Dear  Reviewers,

Re: Degradation of Polycyclic Aromatic Hydrocarbons in Contaminated Soil by Immobilized Laccase (No.: 5946-30286-2-SM)

By Xin Wang, Shiyu Sun, Zijun Ni, Zhaoxing Li and Jia Bao

Many thanks for your email of 17 November 2017, regarding the revision and advice of the above paper. Overall the comments have been fair, encouraging and constructive. We have learned much from it. After carefully studying the reviewer’ comments and advice, we have made corresponding changes to the paper. The relevant regulations had been made in the original manuscript according to the comments of reviewers, and the major revised portions were marked in red and some grammar and spelling errors had also been corrected. Moreover, we have added some important details,and the new contents are enclosed.

Thank you very much for the excellent and professional revision of our manuscript.

Sincerely yours,

Jia Bao

(1) *Preparation of crude laccase.* A certain quantity of straws (each about 3 cm long) was weighed into a conical flask, then water was added to soak for one day and the wet straws were sterilized at high temperature and high pressure for 30 min. After cooling, the straws were used as raw material of producing enzyme culture medium. We grafted white-rot fungi (Lenzites betulinus) into this culture medium and fostered it in an incubator at 26ºC, then added 100 ml aseptic abstract liquid and soaking-draw under 26ºC and 120 revolutions per minute (rpm) for 24 h. For removing the impurity, the mixture was centrifuged for 15 min at 4,000 rpm and the supernatant was collected as crude laccase.

(2) *Determination of free laccase* *activity.* ABTS was used to determine the activity of free laccase and immobilized laccase. Reaction solution (1 mL) was made of HAc-NaAc (500 μL, 50 mM, pH=4), H2O (390 μL), ABTS (100 μL,500 μM) , and laccase liquid (10 μL). The reaction solution was placed in UV-V spectrophotometer and the temperature was set as 28℃, then the increase in absorbance during 3 min at 420 nm was used to calculate the free laccase activity (ε420=36000 L/mol·cm). The free laccase activity was calculated by the following formula:

 

 △A was increment of absorbance during 3 min, V was reaction solution (1 mL) and Vl was the sterile leach liquor, △t was 3 min, V0 was laccase liquid (0.01 mL) . l was the inner diameter of cuvette, l = 1 cm, and ms was the quality of the medium. One unit (U) of laccase activity was defined as the amount of laccase used for catalytic oxidation of ABTS (1 μM) after 1 min.

 Determination of immobilized laccase activity. Immobilized laccase (0.01 g) was accurately weighed and added into beaker, reaction solution (2 mL) was made of HAc-NaAc (1000 μL, 50 mM, pH=4) , H2O (780 μL), ABTS (200 μL, 500 μM) and laccase liquid (20 μL), and reaction solution was mixed for 10 min at room temperature. Then the supernatant was taken into UV-V spectrophotometer and starting the reaction. as well as determining free laccase activity and increasing the absorbance to 420 nm during 3 min. Measurement data was used to calculate the immobilized laccase activity. The activity was calculated by the following formula:



ΔA was increment of absorbance during 3 min, V was 2 ml of reaction solution, t was 3 min, M0 was 0.01 g immobilized laccase, and Ui was the activity of immobilized laccase.

(3) *Experimental design.* The immobilized lactase of the two methods was used to repair the soil contaminated by polycyclic aromatic hydrocarbons. Mud and soil in the soil were 3:1, solution was HAc-NaAc buffer (pH=4), and the initial concentration of pollutants was 100 g/ml. The immobilized lactase was put into the soil was 1g/50ml, and the free laccase was used as the control. Some of the environmental factors that may be encountered in the experiment need to studied. First was the different temperature, including 20℃ (room temperature) and 40℃, at which pH was 4. Secondly, the different pH values were set as 4 and 6, at this time the temperature was 40. The running time of the experiment was 72 h.

(4)*The recovery rates of immobilized laccase and free laccase*

As shown in Fig 1, in the case of different environmental conditions or different carriers, the recovery rates of laccase had obvious difference. From the point of recovery rate, the free laccase had loss more activity than immobilized laccase, however, during the immobilization process, the laccase had loss about 50% activity.

With the constant development of immobilized technology and the deepening of the study on laccase, the application of immobilized laccase has shown a bright future. At present, the immobilized laccase has been emmploed in the areas of decolorization of dyes[25,26], degradation of various pollutants[19,27], and biosensing[28,29]. So far, most of the studies have been performed on immobilized laccase aimed at the degradation of organic contaminants in water[25,26,30]. In this study, fungal laccase was immobilized on nylon net and chitosan, and then applied the immobilized laccase on the degradation of Pyr and Bap in contaminated soil. Results have indicated that the methods adopted in this study were effective. In addition, laccase have been successfully immobilized on many different materials, such as silica gel[31], non-porous acrylate beads[26] and magnetic chelator particles[32]. In this study, the nylon net and chitosan as immobilized carriers have the advantages of cheap, practical and readily availability, which could reduce the cost of the application and simplify the operation processes of immobilized laccase. Previous studies[33] have showed that through immobilization, the loss of the laccase activity decreased greatly, and the activity of laccase was considerably stable in general. Under different pH, it could be seen that the laccase can maintain certain activity in near neutral or acid condition.

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Fig. 1 The recovery of immobilized laccase and free laccase in different conditions

1)left: different pH,2)right: different temperature)