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Mass culture strategy for bacterial yeast co-culture for degradation of petroleum hydrocarbons in marine environment

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ABSTRACT

In the present study a metabolically versatile co-culture with two *Bacilli* and one yeast strain was developed using enrichment culture techniques. The developed co-culture had affinity to degrade both aliphatic and aromatic fractions of petroleum crude oil. Degradation kinetics was established for designing the fermentation protocol of the co-culture. The developed mass culture strategy led to achieve the reduction in surface tension (26 dynes cm^{-1} from 69 dynes cm^{-1}) and degradation of 67% in bench scale experiments. The total crude oil degradation of 96% was achieved in 4000 l of natural seawater after 28 days without adding any nutrients. The survival of the augmented co-culture was maintained (10^9 cells ml^{-1}) in contaminated marine environment. The mass culture protocol devised for the bioaugmentation was a key breakthrough that was subsequently used for pilot scale studies with 100 l and 4000 l of natural seawater for potential application in marine oil spills.
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1. Introduction

Every year over 1500 million tonnes of crude oil are transported over an extensive network of marine transshipment. Even with the best safeguards adopted, a substantial amount of oil is released into the marine environment, either as a result of operational discharges or due to accidents. The Indian subcontinent has a mainland coastline of 5500 km in addition to its offshore coastline of 2500 km. The Strait of Hormuz and the Strait of Malacca, located on the west and east coasts of India have been considered two of the prone areas of oil spill (Pasumarthi et al., 2013; Dasgupta et al., 2013). Recent examples are the Mumbai oil spill and Paradip (Odisha) oil spill in the year 2010. The hazardous oily waste is composed of a complex mixture constituting the total petroleum hydrocarbons (TPHs) comprising of various aliphatic, aromatic, NSO (nitrogen, sulphur, oxygen compounds) and asphaltene fractions. Aliphatics and aromatics are the two major components reported for their toxic nature to the environment (Bhattacharya et al., 2003; Sarma et al., 2004). The long term environmental and health impacts due to an oil spill are well documented. As a consequence there have been several studies reporting the development of methods for mitigation or remediation and restoration protocols to salvage the environmental damage of oil spill site (Vidali, 2001; Kadali et al., 2012). After the occurrence of an oil spill in marine environment, conventional oil remediation methods such as physical removal with booms, skimmers and absorbent materials must be deployed as these can help in the removal of the oil from the surface water layer, although complete removal is very rare. The use of chemical methods such as surfactants and solvents, disperse the oil into droplets relieving stress on the marine ecosystem. However, these methods do not remove the oil from the environment and has toxic side effects (Nikolopoulou and Kalogerakis, 2011; Sheppard et al., 2014). Unlike the conventional methods bioremediation is an easy to implement, economical and environmentally friendly solution for a marine oil spill. Moreover, it also offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it uses relatively lesser techniques, which generally have high public acceptance and can be easily implemented on site (Vidali, 2001).

Bioremediation utilizes microorganisms to accelerate biological degradation and/or precipitation of pollutants (Osborne et al., 2004). Biostimulation and bioaugmentation are distinct strategies of bioremediation used to treat hydrocarbon-contaminated environments. Biostimulation involves nutrient addition to stimulate the growth of the indigenous microbial community. Bioaugmentation is a technique that improves the degradation rate through the introduction of specific microbial strains or multiple strains known to be efficient degraders.

Microbial populations consisting of strains belonging to various genera such as *Pseudomonas*, *Acromobacter*, *Acinetobacter*, *Alcalogenes*,

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Arthrobacter, *Bacillus*, *Brevibacterium*, *Cornybacterium*, *Flavobacterium*, *Nocardia*, *Pseudomonas*, *Dietzia*, *Methylobacterium*, *Rhodococcus* and *Vibrio* species have been reported in petroleum-contaminated soil and water. The selection of appropriate microbes is a key factor that may affect the success of the bioremediation process, including the capacity to degrade contaminants, competition with autochthonous microorganisms, loss of microbial viability (Cappello et al., 2007; Tyagi et al., 2010; Dellagnezze et al., 2014). It has been established that virtually all types of hydrocarbons are susceptible to microbial degradation (Atlas and Cerniglia 1995; Pacheco et al., 2010; Kadali et al., 2012; Verde et al., 2013; Dellagnezze et al., 2014). The role of augmented microbes in commercial microbial products is often undetermined or questioned (Thouand et al., 1999). One of the biggest challenges, for a successful bioaugmentation process to be implemented in the field lies in preparing an effective inoculum of the strains to be augmented and thereafter maintaining the activity of the inoculants for a sustained duration of time in a particular environment (Mishra et al., 2001; Tyagi et al., 2010). This makes it imperative to monitor the physiological state of the augmented microbes as the metabolic state of a specific bacterial strain can be affected by biotic and abiotic variables associated within the contaminated site. Microcosm and pilot scale studies are thus necessary to establish the efficiency of developed culture in degradation of petroleum hydrocarbons (Mishra et al., 2001; Sarma et al., 2004; Simons et al., 2013).

Based on the points highlighted above, the main objective of the study was to develop a co-culture that has the capability to degrade a broad range of hydrocarbons in marine environment and to devise a protocol for its mass culture for successful bioaugmentation. Moreover the focus of the study was on development of an effective inoculum in large scale via mass culture strategy in 150 l bioreactor for successful application of petroleum hydrocarbon degradation in marine environment.

2. Materials and methods

2.1. Microorganisms and medium composition

Microorganisms used for the study were isolated by enrichment technique from seawater and soil samples contaminated with petroleum hydrocarbons from Vishakhapatnam coast (17° 41′ 18″ N, 83° 13′ 7″ E) Andhra Pradesh and Mehsana (23.60°N, 72.40°E), Gujarat, India respectively. The source of petroleum crude oil used in the present study was from the oil wells of Mehsana, Gujarat. In the initial degradation experiments Artificial seawater (ASW) medium (Kester et al., 1967) was used with petroleum crude oil (1% w/v) as the sole source of carbon. Similarly, ASW medium with the respective model aliphatic and aromatic compounds as sole carbon source (0.1% w/v) from Sigma-Aldrich (USA) was also used to evaluate individual hydrocarbon degradation. For growth kinetics and subsequent mass culture studies in the bioreactor (Mishra et al., 2001), fermentation medium was used consisting of ASW and 0.5% (w/v) sucrose as carbon source. Natural seawater from R.K beach, Vishakhapatnam (17° 41′ 18″ N, 83° 13′ 7″ E), Bay of Bengal and Dauna Paula coast (15° 27′ N, 73° 48′ E), Arabian Sea, Goa, with 1% (w/v) petroleum crude oil (sole carbon source) was used for the augmentation studies at pilot scale as described by Simons et al., 2013; Sheppard et al., 2014.

2.2. Isolation and enrichment of microorganisms

Isolation of microbes was done by selective enrichment of the samples using petroleum crude oil as sole source of carbon in simulated seawater conditions. Portion of soil (1 g) or water (1 ml) samples were added to 250 ml Erlenmeyer flasks containing 100 ml ASW media with 3% (w/v) NaCl at 30 °C and 180 rpm. Further, all the samples were enriched in ASW media with 1% (w/v) of petroleum crude oil as the sole source of carbon. After five cycles of enrichment in ASW, 1 ml of culture was diluted 10^5 fold, and 100 μl was plated on ASW medium plates with petroleum crude oil (1% w/v) as the carbon source to obtain phenotypically different colonies. Phenotypically different colonies obtained from the plates were transferred to a fresh ASW medium with petroleum crude oil (1% w/v) as sole carbon source to eliminate autotrophs and agar-utilizing bacteria (Hassanshahian et al., 2012a, 2012b). The procedure was repeated multiple times hence the strains exhibiting pronounced growth and utilization of petroleum crude oil as a sole source of carbon were stored for further characterization (Mishra et al., 2001; Kadali et al., 2012; Hassanshahian et al., 2012b). The enriched culture, as well as the purified isolates was routinely sub cultured and stock cultures were maintained in 25% glycerol at −70 °C for subsequent studies.

2.3. Identification and characterization of microorganisms

The enriched co-culture was microscopically examined by Gram staining and subsequently subjected to DNA extraction for molecular characterization. The bacterial strains were identified by sequencing of the 16S rDNA gene as described by Sarma et al. (2004) whereas the identification of the yeast strains was done by sequencing the ITS region as described by Prasad et al. (2005). Sequences were analyzed and identified using microseq microbial identification and analysis software and nucleotide–nucleotide Blast (Blast N), RDP search of NCBI database. Multiple sequence alignments were performed using CLUSTAL W, version 1.8 (Thompson et al., 1994). Phylogenetic tree of the individual strains in co-culture was constructed with the evolutionary distances using the neighbor-joining method. Tree topologies were evaluated by performing bootstrap analysis of 1000 data sets with the MEGA version 5.1 packages (Tamura et al., 2011).

2.4. Hydrocarbon degradation study

The degradation rate of petroleum hydrocarbons of the individual strains as well as in combination as co-cultures was examined in ASW medium. A volume of 25 ml ASW medium was used in 100 ml flask with 1% (w/v) petroleum crude oil as sole source of carbon. Individual experimental sets of ASW flasks with different model alkane and aromatic hydrocarbons as sole source of carbon were taken. Four different model alkanes (0.1% w/v) with varying carbon chain lengths (C17, C20, C25, C30) and three different model aromatic hydrocarbons (pyrene, naphthalene and fluorene) were selected for the study. These individual authentic model compounds of both aliphatic and aromatic standards were procured from Sigma, USA. The flasks were incubated on rotatory shaker (180 rpm) at 30 °C for 72 h. The strains were grown overnight in Luria Bertani broth to a cell density of 10^8 cells ml^{-1} and 3% v/v was used as an inoculum. Flasks containing uninoculated ASW medium with the addition of the respective hydrocarbons was kept as control to monitor abiotic loss. The reduction in extractable petroleum hydrocarbons was evaluated by comparing with uninoculated controls. This was done to nullify the loss due to vaporization or due to undissolved components such as asphaltene, which was not extracted. The remaining residual crude oil was extracted after 72 h with thrice the volume of hexane. The degradation was calculated gravimetrically by simple weight loss technique evaluating the percentage reduction in petroleum crude oil as compared with control (Lal and Khanna, 1996; Mishra et al., 2001, Mandal et al., 2012). Further, the degradation was analyzed by gas chromatography (GC-FID) as described in Section 2.6. For the individual aliphatic and aromatics hydrocarbons (model compounds as sole source of carbon), the residual undegraded alkane and aromatic flasks were extracted using twice the volume of hexane and toluene respectively. The degradation percentage was estimated by calculating the difference of the area under the curve of the individual model hydrocarbons with their respective control by Gas chromatography (GC-FID as described in Section 2.6) by the method described by Lal and Khanna, 1996; Mishra et al., 2001. All the experiments were done in

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