Bioresource Technology 123 (2012) 664–668

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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

Short Communication

Simultaneous degradation of phenol and *n*-hexadecane by *Acinetobacter* strains

Ji-Quan Sun [a,b], Lian Xu [a,b], Yue-Qin Tang [a], Fu-Ming Chen [b], Xiao-Lei Wu [a,*]

[a] College of Engineering, Peking University, Beijing 100871, PR China
[b] Research Institute of Tsinghua University in Shenzhen, Shenzhen 518057, PR China

HIGHLIGHTS

- Simultaneous phenol and *n*-hexadecane degradation observed in *Acinetobacter* spp.
- Mixture of phenol and *n*-hexadecane enhanced the growth of *Acinetobacter* strains.
- Phenol somehow inhibited *n*-hexadecane degradation by *Acinetobacter* strains.
- *n*-Hexadecane exerted less influence on phenol degradation in *Acinetobacter* strains.

ARTICLE INFO

Article history:
Received 30 April 2012
Received in revised form 22 June 2012
Accepted 24 June 2012
Available online 29 June 2012

Keywords:
Biodegradation
Acinetobacter spp
Phenol
n-Hexadecane
Co-contamination

ABSTRACT

Three phenol- and alkanes-degrading bacterial strains were isolated from a freshwater sample. Upon the 16S rRNA gene analysis, phenotype and physiological features, the three strains were designated as *Acinetobacter* sp. with both phenol hydroxylase gene (*phe*) and alkane monooxygenase gene (*alkM*) detected. They could simultaneously degrade phenol and *n*-hexadecane for growth, but prefer phenol than *n*-hexadecane. Between phenol (400 mg l^{-1}) and *n*-hexadecane (400 mg l^{-1}), *n*-hexadecane enhanced phenol degradation in mineral salt medium (MSM), while phenol affects negatively the *n*-hexadecane degradation. However, combination of phenol (400 mg l^{-1}) and *n*-hexadecane (400 mg l^{-1}) in MSM led to higher growth of the strains than the phenol and *n*-hexadecane separately. The transcription levels of *phe* and *alkM* genes supported the physiological properties of the strains.

1. Introduction

Aliphatic alkanes and aromatic compounds, from the discharge of a wide variety of anthropogenic activities, often co-exist in various contaminated environments (Dawson et al., 2007; Milleman et al., 1984). Simultaneous degradation of these pollutants is therefore very important for bioremediation of the environments co-contaminated by them. Many bacterial strains have been isolated with the abilities to degrade *n*-hexadecane and phenol, which are often used to represent the petroleum and aromatic pollutant (Milleman et al., 1984). They can include bacteria in the genera of *Acinetobacter* (Ahmad et al., 2012; Maeng et al., 1996; Luckarift et al., 2011), *Pseudomonas* (Chung et al., 2003; Zhang et al., 2011), *Rhodococcus* (van Hamme and Ward, 2001; Sun et al., 2011), *Dietzia* (Wang et al., 2011) and *Sphingomonas* (Liu et al., 2009). However, few strains have been reported to have the dual abilities. Therefore, we designed experiments to isolate the bacterial strains that are able to simultaneously degrade aromatic phenol and petroleum alkane, and analyzed the impacts of phenol and alkane on the degradation each other. Our results, for the first time, revealed that *Acinetobacter* strains could simultaneously utilize alkanes and phenolics as carbon and energy source for growth. In addition, the results also revealed the different impacts of phenol and alkane on bacterial growth as well as degradation of the two compounds.

2. Methods

2.1. Isolation and characterization of phenol- and n-hexadecane-degrading strains

Bacterial strains were isolated from a water sample from *Shahe River* in a southern China city, Shenzhen. Firstly, 1 ml of the sample water was inoculated into 100 ml of minimal salt medium (MSM) (g l^{-1}: NaCl, 1.0; NH_4NO_3, 1.0; K_2HPO_4, 1.5; KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2) amended with 100 mg l^{-1} (final concentration) phenol (purity > 99.0%, Sinopharm Chemical Reagent Co. Ltd., Shanghai, China), followed by incubation at 30 °C and shaking at 150 r min^{-1} for 3 days. After the enrichment culture was plated on LB (g l^{-1}: tryptone 10.0, yeast extract 5.0, NaCl 10.0) agar and

* Corresponding author. Tel./fax: +86 10 62759047.
E-mail address: xiaolei_wu@pku.edu.cn (X.-L. Wu).

0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.06.072

incubated at 30 °C for 3 days, bacterial colonies were purified and screened for their abilities to degrade phenol and *n*-hexadecane (purity > 99.0%, Sigma–Aldrich, USA). Three bacterial strains of interest were identified and characterized with the methods described previously (Wang et al., 2007).

2.2. Degradation of phenol and n-hexadecane

After grown in 100 ml of LB medium for 24 h at 30 °C and shaking at 150 r min^{-1}, cells of the three strains were separately harvested by centrifugation (1000×*g* for 5 min at 4 °C), followed by washing twice and re-suspension with aseptic MSM. The resulted cell suspensions (OD_{600} 3.0) were then used as inoculums for inoculation immediately. Three MSM-based media were used to investigate the degradation abilities of the bacterial strains, i.e. the MSM amended with 400 mg l^{-1} phenol and 400 mg l^{-1} *n*-hexadecane (final concentration), respectively and simultaneously. The inoculums were inoculated into the above three kinds of media respectively with the final concentration of 1% (v:v). The cultures were then incubated at 30 °C in the dark and shaking at 150 r min^{-1}. In addition, the media amended with the phenol and *n*-hexadecane but without cell inoculation were used as control to check background phenol and *n*-hexadecane evaporation. In addition, bacterial growth on MSM amended with glucose was evaluated as control to analyze the transcription levels of the functional genes. All the cultures were made in triple. At certain time points, the cultures were sampled for detecting the cell density, the residual phenol and *n*-hexadecane concentrations as well as the transcription levels of phenol hydroxylase gene (*phe*) and alkane monooxygenase gene (*alkM*).

The cell density in the culture was detected as OD_{600}. Concentration of residual phenol in the cultures was determined using the method described previously (Sun et al., 2011). The residual *n*-hexadecane in culture samples (30 ml) was extracted with 15 ml of *n*-hexane. Then 1 ml of the well-mixed *n*-hexane phase was dried over anhydrous Na_2SO_4. After passing through a filter with pore size 0.22 μm, dehydrated hexane solutions were subject to the detection of *n*-hexadecane concentration. The detection was made by a gas chromatography (GC-2014, Shimadzu, Japan) equipped with a flame ionization detector and a Rtx-1 column (30 m × 0.25 mm, film thickness 0.25 μm) (USA) following the protocol described previously (Wang et al., 2011).

2.3. Analyses of functional genes

The primer sets used for amplification of *phe* and *alkM* genes were: Phe1 (AGGCATCAAGATCACCGACTG) and Phe2 (CGCCAGAACCATTTATCGATC) for *phe* gene (Xu et al., 2001) and alkB-1f (AAYACNGCNCAYGARCTNGGNCAYAA) and alkB-1r (GCRTGRTGRTCNGARTGNCGYTG) for *alkM* gene (Kloos et al., 2006). The thermal profiles for the PCRs were the same as described previously (Xu et al., 2001). Correct *phe* and *alkM* gene fragments with the length of approximately 660 and 550 bp, respectively were checked by gel electrophoresis.

The transcription levels of the *phe* and *alkM* genes were analyzed with the reverse transcription real-time PCR approach. Briefly, after sampled, the cells were immediately harvested by centrifugation (12,000×*g* for 1 min), followed by immediate analysis of the transcripts. The total RNA extraction, treatment, synthesis of first strand cDNA, real-time amplification and analysis were carried out with the protocols described previously (Sun et al., 2011) with the primer sets listed in Supplemental Table A.1. The transcription levels of the functional genes were normalized with the 16S rRNA gene transcription of the cells growth on glucose as control (Sun et al., 2011).

2.4. Nucleotide sequence accession numbers

The partial sequences of *phe*, 16S rRNA and *alkM* genes from strains SJ-2, SJ-15, and SJ-16 were deposited in GenBank under the accession numbers JQ346082–JQ346084 and JQ622391–JQ622396, respectively.

3. Results and discussion

3.1. Isolation and characterization of phenol- and alkane-degrading strains

Three bacterial strains with the abilities of simultaneously degrading phenol and *n*-alkane were obtained using the enrichment method. All the three strains were Gram-negative, and oxidase-negative. They could grow at temperatures ranging 20–40 °C and pH ranging 6.0–9.0 with the optimum growth occurred at 30 °C and pH 7.0. Grown on LB agar at 30 °C for 2 days, the strains all formed white, round, hygric and lubricous clones with diameter around 2 mm. Phylogenetic analysis revealed that the *phe* genes from the three strains were clustered differently, i.e. SJ-15 and SJ-16 were closely related with *Diaphorobacter* sp. strain J5-51 (a pyridine-degrading strain) (JQ346083) and a *p*-chloroaniline-degrading strain PCA039 (FJ601374) (Zhang et al., 2010) with the similarities of 99.4–99.8% and 99.2–99.6% respectively, while strain SJ-2 clustered with strain *Acinetobacter* sp. PND-4 (EF601584) with the *phe* gene sequence similarity of 99.8% (Fig. A.1). The phylogeny of *alkM* gene revealed the three strains were closely related to members of *Acinetobacter*, i.e. strains SJ-15 and SJ-16 were closely related to *Acinetobacter calcoaceticus* 69-V, while strain SJ-2 was closely related to *Acinetobacter* sp. strain MUB1 (Phrommanich et al., 2009) (Fig. A.2). In contrast, the phylogenetic analysis based on 16S rRNA genes revealed that these three strains formed a stable clade with the members in the genus of *Acinetobacter* with the 16S rRNA gene sequence similarity of 98.0–99.8% (Fig. A.3). Based on the 16S rRNA gene analysis, phenotype and physiological features, the three strains were therefore designated as *Acinetobacter* spp.

All the three strains could utilize phenol and *n*-hexadecane, as well as some of their analogs as the sole carbon and energy sources for growth (Table A.2). In addition, the strains could grow well in LB containing 50 mg l^{-1} of hexavalent chromium ($K_2Cr_2O_7$) although the chromium removal rates were low (data not shown).

3.2. Dual n-hexadecane and phenol degradation abilities of the Acinetobacter strains

At the optimal conditions for phenol and alkane degradation, i. e. pH 6.0–9.0 and temperature 30 °C, all the three strains preferred to degrade phenol rather than degrade *n*-hexadecane. Phenol (400 mg l^{-1}) could be completely degraded within 40 and 24 h by strain SJ-2 and SJ-15/SJ-16 respectively no matter whether *n*-hexadecane was added or not. It took more than 60 h for strain SJ-15 and SJ-16 to completely degrade *n*-hexadecane (400 mg l^{-1}) and strain SJ-2 could not completely degrade *n*-hexadecane till the end of the experiments (96 h) (Fig. 1). Apparently, combination of phenol and *n*-hexadecane led to more growth of the strains than phenol and *n*-hexadecane separately (Fig. 1). Strikingly, the combination resulted in less *n*-hexadecane degradation and somehow more phenol degradation (Fig. 1), suggesting the remarkable inhibition of phenol on *n*-hexadecane degradation and positive influences of *n*-hexadecane on phenol degradation (Fig. 1).

Alkane monooxygenase encoded by *alkM* gene catalyzes the terminal oxidation of *n*-alkanes to alcohols, which is the initial and limiting step in the aerobic degradation of *n*-alkanes by