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Utilization of nano-gold tracing technique: Study the adsorption and transmission of laccase in mediator-involved enzymatic degradation of lignin during solid-state fermentation

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ABSTRACT

The degradation of lignin waste by ligninolytic enzymes and the effect of mediators on lignin degradation were studied. Moreover, nano-gold tracing technique was innovatively used to study the adsorption and transmission mechanism of ligninolytic enzymes in the process of SSF. The results showed that the effects of three common mediators on the degradation of lignin were in descending order: veratryl alcohol > ABTS > Mn^{2+}, and the optimum dosages were: Mn^{2+} (20 μM g^{-1}), ABTS (0.2 μM g^{-1}), veratryl alcohol (4 μM g^{-1}). A significantly high degradation ratio of lignin (31.8%) was achieved at the optimum dosages of ligninolytic enzymes and mediators. In addition, laccase, as a typical ligninolytic enzyme, was selected to conjugate to nano-gold for the study of adsorption and transmission mechanism. An obvious decrease of nano-gold-laccase conjugates was observed after 10 days of enzymatic hydrolysis and there were few laccase residues on the surface of straw fibers after 30 days of enzymatic hydrolysis. The present findings will advance the understanding of mediator-involved enzymatic degradation of lignin, as well as the adsorption and transmission mechanism of ligninolytic enzymes in lignin degradation, which could provide useful references for developing waste biotreatment technology.

1. Introduction

Agricultural wastes have been used as raw materials to produce value-added products by solid-state fermentation (SSF) [1]. However, agricultural residues usually contain large amounts of lignin, which is a complex macromolecule compound making it fairly difficult to be utilized. Lignin, one of the most abundant organic materials, is present in the cell wall, providing structural support, impermeability, and standing up to oxidative and microbial attack [2,3]. Due to its indigestibility, lignin is regarded as a rate-limiting substance in the process of agricultural wastes degradation in nature [4].

White-rot fungus is characterized by their unique ability to degrade lignin mainly due to the secretion of low-specificity phenol oxidase enzymes. In particular, the representative species *Phanerochaete chrysosporium* (*P. chrysosporium*) has been extensively studied and proved to be able to degrade a range of organic substrates. Ligninolytic enzymes from *P. chrysosporium* can biodegrade lignin substructure model compounds as well as straw and arbor lignin [5–7]. *P. chrysosporium* secretes extracellular peroxidases known as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) that are involved in the degradation pathway, velocity and degree of lignin [8]. Due to the complexity of composting process, the way in which ligninolytic enzymes pass into the substrate and degrade lignin effectively are related to the final treatment effect, therefore it is significant to study the adsorption and transmission of ligninolytic enzyme in the compost substrate [9]. Laccase, a promising biocatalyst compared with LiP and MnP, does not contain heme as the cofactor but copper, and neither does it need hydrogen peroxide as the co-substrate but rather molecular oxygen [10–14]. However, the adsorption and

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transmission mechanism of laccase in the process of enzymatic hydrolysis of lignin during SSF remains unknown.

Recently, the nano-gold labeling technique has been utilized to study the adsorption of enzymes on solid surfaces. Adsorption of enzymes is greatly influenced by substrate characteristics such as pore volume and accessible surface area. In lignocellulosic substrates, lignin content and distribution or hemicellulose content also determine enzyme adsorption and, in turn, degradability [15,16]. Nano-gold can firmly combine with enzymes through electrostatic interactions, and this combination has no obvious effect on their biological activity [17,18]. This technique allows good visualization of enzyme adsorbed on the substrate surface, which makes it possible to study the adsorption and transmission of ligninolytic enzymes in enzymatic degradation of lignin. Laccase can be conjugated to nano-gold particles and the nano-gold laccase conjugates adsorbed to the surface of lignin was visualized by electron microscopy, so tracking the adsorption and transmission of laccase can become a reality [16,19].

Previous research had shown that the bore diameter of cell wall pore was merely 2–5 nm, when the weight loss reached forty percent in the wood rotting process [20]. It is difficult for ligninolytic enzymes to infiltrate into matrix cell to contribute, due to the molecular dimension which is bigger than the surface microporous aperture of agricultural straw and wood. Thus, it can be concluded that in the primary stage of lignin degradation, there are a variety of permeable mediators being involved in reactions. The reported mediators mainly include veratryl alcohol [21], 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) [22], Mn^{2+} [20,23], Hydroxyl radical (•OH)/iron ion [24–26], Oxalic acid [27], etc. Among them, veratryl alcohol acts as the radical cations and has the biological significance as a secondary metabolite in relation to lignin degradation [28]; increasing the concentration of Mn^{2+} can enhance the catalytic activity of MnP, while Mn^{2+} may be a substrate for laccase [20]; low concentration of ABTS may improve the activity of laccase while high concentration may lead to inhibition of lignin degradation [22]. Further studying the mechanism of mediator-involved enzymatic degradation of lignin is extremely significant to guide the practical application of ligninolytic enzymes.

In this study, on the one hand, the mediator-involved enzymatic degradation of lignin during SSF was studied. The optimum dosage of ligninolytic enzymes and low molecular active mediators were investigated to promote lignin degradation. Loss of total organic matter, lignin degradation components and dynamic changes of enzyme activities were measured to study the degradation effect. On the other hand, a nano-gold tracing technique is also developed to investigate the adsorption and transmission of laccase at the optimum dosage of ligninolytic enzymes and mediators via environmental scanning electron microscopy. Additionally, it could reveal the mechanism of enzymatic degradation of natural lignin.

2. Materials and methods

2.1. Overview of methodology

In order to achieve the objectives, a two-part series of experiments were designed to investigate effects of the ligninolytic enzymes and mediators in the process of enzymatic hydrolysis of lignin during SSF. Part I focused on the lignin degradation of straw in the enzymatic hydrolysis system, with the purpose of choosing the optimum dosage of ligninolytic enzymes and low molecular active mediators. Part II focused on the adsorption and transmission of laccase in the process of lignin degradation.

2.2. Material

2.2.1. Rice straw

The rice straw was harvested at the suburban areas of Changsha, China, planted by Hunan Academy of Agricultural Sciences, and a combine harvester was used to harvest the academician Mr. Longping Yuan's hybrid rice. The straw of hybrid rice was air-dried and ground to pass through 2 mm nylon screen. All the straw stalk powder was autoclaved twice for 20 min at 120 °C.

2.2.2. Enzymes

Lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) from *P. chrysosporium* were purchased from Sigma-Aldrich for use in the process of lignin degradation. Laccase (benzendiol: oxygen oxidoreductase, EC 1.10.3.2) from *Trametes versicolor* was also purchased from Sigma-Aldrich for lignin degradation. Lignin peroxidase and manganese peroxidase were dissolved in ultrapure water (18.2 MΩ). Laccase was dissolved in citrate-phosphate buffer (pH 4.8, 0.1 M). The liquid laccase, lignin peroxidase and manganese peroxidase were separated into eppendorf tubes which were stored at −20 °C.

2.2.3. Mediators

Veratryl alcohol, ABTS and Mn^{2+} were selected as mediators in a series of experiments. Veratryl alcohol and ABTS were purchased from Sigma-Aldrich. The other chemicals including Mn^{2+} ($MnSO_4$) were of analytical reagent grade.

2.3. Preparation of nano-gold-laccase conjugates

Nano-gold particles (AuNPs) were prepared by sodium citrate reduction of $HAuCl_4$ according to the reported procedure with minor modification [29–31]. The analysis of experimental results showed that sodium borohydride deoxidization of $HAuCl_4$ produced 3–8 nm AuNPs while sodium citrate reduction of $HAuCl_4$ produced AuNPs with 10–50 nm grain size. For small size AuNPs had larger specific surface area and higher activity and its size and mass had little effect on the adsorption and transmission of laccase, the sodium borohydride deoxidization of $HAuCl_4$ was selected to produce AuNPs. The optimal concentration of laccase and the optimal pH of nano-gold-laccase conjugates had been determined in the previous experiment [16]. With gently stirring, 4.5 mL of laccase (1.0 mg mL^{-1}) dissolved in citrate-phosphate buffer (pH 4.8, 0.1 M) was added dropwise to 20 mL AuNPs solution (pH 4.8, 0.1 M). The reaction mixture was further reacted for 1 h. After low speed centrifugation (1200 rpm for 20 min) for removing large particles and high-speed centrifugation (15,000 rpm for 1 h) for getting the precipitation of nano-gold-laccase conjugates, the precipitation was re-dissolving in 20 ml citrate-phosphate buffer containing PEG-20000 (pH 4.8, 0.2 mg mL^{-1}) to enhance the stability of nano-gold-laccase conjugates. AuNPs and nano-gold-laccase conjugates were characterized by UV–vis absorption spectrophotometer (Shimadzu UV-2550), zetasizer (Zetasizer nano Zs, Malvern, UK), and transmission electron microscope (TEM), JSM-JEOL 3010.

2.4. Solid-state fermentation conditions

Solid-state fermentation was carried out in 1 L flasks containing 40 g straw stalk powder. An orthogonal experiment was designed to study the mediator-involved enzymatic degradation of lignin (Table 1). Each flask was stoppered and autoclaved twice for 20 min at 120 °C. Then nano-gold-laccase conjugates, LiP, MnP and mediators were evenly added under sterile condition, and 0.12 mM g^{-1} hydrogen peroxide was homogeneously added as initiation factor. The solid-state fermentation was carried out steadily in a constant temperature-humidity machine and the moisture content

Table 1
The various added composition in the orthogonal experiment L9 (3^3).

Number	Lac-Au (U g^{-1})	LiP (U g^{-1})	MnP (U g^{-1})	Mn^{2+} (μM g^{-1})	ABTS (μM g^{-1})	Veratryl alcohol (μM g^{-1})	H_2O_2 (mM g^{-1})
1	6.4	3.6	5.6	0	0	0	0.12
2	6.4	3.6	5.6	0	0.2	2	0.12
3	6.4	3.6	5.6	0	0.4	4	0.12
4	6.4	3.6	5.6	20	0	2	0.12
5	6.4	3.6	5.6	20	0.2	4	0.12
6	6.4	3.6	5.6	20	0.4	0	0.12
7	6.4	3.6	5.6	40	0	4	0.12
8	6.4	3.6	5.6	40	0.2	0	0.12
9	6.4	3.6	5.6	40	0.4	2	0.12

of straw substrate was maintained at 75% in the whole fermentation process, via supplementing appropriate amount of sterile distilled water or terminating the humidifying function in the constant temperature-humidity machine. To avoid the effects of taking sample from fermentation substrate, control flasks were prepared under the same conditions, and fermentation process was performed at 37 °C for 50 days. All experiments were performed in triplicates.

2.5. Sampling and analytical methods

During the process of SSF, 1 g of rice straw sample was taken from different flasks periodically to analyze the organic matter (day 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50) and lignin degradation ratio (day 0, 10, 20, 30, 40, 50). In order to study the activity of ligninolytic enzymes, 0.5 g of rice straw sample was taken from different flasks periodically (day 1, 3, 5, 10, 15, 20, 30, 40, 50). To investigate the adsorption and transmission of laccase, straw sample was taken from different flasks periodically (day 1, 3, 5, 10, 30, 50) to track AuNPs by environmental scanning electron microscope.

2.5.1. Moisture content and total organic matter

Moisture content and total organic matter are important factors to evaluate SSF process [32]. They were determined via a loss-on-drying and ignition procedure. Triplicate 1 g samples were dried at 105 °C for 6 h to constant weight and then transferred to a muffle furnace and held at 550 °C ± 10 °C for 6 h. Moisture content was calculated from the ratio of pre- to post-drying sample weights and total organic matter from that of pre- to post-ignition sample weights.

2.5.2. Enzyme activity assay

During the process of fermentation, fresh samples taken under sterile conditions were extracted with deionized water at a ratio of 1:10 (w/v). Each extraction was performed under rotary shaking (150 rpm) for 1 h. Then the homogenate was centrifuged at 4 °C for 20 min, and the supernatant was filtered through filter papers. The filtrate was analyzed for activities of enzymes. Laccase activity was measured according to the previously described method with a UV–vis spectrophotometer [33]. One unit of laccase activity was defined as the amount of enzyme releasing 1 μmol of product per min. Lignin peroxidase (LiP) was measured with a UV–vis spectrophotometer [34]. One unit of LiP activity was defined as the amount of the enzyme required to produce 1 μmol veratryl aldehyde from the oxidation of veratryl alcohol per min. Manganese peroxidase (MnP) was analyzed by monitoring the change in absorbance of reaction mixture at 290 nm [35]. MnP unit activity was defined as the amount of enzyme required for the production of 1 μmol Mn^{3+} per min.

2.5.3. Lignin degradation analysis

To study the lignin degradation of ligninolytic enzymes and mediators, the contents of lignin were analyzed. Acid detergent lignin (ADL) was determined according to the procedures outlined [36]. Lignin was estimated as the difference between ADL and ash content [6]. Lignin degradation ratio was calculated by the following formula:

$$D = \frac{m_0 - m_{50}}{m_0} \times 100\% \quad (1)$$

where m_0 and m_{50} represent the total amount of lignin in untreated straw and that in fermentation substrate at day 50, respectively.

2.5.4. Environmental scanning electron microscopy

In order to investigate the adsorption and transmission of laccase in the process of solid-state fermentation, environmental scanning electron microscope (ESEM) (FEI QUANTA 200) equipped with an energy dispersive X-ray spectrometer (EDS) (EDAX genesis xm-2), was used operated at an accelerating voltage of 10 kV. By observing the amount of AuNPs on the surface of cell wall (1, 3, 5, 10, 30, 50 days), the adsorption and transmission of laccase in the process of solid-state fermentation could be directly observed. The results were reproducible, and high quality images were selected to illustrate different period in the fermentation.

3. Results and discussion

The effects of the ligninolytic enzymes and mediators in enzymatic hydrolysis of lignin during SSF were studied. Detailed changes of straw were measured, and enzyme activity assays were determined to give evidence of lignin degradation. ESEM of nano-gold-laccase conjugates on the surface of lignin was used to support the enzyme activity assays, and it was also used to observe the adsorption and transmission of laccase.

3.1. Selection of the optimum dosage of ligninolytic enzymes and mediators

It's difficult to confirm the effect of a single ligninolytic enzyme in the process of lignin degradation, for the reason that white rot fungi can secrete different ligninolytic enzymes. The degradation effect of single laccase or single MnP is poor; nevertheless, laccase and MnP act synergistically in the degradation of lignin. The proportion of different ligninolytic enzymes directly influences the degradation of lignin, because the synergistic effect of ligninolytic enzymes may exist plus or minus mechanisms [37]. It was found that the cooperation of laccase, LiP and MnP can effectively promote lignin degradation in our previous experiments [6]. However, adding more laccase has no significance in accelerating the degradation of lignin, when the degradation of lignin reached a certain level. Furthermore, if an overabundance of laccase was used for preparing nano-gold laccase conjugates, it would lead to gold particles clumped together and unfavorably characterize the amount of laccase on the surface of straw fibers via ESEM. In our previous experiments about enzymatic hydrolysis of lignin, it was found that by using a combined ligninolytic enzyme system containing 3.6 U g^{-1} of LiP, 5.6 U g^{-1} of MnP and 6.4 U g^{-1} of laccase, the

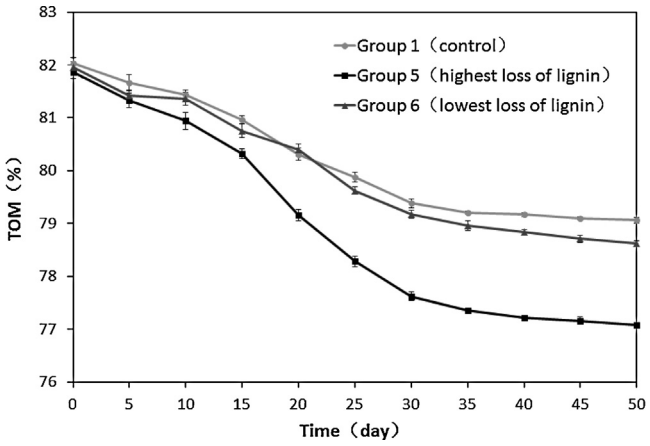

Fig. 1. Changes of total organic matter in mediator-involved enzymatic hydrolysis of lignin during SSF in orthogonal experiment group 1 (control), 5 (highest loss of lignin), 6 (lowest loss of lignin). The bars represent the standard deviations of the means ($n = 3$).

degradation ratio of lignin reached 25.8%. In addition, the optimal mixed ratio was imitated the ratio of LiP and MnP produced by *P. chrysosporium*, while the proportion of added MnP and laccase was according to *Trametes trogii*. The results provided reference for lucubrating of lignin degradation with ligninolytic enzymes.

As seen from Table 2, the lignin degradation effect of group 1 (mediator-free) was significantly lower than other groups after 50 days SSF. Meanwhile the lignin degradation effect of groups without mediators was also poorer. The experimental results confirmed that the low molecular active mediators were involved in lignin degradation. It could be concluded from the results of range analysis that the effects of the three mediators on the degradation of lignin were in descending order: veratryl alcohol > ABTS > Mn^{2+}, the optimum dosages of three mediators were: Mn^{2+} (20 $\mu M\, g^{-1}$), ABTS (0.2 $\mu M\, g^{-1}$), veratryl alcohol (4 $\mu M\, g^{-1}$), respectively. The mediation activity of veratryl alcohol has been attributed to its ability to protect the enzyme from H_2O_2-dependent inactivation. As reported, veratryl alcohol was able to stimulate the oxidation of anisyl alcohol which reacts with compound I of lignin peroxidase, but not with compound II. As a consequence, veratryl alcohol, reacting with compound II, is essential for completion of the catalytic cycle [20]. The ability of LiP to degrade lignin is highly competent than MnP and laccase, and LiP will lead to C—C bound rupture and aromatic ring open [38]. It could explain that veratryl alcohol is the biggest influencing factor of lignin degradation.

3.2. Loss of total organic matter (TOM) during SSF

The TOM content of group 5 (highest loss of lignin), group 6 (lowest loss of lignin) and group 1 (control) is shown in Fig. 1. It could be found that the TOM levels of all the 3 sets of experiments were about 82% at the beginning of SSF and the decreased tendency of TOM was consistent under the different conditions. In the early stage of SSF (first 15 days), the TOM of the 3 sets of experiments decreased at a slower rate, and subsequently speeded up the downtrend until 30 days. Moreover, after 30 days of SSF, the loss of TOM began to stabilize. After 50 days of SSF, most noticeably loss of TOM was found in group 5, and its TOM levels reached about 77%, while the group 8 and control group had higher TOM levels about 79%. From experimental results, it was obvious that in the initial stages matrix structure had not been destroyed and the ligninolytic enzymes were difficult to penetrate inside the matrix. The low molecular active mediators could form the free radicals oxidizing agent to damage the matrix structure, thus opening the channel for the ligninolytic enzymes to enter into the matrix.

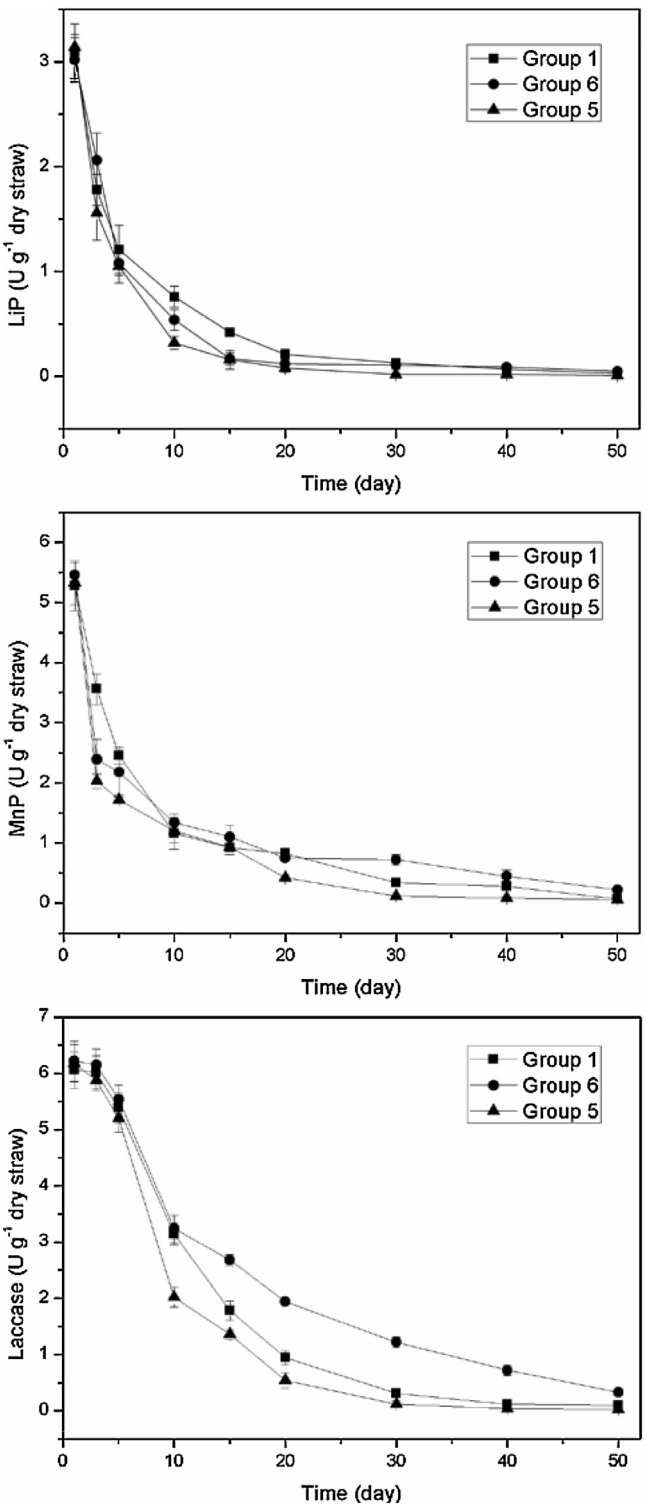

Fig. 2. Enzyme activities of LiP, MnP and laccase in mediator-involved enzymatic hydrolysis of lignin during SSF in orthogonal experiment group 1 (control), 5 (highest loss of lignin), 6 (lowest loss of lignin). The bars represent the standard deviations of the means ($n = 3$).

In this experiment, the change of TOM was mainly caused by the degradation of lignin, so the loss trend of total organic matter could reflect the corresponding degree of lignin degradation. Huang [6] studied the changes of microbial population structure related to lignin degradation during lignocellulosic waste composting and the highest lignin degradation ratio was 26%. Moilanen [39]

Table 2
Results of orthogonal experiment L9 (3^3).

Number	Mn^{2+} ($\mu M\,g^{-1}$)	ABTS ($\mu M\,g^{-1}$)	Veratryl alcohol ($\mu M\,g^{-1}$)	Lignin degradation ratio (%)
1	0	0	0	23.4
2	0	0.2	2	26.6
3	0	0.4	4	27.5
4	20	0	2	27.1
5	20	0.2	4	31.8
6	20	0.4	0	25.2
7	40	0	4	25.7
8	40	0.2	0	26.9
9	40	0.4	2	28.2
K1	25.833	25.400	25.167	
K2	28.033	28.433	27.300	
K3	26.933	26.967	28.333	
R	2.200	3.033	3.166	

studied the laccase-catalyzed modification of lignin for enzymatic hydrolysis. Laccase modification improved the hydrolysis yield of spruce by 12% and reducing the hydrolysis yield by 17% on giant reed. Compared with existing studies, a significantly high degradation ratio of lignin (31.8%) in group 5 was achieved at the optimum dosage of ligninolytic enzymes and mediators. It proved that the mediator-involved ligninolytic enzymes system could effectively promote lignin degradation.

3.3. Dynamic changes of enzyme activities

The changes of laccase, LiP and MnP activities in mediator-involved enzymatic hydrolysis of lignin during SSF were presented in Fig. 2. In the initial phase of SSF, the enzyme activities of laccase, LiP and MnP could reach about $6.2\,U\,g^{-1}$, $3.3\,U\,g^{-1}$ and $5.4\,U\,g^{-1}$ dry straw, respectively. After 50 days fermentation, the activity of three enzymes dropped to extremely low level. From 1 to 5 d of SSF, laccase remained higher activity. However, LiP and MnP had different situations, which gradually declined within 5 days of SSF. It could be hypothesized that the rapid drop enzyme activity of LiP and MnP was due to the addition of hydrogen peroxide, which was added as a promoter of enzymatic reaction. Overall, the downward trend of the activity of laccase was obviously gentler than those of LiP and MnP, and this was why laccase was selected to prepare nano-gold-laccase conjugates.

The activity of laccase in group 5 had a rapid decline from 5 to 10 d, and the enzyme activity of laccase was about $2.1\,U\,g^{-1}$ at the 10th day in fermentation. It turned out that there was a part of laccase entering into straw fibers. In comparison, the enzyme activity of laccase in group 6 maintained a certain amount of activity about 0.5 to $1.5\,U\,g^{-1}$ in the end of fermentation. Perhaps it indicated a poor effect of the degradation, due to a certain amount of laccase staying on the surface of straw fibers. Moreover, in terms of the added content of mediators, it was found that, to some degree, the addition of ABTS influenced the enzyme activity of laccase and the degradation of lignin. In addition, it was found that a small amount of ABTS might promote the degradation by laccase, while a large amount of ABTS would restrain the activity of laccase in the process of degradation in SSF. For the activity of LiP, the dropping rates were different from 3 to 20 days. It is probably due to the added veratryl alcohol, which is one kind reaction substrate of LiP, just like ABTS is one reaction substrate of laccase. For group 1 without veratryl alcohol, the activity of LiP was higher than that in group 5 which was added with $4\,\mu M\,g^{-1}$ veratryl alcohol. It might be because veratryl alcohol has protective effects and dispersive effects. There are three supposes about the effect of veratryl alcohol, one is that it acts as redox mediator of cation radicals for the long distance substrate, while the second one is that veratryl alcohol protects LiP from hydrogen peroxide which can make it lose its activity, and the third one is to assist LiP to accomplish catalytic cycle. Group 5 with $20\,\mu M\,g^{-1}$ of Mn^{2+} had lower enzyme activity of MnP compared with group 1 and group 6. And it also explained that there were more MnP entering into straw fibers in group 5. Analyzing from the addition of mediators, it can be concluded that too many Mn^{2+} restrain the activity of MnP, while a little amount of Mn^{2+} can stimulate the activity of MnP.

Insights can be gained by the analysis of ligninolytic enzymes activity of group 1 (control), group 5 (best degradation of lignin) and group 6 (worst degradation of lignin). It was confirmed that ligninolytic enzymes were able to promote the degradation of lignin. Nevertheless, the mechanism of enzymatic degradation of lignin, the adsorption and transmission of ligninolytic enzymes need further studies. Therefore, nano-gold-laccase conjugates were used to study the whereabouts of laccase, and go a further step to explore the mechanism of enzymatic degradation of lignin.

3.4. Adsorption and transmission of laccase

In order to further understand the enzymatic degradation mechanism of lignin and the synergy between ligninolytic enzymes and low molecular active mediators, laccase was selected to get deep insight into the adsorption and transmission. Lai [16] described the effect of ABTS on the adsorption of *T. versicolor* laccase on alkali lignin and found that ABTS improved the affinity of laccase for lignin in the adsorption procedure. Daniel [40] revealed new details for understanding white-rot decay of lignocellulose by using Cryo-FE-SEM and TEM immune-techniques. However, these instrumentally intensive methods require expensive equipment and costly sample preparations. Thus, a simple and inexpensive method for investigating the adsorption and transmission of laccase in mediator-involved enzymatic degradation of lignin is necessary. Nano-gold tracing technique was developed to obtain the micro positioning of laccase in this study. For the property that nano-gold is easy to be detected unambiguously, especially on the micrographs of back scattered electron mode [41]. Moreover, this characteristic makes it possible that the nano-gold-laccase conjugates can be distinguished from lignin in micrographs, which can be detected as bright spots. The particle size of obtained nano-gold-laccase conjugates was 20 ± 2.8 nm and the polydispersity index (PDI) was 0.193 ± 0.036, which indicated that nanoparticles were well dispersed. The UV–vis absorbance spectrum of nano-gold was shown in Fig. 3. Nano-gold displayed an intense surface plasmon resonance (SPR) peak at 520 nm. However, the UV–vis SPR peak of nano-gold-laccase conjugates shifted from 520 nm to 527 nm. This shift may be attributed to the presence of laccase, which may have altered the nano-gold surface dielectric properties [18]. The inset in

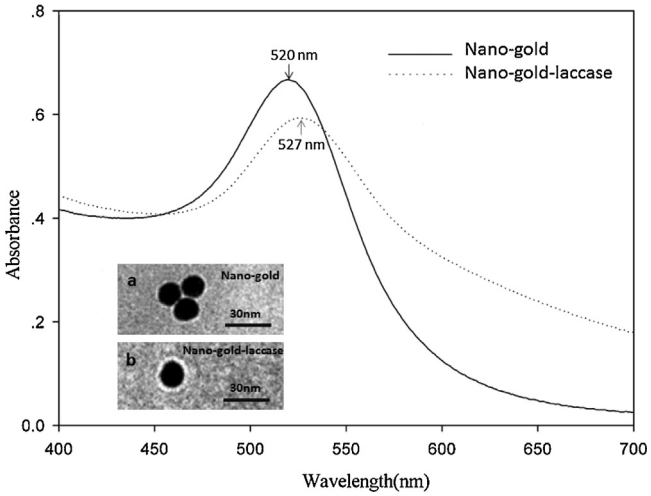

Fig. 3. The UV–vis absorption spectrum of nano-gold (solid line) and nano-gold-laccase conjugates (dash line). The inset is the transmission electron microscope pattern of the nano-gold-laccase nanoparticles.

Fig. 3b shows the TEM image of the nano-gold-laccase conjugates. A thin, bright halo edge (due to the weak electronic absorption of laccase) around the nano-gold (Fig. 3a) was clearly observed compared to bare nano-gold, which demonstrated that the laccase was linked on surface of nano-gold. The interaction between nano-gold and laccase is electrostatic adsorption. Negatively charged nano-gold can firmly combine with positively charged enzyme protein. Furthermore, the interactions between nano-gold and enzyme protein include van der Waals force, hydrophobic interaction, etc.

The adsorption and transmission of laccase in the process of lignin degradation were studied. The SEM micrographs of group 5 (best degradation of lignin) showed that during the initial period of SSF the surface of lignin had not been destroyed (Fig. 4a′ and b′). Along with the fermentation, the surface of straw broke down gradually, and the tiny particle obviously increased. After 30 days of SSF, it could be observed that there were some holes, even small partly collapse and large amounts of tiny particle on the surface of straw, which indicated a certain degree of surface broken (Fig. 4e′). In the final stage, the skeleton of straw fibers could be observed directly on the SEM micrographs (Fig. 4f′). In the process of enzymatic hydrolysis of lignin, the lignin was changed in both appearance

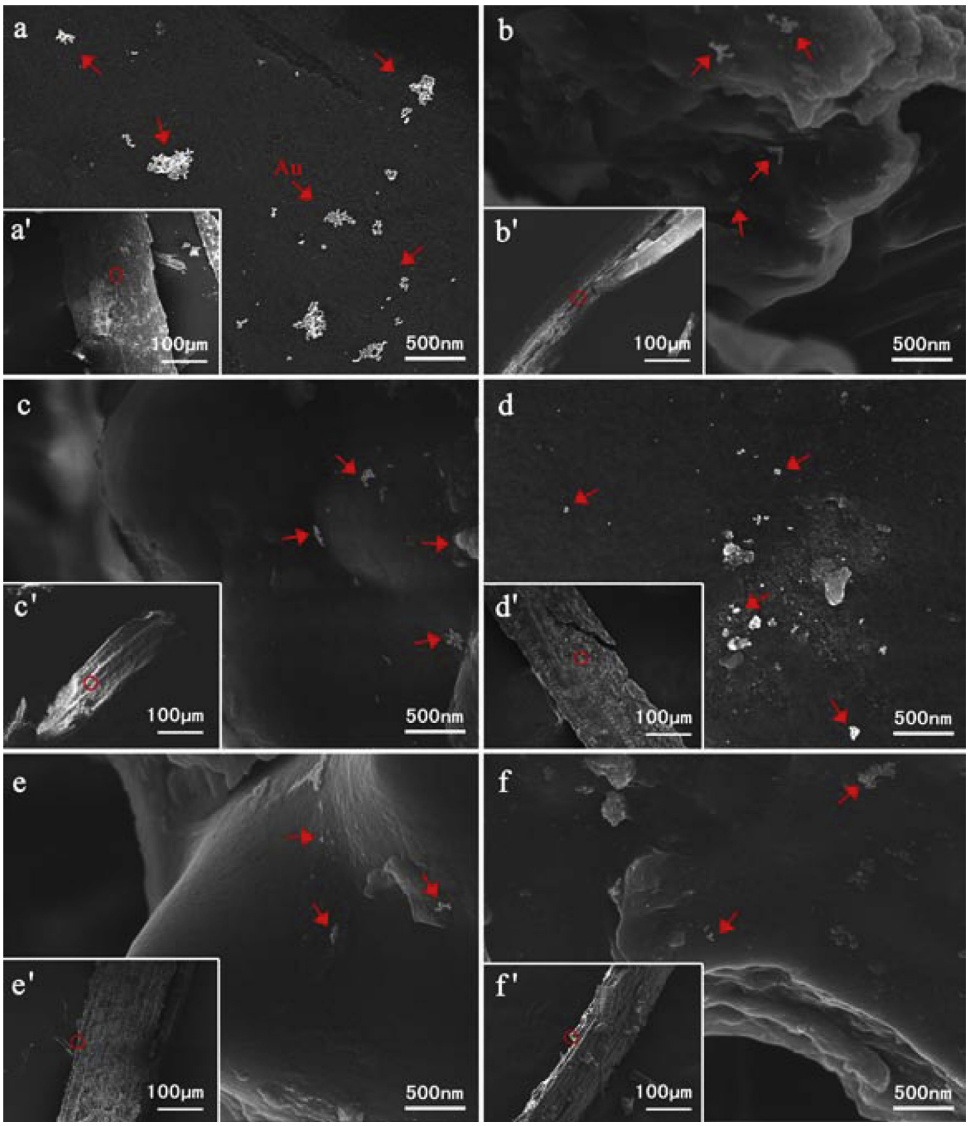

Fig. 4. Scanning electron microscope images of straw surface topography on day 1 (a′), on day 3 (b′), on day 5 (c′), on day 10 (d′), on day 30 (e′) and on day 50 (f′). Scanning electron microscope images of nano-gold-laccase conjugates on surface of straw on day 1 (a), on day 3 (b), on day 5 (c) on day 10 (d), on day 30 (e) and on day 50 (f).

and internal structure, and it inosculated with the degradation of lignin. In addition, it demonstrated that ligninolytic enzymes cooperated with mediators had an effective degradation of lignin. The distribution of nano-gold in the initial stage showed that there was large amount of laccase gathered into a mass on the whole surface of straw fibers (Fig. 4a and b). At 10 day of SSF, an obvious decrease of nano-gold was found (Fig. 4d), which indicated that the amount of laccase went down accordingly. From this result, it can be conjectured that large amount of nano-gold-laccase conjugates entered into the inside of straw fibers. On day 30 (Fig. 4e) and day 50 (Fig. 4f), a small quantity of nano-gold could be viewed, which indicated that there was some laccase residues on the surface of straw fibers. The change trend of the amount of nano-gold was consistent with the variation of enzyme activity of laccase on the surface of straw fibers. In conclusion, eliminating the inactivation of ligninolytic enzymes, the decrease of laccase absorbed on the surface of straw fibers may be due to the gradually transmitting into the matrix. And it provided reliable information that low molecular active mediators would open the matrix channel for the entrance of ligninolytic enzymes.

4. Conclusions

The influence of ligninolytic enzymes combined with mediators on lignin degradation and structure variation was investigated. The effects of three common mediators on the degradation of lignin were in descending order: veratryl alcohol > ABTS > Mn^{2+} and a significantly high degradation ratio of lignin (31.8%) was achieved at the optimum dosage of ligninolytic enzymes and mediators. Moreover, an obvious decrease of nano-gold-laccase conjugates was found after 10 days of SSF and there were few laccase residues on the surface of straw fibers after 30 days of SSF. The present findings could be used as references to promote the treatment of lignocellulosic waste and understand the adsorption and transmission of ligninolytic enzymes.

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References

[1] Y.Q. Lin, D.H. Wang, T.T. Wang, Ethanol production from pulp & paper sludge and monosodium glutamate waste liquor by simultaneous saccharification and fermentation in batch condition, Chem. Eng. J. 191 (2012) 31–37.
[2] J. Perez, J. Munoz-Dorado, T. Rubia de la, J. Martinez, Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview, Int. Microbiol. 5 (2002) 53–63.
[3] D.L. Huang, G.M. Zeng, C.L. Feng, S. Hu, X.Y. Jiang, L. Tang, F.F. Su, Y. Zhang, W. Zeng, H.L. Liu, Degradation of lead-contaminated lignocellulosic waste by *Phanerochaete chrysosporium* and the reduction of lead toxicity, Environ. Sci. Technol. 42 (2008) 4946–4951.
[4] C.L. Feng, G.M. Zeng, D.L. Huang, S. Hu, M.H. Zhao, C. Lai, C. Huang, Z. Wei, N.J. Li, Effect of ligninolytic enzymes on lignin degradation and carbon utilization during lignocellulosic waste composting, Process Biochem. 46 (2011) 1515–1520.
[5] D.L. Huang, G.M. Zeng, X.Y. Jiang, C.L. Feng, H.Y. Yu, G.H. Huang, H.L. Liu, Bioremediation of Pb-contaminated soil by incubating with *Phanerochaete chrysosporium* and straw, J. Hazard. Mater. 134 (2006) 268–276.
[6] D.L. Huang, G.M. Zeng, C.L. Feng, S. Hu, C. Lai, M.H. Zhao, F.F. Su, L. Tang, H.L. Liu, Changes of microbial population structure related to lignin degradation during lignocellulosic waste composting, Bioresour. Technol. 101 (2010) 4062–4067.
[7] G.J. Hua, J.B. Li, G.M. Zeng, Recent development in the treatment of oily sludge from petroleumindustry: a review, J. Hazard. Mater. 261 (2013) 470–490.
[8] A. Hatakka, Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation, FEMS Microbiol. Rev. 13 (1994) 125–135.
[9] M.H. Zhao, Z.T. Zeng, G.M. Zeng, D.L. Huang, C.L. Feng, C. Lai, C. Huang, Z. Wei, N.J. Li, P. Xu, C. Zhang, Z.F. Liu, G.X. Xie, Effects of ratio of manganese peroxidase to lignin peroxidase on transfer of ligninolytic enzymes in different composting substrates, Biochem. Eng. J. 67 (2012) 132–139.
[10] H. Claus, Laccases: structure, reactions, distribution, Micron 35 (2004) 93–96.
[11] P. Widsten, A. Kandelbauer, Laccase applications in the forest products industry: a review, Enzyme Microb. Technol. 42 (2008) 293–307.
[12] M. Fernández-Fernández, M.Á. Sanromán, D. Moldes, Recent developments and applications of immobilized laccase, Biotechnol. Adv. 31 (2013) 1808–1825.
[13] G.M. Zeng, M. Chen, Z.T. Zeng, Risks of neonicotinoid pesticides, Science 340 (2013) 1403.
[14] G.M. Zeng, M. Chen, Z.T. Zeng, Shale gas: surface water also at risk, Nature 499 (2013) 154.
[15] S. Heiss-Blanquet, D. Zheng, N.L. Ferreira, C. Lapierre, S. Baumberger, Effect of pretreatment and enzymatic hydrolysis of wheat straw on cell wall composition hydrophobicity and cellulase adsorption, Bioresour. Technol. 102 (2011) 5938–5946.
[16] C. Lai, G.M. Zeng, D.L. Huang, M.H. Zhao, H.L. Huang, C. Huang, Z. Wei, N.J. Li, P. Xu, C. Zhang, Effect of ABTS on the adsorption of *Trametes versicolor* laccase on alkali lignin, Int. Biodeterior. Biodegrad. 82 (2013) 180–186.
[17] P.K. Jain, K.S. Lee, I.H. El-Sayed, M.A. El-Sayed, Calculated absorption and scattering properties of gold nanoparticles of different size, shape, and composition: applications in biological imaging and biomedicine, J. Phys. Chem. B 110 (2006) 7238–7248.
[18] C. Lai, G.M. Zeng, D.L. Huang, C.L. Feng, S. Hu, F.F. Su, M.H. Zhao, C. Huang, Z. Wei, Detection based on immunogold labeling technique and its expected application in composting, Chin. J. Anal. Chem. 38 (2010) 909–914.
[19] B. Ismail, S.T. Hussain, S. Akram, Adsorption of methylene blue onto spinel magnesium aluminate nanoparticles: Adsorption isotherms, kinetic and thermodynamic studies, Chem. Eng. J. 219 (2013) 395–402.
[20] C. Höfer, D. Schlosser, Novel enzymatic oxidation of Mn^{2+} to Mn^{3+} catalyzed by a fungal laccase, FEBS Lett. 451 (1999) 186–190.
[21] P.J. Harvey, H.E. Schoemaker, J.M. Palmer, Veratryl alcohol as a mediator and the role of radical cations in lignin biodegradation by *Phanerochaete chrysosporium*, FEBS Lett. 195 (1986) 242–246.
[22] S. Kurniawati, J.A. Nicell, Efficacy of mediators for enhancing the laccase-catalyzed oxidation of aqueous phenol, Enzyme Microb. Technol. 41 (2007) 353–361.
[23] J.L. Gong, B. Wang, G.M. Zeng, C.P. Yang, C.G. Niu, Q.Y. Niu, W.J. Zhou, Y. Liang, Removal of cationic dyes from aqueous solution using magnetic multi-wall carbon nanotube nanocomposite as adsorbent, J. Hazard. Mater. 164 (2009) 1517–1522.
[24] M. Cheng, G.M. Zeng, D.L. Huang, L. Liu, M.H. Zhao, C. Lai, C. Huang, Z. Wei, N.J. Li, P. Xu, C. Zhang, F.L. Li, Y. Leng, Effect of Pb^{2+} on the production of hydroxyl radical during solid-state fermentation of straw with *Phanerochaete chrysosporium*, Biochem. Eng. J. 84 (2014) 9–15.
[25] P. Xu, G.M. Zeng, D.L. Huang, C.L. Feng, S. Hu, M.H. Zhao, C. Lai, Z. Wei, C. Huang, G.X. Xie, Z.F. Liu, Use of iron oxide nanomaterials in wastewater treatment: a review, Sci. Total Environ. 424 (2012) 1–10.
[26] W.W. Tang, G.M. Zeng, J.L. Gong, J. Liang, P. Xu, C. Zhang, B.B. Huang, Removal of heavy metals from aqueous solutions using nanomaterials affected by humic/fulvic acid: a review, Sci. Total Environ. 468–469 (2014) 1014–1027.
[27] N.J. Li, G.M. Zeng, D.L. Huang, S. Hu, C.L. Feng, M.H. Zhao, C. Lai, C. Huang, Z. Wei, G.X. Xie, Oxalate production at different initial Pb^{2+} concentrations and the influence of oxalate during solid-state fermentation of straw with *Phanerochaete chrysosporium*, Bioresour. Technol. 102 (2011) 8137–8142.
[28] M. Shimada, F. Nakatsubo, T.K. Kirk, T. Higuchi, Biosynthesis of the secondary metabolite veratryl alcohol in relation to lignin degradation in *Phanerochaete chrysosporium*, Arch. Microbiol. 129 (1981) 321–324.
[29] S.K. Ghosh, Spectroscopic evaluation of 4-(dimethylamino)pyridine versus citrate as stabilizing ligand for gold nanoparticles, Colloids Surf., A: Physicochem. Eng. Aspects 371 (2010) 98–103.
[30] F. Cai, J. Li, J.S. Sun, Y.L. Ji, Biosynthesis of gold nanoparticles by biosorption using *Magnetospirillum gryphiswaldense* MSR-1, Chem. Eng. J. 175 (2011) 70–75.
[31] S. Vial, I. Pastoriza-Santos, J. Pérez-Juste, L.M. Liz-Marzán, Plasmon coupling in layer-by-layer assembled gold nanorod films, Langmuir 23 (2007) 4606–4611.
[32] R.R. Singhania, A.K. Patel, C.R. Soccol, A. Pandey, Recent advances in solid-state fermentation, Biochem. Eng. J. 44 (2009) 13–18.
[33] J.X. Liu, W.J. Zhou, J.L. Gong, L. Tang, Y. Zhang, H.Y. Yu, B. Wang, X.M. Xu, G.M. Zeng, An electrochemical sensor for detection of laccase activities from *Penicillium simplicissimum* in compost based on carbon nanotubes modified glassy carbon electrode, Bioresour. Technol. 99 (2008) 8748–8751.
[34] M. Tien, T.K. Kirk, Lignin peroxidase of *Phanerochaete chrysosporium*, Methods Enzymol. 161 (1988) 238–249.
[35] H. Wariishi, K. Valli, M.H. Gold, Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: kinetic mechanism and role of chelators, J. Biol. Chem. 267 (1992) 23688–23695.
[36] P.J. Van Soest, J.B. Robertson, B.A. Lewis, Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition, J. Dairy Sci. 74 (1991) 3583–3597.
[37] C.A. Reddy, An overview of the recent advances on the physiology and molecular biology of lignin peroxidases of *Phanerochaete chrysosporium*, J. Biotechnol. 30 (1993) 91–107.

[38] G.M. Zeng, D.L. Huang, G.H. Huang, T.J. Hu, X.Y. Jiang, C.L. Feng, Y.N. Chen, L. Tang, H.L. Liu, Composting of lead-contaminated solid waste with inocula of white-rot fungus, Bioresour. Technol. 98 (2007) 320–326.
[39] U. Moilanen, M. Kellock, S. Galkin, L. Viikari, The laccase-catalyzed modification of lignin for enzymatic hydrolysis, Enzyme Microb. Technol. 49 (2011) 492–498.
[40] G. Daniel, J. Volc, M.L. Niku-Paavola, Cryo-FE-SEM & TEM immuno-techniques reveal new details for understanding white-rot decay of lignocellulos, C.R. Biol. 327 (2004) 861–871.
[41] V. Horneffer, R. Reichelt, K. Strupat, Protein incorporation into MALDI-matrix crystals investigated by high resolution field emission scanning electron microscopy, Int. J. Mass Spectrom. 226 (2003) 117–131.