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**SCREENING AND CULTURE MEDIA FORMULATION FOR  
BIOSURFACTANT PRODUCTION BY OIL CONTAMINATED SOIL  
ISOLATE OF *Citrobacter freundii***

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**ABSTRACT**

Increasing contamination of soil with hydrocarbon due to the activities of the chemical and oil refinery industry has prompted severe environmental pollution. The biodegradation of hydrocarbon compounds by the microorganisms has shown promising results to overcome this problem. In the present study, we found stabilized bacterial consortium from the oil contaminated soil samples in Namakkal, Tamilnadu, India. Presently, 6 isolates were showed positive result in all primary screenings methods. Among them, single isolate of *Citrobacter freundii* was potential isolate, which was confirmed with 16sr RNA sequencing. The media has been optimized with potential isolate and increasing the biosurfactant level. Furthermore, the biosurfactant has been identified as a way to reduce hydrocarbon contamination from soil. This study further identified the direct proportional relationship between biosurfactant production and operational variables, showing that biosurfactant are a cheap and viable production pathway map.

**Keywords: Biosurfactant, Bioremediation, *Citrobacter freundii*, Hydrocarbon, CTAB, gravimetric method**

**INTRODUCTION**

Industrial pollution is a major cause of environmental degradation. Several studies have already demonstrated that

areas closest to industrial activity are marked by significant pollution of air, soil, and water. Therefore, such activities may

affect the air we breathe, the water we use, and the soil we stand on and ultimately harm the disease and / or residents of the affected area.

Among various pollutants, hydrocarbon pollutants are one of the major problems in environment. This was occurred from the activities related to the petrochemical industry. Leak and spill were occurred during exploration, production, refining, transport, and storage of petroleum and petroleum products. The contamination of soil by using used lubricating oil is rapidly increasing because of global growth within the utilization of petroleum products. The use of lubricant oil has increased due to the presence of a variety of automobiles and machine [1]. This hydrocarbon contamination of the soil, especially by aromatic hydrocarbons (PAHs) attracts public attention because the metal containing PAHs are toxic, mutagenic, and carcinogenic [2].

The most commonly used technology for soil remediation involves mechanical, burying, evaporation, dispersing and washing. However, these technologies are expensive and can lead to incomplete decomposition of impurities. In this situation urgently needs to better way for remediation. Bioremediation, the degradation of hydrocarbons by the microbes, this method as safe, effective and

low cost then other methods of remediation [3]. Biodegradability by natural populations of microorganisms is one of the primary methods of removing petroleum and other hydrocarbon pollutants from the environment and is cheaper than other solution technologies [4].

A number of microbes were stated to be able to making use of a wide variety of hydrocarbons as carbon and energy. During the bioremediation process, number of factors involved like temperature, composition of the contaminant, soil type, nutrient, and water availability. In addition, important requirement is the presence of microorganisms with the suitable metabolic capabilities [3 & 4].

The present study focuses on this approach, which aims to isolate novel bacterial strains capable of decomposing petroleum hydrocarbon, in addition enhance the biodegradation level with media optimization of different parameters and its effect on growth and biosurfactant yield was determined.

## METHODS

### Collection of soil samples

Surface sediment samples were collected from oil contaminated sites in Namakkal, Tamilnadu, India. The collected samples were pooled and transferred to pre-sterilized, labeled, plastic container and

transported at 4 °C to the laboratory and maintained at 4 °C until analysis.

### **Isolation of bacterial isolates from oil contaminated soil**

The serially diluted soil samples were spread on each of BHA plates overlaid with 100 µl of crude oil and were incubated at 25 °C for 14 days. After incubation period, observed the colonies and sub cultured into nutrient agar for further analysis.

### **Screening of biosurfactant producing isolates**

All colonies were subjected to screening of biosurfactant production with various methods including CTAB agar plate, Blood hemolysis, Drop collapse, oil spreading test, Emulsification test and gravimetric method [5].

### **Characterization and Identification of isolates**

All isolates were identified based on their cultural, morphological, and physiological characteristics in accordance with the Koneman *et al* [6]. The biochemical tests of Sugar utilization; Catalase and oxidase production and Urea hydrolysis; IMVIC tests were performed.

### **Identification of potential isolate by 16sr RNA sequence**

The 16s rRNA genes were amplified using PCR with the universal primer FD1 and RD2. The sequence of FD1 and RD2

were 5'- AGAGTTTGATCTGGCTCAG-3' and 5'-AAGGAGGTCATCCAGCC3' respectively. Sequences obtained were compared against National Center for Biotechnology Information (NCBI) database using BLAST [7].

### **Optimization of biosurfactant production**

The effect of different cultural conditions (pH, temperature, nitrogen source and carbon source) on the growth of the selected bacterial isolate, and the ability of the potential isolate to produce biosurfactant and degradation of oil was determined. The inoculum for the optimization used was first standardized using MacFarlane's standard.

After incubation period, supernatant was acidified with 6 N HCl to pH of 2.0. Then organic solvents of methanol and chloroform were used for the separation of biosurfactant. The dry weights of biosurfactant were determined using the method of Chandran and Das [8]. Simultaneously, degraded oil value also was determined with Anupama method [9].

**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.**

### **Production of biosurfactant with the best parameters**

The optimized parameters were used in setting up the biosurfactant

production media. The production was carried out in a 500 mL conical flask containing 100 mL of the production. The biosurfactant and degraded values were estimated with above mentioned methods.

### Application of biosurfactant

The removal of motor oil from the laboratory contaminated sand was tested through the saturation of 50 g of the standard sand with 10% of motor oil as described by Luna *et al.* [10] with some modification. The laboratory-contaminated soil was placed in 500 mL Erlenmeyer flasks, to which 100 mL of the crude biosurfactants (cell-free broth after fermentation) and isolated biosurfactants (500 mg of biosurfactant dissolved in 100 ml of D.H<sub>2</sub>O) were added. The flasks were shaken at 150 rpm for 12h, 24h, and 48hrs at 28°C. The entire content was then centrifuged at 5000 rpm for 1200 sec. Control assays were performed using distilled water at the same conditions. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of material after extraction with hexane and the % of oil removal was calculated using the equation:

$$\text{Motor oil removed \%} = \frac{O_i - O_r}{O_i} \times 100\%$$

Where  $O_i$  is the initial motor oil in the soil (g) before washing and  $O_r$  is the motor oil remaining in the soil (g) after washing

### Statistical analysis

The data obtained in the resent study was expressed as Mean  $\pm$  SD and

were analyzed using students “t” test and Two- way ANOVA test at 5% level of significance using computer software SAS 9.4 (Statistical Analytical System, North Carolina, and USA).

## RESULTS AND DISCUSSION

### Isolation and screening of hydrocarbon degrading bacteria

Bacterial colonies were observed on BHA agar plate after incubation of 14 days at 25°C on which contaminated soil sample was spread and sprayed of crude oil, only bacterial colonies were chosen for the study. A total of twenty five bacterial isolates could be selected based on colony morphology and color. These were subjected to screening of biosurfactant producing isolates. Among them, 6 isolates were selected based on the primary abilities of the selected bacterial strain to CTAB agar plate method, hemolytic activity, drop collapse, and oil displacement. The **Figure 1** revealed that percentage of surfactant producing isolates through various methods.

Presently, 6 isolates were showed positive result in all primary screen methods, which were subjected to emulsification and gravimetric analysis. It certifies the gravimetric results and suggests that the single isolate (IS 20) was highly effective in degrading crude oil. Similar to studies was accounted by

previous study [5]. They were also confirming the potential isolate of biosurfactant with gravimetric method. Lately, Ali *et al* [11] and Veerapagu *et al.*, [12] also found that biosurfactant producing isolates by gravimetric analysis. The gravimetric method is an effective method to detect the surfactant producing isolates, and accurately estimate the amount of crude oil degradations.

A number of techniques have been reported in literature to screen biosurfactant producers since 1970, although, in recent years, due to automation and miniaturization, high throughput screening strategies, which can lead to the preferred upsurge of novel industrial biosurfactant manufacturers, are being developed. The previous study of Saravanan *et al.*, [13] also observed the biosurfactant producing isolates through blue agar, hemolysis, oil spreading, drop collapse, etc. Ahmed and coworkers [14] emphasized that more than one screening method should be included in the primary screening to identify potential of biosurfactant producers. In recently, Jayathi and Hemashenpagam [5] also obtained the biosurfactant producing bacterial isolates with using 6 types of screening procedure and using BTX as substrate.

Based on the partial 16S rRNA gene sequence analysis, the isolated strain I20

was further identified as a member of the genus *Citrobacter* revealing 100 % identity to *Citrobacter freundii*. Comparison with NCBI sequences after neighbor-joining analysis of different *Citrobacter* indicated that the closest relatives of this strain (IS 20) was *Citrobacter freundii* strain CPO16762.1 (100 %) (Figure 2). The 16S rDNA sequence data has been submitted to Gen Bank and accession no. was MH685405. In study of Eman *et al* [15] also observed the biosurfactant producing isolate of *Citrobacter freundii* from waste samples. However, according to the review of literature, this is first report of *Citrobacter freundii* was isolated from oil contaminated soil samples.

Several studies were reported that the production cost of biosurfactant and potency of it's by media optimization with various carbon and nitrogen sources. Carbon and nitrogen are the most important factor in the biosurfactant production medium. The carbon source, particularly the carbohydrate, has a major effect on the type of glycolipids formed. In the present study, *Citrobacter freundii* was able to utilize all the tested carbon and nitrogen sources for growth and production of biosurfactant. In case of carbon source, glucose containing media showed highest biosurfactant, which was produce the  $0.077\pm 0.003\text{gm}$  of biosurfactant,

simultaneously  $1.683 \pm 0.080$  gm of oil was degraded, and second most result was observed while using lactose (**Figure 3**).

One previous [15] study was utilized the *Citrobacter freundii* for the highest production of biosurfactant with glucose containing media. Moreover, an earlier study of Govindammal [16] was producing the highest yield of biosurfactant with using glucose. The use of a soluble substrate such as glucose for biosurfactant production potentially decrease the production costs, making the process more economically and environmentally captivating, and thereby, desirable [17].

The results for evaluation of nitrogen source showed that peptone acted as the best nitrogen sources for BS production (**Figure 3**). While using peptone,  $0.077 \pm 0.010$  gm of biosurfactant was produced and  $1.672 \pm 0.142$  gm of oil was degraded. The previous report of Hu *et al* [18] also, observed the second highest yield of biosurfactant while using peptone containing media. The hand of Moreno *et al* [19] has observed that the production of biosurfactant improved when using in nitrogen limiting conditions.

The effect of different pH values on growth and biosurfactant production showed the optimum pH as 7 for growth and biosurfactant production. The  $0.054 \pm 0.025$  gm of biosurfactant was

yielded and simultaneously,  $1.632 \pm 0.041$  gm oil was degraded. When increase or decreased the pH value from 7, the biosurfactant rate was decreased. This was similar to Nayarisseri *et al* [20], report; they were observed the highest yield of biosurfactant with pH 7. A recent study of also utilized the pH 7 for biosurfactant production [15]. Our result was contrary to previous study of Adamu *et al.*, [21], were observed the highest yield while using pH 8 and *Bacillus* sp. This phenomenon was clearly indicated that biosurfactant production was depended on the microbes and source of isolates (**Figure 3**).

Temperature is a major factor in the growth of isolate and products, so in this study, temperature was seen as a major factor and tested at different temperatures. Among various temperatures tested, the optimum incubation temperature was 37 °C. The  $0.245 \pm 0.240$  gm of biosurfactant was produced and  $1.29 \pm 0.040$  gm of oil was degraded (**Figure 3**). This was correlated to earlier study, they were utilized the 37 °C for production of biosurfactant [22]. This is in agreement with a previous report that maximum biosurfactant production by *Pseudomonas* spp was achieved at 37 °C [23]. After optimization process, best parameters were selected and subjected for improve the biosurfactant ( $0.122 \pm 0.0044$  gm) and degradation level (1.933

±0.0157gm). The degradation level was improved with above 13% and biosurfactant also improved approximately 36%. **Figure 3 shows the optimization of biosurfactant production with various parameters.**

The statistical Two-way ANOVA test revealed that the variation between pH on oil degradation by *Citrobacter freundii* was significant (F = 92.3005; P < 0.0001). Similarly, the variation between temperature on oil degradation was also statistically significant (F = 23.2438; P < 0.001). With reference to the variation between carbon and nitrogen sources on oil degradation by *Citrobacter freundii* demonstrated an F- value of 11.0444 and P < 0.01 and F = 10.3964 and P < 0.01 respectively which is statistically significant (**Table 1**).

The statistical Two-way ANOVA test revealed that the variation between pH on biosurfactant production by *Citrobacter freundii* was significant (F = 126.7644; P < 0.0001). Similarly, the variation between temperature on biosurfactant production was also statistically significant (F = 24.417; P < 0.001). With reference to the variation between carbon sources on biosurfactant production by *Citrobacter freundii* demonstrated an F- value of 11724.98 and P < 0.0001 which is statistically significant. Also, nitrogen

sources variation on biosurfactant production exhibited statistically significant value of F = 7105.463 and P < 0.0001 (**Table 2**).

The statistical student 't' test was conducted on the results of oil degradation and biosurfactant production revealed that both are statistically significant with t- value = 3.2242 to 8.3962 whereas P- value = P < 0.01 to P < 0.0001 for oil degradation. Similarly, for biosurfactant production, t- value = 4.9599 to 108.282 whereas P- value = P < 0.001 to P < 0.0001 for varied different pH, temperature, carbon and nitrogen sources (**Table 3**).

In case of application part, biosurfactant, cell free metabolic liquid and 10% of SDS were utilized for the removal of artificially contaminated engine oil from sand. It is of great interest to compare the efficacy of biosurfactant with other materials. It could be observed that the biosurfactant was the most effective than other materials. The amount of oil residues in the sand was gravimetrically observed by hexane. While using the crude biosurfactant, 78% of oil was degraded same time 60% of oil was degraded while using cell free culture solution (**Figure 4**). Al-Wahaibi *et al.* [24] reported that the crude biosurfactant from *Bacillus subtilis* B30 strain enhanced light oil recovery by 17–26%. In 2017, Bezza *et al* [25] were

recovery the 67% of oil from sand with *Bacillus* producing half diluted cell free supernatant.

From the previous study, over 50% of the oil was extracted after rinsing of the sand with solutions of biosurfactant from *Candida antarctica* [26]. The interesting result was observed while using distilled

water; it showed 23.1% of oil was removed from sand. This was accordance with the study of Bezza *et al* [25]; they were recovery the 26% of oil from sand. According to Khalladi *et al.* [27], water washing of a diesel-polluted soil could eliminate up to 24% of n-alkanes.

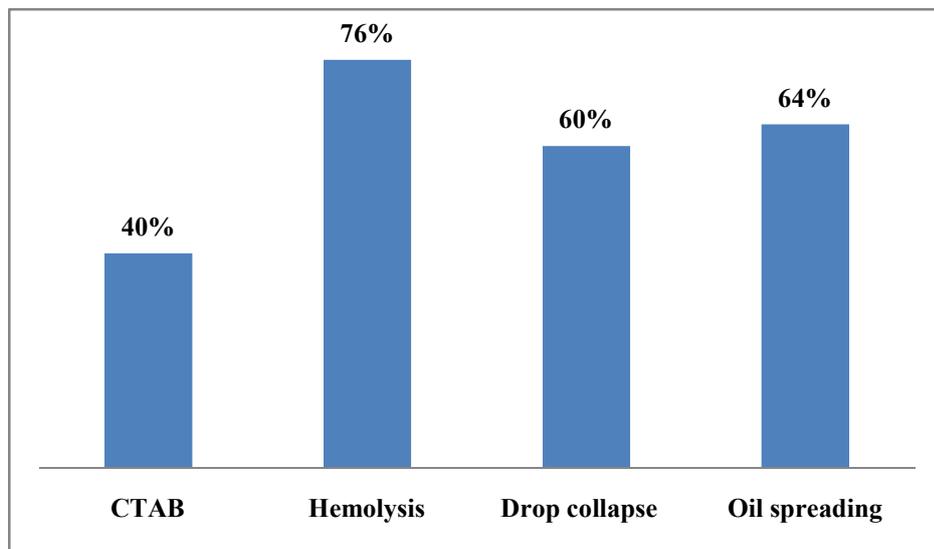


Figure 1: Screening of biosurfactant producing isolates

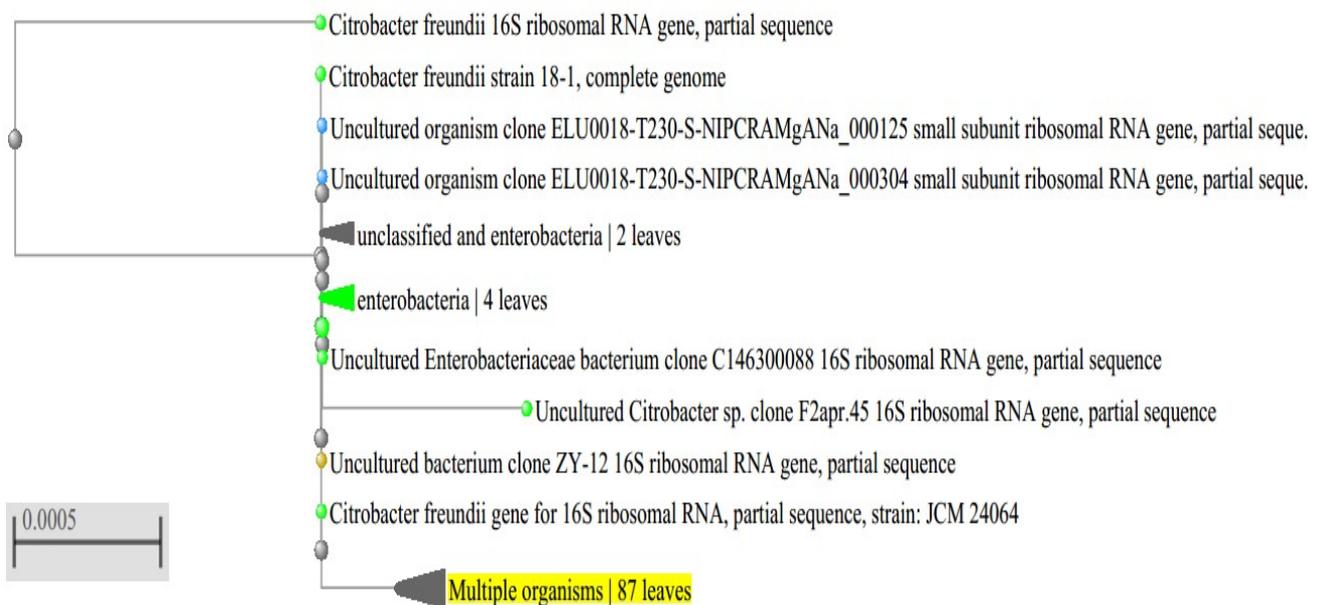


Figure 2: Phylogenetic analysis of *Citrobacter freundii*

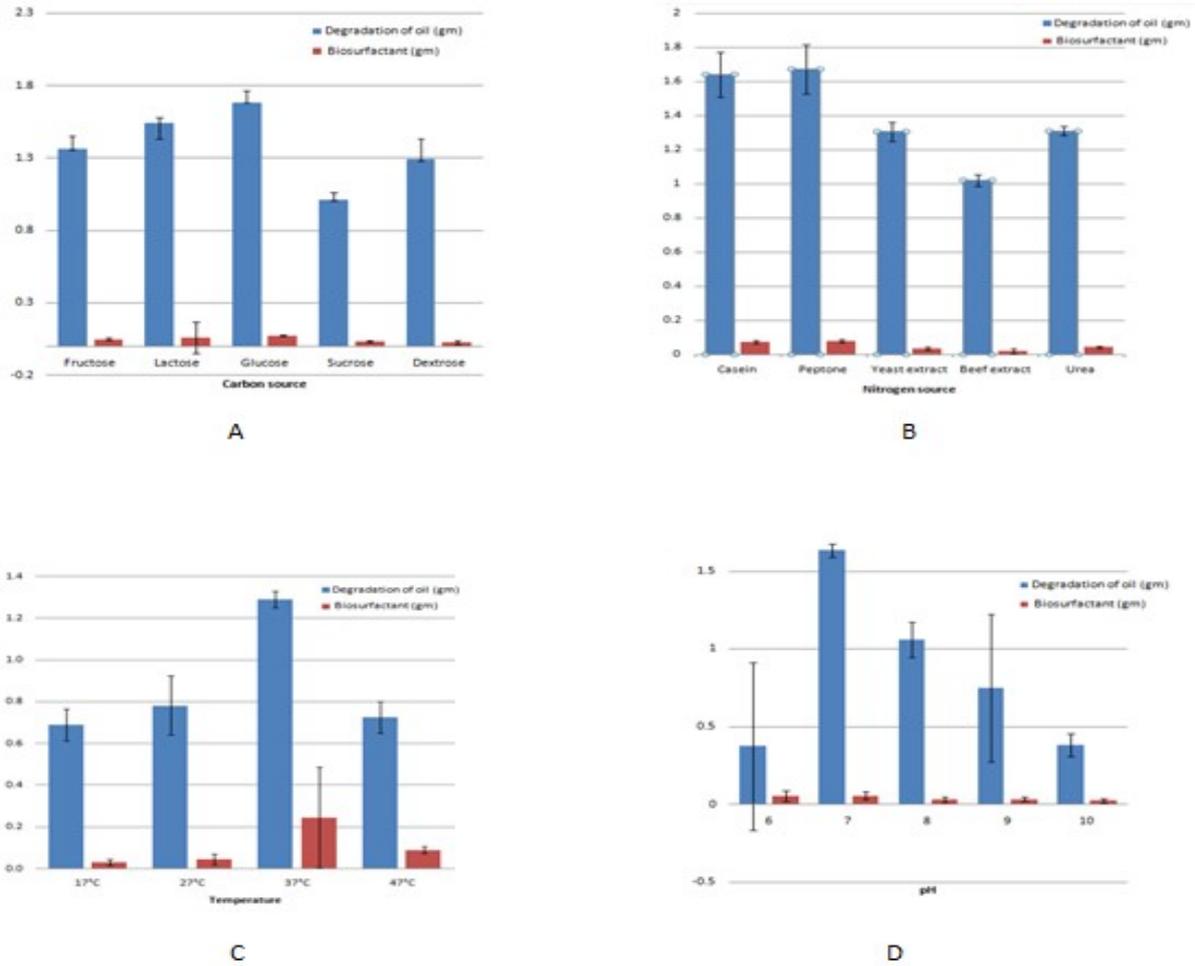


Figure 3: Effect of carbon sources (a) Effect of nitrogen sources (b) effect of temperature (c), and effect of pH on biosurfactant and oil degradation. The error bars represent standard deviations from three independent experiments. SD (n = 3)

Table 1: Two-way ANOVA for the data on oil degradation by *Citrobacter freundii* as a function of variation between pH, temperature, carbon and nitrogen sources

Source of variance	Sum of Square (SS)	Degrees of Freedom (DF)	Mean Square (MS)	F-value	P-value
pH	128.2213	4	32.0553	92.3005	P< 0.0001
Temperature	1937.905	3	645.9683	23.2438	P< 0.001
Carbon	0.3595	4	0.0899	11.0444	P< 0.01
Nitrogen	0.3799	4	0.0949	10.3964	P< 0.01

P< 0.01, P< 0.001 and P< 0.0001 are statistically significant

Table 2: Two-way ANOVA for the data on biosurfactant by *Citrobacter freundii* as a function of variation between pH, temperature, carbon and nitrogen sources

Source of variance	Sum of Square (SS)	Degrees of Freedom (DF)	Mean Square (MS)	F-value	P-value
pH	158.4677	4	39.6169	126.7644	P< 0.0001
Temperature	2034.869	3	678.289	24.417	P< 0.001
Carbon	2.2553	4	0.5638	11724.98	P< 0.0001
Nitrogen	2.2572	4	0.5664	7105.463	P< 0.0001

P< 0.001 and P< 0.0001 are statistically significant

Table 3: Summary table of student 't' test on comparison of oil degradation biomass as well as biosurfactant production by *Citobacter freundii* at different pH, temperature, carbon and nitrogen sources

	Parameters	t-value	P- value
pH	Oil degradation	8.3962	P< 0.0001
	Bio-surfactant	11.1711	P< 0.0001
Temperature	Oil degradation	4.8511	P< 0.001
	Bio-surfactant	4.9599	P< 0.001
Carbon	Oil degradation	3.3233	P< 0.01
	Bio-surfactant	108.282	P< 0.0001
Nitrogen	Oil degradation	3.2243	P< 0.01
	Bio-surfactant	84.2701	P< 0.0001

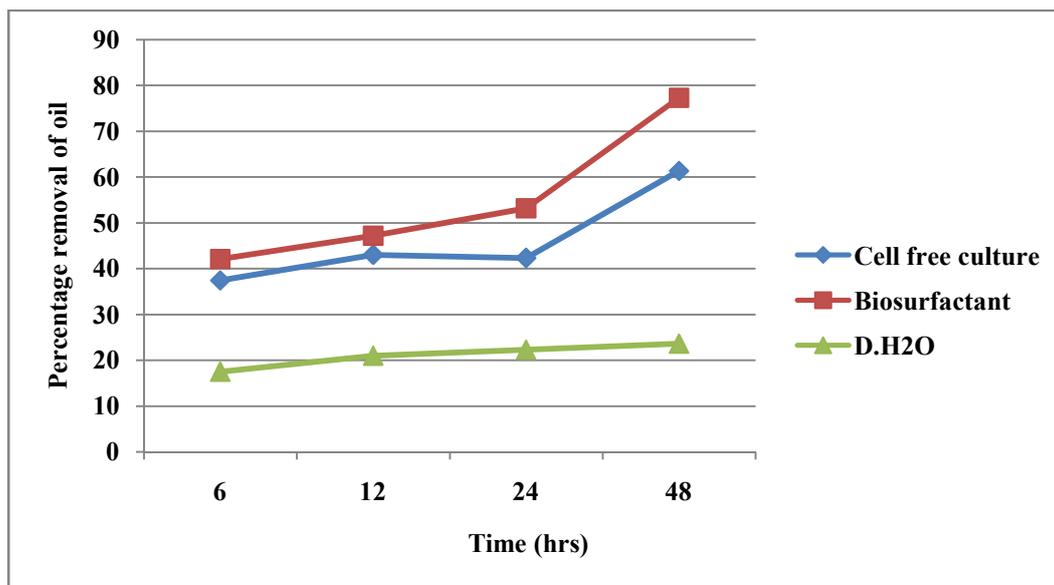


Figure 4: Removal of motor oil adsorbed to sand by the biosurfactant and crude biosurfactant from *Citobacter freundii*. Error bars show the corresponding standard error

## CONCLUSION

The results obtained from the present studies indicated that a promising oil degrading and biosurfactant producing strain has been isolated, characterized and identified as *Citrobacter freundii*. The ability in recovering the oil from oil-saturated sand was also demonstrated. Thus, these characteristics indicated potential use of the biosurfactant in the oil industry. Further research is needed to utilize the *Citrobacter freundii* producing biosurfactant on various fields for the removal of oil from contaminated site.

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