Bioremediation of petroleum oil contaminated water

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Abstract

The wide spread uses of petroleum oil and petroleum based products lead to contamination of soil and aquatic environments causing problems to all life forms including humans. Although there are a number of physical and chemical methods available for treating oil polluted environments, most of them are expensive, time consuming and not environmental friendly at all the time. Therefore, biological remediation systems have evolved as greener alternatives for those "not so much environmental friendly" techniques.

Microbial remediation of a hydrocarbon contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria in soil. In order to develop a successful bioremediation strategy, isolation and characterization of oil degrading microorganisms and optimizing conditions for efficient biodegradation are important aspects.

In the current study, five bacterial isolates and two fungal isolates were isolated using the petroleum oil contaminated soil samples obtained from Spugaskanda oil refinery division. The ability of the isolated bacterial and fungal isolates to degrade crude petroleum oil was monitored by a gravimetric method and a potential bacterial combination was identified which showed a significant effect on biodegradation of crude petroleum oil. The bacterial combination consisted of three different bacteria, which were identified by analyzing the 16s rRNA gene sequences, as *Bacillus aerophilus*, *Bacillus oleronious* and *Pseudomonas* sp. In addition, a conducive nutrient combination was identified as 700 $mgdm^{-3}$ of nitrogen and 1.0 g of glucose per 1L of Bushnell Hass broth medium, which can be applied to enhance the biodegradation of crude petroleum oil by the identified bacterial combination. This study indicates the potential for developing a successful bioremediation strategy for dealing with petroleum oil contaminated environments

Introduction

Petroleum continues to serve as the main source of energy, all over the world. Petroleum and its products such as gasoline, kerosene, diesel/fuel oil have since been used as fuels for land, air and sea transport, for heating purposes, for electric power generation and as petrochemical sources and lubricants. Therefore, petroleum industry is one of the main sectors of the world economy. Petroleum industries bring innumerous benefits to society but are globally recognized as an economic activity with great impacts to the environment, because of leaks and accidental spills during the exploration, production, refining, transport and storage of petroleum and petroleum products lead to environmental pollution. Therefore, one of the major problems faced today is to identify cost effective and environmental friendly strategies to deal with the hydrocarbon pollution experienced by both developed and developing countries.

Although, a number of physical and chemical methods are available to mitigate hydrocarbon pollution, they are time consuming and not environmental friendly at all times. Therefore, the use of biological methods for the remediation of petroleum contaminants can be considered as a greener alternative to environment damaging traditional methods.

Biodegradation can be defined as the biologically catalyzed reduction in complexity of chemical compounds. Accordingly, biodegradation involves the breakdown of complex organic compounds either through biotransformation or mineralization into less complex compounds: H_2O and CO_2 under aerobic mineralization or CH_4 under anaerobic mineralization. Bacteria and fungi are micro-organisms which can degrade organic compounds including petroleum hydrocarbons in soil and water because they are equipped with metabolic machinery to use petroleum hydrocarbons as their carbon and energy sources. Therefore, micro-organisms and microbial processes can be used as potential remediating solutions for petroleum contaminants as cost-effective and environmentally safe methods for treating oil polluted sites.

In order to develop a successful bioremediation strategy for petroleum oil contaminants, isolation and characterization of oil degrading microorganisms from indigenous flora and optimizing conditions for efficient biodegradation of petroleum contaminants are important aspects. This paper deals with the isolation and characterization of hydrocarbon degrading bacteria and evaluation of their efficiency in degrading crude oil to assess their potential field applications.

Materials and Methods

Sample collection

Soil samples were collected from five different oil contaminated sites in oil refinery division of Ceylon Petroleum Corporation, Sapugaskanda, Sri Lanka. Soil samples were obtained from the surface and up to a few centimeters depth from the surface of the contaminated soil. They were collected into separate polythene bags, tightly packed and labeled A, B, C, D and E. In addition to soil samples, a water sample was collected from a drain water tank in the refinery premises into a sterilized glass bottle and labeled as F. All the samples were carefully transported to the laboratory under ambient conditions for the isolation of crude oil degrading microorganisms.

Enrichment and isolation of crude oil degrading microorganisms

From each soil sample, 5.0 g of soil was introduced into 100 ml flasks containing 25 ml of R2B broth (Composition: Peptic digest of animal tissue 0.50 g/l, Magnesium sulfate heptahydrate 0.10 g/l, Sodium pyruvate 0.30 g/l, Casein acid hydrolysate 0.50 g/l, Dipotassium phosphate 0.30 g/l, Yeast extract 0.50 g/l, Soluble starch 0.50 g/l, Glucose 0.50 g/l; Final pH at 25°C 7.2 ± 0.2). From sample F, 5.0 ml of contaminated water was inoculated into 25 ml of R2B broth. Another set of enrichment cultures were prepared, inoculating 1.0 g of each soil sample into test tubes containing 10.0 ml of Bushnell Hass (BH) broth (Composition: Magnesium sulphate 0.20 g/l, Calcium chloride 0.02 g/l, Monopotassium phosphate 1.00 g/l, Dipotassium phosphate 1.00 g/l, Ammonium nitrate 1.00 g/l, Ferric chloride 0.05 g/l, Agar 20.0 g/l, Final pH at 25°C 7.0± 0.2). From sample F, 1.0 ml of contaminated water was inoculated into 10.0 ml of BH broth. Triplicates were prepared for each sample and they were incubated at room temperature for 7 days.

After the incubation period, 0.3 ml of broth culture from each sample was plated on crude oil incorporated Minimal Salt Agar (MSA) medium (Composition: Dipotassium phosphate 7.00 g/l, Monopotassium phosphate 2.00 g/l, Magnesium sulphate 0.10 g/l, Sodium citrate 0.5 g/l, Ammonium sulphate 1.50 g/l, Agar 18.00 g/l, Crude oil 1% (v/v); Final pH at 25°C 7.2 ± 0.2) using the spread plate technique. Two sets of spread plates were prepared for R2B broth enrichment cultures and BH broth cultures. All the plates were incubated at 37°C for 7 days. Fungal colonies grown in the enrichment medium, which were floating on the broth were plated separately on crude oil incorporated MSA and the plates.

The bacterial colonies that formed clear zones around the colonies in spread plates were visually selected based on their colony characteristics and isolated further by streaking on to crude oil incorporated MSA plates using quadrant method. Streaked MSA plates were incubated at room temperature for 7 days. Bacteria that had similar colony morphologies were selected only once, avoiding the possibility of isolating the same strains. The streaking process was repeated twice to obtain pure bacterial isolates. Fungal isolates were also plated on crude oil incorporated MSA and pure colonies were obtained. Pure cultures of bacterial and fungal isolates were maintained on Nutrient Agar (NA) slants and Potato Dextrose Agar (PDA) slants and stored at 4°C in a refrigerator. All the stock cultures were sub-cultured at intervals of 30 days.

Monitoring of crude oil biodegradation

BH broth medium supplemented with 1% (v/v) of crude oil was used to study crude oil biodegradation,. A volume of 50 ml of BH broth was dispensed into 100 ml conical flasks and 0.5 ml of crude oil was added to each flask. After autoclaving and cooling, each flask containing sterilized BH broth supplemented with crude oil was inoculated with 1.0 ml of individual bacterial and fungal spore suspensions. In addition to individual isolates, a bacterial combination was prepared by adding 1.0 ml of three selected bacterial suspensions into BH broth supplemented with crude oil. Three bacterial strains which shown high growth and efficient clear zone formation in oil incorporated MSA plates were selected to be used in the bacteria combination. Triplicates were prepared for each isolate and for the combination. After inoculation, all the flasks were covered with

Bacterial isolate code	Gram reaction	Cell shape and arrangements
2A-1	Gram negative	Rod-shaped, isolated small cells
2B-1	Gram negative	Coccus, arranged into chains
2E-1	Gram negative	Rod-shaped
1F-1	Gram positive	Rod-shaped
2F-2	Gram positive	Rod-shaped, elongated cells

Table 1: Morphological and biochemical characters of isolated bacterial strains

cotton plugs and incubated at 28°C for 14 days on a rotary shaker (TAITEC, BR-300L) at 100 rpm.

As the next step, combined degradation of the same bacterial combination (used in the previous step) was examined with modified BH broth supplemented with 1% (v/v). When preparing the medium, carbon and nitrogen content of the BH broth was modified by adding different concentrations of glucose and urea, respectively. Six different carbon and nitrogen combinations were prepared as follows: no change in nitrogen content in BH broth (N_0), two-fold increase in nitrogen content (N_1), three-fold increase in nitrogen content (N_2) and adding 0.5 g of glucose per 1l of BH broth (C_1) and 1.0 g of glucose per 1l of BH broth (C_2). A set of 100 ml conical flasks were filled with 50.0 ml of prepared broth solutions and triplicates were prepared for each simulation. After autoclaving and cooling, each flask was inoculated with 1.0 ml of three selected bacterial combinations and incubated at 28°C for 14 days in a rotary shaker (TAITEC, BR-300L) at 100 rpm.

Extraction of residual crude oil

For extraction of residual crude oil from the medium, 6 ml of n-hexane was added to the flask containing BH broth and residual oil. The contents were transferred to a separating funnel and residual oil was extracted to a pre-weighed MaCartney bottle. Extraction was carried out thrice for each sample to ensure complete recovery of oil. After extraction, MaCartney bottles containing the extract were dried at 65°C in a dry oven (SANYO, MOV-112) for 2 days to remove moisture.

Analysis of the reduction of crude oil amount

Analysis of the data obtained in the two experiments was carried out using the Minitab 17 software package. One-way ANOVA was performed to test whether, there was a significant difference between the mean reduction of crude oil amount in broth cultures inoculated with individual isolates and to test whether, there was a significant difference between the mean reduction of crude oil amount in modified broth cultures with different combinations of carbon and nitrogen.

Identification of bacteria

Selected bacterial isolates for the preparation of the bacterial combination were subjected to molecular identification using 16s rRNA gene sequencing. Total DNA was extracted from a small colony of 24 h old bacterial culture using Wizard® Genomic DNA Purification Kit. 5 µl of extracted DNA was subjected to Polymerase Chain Reaction (PCR) using 27F/800R and 518F/1492R primer sets. Amplified DNA was subjected to DNA sequencing using 518F (5' CCAGCAGCCGCGGTAATACG 3') and 800R (5' TACCAGGGTATCTAATCC 3') primers. The obtained DNA sequences were compared with known sequences in GenBank using the Basic Local Alignment Search Tool, BLAST (www.ncbi.nlm.nih.gov.blast). DNA isolation, PCR amplification and sequence analyses were done at GENETECH Molecular Diagnostics, Colombo 08, Sri Lanka and DNA sequencing at Macrogen Inc., Korea.

Results and Discussion

The main objective of the present study was to develop a bioremediation strategy using microorganisms for remediation of petroleum oil contaminated water. In order to do so, crude oil degrading microorganisms were isolated from oil contaminated soil habitats and their oil degradation ability was studied under different nutritional combinations, to identify an efficient oil degrading bacterial combination. A total of 12 bacterial strains and 2 fungal strains were isolated from different spread plates prepared by inoculating the enrichment media. Five of the twelve bacterial isolates and the 2 fungal strains were identified as crude oil degraders, avoiding isolates which show similar colony characteristics and very low clear zone formation. The bacterial isolates were coded as 2A-1, 2B-1, 2E-1, 1F-1, 2F-2 and the fungal isolates as 1D* and 2D-1*. Gram reactions, cell

shapes and arrangements of the five bacterial isolates are listed in Table 1.

Monitoring of crude oil biodegradation of isolated bacterial and fungal strains was carried out using BH broth medium supplemented with 1% crude petroleum oil, followed by a gravimetric analysis.

As the first step, the crude oil biodegradation of individual isolates and the selected bacterial combination was measured as the reduction of crude oil amount initially added to the media. Because of the initial amount of crude oil (0.5 g per 50 ml of broth) added to the media is same in all the samples, the measurement of the reduction of crude oil after a certain incubation period gives an indication about the biodegradation of crude oil initially added to the media. After a 14 day incubation period, residual crude oil was extracted from the broth medium to examine the crude oil biodegradation. The highest value for mean reduction of crude oil amount and the highest value for the crude oil degradation % were achieved from bacterial combinations rather than by individual isolates. Respective values for reduction of crude oil amount and % degradation of crude oil in each case are presented in Table 2, which shows the higher efficacy of of bacterial combinations compared with individual isolates (P=0.003). The positive effect of using a bacterial combination, instead of using an individual isolate for the degradation of crude oil has been demonstrated by several authors. A bacterial consortium of five different bacteria was reported to achieve 78% crude oil biodegradation. In a study with bacteria isolated from untreated effluent water a maximum utilization of hydrocarbons indicated by the total discolouration of DCPIP in 53 hours was shown by a bacterial consortium prepared with six isolates. A mixture of two bio surfactant producing bacteria (*Alcaligenes piechaudii* and *Ralstonia picketti*) was reported to achieve 80% crude oil degradation after a 20 day incubation period. The bacterial combination which showed the highest crude oil degradation was used for further study.

As hydrocarbon degradation in most cases is limited by availability of nitrogen and utilizable carbon for co-metabolism, the effect of carbon and nitrogen combinations on biodegradation of crude oil was examined. Co-metabolism is defined as the metabolism of a compound that does not serve as a source of carbon and energy or as an essential nutrient which can be achieved only in the presence of a primary substrate.

Table 2. Degradation of crude oil by individual isolates and in bacterial combination

Isolate	Mean reduction of crude oil amount (g) ± SEM	Mean % degradation of crude oil
Control	0.0617±0.0058	12.29
2A-1	0.1910±0.0070	38.24
2B-1	0.1867±0.0194	37.25
2E-1	0.1810±0.0076	36.25
1F-1	0.1843±0.0068	36.85
2F-2	0.1560±0.0254	30.38
1D*	0.1876±0.0070	36.92
2D-1*	-	-
2E-1, 1F-1 and 2F-2	0.2253±0.0251	45.08

The application of co-metabolism to site-remediation of xenobiotic compounds including petroleum hydrocarbons is required as the compounds cannot serve as a source of carbon and energy due to their molecular structures, which do not induce the required catabolic enzymes. Although a carbon source in the form of petroleum hydrocarbons was added to the BH broth, glucose was added to the media in two different concentrations and available nitrogen increased up to three fold by adding two different concentrations of urea in order to facilitate co-metabolism of bacteria. Urea was used as a nitrogen source as it is a readily available cheap nitrogen source, which would be important when offering bioremediation as a real world solution.

The results obtained for crude oil biodegradation by bacterial combinations at different carbon and nitrogen levels indicated that the combination with a two-fold increase in nitrogen (in this case, 700 mgdm) and 1.0 g of glucose per litre of broth was the best combination of nitrogen and carbon (P=0.001). The respective values for reduction of crude oil amount and % degradation of crude oil in each case are shown in Table 3. Since crude oil contains a large number of different hydrocarbons, the carbon input for the medium is high compared with the nitrogen level present in the normal BH broth. There is the possibility of nitrogen content in the media becoming limited inhibiting degradation of crude oil. With the addition of urea, additional nitrogen was given to the media to avoid a possible limiting effect of nitrogen. In addition, additional carbon in the form of glucose helps switch on the respective pathways in co-metabolism for efficient degradation of crude petroleum oil.

Table 3. Degradation of crude oil by bacterial combinations with different carbon and nitrogen combinations

Nutrient combination	Mean reduction of crude oil amount (g) ± SEM	Mean % degradation of crude oil
N_0C_1	0.2347±0.0046	46.93
N_0C_2	0.2537±0.0123	50.81
N_1C_1	0.2816±0.0014	56.29
N_1C_2	0.3190±0.0108	63.75
N_2C_1	0.2557±0.0233	52.96
N_2C_2	0.2763±0.0109	55.28
Control	0.0807±0.0048	16.59

Finally, the bacterial isolates in the combination were identified by analyzing the 16s rRNA gene sequences. The alignment of the 16s rRNA gene sequence of isolate 2E-1 with sequences obtained by a Blast search revealed 99% similarity to *Pseudomonas* sp. Two other isolates, 1F-1 and 2F-2 revealed 99% and 100% similarity to *Bacillus aerophilus* and *B. oleronius* respectively. The biodegradation efficiency of these bacteria has been reported by several authors. *Pseudomonas aeruginosa* has been reported to degrade 58% of crude oil with direct or indirect addition of the biosurfactant, rhamnolipid. A bacterial consortium with five different bacteria including *Bacillus* sp. and *Pseudomonas* sp. have been reported to effect a 78% of biodegradation of crude oil. In the present work, an effective combination of crude oil degrading bacteria and a combination of carbon and nitrogen to enhance the biodegradation were identified. In order to develop *in-situ* or *ex-situ* bioremediation strategies for the remediation of petroleum oil contaminated environments, such combination of bacteria and nutrients can be used effectively and conveniently.

Conclusion

In the current study, five bacterial strains and two fungal strains were isolated using soil samples contaminated with petroleum oil. The ability of the isolated bacteria to degrade crude petroleum oil was monitored by a gravimetric method. A prepared bacterial combination using three bacterial isolates which showed highest growth and clear zone formation in oil incorporated media was identified as an efficient oil degrading solution. In addition, the favorable nitrogen and carbon combination was also identified as 700 mgdm of nitrogen and 1.0 g of glucose per litre of BH broth medium, which can be applied to enhance the biodegradation of crude petroleum oil by the identified bacterial combination. The bacterial isolates in the combination were identified as *B. aerophilus, B. oleronious* and *Pseudomonas* sp. by analyzing the 16s rRNA gene sequences. Such a bacterial combination and nutrient combination can be effectively used as a bioremediation solution for petroleum oil contaminated environments

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