BIOSURFACTANT PRODUCTION BY INDIGENEOUS PSEUDOMONAS AND BACILLUS SPECIES ISOLATED FROM AUTO-MECHANIC SOIL ENVIRONMENT TOWARDS MICROBIAL ENHANCED OIL RECOVERY

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ABSTRACT

The objective of this work was to study the biosurfactant production of two indigenous organisms isolated from auto-mechanic polluted soil environment and to evaluate their oil recovery efficiency. In this study, six bacteria were isolated from auto-mechanic polluted soil environment. Isolated strains were identified by morphological, biochemical, and physiological characterization. Among these, two isolates *(Bacillus* sp. and *Pseudomonas* sp.) were further selected and used for the production of biosurfactant. *Bacillus* and *Pseudomonas* species were grown in mineral salt medium (MSM) with addition of 3% (w/v) glucose. In the growth kinetic study, the maximum biosurfactant production occurred at 120 h of incubation (2.2 g/l) and maximum biomass was observed at 120 h (3.2 g/l) for *Bacillus* isolate. While for *Pseudomonas* isolate, the maximum biosurfactant production occurred at 96 h of incubation (2 g/l) and maximum biomass at 72 h (2.6 g/l). Different nitrogen sources as well as the effect of salinity and temperature were evaluated for their effect on biosurfactant production. Yeast extract and sodium nitrate was the best nitrogen source for the production of biosurfactant by *Bacillus* and *Pseudomonas* isolates, respectively. The environmental factors such as temperature 30 ^{o}C and salinity (0.2% w/v) were found to be optimum for the biosurfactant production. The stability of the biosurfactant was investigated at different salinities and temperature. The biosurfactant was effective at very low concentrations over a wide range of temperature and salt concentration. The results obtained showed that the biosurfactants have a good oil recovery efficiency thus being more attractive to be applied in microbial enhanced oil recovery.

Keywords: Biosurfactant; Bacillus species; Emulsification index; Pseudomonas species; Surface tension; Microbial enhanced oil recovery.

INTRODUCTION

Microbial enhanced oil recovery (MEOR) is the method of using microorganisms and their metabolic by-products to improve recovery of oil from the reservoirs after secondary oil recovery (Bryant and Lockhart 2002; McInerney et al. 2005; Lazar et al. 2007). This technique is based on in situ growth and metabolism of selected microorganism in the reservoir rock with certain nutrients. The microorganism produce gases and/or chemicals in the formation such as polymer, acids, gases, surfactants and biomass Production of these metabolites in the formation changes fluid and rock properties of the reservoir and improves the sweep efficiency. These changes respectively, increase the oil production from the reservoir. The idea of MEOR was first proposed by Beckmann (1926) when he published results on the possibility to use microbial metabolites to improve the oil production rate. Additional work by Zoebell, 1947 showed that these metabolites are analogue to the chemicals used in chemical enhanced oil recovery and are expected to perform the job of residual oil recovery. There are three major mechanisms by which microorganisms may contribute to enhanced oil recovery: i) microorganisms can produce biosurfactants and

biopolymers on the cell surface, ii) microorganisms produce gases and acids to recover trapped oil and iii) microorganisms can selectively plug high permeability channels into the reservoir (Bryant, 1987). Producing gases, biosurfactants, biopolymers and other non-toxic biochemical may be carried out *in situ* (in reservoir rock) or *ex situ* (in the controlled environment of a fermenter). The mechanisms by which MEOR process operate can be quite complex and may involve multiple biochemical and biophysical process steps (Janshekar1985; Donaldson et al.1989; McInerney et al. 2005; Lazar et al. 2007). These mechanisms can be different from bacteria to bacteria and are normally selected based on reservoir requirement. In general, the mechanisms normally include selective plugging, rock dissolution, emulsification, viscosity reduction, permeability modification and wettability alteration. Recently many investigations on MEOR have used whole cells and their biosurfactants to improve the efficiency of oil recovery (Joshi et al., 2008; Toledo et al., 2008; Jinfeng et al., 2005; Rashedi et al., 2005).

Biosurfactants or surface-active agents are amphiphilic molecules that comprise both hydrophobic and hydrophilic moieties, being the apolar component usually a carbon chain, whereas the polar part, more variable, can be ionic (anionic or cationic) or non-ionic (Nitschike et al., 2005). These compounds are able to reduce surface and interfacial tensions, as well as to form and stabilize oil in water or water in oil emulsions (Desai and Banat, 1997). Among several potential applications of biosurfactants, its use in MEOR represents one of the most promising methods to recover substantial amounts of the residual oil entrapped in mature oil fields (Banat et al., 2010; Simpson et al., 2011). The replacement of conventional synthetic surfactants by these biocompounds appears to be a good and efficient approach; however it still depends on the strategy adopted. The use of biosurfactants in MEOR can be performed in two different ways. In the first one, biosurfactants are produced ex situ, and subsequently injected into the reservoir. The other option is to produce the biosurfactant in situ by stimulation and/or injection of indigenous microorganisms. The first approach is limited by the costs involved in the biosurfactant production and purification processes. On the other hand, the costs involved in the second approach are apparently lower; however it requires that the microorganisms used are properly stimulated and able to produce sufficient amounts of the biosurfactant (Banat et al., 2010). Moreover, taking into account the use of biosurfactants in in situ MEOR processes, the greatest challenge is to isolate microorganisms that are able to grow under anaerobic conditions, high salinities, temperatures and pressures. Several reports have described the isolation of B. subtilis strains from oil reservoirs (Yakimov et al., 1995; Ghojavand et al., 2008; Youssef et al., 2009; Simpson et al., 2011), which suggest that these organisms can be successfully used under reservoir conditions.

Despite the short-coming and limitations of in situ MEOR technology, the production of biosurfactant by diverse microflora has been implicated to explain some of the effects and mechanisms involved in the observed oil displacement. The main objectives of this study are to isolate biosurfactant producing bacteria from auto-mechanic polluted soil environment for use in MEOR, investigate the growth and its metabolic products potential or capability to enhance oil recovery at elevated temperature and salinity. Also, biosurfactant production by the isolated biosurfactant-producing organisms was optimized through a proper manipulation of carbon and nitrogen sources. It is expected that the knowledge gain from this study can give useful information in successful implementation of MEOR technology for enhanced oil recovery.

MATERIALS AND METHOD

Isolation and enrichment of biosurfactant producing microorganisms

Soil samples were collected at subsurface level at five different points, pooled together and stored in closed containers at 4°C prior to use. The spread plate technique (APHA, 2005) using nutrient agar (Oxoid) was employed for their isolation. One (1) gramme of soil sample was taken and serially diluted in 0.85% sterile saline water. All dilutions were performed in triplicates and then the samples were spread on nutrient agar plates The plates were incubated at room temperature for 1 to 2 days. After incubation, plates were enumerated and morphologically different pure bacterial isolates were characterized and identified using various criteria, as described by Krieg et al. (1994). Pure isolates were transferred into nutrient agar slants stored at 4^{o}C and served as the stock cultures for subsequent tests. Six predominant bacterial genera were identified, which include *Alcaligenes, Aeromonas, Bacillus, Micrococcus, Pseudomonas* and *Serratia*. Colonies of Bacillus and Pseudomonas species which possessed biosurfactant-producing activity as reported in the literature (Aparna et al., 2012; Pereira et al., 2013; AnnaJoice et al., 2014) were chosen for further experimentation.

Biosurfactant Production

The inoculums were prepared using Luria Bertani (LB) broth and incubated overnight at 37◦C with 100 rpm agitation. Production was carried out using production medium composed of Glucose – 1(g/l) KH_2PO_4 - 0.5 (g/l), K_2HPO_4-1(g/l), KCl - 0.1(g/l), $MgSO_4$ - 0.5(g/l), $FeSO_4$ – 8 (mg/l), $CaCl_2$ – 50 (mg/l) ,Urea – 6 (mg/l) with the addition of 1ml/l trace elements solution ($ZnSO_4$ - 4.4 mg/l, $MnSO_4$ - 3.3 mg/l, $CuSO_4$ - 0.1 mg/l) at pH 7. The production medium was seeded with 3% inoculum and incubated at 37°C for 48 hours with 150 rpm agitation. The cell free supernatant was used as crude surfactant.

Effect of nitrogen sources on biosurfactant production

The effect of nitrogen sources on biosurfactant production were determined under submerged conditions. The nitrogen sources evaluated in the study were yeast extract, meat extract, urea, sodium nitrate and ammonium nitrate (0.3%). A control flask without nitrogen source was also maintained. The flasks were maintained at 30ºC in an incubator shaker at 150 rpm

Extraction and biochemical analysis of biosurfactant

The production culture was sampled and centrifuged at 3000 rpm for 15 min. The supernatant fluid was decanted and filtered immediately through whatman No. 1 filter paper. The cell free supernatant was acidified to pH 2 using HCl (6 M) and left overnight in a refrigerator for complete precipitation of biosurfactant. The acidified precipitate was then separated and distilled water added. The biosurfactants were extracted from cell-free supernatants using the Folch extraction method that is commonly used to extract lipids from biomolecules (Folch, 1957). The biosurfactant was extracted by adding chloroform/methanol (2:1, v/v) mixture at room temperature and was then concentrated using a rotary evaporator (Liu et al., 2011) and finally freeze dried. Lipid content of the crude biosurfactant was determined according to the method of Folch (1957). Total sugars of the crude biosurfactant were determined by the phenol-sulphuric acid method (Dubois *et al*., 1956).

Characterization of the produced biosurfactant
Oil spreading test

This test involves measuring diameters of clear zones caused when a drop of a biosurfactant-containing solution is placed on an oil-water surface. Fifty milli litre (50 ml) of distilled was poured into a large Petri dish (15 cm diameter) and 20 µl of gasoline + kerosene + diesel oil mixture and 10 µl of culture broth supernatant was added to the water surface. A clear halo was visible under light. The area of the cycle was measured and calculated for oil displacement area (ODA) using the following equation:

$$ODA = \frac{22}{7} \times (radius)^2 (cm^2) \qquad (1)$$

Surface tension measurement

A sample of either the effluent or the mixed liquor taken from the bioreactors was centrifuged at 4 ^{o}C and 8500 rpm for 20 min in order to remove the microbial cells. The surface tension of the obtained supernatant was measured by using a drop shape analysis system (Krüss, DSA10 Mk2) and was carried out at room temperature using the pendant drop method. (For the calibration of the instrument, the surface tension of pure water was first measured.) The measurement was repeated at least 3 times, and an average was used to express the surface tension of the sample. The percentage reduction of surface tension is calculated by the following equation:

$$\%\text{surface tension reduction} = \frac{\gamma_m - \gamma_c}{\gamma_m} \times 100 \qquad (2)$$

where γ_m is the surface tension of the feed solution and γ_c is the surface tension of the centrifuged sample.

Emulsification index (E_{24}) determination

The emulsification activity (or index) of the biosurfactant was measured as described by Cooper and Goldenberg (1987). Three (3) ml of different oil samples was added to 2 ml of the biosurfactant solution in a graduated test tube and vortexed at high speed for 2 min. at room temperature and was subsequently allowed to stand for 24 h. The emulsification index (E_{24}) was then calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100. All emulsification indexes were performed in triplicate.

Biomass estimation

Bacterial cell growth was monitored by measuring the dry cell weight method. It was determined by centrifugation (10,000 rpm for 30 minutes) of a 1 ml culture broth; the cell pellet was washed with distilled water twice and dried by heating at 50 ^{o}C until constant weight was attained.

Biosurfactant stability characterization

To determine the thermal stability of the biosurfactant, cell-free supernatant of *Bacillus* and *Pseudomonas* species were maintained at a constant temperature range of 25 -100 ^{o}C for 15 min, followed by cooling at room temperature (28 ± 2 ^{o}C). To study the effect of different concentration of NaCl on the activity of the biosurfactant, the biosurfactant was re-dissolved

after purification with distilled water containing the specific concentration of sodium chloride (1 to 16 per cent, w/v).

Application of biosurfactants in removal of crude oil from sand

The applicability of biosurfactants produced by Bacillus and Pseudomonas species, respectively, in oil recovery was evaluated using artificially contaminated sand containing 10% (w/w) of gasoline + kerosene + diesel oil mixture. Soil samples of 40 g were mixed with 4 g of gasoline + kerosene + diesel oil mixture in 250 ml Erlenmeyer flasks by shaking and allowed to age for 24 h. Thereafter, 40 ml of biosurfactants and commercial surfactants solutions at a concentration of 1 g/l were added to each flask. The flasks were incubated at 150 rpm and 40 ^{o}C for 24 h. After which, the oil removed was recovered from the surface and its volume was measured. The control experiments were performed using de-ionized water at the same conditions. All the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Effect of nitrogen sources on growth and biosurfactant production

The type of nitrogen present in growth medium influences the biosurfactant produced by microbial cells (Desai et al., 1994). Table 1 shows the effect of nitrogen sources on microbial cell growth and biosurfactant production. The highest biomass production was obtained using yeast extract as the sole nitrogen source for Bacillus isolate, while for *Pseudomonas* isolate sodium nitrate gave the highest biomass production. The lowest surface tension value (37 mN/m) which corresponded to the highest biosurfactant production (2.56 g/l) was obtained with yeast extract for *Bacillus* isolate. The lowest surface tension value (35.8 mN/m) which corresponded to the highest biosurfactant production (2.20 g/l) was obtained with sodium nitrate for *Pseudomonas* isolate. Other authors have reported crude biosurfactant productions between 720 and 2288 mg/l (Makkar and Cameotra, 1997; Abdel-Mawgoud et al., 2008; Pereira et al., 2013). Robert et al. (1989) and Abdel-Mawgoud et al. (2008) have reported the use of sodium nitrate and ammonium nitrate as nitrogen sources for the highest biosurfactant production by *Pseudomonas aeruginosa*. Pereira et al. (2013) reported the use of yeast extract for Bacillus isolate #309; ammonium nitrate and ammonium sulphate for Bacillus isolate #573 and ammonium sulphate and yeast for Bacillus isolate #311; as sources of nitrogen for their highest biosurfactant production, respectively. Other authors have reported the highest biosurfactant production by *Bacillus subtilis* using urea (Makkar and Cameotra, 1997; Ghribi and Ellouze-Chaabouni, 2011). Khopade et al. (2012) reported the use of yeast extract followed by tryptone and urea, respectively, for highest biosurfactant production by Streptomyces species B3. The highest emulsifying index (E_{24}) was obtained with sodium nitrate for Pseudomonas isolate (65.2%) while for Bacillus isolate, the highest emulsifying index was obtained with yeast extract (60.8). Similar observation has been reported (Pereira et al., 2013). Dastgheib et al. (2008) reported that sodium nitrate was the best substrate for emulsifier production, followed by urea, yeast extract and peptone. Taking into account the amounts of biosurfactant produced with the different nitrogen sources, and in order to standardize the medium for all isolates, sodium nitrate was selected for the production of biosurfactants to be further used in the other experiments. Moreover, the use of sodium nitrate represents a good compromise between biosurfactant yields and the costs associated with their production. More complex nitrogen sources provide higher emulsification indexes; however their cost is also higher which makes a potential application in MEOR economically unfeasible (Pereira et al., 2013). Also, nitrate is crucial for microbial growth under the low oxygen concentrations present in reservoirs.

Table 1: Surface tension values (mN/m), emulsifying indexes (%), biomass concentrations (g dry weight/l) and biomass concentrations (g/l) obtained for Bacillus and Pseudomonas isolates grown in glucose (1 g/l) with different nitrogen sources (2 g/l) at 30 ^{o}C for 120 h. Results represent the average of three independent experiments ± standard deviation

Nitrogen source	Biomass Concentration (g/l)	Biosurfactant Concentration (g/l)	Emulsification Index (E_{24}) (%)	Surface Tension (mN/m)
Bacillus isolate				
Control	0.30 ± 0.09	0.11 ± 0.07	10.0 ± 0.11	65.0 ± 0.20
Yeast extract	3.20 ± 0.15	2.56 ± 0.11	60.8 ± 0.17	37.0 ± 0.10
Meat extract	2.00 ± 0.08	1.52 ± 0.13	50.0 ± 0.15	44.6 ± 0.11
NH_4NO_3	2.40 ± 0.12	2.00 ± 0.15	54.5 ± 0.13	41.5 ± 0.14
$NaNO_3$	2.60 ± 0.16	2.42 ± 0.10	59.7 ± 0.12	39.5 ± 0.15
Urea	1.45 ± 0.10	1.19 ± 0.12	45.4 ± 0.14	46.2 ± 0.09
Pseudomonas isolate				
Control	0.45 ± 0.04	0.14 ± 0.11	12.0 ± 0.09	68.0 ± 0.17
Yeast extract	1.70 ± 0.12	1.24 ± 0.13	55.2 ± 0.10	43.2 ± 0.12
Meat extract	1.25 ± 0.10	0.84 ± 0.12	52.0 ± 0.13	46.7 ± 0.10
NH_4NO_3	2.00 ± 0.13	1.65 ± 0.14	60.6 ± 0.15	40.0 ± 0.13
$NaNO_3$	2.40 ± 0.15	2.20 ± 0.13	65.2 ± 0.17	35.8 ± 0.15
Urea	1.10 ± 0.11	0.72 ± 0.15	50.4 ± 0.11	48.5 ± 0.09

Kinetics of biosurfactant production

The biosurfactant production and surface tension was dependent on growth of culture in the fermentation medium. The surface tension dropped rapidly after inoculation, reaching its lowest value

of 25 mN/m for Bacillus species during exponential growth phase after about 120 h of growth (Fig. 1a) and 40 mN/m for Pseudomonas species after about 72 h of growth (Fig. 1b). On the 24 h of growth, biosurfactant concentration starts to increase, reaching its maximum after about 120 h (day 5) (Fig. 1a) and 72 h (day 3) (Fig. 1b) for *Bacillus* species and *Pseudomonas* species, respectively. The increase in surface tension and the decrease in E_{24} after 120 h and 72 h of incubation respectively for Bacillus and Pseudomonas species showed that biosurfactant synthesis has been stopped and may probably be due to the production of secondary metabolites that could interfere with emulsion formation and the adsorption of surfactant molecules at the oil–water interface (Khopade et al., 2012). These results revealed that the biosurfactant biosynthesis occurred predominantly during the exponential growth phase, which suggests that the biosurfactant is produced as primary metabolite accompanying cellular biomass formation (growth-associated kinetics) (Khopade et al., 2012).

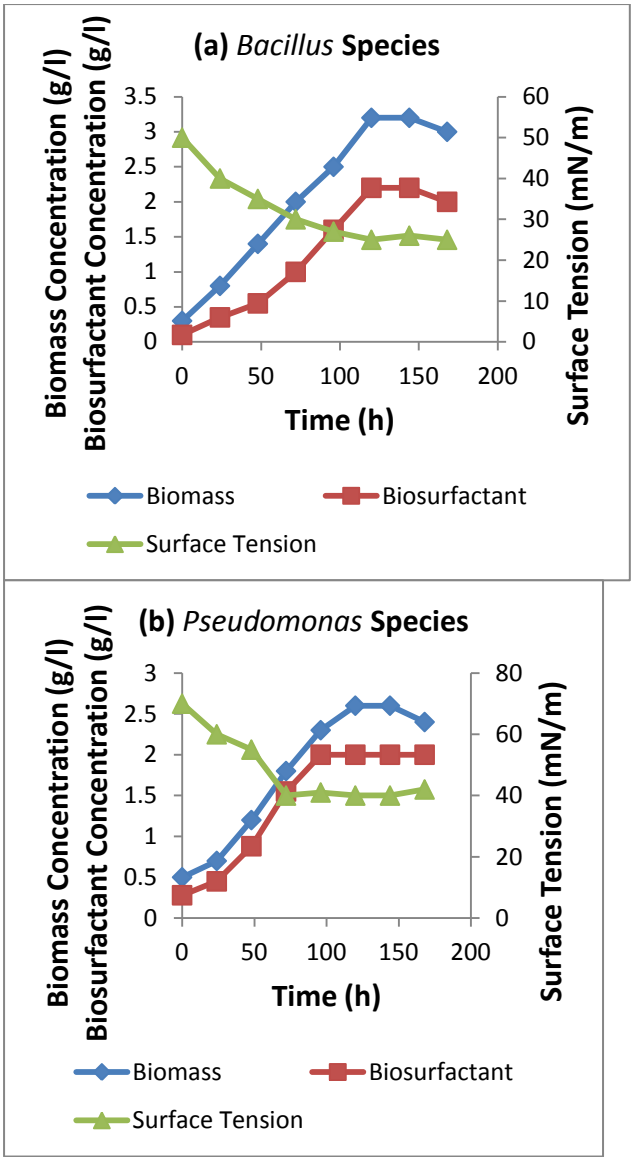

Fig. 1: (a) Time course profile of biosurfactant synthesis, cell growth and surface tension reduction by Bacillus species in 3% glucose (w/v) (b) Time course profile of biosurfactant synthesis, cell growth and surface tension reduction by Pseudomonas species in 3% glucose (w/v)

Effect of temperature and salinity on biosurfactant production

The temperature and salinity of the medium were important characteristics for cell growth of organism and production of secondary metabolites. Temperature was one of the critical parameters that have been controlled in bioprocess. The *Bacillus* and *Pseudomonas* strains showed good growth between the temperature range of 25 – 45 ^{o}C. The results in the present study showed that the biosurfactant activity reached the highest when the *Bacillus* and *Pseudomonas* strains were respectively grown at 30 ^{o}C (E_{24} = 70% for *Bacillus* strain and 79% for *Pseudomonas strain*) (Fig. 2a and 2b), and this clearly indicates moderately thermo stable nature of biosurfactant. A similar observation has been reported for *Streptomyces* bacteria strain (Khopade et al., 2012). However, Youssef et al. (2004) and Bento et al. (2005) have both reported that a change in temperature caused alterations in the composition of the biosurfactant produced by *Pseudomonas* species and *Arthrobacter paraffineus*, respectively. The *Bacillus* and *Pseudomonas* strains were able to show good growth between the NaCl

concentration range of 0.2 to 2% w/v. Fig. 2c and 2d showed that maximum biosurfactant production by *Bacillus* (E_{24} = 70%) and *Pseudomonas* (E_{24} = 79%) strains respectively was obtained in the presence of 0.2% (w/v) of NaCl and remained the same up till 0.8% (w/v) of NaCl. Nevertheless, *Bacillus* and *Pseudomonas* strains showed an emulsification activity (E_{24}) of 52% and 55%, respectively, in the presence of 2% (w/v) of NaCl (Fig. 2c and 2d).

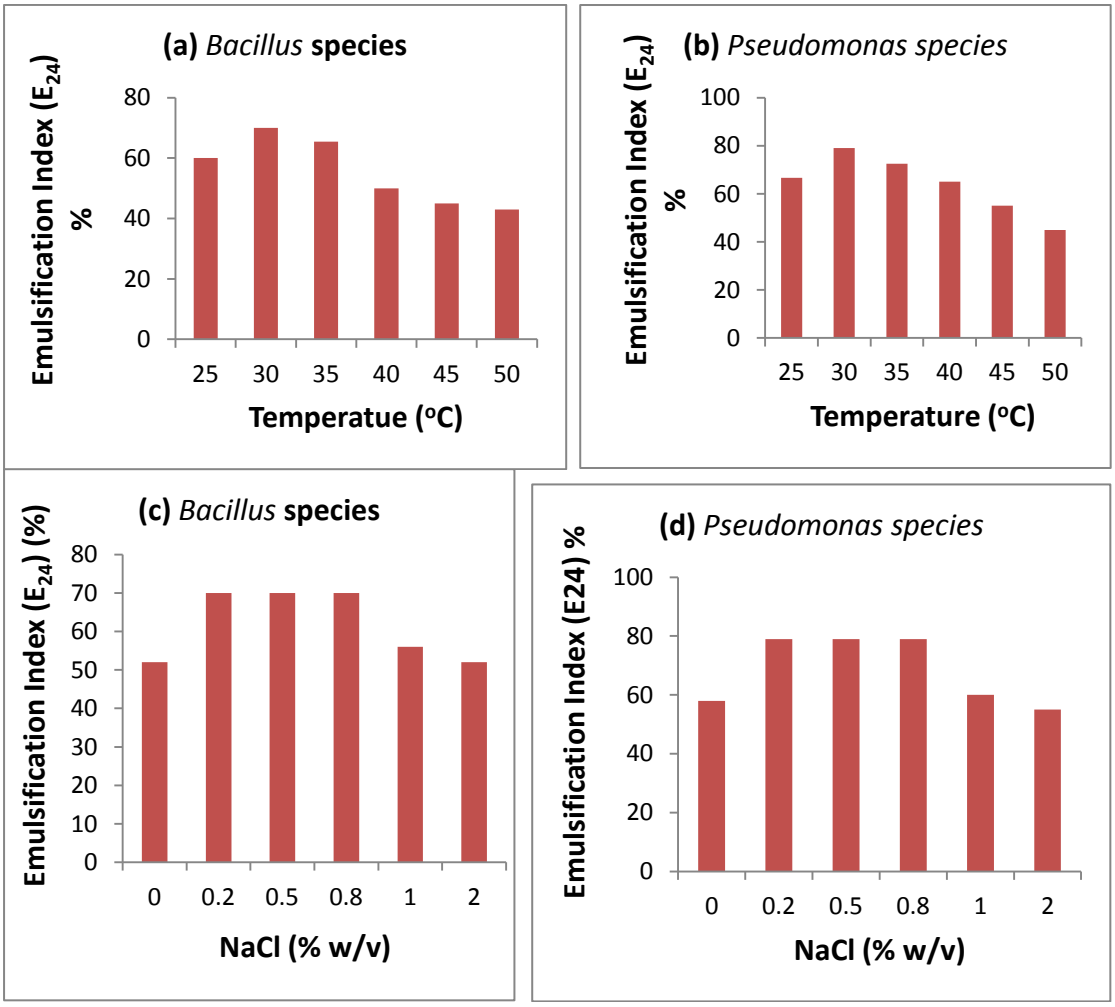

Fig. 2: (a) Effect of temperature on biosurfactant production by *Bacillus* species (b) Effect of sodium chloride (NaCl) on biosurfactant production by *Bacillus* species (c) Effect of temperature on biosurfactant production by *Pseudomonas* species (d) Effect of sodium chloride (NaCl) on biosurfactant production by *Pseudomonas* species

Effect of temperature and salinity on biosurfactant stability

The applicability of biosurfactants in several fields depends on their stability at different temperatures. The stability of biosurfactant was tested over a wide range of temperatures. The biosurfactant produced by *Bacillus* and *Pseudomonas* species respectively was shown to be thermo stable as indicated by the stability in the surface tension values and the oil displacement area (Fig. 3a and 3b). Thus, heating of the biosurfactant to 100 ^{o}C caused no significant effect on the biosurfactant performance. Abouseoud et al. (2008), Techaoei et al. (2011), Khopade et al. (2012) and AnnaJoice and Parthasarathi (2014) hve reported similar observations for biosurfactants produced by *Pseudomonas fluoresens*, *Pseudomonas aeruginosa* SCMU106, *Streptomyces* sp. and *Bacillus aureum* MSA13, respectively.

Therefore, it can be concluded that these biosurfactants maintains their surface properties unaffected in the range of temperatures between 30 and 100 ^{o}C.

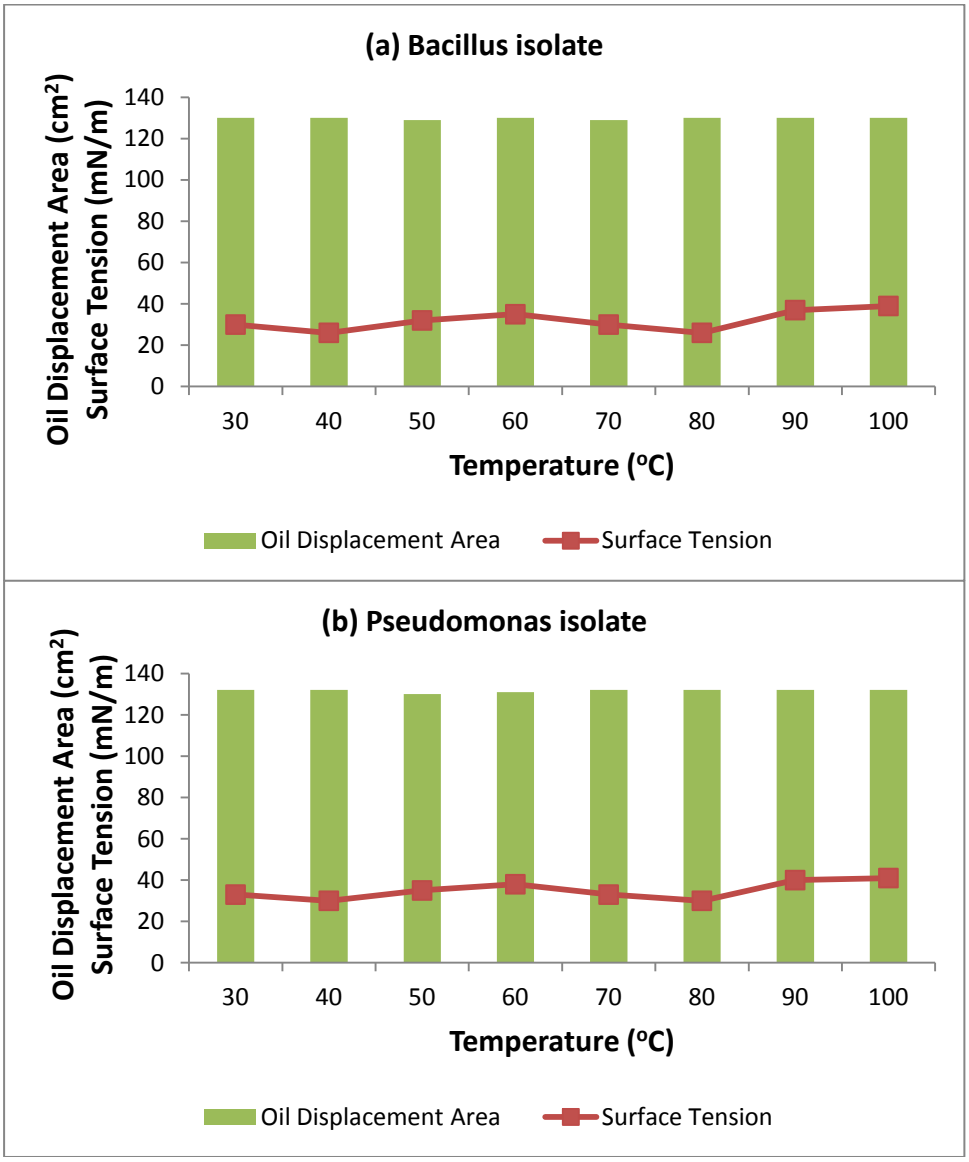

Fig. 3: Effect of temperature on biosurfactant stability for (a) *Bacillus* species (b) *Pseudomonas* species

The effect of sodium chloride addition on biosurfactant produced from *Bacillus* and *Pseudomonas* species was studied. Maximum stability of biosurfactant was observed at 16% (w/v) of NaCl concentration as indicated by the lower surface tension value (Fig. 4a and 4b). Its oil displacement area decreased at high NaCl concentration (above 8%). Techaoei et al. (2011) and Khopade et al. (2012) have reported the stability of biosurfactant produced by *Pseudomonas aeruginosa* at NaCl concentration range of 2 – 20% (w/v) and biosurfactant produced by *Streptomyces sp*. at NaCl concentration range of 1 – 9% (w/v), respectively. Biosurfactants that has stability at high salinity may be useful for bioremediation of spills in marine environment because of its stability in the presence of salt (Prieto et al., 2008) as well as in the enhancement of oil recovery from petroleum reservoirs.

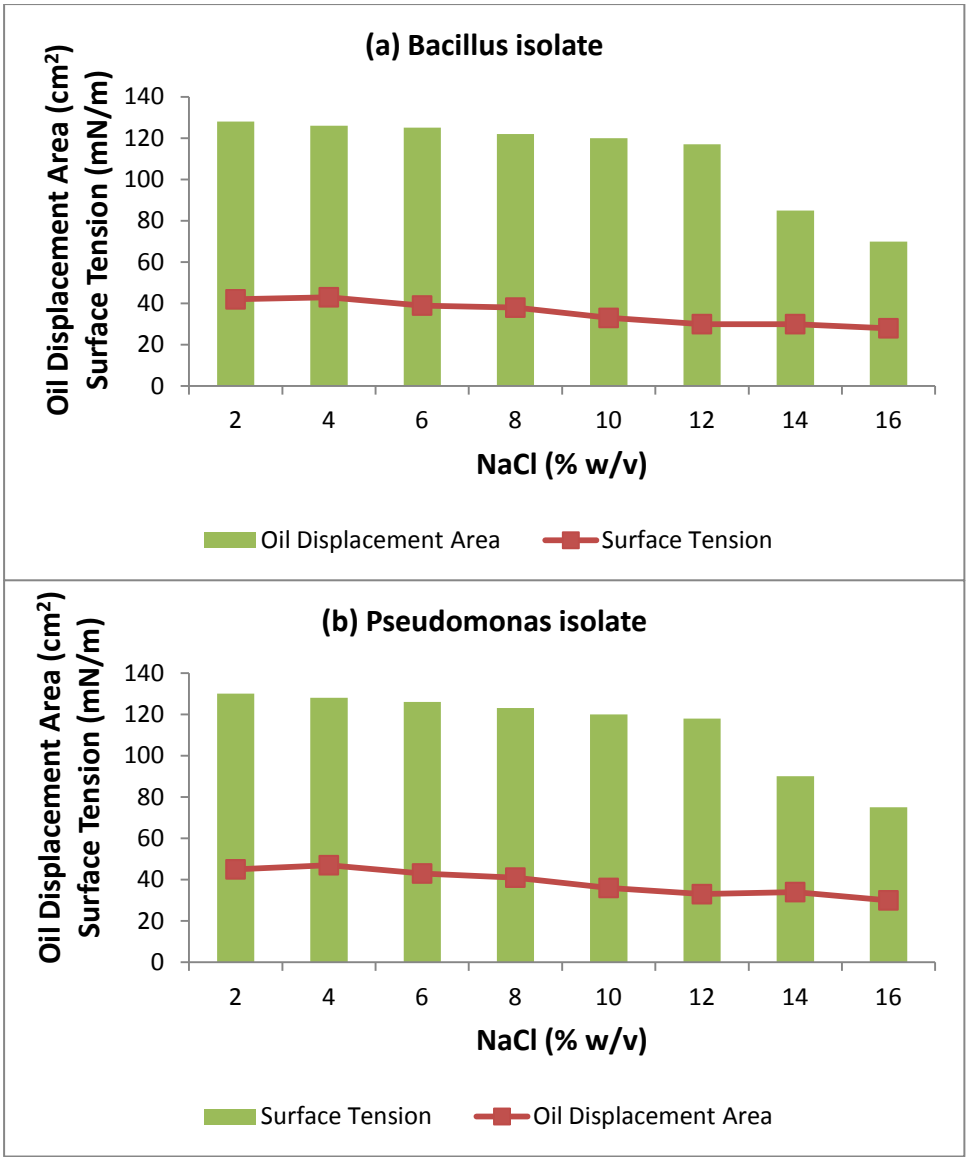

Fig. 4: Effect of salinity on biosurfactant stability for (a) *Bacillus* species (b) *Pseudomonas* species

Application of biosurfactants in removal of oil from sand

The performance of biosurfactants and chemical surfactants in oil recovery was studied using crude oil contaminated sand. As can be seen from the results obtained (Table 2), the different biosurfactants at a concentration of 1 g/l recovered between 45% and 40% of oil.

Table 2: Percentages of oil recovered by different biosurfactants at a concentration of 1 g/l

Biosurfactant	Percentage of Oil Recovered
Bacillus isolate	45
Pseudomonas isolate	40

CONCLUSION

From the experimental investigation of biosurfactant production by indigenous pseudomonas and bacillus species isolated from auto mechanic soil environment towards microbial enhanced oil recovery the following deductions were made:

- ✓ Produced biosurfactants have the highest activities when the *Bacillus* and *Pseudomonas* strains were grown at 30 ^{o}C.
- ✓ Biosurfactants biosynthesis occurred predominantly during the exponential growth phase.
- ✓ Biosurfactant surface tension and oil displacement area are stable over wide range of temperatures and salinities.
- ✓ Biosurfactants have a good oil recovery efficiency thus being more attractive to be applied in microbial enhanced oil recovery.

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