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Pyrene biodegradation with layer-by-layer assembly bio-microcapsules

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ABSTRACT

Biotechnology is considered as a promising technology for the removal of polycyclic aromatic hydrocarbons from the environment. Free bacteria are often sensitive to some biotic and abiotic factors in the environment to the extent that their ability to effect biodegradation of organic pollutants, such as polycyclic aromatic hydrocarbons, is hampered. Consequently, it is imperative to carry out investigations into biological systems that will obviate or aid tolerance of bacteria to harsh environmental conditions. Chitosan/alginate bio-microcapsules produced using layer-by-layer (LBL) assembly method were tested for pyrene (PYR) biodegradation under harsh environmental conditions. Morphology observation indicated that the flake bio-microcapsules could be successfully prepared through LBL assembly method. Surface analysis showed that the bio-microcapsules had large fractions of mesopores. The results of the biodegradation experiments revealed that the 95% of 10 mg L^{-1} PYR could be removed by the bacteria encapsulated chitosan/alginate bio-microcapsules in 3 days, which was higher than that of the free bacteria (59%). Compared to the free cells, the bacteria encapsulated chitosan/alginate bio-microcapsules produced 1–6 times higher PYR biodegradation rates at a high initial PYR concentration (50 mg L^{-1}) and extremely low pH values (pH = 3) or temperatures (10 °C or 40 °C), as well as high salt stress. The results indicated that bacteria in microcapsules treatment gained a much higher tolerance to environmental stress and LBL bio-microcapsule could be promising candidate for remediating the organic pollutants.

1. Introduction

The remediation of polycyclic aromatic hydrocarbons (PAHs) is of great concern for environmental protection and public health (Lu et al., 2010), owing to the persistence, cytotoxicity and bioaccumulation of the PAHs. Bioremediation, having cost and technical advantages over other remedial options (e.g., physical or chemical techniques) (Zeng et al., 2000), is considered as a promising strategy to remove PAHs from the environment. However, the use of free bacteria for pollutant remediation was often sensitive to important biotic and abiotic factors in the environment (e.g., high pollutant concentrations, high/low temperature and pH conditions) (Zheng et al., 2009).

To improve the adaptation of free bacteria and to enhance the bioremediation of organic pollutants, different bacterial immobilization methods have been developed. Among these traditional methods, the physical adsorption of bacteria on different carriers (Liu et al., 2012; Lin et al., 2010) or clay minerals (Biswas et al., in press) and entrapment of bacteria in alginate beads (Zhang et al., 2008; Tan et al., 2014; El-Naas et al., 2009) are commonly used. However, both approaches have shortcomings, e.g., the protection provided by the physical adsorption carriers for bacteria is frequently limited for the bacteria can't be completely coated by the carriers, and the dense gel layers of the entrapment alginate beads often interfere with the mass transfer of the substrates, pollutants and intermediate products (Zhang et al., 2008).

The layer-by-layer (LBL) assembly of microcapsules prepared from polyelectrolyte polymers containing opposite charges was developed as a strategy to immobilize lipase (Jiang et al., 2009). The strategy provided lipase protection and allowed free entrance of substrates and discharge of products via micropores in the capsule wall (Jiang et al., 2009), aiming to resolve some drawback of the conventional methods. Chitosan (CHI) and alginate (ALG) are two types of natural polymers that commonly biodegradable and biocompatible. Furthermore, both are polyelectrolyte polymers, containing different charges that enable them form microcapsules through electrostatic attraction. Many researchers had applied them for preparing oral delivery alginate/chitosan beads or porous scaffolds (Tahtat et al., 2013; Han et al., 2010). Lu et al. (2012) had used them to prepare alginate-chitosan-alginate (ACA) capsules with no sacrificial templates for immobilizing organic pollutants degrading bacteria. Compared to

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the free bacteria, the superiority of the ACA immobilized ones was not significant at different pH conditions, which might be due to the big size of the capsules resulting from using no sacrificial templates in preparation.

To improve bacteria's tolerance to the severe conditions in the environment, the current study seeks to capitalize on and expand the advantages of using LBL assembly microcapsules for bioremediation materials (BMs) preparation. In this study, CHI and ALG were employed to prepare microcapsule-encapsulated bacteria (bio-microcapsules, a kind of BMs) for PAHs biodegradation. Calcium carbonate ($CaCO_3$) particles were used as sacrificial templates. Four-ring pyrene (PYR) was selected as a representative of the PAHs for its common occurrence in the environment. Furthermore, the tolerance of bio-microcapsule to some severe environment conditions (i.e., high initial PYR concentrations, and inappropriate temperature, pH and salt stress conditions) during PYR biodegradation was also investigated.

2. Materials and methods

2.1. Materials and organism

CHI was purchased from Aladdin Chemical Reagent Co. (Shanghai, China; A. R. grade, purity ≥ 98%). The viscosity is 100–200 mPa s, molecular weight (M_w) =179. ALG was purchased from the Tianjin Fuchen Chemical Reagents Factory (Tianjin, China; A. R. grade, purity ≥ 98%). PYR was purchased from Sigma-Aldrich (purity ≥ 98%) and its acetone stock solution at 400 mg L^{-1} was prepared and stored in brown bottle placed at 4 °C. Working solutions were prepared by diluting appropriate amounts of acetone stock PYR in mineral salt medium (MSM) after acetone volatilization. Nutrient broth was purchased from Huankai Microbial Sci. & Tech. Co., Ltd. (purity ≥ 98%).

The microorganism used in this study was *Mycobacterium gilvum* CP13, which was isolated from the activated sludge of a coking plant in Shaoguan, Guangdong, China (Wu et al., 2014). The bacteria were cultured in fresh nutrient broth (aqueous solution; 1.8%) for 2–3 days to an optical density (OD) of 2. After being centrifuged, the bacteria were resuspended with 0.9% NaCl for immobilization according to the method by Deng et al. (2016a). Bacteria were inactivated for the control treatments.

2.2. Preparation of LBL CHI/ALG bio-microcapsules

LBL assembly bio-microcapsules with *Mycobacterium gilvum* CP13 for the experiments were prepared using the layer-by-layer assembly technique by alternately deposition of oppositely charged polyelectrolytes on $CaCO_3$ particles according to the method described by Deng et al. (2016b). Firstly, CHI solutions and bacteria suspension was mixed with $CaCl_2$. Na_2CO_3 was then added drop wise under stirring to form CHI-doped $CaCO_3$ microparticles, which could then adsorb the ALG. Subsequently, the ALG was added and deposited to allow a new layer. The above cycle was repeated, and the multilayer structure was formed, and then the $CaCO_3$ cores were dissolved in EDTA.

2.3. Characterization of LBL CHI/ALG bio-microcapsule

The surface Zeta-potentials of $CaCO_3$ microparticles after the deposition of CHI/ALG layer were measured on a Dalven Zeta-potential analyzer. Five parallel measurements were conducted for every sample and the average values were reported.

Morphological analysis of the LBL CHI/ALG bio-microcapsule were performed by a scanning electron microscope (SEM QUANTA 400) with the acceleration voltage (Acc. 5 kV) and a transmission electron microscope (TEM). Prior to being observed by the SEM, the samples were freeze dried and then coated with thin layer of gold. Thin sections of the samples were stained with 2% uranyl acetate and lead citrate, and then observed in a PHILIPS TECNAI 10 TEM (see Supporting Information).

The specific surface area (BET method) of the bio-microcapsule samples were measured by a surface area analyzer (ASAP 2020M). 300 mg dried LBL samples were degassed under a nitrogen atmosphere at 80 °C for 6 h.

X-ray photoelectron spectra (XPS) were obtained using a PHI X-tool X-ray photoelectron spectrometer (Ulvac-Phi, Inc., Kanagawa, Japan) equipped with a non-monochromatic Al Kα X-ray source operating at 15.0 kV, 51 W for all data acquisitions. Survey spectra were recorded for the 0–1000 eV binding energy range, at pass energy of 140 eV. Analyses were performed at emitted photoelectron take-off angles of 45°. The radius of the analyzed area during XPS analysis was 0.2 mm.

2.4. PYR biodegradation experiments

PYR degradation in an aqueous solution using CP13 as free cells (FCs) and LBL bio- microcapsules was conducted in batch experiments in 100-mL shake flasks containing 20 mL of mineral salt medium (MSM) with PYR as the sole carbon source. The MSM consisted of the following: 2.5 g L^{-1} K_2HPO_3, 0.77 g L^{-1} KH_2PO_4, 100 mg L^{-1} $(NH_4)_2SO_4$, 20 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 10 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 1.2 mg L^{-1} $FeSO_4{\cdot}7H_2O$, 0.3 mg L^{-1} $MnSO_4{\cdot}H_2O$, 0.3 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, and 0.1 mg L^{-1} $CoSO_4{\cdot}7H_2O$, 0.1 mg L^{-1} $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. The effects of the initial PYR concentration (10, 30, and 50 mg L^{-1}), as well as the pH (3, 7, and 10), temperature (10 °C, 30 °C, and 40 °C) and salt concentration (NaCl, *w/V*, 0, 1%, 2%), on the PYR biodegradation were investigated in the FC and LBL bio-microcapsule systems, respectively. The PYR in the FC and LBL bio-microcapsule systems was ultrasonic dissolved in isovolumetric methanol for 30 min, respectively. The samples were then filtrated through 0.22 μm filter units and the concentration of PYR was determined using an Agilent 1200 HPLC with ultraviolet detector set at 234 nm.

3. Results and discussion

3.1. Characterization

3.1.1. The surface potential of the LBL CHI/ALG bio-microcapsules

The LBL assembly process was monitored by measuring the surface potential of $CaCO_3$ microparticles after the deposition of each layer. As shown in Fig. S1, the initial Zeta-potential of CHI-doped $CaCO_3$ microparticles was about 20 mV. With the alternative deposition of ALG and CHI, the surface potential changed between −23 and 18 mV. This periodical and reproducible change was indicative of multilayer deposition of charged materials on the surface of $CaCO_3$ microparticles.

3.1.2. Morphology of LBL CHI/ALG bio-microcapsules

The SEM micrographs of the LBL bio-microcapsules are shown in Fig. 1(a-c). The LBL bio-microcapsules with the $CaCO_3$ microparticle templates maintained a regular spherical shape, with diameters of around 3 μm. The shape of the bio-microcapsule was larger than that of the bacteria used in this study, which had an average of 600 nm length and a width of 200 nm, indicating the bacteria could be encapsulated into the bio-microcapsules. The surface of the bio-microcapsules had a lot of wrinkles, which was good for the adsorption of the pollutant, and thereby improving the biodegradation. After the $CaCO_3$ microparticle templates were removed, the bio-microcapsules still maintained in the original shape of regular sphere.

The TEM micrographs of the LBL bio-microcapsules are shown in Fig. 1(d-f). LBL bio-microcapsules with cells and $CaCO_3$ template showed a shape of long stick with a white sphere (Fig. 1e), demonstrating the existence of the $CaCO_3$ templates. Combining the results of the TEM and the SEM, the bio-microcapsule looks like a flake with a length of around 3 μm and a height of 200–400 nm. The black substances at the two sides indicated the encapsulated-cells. The shape of the LBL bio-microcapsule was not collapsed and showed wholly dark after the