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Original Research

Studies on Hydrocarbon Degradation by the Bacterial Isolate *Stenotrophomonas rhizophila* (PM-1) from Oil Spilled Regions of Western Ghats of Karnataka

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Abstract

The hydrocarbon utilizing capability of *Stenotrophomonas rhizophila* (PM-1), isolated from oil contaminated soil composts from Western Ghats region of Karnataka was analyzed. In the bioremediation experiment, ONGC heavy crude oil and poly aromatic hydrocarbons (PAHs) utilization by the bacterial isolate was studied. Preliminary studies by DCPIP method suggests, the isolate PM-1 having the potential PAHs and crude oil utilization ability. The degradation of 2 % heavy crude oil and other PAHs from the isolate PM-1 was assessed by Total Plate Count (TPC), Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) level at regular time intervals. Increased in population densities with simultaneous increase in BOD and COD ratio correlates with the UV spectrophotometry result by change in the λ_{max} (from 249 nm to 278 nm), gas chromatographic analysis (25.00 %) and total petroleum hydrocarbon (TPH) (65.78 %). A substrate specificity test of the isolates on different hydrocarbons (PAHs) showed that the isolate PM-1 had good growth on decanol, hexadecane, toluene, dodecane, engine oil, benzene, phenol, ethyl benzene, pentadecane, tetradecane, octane, oleic acid and naphthalene. The breakdown of the molecular structure in heavy crude oil and in PAHs indicates, the loss of conjugation in parental hydrocarbons shows the degradation potency of the bacterial isolate (PM-1).

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INTRODUCTION

Environmental pollution, especially with hydrocarbons is a major environmental and health concern, hydrocarbons such as crude oil are highly toxic and can affect plants, animals and human (Alkhatib *et al.,* 2011). Bioremediation is the microbial degradation of organic pollutants such as petroleum in soil and ground water. This technique has the benefits of high treatment efficiency, low cost, relatively quick action, *in situ* and *ex situ* application and compatibility with other techniques. Nevertheless, the application of hydrocarbon-degrading bacteria in oil-contaminated soil does not remove all components of crude oil because some components are difficult to degrade, such as unsaturated hydrocarbons ($<C_{10}$ and C_{20}-C_{40}) and polycyclic aromatic hydrocarbons (PAHs). Hence there is a need to find new bacterial strain that can metabolize a broad range of hydrocarbons contained in crude oil, especially the highly persistent components (Hong *et al.,* 2005). Crude oil hydrocarbons are often difficult to remove from the environment because of their insolubility in water. The addition of surfactant results in dispersion of hydrocarbons in aqueous phase, thereby facilitating transportation across the cell membrane (Kaczorek and Olszanowski, 2010). It is noted that someorganisms capable of producing bio-surfactants are likely to fairly better in a harsh environment such as the oil-contaminated region.One of the key success factors in using bacteria for bioremediation is the preparation of bacteria in a ready-to-use form appropriate to the contamination site. However, the use of free oil degrading microbes shows that the microbes have limited efficacy and are not reusable in a continuous treatment system. One approach to overcoming such an obstacle is to immobilize the cells on a solid support material (Nopcharoenkul*et al*., 2013). Even though much research has been carried out with several species of microbes, there still exists a scope for the isolation of novel strains which can degrade various kinds of hydrocarbons in oil sample. By considering all these factors, in present investigation we made an effort to isolate a microorganism which degrade the hydrocarbons efficiently in an oil sample. The results of the oil degradation were confirmed by spectral chemical and microbiological methods.

MATERIALS AND METHODS

Soil Sampling and Pre-treatment

Soil samples were collected from different localities of Western Ghats of Karnataka State covering oil spilled

areas. Each collection was made from 10-15 cm depth of the soil. Collected soil samples were transported to the laboratory in air tight pouches and stored in cold condition. Further processing was carried out by reported method (Al-Nasrawi, 2012) and used for isolation studies.

Media and Chemicals

Reasoner's 2A Agar (R_2A)containing Casein Hydrolysate (0.5 g/L),Yeast Extract (0.5 g/L), Protease Peptone (0.5 g/L), Dextrose (0.5 g/L), Starch (0.5 g/L), Di-potassium Phosphate (0.3 g/L), Magnesium Sulphate (0.024 g/L), Sodium Pyruvate (0.3 g/L), Agar (15 g/L), Trace salt solution (1 %), pH 7.2 and Nutrient agar (Hi-media) were used for the isolation of bacteria. Bacto Bushnell-Hass broth containing $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.02 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (1 g/L), $FeCl_2$ (0.05 g/L), NH_4NO_3 (1 g/L) was used for the degradation studies. Redox indicator (2% 2, 6 Dichlorophenol indophenol (DCPIP)) and 2% ONGC crude oil were all incorporated into the broth.The media and chemicals used in this study were obtained from Merck, Sigma and Himedia. All the chemicals used were of analytical grade and the heavy crude oil was collected from ONGC Cauvery Asset Karaikal, Tamil Nadu.

Isolation and Screening of Bacteria from Oil Contaminated Soil

Hydrocarbon degrading bacteria were isolated using R_2A agar media employing pour plating technique. The soil samples were serially diluted and 1 ml aliquot of each serially diluted sample was transferred to different sterile petriplates and over that 20 ml of R_2A agar waspoured. The plates were incubated at 37 ^oC for 24-48hrs. After incubation, morphologically different colonies were selected and isolated. The isolated colonies were picked up and maintained as pure culture in agar slants (Veena *et al*., 2011).

Preliminary Degradation Studies

The method of Youseff *et al.,* (2010) was adopted for the biodegradation studies. 24 hour old bacterial culture was adjusted to10^5 cfu using McFarland standards and one ml of the bacterial isolate was inoculated into BH broth incorporated with sterile 2 % crude oil and redox indicator 2 % DCPIP. The uninoculated flask serves as control. The flasks were incubated at 37^o C with constant shaking at a 180 rpm/min for 10 days. The aliquots in the flasks were monitored daily for color change (from deep blue to colorless).

PAHs and Crude Oil Utilization Studies

Each of the potential bacterial strains obtained from the preliminary degradation studies were inoculated in 150 ml of Bacto Bushnell Hass broth containing 2 % different PAHs viz., decanol, hexadecane, toluene, dodecane, engine oil, benzene, phenol, ethyl benzene, pentadecane, tetradecane, octane, oleic acid, naphthalene and ONGC crude oil. The flasks were incubated at 37 ^0C in an orbital shaker at 180 rpm for 15 days. The PAHs and crude oil utilization by the isolate PM-1 was confirmed by % TPH analysis,total plate count, BOD, COD level using the standard protocol and by UV spectrophotometric studies at regular intervals of time (0^{th}, 2^{nd}, 5^{th}, 10^{th} and 15^{th} day) respectively.

Extraction of Residual Oil

Residual crude oil was extracted using the method followed by Mittal and Singh (2009) with suitable modification. The residual crude oil was subjected for liquid-liquid extraction using equal proportion (1:1) of hexane and acetone in a separating funnel. Acetone was then added to it and shaken gently to break the emulsification, which results in formation of three layers. Top layer was a mixture of hexane, crude oil and acetone; clumping cells make the middle layer and the bottom aqueous layer contains acetone, water and biosurfactant in soluble form. The lower two layers were separated out while top layer containing hexane mixed with crude oil and acetone was taken out in a clean beaker. The extracted oil was dehydrated byusing anhydrous sodium sulphate. The hexane and acetone were evaporated on a water bath. The gravimetric estimation of residual oil left after biodegradation was made. The biodegraded crude oil was further used for gas chromatographic analysis.

Gas Chromatographic Analysis

Crude oil was extracted with chloroform and estimated by Chemito-2100 gas chromatograph equipped with capillary column of 30 m length and made of carbowax column material. The carrier gas used for the analysis was nitrogen and a FID was used as the detection system. The injector temperature was maintained at 160 ^0C, oven at 120 ^0C and detector at 300 ^0C. The Percentage of Biodegradation was obtained by using the formula:

$$\%\ \textbf{crude oil degradation} = \frac{(\text{total area})\text{control} - (\text{total area})\ \text{sample}}{(\text{total area})\text{control}} \text{X}100$$

PAHs Degradation

2% (v/v) PAHs were added to 250ml Erlenmeyer flasks containing 150 ml of BH media. Thirteen hydrocarbons were used in the experimental study; the media was inoculated with potential isolates and studied for utilization of these PAHs as sole carbon source over a period of 15 days (Al-Awadhi *et al.,* 2007). The ability of the isolate (PM-1) to degrade the thirteen hydrocarbons was determined spectrophotometrically and via visual observation.

Screening for Bio-Surfactant Activity

The screening of bio-surfactant activity was carried out by using 24 hours old bacterial culture. The cultures were centrifuged at 12,000 rpm for 10 minutes to obtain a cell free supernatant. The tests viz., Oil spreading technique, Emulsification test, Drop collapse test and Hemolytic activity were carried to determine bio-surfactant activity.

Identification and Characterization

Bacterial Genomic DNA was isolated by using the InstaGeneTM Matrix Genomic DNA isolation kit. The 16S rRNA was amplified in MJ Research PTC-225 Peltier Thermal Cycler. The 27F primer with the sequence (AGAGTTTGATCMTGGCTCAG) having 20 bases and primer 1492R with the sequence (TACGGYTACCTTGTTACGACTT) having 22 bases was used.1ng of template DNA was added to 20 ng of PCR reaction solution. 27F/1492R primers were used for the bacteria, and 35 amplification cycles at 94 ºC for 45 sec, 55 ºC for 60 sec, and 72 ºC for 60 sec were performed. A positive control (*E.coli* genomic DNA) and a negative control were included in the PCR. Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore).The PCR products were sequenced by Sanger's dideoxy chain

termination method. Sequence data was aligned and analyzed for identifying the bacteria. BLAST analysis performed using NCBI blast tool to identify the closely related sequence. The phylogeny analysis of the sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar, 2004). The resulting aligned sequences were cured using the program G-blocks 0.91b. These G-blocks eliminated poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana, 2007).Finally, the program Phy-ML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree-Dyn 198.3 was used for tree rendering. (Dereeper *et al.,* 2008)

RESULTS

Isolation of Oil Degrading Bacteria and Screening for Crude Oil Degradation Ability

Several bacterial isolates were successfully isolated from the oil contaminated soil samples. A total of 45 isolates were initially obtained from R_2A and Nutrient agar, of which 23 isolates were selected for crude oil utilization studies based on their ability to produce a color change in the medium (blue to colorless), this may be due to the reduction of the redox indicator by the oxidation reactions carried out by the isolate in the medium (Figure 1a). Minimal Salt Medium was used for screening the hydrocarbon degrading microbes, as the media was minimal and it supports the culture for a few hours and after that the available source of carbon would be the hydrocarbons present in the crude oil/ PAHs, a growth throughout the incubation indicated that the culture was able to utilize hydrocarbon by degrading it into its basic components (Figure 1b). One of the promising isolate (PM-1) was identified by 16S rDNA sequencing and was identified as *Stenotrophomonas rhizophila sp.*

Crude Oil Utilization Studies and Assessment of Degradation

Among 23 potential isolates, only 6 showed prominent utilization of the crude oil. This could be visually inferred, as those isolates that utilized crude oil were able to produce a turbid mixture with sand like color and consistency (fig 1b); while the rest of the flasks retained considerable amounts of crude oil which formed an immiscible disc like layer in the BH medium. Among the 23 isolates PM-1 showed the fastest extent of color change which concurred with the results of the crude oil utilization study. The degradation potency of the isolate PM-1 was assessed continuously by measuring the BOD, COD and total plate count (Table1). The BOD values increased from 86 mg/L, 236 mg/L, and 798 mg/L at the end of 5^{th}, 10^{th}, and 15^{th} days of incubation. COD values were also increased with respect to incubation period from 95 mg/L, 153.6 mg/L and 196.5 mg/L at the end of 5^{th}, 10^{th} and 15^{th} days, respectively.

Table 1: Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Plate Count (TPC) and TPH analysis to assess the rate biodegradation by PM-1 bacterial isolate

	5^{th} day	10^{th} day	15^{th} day	
BOD(mg/L)	86	236	798	
COD(mg/L)	95	153.6	196.5	
TPC (cfu/ml)	**0^{th} day**	**24 hrs.**	**48 hrs.**	**72 hrs.**
	$1.0x10^4$	$6.2X10^3$	$1.2 x 10^5$	$3.5x 10^7$
% TPH	Final weight	% of TPH (at the end of 15^{th} day of incubation)		
Control	3.560 gm			
PM-1	1.218 gm	65.78 %		

Figure 1 (a): Preliminary screening of PM-1 bacterial isolate for crude oil degradation using DCPIP as indicator. From left: Media Control, Media control+2 % crude oil, Media +2 % crude oil + bacterial isolate(PM-1).

Figure 1 (b): Degradation studies for the of PM-1 bacterial isolate using minimal salt medium (MSM).From left: Media Control, Media control+2 % crude oil, Media +2 % crude oil + bacterial iso:ate (PM-1).

PAHs and Crude Oil Utilization Studies by UV-visible Spectrophotometer

The increase in the biomass/total bacterial count was evaluated by total plate count method and the results shows that the increase in bacterial count by $1.0x10^4$, $6.2X10^3$, 1.2×10^5, $3.5x\ 10^7$ at 0^{th} day, 24 hrs, 48 hrs and 72 hrs respectively (Table 1). The UV-Visible studies of the crude oil were recorded for the control and for the PM-1 sample after 15 days of incubation period. The λ_{max} for the control and MP-1 sample found to be 278 nm and 249 nm respectively (Figure 2).

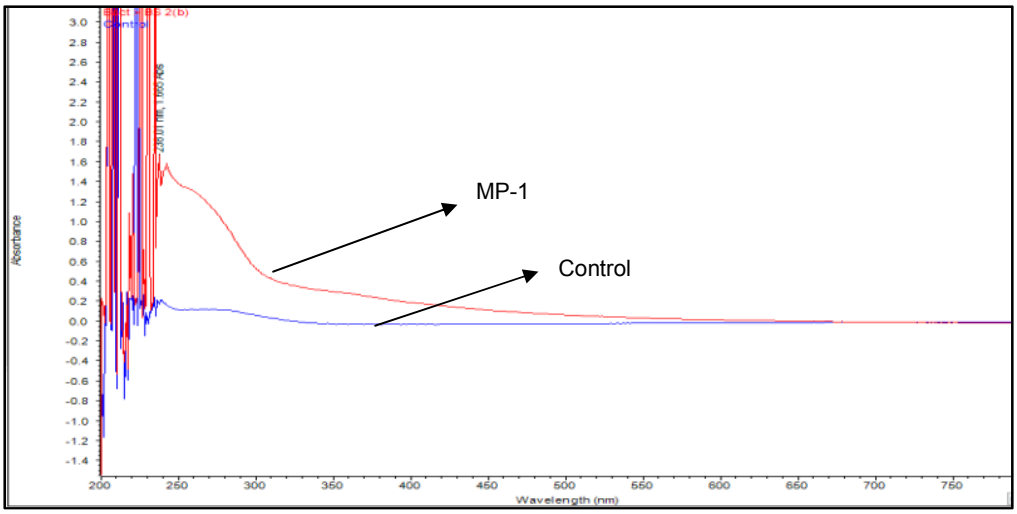

Figure 2: Crude oil utilization studies from the isolate PM-1, using UV-Visible Spectrophotometer by scanning the residual hydrocarbon between 200-800 nm

Crude Oil Utilization Studies by Gas Chromatography

Although, the strain MP-1 was one of the best potential isolates from the preliminary PAHs degradation studies and crude oil utilization studies, the gas chromatographic analysis of the residual crude oil revealed that the degradation was merely 25 %, where the total peak area for the control (2% heavy crude oil) and sample (PM-1) was found to be 34598.35 and 25949.14 m.Vs (Figure 3).

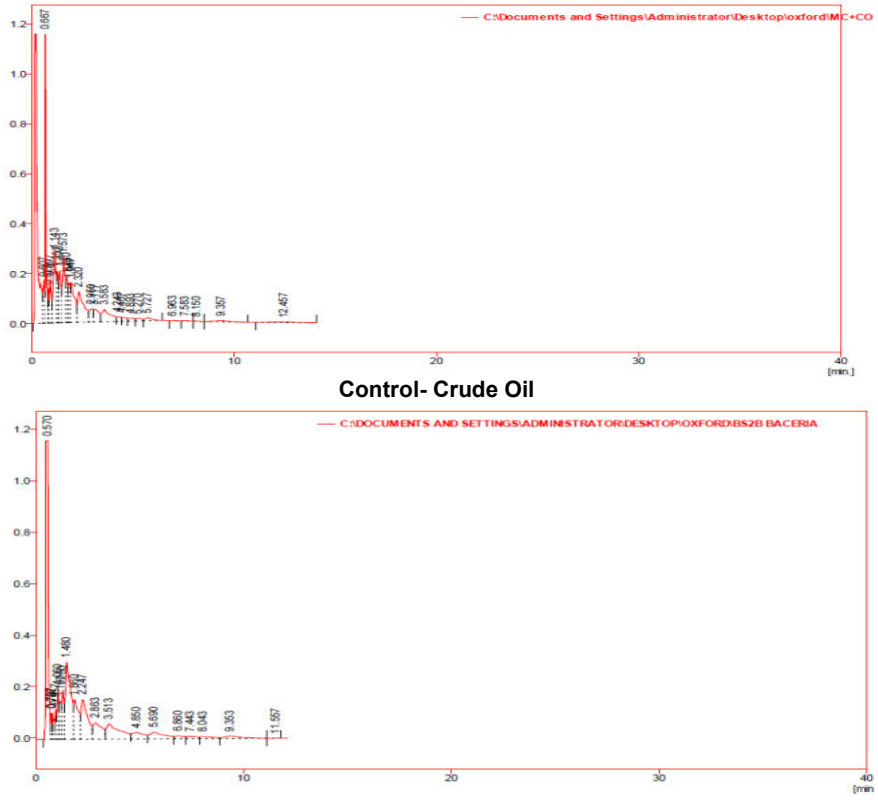

Figure 3: Gas chromatographic analysis of residual crude oil after bioremediation experiment using an soil isolate PM-1

PAHs Degradation by the Isolate PM-1

A substrate specificity test of the isolate PM-1 on different hydrocarbons (PAHs) showed that the isolate PM-1 had good growth on decanol, hexadecane, toluene, dodecane, engine oil, benzene, phenol, ethyl benzene, pentadecane, tetradecane, octane, oleic acid and naphthalene and the isolate proves its potency to degrade of various forms of hydrocarbon (Figure 4).

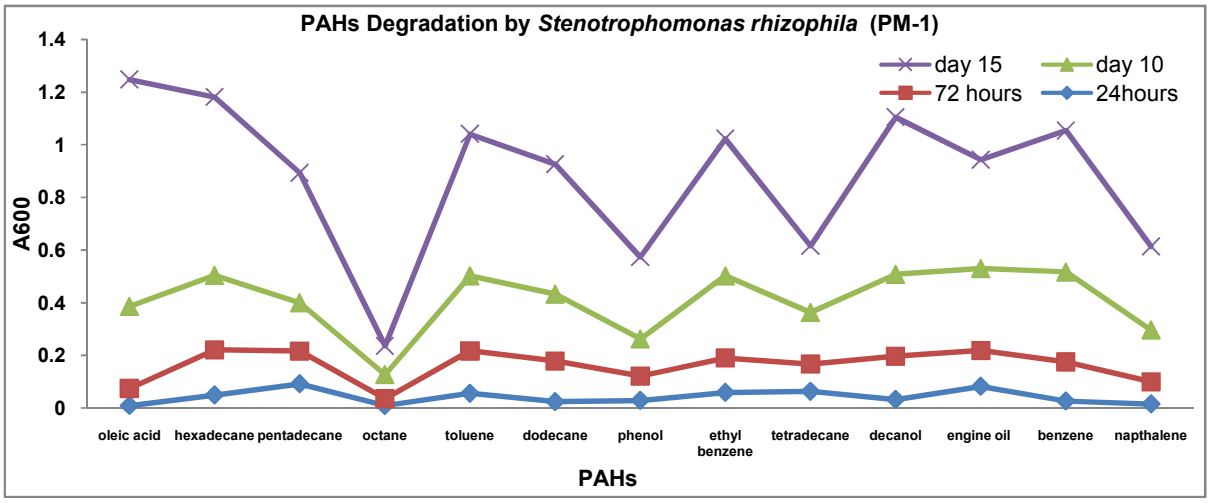

Figure 4: Showed the degradation ability of the isolate PM-1 to utilize various PAHs as sole carbon source

Screening for Bio-surfactant Activity

However, the selection of the strain MP-1 was based on the fact that the strain showed the presence of bio-surfactant activity. The strain tested positive for oil dispersion, oil drop collapse and hemolytic activity. The production of green color around the colonies indicated α-hemolysis which was in turn indicative of bio-surfactant synthesis. α-hemolysis is caused by the biosurfactant, in this case which oxidizes hemoglobin to green methemoglobin.

Identification and Characterization

>BS-2b_contig_1
TGGGCTCAGAGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCAGCACAGGAGAGCTTGCTCTCTG
GGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGATAACGTAGGGAAACTTACG
CTAATACCGCATACGACCTTCGGGTGAAAGCAGGGGACCTTCGGGCCTTGCGCGGATAGATGAGCCGATGTCGGA
TTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCA
GCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAGCCAGTTAATA
CCTGGTTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGT
GCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCTGTTGTGAAAGCCCTGGGC
TCAACCTGGGAATTGCAGTGGATACTGGACGACTAGAGTGTGGTAGAGGGTAGTGGAATTCCTGGTGTAGCAGTG
AAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAAT
TTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTT
GACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGT
GGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGG
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CTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGT
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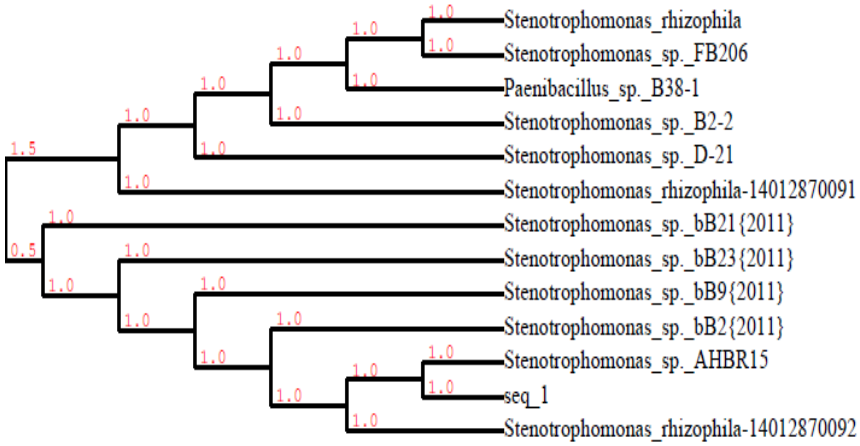

Figure 5: Assembled sequence and Neighbor-joining tree, based on 16S rDNA sequences showing the relations between strain PM-1 and type species of the genus *Stenotrophomonas rhizophila*

DISCUSSION

Bacteria are known to be efficient degraders of a variety of compounds, both simple and complex. In this study we were able to isolate a number of potential oil degrading bacterial strains. Among all the isolates six isolates were able to degrade 2% ONGC crude oil effectively, whereas rest found to be potential sources for bio-surfactant synthesis. The indigenous bacteria inoculated into the BH media utilize PAHs and crude oil as the sole carbon source since the media is devoid of any other source of hydrocarbon. The microbes that are able to utilize crude oil as the carbon source eventually degrade oil and thus reduce the oil into turbid sand like mixture. The potential of this bacteria to use 2% ONGC crude oil and PAHs as sole carbon source confirms with the reports of several other research works carried out in the same direction towards bioremediation of crude oil, wherein it has been reported that indigenous microorganisms can play a effective role as biological agents for cleanup of oil spills.

Absorbance at 600 nm (A_{600}) was measured; the increase in cell mass in terms of turbidity directly indicates the utilization of crude oil as the sole source of carbon. Inoculated BHM medium without hydrocarbon served as control. Cultures without any increase in turbidity over the initial O.D of test and control were scored as no growth (-), culture with slight increase over initial O.D significantly greater than the control O.D was scored as poor growth (+). Cultures with growth well above the initial were scored as moderate (++), while cultures with luxuriant growth were scored as heavy growth (+++) and (++++) (Santhini *et al*., 2009).

Similarly the UV spectral studies of the crude oil were recorded and compared with control. The change in λ_{max} value for the control and for the sample indicates the loss of conjugation and breakdown in the molecular structure of the oil and it confirms the degradation of crude oil by bacterial isolate. Another explanation to the appearance of these peaks as suggested by Evdokimov and Losev, (2007) is that, as the long chain hydrocarbons break, the C-C linkage at which the breaking occur results in the shift of the atom's energy state and hence the peak symbolizes the highest absorption for that atom. The result shows that the change in the λ_{max} from 278 nm to 249 nm indicates the breakdown of the molecular structure in the oil and it confirms the degradation of crude oil by MP-1 bacterial isolate due to the breaking of conjugates in the molecular structure of the crude oil.

CONCLUSIONS

Present study reveals that *Stenotrophomonas rhizophila (PM-1)* is a natural biosurfactant producer, showed degradation potency for various hydrocarbons. The isolated species holds promise for the development and discovery of novel strains which can serve as effective agents for Bioremediation in oil spilled regions.

Conflict of Interest

Conflict of interest none declared.

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