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Characterization of Potential Oil Degrading and Plant Growth Promoting Bacteria from the Soils of Himachal Pradesh

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Biodegradation by natural populations of microorganisms is easy, cheap and environmental friendly than other remediation technologies. Keeping this in view, 182 oil utilizing bacteria were isolated. These isolates were screened for biosurfactant production and only 25 isolates were showed biosurfactant production. Further, these isolates were tested for nitrogen fixing capability and phosphate solubilization. The isolate OBD 17 showed 756 μg P/ml of solubilization and 921 nmole C_2H_4 h^{-1} mg^{-1} protein of nitrogen fixation. Whereas, isolate OBD 36 showed 697 μg P/ml of solubilization and 781 nmole C_2H_4 h^{-1} mg^{-1} protein of nitrogen fixation. These two most efficient isolates were grown in three different hydrocarbons (kerosene, diesel and engine oil) and results showed that both isolates efficiently grow in diesel followed by engine oil. Finally, these two isolates were tested under different temperature and pH conditions. These isolates survived well under various conditions and showed maximum growth at 30°C at pH 7.0. The 16S rRNA gene sequencing showed that isolate OBD 17 was *Pseudomonas fluorescens* and isolate OBD 36 was *Micrococcus luteus*. Since, these native isolates not only have biodegradation activity but also, have high nitrogen fixing and phosphate solubilization capability. So, these isolates have immense potential to be used for bioremediation purpose as well as biofertilizers in agriculture for increasing crop production.

Key words: Bioremediation, Biosurfactant, Nitrogen fixation, Phosphate solubilization, 16S rRNA.

Bioremediation is an alternative that offers the possibility to destroy toxic pollutant using natural biological activity. By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment (Atlas 1981). The soil is habitat to many living organisms, any contamination with petroleum products can change their number or form may upset or cause a total collapse of the ecosystem. The effect of oil spills on soil can lead to an enrichment of the oil-degrading microbial population (Akoachere *et al.* 2008). The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes. When microorganisms are imported to a contaminated site to enhance degradation we have a process known as bioaugmentation (Vidali 2001). These microorganisms can degrade a wide range of target constituents present in oily contamination site (Barathi and Vasudevan 2001). A large number of *Pseudomonas* strains capable of degrading PAHs

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[Poly Aromatic Hydrocarbons] have been isolated from soil and aquifers (Kiyohara *et al.* 1992). Other petroleum hydrocarbon degrading bacteria include *Yokenella* spp., *Alcaligenes* spp., *Roseomonas* spp., *Flavobacter* spp., *Acinetobacter* spp., *Stenotrophomonas* spp., *Corynebacterium* spp., *Streptococcus* spp., *Providencia* spp., *Sphingobacterium* spp., *Capnocytophaga* spp., *Moraxella* spp. and *Bacillus* spp. (Bhattacharya *et al.* 2003).

In general, surface tension of water and other liquids are reduced in the presence of synthetic organic chemicals, such as surfactants or wetting agents. Though, use of these agents may solve the oil spill problem, however, adding these synthetic agents to the site, is amplify the percentage level of non-degradable agents, which, further intensify the problem (Maneerat and Phentrong 2007). Thus, serious attention is being given at global level to have non–toxic, non-hazardous surface-active agents. Most of the microbial surfactants are lipid in nature and grouped into glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids and lipopolysacharides (Parkinson 1985). Nevertheless, the origin and the strain types decide the nature and the surface-active property of biosurfactants.

In the present study, we aimed to isolate the indigenous bacteria which can degrade/use various petroleum products as carbon source. It is well known that indigenous isolates performed better then the exotic strains due to their better adaptability to the local climatic conditions. Simultaneously, organisms producing biosurfactants were screened which help in accelerating the process of bioremediation. As well as the isolated strains were tested for nitrogen fixation and phosphate solubilization. Since, these activities help in establishment of isolates in the contaminating sites. Also, various parameters were optimized for the growth of screened isolates on medium supplemented with petroleum products.

MATERIALS AND METHODS

Sample collection

Soils contaminated by different petroleum hydrocarbons were collected from mechanic workshops at Kangra (H.P), India. All soil samples were collected in triplicates.

Isolation of oil utilizing bacteria

Microorganism isolation was carried out using minimal salt medium (MSM) containing unique carbon sources (diesel, kerosene and engine oil). The selective medium used for isolation contained 1.5 g of agar-agar, 10 g NaCl, 0.29 g KCl, 0.42 g $MgSO_4$, 0.83 g KH_2PO_4, 0.42 g $NaNO_3$, 1.25 g $NaHPO_4$, 100 ml distilled water, pH 7.2 and 1 ml of carbon source (petroleum hydrocarbons) (Okpokwasili and Okorie 1988). After dilution of soil samples, selective agar media were spreaded with appropriate dilutions and incubated at 30°C. For the bacterial strain selection, the following rules were considered: colonies of microorganisms grown within 48-72 hr period and colonies with the bigger size at the end of incubation period.

Screening of biosurfactants producing microorganism

For screening of biosurfactants producing organisms, all the obtained pure cultures were grown in Zobell medium individually at 30°C under 150 rpm for 48 h and the biosurfactants activity of the cell free medium was assessed according to the methods of Tugrul and Cansunar, (Tugrul and Cansunar 2005) and the isolates exhibiting appreciable surfactant activity was selected.

Screening for Nitrogen fixing and Phosphate solubilizing activity

The isolates were tested for phosphate solubilizing ability on Pikovskaya's medium incorporated with tricalcium phosphate ($Ca_3(PO_4)_5$). Isolates showed halo zones around the colony considered as phosphate solubilizer. Diazotrophic microorganisms were isolated using Nitrogen free Jensen's medium throughout the study. Microorganisms growing on nitrogen free medium were considered as nitrogen fixers.

Solubilization of Inorganic Phosphate

Quantitative estimation of inorganic phosphate solubilization by different isolates was done in National Botanical Research Institute's Phosphate (NBRIP) broth containing 1000 µg/ml tricalcium phosphate (TCP) using vanado-molybdate method as described by Jackson (1973).

Acetylene reduction assay [ARA]

Nitrogen fixation of the isolates was determined in nitrogen free medium by the acetylene reduction assay (Hardy *et al.* 1968). Ethylene

production was measured using a Hewlett Packard gas chromatograph (Model HP Series 5890, USA) fitted with flame ionization detector and a Porapak-N column. After completion of the ARA, the protein concentration in the cells was determined by Lowry *et al*. (Lowry *et al.* 1951). The isolates showed ARA activity more than 150 nmole C_2H_4 h^{-1} mg^{-1} protein were stocked for further study.

Survivability of efficient strains in various petroleum hydrocarbons

The survivability of efficient oil utilizing bacterial strains (ODB 17 and ODB 36) were studied in different petroleum hydrocarbons i.e. kerosene, diesel and engine oil separately. Erlenmeyer flask containing liquid minimal salt medium with 1% v/v of petroleum products as sole carbon source were inoculated by isolated efficient strains and incubated in shaking incubator at 250 rpm at 30°C. The viable count was taken at 4^{th}, 8^{th}, 12^{th} and 16^{th} days of incubation.

Impact of Abiotic Factors on the growth of efficient strains

The experiment was set up to determine the effect of pH and temperature on the growth of efficient isolates.

Effect of pH

In order to optimize the effective pH, isolates were grown on nutrient agar medium at varied pH's viz., 4.0, 5.0, 6.0, 7.0 and 8.0.

Effect of temperature

To have optimum temperature for maximum growth, cultures were grown at four different temperatures, viz., 15, 30, 35 and 40°C on nutrient agar medium.

Phenotypic characterization of oil utilizing bacteria

Individual cultures were identified by morphological and biochemical techniques using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994).

16S rDNA gene amplification and sequencing

Genomic DNA of isolates was extracted with DNA isolation kit (Real Biogenomics) following the manufacturer instructions. The following primers were used for PCR amplification of the 16S ribosomal DNA: 27F (5'AGAGTTTGATC ATGGCTCAG 3') and 1487R (5' TACCTTGTT ACGACTTCACC 3') (Heddi *et al.* 1998). Each PCR mixture (25 µl) contained primers (each at a concentration of 20 pmol), a mixture of dNTPs mix (Promega Co., Southampton, England) (each at a concentration of 10 mM each), Taq polymerase buffer (2.5 µl), $MgCl_2$ (1.5 µl) and chromosomal DNA (10 ng). Taq polymerase (5U/µl) (Life Technologies India, Pvt. Ltd.) was added to each of the reaction solutions. The thermo cycling conditions consisted of an initial denaturation step at 94°C for 5 min, 30 amplification cycles of 94°C for 45 sec., 53°C for 45 sec., and 72°C for 30 sec. and a final extension step of 72°C for 30 sec. with GeneAmp PCR System (Applied Biosystems, USA). PCR products were run and visualized on a 1% agarose gels. PCR products of 16S rRNA gene of bacterial isolates were freeze dried in a lyophilizer (CHRIST *ALPHA I-2LD*) and sent for custom sequencing using the upstream and downstream primers used for amplification of 16S rRNA gene (Ocimum Biosolutions, Pvt. Ltd.). Related sequences were obtained from GenBank database National Center for Biotechnology Information (NCBI), using BLAST (Altschul *et al.* 1997). The sequences were aligned by using Clustal W software (Higgins *et al.* 1994). Evolutionary trees for the data sets were inferred by the neighbor-joining method of Saitou and Nei (1987) by using the neighbor-joining program, MEGA version 4 (Tamura *et al*. 2007). Confidence limits on grouping were done by the bootstrapping technique (1,000 repeats).

Gene submission

The nucleotide sequences of efficient oil degrading isolates were deposited in Gen Bank under accession numbers: ODB 17 [GenBank: JN709477] and ODB 36 [GenBank: JN709478].

Statistical analysis

Results of the measurements were subjected to analysis of variance (ANOVA) and significance at the 1% level was tested by Least Significant Difference (LSD) using Windowstat package, Version 8.0. All treatments were in triplicate.

RESULTS AND DISCUSSION

From all the soil samples collected from Kangra (H.P), India, we obtained a total of one hundred eighty two oil degradating bacterial strains. In the present study, only oil-degrading bacteria were isolated because bacteria are the most active agents in petroleum degradation, and they

work as primary degraders of spilled oil in environment (Brooijmans *et al.* 2009). Earlier, workers also isolate oil degradating bacteria from oil contamination sites (Nwachukwu 2001; Kafilzadeh *et al.* 2011). Out of one hundred eighty two strains, seventy three, forty eight and sixty one morphologically distinct bacterial colonies were obtained on selective medium containing diesel, kerosene and engine oil, respectively. Out of 73, 48 and 61 isolates only twelve (ODB 06, ODB 12, ODB 17, ODB 22, ODB 36, ODB 43, ODB 49, ODB 53, ODB 59, ODB 62, ODB 67, ODB 71), four (ODB 87, ODB 104, ODB 109, ODB 118) and nine (ODB127, ODB 138, ODB 152, ODB 157, ODB 164, ODB 169, ODB 173, ODB 177, ODB 180) strains grown on medium containing diesel, kerosene and engine oil, respectively showed biosurfactant production. Biosurfactants are produced by a wide variety of microorganisms, such as bacteria, yeast and fungi and the majority share goes with bacteria (Healy *et al*. 1996). To effectively degrade hydrocarbons of crude oil, emulsification with a surfactant is of importance due to their low water solubility, especially polyaromatic components in solid and liquid discharges of petroleum (Oberbremer *et al.* 1990). According to Abdul and Gibson (1991) and Bai *et al.* (1997) removal of oil/ hydrocarbons from contaminated soil using commercial biosurfactants in its purified form, showing better results than that of chemical surfactants such as sodium dodecyl sulphate (SDS) and Tween 80.

Another important factor in bioremediation of contaminated soils is the availability of nitrogen and phosphorus, which allows the necessary increase in the size of the hydrocarbon-degrading microbial populations (Vinas *et al.* 2005). The twenty five biosurfactant producing isolates were further tested for phosphate (P) solubilization and nitrogen fixing capability. Isolates ODB 12, ODB 17, ODB 36, ODB 59, ODB 109, ODB 138, ODB 169 and ODB 177 showed P-solubilization on Pikovskaya's medium whereas, isolates ODB 17, ODB 36, ODB 49, ODB 67, ODB 109, ODB 138 and ODB 180 showed growth on nitrogen free Jensen's medium.

As the direct measurement of phosphate solubilization in broth assay is likely to give more reliable results (Johri *et al.* 1999) than a regular plate assay, the screened 8 phosphate-solubilizing strains were further tested for their ability to solubilize tricalcium phosphate in NBRIP broth (Table 1). The results showed that highest P-solubilization was observed in case of isolate ODB 169 (782.55 µg P/ml) followed by ODB 17 (756.49 µg P/ml) and ODB 36 (697.17 µg P/ml). The least activity was showed by isolate ODB 138 (127.50 µg P/ml). Though the ability to reduce acetylene is an indirect measure of N_2-fixation, it is specific for monitoring functional nitrogenase activity, and is indicative of N_2-fixing potential (Andrade *et al.* 1997; Mehnaz *et al.* 2007). Hence, for selection of prospective strains, ARA was used as a test for diazotrophy The results showed that the most efficient nitrogen fixing isolate was ODB 17 (921.58 nmole C_2H_4 h^{-1} mg^{-1} protein) followed by ODB 138 (846.49 nmole C_2H_4 h^{-1} mg^{-1} protein) and ODB 36 (781.45 nmole C_2H_4 h^{-1} mg^{-1} protein). The least activity was showed by isolate ODB 109 (249.37 nmole C_2H_4 h^{-1} mg^{-1} protein).

Table 1. Showing P-solubilization and Nitrogen fixation by oil degradating bacterial isolates

S. No.	Isolate	P-solubilization (µg P/ml)	Isolate	Nitrogen fixation (nmole C_2H_4 h^{-1} mg^{-1} protein)
01.	ODB 12	519.58	ODB 17	921.58
02.	ODB 17	756.49	ODB 36	781.45
03.	ODB 36	697.17	ODB 49	374.31
04.	ODB 59	639.38	ODB 67	537.65
05.	ODB 109	361.60	ODB 109	249.37
06.	ODB 138	127.50	ODB 138	846.49
07.	ODB 169	782.55	ODB 180	484.60
08.	ODB 177	449.32		
CD at 5%		3.47		5.21

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. The oil degrading bacteria have wide metabolic versatility in utilizing various petroleum hydrocarbons as food and energy sources for growth and reproduction (Wang *et al.* 2011). The Figure 1 and Figure 2 clearly showed that both efficient isolates (ODB 17 and ODB 36) grow well in medium containing diesel. Least growth was observed in minimal salt medium containing kerosene as carbon source.

Apart from carbon and nitrogen, environmental factors such as volume, aeration or agitation, incubation period, pH and temperature, also play an important role in the activity of oil degrading bacteria and production of biosurfactants (Syldatk and Wagner 1987). The efficient isolates (ODB 17 and ODB 36) survived well under different pH (except for pH 4.0) and temperature conditions. Both the isolates showed more growth at pH 7.0 as compared to the other tested pH, whereas least growth observed at pH 8.0. More growth was observed at 30°C as compared to the other temperatures tested. The least growth was observed at 40°C.

The most efficient isolates ODB 17 and ODB 36 were biochemically characterized by following biochemical tests of Bergey's Manual of Determinative Bacteriology. The isolate ODB 17 was found gram negative rods, motile in nature. This isolate was found positive for catalase, oxidase, gelatin liquefaction, denitrification and negative for egg-yolk reaction. This isolate used adonitol, fructose, galactose, glucose, mannose and sucrose. These results showed the broad similarity of ODB 17 with genus *Pseudomonas*. Whereas, the isolate ODB 36 was yellow pigment

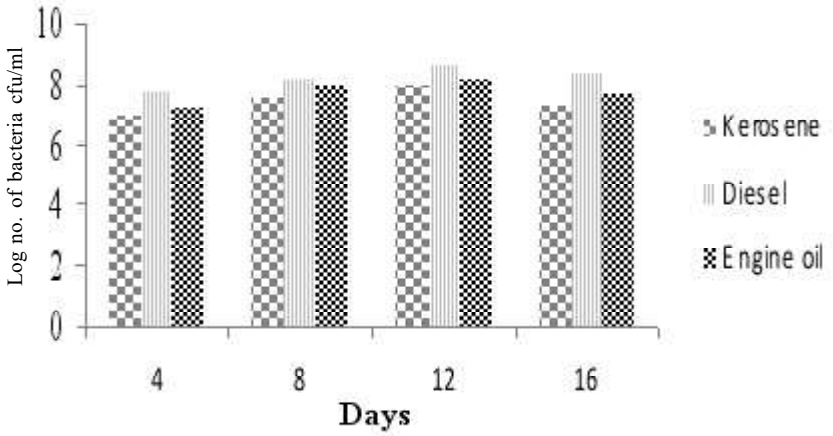

Fig. 1. Survivability of efficient isolate ODB 17 (*Pseudomonas fluorescens*) in various hydrocarbons

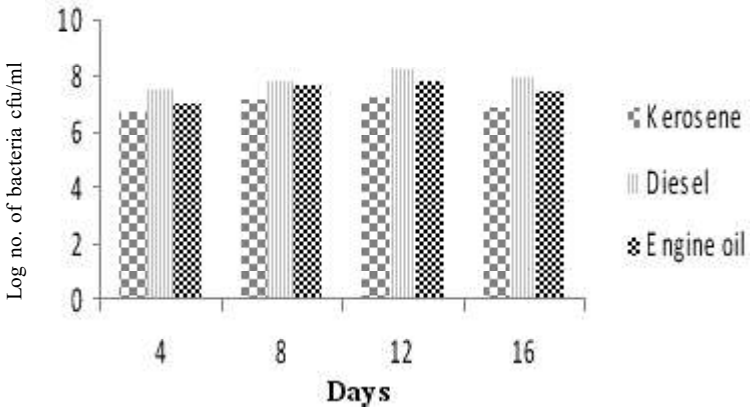

Fig. 2. Survivability of efficient isolate ODB 36 (*Micrococcus luteus*) in various hydrocarbons.

producing, gram positive cocci, non-motile, catalase and oxidase positive. This isolate was found positive for gelatin and Urease hydrolysis, whereas negative for simmone citrate agar, reduction of nitrate to nitrite, starch hydrolysis, egg-yolk reaction. Produced acid from glucose, galactose, lactose, mannose, and sucrose. Thus, the results of biochemical characterization showed that isolate ODB 36 belonged to genus *Micrococcus*.

To further, confirm the results of biochemical characterization these efficient isolates were characterized by 16S rRNA gene sequencing. This gene has universal distribution, highly conserved nature, fundamental role in protein synthesis, does not horizontally transfer and its slow rate of evolution which represents an appropriate level of variation between organisms, thus this gene is used for inferring the phylogenetic relationship among bacteria (Stackebrandt and Goebel 1994; Mora and Amann 2001)**.** Nucleotide sequence analysis of test isolates using clustalW program revealed that isolate ODB 17 showed maximum homology (98%) with *Pseudomonas fluorescens* (AY785748), isolate ODB 36 showed maximum homology (98%) with *Micrococcus luteus* (FR848405). The 16S rRNA gene analysis revealed that isolates ODB 17 and ODB 36 clustered with *Pseudomonas,* and *Micrococcus* which belong to Proteobacteria, and Firmicute (Figure 3). Previously these genera are reported to be oil degraders, nitrogen fixers and phosphate

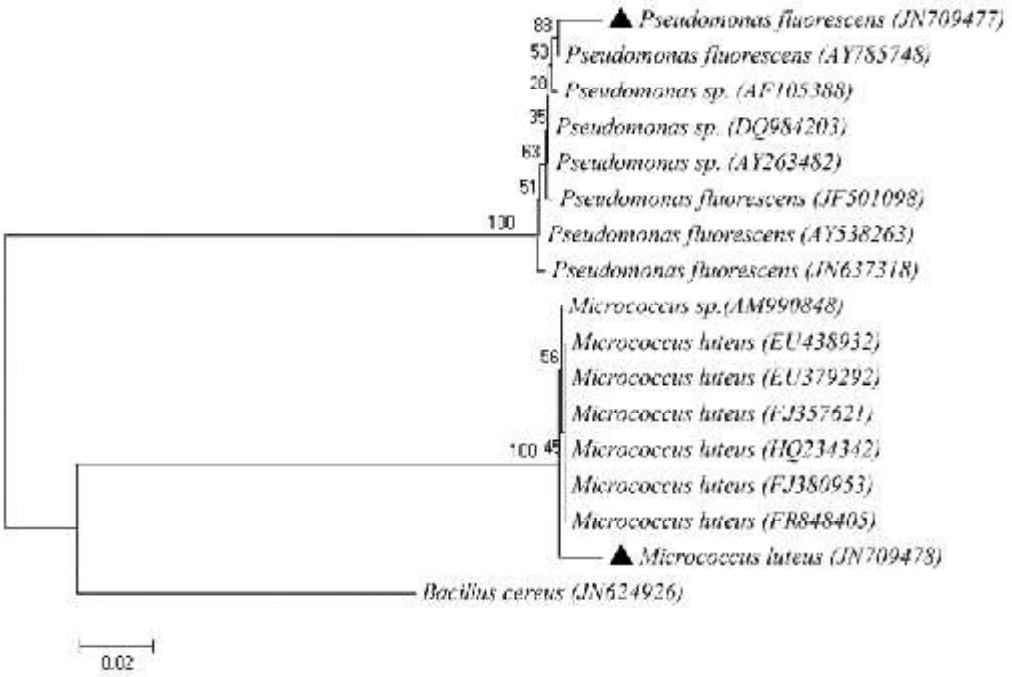

Fig. 3. Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 16S rRNA gene sequences of native isolates and related sequences obtained from NCBI. Scale bar, 0.02 substitutions per nucleotide position (▲ represents native isolates)

solubilizers (Thakuria *et al.* 2004; Park *et al.* 2005)**.** Cleaning up of petroleum hydrocarbons in the subsurface environment is a real world problem. Most studies showed that the indigenous microbial populations degrade hydrocarbons more efficiently than the introduced strains (Margesin and Schinner 2001). In the present study, isolated bacterial strains have not only biodegradation property but also have high nitrogen fixation and phosphate solubilization potential. So, these native isolates can be used for bioremediation as well as these isolates have potential to be used as biofertilizers for increasing crop production under local agro-climatic conditions.

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