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Oxalate production at different initial Pb^{2+} concentrations and the influence of oxalate during solid-state fermentation of straw with *Phanerochaete chrysosporium*

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ABSTRACT

The production of oxalate at different initial Pb^{2+} concentrations during solid-state fermentation of straw with *Phanerochaete chrysosporium* was investigated. It was found that the maximal peak value of oxalate concentration (22.84 mM) was detected at the initial Pb^{2+} concentration of 200 mg kg^{-1} dry straw, while the minimum (15.89 mM) at the concentration of 600 mg Pb^{2+} kg^{-1} dry straw, and at moderate concentration of Pb^{2+} the capability of oxalic acid secretion was enhanced. In addition, it was also found that more oxalic acid accumulation went together with better Pb^{2+} passivation effect and higher manganese peroxidase (MnP) activity. The present findings will improve the understandings of the interactions of heavy metals with white-rot fungi and the role of oxalate in lignin degradation system, which could provide useful references for more efficient treatment of Pb-contaminated lignocellulosic waste.

1. Introduction

In recent years, there has been an increasing trend towards the utilization of agricultural wastes as raw materials to produce value-added products by solid-state fermentation (SSF) technique (Graminha et al., 2008). The use of agricultural residues besides providing alternative substrates helps to solve the disposal problem considering their large quantities in environment. Moreover, agricultural residues usually contain lignin, a very complex polymer and resistant to biodegradation, which adds difficulties to their disposal (Pérez et al., 2002). While it has been well known that white-rot fungi are effective on decomposing lignin into carbon dioxide, using very nonspecific mechanisms (Barr and Aust, 1994). Therefore, much attention has currently been drawn to the inoculation of white-rot fungi in SSF process for more efficient treatment of lignocellulosic waste (Huang et al., 2010).

Nowadays, Pb pollution of air and agricultural soils has been one of the most important ecological problems on a world scale. Accordingly, plants and crops are widely polluted by Pb to different extent and the wastes from those plants and crops also contain Pb contamination. During biodegradation of agricultural wastes, the presence of Pb would pose threat to fungal survival and biodegradation ability (Baldrian, 2003). However, wood-rot fungi still could survive and grow even with Pb^{2+} level in the substrate up to 400 mg kg^{-1} dry mass (Huang et al., 2008) or 400 mg L^{-1} (Falih et al., 1997). And at certain Pb^{2+} concentration, it can degrade lignin even more effectively (Huang et al., 2008). The above facts indicates that fungi must have evolved some more efficient mechanisms connecting Pb^{2+} toxicity alleviation with the biodegradation system. During SSF process with white-rot fungi, lignocellulolytic enzymes are unable to penetrate into the cell wall structure in the early stages of the decay process, so low-molecular weight compounds have been proposed to be involved in wood decay (Enoki et al., 1997). Among these compounds, oxalic acid is included and also plays an important role as a metal chelator. So more detailed investigation on oxalate production during SSF process of lignocellulosic waste with white-rot fungi in the presence of Pb^{2+} is essential.

Oxalic acid can be produced by both white-rot fungi and brown-rot fungi. However, the production of oxalic acid by fungi is species-specific. Oxalate production by wood-rot fungi was found in both liquid and solid culture medium (Mäkelä et al., 2002). Among white-rot fungi, *Phanerochaete chrysosporium* (*Pc*) can produce higher amounts of oxalate (Baldrian, 2003). Most previous studies on the production of oxalic acid or oxalate by fungi have been carried out in medium with single concentration of heavy metal (Jarosz-Wilkolazka and Gadd, 2003) or in substrate without toxic

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metal ions (Dutton et al., 1993; Mäkelä et al., 2002). No details on the oxalate production by fungi at different initial Pb^{2+} concentrations during SSF have been reported so far.

The primary aim of this study was to investigate the oxalate production at different initial Pb^{2+} concentrations during SSF of straw with *Pc*. Also, pH, GI (a factor of relative seed germination and relative root elongation) of rapeseed and two main ligninolytic enzymes, (lignin peroxidase) LiP and MnP, produced by *Pc* BKM-F-1767 during SSF of straw were determined to follow their connection with the secretion of oxalic acids.

2. Methods

2.1. Microorganism cultivation and inoculum preparation

The fungus *Pc* strain BKM-F-1767 was used. It was maintained at 4 °C on potato dextrose agar (PDA) slants and then transferred to PDA plates at 37 °C for 6 days. Inocula consisted of spore suspension which was prepared by scraping the spores on the agar surface and then diluting them in sterile distilled water. Spore concentration was measured and adjusted to 2.0×10^6 CFU mL^{-1}.

2.2. Solid-state fermentation conditions

The straw obtained from suburban areas of Yuelu District (Changsha, China) was air-dried and ground to pass through 2 mm nylon screen. The concentration of total Pb in the straw was 2.3 mg kg^{-1} dry weight. SSF was carried out in 500 mL flasks containing 35 g of straw powder and flasks were labeled as A(0), B(50), C(100), D(200), E(400) and F(600), respectively. $Pb(NO_3)_2$ solutions were supplemented and mixed thoroughly with straw powder till the final Pb^{2+} levels in the substrates were 0, 50, 100, 200, 400 and 600 mg kg^{-1} dry straw correspondingly. Each flask was stoppered and autoclaved twice for 20 min at 121 °C. Then 5 mL spore suspensions were inoculated at room temperature. The fermentation process was undertaken statically in a constant temperature-humidity machine and the moisture content in straw substrate was maintained at 75% in the entire fermentation period, by adding appropriate amount of sterile distilled water or closing the humidifying function in the constant temperature-humidity machine. For a better comparison, control flasks containing 200 mg kg^{-1} dry straw without incubation of fungus were also prepared under the same conditions. Fermentation processes were performed at 37 °C for 45 days. To avoid the effects of sampling on fermentation, 9 alike conical flasks for each concentration were started and used for sampling on 0, 3, 6, 9, 15, 20, 24, 36 and 45 day, respectively. All experiments were performed in triplicates.

2.3. Sampling and analytical methods

After inoculation, 7 g of fermented straw was harvested from different sites in the flask periodically (0, 3, 6, 9, 15, 20, 24, 36 and 45 day) and mixed together homogeneously for routine analysis. Final results were the means of three replications.

2.4. Moisture content and total organic matter (TOM)

Moisture level and organic matter are both important factors of SSF processes (Singhania et al., 2009). They were determined using a classical loss-on-drying and ignition procedure. Triplicate 2.0 g samples were dried at 105 °C for 6 h to a constant weight and then transferred to a muffle furnace and held at 550 ± 10 °C for 6 h. Moisture content was calculated from the ratio of pre- to post-drying sample weights and TOM from that of pre- to post-ignition sample weights.

2.5. Extraction and analysis of oxalate

2.5.1. Extraction of oxalic acid from fermented straw

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 used for HPLC analysis (Dutton et al., 1993; Hofrichter et al., 1999).

2.5.2. High performance liquid chromatography (HPLC) analysis of oxalate

Oxalate in the straw extract was analyzed by HPLC using an Agilent 1100 apparatus equipped with UV–vis variable wavelength detector (VWD). Phosphoric acid (0.15% v/v) was used as the solvent at a flow rate of 0.5 mL min^{-1}, and detection wave length was 210 nm. The column was maintained at 30 °C. Oxalic acid was used as external standard for quantization. Oxalate concentrations were expressed in relation to the moisture content of fermented straw.

2.6. pH determination

pH is not only an important factor of SSF processes, but also involved in Pb^{2+} immobilization (Chen and Lin, 2001; Kumar and Nagendran, 2007). Suspension at a 1:20 (w:v) ratio of sample-to-water was shaked at 180 r min^{-1} for 45 min and then centrifuged at 5000 r min^{-1} for 10 min. The supernatant fluid was filtered through fine filter paper and then pH of the filtrate was measured with a Mettler Toledo FE 20 pH meter.

2.7. Toxicity analyses and ligninolytic peroxidases activities

Sample was suspended at a 1:10 (w:v) ratio of sample-to-distilled water on a rotary shaker at 180 r min^{-1} for 30 min and then centrifuged at 3500 r min^{-1} for 15 min.

2.7.1. Toxicity analyses

Part of the supernatant fluid was filtered through filter paper and the aqueous extract was for toxicity analysis, which was undertaken in a Petri dish by placing a filter paper in it. Water was subsequently added until the filter paper was completely submerged (Zeng et al., 2007). Bird rapeseeds (*Brassica campestris* Linn) were then placed on the filter paper. The percentage of germination and root elongation was measured after incubating the covered Petri dishes in the dark at 22 °C for 4 days. As a control, the same amount of distilled water was used for the germination experiment. GI was calculated according to Zucconi et al. (1981).

2.7.2. Enzyme assays

Spare supernatant fluid was filtered through 0.45 μm filter paper. Substrate filtrate was used for ligninolytic peroxidase activity analyses. In this study, two main ligninolytic peroxidases (Arora et al., 2002), LiP and MnP, were measured with a Shimadzu 2550 UV–visible spectrophotometer.

LiP activity was measured by a modified method as described by Tien and Kirk (1988), with one unit (U) representing 1 μmol veratryl alcohol oxidized to veratraldehyde per minute at pH 2.5 and 25 °C. Each reaction mixture (total volume 3 mL) contained 1.5 mL of 100 mM sodium tartrate (pH 3.0), 1 mL of 10 mM veratryl alcohol which was replaced by the same volume of sodium tartrate in the control mixture, 0.4 mL of enzyme extract, and 0.1 mL of 10 mM H_2O_2. The reaction was started with H_2O_2, and the formation of veratraldehyde was monitored at 310 nm (ε_{310} = 9300 mol^{-1} cm^{-1}).

MnP activity was determined according to the method described by Wariishi et al. (1992), which was based on the oxidation of Mn^{2+} to Mn^{3+}. Each 3-mL reaction mixture contained 2.4 mL of 50 mM sodium succinate (pH 4.5), 0.1 mL of 15 mM $MnSO_4$ replaced by 0.1 mL of sodium succinate in the control mixture, 0.4 mL of crude enzyme solution, and 0.1 mL of 10 mM H_2O_2. The reaction was initiated at 37 °C by adding H_2O_2, and the rate of Mn^{3+}-succinate complex formation was monitored by measuring the increase in absorbance at 240 nm (ε_{240} = 8100 mol^{-1} cm^{-1}).

3. Results and discussion

3.1. Loss of TOM during SSF process

As shown in Fig. 1, TOM levels of all groups were 78–79% at the beginning of SSF and decreased rapidly in the early stage of SSF (first 15 days). After 36 days, TOM was decreasing slightly. After 45 day fermentation, the decrease of TOM level was the most in the treatment with 200 mg Pb^{2+} kg^{-1} dry straw, reaching about 34.8%, whereas when Pb^{2+} was 600 mg kg^{-1} dry straw, the treatment kept the highest TOM level (57.8%).

TOM loss is mainly due to the mineralization of carbohydrates by *Pc*, one kind of well studied white-rot fungi which is characterized by its unique ability to degrade lignin (Zeng et al., 2007). During the entire period of experiment (Fig. 1), it was shown that SSF process in each treatment was undertaken successfully. In the initial stage, fewer TOM losses were found in treatments than that in A(0). It was probably due to biological activity inhibition by the high concentration of Pb^{2+} (Falih, 1997) until day 3. After 6 day fermentation, the loss of TOM was not reduced even Pb^{2+} concentration was high up to 200 mg kg^{-1} dry straw (Fig. 1). This might because Pb^{2+} toxicity was weakened due to the production of metal chelating compounds by *Pc*, such as oxalic acid (Baldrian, 2003), and in D(200), *Pc* formed a more effective defense mechanism against Pb^{2+} toxicity and a better organic matter decomposing system than that in any other treatments.

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

Oxalate concentrations in all treatments varied during incubation (Fig. 2). Oxalic acid was the primary organic acid detected in the extracts from the solid-state fermentation cultures with different initial Pb^{2+} concentrations. The maximum concentration of oxalate (22.8 mM) was detected on day 9 in the extract from D(200), while that of A(0), B(50), C(100) were 16.8, 18.8 and 20.4 mM, respectively. The oxalate production in E(400) and F(600) did not reach a peak until day 15, and the concentrations were respectively 13.3 and 12.0 mM. Then a decrease appeared which may indicate the degradation of oxalate. A new faint fluctuation in oxalate concentrations of all the cultures was observed in the later stage of SSF. Oxalate accumulations in E(400) and F(600) were higher than that in the other four treatments at the end of experiment (Fig. 2).

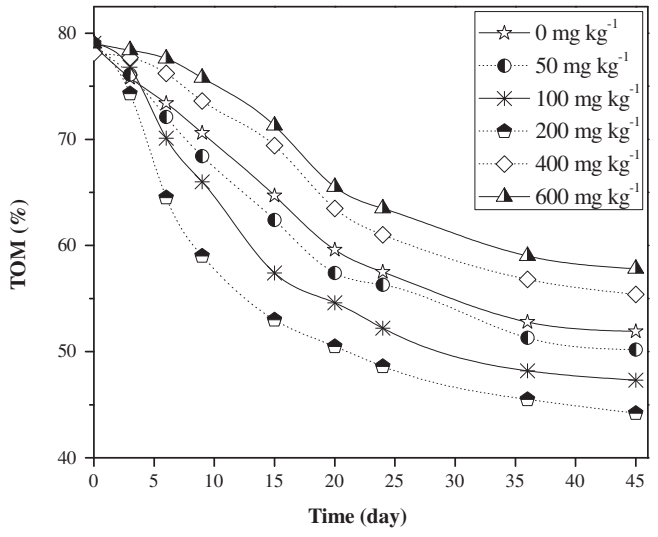

Fig. 1. Changes of TOM during SSF of straw with *Pc* in the treatment without Pb^{2+} addition and the treatments with Pb^{2+} at concentrations of 50, 100, 200, 400 and 600 mg kg^{-1} dry straw, respectively.

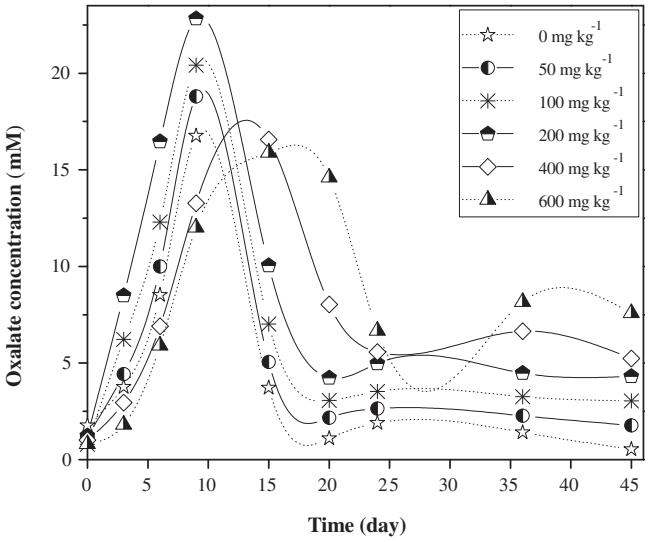

Fig. 2. The concentrations of oxalate detected in extracts during SSF of straw with *Pc*. Oxalate concentrations are expressed in relation to the moisture content of fermented straw (in units per liter).

In this study, the peak value of oxalate concentration was detected with initial Pb^{2+} concentration of 200 mg kg^{-1} dry straw (Fig. 2). And the peak value in treatment A(0) and F(600) showed a moderate and lowest level, respectively. It was demonstrated that an appropriate addition of Pb^{2+} could induce more production of oxalic acid during 45 day SSF. The reason was that the presence of heavy metals probably can interfere with the carbon and energy supplying system (Baldrian, 2003), in which the oxalate biosynthesis is included. 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 (Mäkelä et al., 2002). In this study, moderate Pb^{2+} concentration made *Pc* formed a better environment for oxaloacetase and glyoxylate oxidase to carry out the catalysis so that *Pc* secreted more oxalate. Peak values of oxalate concentrations in all the six treatments were higher than the value 10 mM which was the maximum oxalate concentration reported by other researchers (Dutton et al., 1993; Hofrichter et al., 1999). Both of the following reasons can help to make it clear that such high oxalate levels in this study. For one thing, previous studies showed that because of the added advantages of fast oxygenation and a static process without mechanical energy expenditures (Pandey, 2003), SSF produces metabolites in a more concentrated form than liquid (submerged) fermentation (Li et al., 2006). Additionally, both deionized water extract and HCl extract were performed in the extraction of oxalic acid from fermented straw in the study. Acid extraction could release strongly adsorbed oxalate and insoluble oxalate salts, such as calcium oxalate (Jarosz-Wilkolazka and Gadd, 2003).

3.3. Connection of oxalic acid or oxalate with pH, Pb^{2+} phytotoxicity and ligninolytic enzymes

To make a better discussion of the connection of oxalate with pH, Pb^{2+} phytotoxicity and ligninolytic enzymes, treatments with different level of oxalate detected were taken into account, i.e. A(0), D(200) and F(600).

3.3.1. Connection of oxalic acid or oxalate with pH

During the early stage of SSF, pH was raised rapidly from initial value of about 7.2–8.27 in A(0), 7.92 in D(200) and 7.48 in F(600). Afterward, it did not increase again until day 15 in A(0), day 20 in D(200) and day 24 in F(600). The pH values on last day of SSF were 7.33, 7.53 and 7.89, respectively. The pH in A(0) was higher than that in D(200) and F(600) during 45 days of fermentation (Fig. 3).

pH is known to affect heavy-metal toxicity and the ionic form and chemical mobility. Oxalic acid is an excellent chelator of heavy metal, but the accumulation of oxalate was associated with a decrease in pH which can increase the solubility of metals in the medium (Kumar and Nagendran, 2007). Nevertheless, it is interesting to note that pH values during entire processes were nearly all over 7.0 (Figs. 2 and 3), which is still advantageous for heavy metal passivation (Chen and Lin, 2001). pH still descended for a while when the oxalate concentration decreased, which is probably due to the effect of other acidic production in oxalate degradation metabolism. This indicates that there is no significant relationship between oxalate concentration and pH in these medium. The reason for pH increase in initial and later stages may lie in little accumulation of oxalic acid and ammonia matters produced during organic matter catabolism by *Pc*, e.g. NH_4^+ released from L-phenylalanine at the initial step in VA biosynthesis (Hattori et al., 1999). It is well-known that the activities of LiP and MnP are both pH-dependent. As reported previously (Singh and Chen, 2008), the optimum pH range for lignin decomposition was between 4.0 and 4.5, with marked suppression detected above 5.5 and below 3.5. However, pH of the growth medium is higher than the pH ranges of the enzymes notwithstanding oxalate accumulation can make pH in the medium closer to that. It is unclear how the two extracellular enzymes *Pc* produced perform functions in the media with such high pH values. The same phenomenon for the other enzyme was also reported by Dutton et al. (1993).

3.3.2. Connection of oxalic acid or oxalate with Pb^{2+} phytotoxicity

On day 0, GI of unfermented straw in A(0), D(200), F(600) were different (Fig. 4). Then a fast increase in GI values was observed and the increase slowed down as fermentation went on. The GI value was 106.1%, 95.8% and 85.8% for A(0), D(200) and F(600) on day 45, respectively (Fig. 4).

GI analysis was utilized to ascertain Pb^{2+} toxicity of fermented straw extracts on seed germination and root elongation (Salvatore et al., 2008). The toxicity of Pb^{2+} is mainly depended on active Pb concentrations in the soluble and exchangeable fractions, which can be taken up by living organisms (Liu et al., 2009). The differences of GI values in day 0 indicated that phytotoxicity increased with the increasing initial Pb^{2+} level in the extract. As oxalate accumulation took place, GI values increased rapidly, independently of the high initial Pb^{2+} concentration (Figs. 2 and 4). It is probably because of the production of Pb oxalate that active Pb^{2+} concentrations were reduced. When oxalate concentrations began to decline, the uptrends of GI became slow (Fig. 4). These findings gave an indication that oxalate production could be involved in the detoxification of Pb^{2+}. The latter slow-motion increase may be due to the reactive species produced in organic matter decomposition by white-rot fungi which probably can promote the formation of humus, which can perform the chelation and thus immobilization of Pb^{2+} (Tien and Kirk, 1988; Huang et al., 2006). Furthermore, GI is an important factor to judge the maturity of SSF substrate and GI below 80% indicates phytotoxicity (Lasaridi et al., 2006). It was apparent that GI in D(200) mounted up to above 80% after 24 days of fermentation, which was observed in A(0) after 20 day incubation, while 36 days are needed for F(600). On the final day, no significant phytotoxicity differences were evidenced among all the treatments, indicating that SSF substrates in all treatments were almost mature.

3.3.3. Connection of oxalic acid or oxalate with ligninolytic enzymes

High levels of MnP activity and minor levels of LiP activity were detected in extracts of fermented straw (Fig. 5). Both of them were monitored on day 3. After 9 day fermentation, MnP activity of A(0) and D(200) displayed the highest level (3.27 and 4.92 U g^{-1} dry straw, respectively), while in F(600), it did not reach a peak value (3.05 U g^{-1} dry straw, respectively), until day 15 (Fig. 5b). Subsequently, the levels of MnP activity decreased significantly and tended to be minor after about 20 days. LiP activities detected in

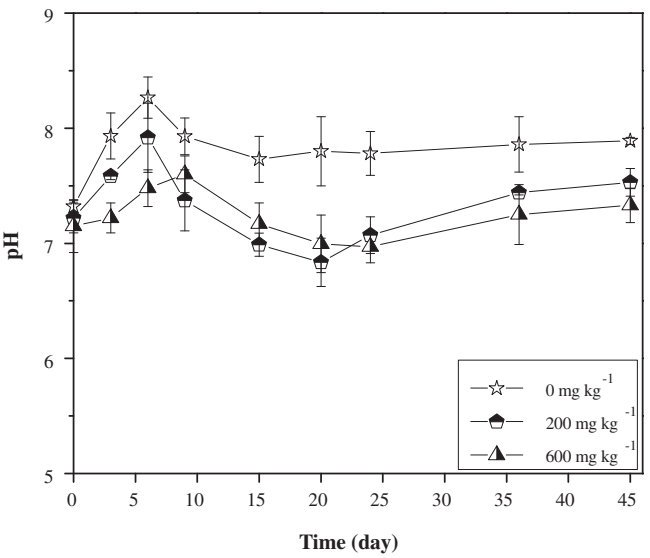

Fig. 3. pH changes in A(0), D(200) and F(600) during SSF of straw with *Pc*. The bars represent the standard deviations of the means ($n = 3$).

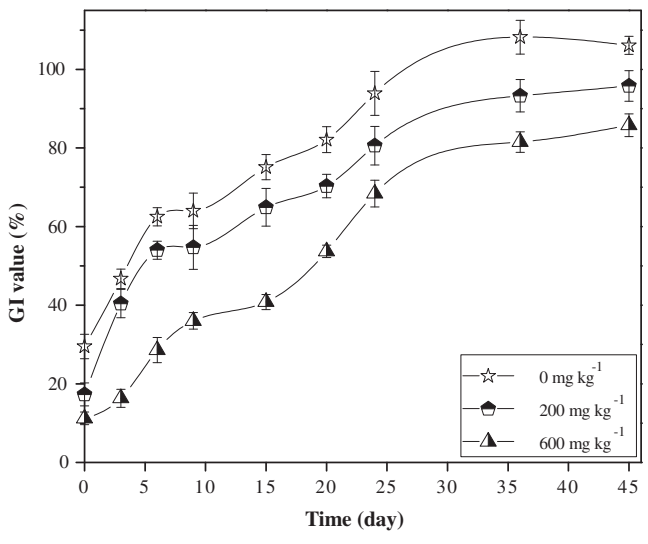

Fig. 4. Change of germination index (GI) in treatment A(0), D(200) and F(600) during SSF of straw with *Pc*. The bars represent the standard deviations of the means ($n = 3$).

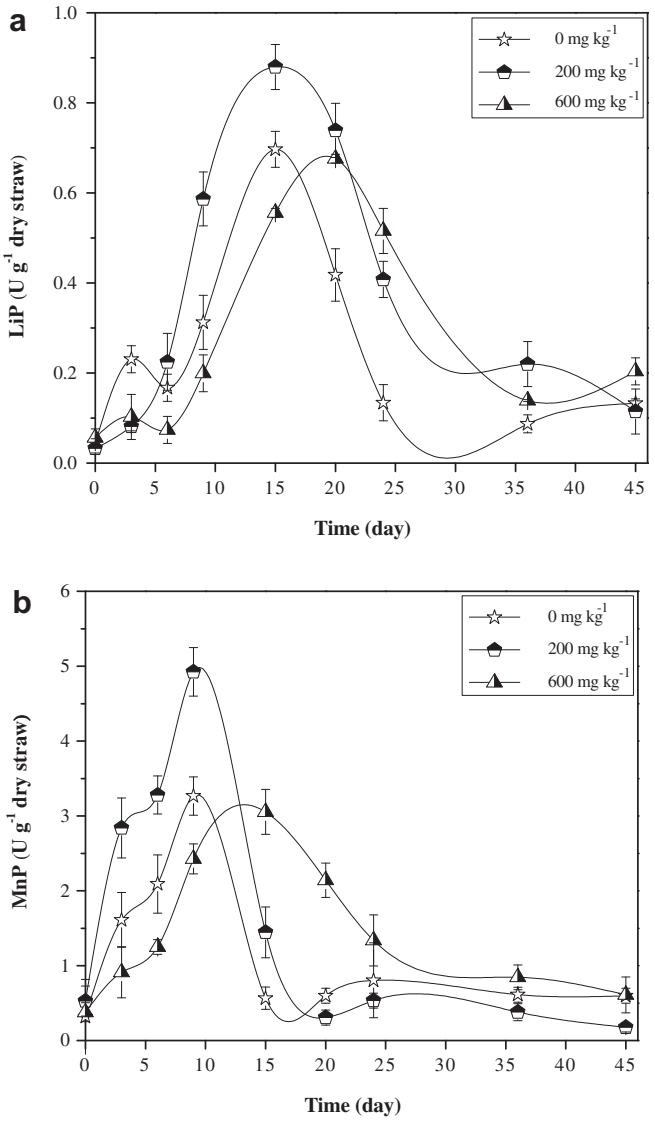

Fig. 5. Activities of LiP (a) and MnP (b) during SSF of straw with *Pc*. Enzyme activities are expressed in relation to the dry weight of fermented straw. The bars represent the standard deviations of the means ($n = 3$).

the straw extracts peaked (0.70, 0.88 and 0.68 U g^{-1} dry straw, respectively), after oxalate degradation, approximately in 15 days of fermentation (Figs. 2 and 5a). Low levels of MnP and LiP activities were detected until the end of incubation.

Oxalic acid plays an important role in white-rot decay system. From Fig. 5, it was displayed that MnP displayed a higher activity than that of LiP, and similar results were also shown by Arora et al. (2007). MnP activity peaked together with oxalate concentration (Figs. 2 and 5b), which was probably due to the chelation and stabilization of Mn^{3+} by oxalic acid (Hofrichter et al., 1999; Pérez and Jeffries, 1993). Besides, detoxification of Pb^{2+} by oxalic acid in MnP system may be another reason, after all heavy metals in general are potent inhibitors of enzymatic reactions, especially for extracellular enzyme. In the early stage, low LiP activities may be due to the lack of hydrogen peroxide in the cultures, whose production depends on the oxidation of oxalate by MnP (Urzúa et al., 1998), and low levels of oxalate and MnP were observed in this period (Figs. 2 and 5). In other words, oxalate production probably could inhibit LiP in the extracellular site, which was also proved by Akamatsu et al. (1990).

4. Conclusions

More oxalate was produced when Pb^{2+} concentration in the substrate of SSF inoculated *Pc* was 200 mg Pb^{2+} kg^{-1} dry straw than that detected in the substrate without Pb^{2+} or with Pb^{2+} high up to 400, 600 mg kg^{-1} dry straw. The present report also demonstrated that production of oxalate was able to bring about active changes in Pb^{2+} passivation effect and lignin degradation system. The present findings could be used as references to promoting more efficient treatment of Pb-contaminated lignocellulosic waste.

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