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RESEARCH PAPER

Contribution characteristics of the in situ extracellular polymeric substances (EPS) in *Phanerochaete chrysosporium* to Pb immobilization

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Abstract White rot fungi have been extensively reported to have strong adsorption capacity to heavy metal ions, whereas the knowledge of extracellular polymeric substances (EPS) from the fungus has been rarely involved. In this study, the contribution characteristics of 'the in situ EPS in *Phanerochaete chrysosporium* to Pb immobilization were investigated. First of all, the component and amount of EPS were investigated. It was found that the main component of EPS was carbohydrates, and highest EPS amount was produced at 5 days. In the Pb^{2+} immobilization experiments, EPS was demonstrated to play a more important role in immobilizing Pb^{2+} at lower initial Pb concentration. pH increase was beneficial for EPS to immobilize Pb. Higher EPS amount increased the Pb removal efficiency at a certain extent, while the specific uptake decreased. The Pb^{2+} immobilization by EPS produced at 7 days was most successful.

Keywords *Phanerochaete chrysosporium* · EPS · Heavy metals · In-situ immobilization

Introduction

In recent years, many studies have demonstrated that the white rot fungi have strong adsorption capacity to heavy metals [1, 2]. Along with the research, more scholars began to study the interaction mechanism of white rot fungi with heavy metals [3–6]. White rot fungi can form effective defense systems against the stress of toxic heavy metals. These defense systems are based on the intracellular and extracellular material which has the ability to complex heavy metals [7]. As the first interaction environment, the extracellular defense system is the most important for the passivation of heavy metal ions [8, 9].

In the biotreatment, extracellular polymeric substances (EPS) could be excreted by microorganisms produced from lysis and hydrolysis and adsorbed organic matters from wastewater [10–12]. Generally, the main components of EPS were comprised of polysaccharides, proteins, nucleic acids and other cellular components [13]. Part of EPS is dispersed in the solution (known as soluble polymer), the other part is located around the surface of microorganisms (known as the adsorbed polymers) [13]. The adsorbed polymers have multiple physiological functions for the living cells, including enhancing the cell's adhesion, protecting cells from the harsh external environment, as well as adsorbing and storing the nutrient [14–16]. EPS is rich in a variety of functional groups, such as hydroxyl, amide, amino, carboxyl, thiol, etc., providing important basis for the removal of heavy metal ions in the environment [17, 18]. A number of studies have demonstrated that directly or indirectly proved the contribution of EPS to heavy metal immobilization [19, 20]. However, those studies were mainly focused on EPS produced by bacteria, biofilm and the activated sludge, knowledge of EPS from fungi, including white rot fungi, has been rarely involved.

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Until now, the researches on the mechanisms of white rot fungi to remove heavy metal displayed some findings related with EPS. For example, heavy metal has always been explained to be ion exchanged by the fungal cell wall where EPS probably also participated in metal ion exchange [21]. Previous reports revealed that heavy metal oxalate crystals were found outside the mycelium of white rot fungi indicating that EPS probably plays a role in immobilizing metal chelate [4, 22]. However, the effect of the fungal EPS has not been directly studied. Those ambiguous descriptions would lead to inaccurate assessment of EPS contribution to heavy metal sorption or immobilization, and would hinder the further use of white rot fungi, in particular, some direct application of the fungal EPS in heavy metal treatment. Therefore, it is necessary to study the contribution characteristics of EPS when white rot fungi coping with toxic heavy metal.

In this study, the basic property of EPS produced by the model white rot fungi *Phanerochaete chrysosporium* (*P. chrysosporium*) was researched, including the component and the amount changes of EPS. Investigations on the contribution characteristic of the in situ EPS to Pb immobilization was carried out in batches of Pb contact experiment, including the effect of Pb concentration, pH, EPS amount and EPS age.

Materials and methods

Culture experiment

Phanerochaete chrysosporium (BKMF-1767) (CCTCC AF96007) was grown on potato dextrose agar plates at 37 °C for several days. Spores on the agar surface were diluted in sterile distilled water and controlled at 2.5×10^6 spores per mL [8]. 1.5 mL of spore suspension was inoculated into 200 mL of sterile potato dextrose broth in 500 mL flasks at room temperature. The culture process was undertaken in a constant temperature incubator shaker at 150 rpm, 30 °C.

EPS extraction

Mycelia in one flask were separated from the culture medium with filter paper and washed three times with ultrapure water. The obtained mycelia were re-suspended in 50 mL of ultrapure water and then centrifuged at 10,000 rpm for 15 min. The centrifuged suspension was separated from the fungal mycelia and used as EPS solution. The mycelia after centrifugation were seen as mycelia without EPS. There were additional compounds to EPS in the solution even if EPS might be the major component, such as oxalate as reported in the previous study [8]. However, that kind of oxalate was most likely to be oxalate metal which nearly has no ability to immobilize Pb^{2+}. From this point, the additional compounds to EPS in the solution had little influence on the calculation of all the results in the manuscript.

Characterization of EPS

The carbohydrate content in EPS solution was determined with the phenol–sulfuric acid method, using glucose as standard. The protein content was determined by coomassie brilliant blue G-250 method, using bovine serum albumin as standard.

Pb^{2+} immobilization by mature mycelia with EPS and mature mycelia without EPS

The white rot fungal mycelia were cultured for some days and then separated from the medium for Pb^{2+} immobilization experiment. Mycelia with EPS or mycelia without EPS were exposed to Pb^{2+}. $Pb(NO_3)_2$ was used as the source of Pb^{2+}.

Choose of exposure time between mycelia and Pb^{2+} in the preliminary experiment

In the preliminary experiment with mycelia exposed to 50 mg L^{-1} of Pb^{2+}, Pb concentration in the solution was sampled at different exposure time. It was found out the saturation point existed at 4 h which was used as the exposure period for the following experiments (Online Resource 1).

Effect of Pb^{2+} concentration

Mycelia with EPS or mycelia without EPS were exposed to different concentrations of Pb^{2+}, i.e. 10, 50 and 100 mg L^{-1}.

Effect of pH

pH may influence the protonated state of the functional groups in EPS, which further affect the contribution of EPS to Pb^{2+} immobilization [23]. Therefore, the effect of pH was considered in this study. Mycelia with EPS or mycelia without EPS were exposed to 50 mg L^{-1} of Pb^{2+} for 4 h. The pH condition was adjusted to a range from 3.0 to 7.0 with 0.1 mol L^{-1} of NaOH or 0.1 mol L^{-1} of HCl.

Effect of EPS amount

Different amount of 5-day-old fungal mycelia with EPS were exposed to 50 mg L^{-1} of Pb^{2+} for 4 h. With the same

original amount of mycelia with EPS, EPS extraction was carried out and obtained the corresponding portion of mycelia without EPS, which were also exposed to 50 mg L^{-1} of Pb^{2+} for 4 h. EPS amount was calculated with the content summation of carbohydrates and protein and expressed with the unit of mg L^{-1}.

Effect of EPS age

Based on the knowledge of EPS composition during the fungal growth, a certain amount of 3-, 5- and 7-day-old fungal mycelia was used for Pb^{2+} immobilization experiment, ensuring the same EPS amount in each treatment.

Analysis of Pb concentration

The sample was acidified with 3% (v:v) HON for the estimation of Pb concentration, which was determined with atomic absorption spectrophotometer. The instrument was calibrated with Pb^{2+} standard solutions. The final results were expressed in relation to the dry weight (dw) of the harvested mycelia, which was measured after the harvested mycelia were freeze dried.

Statistical analysis

All experiments in this study were performed in triplicates and mean values were used in the analysis.

Results and discussion

Characteristic of EPS

The compositions of EPS extracted from the mycelia are shown in Fig. 1. The results revealed that carbohydrates were always the main component of EPS during the whole fungal culture period. By comparison, there was much less protein which was out of our initial expectation. This may be due to the physical property of carbohydrates made them more easily attached to the cell wall, while most protein secreted by the fungus probably was soluble in solution. We further detected the soluble protein content at 5 days and found it was 40.6 mg L^{-1}, much more than the biosorbed protein when both the two parts of protein were converted into mg as a unit. As displayed in Fig. 1, most of time both of the two compositions of EPS continuously decreased, which was due to the increase of EPS amount was much slower than the increase of fungal mycelia weight. Mycelia used for the batches of Pb^{2+} immobilization experiment was 5-day-old, adsorbed 92.02 mg g^{-1} of carbohydrates and 5.26 mg g^{-1} of protein. The fungal EPS production at different growth stage was different. EPS amount produced by fungus increased during the previous 2-day culture and then kept at a constant level from 3 to 4 days or from 5 to 6 days. At 7 days, the EPS amount decreased. As the two main compositions in EPS, carbohydrates and protein contain functional groups, like carboxyl (–COOH), hydroxyl (–OH) or amino ($–NH_2$) groups, which provide negative charges for the molecules [20] and is important for EPS to bind cations, such as heavy metal ions.

Role of EPS in the immobilization of Pb

As shown in Fig. 2, with initial Pb^{2+} content of 10 and 50 mg L^{-1}, the immobilization rate of Pb^{2+} by mycelia with EPS was close (57.4 and 57.7%, respectively), so was that by mycelia without EPS (41.7 and 41.6%, respectively). However, with 100 mg L^{-1} of Pb^{2+}, the immobilization rate of Pb^{2+} by the two kinds of mycelia decreased obviously (39.7 and 27.6%, respectively). It is clear that the Pb^{2+} immobilization rates by mycelia with EPS were always higher at the three different initial Pb^{2+} concentrations. It was proved that the absence of EPS affected the Pb^{2+} immobilization absolutely, while the mycelia without EPS still displayed efficient removal ability of Pb^{2+}.

Those findings demonstrate that EPS is important for *P. chrysosporium* to immobilize Pb^{2+}. The most significant difference in the immobilization rate of Pb^{2+} between the two kinds of mycelia was 15.7% at 10 mg L^{-1} of Pb^{2+}, with 16.1 and 12.1% at 50 and 100 mg L^{-1} of Pb^{2+}, respectively. It seems that EPS was proportionally more important for Pb^{2+} immobilization at lower initial Pb^{2+} concentration. When initial Pb^{2+} concentration increased, the probability of binding sites in EPS to contact with Pb^{2+} in solution was greater. If there were enough binding sites in EPS, the immobilization rate of Pb^{2+} would be kept at a constant level around 16%. Considering the above result, we speculate that the binding ability of EPS probably reached saturation when mycelia faced with more than 50 mg L^{-1} of Pb^{2+} and in that situation the fungal intracellular immobilization of Pb^{2+} became more vital.

Effect of pH on the EPS contribution to Pb immobilization

Figure 3 presents the immobilization of Pb^{2+} by mature mycelia with EPS and mature mycelia without EPS as a function of pH. For the mycelia with EPS, the removal of Pb^{2+} was more efficient at higher pH. However, the situation for the mycelia without EPS was apparently different, with highest removal rate of Pb^{2+} at pH 5.0. Comparison between the two kinds of mycelia found out that Pb^{2+} was more efficiently removed by mycelia with EPS, and the difference as presented in Fig. 3 became more obvious as the increase of pH.

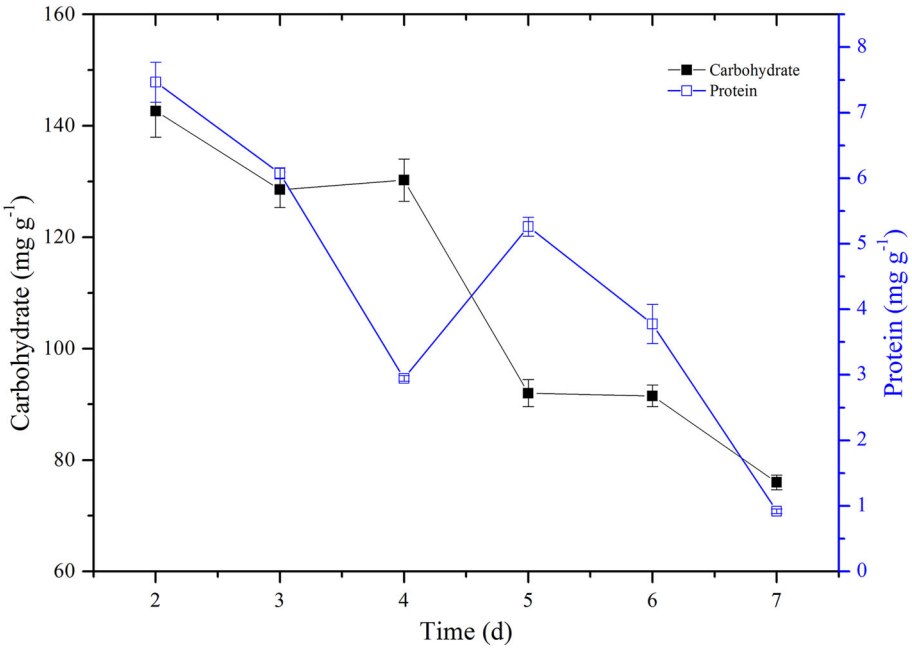

Fig. 1 Characteristic of EPS produced by *Phanerochaete chrysosporium* (150 rpm, 30 °C)

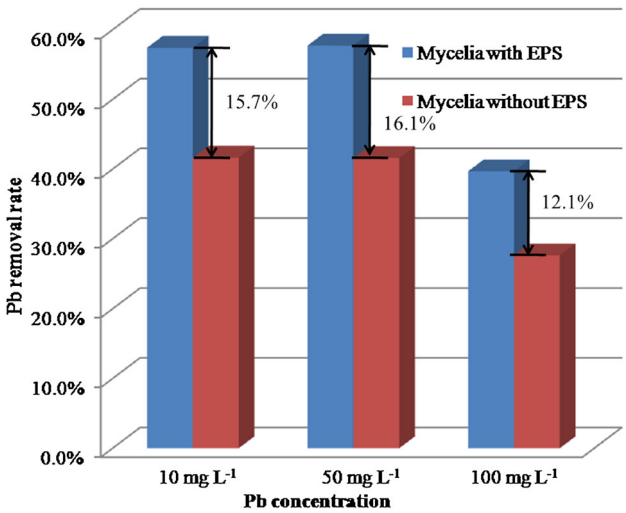

Fig. 2 Effect of Pb concentration on Pb removal ability by EPS (150 rpm, 30 °C, 5-day-old mycelia)

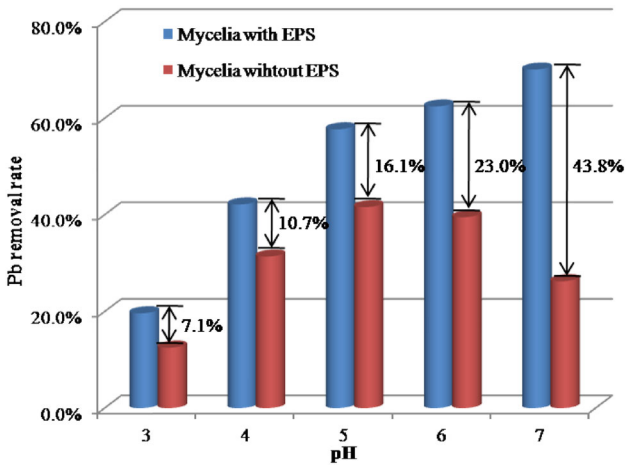

Fig. 3 Effect of pH on Pb removal ability by EPS (50 mg L^{-1}, 150 rpm, 30 °C, 5-day-old mycelia)

In aquatic system, pH is considered to be the main parameter affecting the affinity of metal ions to biological surfaces due to the competition between the proton and metal ions for available binding sites [24]. The results obtained above proved that the binding sites in EPS contributed to the immobilization of Pb in the whole pH range of 3.0–7.0 and the contribution became greater when pH increased. Functional groups, such as carboxylic (p*K* 4.0–6.0), phosphoric (p*K* 7.0–7.4), thiol and amino (p*K* 7.0–9.0), hydroxyl groups (p*K* 11.0) [25], display different deprotonated or protonated states with pH changes. From the pH range investigated in the study, we can suppose carboxylic group in EPS is probably the main functional group involved in Pb immobilization. Within the pH range from 4 to 6.0, most carboxylic units are deprotonated and present high metal retention ability. When pH in the solution was higher than 6.0, the functional groups seemed not to be so effective from the aspect of p*K* values. Even though, the mass removal of Pb^{2+} by EPS was also obtained. At pH 7.0, Pb^{2+} was prone to react with hydroxide ions in the solution and form lead hydroxide particles, which cannot be ignored. Because of the mucilaginous characteristic of EPS, the original thought Pb^{2+} immobilization probably was accomplished by the adhesion of lead hydroxide particles to EPS.

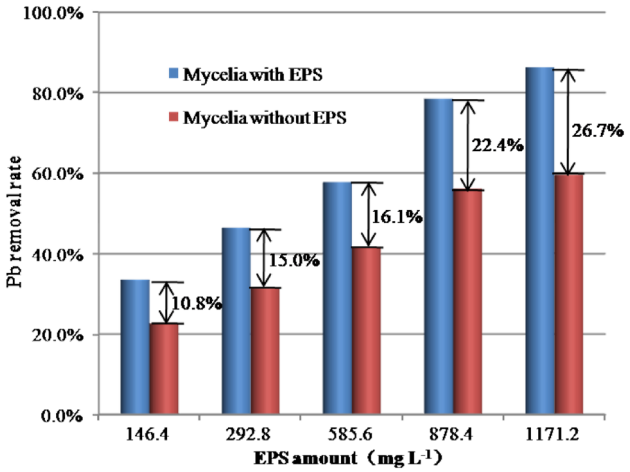

Fig. 4 Effect of EPS amount on Pb removal ability by EPS (50 mg L^{-1}, 150 rpm, 30 °C, 5-day-old mycelia)

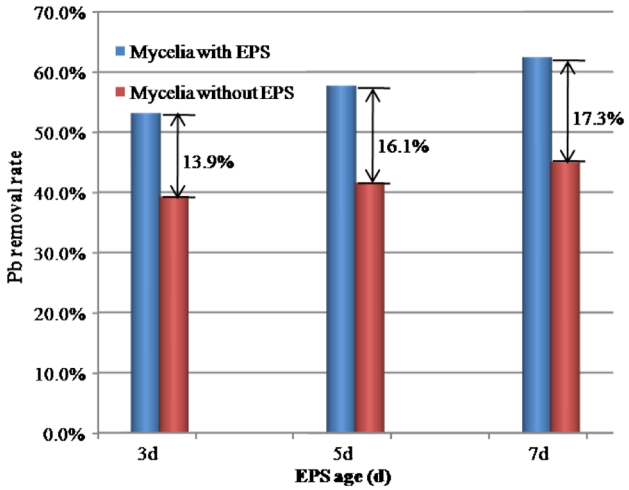

Fig. 5 Effect of EPS age on Pb removal ability by EPS (50 mg L^{-1}, 150 rpm, 30 °C)

Effect of EPS amount on Pb immobilization

The results of Pb^{2+} immobilization on 5-day-old EPS when the in situ EPS amounts ranging from 146 to 1171 mg L^{-1} are presented in Fig. 4. The increase of the in situ EPS amount led to a higher Pb immobilization efficiency by EPS, increased from 10.8 to 26.7%, which probably is mainly due to the increase of immobilization sites and the surface area. Nevertheless, the Pb^{2+} specific immobilization decreased from 37 to 11.4 mg g^{-1} EPS. That is because that with constant initial Pb^{2+} amount and the increase of EPS amount, the proportion of immobilization sites to contact with Pb^{2+} became lower. Similar trend in EPS sorption characteristics was also reported by Dogru et al. [26].

Effect of EPS age

In this study, we chosen same amount of EPS at 3, 5 and 7 days and analyzed the Pb^{2+} immobilization characteristic by in situ EPS produced at different period. The result is presented in Fig. 5. It can been seen that the immobilization rate of Pb^{2+} was least for EPS at 3 days (13.9%), that by EPS at 5 days was 16.1% and at 7 days was 17.3%. As the growing curve of *P. chrysosporium* reported before [8], the 3-day-old fungus was at its accelerate phrase, the 5-day-old fungus was at its stationary phrase. At 7 days the fungal biomass began to decrease, which was at the decline phrase and the sign of mycelia hydrolysis due to the lack of nutrient in the environment. The result implied that EPS resourced from mycelia hydrolysis was most beneficial to the immobilization of Pb^{2+}, EPS from resting mycelia the second and EPS from accelerate phrase the last. The main reason for the effect of EPS age on Pb immobilization probably lies in the composition change of EPS. On the one hand, the proportion of carbohydrates and protein in EPS changed during fungal growth as proved in Fig. 1. On the other hand, the specific material species of EPS probably changed and affected its contribution to Pb removal. The secondary metabolite was secreted by wood rooting fungi at decline phrase, such as polyketides, nonribosomal peptides, and terpenoids, which play a critical role for fungal survival and success, and the array of secondary metabolism genes has also been reported [27].

Conclusion

This study investigated the contribution characteristics of the in situ EPS in *P. chrysosporium* to Pb immobilization. Highest EPS amount was produced after 5-day culture. The main component of EPS was carbohydrates, with only a small part of protein and the ratio of carbohydrates to protein changed during the fungal growth. EPS was demonstrated to play a more important role in immobilizing Pb^{2+} at lower initial Pb concentration. The increase of pH in the solution was beneficial to the Pb immobilization in EPS. Higher EPS amount increased the Pb removal efficiency at a certain extent, while the specific uptake decreased. EPS produced at 7 days was most beneficial to the Pb^{2+} immobilization, which probably lies in the composition change of EPS, including not only the proportion of carbohydrates and protein, but also the existence of the secondary metabolite. This investigation could provide detailed information for the biology of white rot fungi.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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