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Fabrication of a novel immobilization system and its application for removal of anthracene from soil

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ABSTRACT

The remediation of anthracene (ANT) polluted soil has become a concern due to its difficult diffusion from soil particles and adverse impacts on environmental and human health. To remove ANT from soil efficiently, *Ganoderma lucidum* (*G. lucidum*) mycelia pellets combined with corn-cobs were encapsulated in hydrophobically modified Ca-alginate (CA) by poly-ϵ-caprolactone (PCL), without the need for enzyme purification. The optimum PCL concentration in CA/PCL/corn-cobs bead was 12%, which provided enzymes secreted by the *G. lucidum* mycelia with a good storage stability and a similar activity to the immobilized laccase. Scanning Electron Microscope and Brunauer–Emmett–Teller method were applied to characterize the bead. CA/PCL/corn-cobs ensured the highest ANT removal efficiency regardless of different pH values and temperatures. The ANT removal from soil followed third-order reaction model. Removal of ANT reached 96.2% ± 2.0% from soil after 20 days of incubation at pH 5.0 and 45 °C, which is attributed to the fast secretion of enzymes stimulated by lignocellulosic substances of the corn-cobs, the protection of enzymes by matrix capsules, and the strong adsorption of ANT on the relatively hydrophobic surface of bead. The efficient ANT removal was also observed in aged soil samples simulated by addition of soil organic matter.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have become ubiquitous pollutants in the ecosystem, as a result of fossil fuel combustion, the release of petroleum, and improper disposal and accidents during transport [1,2]. Since their toxicity, carcinogenicity and mutagenicity with respect to both environment and human health [3,4], 16 PAHs have been listed as priority pollutants by the U.S. Environmental Protection Agency (USEPA). Anthracene (ANT), a nonpolar and hydrophobic PAH, has relatively serious toxicity [5]. Once ANT enters the body, it appears to target the skin, stomach, intestines and the lymphatic system, and it is a probable inducer of tumors [6]. Moreover, ANT, whose basic structure is repeated in other high-molecular-weight PAHs, such as benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene, is much less susceptible to degradation than phenanthrene and pyrene due to its low solubility (0.05 mg L^{-1}) in aqueous phases [7,8]. Hence there is a great demand for the removal of ANT from contaminated soil.

Several white rot fungi, such as *Phanerochaete*, *Trametes* and *Pleurotus*, have been applied for the cleanup of pollutants depending on a group of extracellular enzymes secreted by them [9,10]. The enzymatic oxidation of ANT by these extracellular ligninolytic enzymes, i.e., laccase, lignin peroxidase, and manganese peroxidase (MnP), has been suggested as a method to degrade the anthropogenic contaminant [11,12]. It has been reported that the laccase secreted by mycelia of mushroom *Ganoderma lucidum* (*G. lucidum*) shows a tremendous potential for degrading PAHs in the environment [13]. Immobilization has been demonstrated for purified ligninolytic enzymes to protect them against deactivation and increase their stability [14,15]. However, pure ligninolytic enzymes are too expensive to produce on a large scale required to be used for catalytic ANT degradation. It is clear that cost reduction is of great significance to the industrial application, and this disadvantage of high cost may be mitigated by immobilizing mushroom mycelia.

Immobilization of white rot fungi provides the possibility to avoid the loss of enzyme activity during their purification, to ensure a highly effective degradation of pollutants, and to facilitate their handling, separation, and environmental friendliness [16]. Corn-cobs are inexpensive and biodegradable lignocellulosic materials from agricultural residues, and are quite a lot available in China [17]. Experiments have demonstrated that employing inside corn-cobs as the carrier of *Phanerochaete chrysosporium* and *Mucor* sp. resulted in considerably high ligninolytic activity [18]. In another work, highly purified metabolites of filamentous fungi are obtained

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though the entrapment of mycelia fragments in Ca-alginate (CA) [19]. To date, although there are a few reports covering the degradation of PAHs by mushroom *G. lucidum*, the immobilization of *G. lucidum* mycelia has not been studied yet. Considering the practical application, a number of limitations still restrict the use of traditional immobilized carrier in complex soil environment. First, corn-cobs cannot offer adequate protection for *G. lucidum* mycelia from long exposure to extremes of pH or temperature, toxic compounds and microbial infection. Second, PAHs tend to interact with non-aqueous phases and soil organic matter and, as a consequence, become potentially unavailable for degradation [20]. Third, it is required that the immobilized carriers should be biodegradable and eco-friendly instead of being reusable for bioremediation of PAH-contaminated soil. Fourth, release of enzymes from the CA bead, including their fabrication, is an issue which lowers immobilization efficiency and removal efficiency. These problems may be solved by a modification of traditional immobilized system.

Poly-ϵ-caprolactone (PCL) is a semi-crystalline, hydrophobic polymer material [21]. Owing to its high biodegradability, biocompatibility and permeability, PCL is ideally suitable for long-term drugs delivery and widely used in the fields of nanotechnology and medicinal chemistry [22,23]. However, no application is associated with PAH-contaminated soil treatment.

The purpose of this work is to study the highly efficient removal of ANT by *G. lucidum* from contaminated soil samples. Due to the hydrophobic character of PCL, modified alginate hydrogels have been utilized as carriers of *G. lucidum* mycelia to achieve the goal of strong adsorption of ANT. In order to reduce leaching of enzymes from carriers, an optimum concentration of PCL in the modified hydrogel is also analyzed. Since corn-cobs have a stimulatory effect on the secretion of extracellular ligninolytic enzymes, we choose the corn-cobs as inoculated matrix of mycelia inside the immobilization system. The mechanism of ANT removal from soil samples through the immobilized mycelia was analyzed. To investigate the applied potential of the immobilized mycelia in CA/PCL/corn-cobs beads, the extremely environmental effects on removal efficiency were also examined. Moreover, the ANT removal efficiency of immobilized mycelia in the aged soil samples simulated by addition of soil organic matter (SOM) was also observed.

2. Materials and methods

2.1. Materials

A *G. lucidum* strain was obtained from the Soil and Fertilizer Research Institute affiliated to Sichuan Academy of Agricultural Sciences (Chengdu, Sichuan Province, China). The fresh corn-cobs were acquired from a local supermarket. PCL (M.W. = 60,000, Sigma–Aldrich) was employed as the modified material. The laccase (E.C. 1.10.3.2) with a 29 U mg^{-1} solid activity, manganese peroxidase (MnP, E.C. 1.11.1.13) with an activity of 18 U mg^{-1} protein and their substrate 2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS, ≥99% purity), ANT (≥97% purity), sodium alginate and humic acid were purchased from Sigma–Aldrich. Other reagents were of analytical grade and purchased from Kelong Chemical Reagent Factory (Chengdu, Sichuan Province, China).

2.2. Soil preparation

Soil samples without any detected PAHs were collected from Sichuan University, Chengdu, China (30°37′54″N, 104°05′1″E), at a depth of 0–20 cm. After the removal of stones and visible plant material, the soil was air-dried, ground, and sieved though 2-mm mesh. The basic physico-chemical properties of soil are as follows: total organic matters (TOC), 1.24%; total nitrogen (TN), 0.19%; total phosphorous (TP), 0.046%; total potassium (TK), 2.48%; sand, 10.8%; silt, 79.0%; clay, 10.2%, and pH 6.8. The pH of the soil samples was measured by a pH meter (Model pHS-25) in 1:1 soil solution in distilled water. To adjust the pH of soil, 0.1 M NaOH and 0.1 M HCl was added to the soil solution to obtain the proper pH values and then the soil solution were equilibrated under stirring (120 rpm) for 24 h, then the solid phases were obtained by centrifugation for 10 min at 1200 × *g* (D-37520 Osterode, Germany), and were air-dried using for the following experiments.

Table 1
Compositional data of corn- cobs (wt%, oven-dry basis).

Fraction	Corn-cobs (%o.d. weight)	SD[a]
Cellulose	32.5	0.42
Hemicellulose	41.8	0.36
Lignin	19.6	0.18
Extractives	6.1	0.12

Results have been calculated from six replicate determinations.
[a] SD: standard deviation.

The above soil was spiked with ANT in a manner that was optimized for uniformity of contamination and minimal damage to soil environment [24]. The procedure mixed clean and non-sterilized bulk soil with PAH-spiked soil to bring the final concentration of ANT to 200 mg kg^{-1}. To simulate environmental conditions, we selected acetone as the optimal solvent for the maximal solubility of ANT [11]. 1.44 g of ANT was dissolved in acetone and added into a 25% fraction (1.8 kg) of the total soil samples. After thorough mixing, the slurry was allowed to evaporate for 2 days in a dark fume hood, and then separated into eighteen 0.1 kg aliquots. For each pot (bottom diameter 8.0 cm, top diameter 10.5 cm, depth 9.0 cm), 0.1 kg of soil was added to 0.3 kg ANT-free soil. All the spiked soil considered as newly contaminated soil was mixed thoroughly and stored in dark at room temperature.

2.3. G. lucidum mycelia culture

Fresh corn-cobs were air-dried, milled mechanically, and sieved through 1-mm and then 0.9-mm mesh to obtain particles with a size of approximately 1.0 mm for mycelia immobilization. The corn-cobs, moistened with ultrapure water, were sterilized at 121 °C for 30 min before use. Corn-cob composition was determined according to standard methodology [25] and is shown in Table 1.

The *G. lucidum* was transferred from slant tubes maintained at 4 °C to malt extract agar plates composed of (L^{-1} of distilled water) malt extract 15 g, glucose 15 g, polypeptone 2 g, KH_2PO_4 2 g, $MgSO_4{\cdot}7H_2O$ 1 g, VB_1 1.5 mg, agar 20 g. The pH of the medium was adjusted to 4.5 with 20 mM sodium acetate and kept at 25 °C for 5–7 days. The liquid growth medium was conducted in 100-mL Erlenmeyer flasks containing 50 mL malt extract medium. Five agar disks (1.5 mm in diameter) of active mycelia of *G. lucidum* from 6-day-old cultures were inoculated on corn-cobs in the flask. All the flasks were agitated (160 rpm) in the dark at 28 °C for several days to achieve similar radical growth. All of the culture media above were sterilized at 115 °C for 30 min before use.

2.4. Immobilization in CA/PCL matrix capsules

The immobilization of *G. lucidum* mycelia via entrapment was performed according to procedures described previously [26]. Briefly, 2 g sodium alginate was dissolved in 100 mL deionized water utilizing an agitator. PCL solution which was prepared using 99.5% pure chloroform at a concentration of 13.2% (w/v) PCL [27], was then added and stirred for 8 h for homogenization as well as to evaporate the chloroform. Finally, the mycelia pellets at the