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Horizontal gene transfer versus biostimulation: A strategy for bioremediation in Goa

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ABSTRACT

Bioaugmentation, Biostimulation and Horizontal gene transfer (HGT) of catabolic genes have been proven for their role in bioremediation of hydrocarbons. It also has been proved that selection of either biostimulation or bioremediation varies for every contaminated site. The reliability of HGT compared to biostimulation and bioremediation was not tested. The present study focuses on reliability of biostimulatiion, bioaugmentation and HGT during biodegradation of Diesel oil and Non aqueous phase liquids (NAPL). *Pseudomonas aeruginosa* (AEBBITS1) having *alk*B and NDO genes was used for bioaugmentation and the experiment was conducted using seawater as medium. Based on Gas chromatography results diesel was found to be degraded to 100% in both presence and absence of AEBBITS1. Denturing gradient gel electrophoresis result showed same pattern in presence and absence of AEBBITS1 indicating no HGT. NAPL degradation was found to be more by Biostimulated Bioaugmentation compared to biostimulation and bioaugmentation alone. This proves that biostimulated bioaugmentation is better strategy for oil contamination (tarabll) in Velsao beach, Goa.

1. Introduction

Oil pollution is one of the major concerns of Goa tourism. Goa is located on the Arabian stretch of Indian peninsular region (15°29′N, 73°48′E) and its shoreline provides site for public recreational activities. Marine accidental oil spills or intentional disposal of oil is a major concern, as the spilled oil turns into tarballs and floods the beaches. It has been stated by National Institute of oceanography (NIO, Goa) that the occurrence of tarballs or mats of oil on beaches is a seasonal process. The trajectory of tarballs or oil slicks has been studied according to the wind currents and action of waves and found that the chances of oil and tarballs flooding coast of Goa is very high (Vethamony et al., 2007). Horizontal gene transfer (HGT) helps the bacteria to adapt to selection pressure prevailing in the contaminated environment. For instance the development of antibiotic resistance in bacteria is an example for the adaptation of bacteria by horizontal gene transfer (Tschape, 1994, Mazel and Davies, 1999a, 1999b). It has been proposed in several instances that bioaugmentation fails as the introduced bacteria could not survive due to nutrient deficiency at the contaminated site (Hamdi et al., 2007; Bento et al., 2005). Horizontal gene transfer of catabolic genes responsible for hydrocarbon degradation to the indigenous bacteria could be a solution and several studies have been conducted to prove the same. Naphthalene dioxygenase (NDO) is the frequently reported catabolic gene for horizontal gene transfer either by retrospective or mechanistic approach. Herrick et al. (1997) have found the presence of *nahAC* allele of NDO gene in different bacteria which are phylogenetically diverse. Stuart-Keil et al. (1998) have used TRFLP and Southern hybridization to study similarity between different size plasmids isolated from coal tar contaminated site. It was found that these naphthalene catabolic plasmids are closely related to each other and pDTG1 plasmid from *P. putida* NCIB 9816. TRFLP studies conducted by Giebler et al. (2013) on 400 alkane degrading soil isolates revealed the possibility of high genetic mobility of alkane hydroxylase gene (*alkB)*. Addition of required nutrients to the contaminated site (Biostimulation) can also result in successful bioremediation as the nutrient deficiency is dealt. Co-metabolism of hydrocarbon also results in better degradation (Arulazhagan et al., 2014; Pugazhendi et al., 2015). There were bioaugmentation studies conducted in lab scale and *Pseudomonas aeruginosa* (AEBBITS1), *Pseudomonas mendocina* (isolated from crude oil and bilge oil contaminated samples respectively) were proposed to be used for bioremediation via augmentation (Pasumarthi et al., 2013; Sivaraman et al., 2010; Prakash et al., 2008). In the incident of oil spill even though high content of oil is available, the rate of degradation was limited due to low availability of nitrogen and phosphorus (Atlas and Bartha, 1972). Though HGT and Biostimulation are reported to overcome the situation of failed bioaugmentation, HGT versus Biostimulation has been never investigated. The present study focuses on role of HGT and Biostimulation in successful bioremediation using *Pseudomonas aeruginosa* (AEBBITS1) as hydrocarbon degrading bacteria.

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2. Material and methods

2.1. Catabolic gene profile of Pseudomonas aeruginosa (AEBBITS1)

Genomic DNA was extracted as per the protocol by Rochelle (Rochelle, 2001) and catabolic genes for degradation of alkanes (*alk*BF: AAYACNGCNCAYGARCTNGGNCAYAA, *alk*BR: GCRTGRTGRTC NGARTGNCGYTG) (Wasmund et al., 2009), naphthalene (NDOF: CAC TCATGATAGCCTTGATTCCTGCCCCCGGCG, NDOR: CCGTCCCACAACAC ACCCATG CCGCTGCCG) (Kurkela et al., 1988), pyrene (NidAF: ATCTTCGGGCGCGCCTGGGTG TTTCTCGG, NidAR: AATTGTCGGCGGCT GTCTTCCAGTTCGC) (Brezna et al., 2003), polyaromatic hydrocarbons (Pdo F: GTTCTACCTCGACCTCATTGCG, Pdo R: CTGACC CATGTATTCCAG CC) (Krivobok et al., 2003) and Glutathione S transferase (Lloyd-Jones and Lau, 1997) were checked for their presence using Eppendorf flexi lid PCR. PCR was run for 30 cycles for all the genes mentioned.

2.2. Microcosm studies

2.2.1. For HGT

Three five liter sterile containers were filled with four liters of sea water. Every container has an inlet, outlet and a provision for aeration. Sterile air was supplied using 0.22 micron filters. First microcosm contains only sea water and the other two microcosms were supplied with 0.5% diesel oil as carbon source and also supplied with 3.27 g/l of Bushnell Hass medium to support microbial growth. The third setup was inoculated with *Pseudomonas aeruginosa* (AEBBITS1) to monitor the process of HGT while other two setups act as controls. The change in microbial diversity by addition of diesel oil and *Pseudomonas aeruginosa* (AEBBITS1) was studied using DGGE (Pasumarthi et al., 2013).

2.2.2. For NAPL degradation

The model non aqueous phase liquid (NAPL) was prepared as described by Mukherji et al. (1997). The NAPL was prepared by maintaining the mole fraction of each component less than its fugacity ratio. The mole fraction of each hydrocarbon used is as follows: n-Tetradecane (0.25), n-Hexadecane (0.45), n-Octadecane (0.11), n-Naphthalene (0.15), and n-Phenanthrene (0.04). As NAPL should represent diesel, hexadecane to Naphthalene ratio has been maintained in 3:1 ratio. Microcosm setup has been designed in 100 ml serum vials so that a comparison will be possible between natural attenuation, biostimulation and bioaugmentation (Sivaraman et al., 2010). In each microcosm 20 ml of sea water was taken and supplied with 100 μl of NAPL. Abiotic setup having sterile seawater and NAPL acts as control. 0.077 g/l of KH_2PO_4, 0.2 g/l of NH_4Cl, 0.1 g/l of $NaNO_3$were added as additional nutrients in the biostimulated microcosm setup (Hassanshahian et al., 2014). In setup of biostimulated bioaugmentation the above mentioned nutrients were added to sea water and also inoculated with *Psedomonas aeruginosa* (AEBBITS 1). In bioaugmented setup, sea water was enriched with NAPL and inoculated with *Pseudomonas aeruginosa* (AEBBITS 1).

Gas chromatography was performed for both diesel degradation setup and NAPL degradation setup. The residual diesel and NAPL were extracted by addition of equal volume of ethyl acetate and stirring for 15 min. For estimating residual diesel, ethyl acetate was evaporated and the sample was dissolved in 1 ml of ethyl acetate. 1 μl of sample was injected into Gas chromatograph to estimate the residual hydrocarbons (Pasumarthi et al., 2013). Total protein biomass was estimated as per the protocol mentioned in our previous study (Pasumarthi et al., 2013).

2.3. DNA extractionand DGGE

Genomic DNA was extracted from all the experiment setups as per the protocol suggested by Rochelle (2001) but with minor changes (Pasumarthi et al., 2013). The obtained DNA was confirmed with gel electrophoresis. Nested PCR was applied to amplify 16SrDNA by Eppendorf flexi lid thermal cycler. The primers used are 27F (AGA GTT TGA TCM TGG CTC AG), 1492R (CGG TTA CCT TGT TAC GAC TT) for first PCR and 968FGC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT), 1492R for GC clamping in second PCR (Nikolausz et al., 2008). DGGE was performed to understand the role and survivability of different bacteria from sea water, possibility of HGT, survivability of bacteria used in augmentation and as well as the difference between biostimulation, bioaugmentation and combination of biostimulation and bioaugmentation. 7.5% acrylamide gel was used with denaturant gradient ranging from 30% to 60% and 10 μl of sample was loaded and run for 14 h at 70 V.

2.4. Different parameters (pH, temperature, optical density, nitrogen, phosphorus)

Nitrogen by total kjeldahl (TKN) and Phosphorus by Vanadomolybdophosphoric acid were estimated as per the standard methods of APHA (2005). The samples were centrifuged to get rid of cell biomass before estimating nitrogen and phosphorus. Temperature was measured using thermometer and salinity was measured by Baume hydrometer (LEMICO). pH was measured by Equiptronics pH meter.

3. Results

3.1. Catabolic genes

The amplification of *alk*B and NDO genes from the bacteria showed that *Pseudomonas aeruginosa* (AEBBITS1) has both the genes but no other catabolic genes were present. The amplified product was confirmed based on size (Fig. 1) and*Pseudomonas aeruginosa* (AEBBITS1) has been selected for further studies.

3.2. Biodegradation of diesel

In the microcosm setup having diesel and the microcosm setup inoculated with AEBBITS1 there were about 13 different fractions of diesel observed. Gas chromatography was performed on every seventh day followed by addition of 0.5% diesel. The Gas chromatography results showed that there was no residual diesel present in both the microcosms (Figs. 2 and 3). There was no difference in degradation between the microcosm just with diesel and the one inoculated with *Pseudomonas aeruginosa* (AEBBITS1).

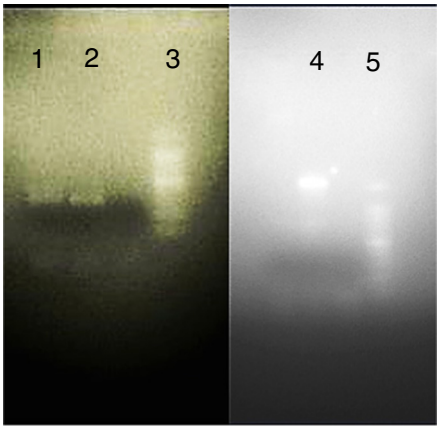

Fig. 1. a) alkB (Alkane hydroxylase) and b) NDO (Naphthalene dioxygenase) genes on gel electrophoresis. Lanes 1,2 represents alkB. Lane 4 represents NDO. Lanes 3,4 represents 100 bp ladders.

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