Bioresource Technology 134 (2013) 17–23

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Evaluation of the application potential of bentonites in phenanthrene bioremediation by characterizing the biofilm community

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HIGHLIGHTS

- First characterization of biofilm community on bentobites during Phe remediation.
- Major members in biofilm were *Sphingomonadaceae* and *Rhodobacteraceae.*
- Most dioxygenase genes match to those of *Sphingomonas.*
- Selective effect of COB was evident.

ARTICLE INFO

Article history:
Received 5 December 2012
Received in revised form 30 January 2013
Accepted 1 February 2013
Available online 9 February 2013

Keywords:
Bentonites
Phenanthrene
Biofilm
PAH-RHDα genes

ABSTRACT

Application of clay minerals in bioremediation has emerged as a new and promising research field. In this study, the application of calcinated bentonite (CB) and calcinated organobentonite (COB) in phenanthrene (Phe) bioremediation showed high Phe removal efficiency. Clone libraries based on 16S rRNA gene and scanning electronic microscopy showed that diverse taxa of bacteria formed biofilms on both COB and CB particles. The family *Sphingomonadaceae* was the major group and made up 18% and 23% of the COB and CB biofilm composition, respectively. All and 80% of dioxygenase genes from COB and CB biofilms were closely related to that of *Sphingomonas* sp., and others matched to that of *Comamonas* and *Mycobacterium*. The selective effect of COB on bacterial community was also evident. This study characterized for the first time the bacterial diversity of biofilm community and functional Phe degrading groups on bentonites particles, and provided useful information for future applications.

1. Introduction

Bentonite is a naturally occurring clay mineral, mainly consisting of montmorillonite. Due to its high cation exchange capacity and remarkable sorption capacity, bentonite is extensively studied for application in the environmental remediation (Santi et al., 2008; Smith and Jaffe, 1994). By replacing its hydrated cations with organic cations, it can be modified to organobentonite, which exhibited special advantages in organic pollutants abatement (Tian et al., 2004; Zhu et al., 1997). The calcinated organobentonite (COB), on the other hand, had been shown to be less bio-toxic (Sarkar et al., 2012). Studies on pollutants removal by these materials are generally focused on the physical and chemical aspects, whereas their application in bioremediation has been largely ignored (Sarkar et al., 2012; Zhu et al., 2003). Although physical and chemical remediation showed high efficiency in removal of pollutants, these processes usually did not break down pollutant to non-toxic substrates (Sarkar et al., 2012). In recent years, the bioremediation is emerging as an alternative or complementary process to physical and chemical remediation (Peng et al., 2008). The bio-reactive properties of clay minerals have attracted great interests recently in the field of bioremediation study (Sarkar et al., 2012). Bentonites possess high cation-exchange capacities and surface area. These special properties make them perfect materials to carry out combinations of any chemical, physical and biological remediation.

Polycyclic aromatic hydrocarbons (PAH) are one group of typical persistent organic pollutants. Microbial degradation of PAHs was extensively studied and the metabolic pathways for some PAHs such as phenanthrene (Phe) and acenaphthene were illustrated (Peng et al., 2008). Factors affecting the biodegradation of PAHs were the key topics in many researches (Haritash and Kaushik, 2009). Several studies reported that biofilm formation, a surface life style for many bacteria (Watnick and Kolter, 2000), was a promoting factor for biodegradation of PAHs in that biofilm ensured higher bioavailability of PAHs (Johnsen and Karlson, 2004; Wick et al., 2001), and improved PAHs degradation rate (Leglize et al., 2008).

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http://dx.doi.org/10.1016/j.biortech.2013.02.009

Combination of sorption and biodegradation has evolved as a new remediation approach and held a lot of promise. The crucial part of this new technology was microbial survival and activities on the mineral materials (Sarkar et al., 2012). In this study, the application potential of bentonites in Phe bioremediation was investigated by characterizing the diversity of bacterial community and the degrading group in biofilm community. In addition, the selective effect of COB was explored.

2. Methods

2.1. Phenanthrene bioremediation treatments

Soil samples collected from a PAHs contaminated site (Hangzhou coking plant, Zhejiang, China) were used as microbial resource. To enrich Phe-degrading bacterial community, one gram of soil sample was inoculated into a Erlenmeyer flask containing 100 ml Bushnell-Haas (BH) minimal medium with 5 mg Phe (J&K Chemical Ltd, 98% purity) as the sole carbon and energy source for growth (Hilyard et al., 2008). The cultures were incubated aerobically at 28 °C for 10 days with shaking at low speed (120 rpm) to prevent grinding.

The calcinated organobentonite (COB) was produced by calcinating cetyltrimethylammonium-bentonite at around 500 °C for 8 h. The calcinated bentonite (CB) was prepared by calcinating bentonite at 580 °C for 6 h. Particles of COB and CB with size between 0.5 mm and 1 mm diameter were collected by mesh sieves. Particles were rinsed by deionized water and sterilized before use. For each treatment, the enriched soil bacterial culture was inoculated by 10% (V/V) to fresh BH media contained 5 mg Phe and 1 g COB/CB particles. The treatments were cultured in two continuously batches of 10 and 15 days at 28 °C with shaking at low speed (120 rpm) to prevent grinding. The culture flasks without COB/CB particles served as controls.

2.2. Phenanthrene measurement

At each sampling point (0, 1, 2, 3, 4, 5, 7 days), 10 ml culture broth was extracted twice with equal volume of dichloromethane in ultrasonic bath for 30 min. The combined extractions were dried over anhydrous sodium sulfate. The solvent was removed by vacuum evaporation at 40 °C and the residue was re-dissolved in 1 ml methanol for HPLC analysis (Chen et al., 2004). The HPLC analysis was conducted with an HPLC system (Agilent 1100 series) using a C18 special column (Φ 4.6 × 250 mm, Qrace Vydac, USA) and in a linear gradient program at a 1.0 ml/min flow rate of mobile phase. Determination of Phe concentration was achieved by an ultraviolet detector (Gl314A, Agilent) at 250 nm.

2.3. DNA extraction

The genomic DNA from bacterial community was extracted using a PowerSoil DNA extraction kit (MoBio, USA) according to the manufacturer's instructions. COB/CB particles were washed with deionized water before subjected to biofilm DNA extraction. Broth from COB treatment was centrifuged at 2500 rpm to collect bacterial community for broth DNA extraction.

2.4. Amplification of 16S rRNA gene and dioxygenase gene

The primers PAH-RHDα-396F and PAH-RHDα-696F were chosen to amplify PAH Ring-Hydroxylating dioxygenase (PAH-RHDα) genes in DNA samples (Ding et al., 2010). Universal primers 27F and 1492R were used to amplify bacterial 16S rRNA gene. PCR products with correct sizes were purified by Polygel Extraction kit (TaKaRa, CN) for clone library construction.

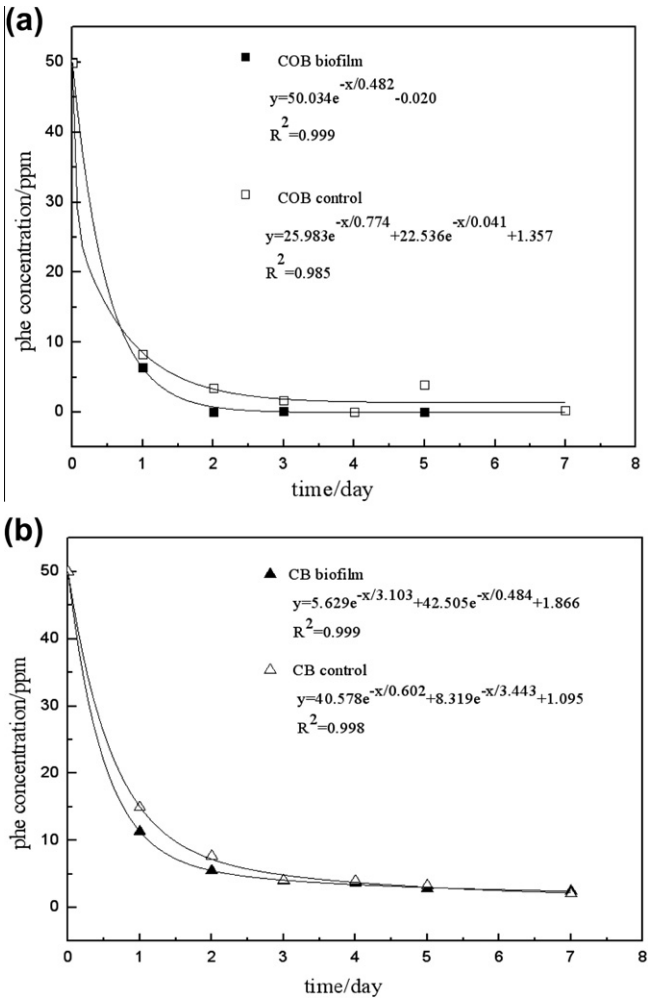

Fig. 1. The phenanthrene removal kinetics in COB/CB treatments was plotted by remained phenanthrene concentration over time. Correlation between phenanthrene concentration and time was fitted with exponential equation using Origin v8.0. COB treatment was marked with filled square (a) and CB treatment with filled triangle (b), respectively. Controls were marked with open symbols.

2.5. Clone library construction and sequencing analysis

The purified 16S rRNA and PAH-RHDα gene amplicons were ligated into pMD19-T simple Vector (TaKaRa, Dalian, CN), and transformed into competent cells of *Escherichia coli* DH5α (Beyotime, CN) according to the manufacturer's instruction. Correct size fragments in the clone library were sequenced and the sequences were analyzed by BLAST programs in NCBI. The obtained 16S rRNA sequences were manually aligned with reference taxa by using ClustalW in BioEdit software. Phylogenetic trees were constructed according to the neighbor-joining method and tested with bootstrap analysis (1000 replicates) by using the MEGA software (version 5.1).

2.6. Visualization of COB/CB particles and their biofilms by scanning electronic microscopy (SEM)

To view the biofilm on the COB/CB particles, COB/CB were fixed in 4% formaldehyde at 4 °C, dehydrated in an ethanol series, and dried in liquid CO_2 with a critical point dryer. Dried particles were mounted on stubs with double-stick adhesive, and gold coated with a sputter coater. The samples were then viewed with a XL30-ESEM microscope (Philip, Netherlands).2