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# Biodegradation of crude oil using efficient biosurfactant producing microorganisms

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## Abstract

*Pollution of sea by crude oil caused by stranding of tankers is one of the urgent and serious environment issues over the world. Biosurfactant are formed as extracellular compounds or localized on cell surface of microorganisms. Therefore in the present study focus of attention was given with the foresight of using biosurfactant as a promising tool to emulsify the polluted oils prior to biodegradation. When microbes grow in hydrocarbon contaminated site it undergoes may adaptations. It influences the uptake of hydrocarbon as substrate. Crude biosurfactant helps the biodegradation of hydrocarbon using biosurfactnat producing bacteria to gain better access to their hydrophobic substrates since it bring reduction of surface tension of the marine ecosystem around the bacterium. In the present study, crude oil degradation with mineral salt medium (MSM) the Infrared spectrum of Pseudomonas aeruginosa, Pseudomonas stutzeri, Bacillus cereus, Bacillus licheniformis and control which produce biosurfactant was confirmed by FTIR analysis. The result on bacterial cell growth in all the experiments revealed that biosurfactant producing bacterial cells utilized crude oil as the carbon and energy source which was evident from cell growth observed with experimental Set up exhibited by extraction of crude oil revealed 81.4%, 85.6%, 77.2% and 68.3% of crude oil biodegradation by Pseudomonas aeruginosa, Pseudomonas stutzeri, Bacillus cereus, Bacillus licheniformis, standard and control. Synthetic biosurfactant as standard n-hexane revealed 88.6% during biodegradation of crude oil and complete absence of microorganisms served as control Set exhibited 12% respectively.*

**Keywords:** Crude oil, FTIR, *Pseudomonas aeruginosa, Pseudomonas stutzeri, Bacillus cereus, Bacillus licheniformis* and MSM.

## Introduction

Biosurfactants were amphiphilic biochemical compounds produced on microbial cell surfaces, (or) are extracellular secreted and contain hydrophobic and hydrophilic groups that confer the ability to accumulate between fluid phases. A biosurfactant may have one of the following structures: mycolic acid, glycolipids, polysaccharide–lipid complex, lipoprotein or lipopeptide, phospholipids, or the microbial cell surface itself[1].

The unique properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in a great number of industrial operations. Surfactants are used by many industries and one could easily infer that there is almost no modern industrial operation where properties of surfaces and surface active agents are not exploited[2].

The more biosurfactant producing microorganisms possessed the enzymatic activity to degrade such hydrocarbons like petroleum, diesel, and crude oil. Few microorganisms were degraded by alkanes, ketones and other aromatic properties, both paraffinic and aromatic (aliphatic) hydrocarbons. The normal alkanes in the range $C_{10}$ to $C_{26}$ are viewed as the most readily degraded, but low-molecular-weight aromatics, such as benzene, toluene and xylene, which were among the toxic compounds found in petroleum and also crude oil, biodegraded by more efficient marine born microorganisms[3].

The bioremediation of petroleum and crude oil in the marine ecosystem is carried out largely by diverse microbial populations, including various *Pseudomonas* species. The hydrocarbon-bioremediation populations were widely transferred into the world's oceans; surveys of marine born bacteria indicate that hydrocarbon-degrading microorganisms were widely distributed in the marine ecosystem[4]. Recently, in pristine environments, the hydrocarbon-degrading bacteria astonishing < 1% of the total bacterial population. These efficient bacteria presumably utilize hydrocarbons that are naturally produced by plants, algae, water sources and other living organisms. It's also utilizing the other substrates, like lipid, carbohydrates and proteins[5].

Bioremediation of a given hydrocarbons depends on its dispersion state. The bioremediation is maximized when the water-insoluble substrate is dissolved, solubilized, or emulsified. Synthetic detergents used to clean up these spillages have often led to more destruction of the environment. Bioremediation of hydrocarbons by native microbial biosurfactants is the primary mechanism by which hydrocarbon contaminants are removed from the environment. Proposed

roles for biosurfactants with respect to their interactions between microorganisms and hydrocarbons in the content of modulation of cell surface hydrophobicity[6].

## Materials and methods

**Biodegradation of crude oil:** The 100ml of Mineral Salt Medium containing (g/1), $K_2HPO_4$ - 1.0, $MgSO_4.7H_2O$ - 0.05, $FeSO_4.7H_2O$- 0.05, $CaCl_2.2H_2O$ - 0.1, $Na_2MoO_4.27H_2O$ - 0.001, NaCl - 30g, Glucose - 2%, Yeast extract - 3% and crude oil (2% w/v). The flasks were inoculated with 5% inoculum and kept in shaker for 720hours (300days) at 200rpm and $30^0$C. The different biodegradation experiments were conducted to using above the culture medium.

**Biodegradation of experiments:** i. Experiment–I: Bacterial cells (S2, S3, S6 and S7) + Mineral salt medium + Crude oil (Normal), ii. Experiment –II: Mineral salt medium + Crude oil (Control).

**Extraction of crude oil from control flask:** Flask containing 100ml of Mineral salt medium with 2% crude oil was acidified with 0.1N of concentrated HCl was added and pH: 2.10ml Hexane was added and flask was placed on shaker at 120rpm for 20min to enhance the mass transfer rate of crude oil from water to hexane. This solution was then transferred in separating funnel, mixed well and then the aqueous the hexane phases were allowed to separate. The lower layer of $H_2O$ was drained and the extraction was repeated with 10ml of solvent. The combined solvent extract was drained through a funnel containing 1g of anhydrous $Na_2SO_4$ in an ordinary filter paper, than it was completely evaporate the hexane. The extract was kept in hot air oven in temperature at 72°C.

**Extraction of crude oil from experimental flask:** Cells and crude oil was centrifuged at a speed of 5000rpm for 35min such that the biomass settled at the bottom and the supernatant aqueous phase containing bulk of the oil separated biomass pellet was extracted by adding 2ml of hexane, The procedure were repeated twice and reduction amount of crude oil due to microbial action and biotic losses were determined.

**Standardizing time required for evaporation of hexane:** Removing of hexane by evaporation in a heated hot air oven is difficult. To determine the time for complete evaporation of hexane. Two beakers of 50ml capacity were weighted individually. In the first beaker, 20ml of hexane was taken and second beaker 20ml of hexane along with 1ml crude oil was taken and kept in an oven set at a constant temperature of $72^0$C. The weight of both beakers was measured overtime until there be no further in weight. At $72^0$C, 20ml of hexane is evaporated in 126min, Crude oil degradation percentage was calculate by formula

$$\% \text{ of crude oil degradation} = \frac{\text{Initial concentration of crude oil} - \text{Final conc.of crude oil}}{\text{Initial concentration of crude oil}} x\ 100$$

**Biodegradation of crude oil by FTIR analysis:** The biodegradation of crude oil (hydrocarbons) degraded by *Bacillus* sp and *Pseudomonas* sp and purified biosurfactants was collected after 30days of incubation. After incubation period the samples were centrifuged at 10000rpm for 30minutes. The hydrocarbon residue was air dried and used for FTIR analysis. Sample for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride (salt). Salt is transparent to infrared light. The drop forms a thin film between the plates. Liquid hydrocarbon samples were milled with potassium bromide (KBr) to form a very fine powder. This powder is then compressed into a thin pellet which can be analyzed. KBr is also transparent in the IR. Alternatively, powder samples can be dissolved in a solvent such as methylene chloride and the solution placed onto a single salt plate. The solvent was then evaporated off, leaving a thin film of the hydrocarbon residue on the plate.

## Results and discussion

This study investigated the potential application of biosurfactant producing microorganisms and control was compared for enhanced removal capability and biodegradation of crude oil. The biosurfactants were produced by both gram negative and gram positive bacterium *Pseudomonas* sp and *Bacillus* sp were cultivated in low-cost substrates.

**Biodegradation of crude oil by extraction method:** In the present study was conducted to investigate the effect of biosurfactant on biodegradation of crude oil by four bacterial strains namely *Pseudomonas aeruginosa, Pseudomonas stutzeri, Bacillus cereus* and *Bacillus licheniformis.* Six set of experiments were carried out in shake flask conditions with same concentration of crude oil as follows. Set-I designated as control un inoculated microorganisms which include crude oil and mineral salt medium, Set-II denote standard n-Hexane as solvent synthetic surfactants with mineral salt medium and crude oil, Set-III exposed to crude oil, mineral salt medium amended with *Bacillus licheniformis* only. Set-IV include crude oil, mineral salt medium amended with *Bacillus cereus.* Set-V treated crude oil, mineral salt medium amended with *Pseudomonas stutzeri* and Set-VI includes crude oil, mineral salt medium amended with *Pseudomonas aeruginosa* all the above experimental sets were incubated for duration of 30days exposure.

The result on bacterial cell growth in all the experiments revealed that biosurfactant producing bacterial cells utilized crude oil as the carbon and energy source which was evident from cell growth observed with experimental Set-III exhibited by extraction of crude oil revealed 68.3% of crude oil biodegradation by *Bacillus licheniformis.* Similarly on analysis with experimental Set-IV biosurfactant producing microbes by *Bacillus cereus* revealed an enhanced crude oil extracted and biodegradation was revealed 77.2% of crude oil degradation.

Simultaneously experimental Set-V tested with surfactants producing microbes *Pseudomonas stutzeri* with the incubation period of 30days found to exhibit a drastic increase biodegradation and extraction of crude oil expressed in 81.4%, while, the experimental Set-VI treated with biosurfactant producing bacteria of *Pseudomonas aeruginosa* revealed highest growth rate and extracted of crude oil was exhibited 85.6% a highest impact of biodegradation. The further study was extended to analysis Set-II denoted synthetic biosurfactant as standard n-hexane, crude oil and mineral salt medium were treated with a period of 30 days extracted revealed an elevated level percentage of 88.6% during biodegradation. The results on biodegradation of crude oil complete absence of microorganisms served as control Set –I the percentage revealed 12% respectively. The exhibited showed in Table-1 and Figure-1.

Figure-1 exhibited biodegradation of crude oil with biosurfactant producing microorganisms S2-*Bacillus licheniformis,* S3-*Bacillus cereus,* S6-*Pseudomonas stutzeri*, S7-*Pseudomonas aeruginosa* and the experiments were compared with control.

**Biodegradation of crude oil using mineral salt medium treated as control by FTIR analysis:** The present study, on spectral analysis using FTIR to assess biodegradation experiments and in specific the control treated systems were monitored which contain a number of bands with different intensity of wavelength with a prominent peak at 1241.25cm$^{-1}$ indicated stretching vibration of C-O and C-O-C. They can also indicate bending vibration of O-$CH_3$ in the spectrum. Another prominent spectrum which appeared between 1370.48cm$^{-1}$ to 1458.25cm$^{-1}$ can be ascribed to the bending vibration of C-H methyl groups in the hydrocarbon. At lower wave length of 678.01cm$^{-1}$ indicate the presence of =C-H functional groups. They posses bending type of vibrations appearing at low frequency region. The results were recorded in Figure-2.

Figure-2 showed biodegradation of crude oil treated with control, the functional group modifications were analyzed by FTIR.

**Biodegradation of crude oil using biosurfactant producing strain *Pseudomonas aeruginosa* by FTIR analysis:** The Infra Red spectrum of purified biosurfactant indicated a broad spectrum of 3531.81cm$^{-1}$ which is a characteristic of OH stretching vibration and the chemical structure of *Pseudomonas species* biosurfactant consist of CHO moiety bonded to a fatty acid through a carbonyl ester group which is assigned by a band value of 1650.17cm$^{-1}$. It is also correspond to C-O deformation vibrations. The presence of carboxylic acid functional group in the molecules was also confirmed by the medium intensity band in the regions of 1455.35cm$^{-1}$ for bending of hydroxyl groups (O-H) stretching band. The absorbance peak around 1070.54 cm$^{-1}$ was reported as C-O-C stretching in rhamnose moiety. The other characteristics absorption band at 870.9cm$^{-1}$ showed the presence of di-rhamnolipid in the mixtures. Based on the above observation of absorption band with the chemical structure identical to these of rhamnolipids with rhamnose rings and long hydrocarbon chains which is produced due to biodegradation of crude oil by biosurfactant producing *Pseudomonas aeruginosa*. The results were exhibited in Figure-3.

Figure-3 showed biodegradation of crude oil treated with *Pseudomonas aeruginosa*, the experiments were analyzed by FTIR.

**Biodegradation of crude oil using biosurfactant producing strain *Pseudomonas stutzeri* by FTIR analysis:** *Pseudomonas stutzeri* a gram negative bacterium synthesize rhamnolipids when grown under appropriate conditions, such as media composition, type of strain, culture conditions and age of culture. Although rhamnolipids is not strongest biosurfactant available, but it is well suited for bioremediation of oil pollutants due to possession of high emulsification activity. In the present study *Pseudomonas stutzeri* was employed after partial purifications to perform structural characterization and to determine functional group modification, properties by FTIR studies.

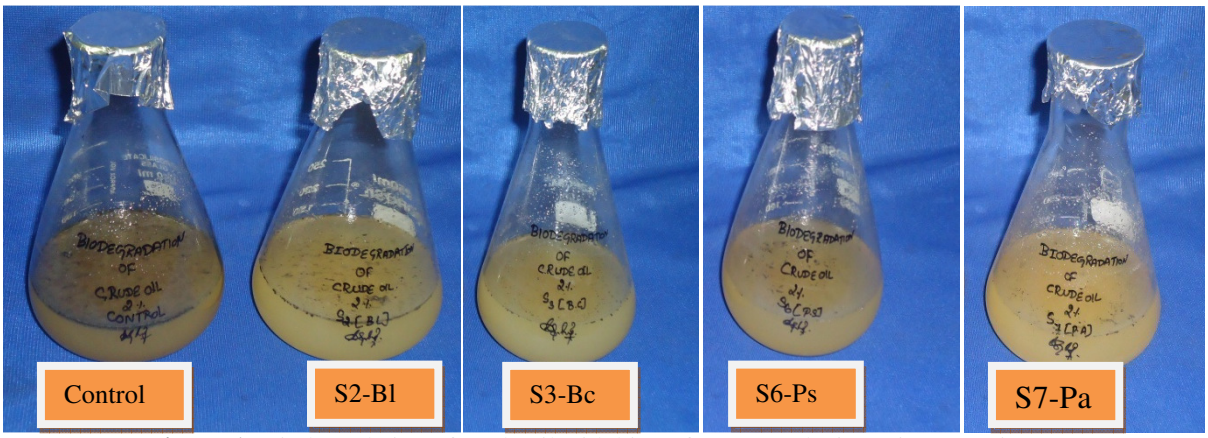


**Figure-1:** Biodegradation of crude oil with biosurfactant producing microorganisms.

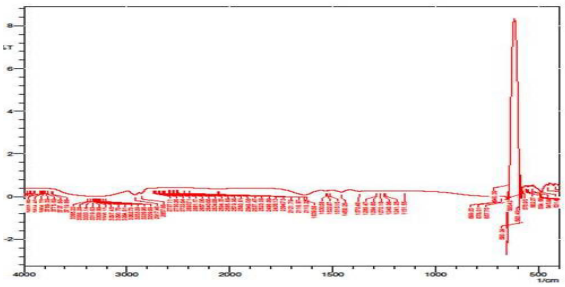

**Figure-2:** FTIR analysis for control.

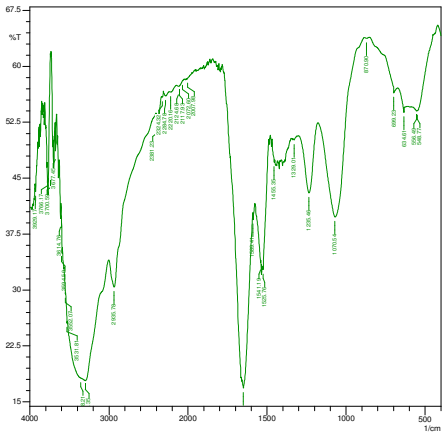

**Figure-3:** FTIR analysis for *Pseudomonasaeruginosa* (S7).

The Infrared spectrum of purified biosurfactant obtained from *Pseudomonas stutzeri* indicated a broad peak at 3594.5cm$^{-1}$ characteristic of O-H stretching vibrations. Absorption band around 2931.93cm$^{-1}$ is assigned to asymmetric C-H stretch of $CH_2$ and $CH_2$ groups of aliphatic chain.The corresponding symmetric stretch is seen at 2877.92cm$^{-1}$. The presence of carboxylic acid functional group in the molecule was confirmed by medium intensity band in the region of 1455.16cm$^{-1}$for bending of hydroxyl group (O-H).The another prominent absorption peak around 1074.4cm$^{-1}$ reported as C-O-C stretching in Rhamnose. The results were depicted in Figure-4.

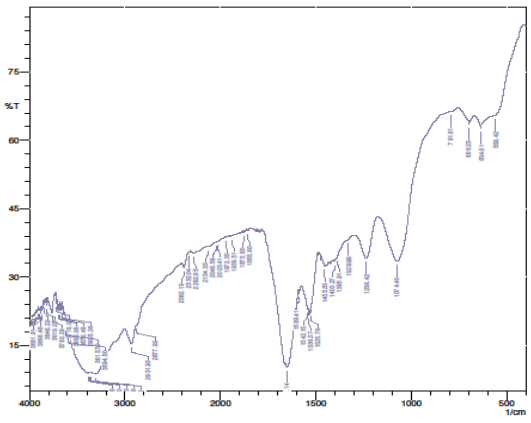

**Figure-4:** FTIR analysis for *Pseudomonasstutzeri* (S6).

Figure-4 exhibit biodegradation of crude oil treated with *Pseudomonas stutzeri*, the experiments were analyzed by FTIR.

In specific, and in accordance with FTIR results it could be identified as Rhamnolipids which here Rhamnose rings with long hydrocarbon chains. These Rhamnolipids produced by *Pseudomonas stutzeri* belong to glycolipid group which comprised of aliphatic acids and esters. The results obtained were consistent with structural modifications due to bioremediation leading to formation of aliphatic and glycolipid moieties.

**Biodegradation of crude oil using biosurfactant producing strain *Bacillus cereus* by FTIR analysis:** Biodegradation of crude oil as hydrocarbon was aimed to investigate the impact of biosurfactant production and bioremediation process. It is frequently reported that various types of microorganisms can produce biosurfactant using hydrocarbon as sole carbon sources. Biosurfactant have certain advantage over certain chemical counterparts. That is lower toxicity, biodegradability pruning them into the potentially effective agent using bioremediation process. In the present an attempt to address the impact of biosurfactant producing species and hydrocarbon degradation rate.

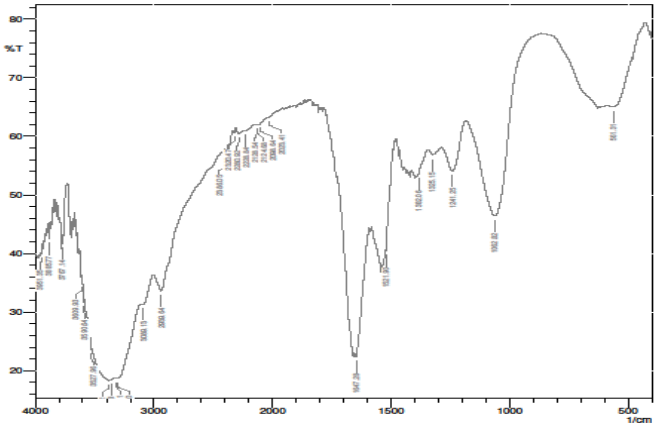

**Figure-5:** FTIR analysis for *Bacillus cereus (*S3).

Figure-5 exhibit biodegradation of crude oil treated with *Bacillus cereus*, the experiments were analyzed by FTIR.

FTIR study indicated the spectrum for the biosurfactant of *Bacillus cereus* showed to have a peptide section in it is structure. Figure-5, exhibited Infrared results of biosurfactant of *Bacillus cereus* seems to be a lipopeptide a wavelength of 1647.28cm$^{-1}$ because appearance of peptide band characteristics revealed in the spectrum 1647.28cm$^{-1}$ carboxyl group and 3382.32cm$^{-1}$ for amine groups. Other peaks prominently seen with the wave number of 1382.03cm$^{-1}$ for related to aliphatic group in the lipid tail of functional groups. The aliphatic components of lipid moiety were visualized by the peaks 2939.64cm$^{-1}$ is an indication of functional group modifications exhibited by Infra Red results, where most similar to *Bacillus cereus.*

**Biodegradation of crude oil using biosurfactant producing strain *Bacillus licheniformis* by FTIR analysis:** The Infra Red spectrum analysis of biosurfactant from *Bacillus licheniformis.* A prominent spectral wavelength band characteristics of peptides appeared at wavelength of 3430.55cm$^{-1}$ with N-H stretching and another prominent wavelength of 1653.07cm$^{-1}$ indicated carboxyl groups. Figure-6, revealed the Infra red spectrum of crude oil degradation.

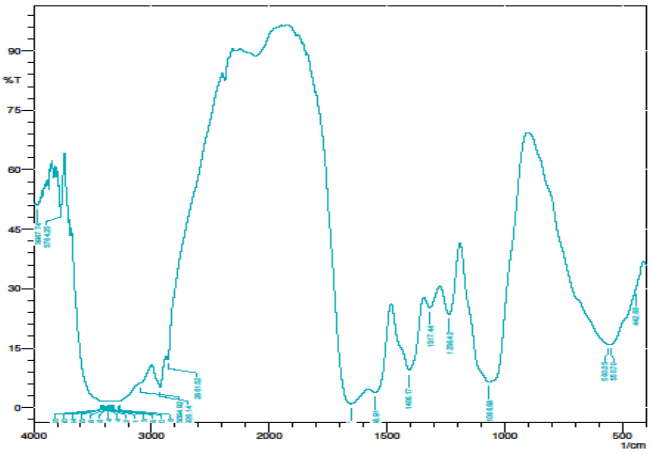


**Figure-6:** FTIR analysis for *Bacillus licheniformis (S2).*

Figure-6 exhibit biodegradation of crude oil treated with *Bacillus licheniformis* the experiments were analyzed by FTIR.

The presence of another absorption band at wavelength 1548.91 cm$^{-1}$ indicated C-N stretching, thus according to the results of Infra Red spectrum indicating the presence of aliphatic chain with the wavelength of 2861.52cm$^{-1}$ and 2926.14cm$^{-1}$ indicated that this compound was found as lipopeptide. The biosurfactant produced from *Bacillus licheniformis* showed the ability of bacteria adhering to hydrocarbons showing high hydrophobicity were concretely found to be more efficient degrades from the FTIR analysis it is concluded that biosurfactant produced by *Bacillus licheniformis* can be used as eco-friendly novel organism for enhancement of bioremediation consider to be a value added product.

**Discussion:** Pollution caused by crude oil and its products is the most prevalent problem in the environment. Biosurfactants producing microbes such as *Pseudomonas sp, Bacillus sp, Sphingomonas, Rhodococcus* are distributed among wide variety of genera even *Candida* have been reported to produce biosurfactants. Biosurfactants are used in processes of oil removal from contaminated sites and for enhancement of hydrocarbon degradation[7]. Recently, increased attention has been focused on hydrocarbon degrading microorganisms with biosurfactant producing capability. Since, these microorganisms were used in present study can able to produce biosurfactant, when grown in oil supplement along with other carbon, nitrogen sources. They are highly promising for bioremediation purposes[8].

Biodegradation of crude oil with biosurfactant producing microorganisms observed on 0$^{th}$ day to a duration exposure of 30days revealed in control and non-inoculated experiment a drastic difference between inoculated experiments and non-inoculated experiments were studied. Although many studies have focused on enhancement of biodegradation as a procedure for cleaning the environmental oil, only few studies on using biosurfactants for degradation have been conducted.

In the present study, gram negative biosurfactant producing isolates were found in hydrocarbon contaminated soils and marine waste water samples, especially *Pseudomonas aeruginosa* was screened to assess the bacterial isolates based on the ability of utilization of crude oil with same concentration of 2% revealed a recovery of 85.6% of degradation, on comparison with *Pseudomonas stutzeri* exhibited 81.4% of degradation. Similar study was carried out by Penet and Marchal[9]. Microorganisms possess mechanisms by which they degrade the crude oil compounds by utilizing them as carbon and nitrogen sources.

**Table-1:** Biodegradation of crude oil with biosurfactant producing microorganisms.

| Experimental Setup | | Crude oil concentration of ML Initial Day of Exposure (0$^{th}$ day) | Crude oil concentration of ML Duration of exposure (30$^{th}$ Days) | Percentage (%) of Crude oil Degradation |
|---|---|---|---|---|
| Setup-I | Control - MSM+ Crude oil | 2 | 1.93 | 12% |
| Setup-II | Standard(Hexane)+MSM+ Crude oil | 2 | 0.87 | 88.6% |
| Setup-III | *Bacillus licheniformis* + MSM + Crude oil | 2 | 0.73 | 68.3% |
| Setup-IV | *Bacillus cereus* + MSM + Crude oil | 2 | 0.79 | 77.2% |
| Setup-V | *Pseudomonas stutzeri* + MSM + Crude oil | 2 | 0.83 | 81.4% |
| Setup-VI | *Pseudomonas aeruginosa* + MSM + Crude oil | 2 | 0.89 | 85.6% |

The pattern of degradation varies with different degrading microorganism because different microbes possess different catabolizing enzymes. In degradation of crude oil, *Pseudomonas sp* advocate for high level degradation compared to other test organisms. Several studies have reported the role of *Bacillus cereus,* more tolerant to high level of hydrocarbon degradation due to their endospore nature. In our studies, *Bacillus cereus* supplemented with 2% crude oil revealed 77.2%, followed by *Bacillus licheniformis* found to degrade crude oil to as extent of 68.3%. In tune with above discussion[10]. *Pseudomonas sp* and *Bacillus sp* degrade oil contaminants which was achieved at 2% of crude oil.

Simultaneously, on analysis of crude oil with standard hexane into mineral salt medium showed maximum degrading ability with 88.6% of recovery surfactant with crude oil degradation. The above result were in total accordance with Atlas and Bartha[11] wherein he emphasized in that control ie., non-inoculated mineral salt medium along with crude oil exhibited only 12% of degradation.

## Conclusion

In the present study suggested the biodegradation of crude oil by *Pseudomonas aeruginosa, Pseudomonas stutzeri, Bacillus cereus* and *Bacillus licheniformis* were biosurfactant producing microorganisms. These strains were found to be an efficient crude oil degrader and both *Pseudomonas sp* could produce rhamnolipid biosurfactant using crude oil as the sole carbon and energy source in the course of degradation. The both *Bacillus sp* strains exhibited excellent degradation of various crude oil components including a number of recalcitrant PAHs. The biosurfactant possesses high surface activity and exhibited excellent emulsification activities against different hydrocarbon substrates. The both gram negative and gram positive bacteria have all these favorable properties facilitate the strain as an efficient tool in various environmental applications, particularly in the bioremediation of crude oil contamination sites.

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