ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2011; 2(2): 784-790

ORIGINAL RESEARCH ARTICLE

Biosurfactant Production And Plasmid Isolation From Newly Isolated Hydrocarbonoclastic Bacteria *Proteus inconstans*

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Received 20 Feb 2011; Revised 02 Apr 2011; Accepted 12 Apr 2011;

ABSTRACT

Biosurfactant are surface active agent that are produced extracellularly or as part of the cell membrane by bacteria, yeast and fungi. In the present study, hydrocarbonoclastic bacteria were isolated from petroleum contaminated sites nearby petrol bunks in Chidambaram. Screening of biosurfactant producing potential strain was done by haemolytic assay and bath assay and the potential strain was identified by biochemical tests as *Proteus inconstans.* After optimizing the biomass production in different physical and chemical parameters, the mass production of biosurfactant was carried out along with biodegradation and emulsification of crude oil. The ability of strain *Proteus inconstans* to grow and produce biosurfactant on different carbon sources, temperatures and pH was studied. In this study, the biosurfactat productivity was 0.8mg/ml in cultures grown in a medium supplemented with glucose adjusted to pH 7.0 and incubated at 37º. The productivity at the optimum condition. Fourier Transform Infrared Spectroscopy & Mass Spectrometric Analysis were carried out which confirmed the glycolipid nature of the biosurfactant. The plasmid with 1.8Kbp was isolated from the strain and it was cured by acridine orange. Biodegradation and biosurfactant activities were totally inhibited. Hence it was determined that the biosurfactant production was purely plasmid mediated.

Key words: Biosurfactant, hydrocarbanoclastic, *Proteus inconstans*, Emulsification, Biodegradation.

INTRODUCTION

Bioremediation is the degradation of toxic pollutants through biological means. Biosurfactants are attractive for bioremediation because they are biodegradable and relatively non-toxic, making it an attractive compound to be released in bulk at a remediation site. Biosurfactants act by emulsifying hydrocarbons, increasing the solubilization of crude oil and subsequent availability for microbial degradation [1].

Biosurfactants are naturally occurring surface active compounds. Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively. Biosurfactants are produced by different microorganisms such as bacteria, fungi and yeast. Biosurfactant producing microorganisms were naturally present in the oil contaminated soil. Oil contaminated environment contain large amount of hydrocarbons. Hydrocarbons are composed of complex chemical structure i.e. aliphatic and aromatic hydrocarbons. Microorganisms exhibit emulsifying activity by producing surfactants and utilize the hydrocarbons as substrate often mineralizing them or converting them into harmless products [2].

The vast majority of commercially employed surfactants are synthesized from petroleum derivatives. Crude oil is mostly composed of hydrocarbon, alkanes, aromatics and hetero aromatic compounds containing sulfur or nitrogen. Biosurfactants are categorized mainly by their chemical composition and microbial origin. Generally, their structures include a hydrophilic moiety consisting of amino acids or peptides, mono-, di- or polysaccharides and hydrophobic

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moiety comprising unsaturated or saturated fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopeptides, lipoprotein, phospholipids, fatty acids, Polymeric biosurfactant and particulate biosurfactants. Biosurfactant producing microorganisms belong to different genera including: *Arthrobacter spp., Bacillus spp., Candida spp., Clostridium spp., Corynebacterium spp., Nocardia spp., Pseudomonas spp., Rhodococcus spp.* and more other genera have been reviewed [3].

Biosurfactants are increasing interest for commercial use because of the continually growing spectrum of available substances. There are many advantages of biosurfactants compared to their chemically synthesized counterpart [4]. The enormous market demands for surfactants are currently met by numerous synthetic mainly petroleum-based, chemical surfactants. These compounds are usually toxic to the environment and non-degradable. Tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternative to chemical surfactants [5].

Furthermore, biosurfactants have the potential to be used as anti adhesive biological coatings for medical insertional materials, thus reducing hospital infections and use of synthetic drugs and chemicals. They may also be incorporated into probiotics preparations to combat urogenital tract infections and pulmonary immunotherapy [6]. In the present study, hydrocarbonoclastic bacteria were isolated from petroleum contaminated sites nearby petrol bunks in Chidambaram. Screening of biosurfactant producing potential strain was done by haemolytic assay and BATH assay and the potential strain was identified by biochemical tests as *Proteus inconstans.* After optimizing the biomass production in different parameters, the mass production of biosurfactant was carried out along with biodegradation and emulsification of crude oil.

MATERIALS AND METHODS

ISOLATION & IDENTIFICATION OF HYDROCARBONOCLASTIC BACTERIA (HCB)

Soil samples were collected from oil polluted site in the vicinity of petrol bunks near Chidambaram. Hydrocarbono-clastic bacteria were isolated using 50 ml of Bushnell Hass broth supplemented with 0.1% crude oil as sole carbon source in 250 ml conical flasks. One ml of water sample was inoculated. Flasks were maintained in a shaker with 150 rpm at room temperature for 4 days. Serial dilutions were made from the broth and the diluted samples were plated on Bushnell Hass agar [7]. Plates were incubated at 37°C for 4 days. Apparently different colonies were isolated based on their colony morphology.

The most promising strain was identified following Bergey's manual of determinative bacteriology [8]. Morphologically distinct colonies were isolated and purified by replicating on the same solid medium to obtain pure cultures.

SCREENING FOR BIOSURFACTANT PRODUCING BACTERIA

Haemolytic Activity:

Hydrocarbonoclastic bacteria was isolated from oil polluted site in the vicinity of petrol bunks near Chidambaram and screened for biosurfactant production in blood agar plates [9]. Strains were streaked on the plates containing blood agar and incubated for 48 hr at 37°C. The plates were visually inspected for zone of clearance around the colonies, which is an indication of biosurfactant production. The diameter of the clear zone was taken as an indicator of biosurfactant production. The strains showed maximum zone of clearance was taken as the highly potential biosurfactant producing strains.

Bacterial Adhesion to Hydrocarbons (BATH Assay) :

Based on the results obtained in the hemolytic activity three HCB strains were tested for BATH assay [10]. The optical density of cells in the mineral salt medium was determined initially at OD 660 nm.

Emulsification capacity (E24):

Emulsification activity experiment was done using 7 days old culture of the identified bacteria grown on Bushnell Hass broth medium with crude oil as the carbon source, broth culture was centrifuged at 6000 rpm for 20min and emulsification factor was precipitated using chilled acetone and vacuum dried. 3mg of dried precipitate was dissolved and suitably diluted Tris buffer (pH 8.0) in 30ml screw capped test tube. 0.1g of crude oil was added and shaken for 20min in a shaker at 150 rpm and the mixture was allowed to stand for 20min. Turbidity of stabilized emulsion was read at 610 nm in a spectrophotometer. Results were expressed as D610 [11]. Statistical analysis was performed using Minitab (Version 15) software.

Degradation of crude oil [12]

The degradation of crude oil was done by inoculating the bacterial culture in conical flasks

containing 50 ml of seawater medium, 450 mg of urea supplemented with 8.5 mg of crude oil for 7 days in a shaker.

The cultures in the flasks were centrifuged at 6000 rpm and the cell free aqueous solutions were collected. Then hydrocarbons in the solution were extracted three times using hexane. The extracts were stored at dark at a low temperature (5°C) before analysis. Estimation of crude oil in the samples was done following the method described in [13]. The solvent was evaporated to dryness in a rotary evaporator at 30°C under reduced pressure.

The hexane fraction was used for fluorimeter assay. The fluorescence of the sample was measured at 310 nm excitation and at 374 nm emission wavelength respectively (Vairian, Cary Eclipse, Fluorescence spec, Australia). Cresol oil was used as standard. Content of hydrocarbon in the samples after degradation were calculated from their OD values.

OPTIMIZATION OF BIOSURFACTANT PRODUCTION IN SHAKE FLASKS

Based on hemolytic activity and BATH assay all the 6 strains tested were found to be potential biosurfactant producers as per the criteria explained by [9]. Optimization study was conducted for the strains to find out the optimum conditions for the large scale production. Biosurfactant production was estimated at various temperatures (25, 30, 35 and 40°C), various substrates (Glucose, sucrose and glycerol) and pH (5.0, 6.0, 7.0, 8.0, 9.0and10).The impact of salinity on biosurfactant production was evaluated using various concentration of NaCI (0, 0.5, 1 and 1.5%). All the experiments were performed in 250ml conical flasks with 50ml of mineral salt medium [12]. Cultures were maintained in a water bath shaker at 150rpm.

FOURIER TRANSFORM INFRARED SPECTROSCOPY AND MASS SPECTROMETRIC ANALYSIS

FTIR is most useful for identifying types of chemical bonds (functional groups) and therefore can be used to elucidate some components of an unknown mixture. Ten milligrams of freeze-dried pure biosurfactant was ground with 100 mg of KBr and pressed with 7,500 kg for 30 s to obtain translucent pellets. Infrared absorption spectra were recorded on a Thermo Niocolet, AVATAR 330 FTIR system with a spectral resolution and wave number accuracy of 4 and 0.01 cm−1, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as background reference.

PLASMID ISOLATION AND CURING

The plasmids were isolated from oil degrading bacteria adopting the method of [14]. The DNA bands were viewed under UV trans-illuminator in 2D Gel-documentation system.The role of plasmids in the bacteriocin production was confirmed by curing the plasmid with acridine orange at a concentration of 500 µg /ml which was added to the culture broth and incubated for 12hrs [15]. The plasmid cured strains were screened antibiotic resistance activity.

RESULTS

To depict the environment from which the bacteria were isolated, physical and chemical characteristics of soil samples were determined. The grown populations of the oil-polluted site 3.43 $\times 10^4$ CFU/g was observed. Morphologically six different colonies were isolated from oil polluted site near petrol bunk and were screened for their ability to produce biosurfactant.

Haemolytic activity:

The morphologically different colonies were tested for hemolytic activity. In which maximum zone of clearance was observed as 19 mm and shown in (**Figure 1**).

Figure 1: Blood agar plate

BATH assay:

The positive strains of hemolytic assay were tested for BATH assay and maximum activity of 92 % was observed.

Identification of potential biosurfactant producing microbes:

The most potential biosurfactant producing microbe determined by hemolytic assay and BATH assay was identified based on the biochemical tests according to Bergey's Manual of Determinative Bacteriology. The results showed that the strain was *Proteus inconstans* (**Table 1**).

Table 1: **Morphological and Biochemical identification of *Proteus inconstans***

TESTS	RESULT
Gram's reaction	Gram negative
Shape	Rod
Motility	motile
Spore	Non sporing
Oxidase	-
Lactose	-
Glucose	+
Mannitol	-
KCN Test	+
H_2S	-
Phenyl alanine -deaminase	+
Arginine decarboxylase	-
Lysine decarboxylase	-
Methyl red	+
Indole	+
Urease	+
Citrate	+

+ indicates Positive , - indicates Negative reactions

Optimization of Biomass production and mass scale culture:

Optimization studies for biomass production was carried out and different incubation times (0-90 hours), pH (5-10), temperature (25-40^{o}C), salinity (0.1-1.5%) and different concentrations of crude oil as substrate (0.1-5%) were tested. Incubation period seems to be the important factor for the biomass production and maximum growth was observed at 72 hrs. Growth in different pH values showed maximum at pH 7. Maximum growth was observed in the glucose supplemented medium at temperature 35^{o}C and pH 7 with 0.3% salinity, glucose within 72hrs of incubation (**Figure 2, 3, 4, 5**).

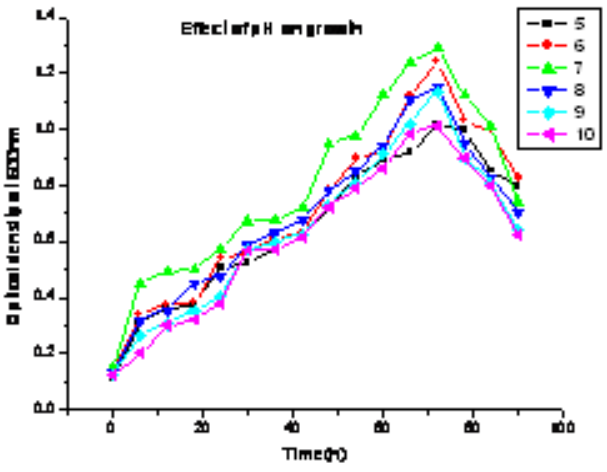

Figure 2: Effect of pH on growth

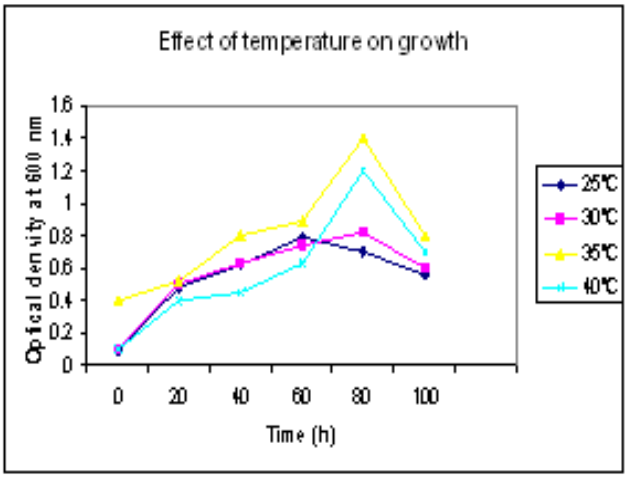

Figure 3: Effect of temperature on growth

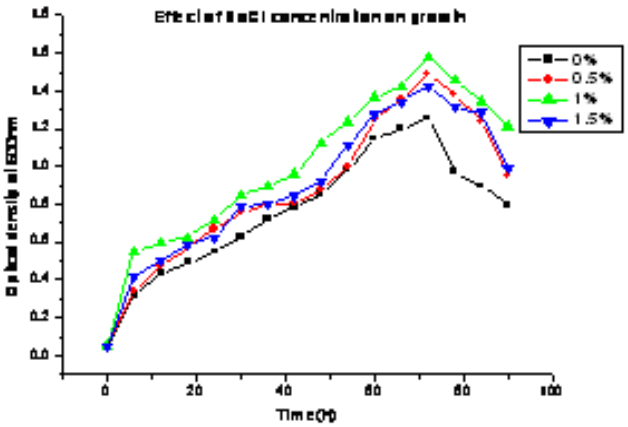

Figure 4: Effect of Nacl concentration on growth

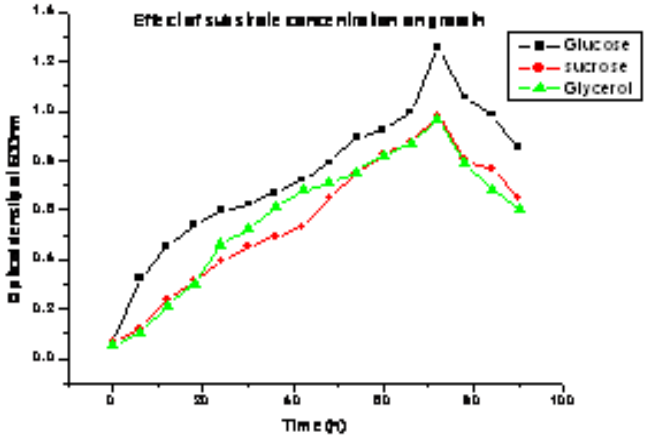

Figure 5: Effect of substrates on growth

Estimation of biodegradation and emulsification activity:

In the optimal conditions, 0.8 mg/mL of biosurfactant was produced. Emulsification activity of D_{610} was found to be 1.4 crude oil degradation was studied for 10 days and degradation activity was increased on incubation time and maximum degradation was observed in

168hrs (72%) where as 59% degradation activity was observed at 48hrs at temperature 35°C by Proteus *inconstans*.

Fourier Transform Infrared Spectroscopy and Mass Spectrometric Analysis:

The wave numbers 3515, 3481, 3371 indicated the presence of O-H bonds which showed the presence of carboxylic acids. The C-H bonds were observed at 2961 and 2925. C-H bonds of CH_2 group was observed at 1458 (Fig 6). The N-H bonds was referred by the wave number 883. These bonds confirmed the glycolipid nature of the biosurfactant (**Figure 6**).

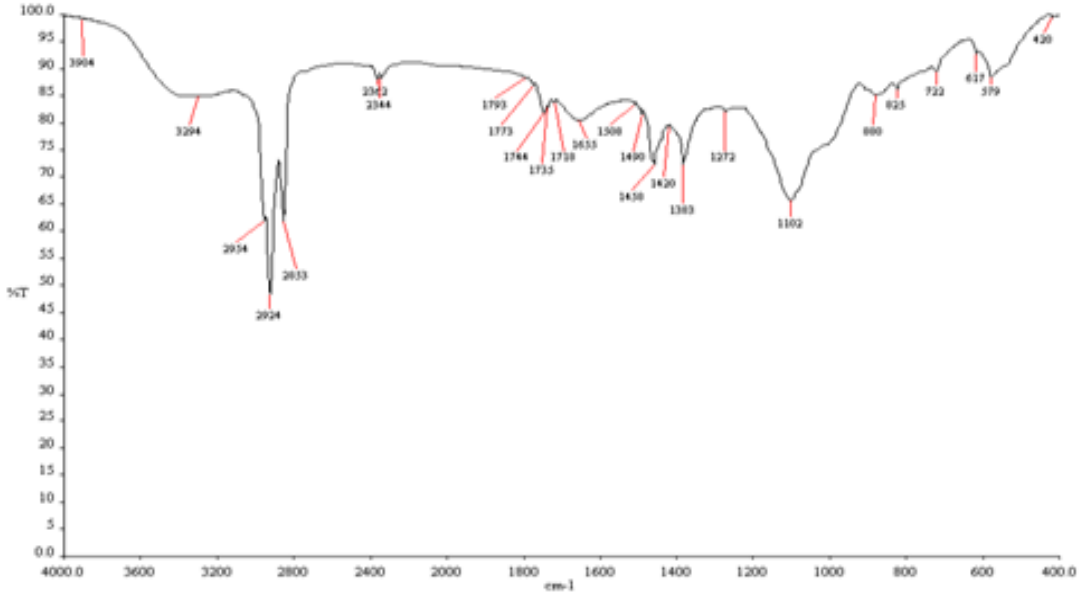

Figure 6: FT-IR Spectral assignment

Plasmid isolation and curing:

The *Proteus inconstans* strain screened to possess a single plasmid of 1.8 Kb.

After curing, the strain lost biodegradation and biosurfactant production ability (**Figures 7, 8**).

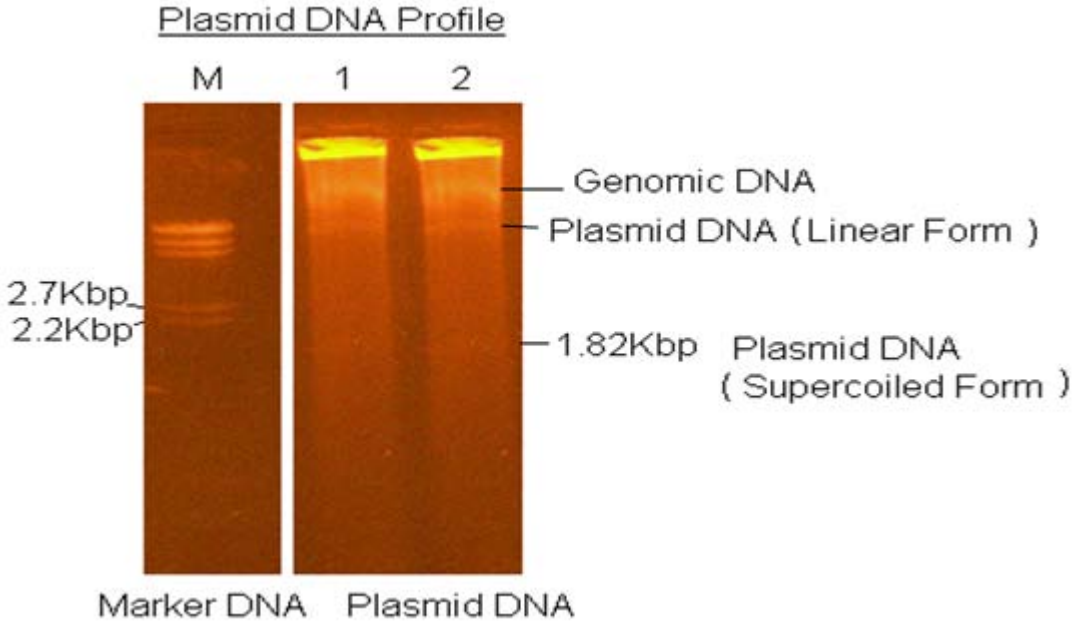

Figure7: Plasmid DNA isolation using Agarose gel electrophoresis

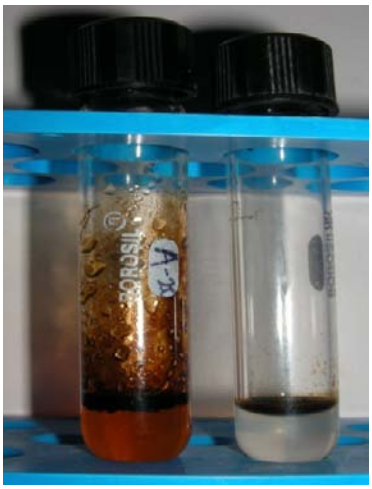

Figure 8: Plasmid curing

DISCUSSION

Soil is a known habitat and source of versatile microorganisms and since the microorganisms capable of emulsifying and solubilizing hydrophobic agents have an apparent advantage over their competitors, sampling of this nature provides a source rich in microorganisms with desired characteristics. Pure isolates were cultured in production medium and following centrifugation, supernatants were used for preliminary screening since excretion type bacteria that release biosurfactants to the culture medium are more interesting from the industrial point of view than bacteria with adherent biosurfactants due to the simplicity and economical aspects of the recovery Process.

The maximum density of hydrocarbanoclastic bacteria observed was 3.43×10^4 CFU/g. The higher counts of Hydrocarbon Utilizing Bacteria (HUB) in crude oil polluted soils ($3.60x10^3$ to $7.60x10^4$ CFU/g of soil) than crude oil free soil ($1.33x10^3$ to $1.66x10^4$ CFU/g of soil) [16]. The reason for higher counts in crude oil polluted soil may be due to the presence of residual crude oil in the polluted soil which boosts the carbon supply in the soil. Hence favour the growth of the hydrocarbon utilizing bacteria as compared to crude oil free soil [17, 18]. Similar experimental studies conducted shows the maximum biomass under the optimal condition using *Nocardia amarae*[19].High affinity of cells with crude oil was revealed by BATH assay for *Proteus inconstans* was reported as 95.3% [20].

In the present study, optimization studies were carried out for maximum biomass production. Incubation period seems to be the important factor for the biomass production and maximum growth was observed at 72h, growth in different pH values showed maximum at pH 7 and minimum at 10, maximum growth at temperature 35˚C and minimum at 25˚C, higher growth was observed at 0.3% salinity and minimum at 1.5% and maximum growth at glucose and minimum at glycerol was observed. The best environmental conditions for *R. ruber* AC 239 growing in M2 were 37°C, 200 rpm, initial pH 7.0, and 1% Diesel (v/v) [21]. Degradation of alcohol and hydrocarbons at different concentrations ranged from 0.13% to 2.0% and found better growth rate at 0.5% [22]. The results obtained from FTIR reveals the presence of glycolipid nature of the biosurfactant. Biodegradation of crude oil by *Pseudomoas aeruginosa* in the presence of rhamnolipids as been previously proved [23].

Results obtained in the plasmid analysis revealed that, *Proteus inconstans* was found to harbor single plasmid with molecular weight of 1.8 kb. The other bacterial strains namely *L. delbrueckii, C. kutscheri* and *B. megaterium* were also found to have single plasmid with respective molecular weight of 3.8, 4.2 and 4.1 kb. Presence of catabolic genes responsible for the degradation of naphthalene in plasmid of *P. putida* [24, 25].

These results conclude that, the *Proteus inconstans* isolated was identified as highly potential strain in the degradation of crude oil. It was also found to be suitable for bioremediation of oil contaminated sites. Their emulsion capacity makes them new potential candidates for biosurfactant and bioemulsion production. Further studies have been initiated to identify their properties and consequently determine the potential of their different industrial applications in particular enhanced oil recovery application.

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