International Biodeterioration & Biodegradation 121 (2017) 107–113

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

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

Biodegradation of phenol by a native mixed bacterial culture isolated from crude oil contaminated site

Sounak Bera [a], Abhijit Sarma Roy [a], Kaustubha Mohanty [a, b, *]

[a] Center for Energy, Indian Institute of Technology Guwahati, Guwahati, 781039, India
[b] Department of Chemical Engineering, Indian Institute of Technology Guwahati, Guwahati, 781039, India

ARTICLE INFO

Article history:
Received 20 May 2016
Received in revised form
3 April 2017
Accepted 4 April 2017

Keywords:
Mixed bacterial culture
Phenol degradation
Inhibition
Maximum specific growth rate
Dynamic profiles
Ortho-cleavage pathway

ABSTRACT

An efficient phenol degrading mixed bacterial culture was isolated from sludge sample collected from one of the refinery located in Assam, India. The mixed culture was found to consist of three bacterial strains. These were identified as *Stenotrophomonas acidaminiphila*, *Brevibacterium sp.* and *Brucella sp.* Batch phenol biodegradation experiments were carried out for a wide range of initial phenol concentrations after pH and temperature optimization. It was found that the mixed culture was able to degrade a maximum phenol concentration up to 1000 mg L^{-1} within 96 h while the maximum specific growth rate (μ_{max}) was observed at 100 mg L^{-1}. The pH and temperature required for optimal phenol degradation was 6.5 and 37 °C respectively. The mixed culture degrades phenol via *ortho*-cleavage pathway by formation of an intermediate (*cis*, *cis*-muconate) which was detected spectrophotometrically at 260 nm. The experimental data were validated by fitting the growth and substrate utilization curves with their corresponding simulated dynamic profiles obtained by solving Haldane's equation via MATLAB R2015a with $\mu_{max} = 0.155$ h^{-1} and $K_I = 400$ mg L^{-1}.

1. Introduction

Crude oil drilling and refining activities in North Eastern region of India, particularly Assam, dates back to early part of the nineteenth century. The natural environment in and around these sites have been continuously exposed to different hydrocarbon components present in crude oil since those days. The various hydrocarbons present in crude oil can be classified into aliphatic, aromatic and polycyclic aromatic hydrocarbons (PAHs). Among the different aromatic hydrocarbon contaminants present in crude oil, phenol is of utmost importance due to its recalcitrant nature and widespread prevalence in soil and water ecosystems near the drilling sites and refinery fallouts. This is attributed to its high solubility in water with reports of up to 10,000 mg L^{-1} (Bajaj et al., 2009) whereas its permissible limit in potable water is 10^{-3} mg L^{-1} as recommended by World Health Organization (WHO) (Kumaran and Paruchuri, 1997). Apart from oil drilling sites and refinery fallouts, high concentration of phenol has also been reported in wastewater discharges of other industries like phenol-formaldehyde resin, coal conversion, coking plant, leather, textiles, pharmaceutical etc by various research groups across the world (Huang et al., 2014; Kumaran and Paruchuri, 1997; Pinto et al., 2003; Wang et al., 2014). Phenol is toxic to a host range of beneficial soil and water microbes, aquatic life and plants and has adverse effect on human health even at low concentrations. It has been reported to cause liver and kidney damage, cardiac toxicity, reproductive and developmental toxicity, neurotoxicity, cardiac depression and reduced blood pressure in humans and therefore must be removed from the environment (Huang et al., 2015; Nuhoglu and Yalcin, 2005). Several research groups across the world have reported the degradation of phenol and its derivatives by various physical and chemical methods. However, these methods are quite energy consuming and are not cost effective and also cause secondary pollution (Shourian et al., 2009). On the other hand, biological methods involving potent microorganisms with phenol degrading ability are becoming increasingly popular, as they are inexpensive, eco-friendly and do not cause secondary pollution (Liu et al., 2016). Numerous approaches like immobilization of microbial cells, addition of readily utilizable carbon source as co-substrate or adaptation of microbial cells to high phenol concentrations have been put forward in order to resist the toxicity. However, adaptation of microbial cells to high phenol concentration has been

* Corresponding author. Center for Energy, Indian Institute of Technology Guwahati, Guwahati, 781039, India.
E-mail address: kmohanty@iitg.ernet.in (K. Mohanty).

http://dx.doi.org/10.1016/j.ibiod.2017.04.002
0964-8305/

described as a more effective way to overcome substrate inhibition (Acikgoz and Ozcan, 2016). Phenol degradation using pure and mixed bacterial cultures have been widely studied. Some of the widely reported bacterial genus for phenol degradation are *Pseudomonas* and *Bacillus* (Bandyopadhyay et al., 1998; Banerjee and Ghoshal, 2010a; Shourian et al., 2009). Mixed bacterial cultures have also been used by several research groups for efficient phenol degradation (Bajaj et al., 2009; Kumar and Mohanty, 2012; Ma et al., 2015; Pradhan et al., 2012; Saravanan et al., 2008).

The present research reports the isolation of phenol utilizing bacterial strains from several oil refining and drilling sites of Assam, India with diverse history of contamination and exposure to crude oil spills. A mixed bacterial culture comprising of three bacterial strains having high phenol degradation ability was obtained. Further, the strains in the mixed culture were identified and its phenol degradation ability was tested. Effects of initial phenol concentration, pH and temperature were studied to obtain the optimal conditions for bacterial growth and phenol degradation. Phenol degradation pathway was also determined. Experimental data for microbial growth and substrate utilization were fitted against simulated growth and substrate utilization curves obtained from Haldane's Model in MATLAB R2015a.

2. Materials and methods

2.1. Sampling from hydrocarbon contaminated sites and enrichment of the samples in high phenol concentrations

Samples were collected from four hydrocarbon contaminated environments of Assam, India. A total number of 15 samples were collected from these sites. The samples were mainly of three types, a) oily sludge, b) hydrocarbon contaminated wastewater and c) crude oil (Refer Table S1 in supplementary file). The samples were collected in aseptic plastic bags and sample bottles and were stored at 4 °C until further use. The pH, electrical conductivity, temperature, ORP (Oxidation reduction potential) and dissolved oxygen content of these samples were recorded.

For enrichment of the samples, 1 g each of solid samples and 1 mL each of liquid samples were added to sterile mineral salt medium (MSM) in 250 mL Erlenmeyer flasks. MSM contains $NaNO_3$ (4.0 g L^{-1}), Na_2HPO_4 (3.61 g L^{-1}), KH_2PO_4 (1.75 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), $CaCL_2.2H_2O$ (0.05 g L^{-1}), $FeSO_4 \cdot 5H_2O$ (0.001 g L^{-1}), $CuSO_4 \cdot 5H_2O$ (50 μg L^{-1}), Na_2MoO_3 (10 μg L^{-1}) and $MnSO_4$ (10 μg L^{-1}). 100 mg L^{-1} of phenol was added as the sole source of carbon and incubated at 37 °C and 120 rpm for 48 h in an incubator shaker (ZHICHENG Instruments ZHWY-2112B). All the chemical components of mineral medium and pure phenol crystals were obtained from MERCK India and HiMedia®. The culture flasks were regularly monitored for visual bacterial growth. Gradual increment in phenol concentration of up to 1000 mg L^{-1} was done for the samples. The initial pH of the medium was maintained at 7.0 and the working volume was maintained at 100 mL. The enrichment process was carried out until a high phenol (1000 mg L^{-1}) tolerant mixed culture was obtained.

2.2. Isolation and identification of the bacterial strains

Isolation of the culturable bacterial strains from the enrichment cultures was carried out by serial dilution and repeated subculturing on solid MSM containing yeast extract (3 g L^{-1}) as carbon source and 1.8% agar as gelling agent. Identification of the bacterial isolates was done by PCR amplification of the 16S rDNA conserved region. Genomic DNA was isolated using a column based DNA isolation kit. The isolated genomic DNA was subjected to 16S rDNA PCR amplification using 16S universal forward primer fD1 (5′ - AGAGTTTGATCCTGGCTCAG-3′) and 16S universal reverse primer rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). The conditions for PCR reactions involved an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, an extension/elongation step at 72 °C for 90 s and a final elongation step at 72 °C for 10 min. The PCR thermal cycler used for the reactions was obtained from Applied Biosystems (Model: 2720 Thermalcycler). PCR Master Mix, DNase and RNase free water and DNA isolation kit was obtained from MERCK Bangalore GeNei™. Isolated DNA fragments were sequenced and the sequences were analyzed using BLAST (http://www.ncbi.nih.gov/BLAST) and submitted to NCBI Nucleotide database using the BankIt sequence submission tool (www.ncbi.nlm.nih.gov/BankIt). The sequences were aligned using ClustalW program (http://www.ebi.ac.uk/clustalw) of the European Bioinformatics Institute (EMBL-EBI) and BioEdit Sequence Alignment Editor Software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The construction of phylogenetic tree was done using MEGA6.

2.3. Biochemical characterization and field emission scanning electron microscopy

For all the isolated bacterial strains several biochemical characteristics were analyzed according to Bergy's manual. The biochemical tests included Gram's staining, Gelatin hydrolysis, Starch hydrolysis, Catalase test, E_{24} Assay and oil spread assay.

Scanning electron microscopy was done for observing the mixed culture which was able to degrade phenol up to a concentration of 1000 mg L^{-1}. 24 h old phenol grown cells were collected and sample preparation was done by glutaraldehyde treatment and acetone gradation. Sample was then taken on a slide and dried overnight in a vacuum desiccator. Subsequently the sample was coated with a gold film and community morphology was observed and recorded in Zeiss Field Emission Scanning Electron microscope (model: Sigma) at a magnification of 32.77 KX.

2.4. Batch studies for optimization of growth parameters: inoculum dosage, substrate concentration, pH and temperature

2.4.1. Optimization of inoculum dosage

Inoculum dosage optimization studies were carried out in 250 mL Erlenmeyer flasks containing 100 mL MSM supplemented with 1000 mg L^{-1} phenol as the sole source of carbon. Using the hyper phenol tolerant mixed bacterial culture, experiments were conducted for inoculum volumes of 0.5%, 1.0%, 1.5%, 2.0% and 2.5% v/v respectively. Optical density (at 600 nm) of the inoculum (3 days old) was recorded to determine the amount of bacterial cells being used for the experiments. Cell growth and residual phenol concentrations were measured at regular intervals. Experiments were also conducted to obtain a calibration curve to convert optical density values into corresponding dry biomass or DCW (dry cell weight) (data not shown here): one OD = 0.866 g dry cells L^{-1} ($R^2 = 0.97$).

2.4.2. Growth under different initial phenol concentrations

Experiments were carried out with an optimal inoculum dosage at different increasing concentrations of phenol (75–1000 mg L^{-1}) to find out the optimum substrate concentration for growth and to observe the growth and substrate utilization patterns. At first, batch experiments were conducted for three different initial phenol concentrations (75, 300 and 900 mg L^{-1}) and the data obtained was used for model development. In the next step, the model was validated by cultivating the mixed culture under the remaining phenol concentrations. All the experiments were conducted in