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BIODEGRADATION OF 2T ENGINE OIL BY BREVIBACILLUS AGRI STRAIN ISOLATED FROM PETROLEUM CONTAMINATED SOIL

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ABSTRACT

Petroleum has been used for many decades for illumination and, on a smaller scale, as lubricant. Environmental pollution by petroleum hydrocarbons has become a serious problem all around the world. The contaminations caused by petrol and petroleum products have been proved a great disaster on ecosystem. Bioremediation is one of the economical methods used to treat this threat and an approach to save the environment from petroleum related issues. This study was aimed to determine the degradation potential of *Brevibacillus agri* strain isolated from petroleum contaminated soil which was collected from arid region of Rajasthan (Churu). Enrichment culture technique was used for isolation and some biochemical tests were performed at laboratory scale. Bacterial cell density was monitored by measuring the OD at 620nm. This culture could utilize engine oil as sole carbon and energy source in aerobic conditions, highest 59% degradation of 2T engine oil was observed after 28 days. Gravimetric analysis method and GC-MS were used to determine the biodegradation of 2T engine oil with the aromatic and aliphatic fractions. Identification and characterization of isolated bacterial strain biochemical, morphological characterization and 16s sequencing technique were performed. *Brevibacillus agri* has good potential for decontamination of oil contaminated soil in the environment.

KEYWORDS: ***Pollution, Enrichment Culture, Aromatic, Potential, Petroleum Hydrocarbon***

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INTRODUCTION

Ecosystems have been changed due to the increasing influence of human actions. Accordingly, public have become aware to require save ecosystem and additionally, assess the deterioration formed by the pollution[1]. Hydrocarbon pollution is one of the most important contamination sources around the worldwide. Due to oil spills, hydrocarbon contamination becomes a worldwide problem particularly in growing and industrialized countries. Petroleum is used as a predictable energy source even although it has been consequence as a global ecological contaminant[2]. Crude oil is transformed to diesel oil and gasoline in oil refineries[3]. As an oil product, diesel oil has aromatic hydrocarbon as well as normal, branched and cyclic alkanes. Diesel oil can affect groundwater and surface quality with the harmless compounds[2]. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants generated by the incomplete combustion of organic materials such as coal, wood, oil, gas and garbage. They belong to the group of persistent organic pollutants (POPs), which, according to the protocol on POPs established in 1998 in Denmark, is characterized with toxic, persistent, bio accumulative properties as well as long-range trans boundary atmospheric transport and deposition with negative effects on environment and human health,[4] known for its mutagenic and carcinogenic effect (PAHs) constitute a group of priority pollutants, which are present in the soil of many industrially contaminated sites, particularly those associated with the petroleum industry[5]. The biodegradation of petroleum and other hydrocarbons in the environment is complex process, who's quantitative and qualitative aspects depend on the nature and amount of the oil or hydrocarbons present, the ambient and seasonal environmental conditions, and the composition of the microbial community [4, 6, 7,]. Biodegradation exploits the ability of microorganisms to degrade and/or detoxify organic contamination. It has been established as one of the efficient, economic, versatile and environmentally sound treatment[8]. Hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response to the presence of hydrocarbons. Bacteria and fungi are the key agents of degradation. Biodegradation of petroleum hydrocarbon pollutants and petrochemicals by bacteria have been extensively investigated[9, 10, 11] assuming the dominant role in marine ecosystems. Other organisms such as fungi are also capable of degrading the hydrocarbons in engine oil to a certain extent. However, they take longer periods of time to grow as compared to their bacterial counterparts, fungi becoming more important in freshwater and terrestrial environments as well[12]. Biodegraders of one of the petroleum based product, engine oil, across strains of genera such as *Achromobacter, Bacillus, Enterobacter, Escherichia, Gordonia, Mycobacterium, Pseudomonas, Rhodococcus, Serratia and Staphylococcus* were reported abundantly during previous year[13,14,15,16,17,18,]. Also, there had been some reports particularly detailing degradation of used engine oil by bacteria[13,19]. The microorganisms generally isolated from hydrocarbon contaminated sites, having engine oil degrading ability, belong to the genera *Pseudomonas, Rhodococcus, Alcaligenes, Acinetobacter, Arthrobacter, Citrobacter, Serratia, Micrococcus, Bacillus*, etc,[20,21] but single bacterial species has only limited ability to degrade all the fractions of hydrocarbons present in the engine oil[22,23,24,25]. The present work have been originated due to the spills of petroleum products which are contaminated the environment, and a systematic laboratory experiments were design for the elimination of petroleum hydrocarbons from contaminant soil by using the various aspect of Biodegradation technique.

MATERIAL AND METHODS

Sample collection

The oil contaminated soil samples extending from the ground surface to a depth of 5-10 cm were collected from automobile workshops from different areas of arid region of Rajasthan (Churu). Samples were then kept in sterile poly bags and were transferred to the laboratory where petroleum degrading bacteria were isolated from them by enrichment culture technique. Aseptic 2T engine oil was used in the assay for ability of the isolated bacteria to degrade petroleum hydrocarbons.

Culture media

Isolation of hydrocarbon-degrading microorganism from the contaminated soil samples were done on Bushnell – Hass medium (0.4g of KH_2PO_4, 0.4g of K_2HPO_4, 0.4g of $(NH_4)_2\ SO_4$ or NH_4NO_3, 0.002g of $FeCl_3$, 0.4g glucose). 1%(v/v) of 2T engine oil was added as carbon and energy source[26, 27].

Isolation of oil-degrading bacteria

1 gm of soil samples was weighed and transferred into 250ml Erlenmeyer flask containing 100ml of Bushnell-Hass medium with 1%(v/v) of oil as carbon and energy source. These flasks were incubated at 30^0C on a rotary shaker at 170rpm for 7 days. After 7 days, 1.0ml of the bacterial culture was transferred to fresh media containing 1% oil and reincubated at 30^0C for next 7 days[28]. The incubated samples were subjected to serial dilution and serially diluted samples were placed on Bushnell hash agar using spread plate method. The spread plates were incubated at 37^oC for 5-7 days for the isolation of bacteria that have the ability to degrade oil spill. The bacterial colonies from the spread plate were subjected to quadrant streaking for single colony isolation. The individual bacterial colonies were streaked on BHM agar plates and incubated at 37^oC for 5-7 days[29].

Biodegradation of 2T engine oil by isolated bacteria *Brevibacillus agri*

Brevibacillus agri bacteria was isolated and later inoculated into 100 ml Erlenmeyer flask containing 50 ml of Bushnell-Hass medium at 37^0C for 48 hours. After 48 hours 1 ml of bacterial inoculum was transferred to 250 ml conical flask which containing 100 ml of Bushnell hash medium with 5% (v/v) 2T engine oil. The flask was incubated on a rotary shaker at 170 rpm at 30^0C for 7, 14, 21, 28 and 35 days respectively. The uninoculated Bushnell hash medium containing the same amount of 2T engine oil was kept as control under the same investigational conditions.

Growth rate determination by turbidometry

Growth of the bacterial culture in the flasks was determined by measuring optical density of flask measured at 7 days interval using spectrophotometer at 620 nm.

Determination of 2T engine oil biodegradation by Gravimetric method

Organic hydrocarbon phase (engine oil) was extracted with 50 ml of n-hexane from the aqueous culture phase using a separating funnel. Extraction was carried out twice to make sure oil recovered completely. The extract was then treated with 0.4g of anhydrous sodium sulphate to remove the moisture and decanted into a beaker leaving behind sodium sulphate. The solvent was evaporated at 60^0C. After the complete evaporation, the oil residue obtained was weighed and taken as the gravimetric value for further calculation[30]. The % of degradation was calculated as follows;

Weight of residual oil = weight of beaker containing extracted oil – weight of empty beaker

Amount of oil degraded = weight of oil added in media – weight of residual oil

$$\% \text{ degradation} = \frac{\text{Amount of oil degraded}}{\text{Amount of oil added in the media}} \times 100$$

Gas chromatography mass spectroscopy (GC-MS) analysis

The degradation potential was checked by GC-MS analysis. After the analysis of biodegradation by gravimetric method 1 ml of extracted residue oil was used for the GC-MS analysis. The amount of hydrocarbons present in 2T engine oil at different time interval was determined by GC-MS technique[31].

Molecular identification of oil degraders

DNA Isolation of oil degrading bacteria

1.5 ml of freshly prepared broth sample was centrifuged at 6000rpm for 10 min and the respective bacterial pellets were processed for genomic DNA extraction using GeNei™ Genomic DNA Extraction kit with slight modification. The extracted DNA was confirmed by running isolated DNA samples on gel electrophoresis set at 50V for 45 minutes. The resultant DNA bands were observed using UV- Transilluminator (Bio-rad).

PCR amplification of oil degrading bacteria

16S rRNA gene fragments of DNA sample was amplified by using universal 16S rRNA gene primers (forward primer 8F: AGAGTTTGATCCTGGCTGAG & reverse primer 1492R: ACGGCTACCTTGTTACGACTT). The PCR amplification was carried out in Thermal cycler with 25 µl of final reaction volume containing - 7.5 µl ddH_2O, 12.5 µl 2X PCR master mix (GeNei™ PCR amplification kit), 1.0 µl forward primer 8F, 1.0 µl reverse primer 1492R and 3.0 µl diluted DNA (30ng/µl). The PCR was initiated with denaturation of DNA at 95^oC for 2min and subsequently the number of cycles (denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 90 sec) were set to 30 and the final extension was performed at 72^oC for 10min. 5 µl from the resulting PCR amplicons of DNA samples were separately mixed with 1µl of 5X gel loading dye and electrophoresis was carried out on 1.5% agarose gel containing ethidium bromide (EtBr) at constant electric field of 5V/cm for 30min in 0.5X TAE buffer. The amplified PCR products were confirmed at 1500bp as compact single band. DNA was visualized separately under UV-light using gel documentation system (Bio-Rad).

Sequencing and analysis of 16S rRNA gene

The purified 16S rRNA gene of selected isolate was subjected to automated DNA sequencing. Sequence data was generated by primer walk using BDTv3.1 chemistry on ABI 3730xl (XcelrisLabs Limited, Ahmedabad) and were then used to identify the bacterial isolate with BLASTn analysis using the NCBI GenBank nucleotide database and the homology to the closest bacterial organism with maximum similarity ranging from 96% -100% was obtained. The best first fifteen to twenty 16S rRNA gene sequences in BLASTN result were chosen for the analysis of homology match using Neighbor-Joining method and aligned using multiple sequence alignment software, Clustal W. These alignment results were used to construct phylogenetic tree using MEGA6 software tool[32].

RESULTS AND DISCUSSION

Soil samples were collected from 5 different motor garage of “churu” which is an arid region of Rajasthan. Seven morphologically different bacterial cultures (I^1, I^2, I^3, I^4, I^5, I^6, I^7) isolated by enrichment pour plate method. I^2 was selected as the good isolate capable to utilizing the hydrocarbons present in 2T engine oil as a solitary source of carbon and energy and selected for further study.

Degradation potential of isolated bacterial strain by gravimetric method

The isolated bacterial strain *Brevibacillus agri* tested up to 14 days grow well. 1% w/v of 2T engine oil was used as the sole carbon and energy source. The percentage of degradation of 2T engine oil by the *Brevibacillus agri* reached a maximum of 59% after 28 day under optimized culture conditions (table 1). The degradation of 2T engine oil after 7, 14, 21, 28 and 35 day respectively, shown in table no. 1 and figure 1. The absorbance of bacterial strain *Brevibacillus agri* at different time interval (7, 14, 21, 28 and 35 day) was respectively at 620 nm as shown in table no. 2 and figure 2.

Table 1
Biodegradation % of oil by Brevibacillus agri at different time period 7, 14, 21, 28, and 35 days

Sr. no	Days	weight of empty beaker	Weight of beaker containing oil	Weight of residual oil	Amount of oil degradation	% degradation
1	7	29.67	30.31	0.64	0.36	36%
2	14	29.67	30.18	0.51	0.49	49%
3	21	29.67	30.14	0.47	0.53	53%
4	28	29.67	30.08	0.41	0.59	59%
5	35	29.67	3016	0.49	0.51	51%

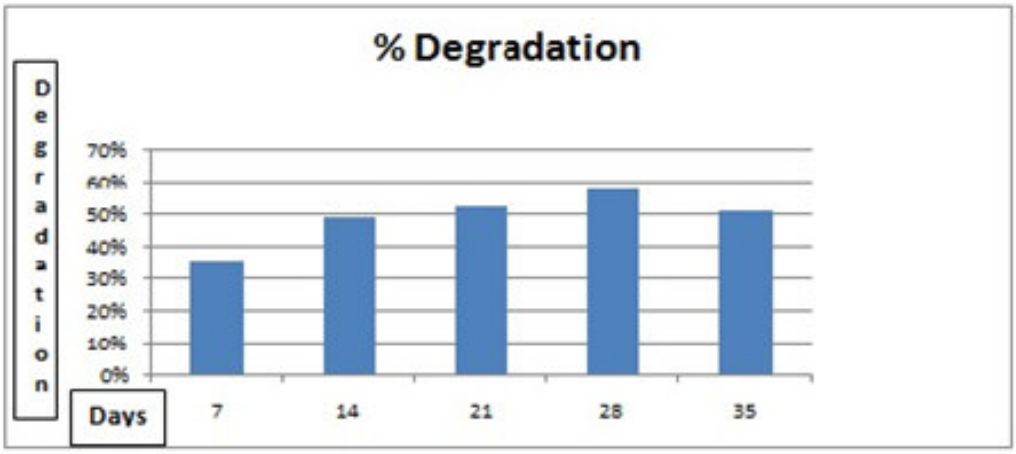

Figure 1
Biodegradation % of oil by Brevibacillus agri at different time period 7, 14, 21, 28, and 35 days

Table 2
Growth Rate of Brevibacillus agri during biodegradation at different time period 7, 14, 21, 28, and 35 days

Sr. No	DAYS	GROWTH
1	0	0
2	7	0.0655
3	14	0.6747
4	21	1.9223
5	28	2.0572
6	35	0.2851

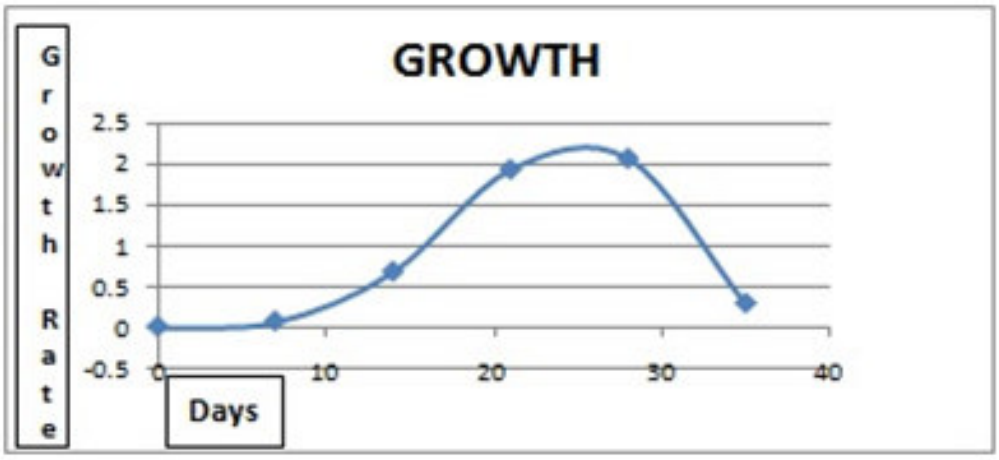

Figure 2
Growth Rate of Brevibacillus agri during biodegradation at different time period measured by turbidometry method

Hydrocarbon degradation analysis by GC-MS

The biodegradation of different hydrocarbons in contaminated environments is mainly through the breaking down activity of bacteria. Biodegradation of these hydrocarbons is one of the means by which contaminants can be removed from the environment[33]. In general, individual microbes may degrade only a limited range of hydrocarbons. Bacteria are the most

dynamic agents in hydrocarbon degradation, and they work as chief degrader of spilled oil in the soil[1]. Several other bacteria are even known to feed solely on hydrocarbons[34]. The Bacterial genera, namely, *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia,* and *Mycobacterium* isolated from oil contaminated soil proved to be the potential organisms for hydrocarbon degradation[13]. Effect of biodegradation on 2T engine oil derivatives (alkane, aromatics and NSO+ asphaltene fractions) by bacterial strain *Brevibacillus agri* has been studied in different time interval (7, 14, 21, 28 and 35 day). Aliphatic and aromatic fractions were analyzed by the gas chromatography – mass spectroscopy. The changes in GC chromatograms of 2T engine oil during degradation by *Brevibacillus agri* showed in figure 4 whereas the oil had full array of hydrocarbon fractions C1 to C30, with peaks normally distributed around C26, which was highest, on the day zero there was extreme reduction of most of the peaks by day 7(figure 5), with vanishing of C21 to C40. In addition, there was a marked reduction from C13 to C20, which were the most abundant. By 21, 28 and 35 day, (figure 6-9) all the peaks had been reduced to an average of 5%. Furthermore, peaks higher then C20 had disappeared. The percentage of various hydrocarbon compounds of fresh and used 2Tengine oil degraded by *Brevibacillus agri* after 35 days (7, 14, 21, 28 and 35 day) of incubation are shown in table no. 3 and figure no.3. The reduction of peaks between 0 day to 35 day (figure 6-9)) concomitant with rapid growth shows that the organism metabolized vigorously within this period. Hydrocarbons such as C20 to C40 reduced drastically by day 14 (figure 5) this may be due to the breakdown of larger Hydrocarbon molecule to smaller units. The biodegradation of petroleum hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) and n-branched alkanes etc. were studied in aqueous media using strains of bacteria isolated from petroleum contaminated soil[35]. A consortium was consisted of *Rhizobiales sp., Pseudomonas sp., Brucella sp., Bacillus sp., Rhodococcus sp., Microbacterium sp.* and *Roseomonas sp*. and removed nearly 52.1% of crude oil at 30 °C within 7 days, with removal of aliphatic hydrocarbons by 71.4% and aromatic hydrocarbons by 36.0%, respectively[36]. In the present study the bacterial strain *Brevibacillus agri* was able to degrade 40 to 60% of hydrocarbons between 7 to 35 days of incubation period at 37°C respectively. *Brevibacillus agri* species represents one of the most adaptable groups of organisms involved in the degradation of hydrocarbons. Whereas the *Pseudomonas* species degraded 90.2% of fresh oil in 30 days reported by Jayashree[15]. *Pseudomonas* sp. and *Achromobacter* sp. species degraded 73.3% and 80.0% of fresh oil reported by Adelowo[19].The 28% degradation of 2T engine oil by *Achromobacter* sp in the first 7 days studied by Harikrishna[37]. These data advance highlight the adaptability of *Brevibacillus agri*. The early slow rate of degradation observed on 2T engine oil may be due to existence of extra recalcitrant fractions of hydrocarbons and toxicity exerted by contamination such as metals and burning products, which are present in the oil, necessitating longer period of adaptability. The microorganism used in this study degraded all the hydrocarbon fractions in 7 to 35 days as shown in the chromatograms (Figures 4 to 8). This is like to previous trends recorded in hydrocarbon degradation by other authors[22]

Table 3
Showing biodegradation % of hydrocarbon compound by Brevibacillus agri at different time period

Days	C1-10	C11-20	C21-30	C31-40	C41-50	C51-60
7	6.4%	61.29%	32.25%	0	0	0
14	6.8%	61.39%	18.18%	11.36%	2.2%	0
21	19.44%	38.88%	27.77%	13.88%	0	0
28	34.92%	41.26%	12.69%	3.1%	7.9%	0
35	59.67%	17.74%	12.9%	8%	1.6%	0

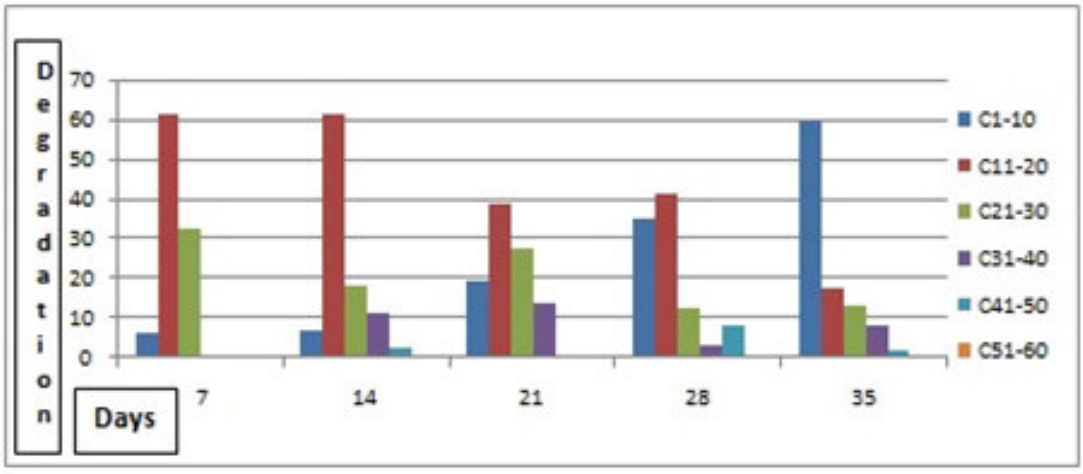

Figure 3
Showing degraded hydrocarbon compounds % (in different range) of 2T Engine oil by Brevibacillus agri

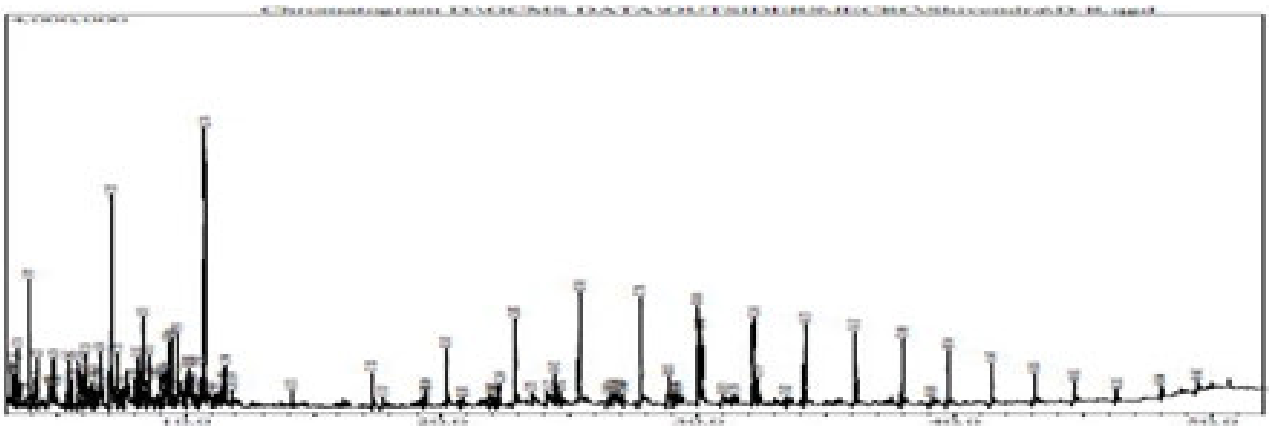

Figure 4
GC-MS analysis showing degradation of hydrocarbons after 7 days by Brevibacillus agri

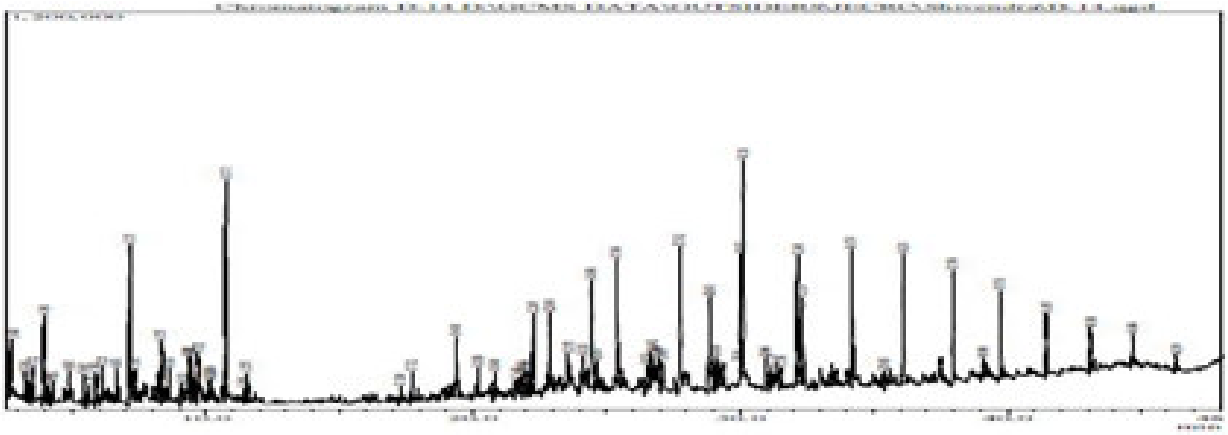

Figure 5
GC-MS analysis showing degradation of hydrocarbons after 14 days by Brevibacillus agri

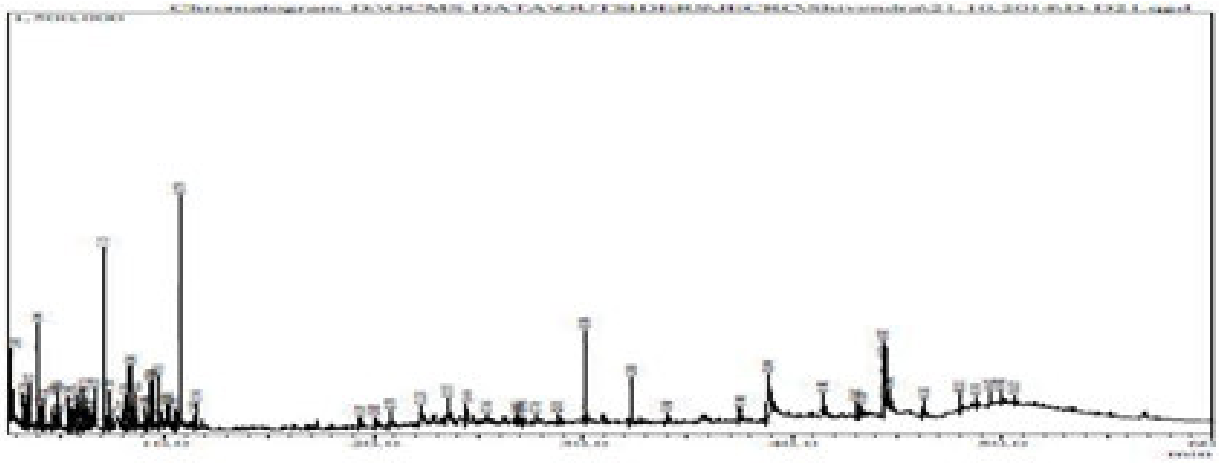

Figure 6
GC-MS analysis showing degradation of hydrocarbons after 21 days by Brevibacillus agri

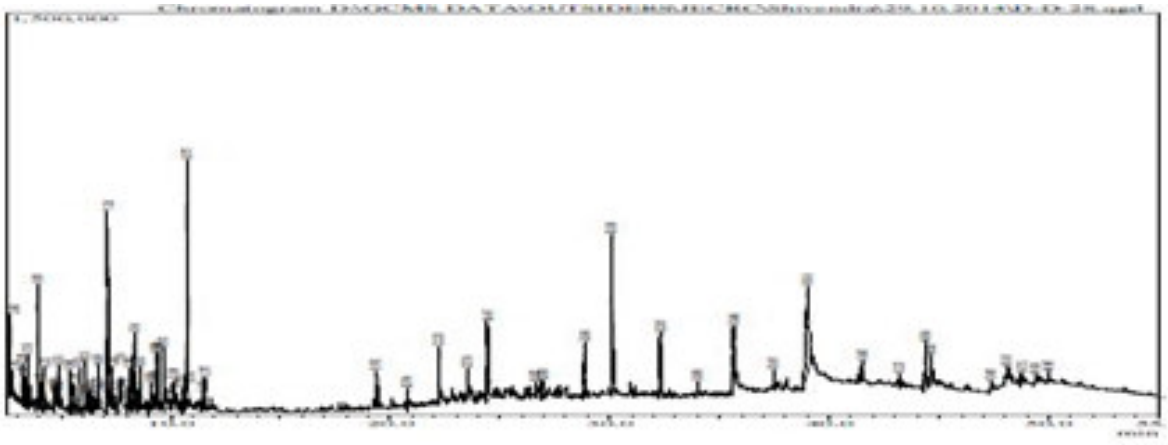

Figure 7
GC-MS analysis showing degradation of hydrocarbons after 28 days by Brevibacillus agri

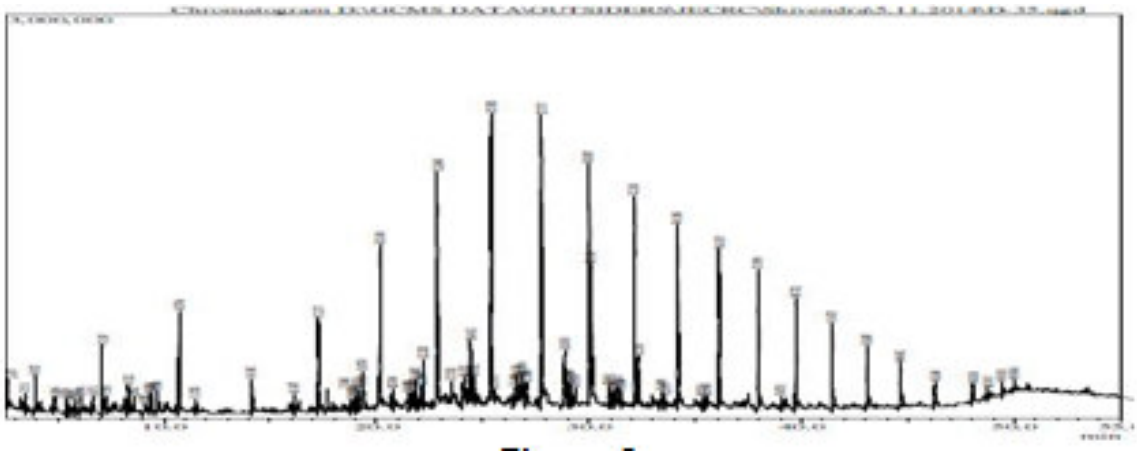

Figure 8
GC-MS analysis showing degradation of hydrocarbons after 35 days by Brevibacillus agri

Molecular identification of Brevibacillus agri

DNA Isolation and PCR amplification

Genomic DNA of the isolate I^3 was extracted and visualized by running it on 1.2% (w/v) agarose gel. The gel was visualized in UV Transilluminator resulting in single band of high molecular weight. The isolated genomic DNA was further quantified by Nanodrop spectrophotometer as 520 ng/µl. The 16S rRNA gene of isolate I^3 was amplified by PCR and the resulting amplified gene was electrophorized and visualized as a compact band of expected 1500 bp using gel documentation systems (Figure 9)

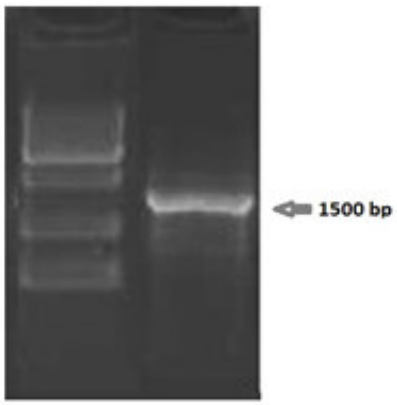

Figure 9
Visualization of PCR amplified 16S rRNA gene under Gel documentation system

Bioinformatic analysis

The gene sequence data obtained from XcelrisLabs for isolate (I^3) was analysed with BLASTN search tool using nucleotide database of NCBI GenBank for the identification of bacterial isolate. The best homology microorganism was selected. The homologous 16S rRNA gene sequences of selected strain were obtained from the nucleotide databases of NCBI with respect to the isolate. The selected homology sequences of 16S rRNA gene were aligned respectively using multiple sequence alignment tool ClustalW and the aligned results was used to identify the organism through MEGA6 software tool (figure 10). Based on the existing database in GenBank, the isolate was identified as *Brevibacillus agri*. The 16S rRNA gene sequence of isolate (I^2) was submitted to GenBank database using Bankit submission tool of National Center for Biotechnology Information (NCBI), USA. The GenBank accession numbers was assigned to the submitted sequence of (I^2) as KT923058.

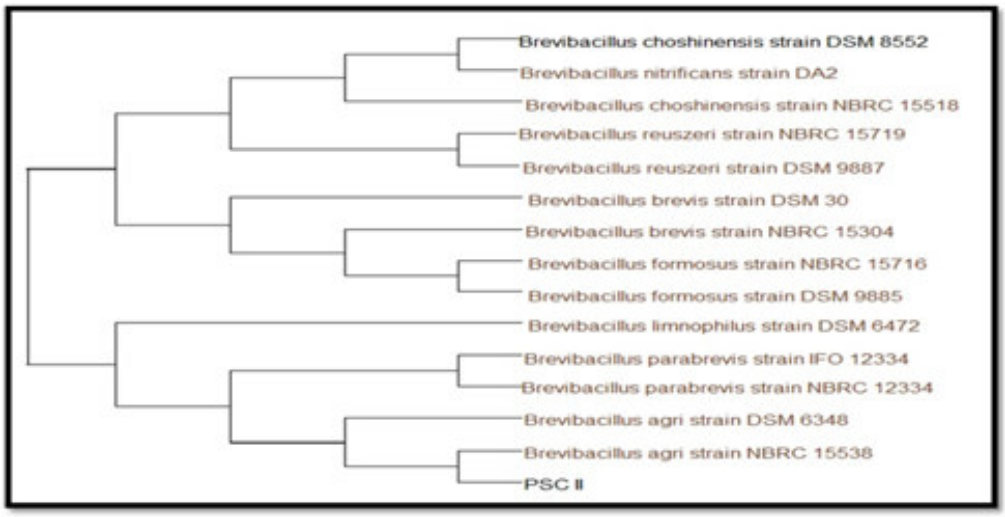

Figure 10

Phylogenetic tree of I^2 with the selected best homologous known bacterial strains

CONCLUSION

This study shows that *Brevibacillus agri,* is isolated from contaminated soil based on its capability to degrade 2Tengine oil, has vast range of degradative potentials for fresh and used engine oil. Hence, *Brevibacillus agri* can be used in bioremediation of hydrocarbon-polluted sites.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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