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Native hydrocarbonoclastic bacteria and hydrocarbon mineralization processes

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ABSTRACT

In the present study we have isolated fourteen hydrocarbonoclastic bacterial strains, from the crude oil contaminated soil of Assam, India. Each of these strain utilized crude oil as sole carbon source. The samples were found to produce crude oil deposition in a range of 0.04%–89.36% and moisture content 1.25%–66.6%. The isolated strains were designated as RC1-RC14 that includes *Alcaligenes*, *Bacillus*, *Enterobacter*, and *Pseudomonas* species; which were able to metabolize crude oil to different extents. Interestingly, we have found that *Enterobacter* sp. RC4 and *Bacillus aryabhattai* RC6 were capable of reducing crude oil content by 80% and 64%, respectively. Further by using different techniques such as GC-MS, FT-IR, NMR and viscosity analysis altogether had confirmed the mineralization of crude oil by the isolated strains. Furthermore, we have found that these strains were distinctively utilized polar fraction, resin by 78% and 64% respectively. Catechol dioxygenase gene was detected in RC4 genome indicating its potential catabolic capabilities for degrading aromatic oil fraction. From the investigation, it was concluded that utilization/mineralization process of crude oil by the investigated HCB was achieved by five independent ways. Our findings of the present study clearly indicate the prospect to develop an environment friendly mitigation technology against crude oil pollution using indigenous HCB.

1. Introduction

The non-degradability and persistent nature of hydrocarbon pollutants is a significant environmental problem (Hu and Zeng, 2013; Oudot et al., 1998). Having saturated and aromatic hydrocarbons, polar compounds, resins and asphaltene as constituents, crude oil possesses diverse toxic effects on plants, animals and human health (Atlas, 1981). The manifold effects of carcinogenic, mutagenic and immuno-toxic properties of crude oil components pose major threat to environment (Ting and HuTan, 1999; Vasudevan and Rajaram, 2001). Compared to conventional methods of hydrocarbon decontamination (Phillips et al., 2009), hydrocarbonoclastic bacteria (HCB) aided cleanup is not only cost effective but also complete recovery of contaminated environment is achieved (Molina-Barahona et al., 2004; McFarlin et al., 2014). Interestingly, studies have reported hydrocarbon contaminated environments to harbour greater and more diverse microbial communities predisposed to hydrocarbon metabolism (Teramoto et al., 2013; Mahjoubi et al., 2013). Thus targeting such sites would increase the likelihood of getting potent bacteria with high crude oil degradation/utilization capabilities. Currently, HCB are being given much importance by researchers and have been found to be appreciable alternatives in controlling the fate of natural and anthropogenic crude oil seepage (Allen et al., 2007).

Assam, in North East (NE) India produces and houses large reserves of crude oil since its discovery in 1867. Various studies have been undertaken to chalk-out biological ways of decontaminating sites polluted as a result of exploration, production, maintenance, transportation, storage and abandonment (Yenn et al., 2014; Roy et al., 2014; Das et al., 2015). In the present study, the main aim was to assess the HCB from hydrocarbon contaminated

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environment of Duliajan and Jorajan, Assam. Emphasis was given to observe how the HCB utilise or mineralise crude oil in entire process of degradation.

2. Methods and materials

2.1. Media and chemicals

Media used in this study were purchased from HiMedia, Mumbai, India. Molecular grade chemicals were from Sigma Aldrich, USA. Crude oil used for the entire study was collected from OIL, Duliajan, Assam.

2.2. Soil sampling and analysis

Sampling was done randomly by disturb sampling at ten oil drilling contaminated sites of Duliajan (27°21′42″ N, 95°19′ 6″ E) and Jorajan (95°11′ – 95°30′E; 27° 06′ – 27° 22′ N) of Dibrugarh and Tinsukia, Assam. All the samples were immediately stored at 4 °C. Samples were analyzed for their total hydrocarbon, pH, moisture content, soil organic carbon (SOC), and total bacterial count. For total hydrocarbon content, 5 g of contaminated sample was extracted with diethyl ether and filtered. The process was repeated minimum three times. The filtrate obtained was dried and weighed. pH of the contaminated sample was estimated in sample: water suspension (1:2.5) using pH meter (Eutech, Malaysia), moisture content was determined gravimetrically by drying the samples at 70 °C until constant weight. Total SOC was determined according to Walkley and Black (1934). Crude oil fractionation *i.e.*, asphalt, resin, wax, aliphatic/aromatic fraction was separated according to Hubbard and Stanfield (1948).

2.3. Isolation of crude oil degraders

Enrichment technique using mineral media M1 (gL^{-1}) (4.0, $NaNO_3$; 3.61, Na_2HPO_4; 1.75, KH_2PO_4; 0.2, $MgSO_4.7H_2O$; 0.01, $FeSO_4$; 0.05, $CaCl_2$: Trace-element solution 1 mlL^{-1}) with crude oil (1%v/v) as sole carbon source was used for isolating crude oil utilizing strains. Morphologically distinct pure colonies were picked and further tested for hydrocarbon utilization/degradation using agar well diffusion techniques with crude oil as sole carbon source.

2.4. Crude oil biodegradation process capability assessment

Hydrocarbon utilizing ability of the HCB was studied under shake flask using mineral media M1 with 2% (v/v) crude oil. Inoculum size of enriched culture was maintained at 1% (A_{600}~1). The experiment was carried out in triplicate and uninoculated flasks constituted the control. Growth of bacteria was monitored at every 24 h interval for 7 days; growth condition was maintained at 130 rpm with a temperature of 30 °C. Efficiency of crude oil degradation/utilization of the individual bacteria was determined qualitatively by estimating the consumed hydrocarbons and growth of the isolates. Visible changes to the treated crude oil during degradation process were noted at every 24 h interval from 0^{th} hour so as to understand the variation that occurs during utilization of crude oil by individual HCB.

The short-listed strains showing crude oil utilization were further tested for their ability to utilise/degrade crude oil fractions in shake flask using M1 media with asphaltene, resin, wax and aliphatic/aromatic fraction independently as sole carbon source. The flasks were monitored for changes in terms of visual differences, turbidity, bacterial CFU and decrease in hydrocarbon content at every 12 h interval.

2.4.1. Bacterial utilization of crude oil

The utilization of crude oil by all 14 isolates was evaluated using the technique based on the redox indicator 2,6-DCPIP. Modified methods were used to screen potential HCB (Varjani et al., 2013; Kubota et al., 2008). Activated bacterial cultures were inoculated into tubes along with 0.5% v/v DCPIP indicator, and 3% v/v respective crude oil for change in colour of the blue dye. All the tubes were incubated at room temperature and 100 rpm.

2.4.2. Biosurfactant production

Biosurfactant production during crude oil utilization for the isolates was estimated by measuring the emulsification capacity; wherein equal volume of kerosene was added to the bacterial culture supernatant and vortexed vigorously at high speed. The mixture was allowed to stabilize for 24 h. The emulsion index E_{24} was calculated as the ratio of the height of the emulsion layer and the total height of liquid (Cooper and Goldenberg, 1987).

2.4.3. Biofilm formation

Detection of the bacterial adhesion to surface in the presence of crude oil as carbon source was done by tube method described by Mathur et al. (2006).

2.4.4. Viscosity

Viscosity of untreated crude oil compared to bacteria-aided degraded samples at every 24 h interval was measured using Rheometers (Anton Paar Rheolab QC, India). Moreover, crude oil was analyzed for changes in pour point and American Petroleum Institute (API) gravity.

2.4.5. Methane emitted

Gaseous fraction liberated during crude oil utilization/degradation was sampled at every 24 h interval and analyzed in a Gas Chromatograph (Thermofisher Scientific Chemito GC 8610). For this, 1 ml gas sample was injected manually using an SGE gas tight valve syringe and separation was performed on a Porapack Q-S Teflon 6 ft column. The carrier gas was Nitrogen (flow rate of 20 ml/min, pressure of 3.5 Bar). Data acquisition was controlled using IRIS32 ICON software, in full scan/selected ion mode for greater sensitivity. Data was recorded in triplicate.

2.4.6. Gas chromatography-mass spectrometry (GC-MS) analysis

Residual remains of degraded crude oil was characterized via gas chromatography-mass spectrometry (GC-MS) using Elite 5 MS column (Perkin Elmer Clarus 600). The column temperature was kept at 80 °C–280 °C at 8 °C min^{-1} increment and finally held at 280 °C for 10 min. The carrier gas was helium with a flow rate of 1 ml min^{-1}. The mass spectrometric data was acquired in electron ionization mode (70 eV). The interface temperature was 280 °C and mass range was 50–500 m/z. The individual components were determined manually by matching the retention time with authentic standards and from MS library search.

2.4.7. FT-IR and NMR analysis

Similar analysis was also done using (Fourier-transformed infrared spectroscopy) FT-IR and nuclear magnetic resonance spectroscopy (NMR). Sodium chloride (NaCl) polished discs were used to analyze oil samples under FT-IR. Background spectra were obtained by scanning clean disc in the instrument. One drop of sample was placed on the NaCl disc and placed in a Perkin-Elmer Spectrum 100 FT-IR spectrometer. Scans were carried out in the 4000–400 cm-' range.

For NMR, a drop of oil as well as degraded product was placed in a NMR tube. Deuterated chloroform ($CDCl_3$) was added to 5 ml of the oil sample and placed in a Bruker AV500 Avance-III 500 MHz