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Effect of Pb^{2+} on the production of hydroxyl radical during solid-state fermentation of straw with *Phanerochaete chrysosporium*

Min Cheng[a,b], Guangming Zeng[a,b,*], Danlian Huang[a,b,*], Liang Liu[a,b], Meihua Zhao[a,b], Cui Lai[a,b], Chao Huang[a,b], Zhen Wei[a,b], Ningjie Li[a,b], Piao Xu[a,b], Chen Zhang[a,b], Fangling Li[a,b], Yang Leng[a,b]

[a] College of Environmental Science and Engineering, Hunan University, Changsha, Hunan 410082, China
[b] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha, Hunan 410082, China

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ABSTRACT

Hydroxyl radical (•OH) is a radical species highly destructive for lignin during solid-state fermentation (SSF) of straw with *Phanerochaete chrysosporium* (*Pc*). The production of •OH at different initial Pb^{2+} concentrations during SSF of straw with Pc was investigated. The results showed that a modest amount (under 200 mg kg^{-1}) of Pb^{2+} could enhance the production of •OH, while a higher Pb^{2+} concentration resulted in inhibition. The content of •OH reached the peak value at day 12 in the whole tested samples, and the maximal content of •OH was obtained at initial Pb^{2+} concentration of 100 mg kg^{-1}. It was also found that the production of •OH was connected to enzymatic activity and oxalate content in some degree, in particular, a significant positive correlation was found between oxalate concentration and production of •OH.

We found that low concentration of Pb^{2+} can promote the degradation of lignin, and the higher initial Pb^{2+} concentration (400 mg kg^{-1}) resulted in inhibition. In addition, it appeared that there was no significant correlation between lignin degradation rate and the production of •OH when Pb^{2+} concentration was taken into account.

1. Introduction

Rice straw, as one of the most important lignocellulosic biomasses available and a major agricultural by-product, has been widely used as raw materials to produce value-added products by solid-state fermentation (SSF) technique [1]. However, rice straw contains a good amount of lignin, which makes it very hard to be utilized effectively [2]. Lignin is the most abundant organic material on earth after cellulose, whose main function is to cement the cellulose fibers in plants [3]. It was reported that lignin is a three-dimensional phenylpropanoid polymer mainly linked by arylglycerol ether bonds between monomeric phenolic units most of which are not readily hydrolyzable [4].

White-rot fungi are characterized by their unique ability to degrade lignin mainly due to the secretion of low-specificity phenol oxidase enzymes whose natural function is degrading lignin. In particular, the representative species *Phanerochaete chrysosporium* (*Pc*) has been most extensively studied and proved to be able to degrade a wide range of organic substrates [5]. However, during SSF process, ligninolytic enzymes are not able to diffuse into the intact cell walls because the enzymes are too large to penetrate the pores of the cell walls [6]. Hydroxyl radical (•OH) produced by ligninolytic enzymes, is a radical species highly destructive for cellulose and lignin [7], and is proposed as a principal low molecular mass oxidant that erodes wood cell walls to enhance the accessibility of the extracellular enzymes of white-rot fungi to wood cell wall components [8]. Besides, previous studies showed that the generation of •OH in cultures of white-rot fungi is proportional to the rate of lignin degradation [9,10], in contrast, ligninolytic enzymes activity in cultures is not necessarily correlated with the rate of lignin degradation [11].

Heavy metals pollution is one of the most important ecological problems on a world scale. Agricultural waste in the heavy metal-polluted area contains a good amount of heavy metals [12–15]. In recent years, more and more studies concerning heavy metals effects on biotechnological processes performed by white-rot fungi. Lead, as one of the most hazardous heavy metals

* Corresponding authors at: College of Environmental Science and Engineering, Hunan University, Changsha, Hunan 410082, China. Tel.: +86 731 88822754; fax: +86 731 88823701.
E-mail addresses: zgming@hnu.edu.cn (G. Zeng), huangdanlian@hnu.edu.cn (D. Huang).

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to white-rot fungi, has attracted particular attention. In previous studies [16–19], most attention has been paid to the Pb^{2+} toxicity toward extracellular enzymes, but rarely details on the Pb^{2+} toxicity toward the production of •OH have been reported in literature so far. This study will help us to understand how Pb^{2+} affect the production of •OH, which would provide useful information for the development of fungi-based technologies to improve the degradation of heavy metal-polluted lignocellulosic wastes.

In this study, we investigated the production of •OH and soluble-exchangeable Pb^{2+} content at different initial Pb^{2+} concentrations during SSF of straw with *Pc*. The production of two main ligninolytic enzymes including lignin peroxidase (LiP) and manganese peroxidase (MnP), oxalate, a low-molecular-weight compound which is included in lignin biodegradation and also plays an important role as a metal chelator, and the superoxide anion ($O_2^{\bullet -}$) were detected to analyze the probable mechanism of heavy metal interactions with the production of •OH. To determine whether the generation rates of •OH is proportional to lignin degradation rates, lignin degradation rates were also detected.

2. Materials and methods

2.1. Microorganism cultivation and inoculum preparation

The fungus *Pc* strain BKMF-1767 was obtained from China Center for type Culture Collection (Wuhan, China). Fungal cultures were maintained on potato dextrose agar (PDA) slants at 4 °C, and then transferred to PDA plates at 37 °C for several days. The spores on the agar surface were gently scraped and blended in the sterile distilled water as spore suspension. The spore concentration was measured and adjusted to 2.0×10^6 CFU per mL.

2.2. Solid-state fermentation conditions and sampling

The straw obtained from suburban areas of Yuelu District (Changsha, China) was air-dried and cut into small pieces about 5 mm. The concentration of total Pb in the straw was 2.1 mg kg^{-1}. SSF was carried out in 500 mL flasks containing 30 g of straw and 118 mL basic nutrient solution. Flasks were labeled as A (control), B (50), C (100), D (200) and E (400), respectively, was supplemented and mixed thoroughly with $Pb(NO_3)_2$ solutions by adding total Pb^{2+} 0, 50, 100, 200, and 400 mg kg^{-1} (dry straw). They were used to simulate different degrees of Pb pollution. Each flask was stoppered and autoclaved twice for 20 min at 121 °C. Then 2 mL spore suspension was inoculated at room temperature. The fermentation experiments were performed at 37 °C for 40 days. The humidity was maintained at the initial level (85%) in the entire fermentation period. To avoid the effects of sampling on fermentation, 5 alike conical flasks for each concentration were prepared in the same way. After inoculation, fermented straw was harvested from different sites in the flask periodically (0, 4, 8, 12, 20, 30 and 40 day) and mixed together homogeneously for routine analysis. All experiments were performed in three replicates.

2.3. Soluble-exchangeable Pb content determinations

0.5 g sample was extracted for 1 h with 20 mL of deionized water with continuous agitation, then filtered through 0.45 μm filter paper. The filtrate was decanted and the equilibrium concentration of each solution was measured by an atomic absorption spectrometer (AAS, Agilent 3510, USA).

2.4. Extracellular enzymes activity assays

1 g sample was suspended at a 1:20 (w/v) ratio of sample-to-distilled water on a rotary shaker at 200 r min^{-1} for 30 min and then centrifuged at 3500 r min^{-1} for 15 min. The supernatant fluid was filtered through 0.45 μm filter paper. Substrate filtrate was used for ligninolytic peroxidase activity analyses with a Shimadzu 2550 UV–vis spectrophotometer. LiP activity was measured as described by Tanaka et al. [20], one unit (U) of LiP activity was defined as the amount of the enzyme required to produce 1 M veratryl aldehyde from the oxidation of veratryl alcohol per minute. MnP activity was measured as described by Zhao et al. [21], and one unit (U) of MnP was defined as the amount of enzyme required for producing 1 M Mn^{3+} from the oxidation of Mn^{2+} per minute.

2.5. Extraction and analysis of oxalate

The extraction process was carried out in an ultrasound bath sonicator. Fermented straw was firstly incubated with deionized water (10 mL g^{-1} sample) for 30 min. Water extracted straw was further extracted with 1.5 M HCl (5 mL g^{-1} sample) for 15 min. Aqueous and acid extracts were collected, mixed together and filtered through 0.45 μm filter paper. The mixed filtrate was analyzed by HPLC (Agilent 1100) equipped with UV–vis variable wavelength detector (VWD). Oxalate concentration was measured as described by Li et al. [22].

2.6. Analysis of superoxide anion and hydroxyl radical

1 g sample was suspended at a 1:20 (w/v) ratio of sample-to-distilled water on a rotary shaker at 180 r min^{-1} for 30 min and then centrifuged at 5000 r min^{-1} for 10 min. 1 mL supernatant fluid was mixed with phosphate buffer solution (pH 7.8) and hydroxylamine hydrochloride (0.1 mL, 10 $mmol^{-1}$), then warm bathed at 25 °C for 30 min, reacted with 0.5 mL α-naphthylamine for 20 min at 25 °C. At last, 4 mL ether was added to the mixture, centrifuged at 1500 r min^{-1} for 5 min. The supernatant fluid was measured with a Shimadzu 2550 UV–vis spectrophotometer at 530 nm.

The improved thiobarbituric acid (TBA) method was used to assay •OH [23]. TBA-reactive substances (TBARS) were determined as follows. The rest of the sample leach liquor from analysis of $O_2^{\bullet -}$ was collected and filtered through 0.45 μm filter paper, 0.0022 g 2-deoxy-D-ribose was added into 4 mL filtrate and incubated at 37 °C for 1 h, then TBA (Sigma, 0.25%) and trichloroacetic acid (0.7%) were added, and the mixture was heated at 100 °C for 15 min. The absorbance at 532 nm was measured. The control was the same reaction mixture with no sample leach liquor added. The reductive activity of •OH was indicated by the decrease of absorbance at 532 nm compared to that of the control.

2.7. Analysis of lignin degradation

To determine the lignin degradation ability of *Pc* under different content of •OH, the contents of lignin was analyzed. Acid detergent lignin (ADL) was determined according to the procedures outlined by Van Soest et al. [24]. Lignin was estimated as the difference between ADL and ash content. Lignin degradation ratios were calculated by the following formula:

$$D = \frac{m_0 - m_{40}}{m_0} \times 100\% \quad (1)$$

where m_0 and m_{40} represent the total amount of lignin in untreated straw and that in fermentation substrate at day 40, respectively.

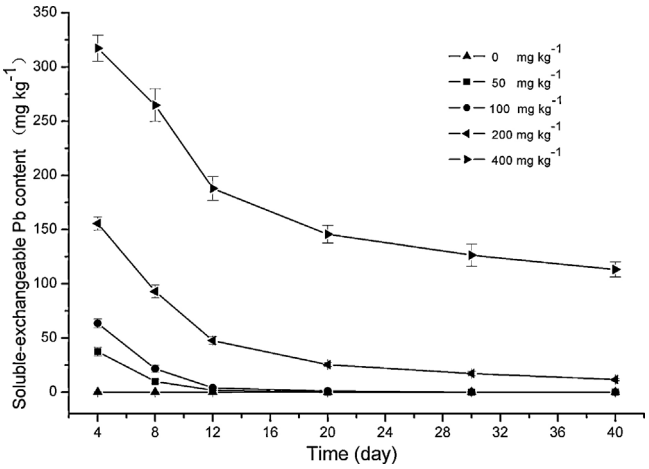

Fig. 1. Soluble-exchangeable Pb content in the control without Pb addition and in treatments with Pb addition (50, 100, 200 and 400 mg kg^{-1} dry straw, respectively) during SSF of straw with *Phanerochaete chrysosporium*. The bars represent the standard deviations of the means ($n = 3$), $^{*}p < 0.05$ vs. the control.

2.8. Statistical analysis

Data are presented as the means of three replicates, and the standard deviations were used to analyze the experimental data. Statistical analyses were performed using the software package SPSS (version 18.0). One-way analysis of variance (ANOVA) was used for testing difference among the content of soluble-exchangeable Pb, the production of oxalate and the content of radical, differences were considered statistically significant at $p < 0.05$. Correlation analyses were used to determine the relationships between the production of •OH with enzymatic activity, the production of •OH, oxalate content and $O_2^{\bullet -}$ concentration.

3. Results and discussion

3.1. Soluble-exchangeable Pb content

The changes of Pb^{2+} concentrations are shown in Fig. 1. It was found that most of soluble-exchangeable Pb^{2+} was immobilized in the flask even at the initial Pb^{2+} concentration of 400 mg kg^{-1} dry straw, and a fast decline of Pb^{2+} content was observed in every flask from day 0 to day 12. We also found that after 30 days of fermentation, Pb^{2+} concentrations in B (50) and C (100) decreased to undetectable level (Fig. 1), however, about 28% of soluble-exchangeable Pb^{2+} still existed in E (400) even after 40 days of fermentation.

Free forms of metals in solution are generally supposed to be more toxic to micro-organisms than complexed or sorbed forms [15]. The results in this study revealed that most of the active Pb ions had been transformed into inactive Pb forms, which indicated the reduction of toxicity of Pb^{2+} after 40 days SSF. The tolerance of *Pc* to heavy metals has been studied for decades, and it was postulated that the defense is usually based on immobilization of heavy metals using extracellular and intracellular chelating compounds, one of the typical metal chelators produced by *Pc* is oxalate, another group of heavy metal-binding compounds produced by fungi which are phenolic molecules associated with the cell wall, besides, there are some less important metal-binding compounds such as ligands and peptides [25]. In this study, after 40 days SSF, soluble-exchangeable Pb^{2+} content of all the 5 systems except E (400) were decreased to a very low level is likely attributable to the extracellular and intracellular immobilization. One possible explanation for the lower Pb^{2+} immobilization efficiency in E (400) is that a high Pb^{2+} concentration in the medium causing extensive damage to the *Pc* cells and leading to a decrease in cellular bioactivity.

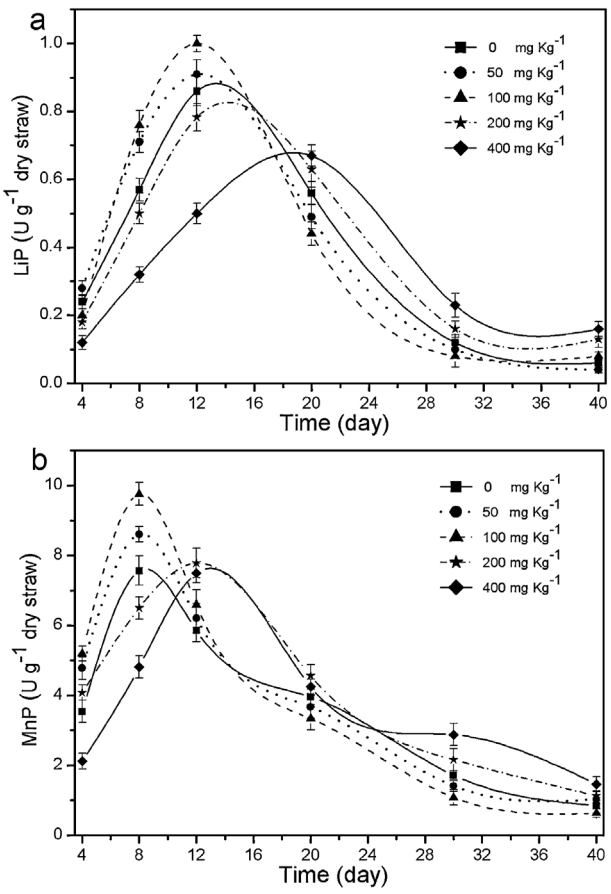

Fig. 2. Activities of lignin peroxidase (a) and manganese peroxidase (b) during SSF of straw with *Phanerochaete chrysosporium* in the control without Pb addition and in treatments with Pb addition (50, 100, 200 and 400 mg kg^{-1} dry straw, respectively). Enzyme activities are expressed in relation to the dry weight of fermented straw. The bars represent the standard deviations of the means ($n = 3$).

3.2. Dynamic changes of enzyme activities

Fig. 2 demonstrates dynamic changes of LiP and MnP activities during SSF of straw with different initial Pb concentrations. The highest LiP activity (1.02 U g^{-1} dry straw) was found in C (100) on day 12. Besides, it was shown that the LiP activities of all the five systems reached the peak on day 12, except that in E (400) did not reach 0.67 U g^{-1} until day 20 (Fig. 2a). After 30 days fermentation, LiP activities decreased significantly and maintained at relative low values. MnP activity in A (0), B (50) and C (100) displayed the highest level (7.57, 8.71 and 10.76 U g^{-1}, respectively) on day 8, and that in D (200) reached the peak (7.79 U g^{-1}) on day 12. Peak value (7.50 U g^{-1}) in E (400) was found on day 20 (Fig. 2b). As a whole, C (100) showed the highest MnP activity, while E (400) presented the lowest.

These results indicated that *Pc* could maintain good activities of ligninolytic enzymes (LiP and MnP) under Pb stress. These data were in good agreement with the Pb-resistant character of *Pc*. The earlier study has shown that *Pc* is able to survive and grow at concentration of Pb^{2+} in Czapek-Dox medium up to 400 mg mL^{-1} [26]. In this study, low or no inhibition of Pb^{2+} to ligninolytic enzymes activities was observed, especially when the initial Pb^{2+} concentration was under 200 mg kg^{-1} dry straw, which might be because that a considerable amount of the soluble-exchangeable Pb was immobilized during the SSF (Fig. 1). The result also showed that under low

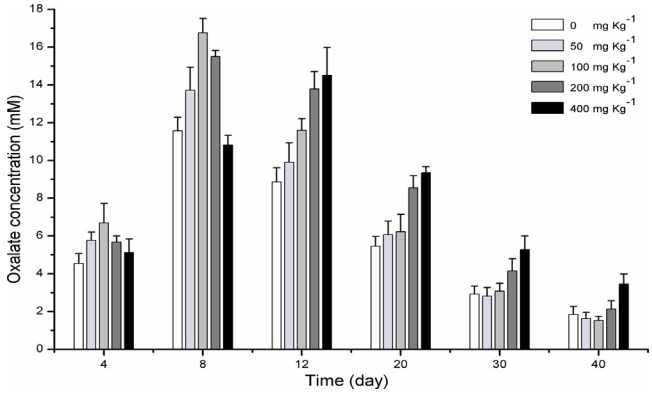

Fig. 3. The concentrations of oxalate detected in extracts during solid-state fermentation of straw with *Phanerochaete chrysosporium* in the control without Pb addition and in treatments with Pb addition (50, 100, 200 and 400 mg kg^{-1} dry mass, respectively). Oxalate concentrations are expressed in relation to the moisture content of fermented straw (in units per liter). The bars represent the standard deviations of the means ($n = 3$), $^{*}p < 0.05$ vs. the control.

Pb^{2+} concentrations the enzymes activities even increased, which might be because a modest amount of Pb^{2+} could stimulate fungi to secrete some small molecule like oxalate to increase enzymes activities. The activities of LiP and MnP decreased under 400 mg kg^{-1} of Pb^{2+} (Fig. 2), which was probably because the influence of high concentration of Pb^{2+} in the extracellular environment, since they are not protected by the cell-associated metal-detoxication mechanisms. This could be the rational explanation for that the enzyme activities in D (200) and E (400) did not reach the peak value until the Pb toxicity was decreased after several days of SSF by *Pc*.

3.3. Oxalate production at different initial Pb^{2+} concentrations

As shown in Fig. 3, oxalate productions varied with different initial Pb^{2+} concentrations during the SSF. Only a small amount of oxalate was found in the extracellular fluid during primary growth (day 0 to day 4). However, on day 8 the highest oxalate concentrations (15.76 mM) was found in extract from C (100), the maximum oxalate concentrations of A (0), B (50), D (200) detected on day 8 were 11.57, 13.71 and 15.50 mM, respectively, and the peak value (14.48 mM) of E (400) was detected on day 12. Oxalate productions increased rapidly during the initial stage followed by the fast decline along with the time shift. We also observed that D (200) and E (400) present higher oxalate concentrations after 10 days of growth.

It was reported that oxalate was produced extracellularly by *Pc*. Oxaloacetase and glyoxylate oxidase had been reported to be the two enzymes involved in the biosynthesis of oxalate from the tricarboxylic acid (TCA) cycle and glyoxylate cycle by white-rot fungi [27]. Oxalate is a secondary metabolite of *Pc*, and takes an important part in degrading of lignocellulose, a significant amount of oxalate has previously been found during SSF of straw with *Pc* [22]. In this study, the productions of oxalate in low initial Pb^{2+} concentrations (50, 100 and 200 mg kg^{-1}) were a little higher than that in control. It is suggested that *Pc* formed a better environment for oxaloacetate and glyoxylate oxidase to carry out the catalysis to secrete more oxalate under a moderate Pb^{2+} concentration. The reason may be that the presence of Pb^{2+} can interfere with the carbon and energy supplying system of oxalate biosynthesis. It was also noticed that a higher Pb^{2+} concentration (400 mg kg^{-1}) could not only decrease the oxalate production but also delay the peak of oxalate. This is likely attributed to the high Pb^{2+} concentrations restrained the growth and reproduction of *Pc*. After 12 days fermentation, quite a number of Pb^{2+} was immobilized (Fig. 1), Pb toxicity dropped so the oxalate production restored to a certain extent. Oxalate concentrations in D (200) and E (400) surpass the others in the latter stage of the fermentation. We presume that is mainly due to constant stimulation of the higher concentration of soluble-exchangeable Pb^{2+} (Fig. 1). Besides, the fungi growths in the other flasks were slower since more nutrients consumed in the earlier stage could be another possible reason.

3.4. The production of superoxide radical

The productions of free radicals were shown in Fig. 4. Both $O_2^{\bullet -}$ and •OH were evaluated by measuring the absorbance of the extract, and the concentration of free radicals was proportional to the absorbance. Fig. 4a shows the absorbance corresponding to the concentration of $O_2^{\bullet -}$. It appeared that the concentration of $O_2^{\bullet -}$ in all 5 flasks except the control one reached a very high level at the very beginning of the fermentation, with absorbance of 0.112, 0.135, 0.145 and 0.101, respectively. Then most of them sharply dropped and decreased to undetectable level after 40 days SSF. It is observed that A (control) presented the lowest production, and higher productions were detected in D (200) and E (400).

The result indicated that the production of $O_2^{\bullet -}$ could be stimulated by a higher concentration of Pb^{2+}. The production of $O_2^{\bullet -}$ sharply dropped from day 4 to day 8. The following two reasons may account for this phenomenon: (i) Pb toxicity reduced since most of the soluble-exchangeable Pb has been chelated as shown in Fig. 1; (ii) *Pc* increased the production of superoxide dismutase (SOD), which could convert superoxide to hydrogen peroxide. A relatively lower production was found in E (400) on day 4, and reached the peak value on day 12, which maybe because the higher Pb^{2+} toxicity decreased the fungal growth rate. In the control group, the production has been rising during the first period, peaked on day 12, and then a larger reduction was observed. We speculate that this maybe affect by LiP activity which also peaked on day 12 (Fig. 2). LiP was demonstrated taking an important part in the produce of $O_2^{\bullet -}$ [28]. In this case, veratryl alcohol was oxidized to cation radical by LiP in the first step, then the cation radical oxidize oxalate to $CO_2^{\bullet -}$, $CO_2^{\bullet -}$ convert O_2 to $O_2^{\bullet -}$. Almost no $O_2^{\bullet -}$ was detected at the end of the SSF, maybe for the reason that few fungal exist due to the insufficient of nutrient.

3.5. The production of hydroxyl radical

Fig. 4b demonstrates the absorbance corresponding to the concentration of •OH. As it happens for all systems, the highest concentration of •OH was observed on day 12, and the maximum absorbance (0.45) was found in C (100). Despite the presence of higher concentration Pb^{2+}, very large enhancements of •OH productions are seen: it is obviously that the productions of •OH in flasks C (100), D (200) and E (400) were higher than the others. As we shall see, •OH production rapidly increased from day 0 to day 8, maintained a high production in the next two weeks, and then a significant decrease was observed, after 40 days SSF the average production dropped to 24% of that detected on day 12.

In biological systems, •OH is thought to be generated in two types of reactions: Fenton reaction and Haber–Weiss reaction [29]. In the Fenton reaction, H_2O_2 is reduced by Fe^{2+} to produce •OH. In the iron-catalyzed Haber–Weiss reaction, superoxide ($O_2^{\bullet -}$) reduces Fe^{3+} to Fe^{2+} which in turn reduces H_2O_2 to form •OH.

3.5.1. Connection between hydroxyl radical and LiP

Comparing Fig. 2 with Fig. 4, we shall see that both LiP activity and •OH production peaked on day 12. In addition, they both present high value from day 8 to day 20. In these respects, there could be some internal relations between LiP activity and •OH production. In order to acquire a precise understanding of the

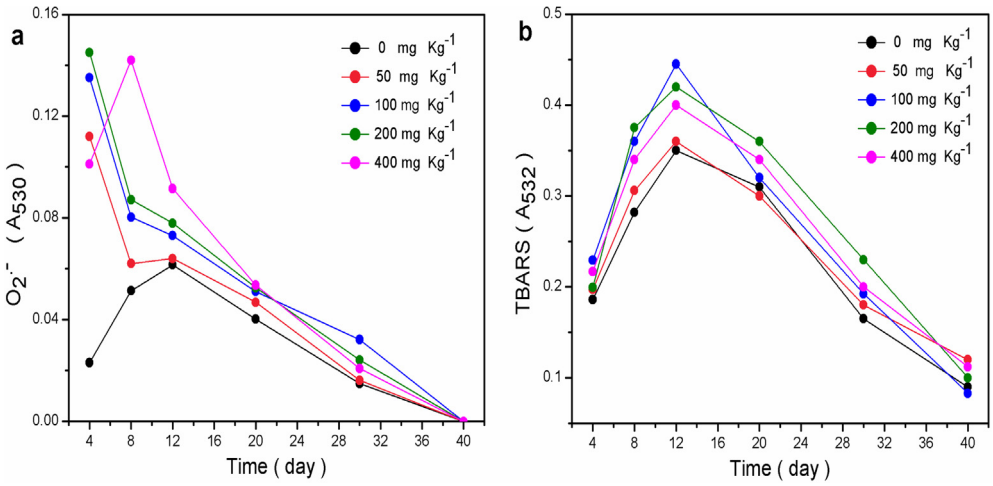

Fig. 4. Effects of different initial concentrations of Pb^{2+} on $O_2^{\bullet-}$ (a) and •OH (b) productions by *Phanerochaete chrysosporium* during SSF of straw. •OH production was estimated as TBARS formation from 2-deoxyribose. Results are mean values of triplicate, and the standard deviations are below 5% ($n = 3$), $^*p < 0.05$ vs. the control.

connection between LiP activity and •OH production, the correlation of them was analyzed (Fig. 5). It turns out that the LiP activity and •OH production were well correlated ($R^2 = 0.945$) in the control group (Fig. 5a). These data suggest that LiP could be the main factor that promotes the •OH production when no Pb^{2+} was added in the flask. Ligninolytic enzymes (LiP and MnP) have previously been found not able to directly catalyze the formation of •OH, however, LiP was demonstrated taking an important part in Haber–Weiss reaction by initiate the produce of $O_2^{\bullet-}$ [28]. As shown in Fig. 5a′, it is also found significant correlation between production of •OH and $O_2^{\bullet-}$, that confirmed our speculation in some degree. On the other hand, the correlation coefficient decreased when the higher Pb^{2+} concentration was taken into account, when the initial Pb^{2+} concentration was 400 mg kg^{-1}, no obvious relationship was observed

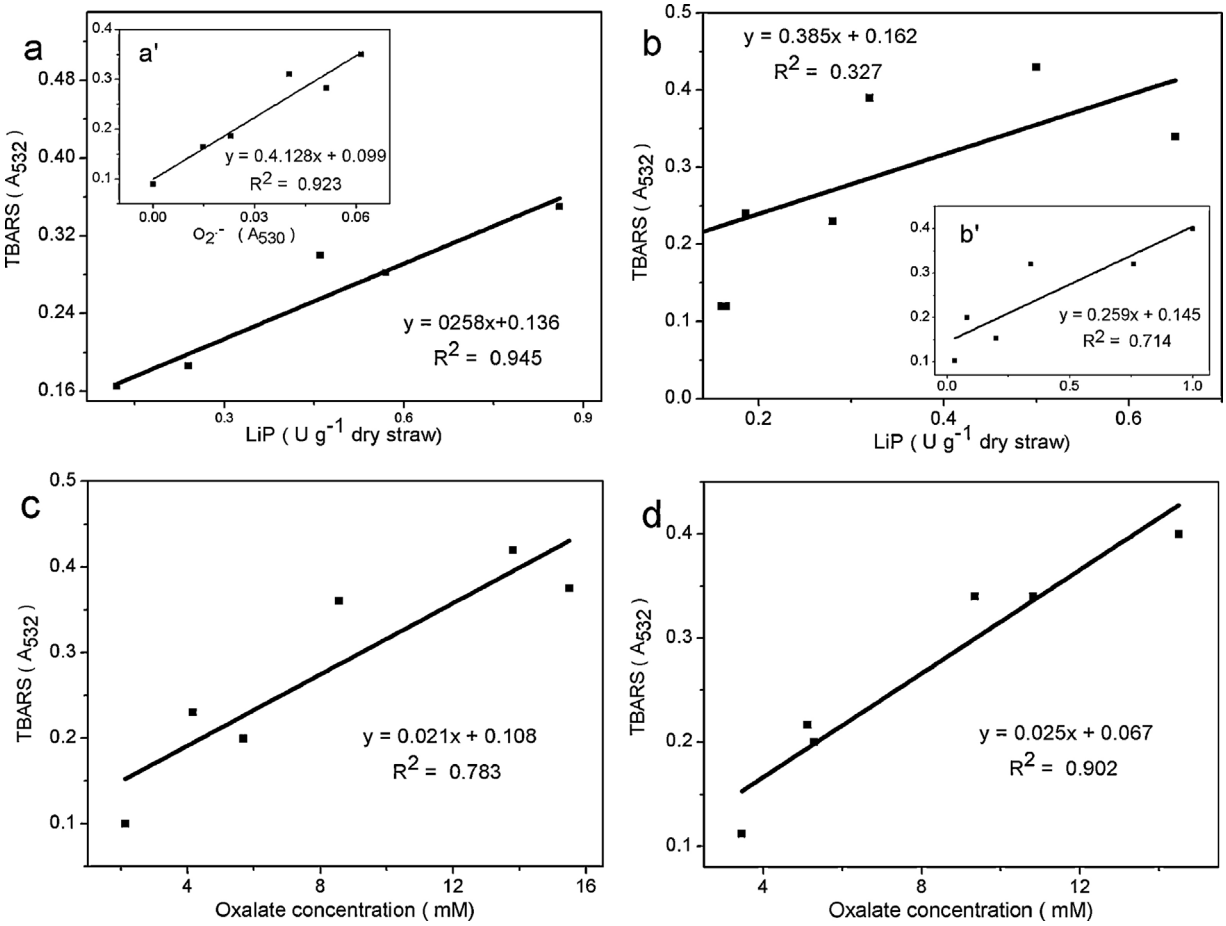

Fig. 5. Correlation between *Phanerochaete chrysosporium* lignin peroxidase (LiP) activity, superoxide anion radical ($O_2^{\bullet-}$) concentration, oxalate concentration and hydroxyl radical (•OH) production (estimated as TBARS formation from 2-deoxyribose) in different medium. (a and a′) In the control without Pb addition; (b, b′, c, d) in treatments with Pb addition (400, 100, 200 and 400 mg kg^{-1} dry mass, respectively).

($R^2 = 0.327$) (Fig. 5b). These observations suggest that when initialed with Pb^{2+} (especially under high concentrations), there must be other pathway existence to stimulate the •OH production.

3.5.2. Connection between hydroxyl radical and superoxide radical

As described above, $O_2^{\bullet -}$ could increase the production of •OH by reduce Fe^{3+} to Fe^{2+}, moreover, $O_2^{\bullet -}$ dismutation ($O_2^{\bullet -} + HO_2^{\bullet} + H^+ \rightarrow O_2 + H_2O_2$) was a way to provide Fenton's reagent H_2O_2 [30]. It is expected that a higher concentration of $O_2^{\bullet -}$ would bring about a higher •OH production, however, apparently when under Pb^{2+} stress, the productions of $O_2^{\bullet -}$ and •OH were not well correlated (Fig. 4): $O_2^{\bullet -}$ concentration in most flasks sharply decreased from day 4 to day 12, while a dramatic increase was observed on •OH production. We speculate that the possible reason is the $O_2^{\bullet -}$ content was sufficient, but other factors restricted •OH production, such as H_2O_2 reduced by catalase and the insufficient of Fe^{2+} chelating agent.

3.5.3. Connection between hydroxyl radical and oxalate

In this study, •OH production was found not quite correlated with oxalate concentration, however, a similar significant increase trend was observed during the primary growth phase (Figs. 3 and 4). This comes about because oxalate leads to the production of Fe^{2+}-oxalate, whose involvement in the reduction of H_2O_2 to •OH has been previously reported [30]. It was also demonstrated that in the presence of oxalate and Mn^{3+}, H_2O_2 production could be promoted [31,32]. Under this circumstances, Mn^{3+} was reduced to Mn^{2+} by oxalate (Mn^{3+} + oxalate $\rightarrow$ $Mn^{2+} + CO_2^{\bullet -} + CO_2$), Mn^{2+} then reduce $O_2^{\bullet -}$ to H_2O_2 ($Mn^{2+} + O_2^{\bullet -} \rightarrow Mn^{3+} + H_2O_2 + 2H^+$). We have already speculated that $O_2^{\bullet -}$ was abundant at this stage, therefore it is very reasonable that the rapid increase of •OH production was mainly contributed to the high concentration of oxalate. Increase rate of •OH slowed down from day 8 to day 12 (Fig. 4b) which is likely due to the concentration of oxalate declined (Fig. 3). Higher •OH productions were detected in flask D (200) and E (400), same results were found in oxalate concentration. The correlations were analyzed, as shown in Fig. 5c, a relatively good linear correlation ($R^2 = 0.783$) between •OH productions and oxalate concentration was observed in D (200). Furthermore, here was a significant correlation ($R^2 = 0.902$) between them when the initial Pb^{2+} concentration was 400 mg kg^{-1} (Fig. 5d). These observations suggest that oxalate makes a good contribution to •OH production during the fermentation when initiated with Pb^{2+}.

3.6. Connection between hydroxyl radical and lignin degradation

After 40 days SSF, lignin in all the 5 flasks was biodegraded at different levels, with the degrading rates of 33.4 ± 0.8% (A 0), 39.6 ± 1.1% (B 50), 42.9 ± 0.4% (C 100), 37.5 ± 0.9% (D 200) and 21.90 ± 0.5% (E 400), respectively. The result showed that low concentration of Pb^{2+} can promote the degradation of lignin, and the higher initial Pb^{2+} concentration resulted in inhibition. The reason might be Pb^{2+} affected the carbon and energy supplying system of ligninase [25]. Previous studies showed that the generation of •OH in cultures of white-rot fungi was proportional to the rate of lignin degradation [9,10]. However, correlations between •OH production and lignin degradation rate were not observed when Pb^{2+} concentration was taken into account. In our study D (200) showed the highest •OH productions (Fig. 4), however, the highest lignin degradation rate was found in C (100). Furthermore, the correlation between lignin degrading rate and the total production of •OH (TBARS) was analyzed, no obvious linear correlation ($R^2 = 0.331$) was found between them. Reasons for these results could be as follows: (i) a high •OH concentration would restrain enzymatic activities and fungal growth as well, and (ii) high Pb^{2+} concentration disturbed the function of the regulators of lignin degradation, for example "*Pc* reducer" which was reported to be able to regulate •OH in selective lignin biodegradation [33].

4. Conclusions

The results showed that an appropriate increase of initial Pb^{2+} concentration could enhance the production of •OH during the solid-state fermentation. The probable reason was that a higher Pb^{2+} concentration leads to a higher generation of oxalate and $O_2^{\bullet -}$, both of which were beneficial to the generation of •OH. However, when the initial Pb^{2+} concentration reached 400 mg kg^{-1}, a decrease of •OH production was observed, which might be due to the inhibition of fungal growth under this condition. In the present of Pb^{2+}, no correlation between •OH production and lignin degradation were observed, and the related mechanism needed further studies.

Acknowledgements

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