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Research Article

RHIZOREMEDIATION OF PETROL ENGINE OIL USING BIOSURFACTANTS PRODUCING MICROBIAL CONSORTIUM IN MUSTARD CROP

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Abstract

Contamination of soil / water resources by petroleum products poses severe threats to underground water and soil quality. In the present study biosurfactant producing bacterial cultures were used to degrade petrol engine oil under *in situ* conditions in the plant rhizosphere system. Two bacterial isolates used in this study were recovered from Haldia oil refinery sites and identified as *Pseudomonas aeruginosa* (JX100389) *and P. moraviensis* (JX149542). Application of consortium C2, (*Pseudomonas aeruginosa and P. moraviensis*) degraded 79.02 % petrol engine oil @ 2% in the soil planted with mustard (*Brassica juncea var. Kranti*) crop after 120 days. GC-MS of biodegraded fuel showed the presence of new product like hexadecanoic acid 2, oxo-methyl ester.

Key words: Rhizoremediation; biosurfactant; consortium; bioavailability.

Introduction

Petroleum is a natural product and formed by anaerobic conversion of dead biomass at high temperature and pressure. After processing it is converted to petrol, diesel, petrol engine oil *etc*. Indiscriminate use and spills of these hydrocarbons lead to contaminate agriculturally important soils and groundwater. The ability of microbes to degrade organic contaminants into harmless constituents has been explored as a means of biological treatment of contaminated environments (Singh *et al.*, 2015). Biodegradation is an environmental friendly and economical process to deal with such problems by using microorganisms and plants. It is a process usually performed by microbial consortia under natural condition facilitating the metabolism of individual microorganism(s) by exchanging substrates and products within the microorganisms, involved in consortia (Trigo and Vaencia, 2009). Such cooperation is observed not only for complex substrates such as petroleum mixtures but also for single compound of complex structures (Ghazeli *et al.*, 2004; Breujelmans *et al.*, 2007). Microorganism producing biosurfactants can be used for mitigation of such polluted sites.

Biosurfactants are amphipathic molecule and consist of a polar (hydrophilic) moiety and a non polar (hydrophobic) moiety which partition preferentially at the interface between fluid phase with different degrees of polarity and hydrogen bindings. Hydrophilic group of a biosurfactant consists of mono-oligo- or polysaccharides, peptides or proteins and hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohol**.** These molecules reduce surface and interfacial tensions in aqueous solutions and hydrocarbon mixtures. Biosurfactants are structurally diverse group of surface-active substances produced by different groups of microorganisms during late log or stationary phase of the growth (Desai and Banat, 1997). Biosurfactant production is regulated by available carbon source, pH, salinity and temperature (Saikia *et al.*, 2012).

The range of pollution in the soil extends from industrial waste including (polychlorinated biphenyls, trichloroethylene, pentachlorophenyl and dioxin) via polyaromatic hydrocarbons, crude oil, refinery products (kerosene, gasoline, diesel fuel, petrol engine oil, benzene, toluene) and pesticide to heavy metals. Pollution of sea water and coast with aromatic containing crude oil resulting from oil tanker discharge and accident is a worldwide problem. Biosurfactants have been reported to biodegrade hydrocarbon and xenobiotic compounds (Muller Hurting *et al.*, 1993; and Finnerty, 1994). In comparison to their chemically synthesized equivalents they have many advantages. They are environment friendly, biodegradable, less toxic and non-hazardous hence preferred over chemical surfactants (Singh *et al.*, 2013).

Biosurfactants have better foaming properties and higher selectivity. They are active at extreme temperatures, pH and salinity (Kebria *et al.*, 2009), and can be produced from

industrial wastes and from by-products. This feature favours their cheap production and also allows utilizing waste substrates and reducing their polluting effect at the same time (Kosaric, 2001; Rahman *et al*., 2003; Das and Mukherjee, 2007; Das *et al*., 2008). Because of their potential advantages, biosurfactants are widely used in many industries such as agriculture, food, cosmetics and pharmaceutics (Muthusamy *et al.*, 2008; Banat *et al.*, 2010; Soberón-Chávez & Maier, 2011). Characterization of bacterial isolates, recovered from the oil contaminated soil from Jhansi and their biosurfactants were characterized on the basis of their emulsification potential for different oil.

Most important species of oil degrading bacteria belong to genera *Pseudomonas* .These microorganisms excrete a variety of biosurfactants which are biodegradable and consequently environmentally safe and help in solubilisation of xenobiotic compounds. Different groups of microorganisms plant growth promotory properties are reported to produce biosurfactants.

Materials and Methods

Isolation and Identification of oil degrading bacteria

All the bacterial strains (H1A= *Pseudomonas aeruginosa and* H1B= *P. moraviensis*) used in this study were isolated from oil contaminated sites (Source: Haldia (22^0 3' 37.65''N latitude and 88^0 06' 35.09'' E longitude. These two strains were selected and characterized on the basis of 16SrDNA sequencing. These two bacterial strains (H1A and H1B) which were previously used for biodegradation of mobil oil hydrocarbon by Kumar *et al.*, (2013).

PGPR properties and biosurfactants production

The plant growth promotory properties *viz*. phosphate solubilization, siderophore production, indole acetic acid production, ammonia production, hydrogen cyanide (HCN) production were tests performed for biosurfactant production and their characterization included turbidity test, foaming, cell biomass and emulsification index (mobil oil). (Kumar *et al.*, 2013).

Biosurfactant extraction and its characterization by FTIR

Biosurfactants from 72 h old bacterial cultures were extracted in diethyl ether according to Bagchi (2008). For FTIR analysis, biosurfactant samples were dried overnight 60^0C with KBr in an oven to remove traces of moisture. Sample (20 mg) was mixed with KBr (120 mg) and grinded using a pastle and mortar. Finely grined sample was analyzed using FTIR model Bruker, vertex 70 Fourier transform IR (FTIR) spectrophotometer at Dept. of Biophysics of the University.

Oil emulsification experiment

Two ml culture supernatant obtained by centrifuging 96 h old bacterial cultures in minimal salt medium at 8000 rpm for 20 min at 4^0 C, was mixed with 2 ml petrol engine oil in a test tube. After measuring the height of the mixture contents were vortexed for 2 min. Tubes were allowed to stand for 24 h. Height of the emulsion layer was measured to determine the emulsion index according to Amiriyan *et al.*, (2004). The equation used to determine the emulsion index E_{24} (%) is as follows:

$$E24\ (\%) = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

Development of consortia

Based on emulsification index and PGPR properties bacterial strains were selected to develop consortium. Bacterial strains showing compatibility on kings B agar medium were selected for consortium. Compatibility among the strains was also checked in MSM broth medium in the presence and absence of hydrocarbons (petrol engine oil 2%). The absorbance was taken from 0 to 120h at an interval of 24h using a spectrophotometer (Lamda35, Perkin Elmer).

Pot experiment

The experimental soil was mixed, allowed to dry to get water content less than 1%, sieved with 2 mm sieve and autoclaved at 121°C for 1hr for three consecutive days. Autoclaved soil (1.5 kg) was transferred to each pot and mixed with 30g (2%) of petrol engine oil hydrocarbons. Microbial consortium were also added to the experimental pots (50 ml i.e. ~ 5×10^5 cells ml^{-1}). Suitable sets without hydrocarbons and/or bacterial consortium were kept as control. Surface sterilized seeds of mustard (*Brassica juncea var. Kranti)* were sown at a depth of 2.5 cm. Observation regarding germination (seedling germination percentage) and plant health parameters (i.e. seedling survival, average plant height, chlorophyll content using Spadmeter (optisciences 2000), plant biomass, average shoot height, average stem dry weight with fruit, average seed weight, average root weight and average yield per pot) (data not shown). This experiment was carried out for 120 days in poly house at room temperature.

GC-MS analysis of residual hydrocarbon

Approximately 5g of samples from petrol engine oil contaminated soil was taken initially (0 days, control) and after cultivation of mustard at 120 days. To each of the soil samples, 15 ml of acetone was added followed by 1hr shaking. Obtained residue was filtered and washed twice with acetone, followed by filtration and concentration on a rotatory vaccum evaporator at 50°C and analyzed for the presence of residual hydrocarbons using FTIR. For Gas Chromatography Mass Spectrophotometry (GC-MS) analysis of the extracted residual hydrocarbons sample was dissolved in 1 ml hexane. The quantification of hydrocarbon compound was done by high resolution gas chromatography using QP-2010 gas chromatograph (Shimadzu) equipped with a HP-DB 5MS column (60 m × 0.25 mm, 0.25 µm film

thickness) coupled with mass spectrometer detector (HP 5972) at AIRF, JNU, New Delhi (India).

Results and Discussion

Identification of Plant Growth Promotory microorganisms

These two bacterial strains were found rod shaped and Gram$^-$ and showed homology with Pseudomonas (homology 97-99%).

All the morphological and Plant growth promotory traits of the recovered test strains were shown in Table 1 &2 (Kumar *et al.*, 2013). Application of these bacterial strains can help in solubilizing and sequester iron from soil and provide it to plant cells under stress. Once these Sid$^+$ strains bind iron and make microbial iron siderophores availability of complex soluble Fe^{++} become carry to plants (Meyer, 2000 and Meyer *et al.*, 2002). Indole acetic acid (IAA) production were best shown by H1A> H1B which can be used to enhance various stages of plant roots and different availability (Kumar *et al*., 2013). Biosurfactant, produced by these organisms, could be rhamnolipid as evident by Sadoudi *et al.*, (2014). Similar kind of study has also been reported by Chandankere *et al.*, (2014).

Consortia are better degraders of hydrocarbons as compared to single organism. So based on the emulsification index, compatibility studies and plant growth promotory properties bacterial consortium were developed in which both organisms were compatible with each other. Under *in vitro* conditions consortium (C2) utilized petrol engine oil as carbon source and showed enhanced exponential growth as compared to control in the absence of any carbon source.

Oil emulsification activity

The biosurfactant produced from the consortium emulsified petroleum hydrocarbon substrates such as petrol engine oil, petrol and diesel oil (Fig 1). Results showed that maximum emulsification observed in case of petrol engine oil hydrocarbon which was 82.32% as compare to other 35.79 % and 75.28% in diesel and petrol after 72 h of time intervals (Fig 1). Similar kind of study also observed in *P. fluorescens* by Barathi and Vasudeven, (2001).

In situ bioremediation studies using mustard crop

In order to investigate the optimum condition for the biodegradation of petroleum hydrocarbons soil was spiked with 2% petrol engine oil and bacterized with efficient consortium. *In situ* experiment was performed to assess the combined impact of phyto & bioremediation in remediating soil from the toxic effect of petroleum hydrocarbon in mustard cropping system under the influence of C2 bacterial consortium.

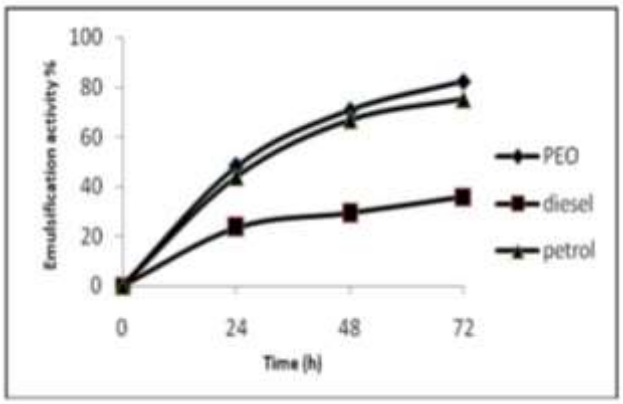

Fig 1: Emulsification of petroleum hydrocarbon substrate (petrol engine oil, petrol &diesel) by consortium C2.

GC-MS Analysis

The GC-MS analysis showed that the compounds appearing in the control sample at 0 DAS in different retention time were absent from the samples after 120 days of treatment (Fig. 2). It indicates that compounds were degraded by the developed bacterial consortium and utilized as carbon and energy sources. The metabolic capability and cell growth were also observed at different time intervals from 0 to 120 days after sowing (DAS). However, during the course of 120 DAS consortium C2 & mustard crop (hyperaccumulator crop) have managed to reduce or mitigate the concentration of petrol engine oil hydrocarbon in polluted soil, as indicated by the increased the biodegradation potential cell growth during the incubation period at different time (0, 20, 40, 60, 80 and 120 DAS) intervals (Table 2). Further, the peaks were characterized for the type of metabolite produced during the biodegradation of petrol engine oil hydrocarbons in mustard rhizospheric soil. In mustard with consortium C2, new peak was observed at Retention Time (RT) 30.258 and identified as hexadecanoic acid, 2-oxo-methyl ester (Fig 3 & Table 1). In addition to this plant may play significant effect on pollution removal as the mustard crop has significant contribution for mobil oil biodegradation (Kumar *et al.*, 2013). The mass fragment and structure of new intermediate products were shown at Fig 3. The average% degradation of petrol engine oil (2%) contaminated soil in C2PE2M (C2= consortia, PE2= 2% petrol engine oil, M= mustard plant) was 79.02 % after 120 DAS (Table 2). Rosado and Pichtel, (2004) have showed similar type of study, at 50 days, methyl ester dodecanoic acid and methyl tetradecanoate, *etc.* were detected from petroleum products hydrocarbon.

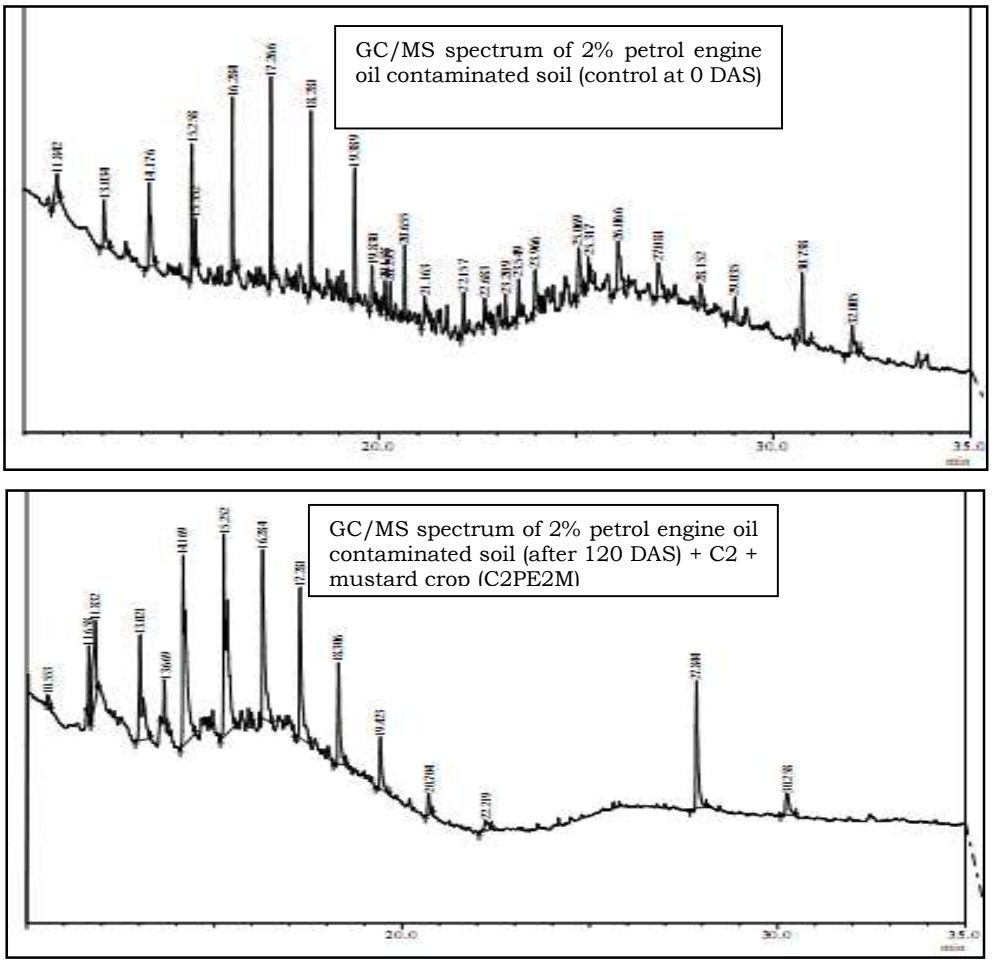

Fig 2: GC/MS spectrum of petrol engine oil contaminated soil at 0 DAS and 120 DAS.

Table 1: Retention time of petrol engine oil at 0 DAS (control) and after 120 DAS.

Cont. (RT)	**C2PE2M (R.T)**
11.842	11.832
13.024	13.021
14.176	14.169
15.258	15.252
15.352	-
16.284	16.284
17.266	17.281
18.281	-
19.389	-
19.830	-
20.158	-
20.293	-
20.655	-
21.163	-
22.157	-
22.683	-
23.209	-
23.549	-
23.966	-
25.069	-
25.317	-
26.066	-
27.081	-
28.152	-
29.035	-
30.738	30.258
32.005	-

Table 2: Metabolic capability and growth (cell count) of microbial consortium

Treatment		Days after sowing (DAS)					
		0	20	40	60	80	120
C2PE2M (Soil+PEO+ Plant+Consortium)	%Degradation	ND	15.89	35.80	57.13	71.72	79.02
	Cell count	$5x10^5$	$1.21x10^7$	$1.5x10^7$	$1.98x10^7$	$2.48x10^8$	$2.79x10^8$

.

Comp.Name: Hexadecanoic acid, 2-oxo-, methyl ester

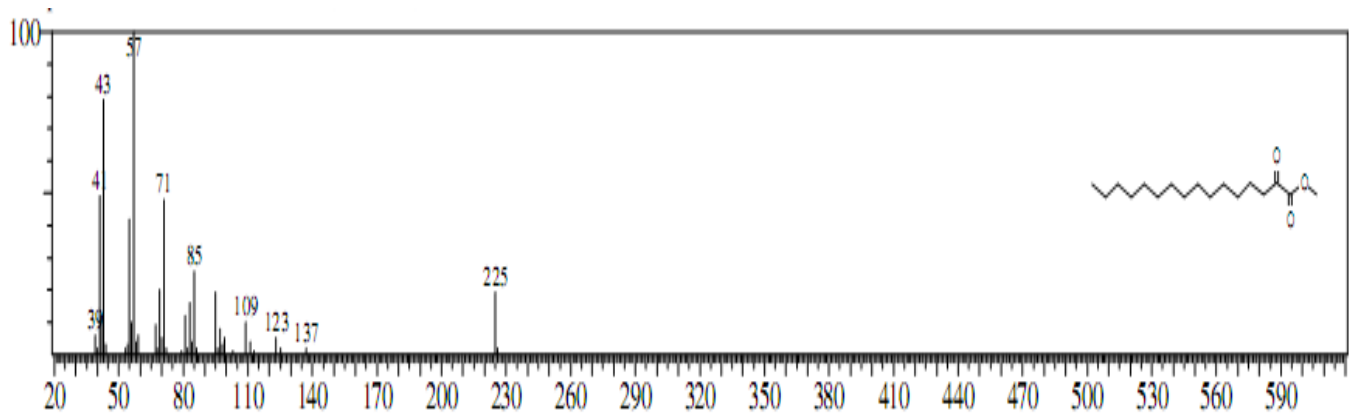

Fig 3: New Peak Observed in C1PE2M treatments and its mass spectrum

Conclusion

After the development of consortium for petrol engine oil (C2= *Pseudomonas aeruginosa* & *P. moraviensis*). Consortium was capable of degradation of 2% petrol engine oil hydrocarbons in mustard (*Brassica juncea var. Kranti*) rhizosphere. Consortium C2 degraded PEO hydrocarbons in mustard rhizosphere with a potential of 79.02% in 120 DAS. Various plants can be used for remediation but owing to the importance of plants and especially mustard (*B. juncea*) the plant was selected for studying the interaction with biosurfactant producing plant growth-promoting rhizobacteria (PGPR) in petroleum products contaminated soil anticipating that this study will be helpful in use of oil contaminated sites for farming and better understanding of remediation mechanism of such sites.

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Conflict of Interest

The authors declare no conflicts of interest.

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