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Enrichment and characterization of hydrocarbon-degrading bacteria from petroleum refinery waste as potent bioaugmentation agent for *in situ* bioremediation

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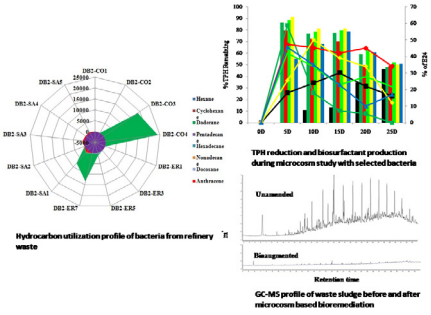
HIGHLIGHTS

- Petroleum refinery waste of Digboi refinery harbours hydrocarbonclastic bacteria.
- Isolation of superior hydrocarbon degrading bacteria achieved only by enrichment.
- Metabolic versatility of *Burkholderia, Pandoraea* and *Enterobacter* spp. explored.
- Microcosm study identified selected strains as potent bioaugmentation agent.

GRAPHICAL ABSTRACT



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ABSTRACT

Intrinsic biodegradation potential of bacteria from petroleum refinery waste was investigated through isolation of cultivable strains and their characterization. *Pseudomonas* and *Bacillus* spp. populated the normal cultivable taxa while prolonged enrichment with hydrocarbons and crude oil yielded hydrocarbonoclastic bacteria of genera *Burkholderia*, *Enterobacter*, *Kocuria*, *Pandoraea*, etc. Strains isolated through enrichment showed assemblages of superior metabolic properties: utilization of aliphatic (C6-C22) and polyaromatic compounds, anaerobic growth with multiple terminal electron acceptors and higher biosurfactant production. Biodegradation of dodecane was studied thoroughly by GC–MS along with detection of gene encoding alkane hydroxylase (*alk*B). Microcosms bioaugmented with *Enterobacter*, *Pandoraea* and *Burkholderia* strains showed efficient biodegradation (98% TPH removal) well fitted in first order kinetic model with low rate constants and decreased half-life. This study proves that catabolically efficient bacteria resides naturally in complex petroleum refinery wastes and those can be useful for bioaugmentation based bioremediation.

1. Introduction

The rapid expansion of petroleum and allied industries has added to the economic prosperity of India (and many other countries as well), but led to the generation of large volume of hazardous oily sludge (>28000 tons/annum in India and >60 million tons/annum globally) that warrants immediate attention (Hu et al., 2013; Solanki et al., 2015). Sustainable and affordable disposal/remediation of all petroleum hydrocarbon (HC) rich waste sludge/contaminants is a prime technological impediment and scope for microbial bioremediation has emerged as the most feasible, yet effective solution to attain complete pollutant degradation (Cerqueira et al., 2011). Hydrocarbon degrading microorganisms

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are ubiquitous in natural as well as impacted environments (Bell et al., 2013; Kostka et al., 2011; references therein) and biodegradation was shown to be successful in remediating oil/HC contamination naturally and/or with engineered strategies (Brooijmans et al., 2009; Fuentes et al., 2014). Bioremediation can be accomplished by either boosting the growth of indigenous microbial community through biostimulation or by introducing organisms with superior catabolic abilities (bioaugmentation) (Agnello et al., 2016). Considering the theoretical limitation of bioremediation posed by the requirement of 'combined capacity of catabolic pathways', bioaugmentation with catabolically superior bacteria has been identified to be more advantageous to achieve improved remediation (Dueholm et al., 2015).

The ubiquity of diverse microbial groups with enzymatic capabilities necessary for the *in situ* breakdown and mineralization of broad range of toxic petroleum hydrocarbons have been widely reported from diverse petroleum HC enriched environment (Fuentes et al., 2014; Kostka et al., 2011; Rodriguez-R et al., 2015). Although isolation-characterization of microbial populations from diverse HC rich environments including oil reservoirs, oil spill sites, contaminated rivers, and other environment relatively well studied (Das and Kazy, 2014; Hilyard et al., 2008; Lamendella et al., 2014), there is a paucity of knowledge on indigenous bacterial communities that simultaneously catalyze alkane and aromatic degradation in petroleum refinery waste sites (Sarkar et al., 2016). Particularly, information on the identity of the cultivable members of the community within petroleum HC enriched waste sludge and evaluation of their abilities for actively degrading the constituent HCs have not been specifically addressed. Isolation and evaluation of strains for degradation of a broad range of HCs present in petroleum wastes are advantageous over culture independent molecular approaches (Hilyard et al., 2008). Isolated strains are useful to (i) elucidate links between phylogeny and catabolic abilities, (ii) explore sources of novel HC degrading genes, (iii) for bioaugmentation in HC contaminated sites/wastes. Introduction of allochthonous bacteria into the contaminated sites often show reduced efficacy due to lack of their adaptability (Abed et al., 2014) and therefore isolation of metabolically superior bacteria from the same habitat and their use as bioaugmentation agent is a sought after technique for developing more effective bioremediation processes (Dueholm et al., 2015; Tao et al., 2016).

With increased economic pressure for producing more oil, priority for sustainable management of HC enriched wastes remained high for the foreseeable future, and hence the development of improved biotechnological processes for their treatment gets prioritized. Isolation and characterization of petroleum HC degrading bacterial groups from refinery waste and evaluation of their candidature as bioaugmentation agent are considered a prerequisite for directing the bioremediation strategies for refinery waste/similar systems (Kostka et al., 2011). Thus, using the refinery waste sludge of Digboi oil refinery, Indian Oil Corporation Limited, Digboi, India, the present study was designed with following objectives: (i) to identify and unravel the ecophysiology of hydrocarbonoclastic bacterial that may serve as bioaugmentation agent (ii) to evaluate performance of the selected strains for *in situ* bioremediation. The entire study depicts the usefulness of bacterial strains as bioremediation candidates for eradicating perilous petroleum enriched refinery wastes and other petroleum contaminated environments.

2. Materials and methods

2.1. Sample collection and characterization

Petroleum refinery waste composed of semi-solid oily sludge (designated as DB2) were aseptically collected from ~30 cm below the top layer of a waste disposition site of Indian Oil Corporation Ltd. (IOCL) oil refinery, Digboi, Assam, India (27° 23′ 33.45″ N and 95° 36′ 55.458″ E) during June 2012. Collected samples were stored at −20 °C in air-tight (Schott-Duran) glass bottle up to the brim and transported to laboratory within 48 h. Enrichment and isolation of cultivable populations were started immediately as the samples reached the laboratory. Major physicochemical parameters (temperature, pH, etc.) were measured on site using OrionStar140™ multiparameter meter (Thermo Electron Corporation, USA). Total petroleum hydrocarbon (TPH) was measured by gravimetric method (Sarkar et al., 2016) followed by gas chromatography (GC) (Perkin Elmer Clarus 580, USA). Major anions were quantified through ion chromatography (IC) (Thermoscientific ICS Dionex 2100, USA) and metals were estimated using inductively coupled mass spectrometry (ICPMS) (Varian Palo Alto CA USA) and /or atomic absorption spectroscopy (AAS) (Perkin Elmer MA, USA). Details of the methods are same as described in Sarkar et al., (2016) and discussed in the later section.

2.2. Enrichment, enumeration and isolation of cultivable bacteria

Bacterial strain enumeration and isolation were done using (a) direct isolation through plating on to Reasoner's 2A (R2A) agar medium and (b) following enrichment with various aliphatic and aromatic hydrocarbons (HCs) and plating on multiple agar media. Heterotrophic bacterial cells from the refinery waste sample were enumerated by dislodging one gram sample in sodium pyrophosphate (0.1% w/v) for 12 h at 30 °C, 150 rpm followed by dilution plating on to Reasoner's 2A (R2A) (Himedia, India) agar plates and incubation at 30 °C. CFUs (colony forming units) were counted at periodic interval till 7 days and morphologically distinct colonies were collected. Enumeration of hydrocarbonoclastic cultivable bacteria and their isolation through enrichment was done in three different media, *viz*., Bushnell-Haas (BH) (Himedia, India) (for isolation of hydrocarbon degrading population), M9 (Ciric et al., 2010) (for assessing the effect of minimal inorganic nutrients on bacterial growth and diversity) and reduced BH (0.1% w/v cystein-HCl added and nitrogen purged, for anaerobic members). Enrichment experiments were set up with one gram waste sample as initial inoculum in Bushnell-Haas (BH), M9 and reduced BH (0.1% w/v cystein-HCl added and nitrogen purged) media with two carbon source supplements:1-crude oil (1% v/v) and mixture of each 1% v/v (hexane, dodecane, pentadecane, hexadecane, docosane and nonadecane) of aliphatic and aromatic 1% v/v (BTEX) or 1% w/v (naphthalene, anthracene) hydrocarbons and 2-crude oil (1% v/v). For the anaerobic enrichments reduced BH medium along with crude oil (1% v/v) was used as the sole carbon source and all the transfers were done inside anaerobic chamber (Coy Labs, USA). Enrichments were incubated at 30 °C in static (anaerobic) or shaking (150 rpm) (aerobic) conditions. Following growth (15 days) each enrichment broth were subcultured (thrice) in the same medium with similar HC mixture and incubated. After 45 days of growth distinct colonies were isolated from the three enrichment setups by plating 100 μl culture on to BH and M9 agars (overlaid with 0.5% v/v crude oil) incubated at 30 °C and on to the anaerobic agar plates incubated at 30 °C within anaerobic jar filled with N_2. Colonies appeared on each plates were inspected for morphological details, and unique colonies were picked up. All colonies obtained were subcultured thrice to check culture purity; all pure cultures were designated and stored (15% glycerol, −80 °C) for future analysis. Bacterial cells obtained from each of the isolation methods were designated as DB2 (direct isolation), DB2AN (enrichment – anaerobic), DB2CO (enrichment –only crude oil in BH medium), DB2ER (enrichment- only crude oil in M9 medium) and DB2SA (enrichment – HC mix in BH medium) along with their strain designations.