figure below, the pretreatment with 11% concentration of two inorganic salts was better than 2% and 8% concentration, and the Lip activity of the Robinia pretreated with 11% concentration of $FeCl_2$ solution reached the maximum at 12 d.

After pretreatment of Robinia with 11% $FeCl_2$ solution, Mnp and Lip activities reached maximum at 15 d (2244 U/L) and 12 d (292.3 U/L), respectively. Compared with the control group (no inorganic salt pretreatment), Mnp and Lip enzyme activities were increased by 3 times and 2.1 times, respectively. For 11% $FeSO_4$ solution, the Mnp and Lip enzyme activities reached a maximum at 18 d (2094 U/L) and 15 d (313.6 U/L), respectively. Compared with the control group, Mnp and Lip enzyme activities were increased by 2.6 times and 2.2 times, respectively. To sum up, after 11% $FeCl_2$ solution pretreatment, Mnp activity was higher than $FeSO_4$ pretreatment, although Lip enzyme activity was lower than $FeSO_4$ treatment, but after $FeCl_2$ solution pretreatment, Lip enzyme activity peak appeared 3 days earlier than $FeSO_4$ pretreatment. Therefore, the higher concentration of $FeCl_2$ (11%) solution pretreatment of Robinia can

promote the production of enzymes and increase the speed of lignin peroxidase production. The optimal pretreatment inorganic salt of *A. Fumigatus* G-13 fermented Robinia was $FeCl_2$ with a concentration of 11%.

3.6 Effect of optimal condition pretreatment and A. fumigatus G-13 fermentation on surface structure of lignocellulosic materials

Scanning electron microscopy has become an important technical means to observe the surface structure characteristics of lignocellulosic substrates. In order to further understand the reasons of $FeCl_2$ solution pretreatment, *A. Fumigatus* fermentation treatment and $FeCl_2$ solution pretreatment after *A. Fumigatus* fermentation treatment to improve the lignin removal rate, the surface structure of Robinia was observed by scanning electron microscope. As shown in Fig. 9 (all Robinia samples have a particle size of 20 mesh).

Figure 9 (a) was Robinia without any treatment. It can be observed in the figure that the physicochemical structure of the untreated Robinia surface was dense and uniform, and the arrangement was relatively neat. Figure 9 (b) was shown that the dense structure of the original Robinia has been destroyed and the surface structure has become irregular after being treated under optimal pretreatment conditions (11% $FeCl_2$ solution). And the fiber bundles on the surface of Robinia appeared spalling or even breaking. Comparing the SEM images of Robinia with or without biological treatment (Fig. 9 (a) and (c)), it can be found that the Robinia treated by *A. Fumigatus* G-13 was obviously loose and the surface becomes rough, and presents a series of irregular micro-holes and cracks. Figure 9 (d) was the SEM image of Robinia that was pretreated with $FeCl_2$ solution and then treated with *A. Fumigatus* fermented. Observing and comparing Fig. 9 (d) and (a), (b), and (c), various treatment methods affect the surface structure of Robinia lignocellulosic substrate, but after inorganic salt pretreatment and then the biological fermentation treatment of the Robinia, the structural damage was more thorough. The possible reason was that $FeCl_2$ solution pretreatment makes the surface of the Robinia rugged, and these structural changes increase the specific surface area of the fiber, thereby increasing the contact area with the enzyme, the contact site of the enzyme and enzyme loading [20]. The change of microstructures enhances the accessibility of ligninase to materials and destroys the natural barrier of lignocellulose, thereby improving the enzymatic efficiency.

3.7 Effect of optimal condition pretreatment and A. fumigatus G-13 fermentation on chemical structure of lignocellulosic materials

3.7.1 Infrared spectra analysis

Infrared spectroscopy is a common means of analyzing the composition and chemical changes of lignocellulose. Table 2 displays the spectra of fundamental chemical bonds and functional groups in the lignocellulose matrix. Figure 10 was an infrared spectrum of Robinia, in which (a), (b), (c) and (d) respectively represent that Robinia has not been pretreated and has not been biodegraded, not biodegraded after optimal pretreatment, not pretreated and biodegraded, and biodegraded after optimal pretreatment.

Table 2. Attribution of key chemical bonds or group characteristics in lignocellulose matrix

Wavenumber /cm^{-1}	Attribution	Lignocellulose Structurally Related
3455-3410	O-H Stretching vibration	Internal hydrogen bond cleavage of cellulose molecules
2920-2850	C-H Stretching vibration	Waxy aliphatic related
2920	C-H Stretching vibration	Cellulose methyl/methylene break
1745	C=O Ester bond	Related to lignin side chain removal
1720	Carboxylic acid and Ester bond	Related to hemicellulose removal
1595-1605	Aromatic benzene ring stretching	Related to lignin removal
1510	β-Glycosidic bond vibration and Benzene ring stretching	Carbohydrate and lignin removal characteristic peaks
1426	CH_2 and CH_3 Bending vibration	—
1329	C-C and C-O Skeleton vibration (Syringyl ring C-O)	Related to lilac-based condensation lignin
1260	Ester absorption (Guaiacyl ring C-O)	Uronic acid removal related
1218-1245	C-O absorption in hemicellulose and lignin	Derived from acetyl group removal
1059	C=O stretching	Related to cellulose/ hemicellulose content
900	Sugar C_1 group or sugar ring frequency, β- glucoside bond	Non-crystalline cellulose

From the Fourier transform infrared spectroscopy (FTIR) results, it was found that the characteristic peaks of cellulose, hemicellulose and lignin in Robinia were all significantly changed after pretreatment. The results are presented in Fig. 10. The absorption peak at 3420 cm^{-1} in the figure was the O-H stretching vibration of the phenolic hydroxyl group and the alcoholic hydroxyl group; the absorption peak at 2920 cm^{-1} was the stretching vibration of C-H; the absorption peak at 1745 – 1655 cm^{-1} represented

C = C, C = O (ketones, esters) (Jahan, 2004), and the absorption peak at 1745 – 1655 cm^{-1} represented C = C, C = O (ketones, esters). The ester bond (C = O) absorption peak at 1736 cm^{-1} was more pronounced in untreated Robinia, and the intensity of this peak was weakened after optimal pretreatment. It was indicated that the key ester bonds between polysaccharide and lignin, such as ferulic acid, p-hydroxybenzoic acid and p-hydroxy cinnamic acid were broken. Further biological treatment of the pretreated sample will significantly reduce the intensity of the peak, indicated that the combined treatment could break more key ester bonds between lignin and polysaccharide [21]. The Robinia has a strong characteristic absorption peak near 1618 cm^{-1} and 1507 cm^{-1}. This peak was the stretching vibration of the benzene ring skeleton, which represents the extension of the lignin component. After pretreatment with $FeCl_2$ solution and then by *A. Fumigatus* fermentation, the absorption peak of Robinia was not obvious here, indicated that most of the lignin structure was destroyed. And the typical lignin infrared absorption peak was around 1618 cm^{-1}, 1507 cm^{-1} and 1319 cm^{-1}. Compared (a) with (b), (c), and (d), it can be seen that with the application of the treatment means, the peak intensity showed a significant weakening, indicated that the pretreatment promoted the production of ligninase by the strain and induced further degradation of the Robinia.

After the Robinia was treated with optimal pretreatment conditions, the chemical changes in its composition were observed. It was found that Robinia contained two types of lignin, guaiacyl and syringyl, and the characteristic absorption peaks were 1248 cm^{-1} (guaiac ring C-O) and 1319 cm^{-1} (syringyl ring C-O). Compared the infrared spectrum of Robinia, it was found that the characteristic peak of cellulose and hemicellulose (1050 cm^{-1}) became wider under the condition of $FeCl_2$ solution pretreatment of Robinia. It was indicated that the pretreatment can further destroy the structure of cellulose and hemicellulose and change the absorption intensity of the binding bond. In addition, change in strength of 897 cm^{-1} can reflect the structural changes of crystalline cellulose. It can also be seen from the figure that after pretreatment with $FeCl_2$ solution and then by *A. Fumigatus* fermentation, the peak shape became slow and the peak width became large, indicated that the characteristic functional groups of crystalline cellulose were also destroyed, resulted in a decrease in absorption intensity.

3.7.2 XRD analysis

The crystallinity of the raw materials of Robinia and the crystallinity of the materials obtained after pretreatment by different methods were studied by X-ray powder diffraction. The results were shown in Fig. 11, in which (a), (b), (c) and (d) respectively represent that Robinia has not been pretreated and has not been biodegraded, not biodegraded after optimal pretreatment, not pretreated and biodegraded, and biodegraded after optimal pretreatment.

Many studies have demonstrated that the reduction of amorphous lignin and hemicellulose affects the proportion of cellulose crystalline regions in the sample [22, 23]. Cellulose crystallinity reflects the relative proportion of crystalline regions in cellulose, rather than the absolute proportion. We found through XRD calculation that the crystallinity of Robinia (20 mesh) without any treatment was 44.84%, and the crystallinity decreased to 43.44% after pretreatment with $FeCl_2$ solution, indicated that $FeCl_2$ destroyed

the crystal structure inside Robinia. The crystallinity of Robinia fermented directly with *A. fumigatus* G-13 without pretreatment was 46.72%, which was higher than that of Robinia without any treatment. The probable reason was that the ligninase produced during the fermentation of *A. fumigatus* degraded the amorphous lignin, but the crystalline cellulose remained in the Robinia structure, resulted in an increase in the CrI of the directly fermented Robinia. Compared with the unpretreated Robinia, the crystallinity of Robinia fermented by *A. fumigatus* after pretreatment with $FeCl_2$ solution decreased from 44.84–43.11%. This was due to the pretreatment of $FeCl_2$ solution, which could break the linkage between the key ester and ether bonds between the linking polysaccharide and lignin, and weakened the hydrogen bonding between hemicellulose and cellulose. After pretreatment, amorphous substances such as lignin and hemicellulose were degraded and dissolved. Subsequently, the ligninase produced by the fermentation of *A. fumigatus* was effective in degrading lignin, thereby exposing the amorphous regions of cellulose. The cellulose in the Robinia was significantly swelled, the crystallinity index of the lignocellulosic structure was lowered, and the cellulose crystallization zone was damaged, so that the crystallinity was lowered (Nakashima et al., 2016).

Conclusion

This study used inorganic salt, a simple, low-consumption, non-polluting chemical reagent to pretreat natural lignocellulose (Robinia). It was found that the $FeCl_2$ solution was an effective pretreatment reagent. The optimal conditions for pretreatment of Robinia by $FeCl_2$ solution were successfully screened by optimization experiments, and the maximum effectiveness of the pre-processing was achieved. SEM and FTIR analysis showed major structure changed after pretreatment, and it was found that the obstinate structure inside the Robinia was destroyed after pretreatment. Meanwhile, it could effectively promote the solid-state fermentation of *A. fumigatus* G-13 to produce enzymes, thereby improved the degradation efficiency of Robinia by biological treatment. To the best of our knowledge, there is no study on the pretreatment of natural lignocellulose by mild conditions to promote the production of enzymes by strains. This work provides a basis and reference for further experimental research in the field of biodegradation of natural lignocellulose.

Declarations

Declarations	Not applicable
Funding	National Natural Science Foundation of China [No: 21776054].
Conflicts of interest	None
Data and Material	Availability and transparency
Contributions	Zijing Zhou: Write drafts, do experiments; Gaijuan Guo: Analyze the data and make graphs; Jinda Li: Propose methods and provide innovation points; Hong Yan: Review articles, revise articles and provide funding; Fen Li: Review articles and revise articles.
Ethics	Approval
Participate	Consent
Consent for publication	All authors agreed to publish

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Figures

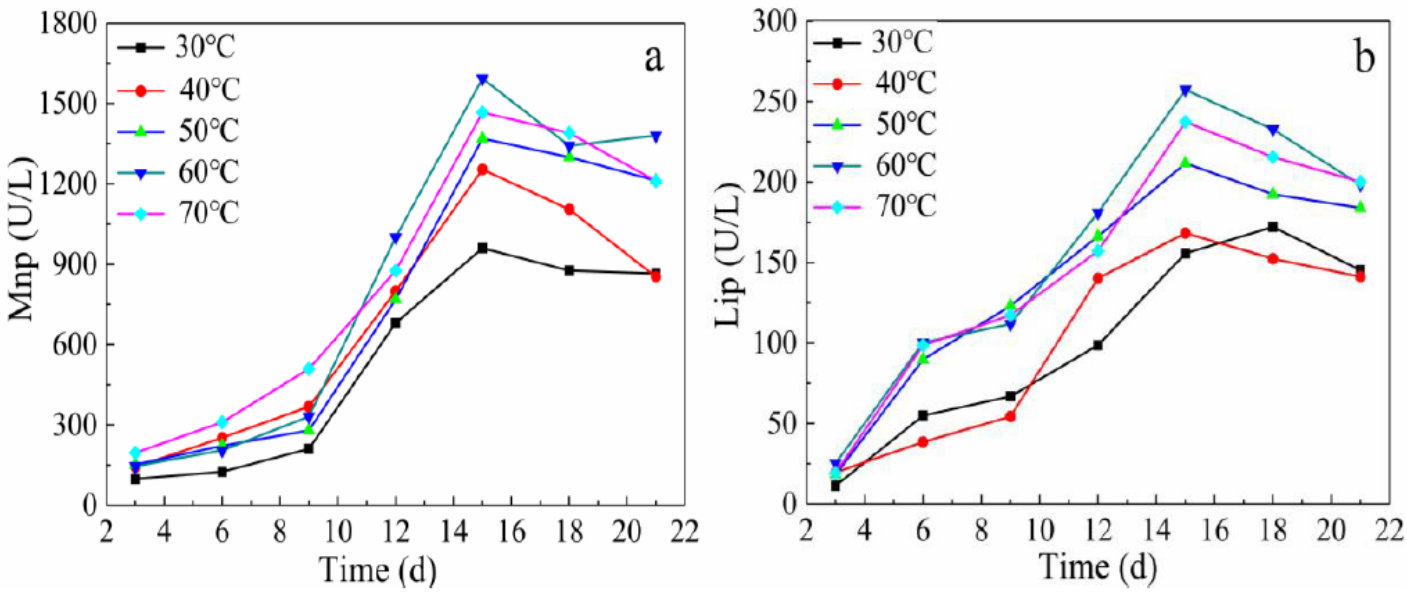

Figure 1

Effect of different temperature conditions of FeCl2 solution pretreatment on ligninase activities produced by A. Fumigatus G 13 on Robinia

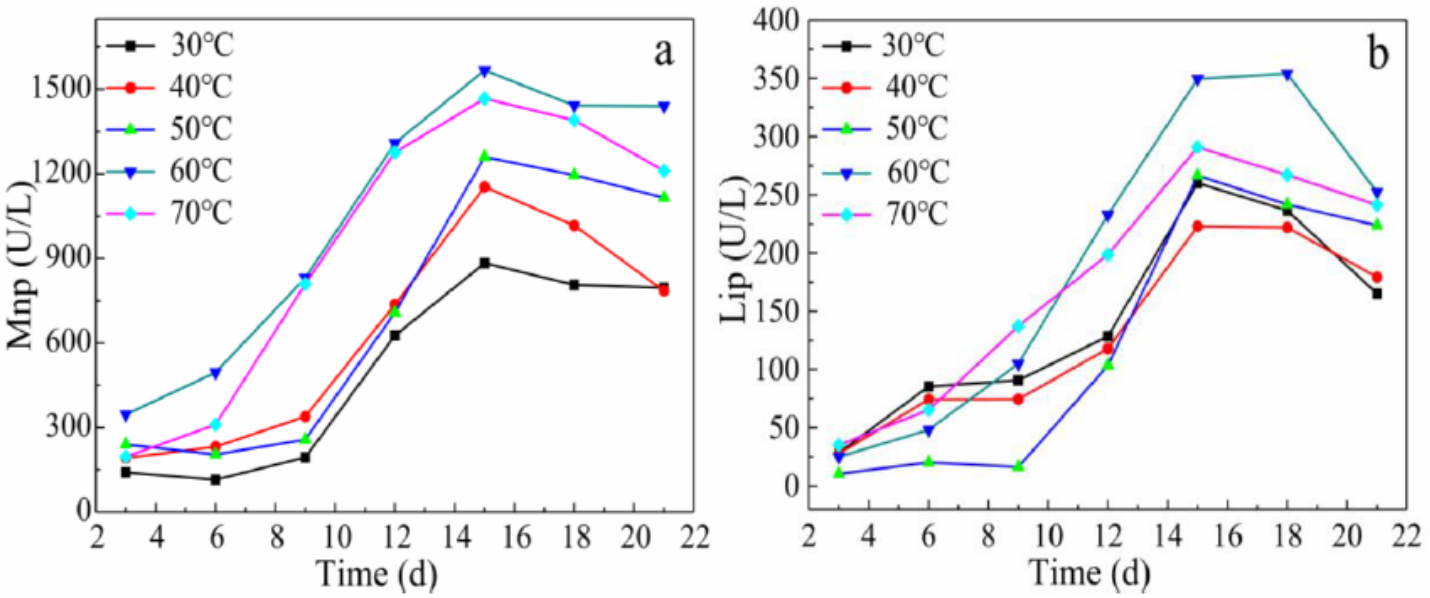

Figure 2

Effect of different temperature conditions of FeSO4 solution pretreatment on ligninase activities produced by A. Fumigatus G 13 on Robinia

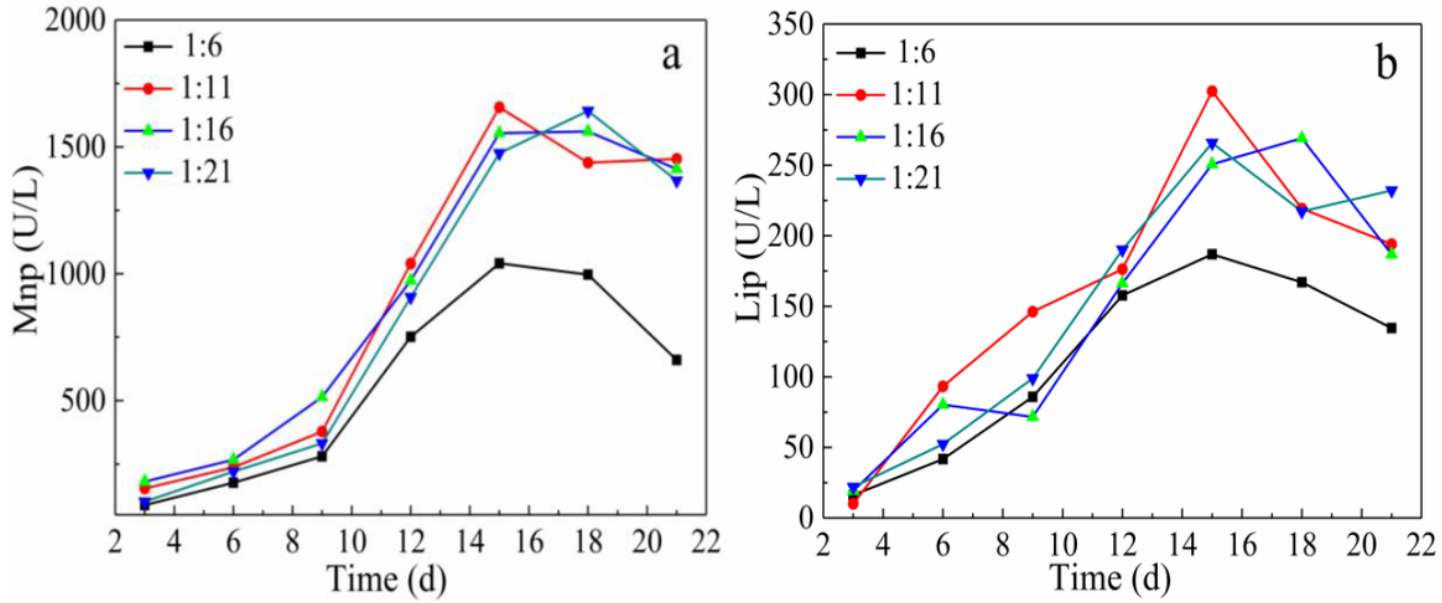

Figure 3

Effect of different solid liquid ratio conditions of FeCl2 solution pretreatment on ligninase activities produced by A. Fumigatus G 13 on Robinia

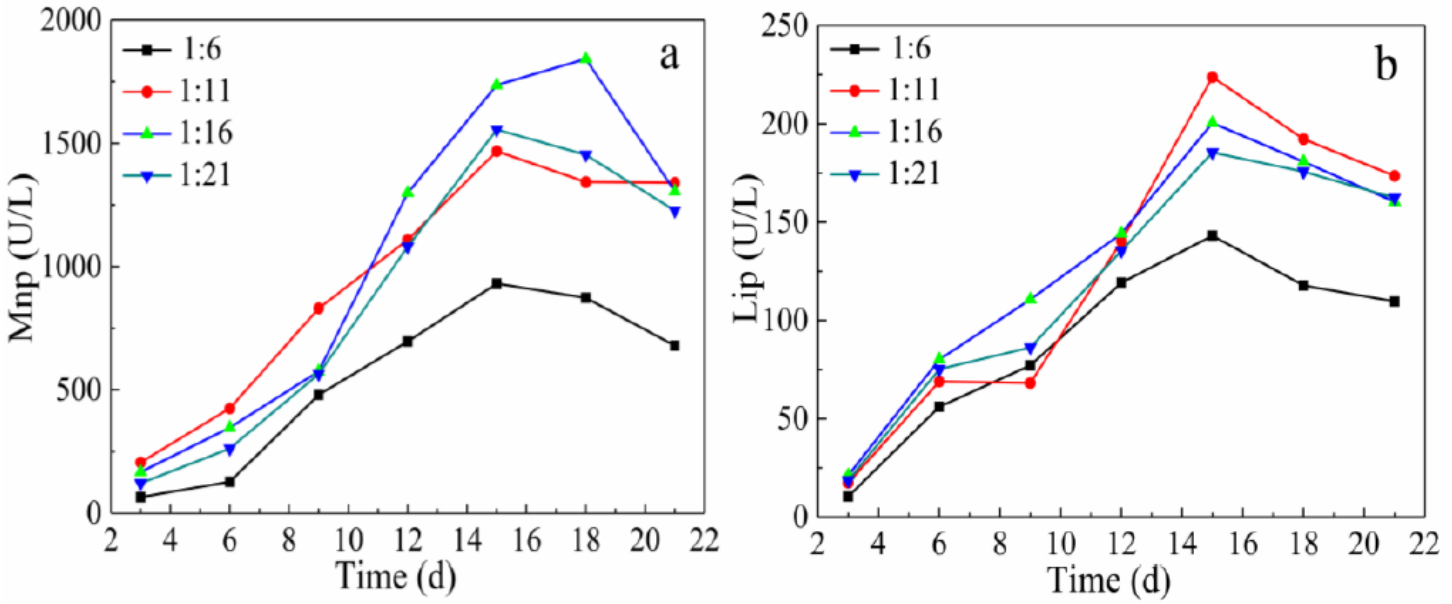

Figure 4

Effect of different solid liquid ratio conditions of FeSO4 solution pretreatment on ligninase activities produced by A. Fumigatus G 13 on Robinia

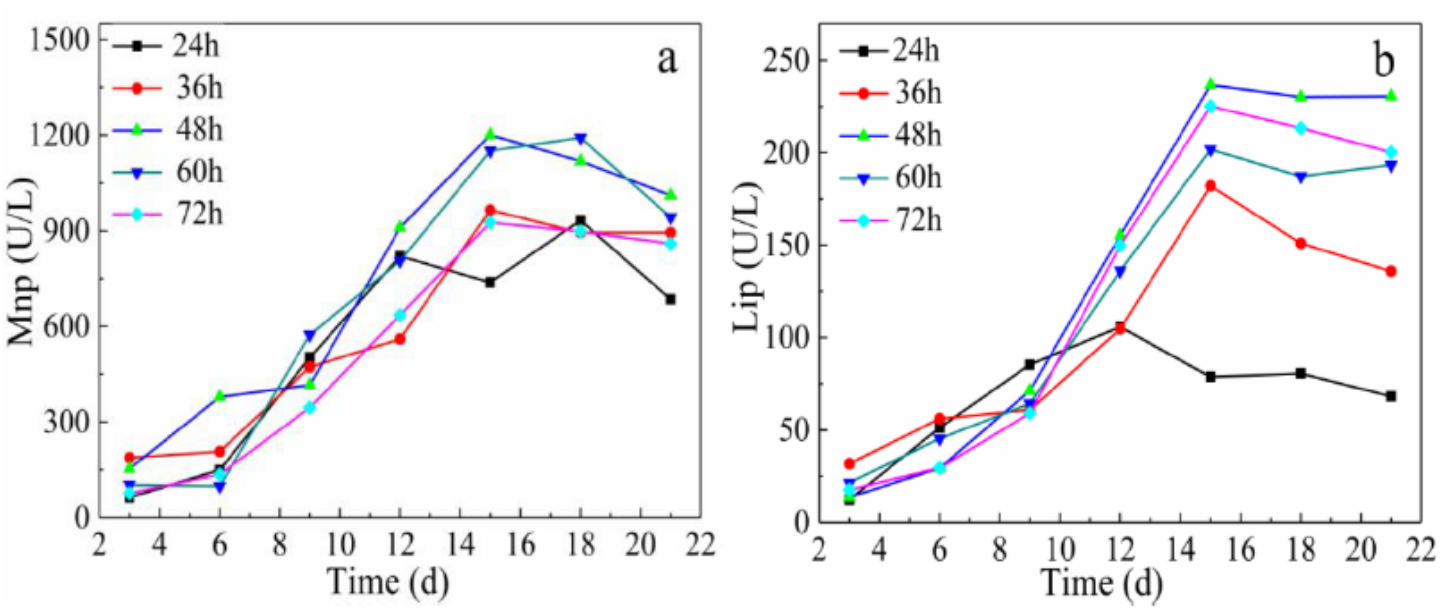

Figure 5

Effect of different time conditions of FeCl2 solution pretreatment on ligninase activities produced by A. Fumigatus G 13 on Robinia

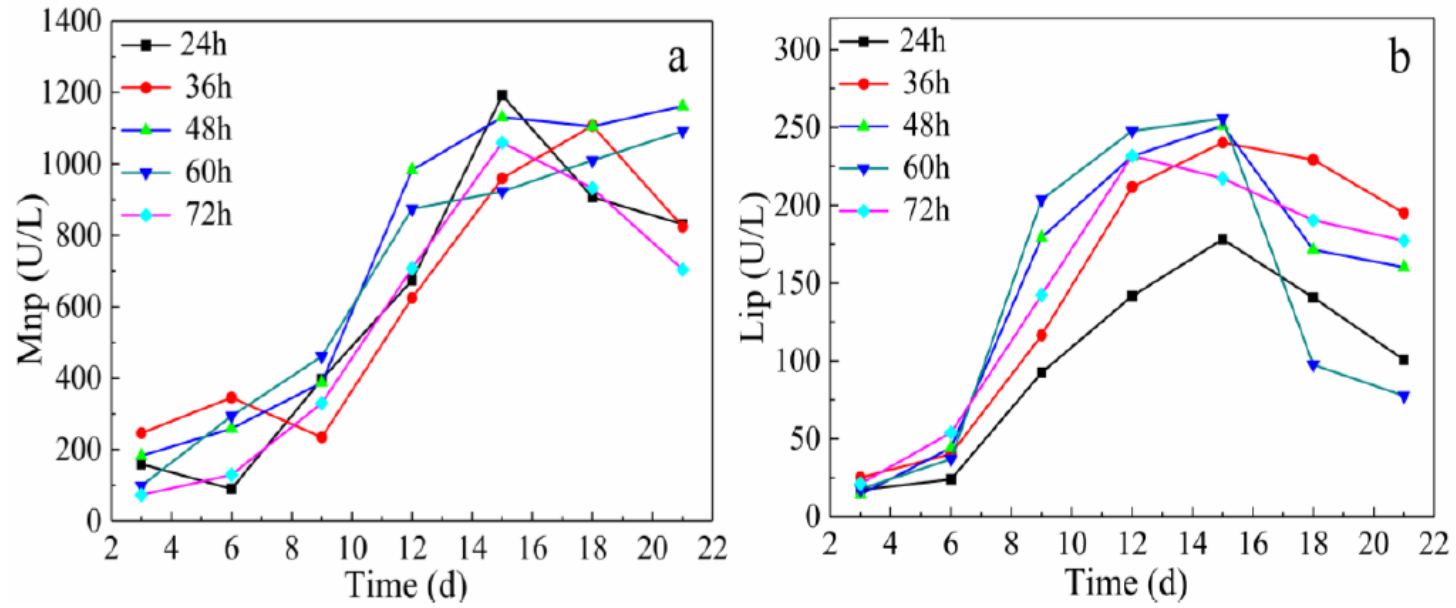

Figure 6

Effect of different time conditions of FeSO4 solution pretreatment on ligninase activities produced by A. Fumigatus G 13 on Robinia

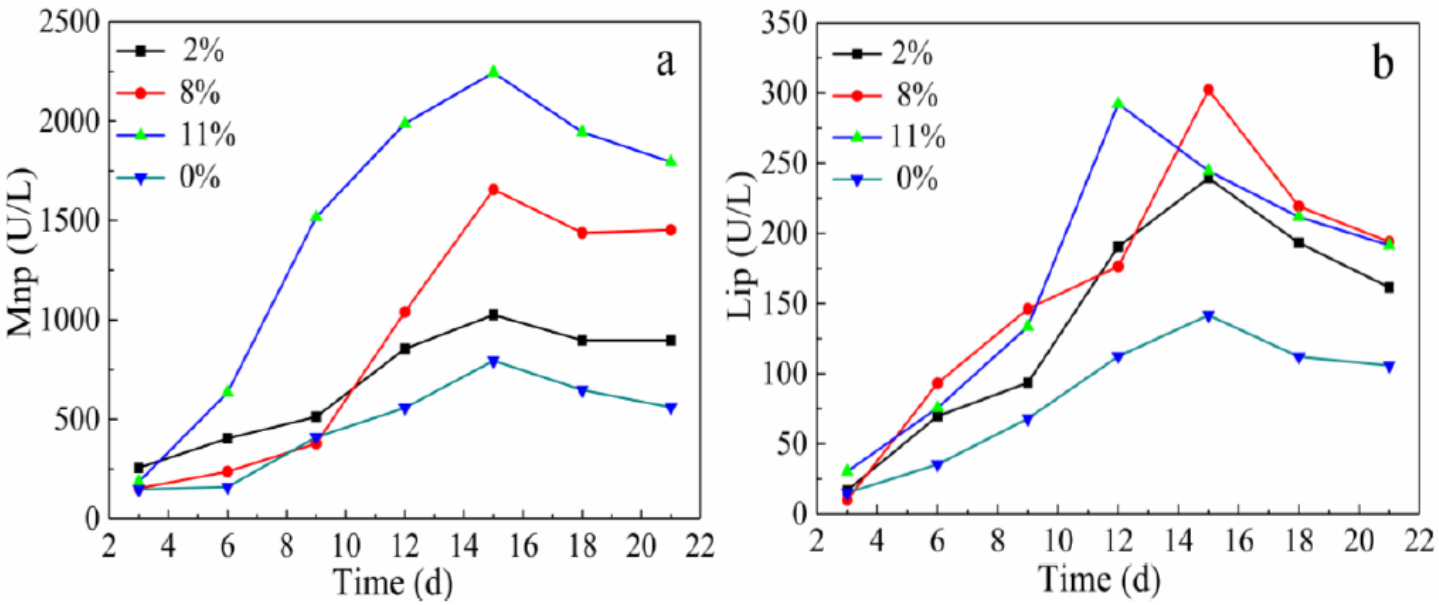

Figure 7

Effect of different concentrations of FeCl2 solution pretreatment on ligninase activities produced by A. Fumigatus G 13 on Robinia

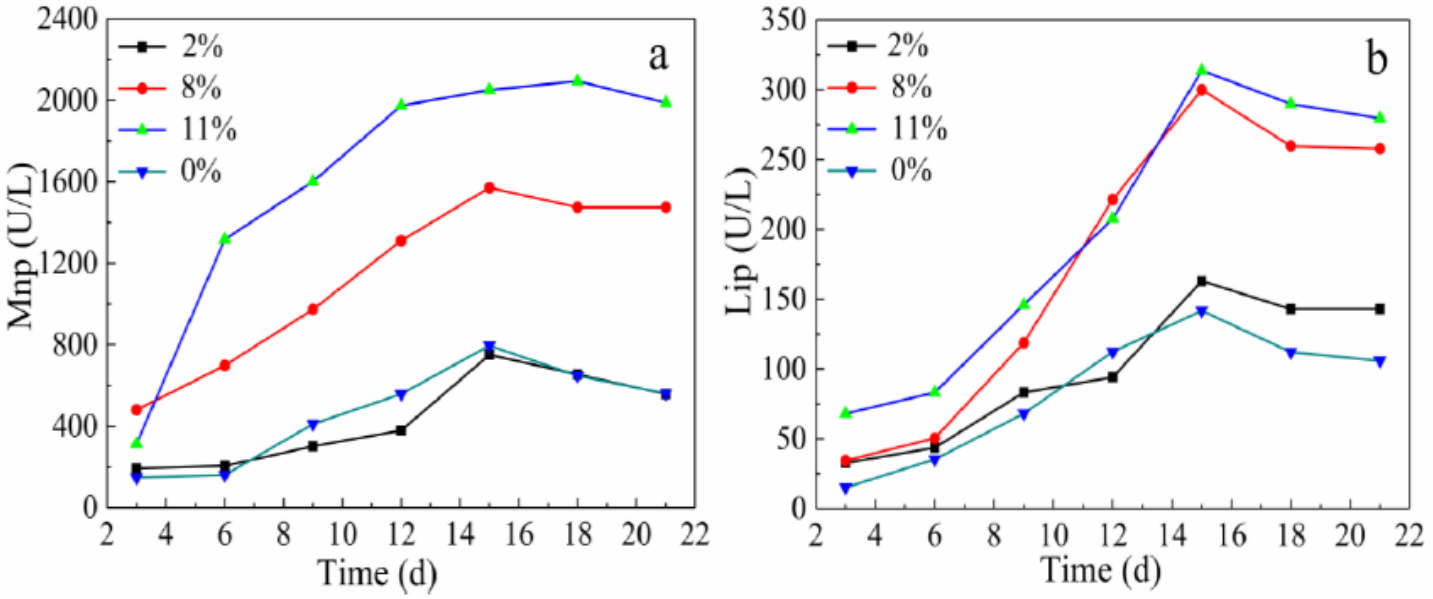

Figure 8

Effect of different concentrations of FeSO4 solution pretreatment on ligninase activities produced by A. Fumigatus G 13 on Robinia

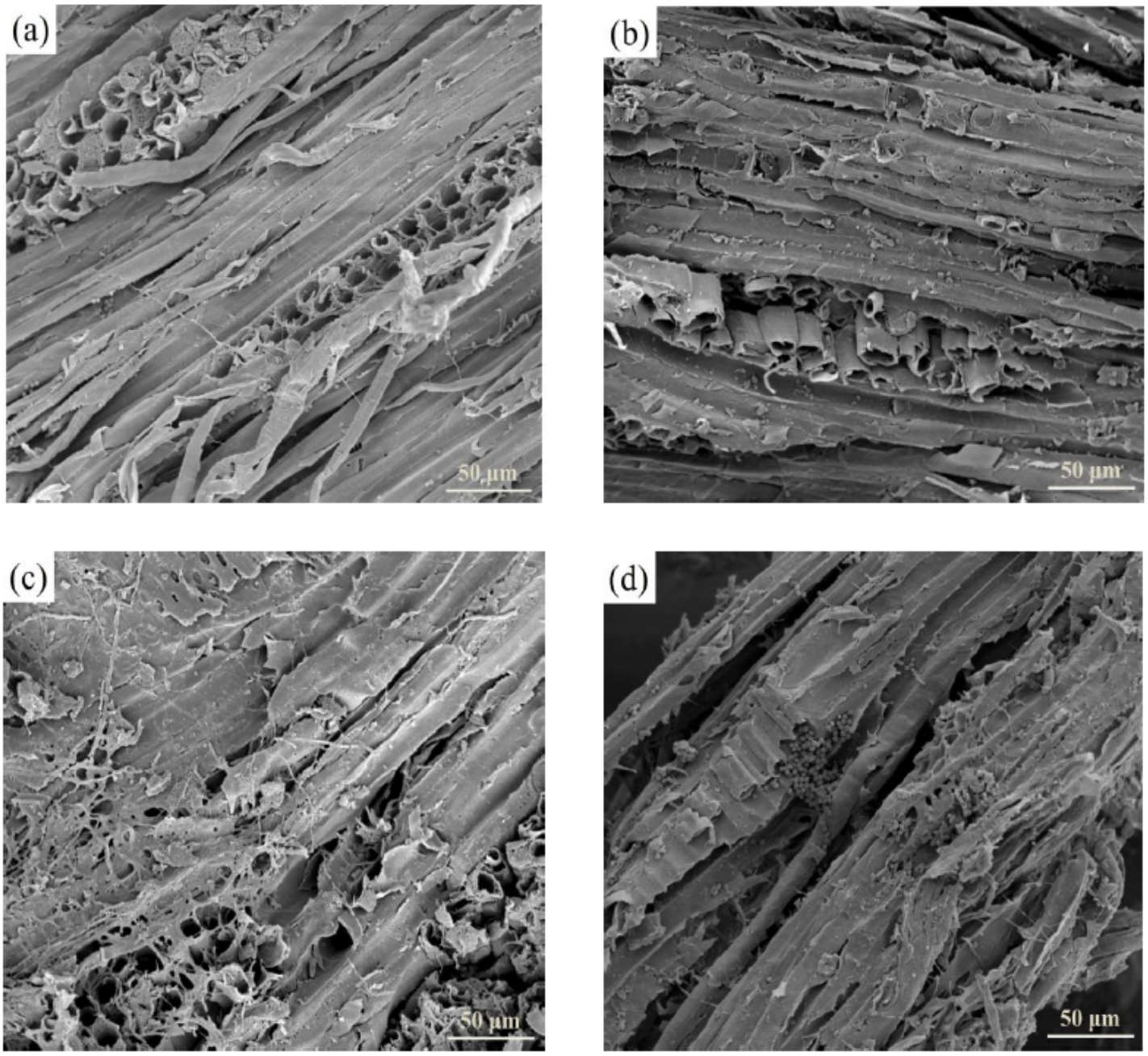

Figure 9

Effects of pretreatment with optimal conditions and A. fumigatus G 13 fermentation (a) Not pretreated and not biodegraded. (b) Not biodegraded after optimal pretreatment. (c) Not pretreated and biodegraded. (d) Biodegraded after optimal pretreatment.

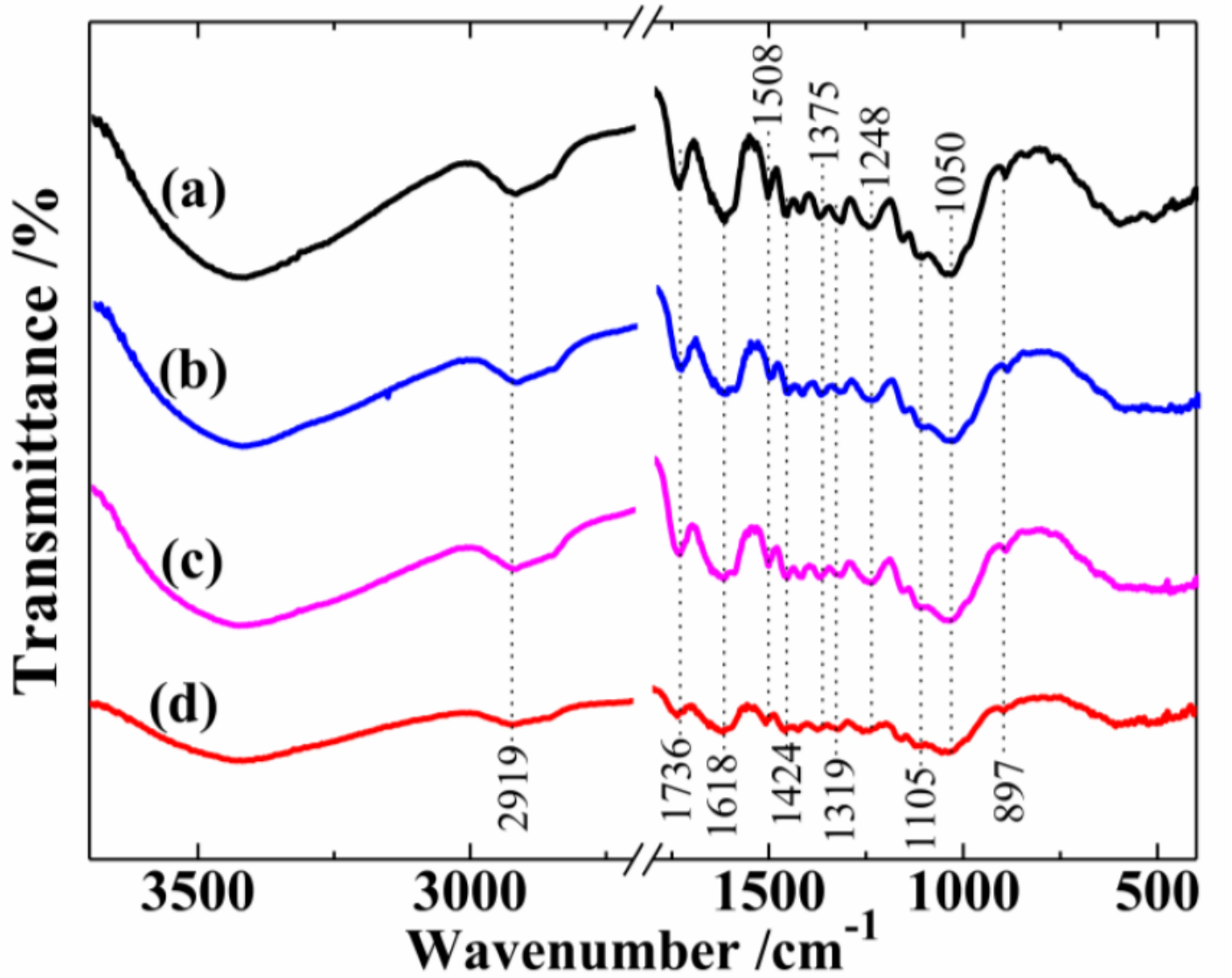

Figure 10

FTIR diagrams of Robinia raw materials and Robinia treated by different methods (a) Not pretreated and not biodegraded. (b) Not biodegraded after optimal pretreatment. (c) Not pretreated and biodegraded. (d) Biodegraded after optimal pretreatment.

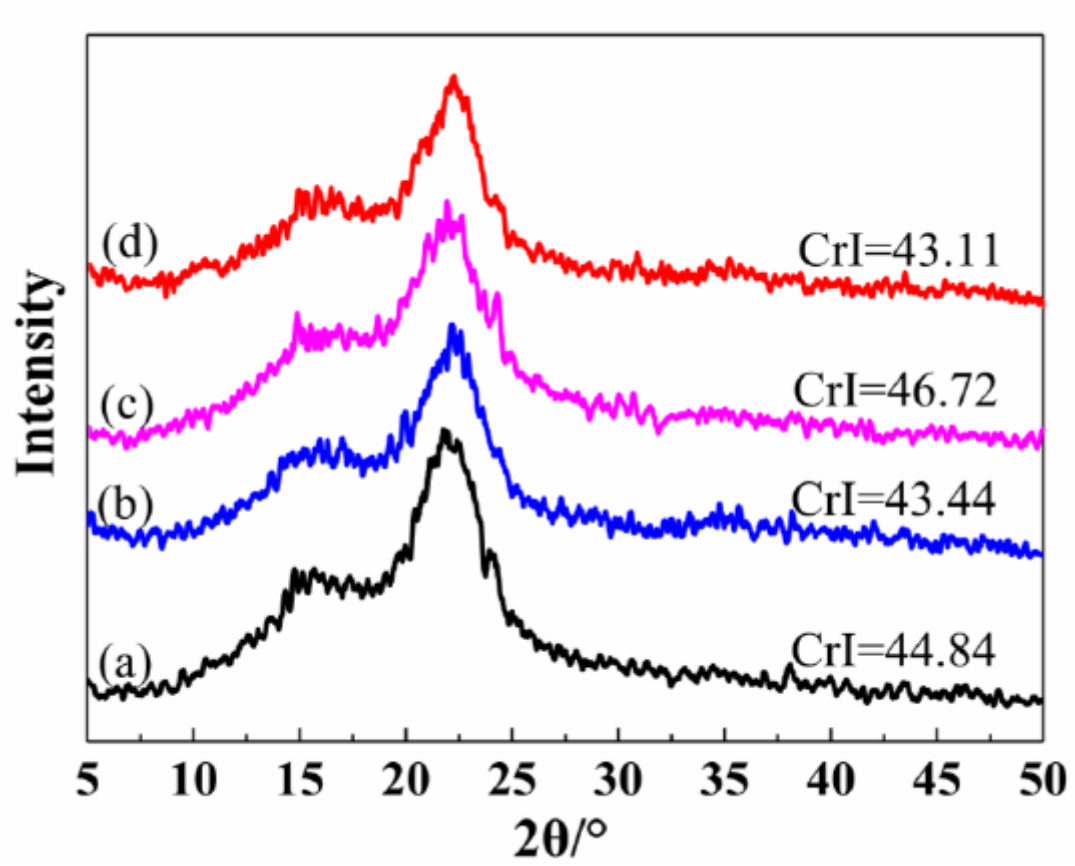

Figure 11

XRD diagrams of Robinia raw materials and Robinia treated by different methods (a) Not pretreated and not biodegraded. (b) Not biodegraded after optimal pretreatment. (c) Not pretreated and biodegraded. (d) Biodegraded after optimal pretreatment.