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Isolation of Effective Hydrocarbon Degrading Bacteria from Hydrocarbon Polluted Sites

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ABSTRACT
Petroleum hydrocarbon contamination is one of the highly dangerous pollution causing hazard factors known today. Bio surfactants are surface active molecule synthesized by many kinds of microorganisms. The purpose of this study was to isolate and identify the hydrocarbon degrading potential biosurfatant producers associated with polluted environments like petrol bunks, four and two wheelers workshop areas at Namakkal district. 25 different isolates were isolated from hydrocarbon polluted sites and subjected to screening for bio surfactant activity by different methods blue agar, emulsification index, drops collapse, oil spreading assay methods and 7 isolates (Bacillus cereus, Pseudomonas stutzeri, Bacillus subtilis, Bacillus subtilis, Pseudomonas putida, Pseudomonas aeruginosa, Citrobacter freundii) and showed bio surfactant activity on all above mentioned standard methods. The isolates were sequenced and submitted to NCBI. The greater potential activity was detected by gravimetric analysis and phenol: sulphuric acid assay. However it was identified that C. freundii as most effective among the seven selected bacterial isolates for hydrocarbon degradation and biosurfactant activity
Keywords:*Bio surfactants, hydrocarbon degradation, pollution*

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INTRODUCTION

Hydrocarbons contamination has become one of the major environmental problem faced globally. It is hazardous to the environment as well as to life forms. Million liters of petroleum products enter into the environment both natural and anthropogenic sources every year (1). In India, Chennai, 2017 40 tonnes of oil sludge and 27 tonnes of oil was got mixed with the water in the north of Chennai harbour(The Hindustan times, 2017). Improper management and disposal of oily sludge wastes causes environmental pollution, particularly to the soil and groundwater systems, due to their low volatility and aqueous solubility. Approximately five million tons of crude oil and refined oil enter into the environment each year as a result of anthropogenic sources such as oil spills (2). The technologies used for soil remediation includes mechanical, burying, evaporation, dispersion, and washing but these methods are not successful, because these can lead to secondary pollutant problems and expensive also. The remediation of hydrocarbon contaminated soil remains a challenge (3). Biosurfactants are amphiphilic biological compounds produced on the microbial cell surface (4,5,6). The microorganisms have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity, and lower CMC. These properties have led to several biosurfactant applications in the food, cosmetic and pharmaceutical industries (7). The biosurfactants such as glycolipids,

lipopolysaccharides, oligosaccharides and lipopeptides are produced by microorganisms (8,9). Many microflora were reported from petroleum contaminated soil sample or PAHc related sites and as well as they were degrade including *Micrococcus, Candida, Brachybacterium, Mycobacterium, Acinetobacter, Enterobacter, Rhodococcus, Flavobacterium, Rhodococcus* and *Pseudomonas* species etc., Strains of *Bacillus* species from a petroleum contaminated soil sample might be efficient hydrocarbon degraders (10,11). There have been very few studies so far that evaluated the presence of natural, indigenous biosurfactant-producing microbes in this Namakkal region. For efficient detection of potential biosurfactant producers, a combination of various screening methods is required, which were successfully evaluated in the present study.

MATERIAL AND METHODS

Soil samples were collected from oil contaminated sites in Namakkal area and screened for bacterial isolates from oil contaminated soil. Biosurfactants were observed by Blue agar plate method (12), Blood agar hemolysis (13), Drop collapse test (14) and modified by (15). The identified isolates were morphologically and biochemically characterized using Bergy's Mannual of classification. Oil spreading assay by (16). The emulsification index (E24) was measured using the method described by (17) and Screening was done by gravimetric analysis (18).

Residual crude oil weight = Weight of beaker containing extracted crude oil Weight of empty beaker.

Degraded crude oil amount= Weight of crude oil added in the media Weight of residual crude oil.

Confirmatory method by Phenol: sulfuric acid method. The efficient isolates were sequenced and they were submitted in NCBI.

RESULTS

The soil sample collected from different sites such as two wheeler, four wheeler workshop and petrol bunks showed that petrol bunks has 36% of hydrocarbon degrading bacteria (Figure 1). Screening of hydrocarbon degrading bacterial isolates for biosurfactant properties was done using four different tests such as Blue agar, Blood hemolysis, drop collapse and oil spreading test in which all the test showed positive results for the isolates 6, 10, 14, 16, 18, 20 and 25 (Table 1).

Morphological characterization of selective bacterial isolates showed that gram and spore staining was positive for Isolates 6, 14, 18 and negative for isolate 10, 16, 20, and 25. The isolates 6, 14, 18 are in the shape of radici form, surface was rough, color was milky white, transparency was translucent, isolates 10, 16, 25 are in the shape of round form, surface was smooth, color was bluish-green, transparency was opaque, isolate 16 are in the shape of round form, surface was rough, color was bluish-green, transparency was opaque, isolate 25 are in the shape of round form, surface was rough, color was bluish green, transparency was opaque and isolate 20 was in the shape of circular, surface was smooth, color was milky white and transparency was opaque. All the isolates were morphologically rod in shape (Table 2).

Biochemical tests for isolate 6 was positive for methyl red, catalase, oxidase, starch, lactose and negative for indole, voges-prosakauer, citrate, urease, alkaline, hydrogen sulphide and identified as *Bacillus sp.* Isolate 10 was positive for methyl red, catalase, citrate, oxidase, starch, alkaline and negative for indole, voges-prosakauer, urease, lactose, hydrogen sulphide and identified as *Pseudomonas sp.* Isolate 14 was positive for voges-prosakauer, catalase, citrate, oxidase, starch, lactose, alkaline, hydrogen sulphide and negative for indole, methyl red, urease and identified as *Bacillus sp.* Isolate 16 was positive for indole, catalase, citrate, urease, oxidase, starch, lactose, and negative for methyl red, voges-prosakauer, hydrogen sulphide and identified as *Pseudomonas sp.* Isolate 18 was positive for methyl red, catalase, citrate, starch, alkaline and negative for indole, voges-prosakauer, urease, oxidase, lactose, hydrogen sulphide and identified as *Bacillus sp.* Isolate 20 was positive for methyl red, catalase, citrate, urease, oxidase, starch, lactose, alkaline, hydrogen sulphide and negative for indole, voges-prosakauer, and identified as C*itrobacter sp.* Isolate 25 was positive for indole, voges-prosakauer, catalase, citrate, urease, oxidase, starch, and negative for methyl red, lactose, hydrogen sulphide and identified as *Pseudomonas sp*(Table 3).

Emulsification percentage of selective hydrocarbon degrading bacterial isolates showed that isolate 20 has high emulsification percentage with 26.33% followed by isolate 14 with 23.83%, isolate 25 has 21.76% and the isolate 6 has lowest emulsification percentage(Figure 2).

Crude oil degradation of selective hydrocarbon degrading bacterial isolates showed that isolate 20 has 0.647gm, followed by isolate 14 with 0.639gm, isolate 25 with 0.601gm and the isolate 10 has least degradation activity (Figure 3).

Conformation of hydrocarbon degrading potential of selective bacterial isolates by Phenol: sulphuric acid method by colour intensity showed that isolate 14 and 20 has high degradation potential next to that is isolate 16 and 25 (Table 4).

The isolates were sequenced and submitted to NCBI, the following are the isolates with name of the organism, blast tree and their accession number, *Bacillus cereus* strain I6 with accession number MH702447 *Pseudomonas stutzeri* strain I10 with accession number MH685402, *Bacillus subtilis* strain I14 with accession number MH685403, *Bacillus subtilis* strain I16 MH685404, *Pseudomonas putida* strain I18 with accession number MH685405, *Citrobacter freundii* strain I20 with accession numberMH685407 (Figure 5-11).

Figure 1. Composition of hydrocarbon degrading bacteria collected from different sites

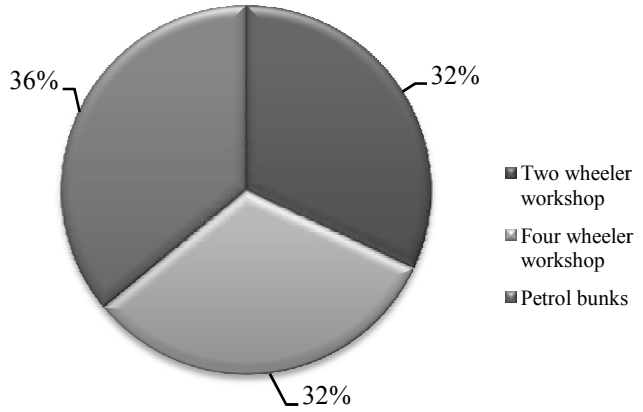


Table 1. Screening of hydrocarbon degrading bacterial isolates for biosurfactant properties

S. No.	Isolates																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Blue agar					■	■		■		■				■		■		■	■	■					■
Blood hemolysis	■				■	■			■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■
Drop collapse test	■	■	■	■		■				■	■			■		■	■	■		■	■		■		■
Oil spreading test	■	■	■	■		■		■	■	■				■		■	■	■	■	■	■				■

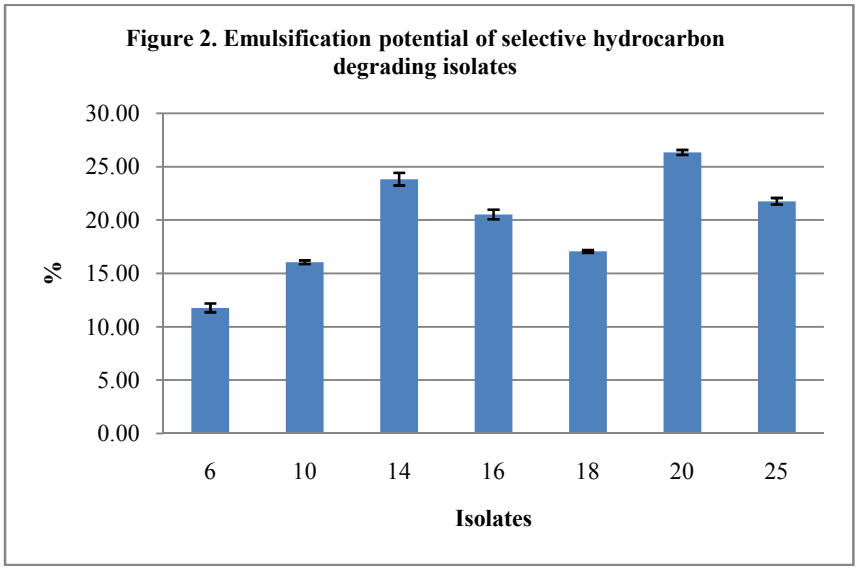
■ Positive; □ Negative

Table 2. Morphological characterization of selective bacterial isolates from hydrocarbon polluted soils

S. No.	Isolates	Gram staining	Spore	Shape	Colony morphology			Indiv. Morp.
					Surface	Color	Transparency	
1	6	+	+	Radiciform	Rough	Milky white	Translucent	Rod
2	10	-	-	Round	Smooth	Bluish green	Opaque	Rod
3	14	+	+	Radiciform	Rough	Milky white	Translucent	Rod
4	16	-	-	Round	Rough	Bluish green	Opaque	Rod
5	18	+	+	Radiciform	Rough	Milky white	Translucent	Rod
6	20	-	-	Circular	Smooth	Milky white	Opaque	Rod
7	25	-	-	Round	Rough	Bluish green	Opaque	Rod

Table 3. Biochemical characterization of selective bacterial isolates from hydrocarbon polluted soils and identified genera

S. No.	Isolates	Ind.	MR	Vp	Cat	Cit.	Ure.	Oxid.	Starch	Lact.	Alk.	H_2S	Species
1	6	-	+	-	+	-	-	+	+	+	-	-	*Bacillus sp.*
2	10	-	+	-	+	+	-	+	+	-	+	-	*Pseudomonas sp.*
3	14	-	-	+	+	+	-	+	+	+	+	+	*Bacillus sp.*
4	16	+	-	-	+	+	+	+	-	-	NA	-	*Pseudomonas sp.*
5	18	-	+	+	+	+	-	-	+	-	+	-	*Bacillus sp.*
6	20	-	+	-	+	+	+/-	+	+	+	+	+	*Citrobacter sp.*
7	25	+	-	+	+	+	+	+	+	-	NA	-	*Pseudomonas sp.*



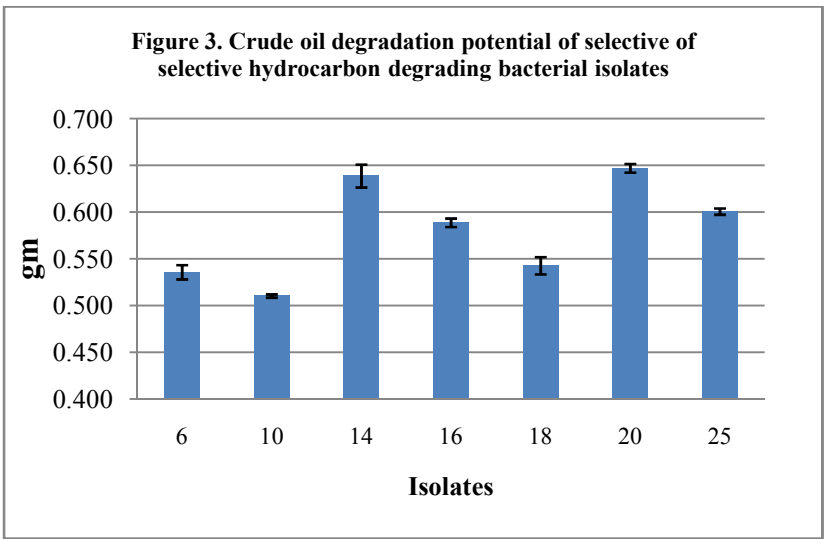


Table 4. Confirmation of hydrocarbon degrading potential of selective bacterial isolates by Phenol: sulfuric acid method.

S. No.	Isolates name	Colour intensity
1.	Isolate 6	+
2.	Isolate 10	+
3.	Isolate 14	+++
4.	Isolate 16	++
5.	Isolate 18	+
6.	Isolate 20	+++
7.	Isolate 25	++

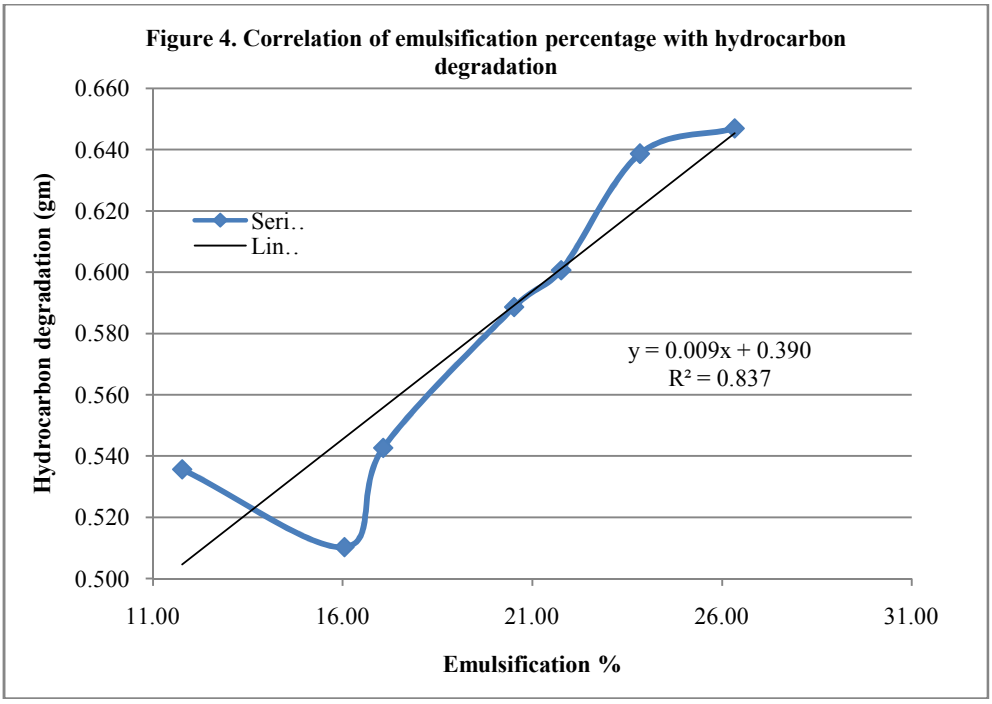


Figure 5. Phylogenetic analysis of *Bacillus subtilis* strain I6

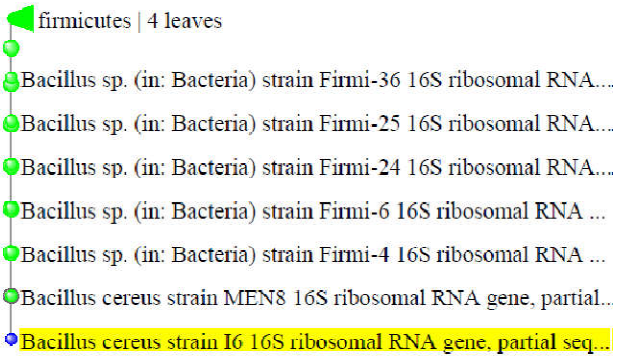


Figure 6. Phylogenetic analysis of *Bacillus subtilis* strain I14

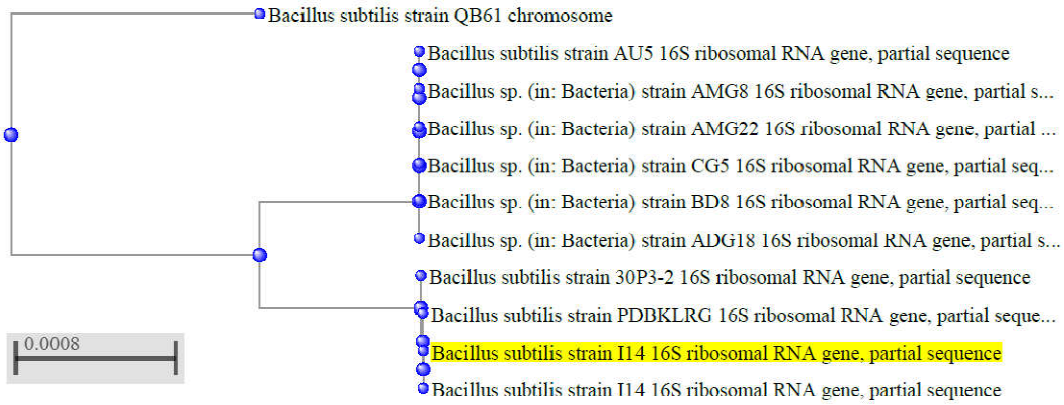


Figure 7. Phylogenetic analysis of *Pseudomonas stutzeri* strain I10

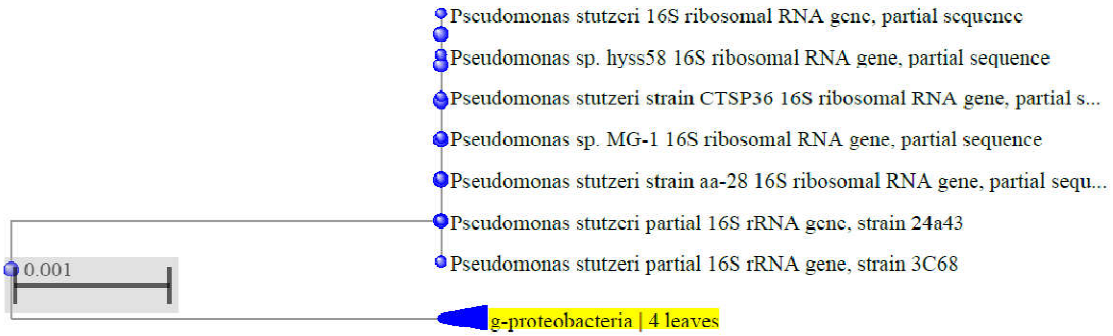


Figure 8. Phylogenetic analysis of *Bacillus subtilis* strain I16

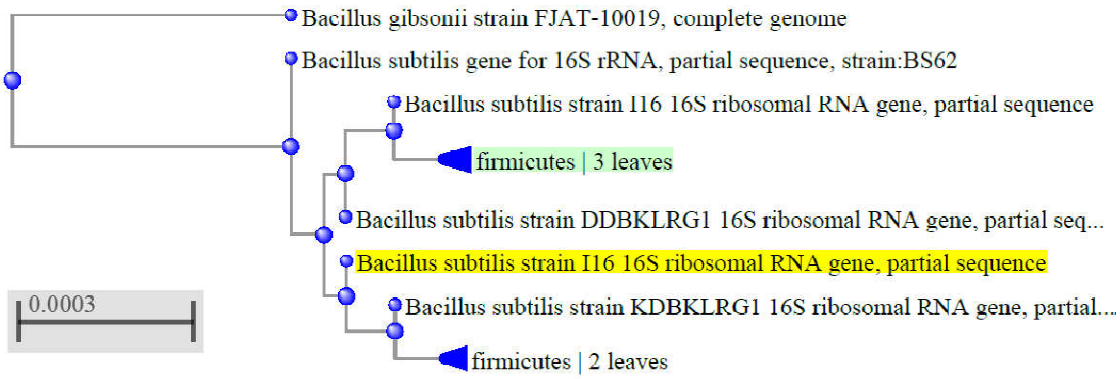


Figure 9. Phylogenetic analysis of*Pseudomonas putida* strain I18

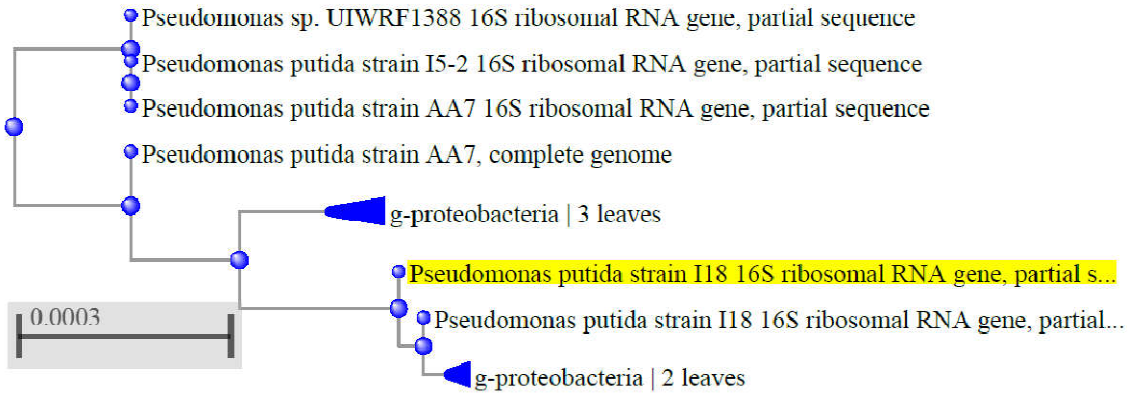


Figure 10. Phylogenetic analysis of *Citrobacter freundii* strain I20

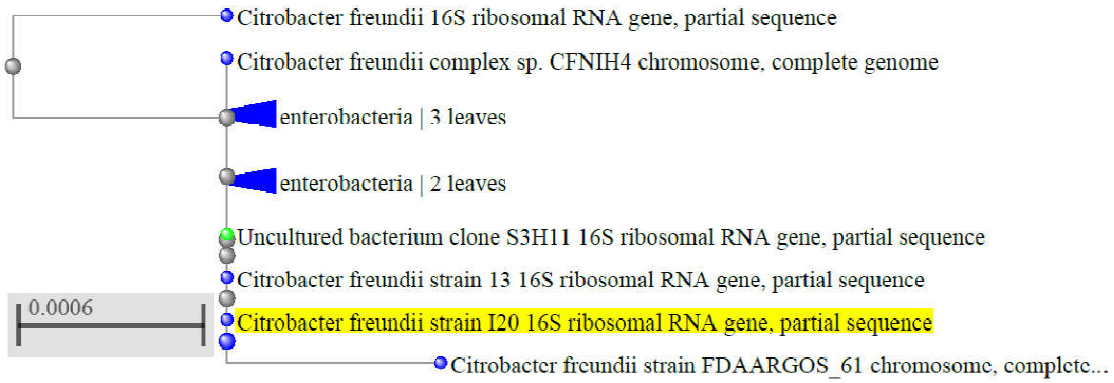
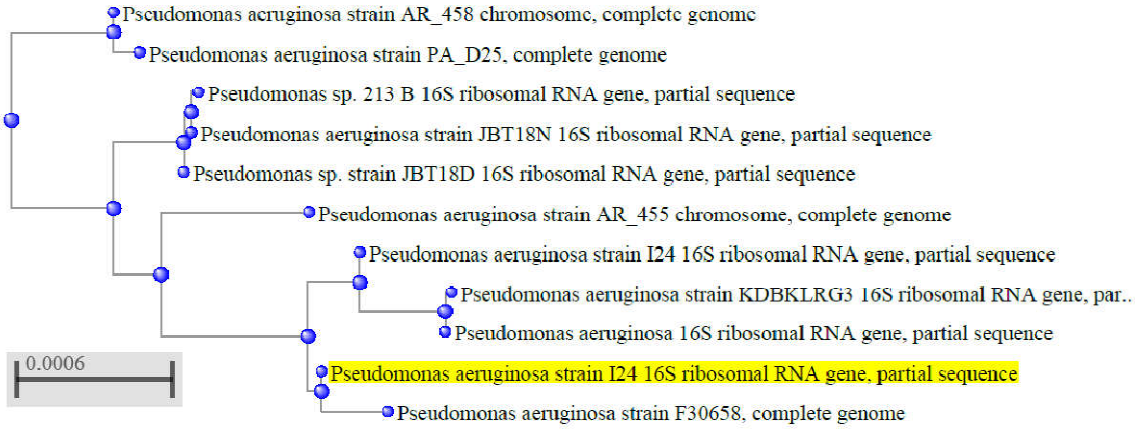


Figure 11. Phylogenetic analysis of *Pseudomonas aeruginosa* strain I24



DISCUSSION

Oil spill due to accidents have become common phenomena which cause ecological and social catastrophes in today's context (19, 20, 21). Environmental contamination of Hydrocarbon contamination caused extensive damage to the soil and water forms major crisis to plants and animals. Poly Aromatic Hydrocarbons (PAHc) in the hydrocarbon contaminant are the recalcitrant in nature have high affinity to soil particulate matter (22, 23, 24).

Bioremediation of hydrocarbon polluted sites are effective with microorganisms, such as bacteria, microalgae, and fungi (25, 26, 27). Number of Bacteria were identified in this process of hydrocarbon degradation since they break the dead materials into organic matter and nutrients(28, 29). Carbon and Hydrogen are the major components of Petroleum

products which are used by many microorganisms as a sole source of carbon energy and these isolates are widely present in soil. In this present study, 25 isolates were observed from 25 oil contaminated soils, which were able to utilize crude oil as a sole source of carbon and energy.

In the recent past biosurfactant property of bacteria was identified as one of the important property that supports biodegradation of pollutants especially hydrocarbons (29, 30, 31, 32, 33). Biosurfactants vary in their chemical compositions and forms fatty acids, lipoproteins, phospholipids, glycolipids and neutral lipids which are amphiphilic nature with hydrophilic and hydrophobic surface area (34, 35). The biosurfactants with their hydrophobic nature increases the surface area of hydrocarbons to water and leads to emulsification and facilitates the increased microbial activity towards solubilization and degradation of hydrocarbon in water (36).

Though numbers of methods are available to screen the biosurfactant property of bacteria most of them are based on their physical modifications (37, 38). The present study used specific screening method Colorimetric CTAB agar assay for qualitative and quantitative analysis of 25 isolates identified as hydrocarbon degrading bacteria. Among the 25 isolates, 10 (40%) isolates were positive. Haemolytic activity is one another methods for tracing the microorganisms with biosurfactant activity (39) out of 25 isolates 19 (76%) isolates were found to be positive, in which eight isolates were negative with CTAB agar assay positive reaction of these isolates may be due to presence of β hemolysins (40), however isolate 8 which was positive in CTAB agar assay is negative with hemolytic activity this is due its poor biosurfactant activity (41).

Drop collapse test is a easier test which could be conduct rapidly determine the presence of surfactants. In the presence of surfactants the drops of culture collapse or spread indicating their hydrocarbon degradation. In the present study 15 (60%) isolates revealed positive in this isolates 2, 3, 4, 21 were positive only in drop collapse test which did not appear positive in the blue agar and hemolysis test these may be due to their weaker biosurfactant production, isolate 1, 17 and 23 were positive in both hemolysis and drop collapse test due to their low biosurfactant production. It is to be noted that isolate 9, 12, 13, 15 and 22 were positive only in hemolysis test confirms their β hemolysins presence.

Oil spreading test which also ensures biosurfactant production of isolated bacteria among 25 isolates 16 (64%) isolates showed positive results in which isolates 1 and 17 showed positive results in haemolysis, drop collapse test and oil spreading test which reveals weak biosurfactant production, isolates 2, 3, 4 and 21 showed positive results only for drop collapse test and oils spreading test which is due to low biosurfactant production further 8, 9 showed positive for only for oils spreading test due to their very low biosurfactant production, isolates 6, 10, 14, 16, 18, 20 and 25 showed positive in all the four tests due to their strong biosurfactant production potential.

All the seven effective biosurfactant producing bacteria were rod shaped in which three isolates (6, 14 and 18) were gram positive spore forming and four gram negative isolates (10, 16, 20 and 25) hence both gram negative and positive organism produce biosurfactants. Four isolates (6, 10, 14, 18 and 20) were indole negative only two isolates (16 and 20) were indole positive whereas all of them were catalase positive, most of them are citrate, oxidase and starch were positive except isolates 6, 18 and 16 respectively shows their effective enzymes systems in hydrocarbon production. 16S rRNA sequencing and identification through sequence blast showed that seven isolates were submitted to NCBI, the following are the isolates with name of the organism and their accession number, *Bacillus cereus* strain I6 with accession number MH702447 *Pseudomonas stutzeri* strain I10 with accession number MH685402, *Bacillus subtilis* strain I14 with accession number MH685403, *Bacillus subtilis* strain I16 MH685404, *Pseudomonas putida* strain I18 with accession number MH685405, *Citrobacter freundii* strain I20 with accession number. Earlier studies identified *Pseudomonas* sp. by (42) and (43, 44) and *Bacillus* sp. by (45) as effective biosurfactant bacterial species.

One of the important biosurfactant is their emulsification capacity where fat globules are broken down into tiny droplets through which it increases the total surface area of the hydrocarbon degradation activity. The emulsification capacity of the seven isolates with effective biosurfactant production showed within the range of 16 to 26% and crude oil degradation ranged from 510gm to 647gm with perfect correlation was observed between emulsification percentage and hydrocarbon degradation with R^2 value 0.837. Among seven

bacterial isolates, isolates 14 and isolate 20 showed effective phenol sulfuric acid confirmation which are *Bacillus subtilis* and *Citrobacter freundii* respectively.

CONCLUSION

It is evident that from the present study hydrocarbon degrading organisms are ubiquitous in the environment and they can be identified through blue agar test, blood hemolysis test drop collapse test and oil spreading test. Such identified bacterial species are effective biosurfactant producing organism and they also have effective emulsification property which in turn correlates with hydrocarbon degradation. It has also been shown that *Bacillus subtilis* and *Citrobacter freundii* species are most effective strains isolated from hydrocarbon contaminated soil. Further studies are needed to explore their biodegradation potential to other polycyclic aromatic hydrocarbons.

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