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Trans-membrane transport of fluoranthene by *Rhodococcus* sp. BAP-1 and optimization of uptake process

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HIGHLIGHTS

- A new strain was identified as *Rhodococcus* sp. BAP-1.
- Demonstrated the mechanism for fluoranthene travel across bacteria cell membrane.
- Transport dynamic combined with RSM to screen the significant variables.
- Plackett–Burman design was used to screen the significant variables.
- Box–Behnken design was employed to optimize the trans-membrane transport process.

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ABSTRACT

The mechanism of transport of ^{14}C-fluoranthene by *Rhodococcus* sp. BAP-1, a Gram-positive bacterium isolated from crude oil-polluted soil, was examined. Our finding demonstrated that the mechanism for fluoranthene travel across the cell membrane in *Rhodococcus* sp. BAP-1 requires energy. Meanwhile, the transport of fluoranthene involves concurrent catabolism of ^{14}C, that leading to the generation of significant amount of $^{14}CO_2$. Combined with trans-membrane transport dynamic and response surface methodology, a significant influence of temperature, pH and salinity on cellular uptake rate was screened by Plackett–Burman design. Then, Box–Behnken design was employed to optimize and enhanced the trans-membrane transport process. The results predicted by Box–Behnken design indicated that the maximum cellular uptake rate of fluoranthene could be achieve to 0.308 μmol min^{-1} mg^{-1}·protein (observed) and 0.304 μmol min^{-1} mg^{-1}·protein (predicted) when the initial temperature, pH and salinity were set at 20 °C, 9% and 1%, respectively.

1. Introduction

Polycyclic aromatic hydrocarbons are a typical type of persistent organic pollutants (POPs) and also a class of petroleum pollutants. (Rehmann et al., 1998; Fernando Bautista et al., 2009; Hua and Wang, 2012). They include a group of potential environmental pollutants and may be present at high concentrations around industrial sites associated with gas production, petroleum, coaltar preservation industries (Kumar and Thomas, 2011). The presence of PAHs in the environment causes a serious of health hazard because of their toxic, mutagenic, teratogenic and carcinogenic properties (Kästner et al., 1998). Currently, more than 100 types of PAH compounds are known worldwide, based on their abundance and toxicity, the US Environmental Protection Agency (US EPA) had classified 28 PAHs compounds as "priority pollutants" on January 2008. Fluoranthene is one of the major component which has five-membered rings in its molecular structure, and it often been taken as a model compound in the research of bioremediation of high-molecular-weight PAHs (Rehmann et al., 2008).

Compared to other treatments, use of microbial technology seems to be more efficient and economical in PAHs contaminated remediation process. Many bacterial species, including *Bacillus*, *Sphingomonas*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus* and *Burkholderia* have a well-known ability to degrade PAHs, they can break the highly hydrophobic organic compounds into less complex metabolites through biotransformation process, then via the mineralization effect into inorganic minerals (Rehmann et al., 1998). *Rhodococcus* species represent one of the most effective PAH-degrading bacterial genera, they are able to utilize PAHs such as phenanthrene, anthracene, benzo[a]pyrene and fluoranthene as carbon and energy sources (Song et al., 2011). Most of the earlier researches focus on the mechanism that how the bacteria contract with the high hydrophobic components (Lang and Philp, 1998; Wick et al., 2002; Hua and Wang, 2012). Meanwhile, some of the

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researches focus on the pathway of the biodegradation process (Annweiler et al., 2000; Balachandran et al., 2012). However, Hearn et al. (2008) proposed that bacterial biodegradation of hydrocarbons requires the passage of hydrophobic substrates across the cell membrane which means outer-membrane transport of aromatic hydrocarbons was the first step in biodegradation. But the transport mechanisms by which aromatic hydrocarbons travel across the outer membranes of bacteria and how the environmental factors performed to effect the trans-membrane transport process have received comparatively little attentions. How hydrophobic compounds travel across the cell membrane was reported by Bateman (Bateman et al., 1986). They found that naphthalene uptake by the *Pseudomonas putida* strain PpG1064 was nonspecific association and the compounds across bacterial membranes by simple diffusions, because the ATP was not essential for association or movement into the cell. However, Beal and Betts (2000) found that the process hexadecane uptake by *Pseudomonas aeruginosa* was energy-dependent, because uptake of hexadecane was susceptible to inhibitors such as CCCP (*m*-chlorophenylhydrazone) and DNP (2,4-dinitrophenol). Bugg et al. (2000) demonstrated two conflicting transport mechanism of PAHs by *Pseudomonas fluorescens* LP6a which were uptake by passive diffusion while excreted by an active efflux mechanism.

Generally, the success trans-membrane transport process was determined by many issues, including the species of microorganism and their properties, types of the pollutants and environmental conditions. Therefore, finding the appropriate reaction conditions could enhance the rate of PAHs uptake by microorganism (Launen et al., 1999). Classical method of optimization involves the change of "one-at-a-time-approach" which is expensive and extremely time-consuming when a number of variables are considered simultaneously (Tang et al., 2004); moreover, they are essentially unable to reflect the relations among multiple parameters (Queiroga et al., 2012). Statistical experimental designs using a response surface methodology (RSM), which includes factorial design and regression analysis, have been most widely applied to select optimum conditions of experiments (Puri et al., 2002). Due to the variety of the advantage to their use, RSM have been widely employed to develop, improve and optimize the processes and evaluate the relative significant affecting factors (Liu et al., 2010; El-Ghenymy et al., 2012; Torres et al., 2012).

To investigate the nature of the trans-membrane transport mechanism and how environmental conditions interact with the process, we examined the uptake of fluoranthene by a new strain *Rhodococcus* sp. BAP-1, a Gram-positive bacterium isolated from crude oil-polluted soil. Meanwhile, combined with trans-membrane transport dynamic and response surface methodology, the significant variables which influence the trans-membrane transport process were screened for.

2. Methods

2.1. Isolation and identification of the bacterial strain

Rhodococcus sp. BAP-1 was isolated from soil at the Beijing Chemical Plant (Beijing, China) that has been contaminated by organic compounds and contains high concentration of PAHs.

The physiological and biochemical characteristics of colonies of BAP-1 were studied following the directions of Bergey's Manual from the China General Microbiological Culture Collection Center. These characteristics mainly included the observation of color and morphology, Gram staining, capsule and spore staining, flagella staining, casein hydrolysis experiments, starch hydrolysis experiments, gelatin liquefaction experiments, aerobic growth, glycolysis tests, glycerol tests, urease tests, Voges–Prokauer tests, methyl red tests, oxidase experiments, glucose-produced acid experiments and glucose-produced gas experiments.

The 16S rDNA gene sequence was determined. The DNA was extracted from bacterial cells using a DNA isolation kit (Biomand, Beijing, China). After quantification by comparison with a DNA marker (Biomand, Beijing, China) using a gel-documentation system (G: Box, Syngene, UK), the 16S rDNA was amplified using PCR (DIO-2AD; Bio-Rad, Hercules, CA, USA) using the following primers: 27F (5′-AGAGTTTGATCCTGGCTCA-3′) and 1492R (5′-CG GTTACC TTGTTACGACTT-3′). The 50 μL reaction conditions were as follows: 25 μL MasterMix [0.1 U Taq Polymerase per μL; 500 μmol l^{-1} dNTP; 20 mmol l^{-1} Tris–HCl (pH 8.3); 100 mmol/L KCl; 3 mmol l^{-1} $MgCl_2$]; 2 μL template DNA; 2 μL 27F; 2 μL 1492R; 25 μL ddH_2O (Biomand, Beijing, China). Cycling conditions were as follows: one cycle of 5 min at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 1.5 min at 72 °C; 10 min at 72 °C; and a hold at 4 °C. Amplified DNA (5 μL) was separated by gel electrophoresis (HB-120, Shanghai Zhenghui Factory and Trade Co., Shanghai, China) on 1.2% (w/v) agarose (in 1×TAE, dyed with goldview) at 80 V. The PCR product after agarose gel electrophoresis was observed with a gel documentation system.

2.2. Cultivation media and conditions

A *Rhodococcus* sp. BAP-1 inoculum from a nutrient agar plate was enriched in 100 ml Luria–Bertani (LB) culture medium (NaCl 5 g/L, yeast extract 5 g/L, tryptone 10 g/L) in a 250 mL Erlenmeyer flask at 30 ± 1 °C on a shaking incubator (120 r/min) for 48 h. Bacterial cells were collected by centrifugation (6000*g*, 10 min), washed twice with mineral salts medium (MSM, pH = 7.0), resuspended in sterile MSM and measured for 600 nm absorbance in a UV–visible spectrophotometer (Varian, Palo Alto, CA, USA). The final cell optical density (OD_{600}) value was adjusted to 1.5. Next, the cells were used as inoculum at 5% (v/v) in a 100 ml Erlenmeyer flask containing 50 ml of MSM. A stock solution of fluoranthene (1 g/L) dissolved in acetone was added before inoculation as the sole carbon source. The composition of MSM was as follows (g/L): 4.0 Na_2HPO_4; 1.5 KH_2PO_4; 1.0 NH_4Cl; 1.0 $NaNO_3$; 0.2 $MgSO_4 \cdot 7H_2O$; 0.02 $CaCl_2$; 0.03 $FeSO_4 \cdot 7H_2O$. One milliliter of a micronutrient solution was added, which contained (g/L): 0.005 $CuSO_4 \cdot 5H_2O$; 0.01 H_3BO_3; 0.01 $MnSO_4 \cdot 5H_2O$; 0.07 $ZnSO_4$; 0.005 $CoCl_2 \cdot H_2O$. The pH of the medium was adjusted to 7.0. Both the LB and MSM liquid culture media were sterilized by autoclaving at 121 °C for 20 min.

2.3. ^{14}C-fluoranthene determination

Fluoranthene uptake was measured with ^{14}C-fluoranthene (ChemDeop, 07-43-060, 99% pure, 60 mCi/mmol). At various time intervals, samples of 1 mL aliquots were filtered using Whatman GF/C glass fiber filter under a vacuum, and filtered cells were washed with 1 mL of the phosphate buffer (pH = 7.0) for six times under a vacuum. Then the filters with cells were transferred into 2 mL of scintillation fluid (nonylphenolethoxylate, 9016-45-9, PerkinElmer Whltham, Massachusetts, USA) The measurements were performed on a PerkinElmer liquid scintillation counter (Wallac Oy 1450 MicroBeta), interfaced to a personal computer for data evaluation.

2.4. Energy dependent trans-membrane transport assay

For energy-dependent transport assay, inhibitors of 30 mmol/L of sodium azide, 0.1 mmol/L DNP, 0.1 mmol/L CCCP and 1 mmol/L CCCP were used to examine whether the transport of fluoranthene by *Rhodococcus* sp. BAP-1 was energy dependent. The inhibitors were added at time zero and at 5 min, respectively. Samples were collected at 1, 3, 5, 8, 10, 12, 15 and 20 min. Control experiment