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Screening, Identification and Characterization of Biosurfactant producing strains from oil contaminated soil- A viable source for degradation of crude oil fraction.

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Abstract : In this study, biosurfactant producing bacteria were isolated from soil samples collected from oil spills at Chennai petroleum corporation limited (CPCL). Micro-organism was screened for biosurfactant production using oil spreading technique, emulsification stability and rapid drop collapse activity. Homology analyses of 16S rRNA sequences with BLAST showed that isolates corresponded to four different genera under same species with genetic similarity values close to 100 % belongs to bacillus species. The emulsification activity was quite stable at temperature125^0 C, pH 13. The Structural characterization of the extracted biosurfactant was determined with FT-IR spectroscopy which revealed the chemical structure of the crude biosurfactant as lipopeptide. The oil recovery in soil column studies revealed that biosurfactant have better oil recovery efficiency, thus being more attractive to be applied in Microbial Enhanced Oil Recovery.

Key words : CPCL,16S rRNA,BLAST,FT-IR, lipopeptide, MEOR.

Introduction:

Crude oil is a naturally occurring, unrefined petroleum product composed of hydrocarbon deposits which can be refined to produce usable products such as gasoline, diesel and various forms of petrochemicals. Oil spills are one of the most prevalent forms of oil pollution[5]. The soluble fraction of crude oil that includes aromatic hydrocarbons such as benzene, toluene, xylene, phenantherene, naphthalene are the one of the main causes of oil toxicity. Oil spillage is a very common factor responsible for reduced growth of plants due to direct toxic effect. The spillage occurs due to petroleum consumption, transportation, exploration and spills. These contaminants are toxic as well as carcinogenic. Oil spillage displaces the air between the soil particles by crude oil which has a direct effect on the germination due to reduced aeration. Due to exposure of high oil concentrations, many liver or kidney diseases are developed and by the effect of this cancer risk is enhanced. The illegal dumping of used motor oil is an environmental hazard with global ramifications is a major environmental concern and attracts the public attention.

Microorganism have the ability to utilize hydrocarbons as the sole source of carbon and hydrogen[3]. Bacteria's like *Arthobacter, Alcaligenes, corynebacterium,Flavobacterium, Achromabacter, Micrococcus, Nocardia, Mycobacterium, Burkholderia, Sphingomonas, and Rhodococcus* were found to be involved for alkyl aromatic degradation[8]. *Pseudomonas* genus is one of the most extensively studied bacterial genera for bioremediation[10,21]. The mechanism of utilization of the hydrocarbons is highly dependent on the chemical nature of various compounds in the crude oil and on the environment[24]. The enzymes present in the microorganisms help to degrade the crude oil and utilize it as a carbon source[16,19]. They produce biosurfactants

which are surface active materials capable of degrading hydrocarbons whether they are short chained or long chained or aliphatic or aromatic hydrocarbons[4,6]. It is the amphipathic compounds that contain hydrophobic and hydrophilic domains which is responsible for reduction in surface tension. The reduction in surface tension between oil and water is seen in low molecular weight biosurfactant which results in enhancement of oil recovery[12]. The high molecular weight biosurfactants are responsible for mobility of oil due to its emulsifying properties. The emulsification activity of the biosurfactant is responsible for the enhancement between water insoluble hydrocarbons and the bacteria that produces emulsifiers[1,2]. Most hydrocarbons are degraded by microorganisms grown on hydrocarbons but some are grown on substrates such as glucose, ethanol which as the carbon source[24]. Biosurfactants have low solubility in water and membrane bound oxygenase is released during degradation of hydrocarbons. This enables the bacteria to come in contact with the hydrocarbons, thereby help to disperse the oil[7,25]. The production of biosurfactant stops the volatilization of small chain hydrocarbons which enhances oil recovery. Hence it is in par with the microbial biosurfactant as emulsifying agents in order to enhance oil degradation and oil recovery[9,13].

Materials and Methods:

Sample procurement:

Sample was collected from Chennai Petroleum Corporation Limited, Manali. Crude oil contaminated soil was collected 5-10 cm near the bioreactor using a sterile spatula and was packed in sterile polybags**.**

Culturing of micro-organism:

The crude oil contaminated soil were serially diluted and plated in duplicates onto modified crude oil medium using pour plate method. The strain was cultured in a mineral medium comprising 1.4 gm K_2HPO_4, 0.2 gm $(NH_4)_2SO4$, 0.6 gm KH_2PO_4, 0.6 gm $MgSO_4.7H_20$, 4 gm agar - agar. The above components were dissolved in 200 ml distilled water and 2 ml crude oil was added. The medium was autoclaved at 121^0C for 15 min. The plates were incubated at 55^0C for 48 h. After incubation, the plates that had 30 to 200 colonies were used. Each bacterial colony type was subcultured repeatedly into nutrient agar plate to obtain pure culture.

Characterisation of Microorganisms:

16S rRNA sequencing and phylogenetic analysis of isolates:

The DNA isolated was amplified using 16S rDNA universal primers and sequenced for the identification of bacterial strain at the molecular level. Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rDNA was determined with a Dye terminator sequencing kit (Applied Biosystems). The gene sequences of each isolate obtained in this study were compared with known 16s rRNA gene sequences in the GenBank database[20].

Silica Gel Fractionation of Crude oil:

Silica gel column preparation:

A Column measuring about 30 cm in height and 10 mm thickness was assembled with a suitable stand with a glass wool as stopper at the bottom. Dichloromethane was added in the column in order to remove any air bubbles along the length of the column. Silica powder moistened with DCM was carefully packed through out the column. Further, about 1 cm layer of anhydrous sodium sulphate was added to the top of the silica gel for removal of any moisture. The stopcock was opened to drain any excess of DCM added. Separation of aliphatics was done using addition of hexane and about 2 ml crude oil to the column. Finally aromatics were separated using 50:50 DCM:Hexane and crude oil.

Crude oil Degradation Studies:

The cultured organisms were further grown in mineral salt medium consisting of (g/l) 3 KNO_3.2 Na_2HPO_4,0.14 KH_2PO_4, 0.01 NaCl, 0.6 $MgSO_4$, 0.04 $CaCl_2$, 0.02 $FeSO_4$and 0.1 mL of trace elements solution consisting of (g/l) 2.32 $ZnSO_4.7H_2O$, 1.78 $MnSO_4.4H_2O$, 1$CuSO_4.5H_2O$, 0.3 $Na_2MoO_4.2H_2O$, 0.4 $CaCl_2.6H_2O$, 1 EDTA, 0.004 $NiCl_2.6H_2O$, 0.6 KI. Growth studies was done in 1 L shake flasks containing 250 mL of

medium at 45 ^{0}C with shaking at 200 rpm. A control flask was prepared under the same conditions and the growth pattern was examined at 600nm for 15 days.

Extraction of Biosurfactant:

About 20 ml of 7th day culture broth was taken and centrifuged at 10,000g for 25 minutes to obtain cell-free broth. The pH of the cell-free broth was adjusted to 2 by using 6N HCl and kept at 4°C overnight to precipitate the biosurfactant. The precipitate thus obtained was pelleted at 10,000 × *g* for 20 min, redissolved in distilled water, adjusted to pH 7.0, freeze-dried, and weighed.

Screening of biosurfactant producers:

Hydrocarbon Overlay Method:

Agar plate was coated with 100 µl of crude oil. Plate was inoculated with the bacterial consortium and incubated at 30ºC for 48-72 hours. Colony that was surrounded by emulsified halo was considered positive for biosurfactant production[18].

Oil Dispersion Assay:

About 20 ml of distilled water was added to a clean petridish followed by addition of 10 µl of crude oil to the surface of water. 10 µl of biosurfactant solution was then added to the oil surface. The biosurfactant producing organism can displace the oil and spread in the water. Development of a clear zone on the oil surface indicated biosurfactant activity.

Drop Collapse Test:

About10 μL of cell free broth was placed on a crude oil coated glass slide. The collapse of the drop indicated biosurfactant activity. The result was compared to a control that had 10 μL of distilled water placed on a crude oil coated glass slide.

Emulsification Activity:

About5mg of biosurfactant was dissolved in 5ml Tris buffer.5ml of crude oil was added to the above mixture and was left to stand for 20 minutes. Absorbance was measured at 610 nm. The same procedure was repeated for diesel oil and olive oil.

Emulsification Index:

About2 ml of cell-free broth is mixed with 3 ml crude oil in a test tube. The mixture is vortexed at high speed for 2 minutes and left at room temperature. The emulsification activity E24 (%) is determined using the following formula after 24 hours :

$$E_{24}\ (\%) = \frac{\text{Height of emulsion layer X 100}}{\text{Total height}}$$

Characterization of Biosurfactant:

FT-IR:

Dried biosurfactant was characterized by transform infrared spectroscopy (FTIR) spectra measurement in the frequency range of 4,000–500 cm−1[7].

Stability Characterization:

pH:

About 30 mg of the Biosurfactant was taken in 5 vials.pH was adjusted to 2,4,7,9 and 13 and left overnight at room temperature. Biosurfactant stability was checked by oil dispersion method[7].

Temperature:

About20 ml of the cell free broth was taken in 3 vials and each exposed to different temperatures - Room temperature (37°C), 125°C and -18°C. The thermal stability of the biosurfactant was tested by oil dispersion method[7].

Soil Column Study

Glass columns (30.0 x 2.0 cm), packed with 80.0 g of loamy soil was added and saturated with 25 ml of crude oil . The efficiency of the biosurfactant solution in releasing the oil from the soil was tested by adding 100 ml of aqueous solution of 0.5% of the biosurfactant solution to the column. Distilled water was used as control. Efficiency of oil recovery was estimated by measuring the volume of oil released.

Results and discussion:

Isolation and screening of biosurfactant producing strain:

Biosurfactant producing bacteria was isolated from soil sample collected from oil refinery. Optimum dilution ranges were selected for pour plate method. After pour plating ,The whitish colony was picked and streaked inorder to obtain a pure culture (Fig: 1).Based on the Biosurfactant screening tests, colonies that showed halos around in the agar medium were selected as potential biosurfactant producers. As emulsifiers it showed rapid drop collapsing reaction, and higher zone formation in oil spreading test and excellent emulsification activity[22,23].In the present study, we report the biosurfactant production potential of the isolate B1.

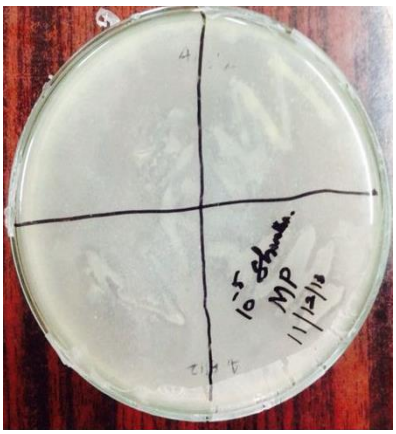


Figure1 : Biosurfactant producing strain B1

Characterisation of Microorganisms:

Homology analyses of 16S rRNA sequences with BLAST showed that isolates corresponded to four different genera under same species with genetic similarity values close to 100 % compared with those reported in GenBank. Isolates belonging to the same species, i.e. *Bacillus lichenoformis, Bacillus amyloliquefaciens, Bacillus aerius, and Bacillus subtilis* were found to have 100 % similarity in their 16S rRNA sequences (Fig: 2). These sequences were aligned with the corresponding sequences of several known hydrocarbon-degrading organisms, and the resulting phylogenetic tree indicated that these isolates was developed (Fig: 3).

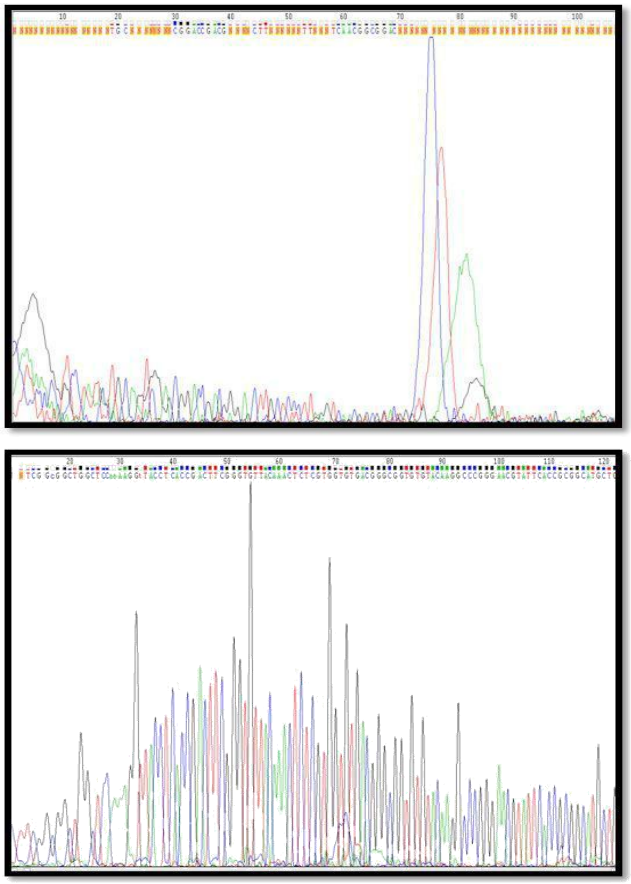


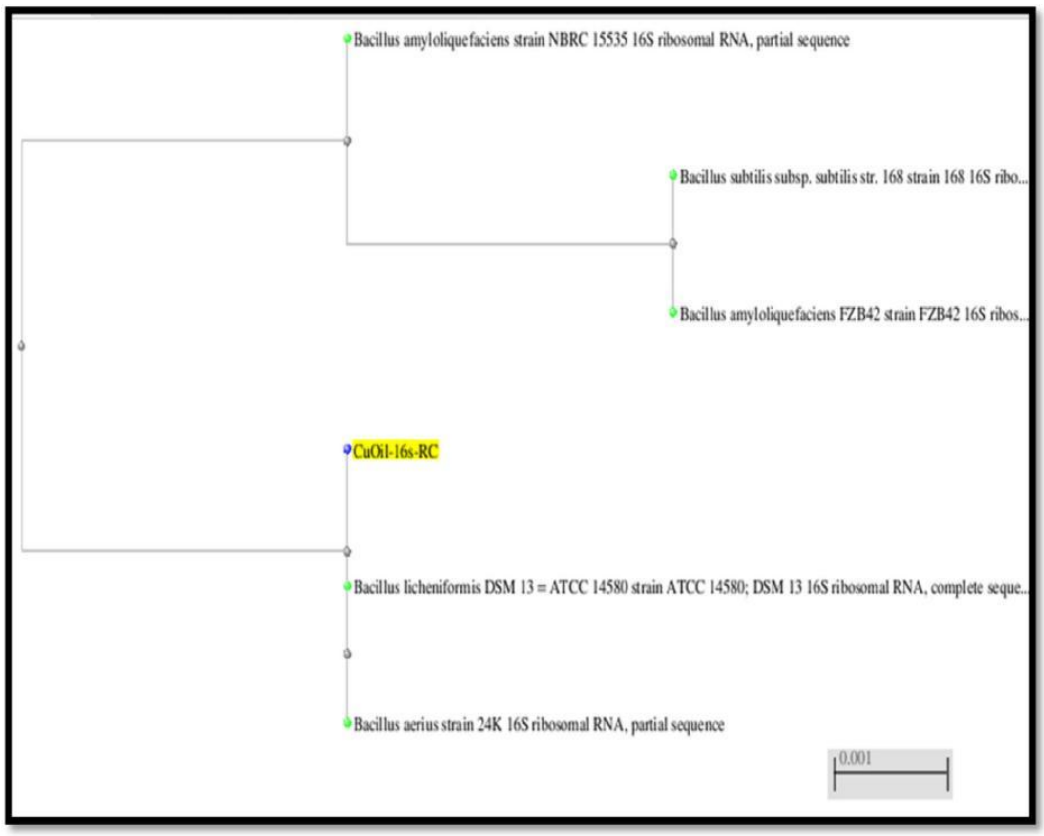
Figure 2: 16S rRNA sequencing primer in BLAST

Forward Sequence:

cTTAGTTgCCagCATTCAGTTGGGCACTCtAAGgtGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAA
GGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGC
AACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAa
CCTTttGGAGCCAGCCgCCGA.

Reverse Sequence:

TCGGcGGCTGGCTCCaaAAGGtTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGG
CGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC
AGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAG
CCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGC
ATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCacCTTaGAGTGCCCAACT
GAATGTCTGGCAACTAAG



Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus licheniformis strain LH-H6 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	KJ148626.1
Bacillus sp. 23SC101Y12 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	KF366704.1
Bacillus sp. 7TBK3Y10 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	KF366692.1
Bacillus sp. 7SBK5Y10 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	KF366691.1
Bacillus sp. 5BBK8Y10 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	KF366689.1
Bacillus sp. 57BK8Y10 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	KF366688.1
Bacillus sp. 43BK8Y10 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	KF366686.1

Figure 3: Consensus phylogenetic tree based on partial bacterial 16S rRNA sequences, GenBank accession numbers of reference sequences are indicated.

Degradation studies:

Degradation using biosurfactant producing consortium was performed on aliphatic and aromatic fractions. On the 7th of degradation, maximum biosurfactant production was observed. The percentage degradation was found to be 13%.On the 10th day there was only little increment in the degradation percentage , which extended upto, 13.33%.

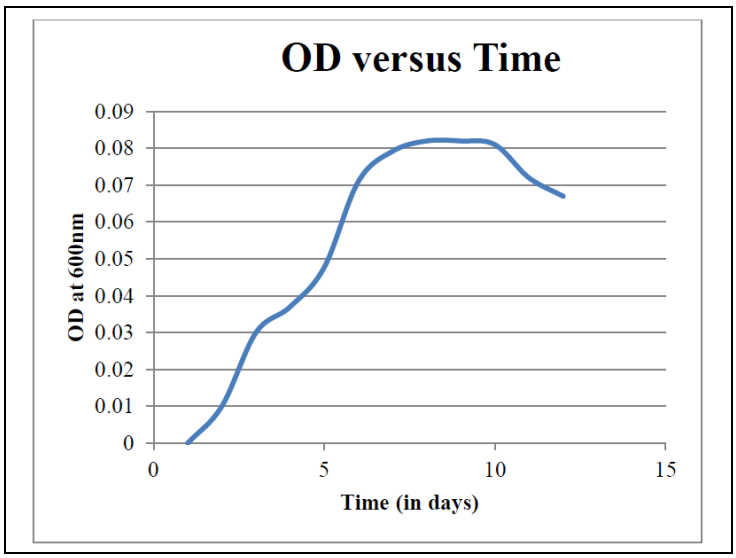


Figure 4: Biosurfactant production and utilization of hydrocarbons.

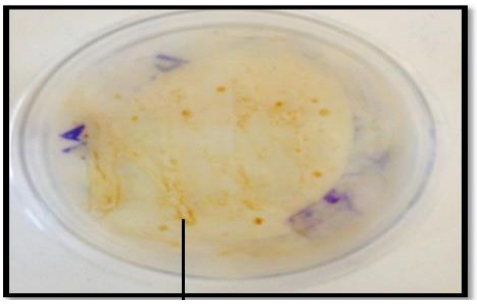
Table1: Degradation of crude oil w.r.t biosurfactant production

Sample	Weight of Oil on 0^{th} day	Weight of oil on 10^{th} day	% degradation
Control	3 g	3 g	0
Crude oil	3 g	2.6 g	13.33

Screening of Biosurfactant producer

Hydrocarbon Overlay Method:

The hydrocarbon overlay agar plate method revealed the isolate consortium had a positive result for emulsified halos around the colony (Figure 5).



Emulsified halo around bacterial consortium is an indication of presence of biosurfactant produced by Bacteria to utilize Crude oil as Carbon source.

Figure 5: Emulsified halo around bacterial consortium in hydrocarbon Overlay Method

Oil dispersion method:

Consortium isolates were positive for the oil-spreading assay and showed the oil-spreading activity (Figure 6).

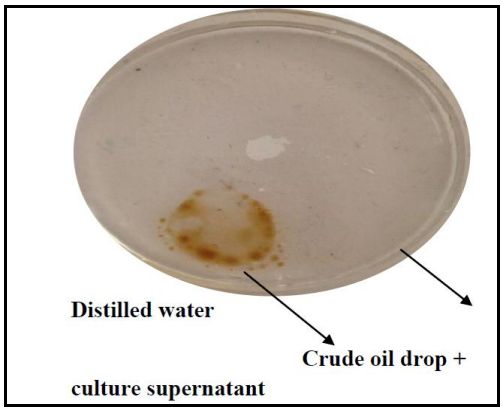


Figure 6: Oil-spreading assay showing the oil-spreading activity.

Drop collapse test:

The addition of biosurfactant to the intact crude oil drop led to the collapse of the drop due to reduction in surface tension.

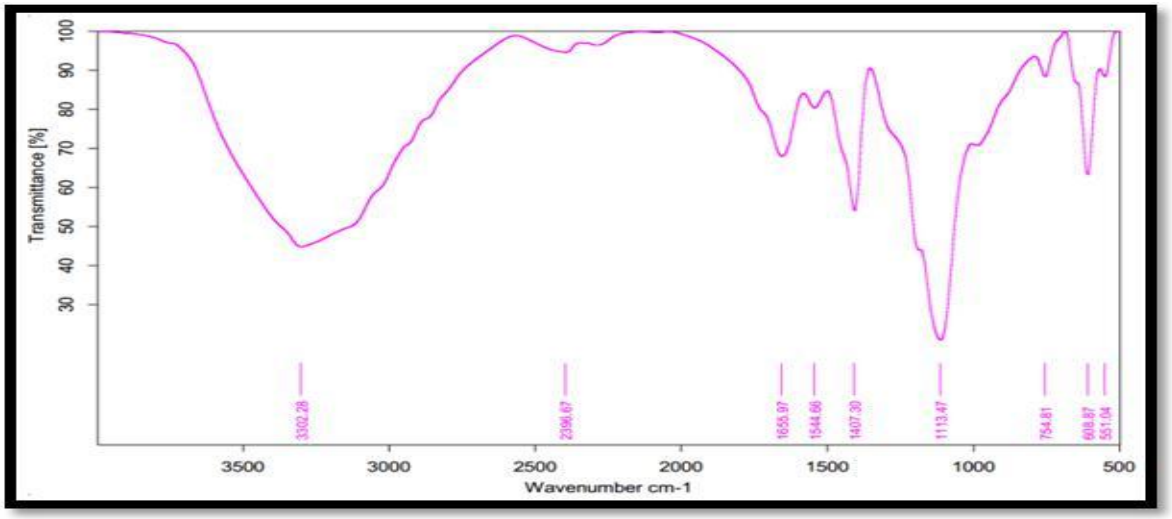
Emulsification Activity:

Emulsification assay of isolates were performed with 4 hydrocarbons: Crude oil, Diesel oil, Olive oil and Aviation Turbine Fluid. Average emulsification units (EU/mL) results for Diesel oil of 6.0 ± 5 EU/mL, Olive oil2.56± 1.4 EU/mL EU/mL, Aviation TurbineFluid4.4 ± 2 EU/mL, and for crude oil of 1.5 ± 0.09 EU/mL were recorded

Characterisation of Biosurfactant

It can be clearly observed characteristic absorbance band of peptides at 3302 cm^{-1} 1544 cm^{-1}. These bands resulted from the stretching mode of N-H, stretching mode of the C=O, and the deformation mode of the N–H bond combined with C–N stretching mode. In addition, the band at 1407cm^{-1} reflect aliphatic chains (-CH3, -CH2) of the sample. These results evidences that the product contains peptide-like moiety as well as aliphatic hydrocarbons. The specific pattern of surface adsorption could be due to exposure of the carboxylic groups on the surface in β-sheet micelles. (Figure 7).

FT-IR Characterisation



ABSORPTION RANGE OF BAND(wave number)	STRETCH VIBRATION
3302 cm^{-1}	N-H stretch
1655 cm^{-1}	CO-N stretch(C=C)
1544 cm^{-1}	N-H stretch
1407cm^{-1}	Aliphatic chain

Figure 7 : FT-IR of biosurfactant produced by *Bacillus species*

5. Stability of Biosurfactant pH

The emulsification activity of the crude biosurfactant at pH 13 showed almost 45% activity, whereas below pH 9 the activity decreased up 34% showing higher stability at alkaline than acidic conditions (Fig 9). This result indicates pH increase has a positive effect on emulsification activity (Fig 8). Studies on biosurfactant production from *Nocardiopsis* showed 66% emulsification at pH 12.

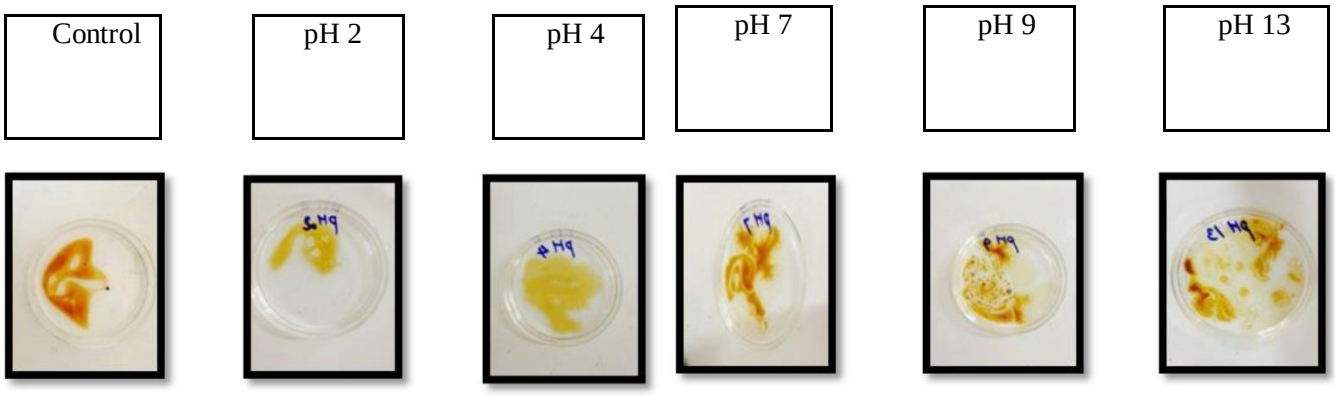


Figure 8 : Stability of pH on emulsifying activity

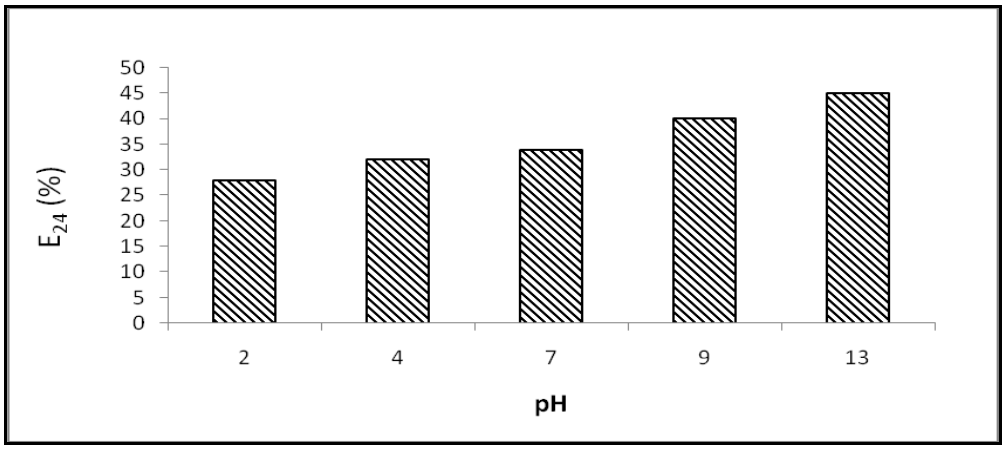
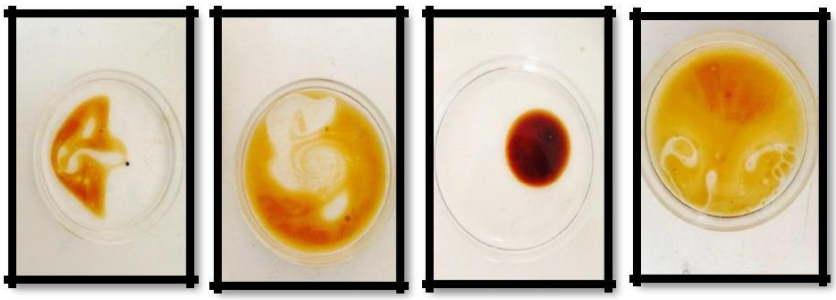


Figure 9 : Graphical representation of effect of pH on emulsifying activity

2. Temperature

The stability of biosurfactant was examined from 0-125° C . The biosurfactant produced by *Bacillus* species in the present study was shown to be thermostable. Exposing the biosurfactant to 100^0 C caused no significant effect on the biosurfactant performance. The emulsification activity was decreased at lower temperatures an started to increase at higher temperature (Fig-1). Biosurfactant produced by *Bacillus methylotrophicus* is thermostable and saved its activity even when exposed to high temperature (120^0 C for 15 min)[14,15].



Control **125° C** **-18° C** **Room temp**

Figure 10 : Stability on temperature for emulsifying activity

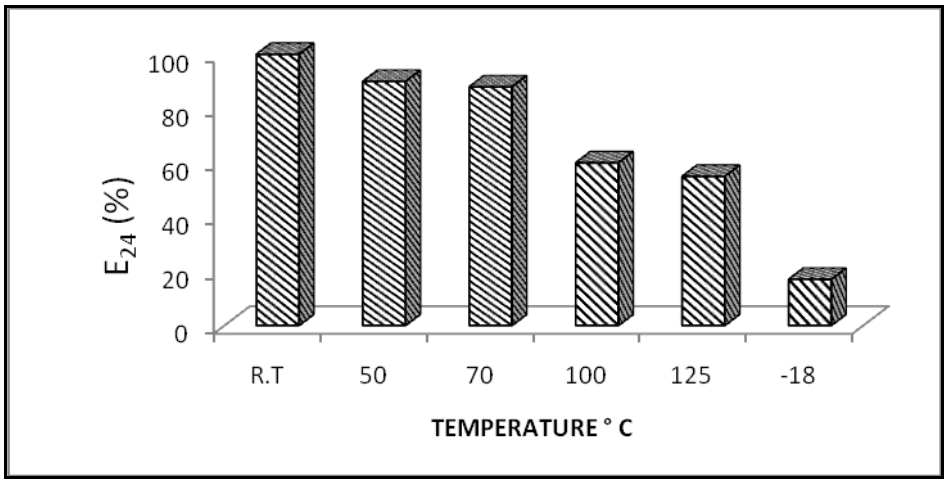


Figure 11 : Graphical representation of effect of temperature on emulsifying activity

8. Oil recovery in laboratory sand-pack column

In soil column study the ability of the crude extracts in oil recovery was observed. Biosurfactant produced by Bacillus species added the factors responsible for decreasing the oil viscosity and making its recovery easier is due to gas and acid production, oil viscosity, plugging by biomass accumulation, reduction in interfacial tension by biosurfactant and degradation by large organic molecules on both environment and oil [11].The oil recovery from our studies was about 12.4% out of total volume of column is 25 ml.

Light Crude Oil	**Formula**	**Control Column**	**Test Column**
PORE VOLUME(PV) (ml)	Empty volume of model	11 ml	11 ml
POROSITY (%)	PV/ TOTAL VOLUME	44 %	44 %
OOIP (ml)	Volume of hydrocarbon retained in the column	8.2 ml	8 ml
S_{OI} (%)	(OOIP/PV)*100	74.5 %	72.7 %
OOIP-S_{ORF} (ml)		4.7 ml	3.7 ml
S_{OR} (%)	[(OOIP-S_{ORF})/OOIP]*100	57.31 %	46.25 %
S_{ORF} (ml)	Oil recovered after media or microbial flooding	0.08 ml	0.46 ml
AOR (%)	Additional oil recovery	1.7 %	12.4 %

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