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Validating the Crude Oil Degrading Ability of Bacillus Subtilis Isolated From Crude Oil Contaminated Soil

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Abstract: Oil spills have caused significant ecological issues. The process of hydrocarbon bioremediation relies on the microbial population's capacity to proliferate within these substrates and breakdown in to harmless byproducts Bioremediation represents a viable and auspicious alternative to conventional cleaning methods. Soil microorganisms possess the ability to mineralize the majority of the organic compounds. Hence, the current study mainly focus on the crude oil degrading bacterial isolation, degradation, molecular analysis and optimization of bacterial isolates present in crude oil contaminated soils from automobile service centre at Namakkal, Tamil Nadu. Through the process of enrichment and screening, bacteria that possess the capability to degrade major crude oil were selected and subsequently subjected to 16s rRNA sequencing method to determine their identity. In order to optimize the cultural conditions a range of factors includes pH, temperature, crude oil concentration and nitrogen source were carried out. Totally thirty six organisms were isolated from the enrichment method. Growth of these isolates was examined and the isolate named PS9 showed higher capacity (56%) for the utilization of crude oil. GC-MS study confirmed an observable crude oil degradation of the samples treated with PS9 when compared to control crude oil. In the analysis of 16s rRNA sequencing data, the isolated strain belong to the species Bacillus subtilis. Analysis of variance was used to ensure statistical accuracy, low $p<0.0001$ was obtained. Based on these studies the above bacterial strain can be used in biodegradation.

Keywords: Soil Bacteria, Enrichment, B. Subtilis, Degradation, Optimization

1. Introduction

Crude oil has a considerable role in all aspects of social life as a chemical raw material and crucial energy source, petroleum oil is the most important source that all nations have been actively seeking to control it [1]. Every single year the accidental crude oil release creates to play a large role in environmental pollution, the normal crude oil leak was likely to be sixty thousand metric tons. Several research studies have shown that hydrocarbon components are neurotoxic and carcinogenic. Hence oil pollution possess a greater threat to all living beings including human and environment. Due to higher usage of petroleum hydrocarbons in our day today life results in persistence of petroleum contamination which ultimately affects the environment microbiome [2].

Recent researchers insists the importance of bacterial usage the capable tool to deal the environmental pollutants due to their eco – friendly nature [3], cost efficiency, its ability to degrade the pollutants by high competence [4, 5] and in oil polluted environments there is a presence of huge number of such bacteria that can degrade hydrocarbons [6, 7]. In occurrence many bacteria are capable metabolizing some alkanes and breakdown the aromatic hydrocarbons, with additional nutrients such as oxygen, nitrogen, phosphorous and sulfur to sustain their growth and metabolism [8]. Bacteria degrade small quantity of environment pollutants inadequately. High amount of hydrocarbon results in the hold back of biodegradation due to oxygen and nutrient restrictions or lethal effects. Biodegradation mostly between the temperature ranges from 30 to 40°C in the soil environment [9]. Bacteria such as *Bacillus* [10], *Pseudomonas* [11] and many other species are involved in hydrocarbon degradation with increased degradation rates. Bacterial enzymes can efficiently degrade the environmental pollutants and its efficiency can be increased by optimizing the growth parameters includes pH, temperature and nutrient

availability [12]. Hence the degradation has been affected by an array of variables, including the added existence of carbon sources, pH, temperature, inoculums size and moisture content. It becomes more crucial to mitigate the impact of PAHs (Poly Aromatic Hydrocarbons) since they have their toxic nature and cancer causing potential [13].

The current work thus focuses on isolating bacteria that degrade crude oil from soils that have been polluted with it. A controlled environment was chosen to investigate the growth and abilities in degradation of crude oil.

2. Materials and Methods

2.1 Sample Collection

Automobile service centers (oil tanker lorrie's service center) located in Namakkal district, Tamil Nadu, India is the sampling site for the bacterial isolation. Four soil samples polluted by crude oil were collected in the depth of 5 cm at different sites in sterile vials. Samples were collected on oil change station, washing and cleaning areas especially in oil tanker lorrie's service centers (from old and new centers) located in different places within the namakkal district. Before being transported, collected samples were kept in ice box. After all samples reached the room temperature they were used for the bacterial isolation that can degrade the crude oil. Study's crude oil source from Chennai Petroleum Corporation limited, Manali, Chennai.

2.2 Enrichment for the isolation of bacteria

The method of selective enrichment was applied for the bacterial isolation. Crude oil serves as the source of carbon for the isolates. Bushnell haas broth was used for the enrichment method enhanced with crude oil and added 1g of collected soil sample in 100 ml of the broth. For 3 days, the soil and broth medium with crude oil were incubated at 37^{o}C with 180rpm orbital shaking. Following incubation, it was serial diluted and then, using spread plate technique, transferred to the sterilized bushnell haas medium that had been enhanced with crude oil. Then the petriplates were incubated for the period of 24 - 48 h at 37°C. After 48 h, incubated plates were seen for the bacterial growth and future studies were carried out.

2.3 Assessment of crude oil degradation

For degradation assessment, bacterial cultures inoculated in sterilized bushnell haas broth with added crude oil (2%/100 ml) then it was incubated at 180 rpm for 7 days at 37°C in a rotary shaker along with the control flask without inoculating the cultures. The remaining crude oil was then extracted using gravimetric analysis. After the completion of incubation, the broth was separated by a separating funnel. Petroleum ether was added to the broth and shaken forcefully. It was left undisturbed to stand for separate layers. Oil and petroleum ether made the top layer. In the bottom layer remaining broth appears. Once removed the moisture, by passing it through sodium sulphate, the oil was collected in a tube that was already weighed and percentage was calculated [14]. After solvent removal, the residual oil and the control oil was analyzed by GC-MS (Gas Chromotography Mass Spectrometry).

2.3.1 GC-MS analysis

Collected extract was analyzed through GC-MS, done using GC clarus 500 Perkin Elmer system. GC-MS loaded with a selected combined silica capillary column measuring 30 mm x 0.25 mm with 1D x 1 µmdf that consists of Dimethyl polysiloxane (100%) and compounds were divided using helium gas in continuous run for 1 ml/minute. Injector temperature was placed at 260°C for the chromatographic run. Temperature for oven was at 60°C for the period of 2 minutes and then it was amplified to 300°C in a speed of 10°C for a minute that was continued for six minutes. Mass spectrum were acquired at 70 eV were recorded in the intervals at the rate of 0.5 seconds, with fragment masses ranges from 45 to 450 Da. The proportion of every component was assessed by relating its regular peaks to the whole area. For handling mass spectra and chromatograms, a turbomass has been used. The compounds spectrums were compared to a known database in GC-MS NIST (National Institute of Standards and Technology) (2008) library.

2.4 Identification of the crude-oil-degrading isolates and PCR amplification and sequencing

The CTAB method was taken to separate DNA from bacteria [15]. The bacterial DNA was isolated and amplified using bacterial Universal-16S rRNA, the primary UF 5′-GAGTTTGCTGCTCAG-3′ and UR 5′-ACGGCTACCTTGACTTT-3′ [16]. The reaction mix for PCR made of DNA (2 µl), taq polymerase (0.2 µl) [2U / µl], 2 µl of 10x PCR buffer, and 2 µl of each [4pmol / µl] main. The amplification began at 94°C for the time of 10 minutes that was followed by 30 number of cycles at 94 °C for a minute, 48°C for 1 minute, 72°C was maintained for 2 minutes, and 72°C was maintained for 10 minutes as the final extension stage. The PCR product was taken using 1% agarose gel maintained in a level of 50 V for 60 minutes in 1 x TAE-buffer. Object amplified was visualized under UV transilluminator. Later, the amplicon was submitted for sequencing; both forward and reverse sequences were determined. The identified sequences were collectively subjected to BLAST analysis to identify the species.

2.5 Optimization of growth conditions

Growth parameters such as pH, temperature, nitrogen supply, crude oil and inoculum concentrations were optimized for the bacteria (crude oil degrading). Standard inoculum was inoculated in sterile mineral salt medium mixed with crude oil (2%) which is used for all the parameters separately. Varied percentages of crude oil such as 0.5%, 1%, 1.5%, 2%, & 2.5%, various pH (5, 6, 7, 8 and 9), different temperatures (32^{o}C, 35^{o}C, 37 ^{o}C, 40^{o}C and 42^{o}C), different concentrations of ammonium sulphate and various inoculum concentrations (0.2%, 0.4%, 0.6%, 0.8%, and 1%) are used. Then prepared flasks with the desired mixture were incubated at 37^{o}C for 7 days at the speed of 200 rpm. The OD value was calculated in UV spectrophometer at 600 nm. All the experiments were carried out along with the control flasks for each parameter.

2.5.1 Data analysis

All the above tests were run with three independent variables. The collected findings were statistically analyzed by ANOVA using prism. The coefficient of determination R2 was used to assess the fit quality of the polynomial model equation, and the F and t tests were used to determine the statistical and regression coefficient significance, respectively. By deriving the regression equation and examining the response surface contour map, the optimal values of the chosen variables were found. Three independent variables were held constant while the other two were changed to create the contour plot analysis. At last with the optimized parameters, a final test was carried out and GC-MS analysis was performed for the collected residual oil along with the control.

3. Results

3.1 Bacterial isolation

In the current study, following enrichment, potential strains that can be used in petroleum biodegradation were isolated. The growth of the bacterial isolates on BH medium with added 1% crude oil indicated that the strains be able to utilize crude oil for the primary carbon source. The labels for the strains are PS7-PS14, NS8-NS15, RS12-20 and R9-R19. Among the above strains labelled as PS7-PS14 six were Gram-positive and other two strains belongs to Gram-negative. In NS8-NS15 half the count belongs to Gram-positive and the remaining belongs to Gram-negative, in the next series RS12-20 three were Gram-positive and six strains were Gram-negative, the last number of strains R9-R19 five was Gram-positive and six were Gram-negative. Through screening, the growth of the isolated strains and their degradative properties were monitored by UV spectrometry at 600 nm and the results were recorded as graphical representation (Figure 1–a, b, c & d). Among the above mentioned strains PS9 showed good growth in crude oil supplemented medium. All the isolates were stored using glycerol that acts as a cryoprotectant and stored under -20°C for further use.

3.2 Validation of the petroleum biodegradation ability of isolated strain

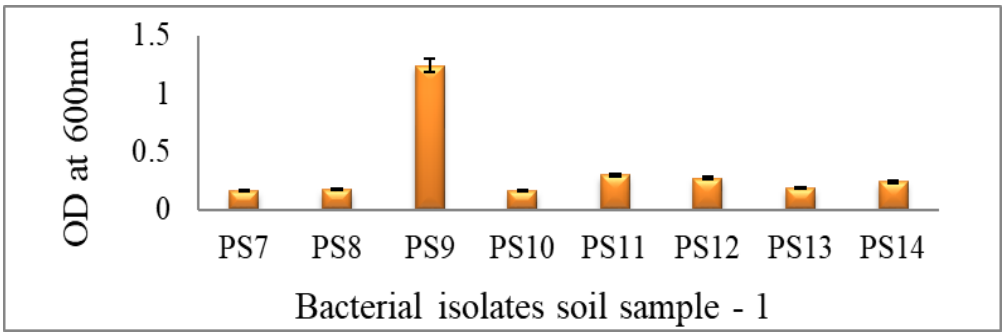
In degradation, PS9 showed higher capacity in crude oil degradation (56%) calculated by dividing the weight of the residual oil treated with PS9 with the initial weight of the control crude oil and multiplied by 100. Figure 2 displays the chromatogram for the strain in comparison to the control (Figure 3). Crude oil degrading ability of PS9 was evaluated by the GC-MS analysis. The lasting crude oil treated with PS9 strain was then separated by gravimetric analysis and GC-MS was done for the untreated and bacteria treated crude oil. Through the results, components variations were analyzed and tabulated (Table 1). Strains considerably decreased many crude oil peaks and several peaks in control crude oil were not evident in the treated oil. The components in the treated oil are broken down into simple forms. Decreases in the peak areas of hydrocarbons in the treated crude oil compared to control were observed. It showed that the strain have metabolized and convert the hydrocarbons in to simpler forms.

3.3 Molecular Identification

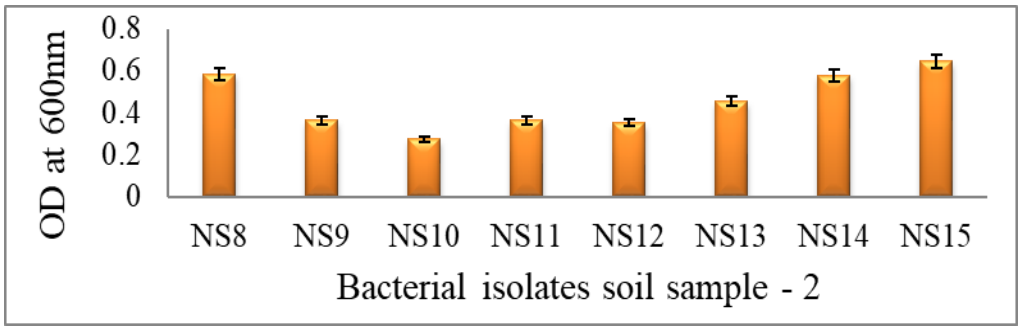
PCR amplification was done to identify the bacterial strain in molecular level and the obtained 16S rRNA sequence was compared in the database. The molecular weight of the amplicon was found to be 1500 bp. To find the bacterial strains neighboring to the studied bacteria, blast was performed between the regularly associated bacterial strains. On the basis of phylogenetic analysis (Figure 4) the bacterial isolate was identified as *B. subtilis* and observed that the amplicon is homologous (90.88%). The sequence of this bacterial strain was deposited in Genetic Sequence Database at the NCBI. The obtained accession number of the sequence is OL312764.

3.4 Optimization

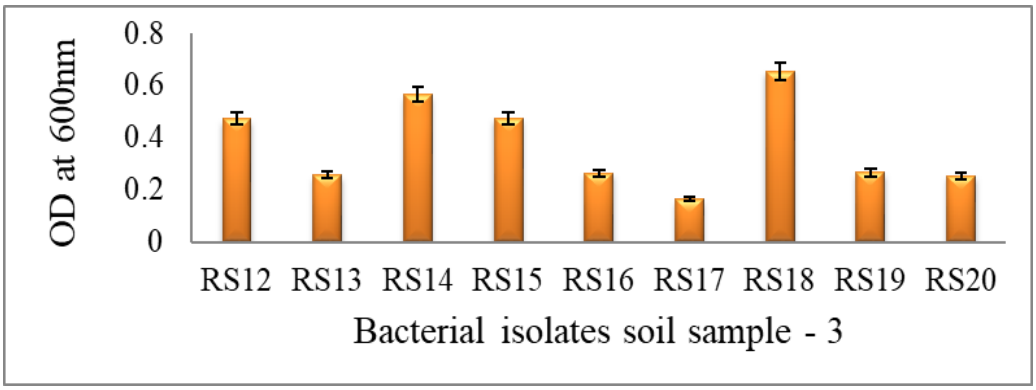
Isolated bacteria were cultivated under varied pH, temperature, nitrogen source and different crude oil concentration to investigate the optimal growth condition. The result showed that *B. subtilis* showed maximum growth at the pH 7.0, temperature at 37 °C, adding 0.1% of ammonium sulphate to the medium as the nitrogen source resulted in higher growth of the strain. And in 2% crude oil the bacteria had good growth when compared to the remaining percentages. Result was analyzed by one way ANOVA. Significance of the present model was confirmed through the low *P* value (*P<0.0001*, mentioned in the figure 5). Counter plots were plotted to explain the individual and interactive effects of the variables (Figure 6). GC-MS were done for the residual oil from optimized parameters (Figure 7).



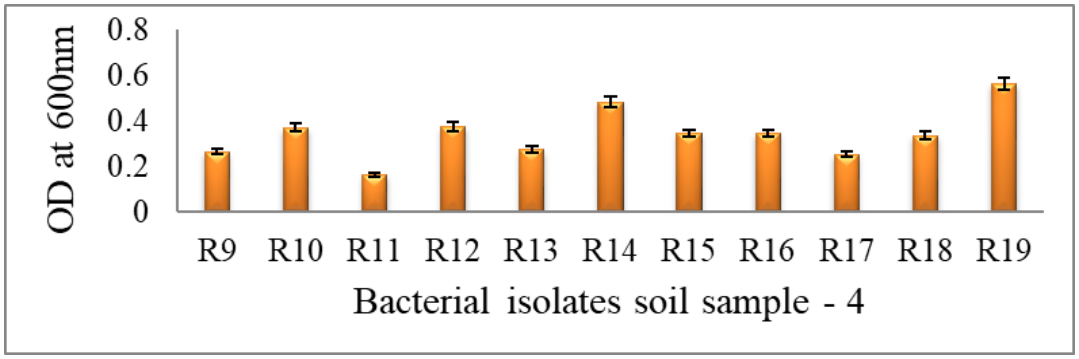
(a) Bacterial isolates PS 7 – PS14 (sample – 1)



(b) Bacterial isolates NS 8 – NS15 (sample – 2)



(c) Bacterial isolates RS12 – RS20 (sample – 3)



(d) Bacterial isolates R9 – R19 (sample – 4)

Figure 1. Isolation of crude oil degrading bacterial isolates from four soil samples.

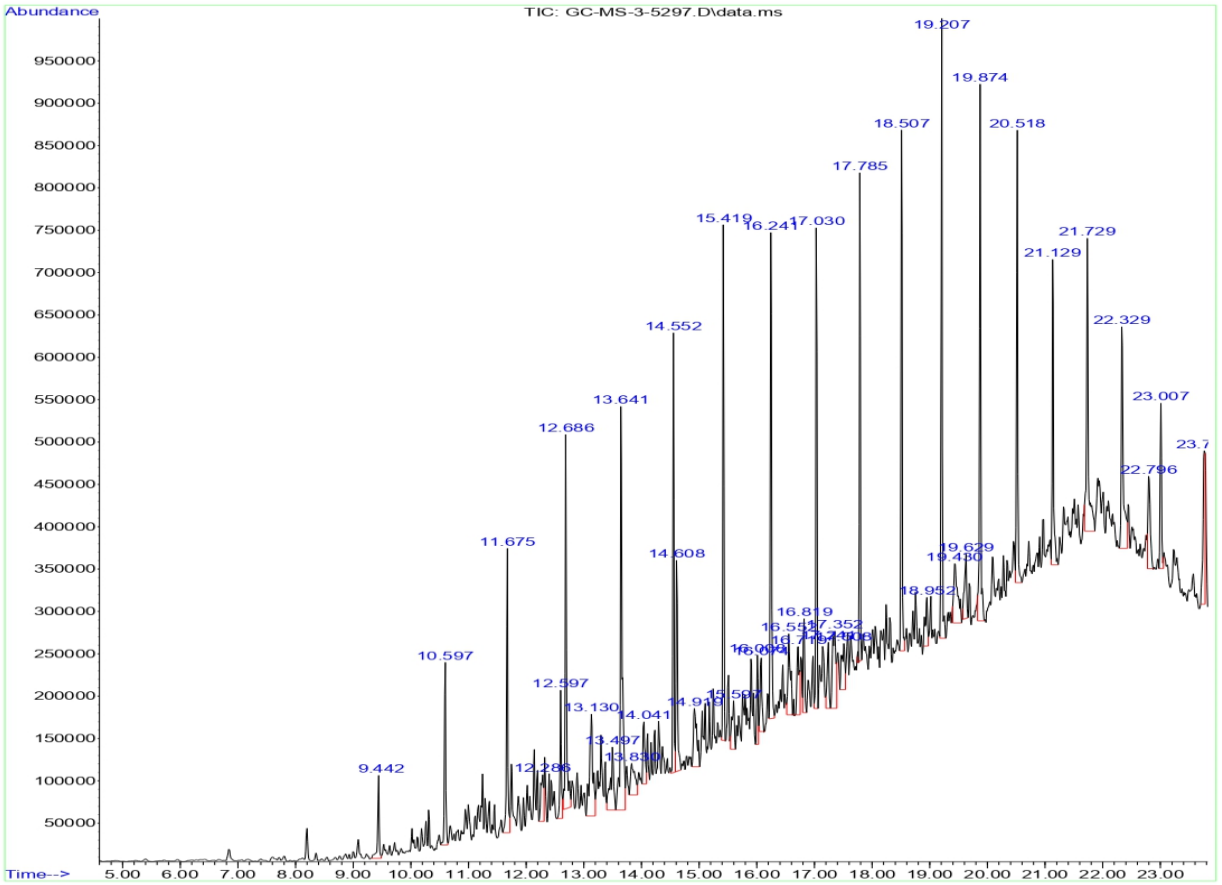


Figure 2. PS9 treated crude oil

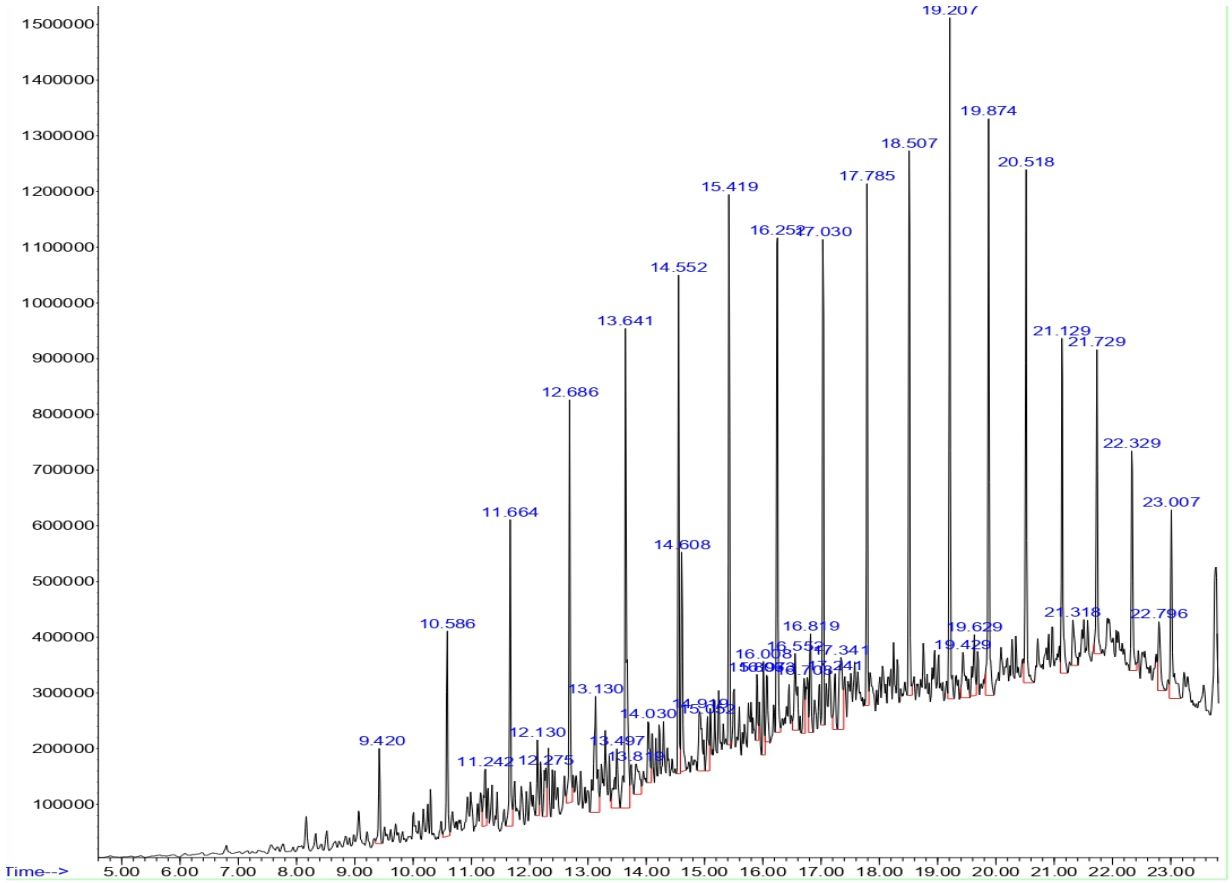


Figure 3. GC-MS spectrum of control crude oil

Table 1. PS9 treated crude oil compounds

S.No	Compounds (PS9) of individual peaks	RT (Retention time)	% (Area)
1	Tridecane	9.442	0.99
2	Tetradecane	10.597	1.88
3	Pentadecane	11.675	2.88
4	Naphthalene, 1,6,7-trimethyl-	12.286	1.02
5	Diethyl Phthalate	12.597	1.35
6	Hexadecane	12.686	3.67
7	Dodecane, 2,6,11-trimethyl-	13.130	2.64
8	Naphthalene, 1,2,3,4-tetramethyl-	13.497	1.43
9	Heptadecane	13.641	6.13
10	Neoisolongifolene	13.830	1.06
11	Dodecane, 2-methyl-8-propyl-	14.041	1.00
12	Octadecane	14.552	3.71
13	Hexadecane, 2,6,10,14-tetramethyl-	14.608	2.42
14	2 –Chloropropionc acid, hexadecylester	14.919	1.48
15	Tetradecane	15.419	4.11
16	1,2-Benzisothiazole, 3-(hexahydro-1H-azepin-1-yl)-, 1-dioxide	15.597	0.87
17	Pentadecane	16.008	0.89
18	Dibenzothiophene, 4,6-dimethyl-	16.074	1.02
19	Pentadecane	16.241	4.97
20	Octadecane, 1-chloro-`	16.552	1.62
21	9,10-Dimethylanthracene	16.719	1.35
22	Nonadecane	16.819	0.99
23	Heneicosane	17.030	4.22
24	N,N-Dimethylindoaniline	17.241	0.92
25	5(10H)- Pyrido[3,4-b]quinolone,7-methoxy-	17.352	2.15
26	Heptadecane, 2-methyl-	17.508	1.13

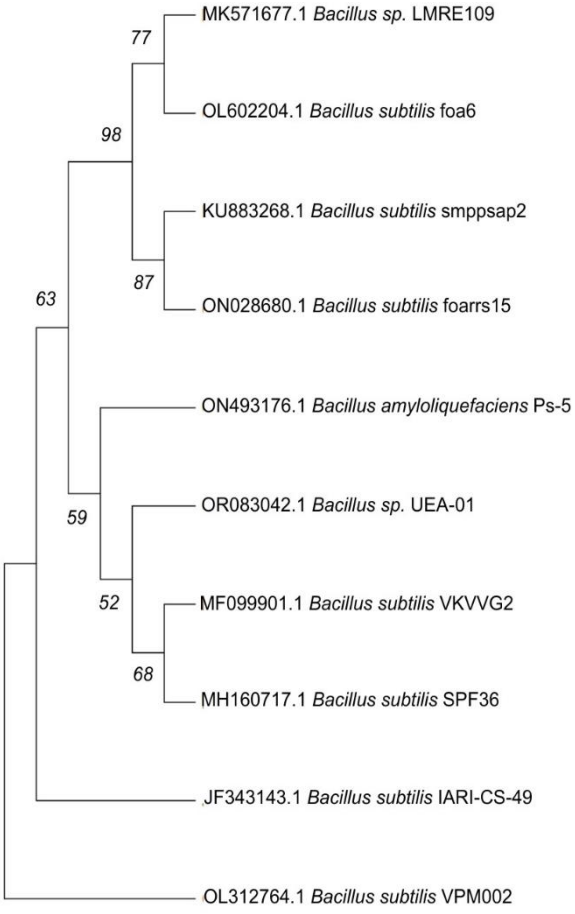
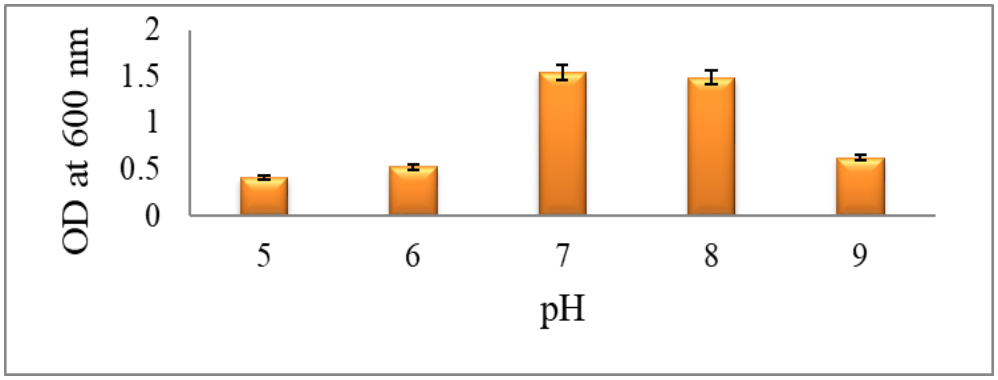
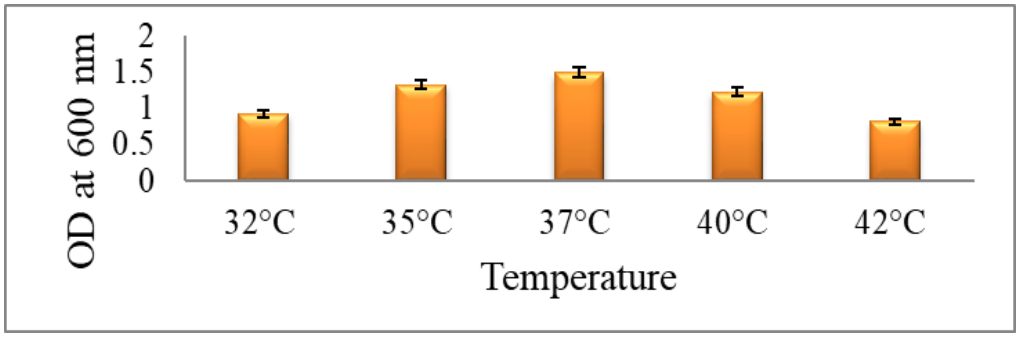


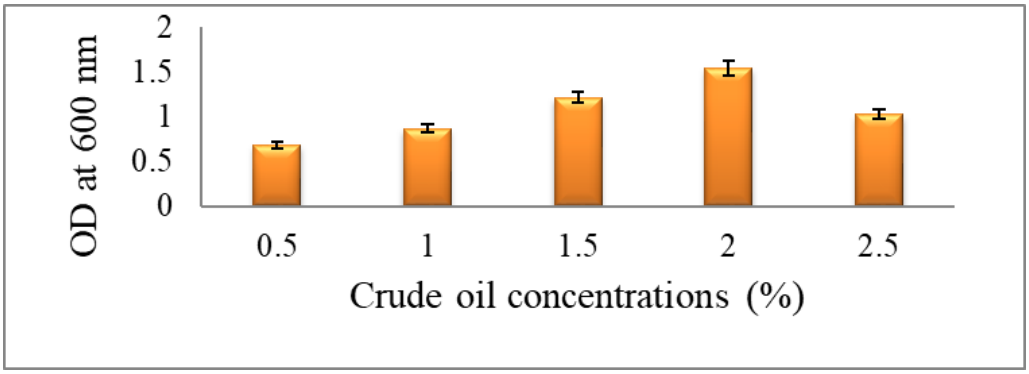
Figure 4. Phylogenetic tree



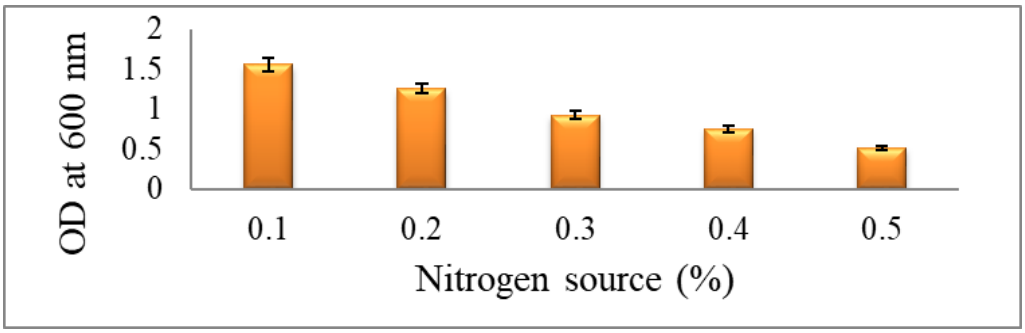
(a) Different pH



(b) Different Temperature

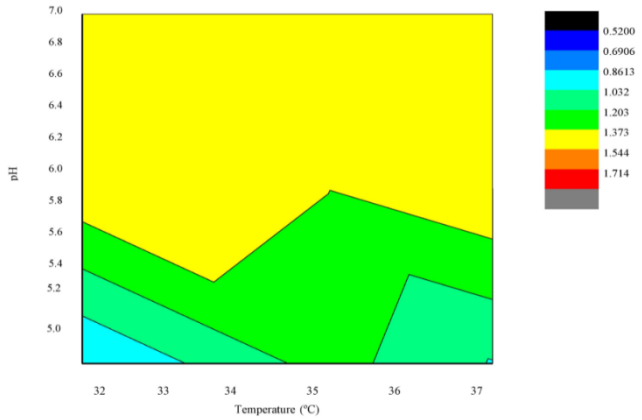


(c) Different crude oil percentage

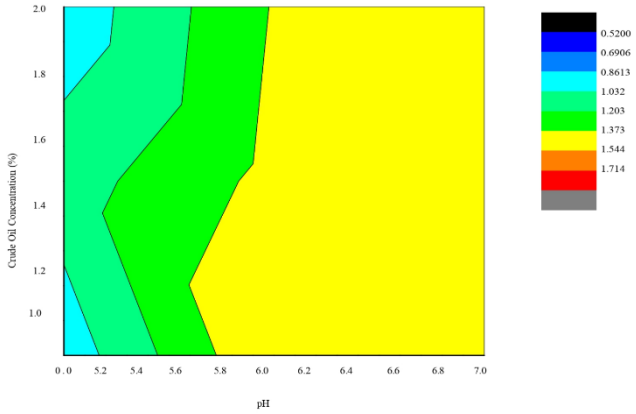


(d) Different concentrations of ammonium sulphate

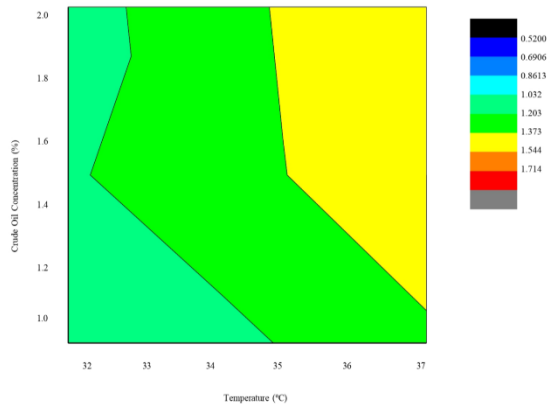
Figure 5. Optimization of growth conditions and significance of the differences was compared with each groups by one-way ANOVA (***p<0.0001).



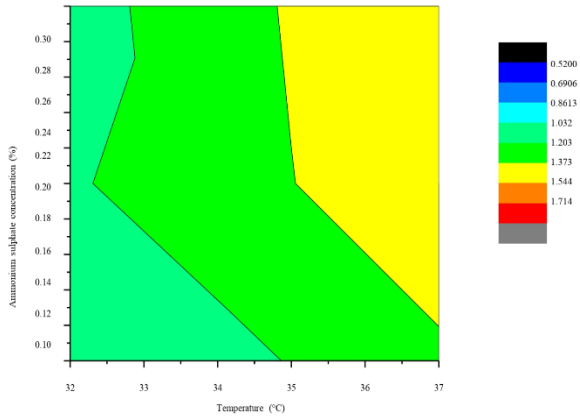
(a) Temperature and pH concentrations



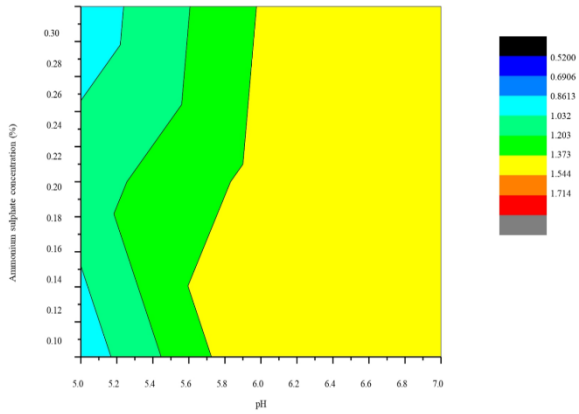
(b) pH and crude oil concentrations



(c) Temperature and crude oil concentrations



(d) Temperature and ammonium sulphate concentrations



(e) pH and ammonium sulphate concentrations

Figure 6. Counter plots and interactive effects of parameters

4. Discussion

Over a century ago, microbes were recognized as crude oil biodegraders. Hydrocarbons are hydrophobic in nature and it is the main factor that influencing in the success of bioremediation. Microorganisms comprise the capability to degrade the crude oil are indeed widespread in the environment and it is the vital part in remediation of polluted soils [17]. Native or exogenous microbes are much more successful in degradation than nonnative microbes. The native microbes adapted to the local environment that has the mechanism to produce metabolites that solubilize and easily convert hydrocarbons [18]. This approach is especially practical and required for the present technologies and social surroundings [19].

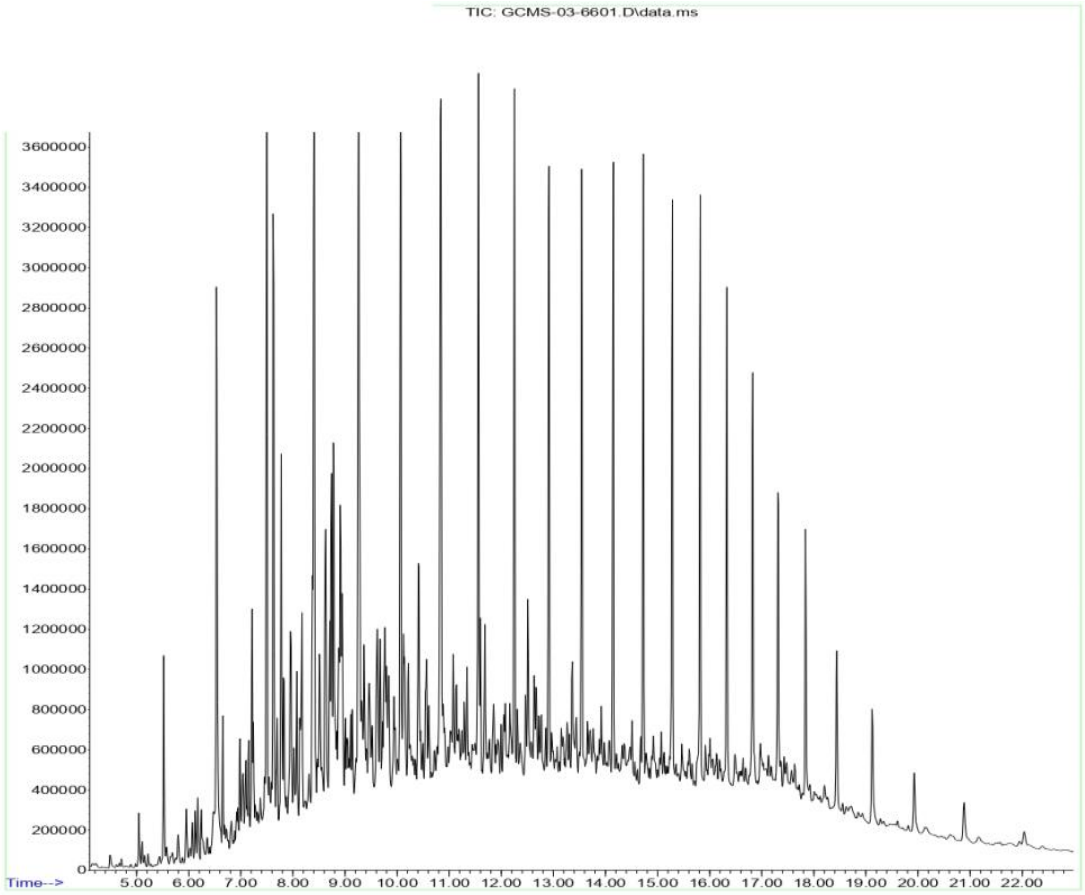


Figure 7. GC-MS spectrum of PS9 treated crude oil in optimized condition

Parallel to the present study some of the hydrocarbon degrading gram positive bacteria were also detected in soil [20]. Similarly, a study reported that when compared to other isolates *Bacillus* have greater efficiency in degradation of diesel and octane [21]. Crude oil degradation rates depend on its availability and strain capacity in degradation of crude oil as their needed energy source. As it converts the crude oil to less toxic and degrade the low molecular weight compounds. Aliphatic hydrocarbons composed of short and long alkane chains and it is readily degradable. Further GC-MS was carried out for validate the strain's potential to make use of the major groups of hydrocarbons. Gas chromatography, common technique used for the evaluation of the quantity of degradation. It was used to compare new and treated crude oil. In a study conducted by Latha *et al*. [22] *B. subtilis* strain degrades the crude oil (0.85mg/ml). *Bacillus* degrades 94% of crude oil and 85% of phenanthrene [17]. According to Das and Mukerjee [23], *B. subtilis* particularly degrade the n-alkanes between the chains of C14 and C30. Gas chromatography give petroleum hydrocarbon distribution charts, fingerprints of principal oil components such as resolved n-alkanes and information on the amount of biodegradation of oil that has spilled [24]. Many reports referred that numerous bacteria capable of degrading hydrocarbons but only very low number of reports showed *Bacillus* as hydrocarbon degraders [25]. Parallel to the present study, in the process of oil recovery *B. subtilis* was isolated from oil reservoirs [26]. Similarly, in a study they isolated the strain *B. subtilis* from automobile garage soil contaminated with oil [27]. It was also reported that *Bacillus* is the most regular form of bacteria occurs in soil that are polluted with crude oil, it has the ability to withstand high amount of crude oil because of its endospores [28]. Accordingly in a study, they isolated *Bacillus subtilis* degrading Benzo [a] Pyrene from automobile workshop soil samples and also reported that the bacteria have the efficiency to degrade anthracene and naphthalene [29]. Several species have the capacity to degrade the hydrocarbons but *Bacillus* effectively degrades the hydrocarbons due to the spore formation that protect the bacteria from the toxic environment. So *Bacillus* can be contributed to oil spill remediation. Similarly in a study they isolated crude oil degrading *B. subtilis* and its optimal pH and temperature was 7 and 45 °C respectively [30]. In another study they also reported *Bacillus* growth was good at pH>5 [31]. Similar to the present study, pH was neutral for the maximum growth of bacteria [18]. In another study they reported that, better degradation observed at pH

between pH 6.9 – 8 [32]. The ideal temperature for the soil bacteria to degrade the oil ranges from 30 °C – 40 °C [9]. With the low oil concentrations, there is a relatively quick deterioration; however with rising oil concentrations, there is a slow degradation. When the oil concentration increases, the biodegradation speed declines and lastly ends [1].

5. Conclusion

Through the present study results, *B. subtilis* in oil contaminated soil has the huge capability to breakdown crude oil in polluted sites. Based on GC-MS significant degradation was done by the strain's efficient use of crude oil as a carbon source when compared to control. To improve the biodegradation efficiency the growth parameters such as pH, temperature, nitrogen source, crude oil concentrations were optimized. According to the statistical analysis, the strain performance was considerably enhanced due to these optimized conditions. A key advantage of this strain is its capacity to produce biosurfactants, which greatly enhances the solubilization and emulsification of hydrophobic substance. Additionally the strain's genetic characteristics serve as an effective bioremediation tool in affected areas. By using *B.subtilis* in bioremediation techniques provides economical and environmentally friendly method to remediate oil spills. The strain can function effectively in various environmental conditions by optimizing the growth conditions. Because of its capacity to adapt to a wide range of environmental factors the strain is a flexible agent for bioremediation in different conditions. In order to evaluate the strain effectiveness in large scale oil spill cleanup, further applications may need. Hence the study highlights *B.subtilis* potential as a useful tool in the remediation of crude oil contamination and their effects in the environment. There are also some limitations that this strain cannot degrade more complex compounds. Degradation rate is low when compared with mixed consortiums. Complete degradation may not happen by this strain.

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Authors Contribution Statement

Priya Rajendran: Investigation, related work, result analysis, writing, original draft. Nirmala Mahendran: Review and editing. Gobianand Kuppannan: Review and editing. Malarvizhi Arthanari: Conceptualization, supervision, review & editing. All the authors read and approved the final version of the manuscript.

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Competing Interests

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Data Availability

The data supporting the findings of this study can be obtained from the corresponding author upon reasonable request.

Has this article screened for similarity?

Yes