Biosurfactant assistance in crude oil degradation by halophilic *Bacillus cereus* ND1

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Moderately halophilic and crude oil degrading strain ND1 was isolated from the Dwarka sea coast, Gujarat, India (22° 14' N, 69° 01' E). 16S rRNA analysis confirmed the isolate ND1 as *Bacillus cereus* which efficiently degraded 90% of crude oil evaluated by gravimetric analysis. Alkanes present in crude oil, degraded by isolate ND1 were identified. Increasing oil displacement activity and reduction in surface tension in the presence of crude oil proved production of biosurfactant and its assistance in oil degradation. It was capable of reducing surface tension up to 30 dynes/cm and shown 72.02% emulsification activity with crude oil. The produced biosurfactant was found to be lipopeptide, characterized by Thin Layer Chromatography and FTIR analysis. Fatty acid component of biosurfactant was identified as n-hexadecanoic acid (C16) with molecular weight of 256.42 Da by GC-MS analysis.

**Keywords:** alkane degradation, *Bacillus cereus*, Biosurfactant, Lipopeptide

**Introduction**

The Mul Dwarka port was polluted with crude oil due to oil spillage during 1998. The pollution impact remained for a long time, where polycyclic aromatic hydrocarbon and alkanes entered into the food chain. Bioremediation is an effective cleanup strategy that makes use of the enzymatic capabilities of hydrocarbon degrading microbes, where the hydrocarbon is degraded to water and CO₂. The hydrocarbons are immiscible in water and utilization of these hydrocarbons present problems for microorganisms. Several bacterial strains produce surfactant which is able to emulsify immiscible hydrocarbons and assist its degradation. Many researchers have added biosurfactants to improve the biodegradation of hydrocarbons.

Biosurfactants are amphipathic molecules produced by several microorganisms. It partitions preferentially between fluid phases with different polarity and hydrogen bonding, such as oil/water or air/water interfaces. The most effective biosurfactant reduce the surface tension of water from 72 dynes/cm to values in the range of 25–30 dynes/cm. Biosurfactants have advantages over chemical surfactants in biodegradability, ecological safety, low toxicity and structural diversity. It seems to be promising to search novel biosurfactants from halophiles and its applications in environmental biotechnology. Halotolerant and Halophilic bacteria have a unique lipid composition which plays an important role as surface active molecules. Note worthy, studies on biosurfactant production by crude oil-degrading bacteria inhabiting the Dwarka coastal region, Gujarat are very scarce.

Present study was to check the application of moderately halophilic isolate ND1 in crude oil degradation and the involvement of biosurfactant was investigated. Further, characterization of biosurfactant that assisted crude oil degradation was also done.

**Materials and Methods**

Halophilic bacteria were isolated from soil and water samples collected at 1-m interval from the Dwarka sea coast, Saurashtra, Gujarat. Growth medium used for the isolation of halophilic bacteria was NaCl (5 to 15%). Tryptone 1g, Glucose 2.5g, Yeast extract 5g and distilled water 1000ml (STGY). The cultures were purified by repeatedly streaking them on solid medium. Typically, the isolate ND1 collected from Dwarka soil sample grew well in the standard Salt Tryptone Glucose Yeast Agar (STGYA) medium at 37°C, pH 8.0 with (7%) NaCl proving its moderately halophilic nature (data not shown).

Crude oil (4%) was added to sterilized Bushnell Haas Mineral salt medium (BHM) (composition: Magnesium Sulfate 0.02g, Calcium chloride 0.002g, Monopotassium phosphate 0.1g, Dipotassium phosphate 0.1g, Ammonium nitrate 0.1g, Ferric...
chloride 0.005 g, distilled water 100ml, pH 7.0) incubated at 37°C and 150 rpm in a shaker for the enrichment of hydrocarbon degrading bacterial isolates. The crude-oil sample was obtained from ONGC, Chandkheda, Gujarat, India. After 1 week of incubation, 1% active inoculum was transferred to the fresh, sterilized BHM supplemented with (4%, w/v) oil. After three transfers in BHM with 4% (w/v) of crude oil, inoculum was used for the isolation of crude oil degrading bacteria by spreading over the BHM plates. Separate colonies were selected for the isolation of bacteria on BHM agar plates. Bacterial isolate ND1 was selected for further study of crude oil degradation as it was found to utilize a number of petroleum hydrocarbons. The identification of ND1 isolate was done on the basis of 16S rRNA analysis (Xcelris Laboratory, Ahmedabad, Gujarat, India).

The BHM medium inoculated with cell suspension of isolate ND1, was containing 4% crude oil as a sole source of carbon and incubated for 12 days. Culture was inspected visually at intervals for turbidity, color change, and oil dispersion. Growth was observed by monitoring optical density (O.D. at 600nm) at regular time interval. Crude oil biodegradation was assessed by Gas Chromatography after n-hexane extraction which is commonly used solvent for the extraction of hydrocarbons from the crude oil. Uninoculated controls were included to monitor sterility and to compensate for the abiotic loss of oil. The degraded components were analyzed by Mass Spectral analysis. Crude oil degradation (%) was also checked as Total Petroleum Hydrocarbons by gravimetric analysis after n-hexane extraction. The amount of residual oil was allowed to dry under reduced pressure in a rotary evaporator and measured10.

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\text{Degradation} \% = \frac{\text{Amount of crude oil degraded}}{\text{Amount of crude oil added in the media}} \times 100
\]

The production of surface active molecules was verified by oil displacement method followed by Morikawa et al11. Production of biosurfactant is noted if the oil is displaced and a clear zone is observed. Diameter of the clearing zone on the oil surface would be measured in light after 30 seconds.

The reduction in surface tension (ST), emulsification and stabilizing capacity are the most important surface-active properties evaluated in the screening of microorganisms with the impending industrial application. Main criterion used for screening biosurfactant producers is the ability to reduce the ST below 40 dynes/cm12. Surface tension values were measured by the ring method in tensiometer 5000M (Anton Paar) using the cell free culture (50 ml) at 28 °C at different time intervals. Isolates testing positive in the oil displacement test were also assessed for emulsion-forming and stabilizing capacity with crude oil, according to the method followed by Das et al9. The criterion used for emulsion-stabilizing capacity is the ability to maintain minimum 50% of the original emulsion volume after 24hrs. Emulsification index (E24) was calculated by dividing the height of the emulsified layer by the height of the liquid mixture layer and multiplying by 10013.

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E24 \% = \frac{\text{Total height of the emulsified layer}}{\text{Total height of the liquid layer}} \times 100
\]

A crude biosurfactant preparation was obtained by centrifuging (10,000 g, 10 min, 4°C) the culture from stationary-phase and adjusting the pH of supernatant to 2 with 1N HCl. The low pH causes the biosurfactant to become positively charged, reducing the effectiveness of the hydrophilic region and causing aggregation. This renders the molecule insoluble and the compound precipitates as a solid. Biosurfactant was purified following the methods of Kebouche-Gana et al14 with some modifications as discussed15. The acidified liquid was kept at 4°C overnight, and the precipitates were collected by centrifugation (17,300 g, 30 min, 4°C). The precipitates were dissolved in distilled water; the pH was adjusted to 7.0 with 1 N NaOH, freeze dried and weighed. The lyophilized material was extracted twice with chloroform/methanol (2:1, v/v) solvent system. The extract was dried in a vacuum rotary evaporator.

According to the method proposed by Siegmund et al16, N-Cetyl-N, N, N-trimethylammonium bromide (CTAB) /methylene-blue agar test was performed. Blue halos surrounding the colonies indicate the presence of a glycolipid type of biosurfactant. Biuret test was applied to check the presence of peptide bond or short-chain polypeptides. Presence of lipopeptide in biosurfactant can be detected by this test17. Okpokwasili et al18 described a method to confirm the occurrence of phosphate as a component of biosurfactant.

The extracted biosurfactant material was characterized by Thin Layer Chromatography (TLC)
plates (Silica gel 60A) were washed three times with chloroform/methanol (1:1, v/v) and activated at 120°C. Partially purified biosurfactant was dissolved in distilled water and spotted on TLC plates. Different solvent systems were checked and chloroform–methanol–acetic acid, 80:18:2 standardized to achieve remarkable separation of components. The components were observed under UV light (280 nm). Peptide components were visualized with ninhydrin components were observed under UV light (280 nm). Lipid components were analyzed as brown spots after spraying with chromosulfuric acid.

IR absorption spectra were attained with a Perkin–Elmer grating 1430 IR in a dry atmosphere. Absorption spectra were plotted in built-in plotter. IR spectra were collected from 400–4000 wave numbers (cm⁻¹) with 2 wave numbers resolution per wave number. Samples were prepared by dispersing the solid, evenly in a matrix of potassium bromide.

The isolated biosurfactant was analyzed by GC–MS (Gas Chromatography Mass Spectral analysis) to identify fatty acid components. Partially purified biosurfactants were analyzed using Perking Elemen (Turbo Mass) Autosystem XL Gas Chromatograph system, equipped with PE-5 ms 30 × 0.25 mm × 0.25 μm column and mass detector that operated in EMV mode. Helium was used as a carrier gas with the flow rate of 1.0 ml min⁻¹. The injection port temperature was operated at 250 °C. The column oven temperature was held at 80 °C for 5 min and then programmed at 10 °C min⁻¹ to 290 °C. Electron impact spectra in positive ionization mode were obtained between m/z 40 and 450.

**Results and Discussion**

Among all the halophilic bacterial isolates, eleven were screened positive as crude oil degrading bacteria. Moderately halophilic isolate ND1 was selected as efficient crude oil degrading and biosurfactant producing strain for further studies. Growth of ND1 in the presence of crude oil was monitored at regular interval. Based on gram’s staining and endospore staining, ND1 has shown gram positive nature and endospore former. Additionally, strain could tolerate salt up to 20%, but maximum growth in halophilic medium with 7% NaCl considered as moderately halophilic. Interestingly, this isolate grown better at pH 8, proves its alkalophilic nature. ND1 was identified as *Bacillus cereus* based on their homology of DNA sequences with the NCBI databases of bacteria on the basis of 16s rRNA analysis. The sequence was deposited in the NCBI database. The strain name and GenBank accession number are Bacillus cereus ND1, KC848678.

To monitor degradation of crude oil in the n-hexane-soluble fraction, Gas chromatography was adopted. From chromatogram of crude oil (control) and after degradation (Figure 1), it is shown that all the hydrocarbons present in crude oil were degraded by the isolate ND1 by using crude oil as a sole source of carbon. From the results of gravimetric analysis, it was found that isolate ND1 shown 90% crude oil degradation. Interestingly, Isolate ND1 could degrade all the alkane components efficiently. The remaining peaks observed in fig.1b also considered as complete crude oil degradation since it covered less than 1% peak area. To identify degraded compounds, Mass spectral analysis was done and compared with standard National Institute of Standards and Technology (NIST) library database available (Table 1).

The alkanes constitute the major fraction of crude oil. Several bacteria can efficiently degrade alkanes and metabolized as a carbon source for growth. *Bacillus* is one of the common hydrocarbon degrading microorganisms found in many diverse environments. Tuleja et al. reported rhamnolipid production by a *Bacillus cereus* strain from naphthalene metabolism. The surfactant production by this microorganism facilitated the substrate uptake and consequently its degradation. Kumari et al. also studied the degradation of aromatics and different length alkanes and its relation to biosurfactant production. Sajna et al. explained identification and percent degradation of crude oil degraded by biosurfactant producing isolate ND1.

<table>
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<tr>
<th>Sr. No.</th>
<th>Retention time</th>
<th>Peak area (%)</th>
<th>Molecular weight</th>
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<td>1.66</td>
<td>604</td>
<td>Tritracontane</td>
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</table>
the alkanes in the crude oil by GC-MS analysis from *P. putida* cultures supplemented with *Pseudozyma* biosurfactant culture.\(^4\)

Biosurfactants naturally synthesize amphiphilic molecules from a variety of microorganisms. These surface active molecules reduce surface tension at surface and interface both and also increases solubility of immiscible hydrophobic components. The production of biosurfactant is found dependent on the type and amount of hydrophobic substrate available.\(^22\) Many hydrocarbon degrading bacteria are described to produce surface active compounds or emulsifying agents extracellularly.

Naturally accessible surfactant molecules from microorganisms, usually facilitate bacterial degradation activity. Strain 2TN-NB isolated from Oil-Contaminated Sites in Vietnam was the efficient petroleum-degrading bacterium and found to produce glycolipid biosurfactant.\(^23\)

According to Yesurethinam et al. the oil displacement test proved one of the effective methods for biosurfactant production in many bacterial strains.\(^24\) ND1 isolate was showing considerable oil displacement activity (5.6cm), selected for further characterization studies.

Isolate ND1 was grown in BHM medium with crude oil as a carbon source and production of biosurfactant was checked. After twelve days incubation, isolation and extraction were done as a maximum reduction in surface tension was observed when cells entered stationary phase (Fig. 2). Madhu et al. also confirmed the maximal reduction in surface tension and production of surfactant in late logarithmic phase and early stationary phase.\(^25\) In this study, acid precipitation method was followed before extraction to obtain maximum yield of biosurfactant. Biosurfactant will present in their protonated form at low pH and less soluble in aqueous solution. The yield of surfactant produced by this isolate was 512 mg/l. The partially purified biosurfactant was further checked for oil displacement activity (Fig. 3a) and surface tension measurement which confirms the presence of biosurfactant as purified.

The most effective biosurfactant reduce the surface tension (ST) of water from 71dynes/cm to values in the range of 25–30 dynes/cm\(^2\). When isolate ND1 was
grown in BHM medium with crude oil as a sole carbon source, it was observed that the surface tension of the medium decreased from 71 dynes/cm to 30 dynes/cm, which is a considerable reduction in surface tension. The ability of biosurfactant produced to emulsify crude oil was tested. Emulsification activity of isolate ND1 with crude oil was observed as 72.02%. The biosurfactant of *Klebsiella* sp. Y6-1 had high emulsification activity and stability with decane, hexadecane and crude oil as substrates. It is indicated in the recent report that the hydrocarbon enriched oils were the suitable sources for oil degradation and the plant oils for biosurfactant production.

In order to determine the type of surfactant produced by efficient isolate ND1, three biochemical tests were performed. CTAB/methylene-blue agar is a method to detect extracellular glycolipid or anionic biosurfactant. In this study, negative result was found when the strain cultured on a CTAB/methylene blue agar plate. Biuret test was applied to detect lipopeptide surfactant in the sample. If Biuret reagent turned to pink or violet ring, it shows the presence of short-chain polypeptides. In this case, positive results were obtained when the sample reacted with Biuret reagent. In phosphate test, colorless phosphate solution changed to yellow if phosphate is present and here the test was negative. It can be interpreted from the primary identification tests that the biosurfactant produced by isolate ND1 could be lipopeptide type.

Jamal et al. used these biochemical tests to reveal the
type of biosurfactant and it was identified as phospholipid from *Klebsiella pneumonia* WMF02\(^{26}\). Okpokwasili et al. also performed a phosphate test to determine phospholipid biosurfactant from *Pseudomonas* sp. in mineral salts medium supplemented with kerosene\(^{18}\).

TLC is the most extensively studied method to determine the nature of biosurfactant. Thin layer chromatography (TLC) data revealed a spot with \(R_f\) value of 0.69 under UV detection. Based on the \(R_f\) value, the spot was concluded as a peptide moiety containing the compound of lipopeptide. Additionally, lipid component was detected as brown spots on the plate when sprayed with chromosulfuric acid and the peptide component was visualized when stained them with ninhydrin as shown in Fig. 3b.

These preliminary results suggest that the partially purified biosurfactant produced by *Bacillus cereus* should contain a lipopeptide. A study conducted by Anyanwu *et al.* substantiated in their study that surfactant with the \(R_f\) values of 0.68 and 0.70, were identified as lipopeptide \(^{27}\). M.B.S. Donio et al. also confirmed that the biosurfactant isolated from halophilic *Bacillus* BS-3 had the \(R_f\) value of 0.68 as lipopetide type\(^{28}\). Yesurethinam *et al.* reported \(R_f\) value as 0.68 in Thin Layer chromatography of lipopeptide biosurfactant collected from halophilic isolate *Kocuria marina* BS-15\(^{24}\).

The Fourier Transform Infrared Spectroscopy (FTIR) method has been widely used to characterize the functional groups, since infrared (IR) transmission spectra present peaks characteristic of specific chemical bonds. Analysis of the FTIR spectrum of surfactant from ND1 sample revealed the composition as lipid and peptide fractions (data not shown). The peaks observed in IR spectrum was analyzed and compared with standard data available. As a result of N–H stretching, a broad absorbance peak (3416 cm\(^{-1}\)) with wave numbers ranging from 3600 cm\(^{-1}\) to 3100 cm\(^{-1}\) was observed. This is probably because of amides. The peak at 1642.24 cm\(^{-1}\) of C=O stretch typically of carbon-containing compounds and stronger in amides than amines. Sharp absorbance peaks are observed at 1422 cm\(^{-1}\), 1513 cm\(^{-1}\), 2743 cm\(^{-1}\) are indicative of aliphatic chains (–CH\(_3\) and –CH\(_2\)-). The peaks observed in FTIR, analyzed in Spectral Data Base for Organic Compounds (SDBS) to identify the nature of compound. It matched with the synthetic surfactant, 2-lauroxyloxyethyl trimethylammonium chloride and presence of quaternary amines in the compound categorized it in cationic surfactant. Joshi et al. analyzed the nature of biosurfactant and presence of hydrocarbons as well as a peptide like moiety in *B. subtilis* 20B\(^{29}\). The results obtained from FTIR spectrum of biosurfactant from *Pleurotus* spp. helped to reveal the complex polysaccharide peptide lipid structure\(^{30}\). The fatty acid composition of biosurfactant was predicted by GC-MS analysis and compared with available data library. It was found that surfactant from ND1 identified as C-16 n-hexadecanoic acid a long chain fatty acids with molecular weight 256.42 Da as shown in Figure 4.

It is reported in *Bacillus* spp. That fatty acid component of biosurfactant is known to offer surface activity to the surfactant produced\(^{31}\). Saravanakumari *et al.* isolated glycolipid type of surfactant from

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Fig. 4 a — GC-MS separation of biosurfactant produced by ND1 mainly showing peaks for n-hexadecanoic acid. b. Structure of n-hexadecanoic acid.
L. lactis which was containing octadecanoic acid as a fatty acid chain. Kiran et al. characterized lipopeptide biosurfactant with nonanoic acid, 9-Oxo-, methyl ester and brevifactin by GC–MS analysis in Brevibacterium aureum MSA13.

Palmitic acid and stearic acid were found as major fatty acid type in biosurfactant from L. pentosus. Based on gas chromatography, the structure of surfactant produced by E. faecium predicted as Xylolipid with a hydrophobic part as β-hydroxydecanoic acid. There is a peptide moiety connected with the hydrocarbon chain in biosurfactant produced by isolate ND1. The peptide compound imparts positive charge on the biomolecule, proving its cationic properties which could be useful for binding and thus remediation anions.

Conclusion

Moderately halophilic bacterial isolate Bacillus cereus ND1 was able to degrade crude oil efficiently. Importantly, the remarkable reduction in surface tension by producing surfactant by isolate ND1 suggests its assistance in crude oil degradation. As this strain is marine originated and could degrade crude oil effectively by producing a biosurfactant warrant its application in enhancing oil recovery at marine oil spillage sites. Further purification and microbial enhanced oil recovery of the biosurfactant is in offing.

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