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"Isolation And Identification Of Biosurfactant Producing Indigenous Microorganism For Bioaugumentation"

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Abstract

The objective of this research was to perform screening of biosurfactant-producing bacteria from diesel contaminating areas in and around Madurai and Sivagangai Districts. Eight sampling sites were chosen in total, four each from Sivagangai and its surroundings and Madurai and its surroundings. The isolates were cultured in nutrient broth and Bushnell Haas (BH) liquid medium with 1% (v/v) diesel, and their extracts were evaluated according to drop collapse, oil dispersion, emulsification, and blood agar tests. From two hundred and eighty four isolates, 17 isolates were selected, screened for biosurfactant production. The extracellular biosurfactant production pointed out the 3 most efficient bacteria that presented high production. The three isolates were identified by 16S rDNA gene sequencing, indicating the presence of two different genera, *Enterobacter* and *Lysinibacillus*. Cluster analysis classified genetically related isolates in different groups, and the isolates were designated as *Enterobacter cloacae* strain GKE1(GenBank Accession Number: OP136122.1), *Lysinibacillus*sp. strain GKL2 (GenBank Accession Number: OP133003.1), and *Lysinibacillusfusiformis* strain GKL1 (GenBank Accession Number: OP133000.1).

Keyword: Biosurfactant, Enterobacter, Lysinibacillus, sequencing, cluster analysis, GenBank

INTRODUCTION

During the extraction, processing, and transportation stages of oil and gas, significant amounts of toxic and non-toxic waste are created. When incorrectly managed, some industry byproducts, such as volatile organic compounds, nitrogen and sulphur compounds, and spilt oil, can pollute the air, water, and soil to toxic levels (Bautista and Rahman, 2016).Because of the difficulty of attaining substantial oil removal through physical washing and collecting, particularly for oil that had gone into the subsurface, bioremediation emerged as a leading contender for ongoing shoreline treatment. Within weeks of the spill, bioremediation was highlighted as a potential emergent technology (Bragg et al., 1992; Pritchard and Costa, 1991).

Because petroleum hydrocarbons occur naturally in all marine settings, many different microbes have evolved the ability to utilise hydrocarbons as sources of carbon and energy for growth. Because of its low cost and eco-friendliness, the use of bacteria to deal

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with environmental pollutants has become a promising technology in recent years (Guerra et al., 2018).Oil-degrading microorganisms are common, however they may constitute only a small part of the prespill microbial community. Hundreds of bacteria, archaea, and fungal species can digest petroleum (Owens, 1990).Many ordinary and unusual bacterial species have been identified and used as biodegraders for petroleum hydrocarbons (Yang et al., 2015).

The hydrophilic - lipophilic balance, which determines the hydrophobic and hydrophilic portions in surface active chemicals, is one of the important properties of biosurfactants. Because of their amphiphilic structure, biosurfactants can not only enhance the surface area of hydrophobic substances, but also change the property of the cell surface of microorganisms. Because of their surface activity, surfactants are effective foaming agents, emulsifiers, and dispersion agents (De et al., 2015). Biosurfactants exhibit substrate selectivity and functional activity in harsh conditions of high temperatures, high salt concentrations, and pH, which can be attributed to industrial products and waste (De et al., 2015).

Surfactants are one of the most promising techniques for dealing with bioavailability issues. Surfactants may improve hydrocarbon mobility and bioavailability, hence accelerating biodegradation (Haftka et al., 2015). According to Haftka et al. (2015), the petroleum industry has relied heavily on surfactants to increase the solubility of petrol and its byproducts. Considering all the factors, the current study was focused on collection of soil samples from diesel contaminating areas in and around Madurai and Sivagangai Districts and to enumerate, and identification of bacteria from soil samples (Total Viable count) that have the diesel degrading capacity and biosurfactant production.

MATERIALS AND METHODS

Collection of soil samples

Totally eight sampling stations were selected, 4 from Madurai and its surrounding area and 4 from Sivagangai and its surrounding area. The sampling stations were: Maruthi Suzuki Service, Meenakshi, Madurai; MyTVS, Madurai; TNSTC SCTC Bus Depot, Madurai; Power Diesel Turbo Service, Madurai; Kalishwari Auto Workshop and Water Services, Sivagangai; Udhayam General Motors, Sivagangai; Tamil Nadu State Transport Corporation, Sivagangai and S*S Car Care, Sivagangai. The soils were collected in a sterile container in triplicate and were transported to the laboratory in aseptic condition. The samples were stored in the laboratory at ambient temperature until further use.

Enumeration of bacteria from soil samples (Total Viable count)

A 5g sample of each soil was placed into a 250ml flask containing 50 ml of tapwater and incubated at 23°C on a shaker at 200 rpm for 21 days. On days 3, 7, 14, and 21, a sample from each soil slurry was serially diluted, plated on Nutrient Agar media using pour plate technique and incubated for 1 week. After incubation, plates were enumerated and the total viable count was calculated (Bodour*et al*., 2003).

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Screening of diesel degrading bacteria

Soils were screened for diesel degrading bacteria using the following procedure. Bushnell Haas (BH) liquid medium (Bushnell and Haas, 1941; Atlas and Bartha, 1992) was used as the enrichment medium with 1% (v/v) diesel as the sole carbon source to isolate diesel degrading bacteria.1g of the diesel contaminated soil sample was added to 100ml of the enrichment medium and incubated at 30°C at 160 rpm. After two weeks, 1ml of enriched medium was transferred to freshly prepared 100ml enrichment media and incubated at the same conditions as described by Mandri and Lin (2007). Serial dilutions (1/10) from the third enrichment process was plated out into BH agar plates by spread plate technique, and covered with 100µl of diesel and incubated at 30°C for approximately one week. The single colonies were streaked into nutrient agar plates and nutrient agar slants. The plates and slants were incubated at 30°C overnight and stored either for short term or long term until further use.

Screening of biosurfactant producing organisms

Primary Screening

Blood hemolysis test

The fresh retrieved isolates were streaked on the surface of Sheep Blood Agar plates and incubated at 30ºC for 48 to 72 hours. After incubation, the bacterial colonies were observed for the presence of clear zone around colonies. Hemolysis activity is indicated by the presence of clear zones around the colony (Carrillo *et al*., 1996).

Oil spreading technique

Oil spreading assay was done as per the method described by Morikawa*et al*. (1993). In this method, culture supernatant was prepared by inoculating a loop full of isolated culture in 10ml nutrient broth and incubated at 30ºC for 24 hours. From the culture broth, 1ml was taken and centrifuged at 10,000rpm in a centrifuge and the supernatant was collected. 30 ml of distilled water was taken in a pre-dried clean petri plate. Then, 1.0 ml of coconut oil/ gingely oil were added to the center of the plate containing distilled water. Then 20µl of the supernatant of the isolated cultures was added. The biosurfactant producing organism could displace the oil and spread in the water. If the oil displaces and clear zone forms then it shows the presence of biosurfactant. The displaced diameter is measured after 30 second. One biosurfactant unit (BS unit) was defined as the amount of surfactant forming 1 cm^2 of oil displaced area (Thaniyavam*et al*., 2003).

Complementary screening

Emulsification index (F24)

The emulsification index (E24) of culture samples will be determined by adding 2 ml of diesel to the same amount of culture media obtained above, mixing with a vortex for 2 min and leaving to stand for 24 h.

The E24 index was given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (Desai and Banat, 1997).

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Quantitative screening of biosurfactant producing organisms

Inoculation and incubation biosurfactant producing selected isolates

A single colony of each selected isolate were inoculated into 10 ml nutrient broth and incubated at 30°C overnight at 160 rpm. The overnight culture was centrifuged for 10 min at 10000 rpm. The cell pellet was washed twice in phosphate buffer saline (pH 7.6) and re-suspended in BH medium until OD at 600 nm was equivalent to 1.1ml of bacterial inoculum (1 OD at 600 nm equivalent). This inoculum was added to 250 ml Erlenmeyer flasks containing 100 ml of liquid BH medium supplemented with 1% carbon source (diesel). Uninoculated control flasks with 1% (v/v) diesel was also incubated in parallel to monitor abiotic losses of the substrate. The culture flasks were incubated at 30°C at 160 rpm for 20 days. After 20 days, growth pattern was determined and the biosurfactant was extracted.

Determination of growth pattern by measuring total viable counts (CFU/ml)

The growth patterns were obtained by measuring total viable counts (cfu/ml) of the isolates, which was enumerated by the spread plate technique using the nutrient agar plates at 30°C for 36 h. All experiments were performed in triplicate.

Extraction of biosurfactant

After incubation of each selected isolates, the supernatant was collected and the pH of the supernatant was adjusted to 2, using 1.0 M H_2SO_4. Then equal volume of chloroform : methanol (2 : 1) was added. This mixture was shake well for mixing and kept for overnight for evaporation. Finally white colored sediment was obtained as biosurfactant. The finally obtained sediment was poured in a pre-weighed petri plate and was then placed on the hot air oven for drying at 100^0C for 30 minutes. After drying, the final weight of the petri plate was measured. By relating the initial and final weight, the dry weight of the biosurfactant was calculated.

Final screening of bio surface producing isolates

From the results obtained from the quantitative screening of biosurfactant producers, four predominant and effective biosurfactant producing isolates were selected. The isolates were streaked on nutrient agar plates, nutrient agar slants, incubated at 30ºC for 24 hours and were identified by morphological, biochemical tests as well as phylogenetic analysis.

Identification of bio surface producing isolates

Morphological and biochemical tests such as, gram staining, motility test, spore staining, indole production, methyl red test, voges-proskauer (vp) test, citrate utilization, nitrate reduction, urease test, oxidase test, catalase test and hydrogen sulfide production test were analyzed following the directions given by Bergey's manual of systematic bacteriology (Locci, 1989). The identification of the isolates were performed by 16S rRNA sequencing and the construction of phylogenetic tree was carried out by BLAST with the nrdatabase of NCBI genbank database. The evolution tree was presented in the form of rectangular cladogram. After identification, the sequences were submitted to Bankit (GenBank) to obtain valid accession numbers.

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Results and Discussion

Enumeration of diesel degrading bacteria

Recent studies have identified bacteria from more than 79 genera that are capable of degrading petroleum hydrocarbons (Tremblay *et al*., 2017). many studies have revealed that there is a large number of hydrocarbon-degrading bacteria in oil-rich environments, such as oil spill areas and oil reservoirs (Hazen et al., 2010; Yang et al., 2015), and that their abundance and quantity are closely related to the types of petroleum hydrocarbons and the surrounding environmental factors (Fuentes *et al*., 2015; Varjani and Gnansounou, 2017). Many normal and extreme bacterial species have been isolated and utilized as biodegraders for dealing with petroleum hydrocarbons. In the present study, bactreria from selected sample soils were enumerated in nutrient agar for total viable count and in Bushnell Haas agar for diesel degrading bacteria. The results are shown in table 1. Highest number of colony forming unit (28 $x10^6$) was found in the soil sample collected from station 1 and the minimum number of colony forming unit was found at station 8 (13 $x10^6$). In the meantime, the colony forming unit of diesel degrading organisms were found to be maximum at station 3 ($78x10^4$) and the minimum number of diesel degrading organisms were found in station 8 ($4x10^4$).

Table 1Enemeration of total viable bacteria and diesel degrading bacteria

S.no	Stations	CFU/ml in Nutrient Agar	CFU/ml in Bushnell Haas Agar
1	Station 1	28 $x10^6$	$8x10^4$
2	Station 2	14 $x10^6$	$60x10^4$
3	Station 3	19 $x10^6$	$78x10^4$
4	Station 4	25 $x10^6$	$53x10^4$
5	Station 5	25 $x10^6$	$58x10^4$
6	Station 6	16 $x10^6$	$10x10^4$
7	Station 7	21 $x10^6$	$13x10^4$
8	Station 8	13 $x10^6$	$4x10^4$

Screening of biosurfactant producing organisms

Primary Screening

Selected bacterial isolates were screened primarily for biosurfactant production. In 1996, Carrillo *et al.* (1996) found the blood agar plate could be used to screen biosurfactant producing bacteria, and this method was widely applied to various biosurfactant producing bacteria screenings.In blood haemolysis test, 9 colonies such as S2K1. S2K2, S3K3, S4K2, S4K3, S4K5, S6K1, S7K1 and S7K2 showed β haemolysis and the other strains such as S1K1, S3K1, S3K2, S4K1, S4K4, S5K1 and S5K2 produces α haemolysis.As biosurfactants have the ability of oil displacement, if there are biosurfactants produced, it will form an oil spreading circle on the oil film (Joshi *et al*., 2008). The oil spreading results of the present study showed high activity by S4K5 and S2K1. Moderate activity was observed in S4K2. The isolates S1K1, S3K1, S3K2, S4K1, S5K1 and S8K1 showed negative results. The results were given in table 2.

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Table 2 Primary screening of biosurfactant producing strains

S.no	Bacterial isolates	Blood haemolysis test	Oil spreading test
1	S1K1	α	-
2	S2K1	β	+++
3	S2K2	β	+
4	S3K1	α	-
5	S3K2	α	-
6	S3K3	β	+
7	S4K1	α	-
8	S4K2	β	++
9	S4K3	β	+
10	S4K4	α	+
11	S4K5	β	+++
12	S5K1	α	-
13	S5K2	α	+
14	S6K1	β	+
15	S7K1	β	+
16	S7K2	β	+
17	S8K1	α	-

- no activity; + low activity; ++ moderate activity; +++ high activity

Complementary screening

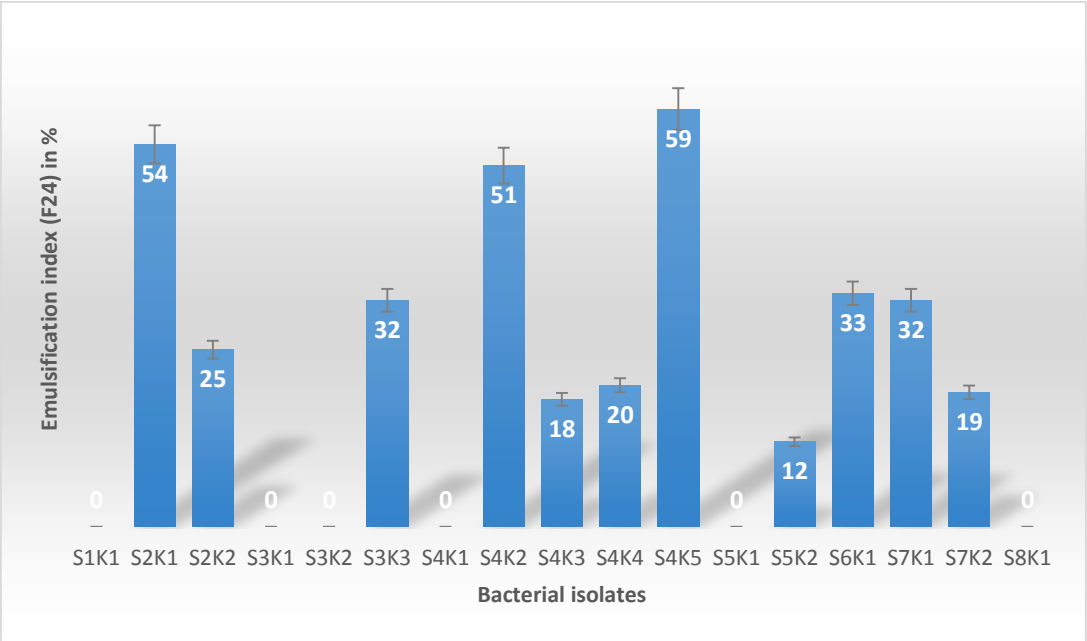

Figure 1 Complementary screening of biosurfactant producing strains

Emulsifying activity is an important property for the performance of biosurfactants. E_{24} is a parameter to measure the emulsifying ability. Cooper (1986) reported that the surface tension could be reduced to less than 40 mN m^{-1}, which might be a promising biosurfactant producer. The strain S4K5 showed the highest emulsification index (E24) ie.,

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59% followed by strain S2K1 (54%) and S4K2 (51%). The minimum emulsification index (E24) was found in S5K2 (12%). From the above results, it could be concluded that the strains S2K1, S4K2 and S4K5 were the best biosurfactant producers from all the isolated strains. The result was given in figure 1.

Quantitative screening of biosurfactant producing organisms

To determine the best isolates which produce maximum biosurfactant, quantitative screening was done by checking their ability for the production of biomass and biosurfactant. The results were given in figure 2.

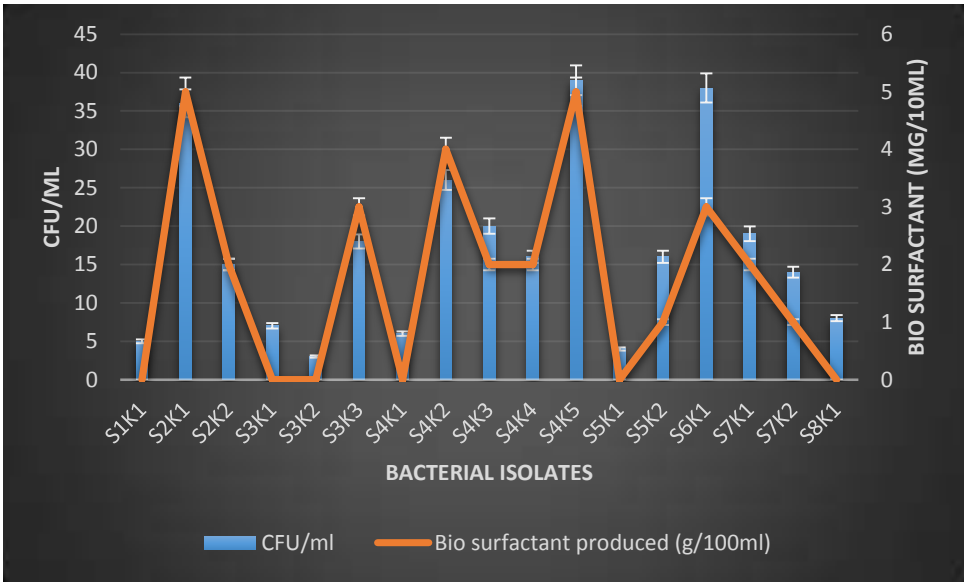

Figure 2 Quantitative screening of colony forming unit (CFU/ml) and biosurfactant production (mg/10ml) by selected isolates

The colony forming unit (CFU/ml) of the isolates were counted in the dilution 10^{-4}. The biosurfactant was extracted from 100ml of fermentation broth. The weight of the biosurfactant was measured in grams. Maximum colony forming unit was obtained in S4K5 and minimum colony forming unit was found in the strain S3K2. In the meanwhile, maximum biosurfactant was produced by S2K1 and S4K5 (5mg/10ml) followed by S4K2 (4mg/10ml). The bacterial isolates S1K1, S3K1, S3K2, S4K1, S5K1 and S8K1 does not produce any biosurfactant (Figure 2).

Final screening of biosurfactant producing isolates

Fardami*et al*. (2022) stated that rhamnolipids are the best studied and industrially important class of biosurfactants mostly obtained from Gram negative bacteria like *Enterobacter cloacae* AYF1. This agreed with the findings of Gamez et al. (2017), who screened and characterized biosurfactant producing bacteria isolated from soil contaminated with oily waste.Ample reports suggest that biosurfactant-producing Bacillus species can be obtained from hydrocarbon-contaminated habitats (Putri and Hertad, 2015; Barakat*et al*., 2017). John *et al*. (2021) screened Lysinibacillusfusiformis from automobile-mechanic-workshop oil contaminated sites, for potential for biosurfactant production. In this concern, three dominant and effective biosurfactant producing isolates S2K1, S4K2 and S4K5 were chosen based on

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the findings of the quantitative screening of biosurfactant producers. The isolates were streaked on nutrient agar plates, nutrient agar slants, and incubated at 30ºC for 24 hours before being identified using morphological, biochemical, and phylogenetic assays.

Morphological and biochemical characterization

The morphological and biochemical tests of the selected strains S2K1, S4K2 and S4K5 were given in table 3. The strain S2K1 was gram negative, motile, rod shaped bacteria. The organism does not produce any spores and showed negative results to indole production test, methyl red test, urease test, oxidase test and H_2S production test. It showed positive reaction to vogesproskauer test, nitrate reduction test and catalase test. The strains S4K2 and S4K5 shared the biochemical results identical. They are gram positive motile rods, produces spores, and were negative to indole production test, methyl red test, vogesproskauer test, nitrate reduction test, and H_2S production test. They showed positive results to the biochemical tests such as citrate utilization test, urease test, oxidase test and catalase test.

Table 3 Morphological and biochemical characterization of selected isolates S2K1, S4K2 and S4K5

S.no	Morphological and Biochemical test	S2K1	S4K2	S4K5
1	Gram staining	Negative rod	Positive rod	Positive rod
2	Motility	Motile	Motile	Motile
3	Spore staining	Negative	Positive	Positive
4	Indole production test	Negative	Negative	Negative
5	Methyl red test	Negative	Negative	Negative
6	VogesProskauer test	Positive	Negative	Negative
7	Citrate utilization test	Positive	Positive	Positive
8	Nitrate reduction test	Positive	Negative	Negative
9	Urease test	Negative	Positive	Positive
10	Oxidase test	Negative	Positive	Positive
11	Catalase test	Positive	Positive	Positive
12	H_2S production test	Negative	Negative	Negative

Identification of the isolates by 16S rRNA sequencing

The 16S rDNA of the selected strains (S2K1, S4K2 and S4K5) was partially sequenced to gather more taxonomic information. To identify the bacteria, a specific portion of gene from the genomic DNA was amplified and only that gene was sequenced. Table 4 contains the sequences of the strains S2K1, S4K2 and S4K5.

Table 416S rRNA sequencing of isolated strains S2K1, S4K2 and S4K5

Isolated strains	16S rRNA sequencing
S2K1	Ccaggggataactactggaaacggtagctaataccgcataacgtcgcaagaccaaagagggg gaccttcgggcctcttgccatcaaatgtgcccagatgggattagctagtaggtggggtaacggctc acctaggcgacgatccctagctggtctgaaaggatgaccagccacactggaactgagacacggt ccagactcctacgggaggcagcagtggggaatattgcacaatgggcgcaagcctgatgcagcc

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	atgccgcgtgtatgaagaaggccttcgggttgtaaagtactttcagcggggaggaaggtgttgtgg ttaataaccgcatcaattgacgttacccgcaaaagaagcaccggctaactccgtgccagcacccg cggtaatacggagggtgcaagcgttaatcggaattactgggcgtaaagcgcacgcaggcggtct gtcaagtcggatgtgaaagtccccgggctcaacctgggaactgcattcgaaagtggcaggctgg agtctttgtacagggggtacaattcct
S4K2	Acgttagcggcggacgggtgagtaacacgtgggcaacctgccctatagtttgggataactccgg gaaaccggggctaataccgaataatctttttgcttcatggttcaaaattgaaagacggtttcggctgt cacttttggatggacccgcgtcgcattagctagttggtgaggtaacggctcaccaaggcgacgat gcgtagccgacctgagagggtgatcggccacactgggactgagacacggcccagactcctacg ggaggcagcagtagggaatcttccacaatggacgaaagcctgatggagcaacgccgcgtgagt gaagaaggttttcggatcgtaaaactctgttgtaa
S4K5	Ttagcggcggacgggtgagtaacacgtgggcaacctgccctatagattgggataactccggga aaccggggctaataccgaataatctcttttgcttcatggttcaaaattgaaagacggtttcggctgtc gcttttggatggacccgcggcgcattagctagttggtgaggtaacggctcaccaaggcgacgatg cgtagccgacctgagagggtgatcggccacactgggactgagacacggcccagactcctacgg gaggcagcagtagggaatcttccacaatggga

The 16S rRNA sequence of isolated strains S2K1, S4K2 and S4K5 was subjected to alignment using Basic Local Alignment Search Tool (BLAST) inorder to find the regions of local similarity between the sequences. With the help of BLAST, the sequences were aligned and phylogenetic trees were created for the isolates S2K1, S4K2 and S4K5 using BLAST pairwise alignments. The phylogenetic tree for S2K1, S4K2 and S4K5 were drawn as rectangular cladogram and were given in Figure 3,4 and 5. The IcI|Query_4905 indicates the query search result for S2K1, IcI|Query_4651 indicates S4K2 and finaly, IcI|Query_26177 indicates S4K5.

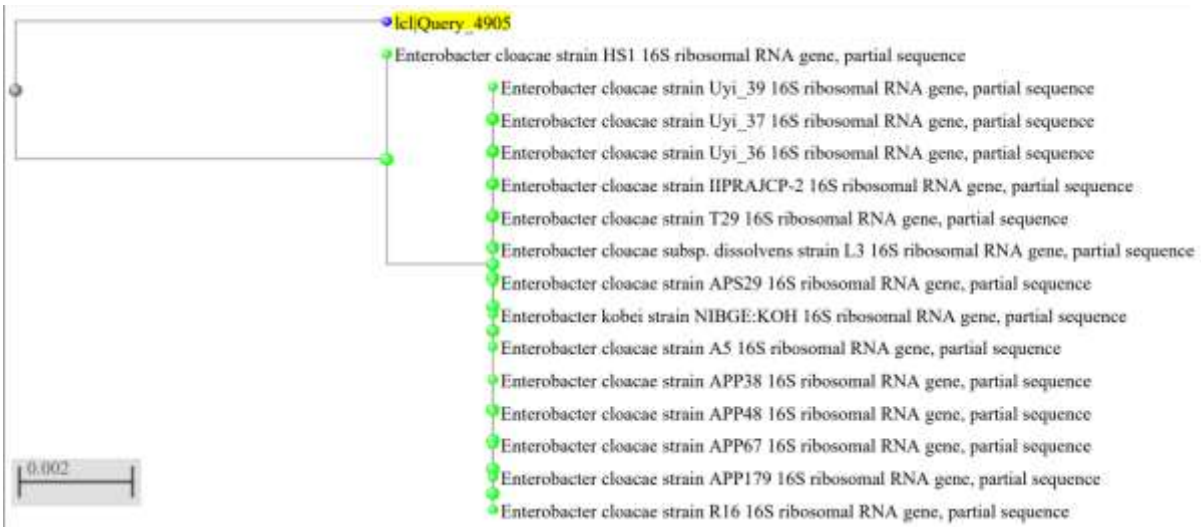

Figure 3 Phylogenetic tree (Rectangular Cladogram) of isolate S2K1

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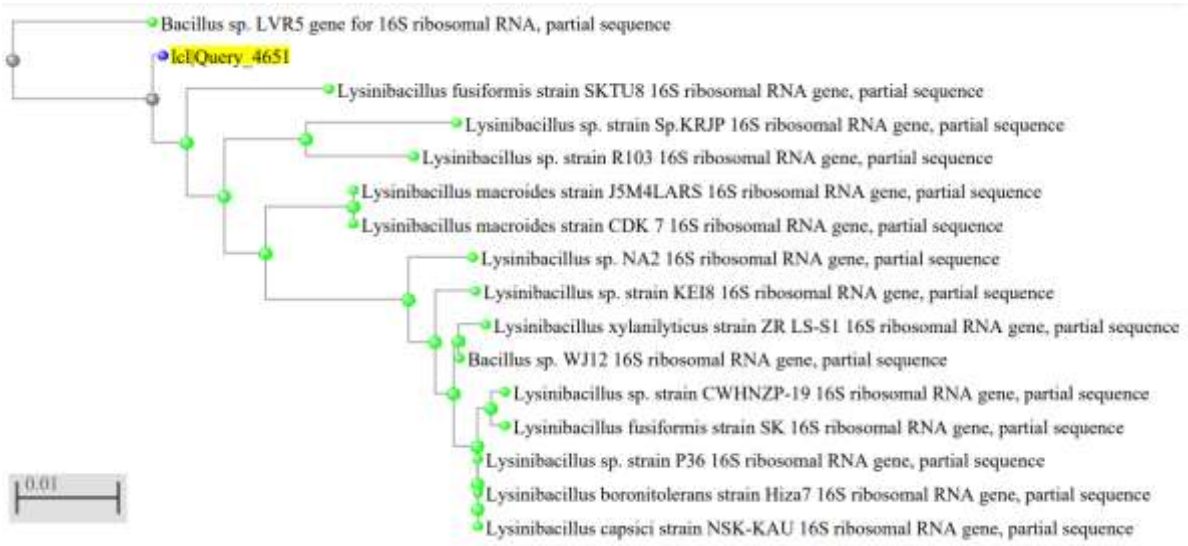

Figure 4 Phylogenetic tree (Rectangular Cladogram) of isolate S4K2

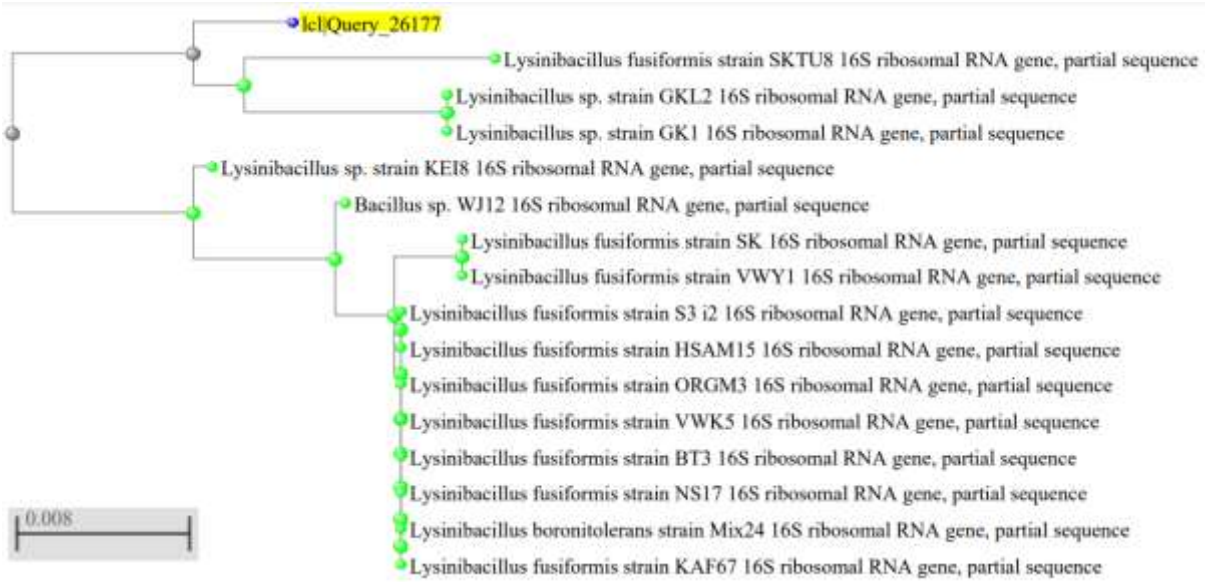

Figure 5 Phylogenetic tree (Rectangular Cladogram) of isolate S4K5

Haritash and Kaushik (2009) demonstrated that microorganisms are the main degraders of hydrocarbons.Based on the nucleotide homology and phylogenetic analysis, the biosurfactant producing strain S2K1 was identified and assigned as *Enterobacter cloacae* strain GKE1(GenBank Accession Number: OP136122.1), strain S4K2 as *Lysinibacillus*sp. strain GKL2 (GenBank Accession Number: OP133003.1), and the strain S4K5 as *Lysinibacillusfusiformis* strain GKL1 (GenBank Accession Number: OP133000.1).

Conclusion

A wide range of techniques have been developed for screening strains that produce biosurfactants as a result of interest in biosurfactants. A effective screening requires a combination of many methods because each has benefits and drawbacks.It's possible that

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different new production strains or new biosurfactants will be discovered in the near future by applying these quick screening techniques and screening a lot of isolates or huge culture collections. Accordingly, the financial barrier of biosurfactants might eventually be overcame if new production strains become accessible.

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