African Journal of Biotechnology Vol. 11(103), pp. 16751-16757, 25 December, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1510
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Isolation of potent biosurfactant producing bacteria from oil spilled marine water and marine sediments

Seema Dhail

Department of Biotechnology and Allied Sciences, Jayoti Vidyapeeth Women's University, Jaipur- 303007, India.
E-mail: seemaresearch21@gmail.com. Tel: +91-9785235164.

Accepted 11 December, 2012

Biosurfactants produced by bacteria are surface-active compounds involved in the degradation of hydrocarbons. They are heterogeneous group of surface active molecules produced by microorganisms, which adhere to cell surface or are excreted extracellularly in the growth medium. These can be divided into low- molecular weight molecules, that lower surface and interfacial tensions efficiently and high-molecular-weight polymers that bind tightly to surfaces. These surfactants, produced by a wide variety of microorganisms, have very different chemical structures and surface properties. It is therefore reasonable to assume that different groups of biosurfactants have different natural roles in the growth of the producing microorganisms. In this study, isolation and identification of biosurfactant producing bacteria were assessed from oil-spilled seawater collected from harbors and docks from Arabian Sea (Mumbai), India. The potential application of these bacteria in microbial enhanced oil recovery (MEOR) was investigated. To confirm the ability of isolates in biosurfactant production, various biosurfactant activity assay tests and measurement of surface tension were conducted. Hemolysis was used as a criterion for the primary isolation of biosurfactant producing bacteria. Effects of different pH, salinity and temperature on biosurfactant production were also studied. Among all of the isolated strains, *Pseudomonas sp.* (MW2) showed high salt tolerance, successful production of biosurfactant in a vast pH and temperature domain and reduced surface tension of the medium to value below 40 mN/m. This strain is a potential candidate for MEOR. The MW2 biosurfactant component was detected as glycolipid in nature.

Key words: Biosurfactant, emulsification, extreme conditions, microbial enhanced oil recovery, marine water.

INTRODUCTION

The large scale production and transportation of petroleum and its products has resulted in dispersion of oil in the marine environment through various activities. A diversified group of petroleum products are amenable to microbial degradation. The activities of microbes involving various complex biodegradation pathways produce a series of primary metabolites which act as synergistic intermediates that accelerate the process of biodegradation. Biosurfactant are surface-active compounds of a heterogeneous group of surface active molecules produced by microorganisms, which either adhere to cell surface or are excreted extracellularly in the growth medium (Fietcher, 1992; Zajic and Stiffens, 1994; Cameotra and Makker, 1998). These biosurfactant molecules reduce surface tension. Several types of biosurfactant have been isolated and characterized, including glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids and lipopolysacharides. Chemically-synthesized surfactants have been used in the oil industry to aid clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment. Due to the amphiphilic structure of biosurfactants they increase the surface area of hydrophobic water-insoluble substances, increase the water bioavailability of such substances and change the properties of the bacterial cell surface. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents (Desai and

Banat, 1997). In comparison to their chemically synthesized equivalents, they have many advantages: they are environmentally friendly, biodegradable, less toxic and non-hazardous. They have better foaming properties and higher selectivity. They are active at extreme temperatures, pH and salinity as well, and can be produced from industrial wastes and from by-products. This last feature makes cheap production of biosurfactants possible and allows utilizing waste substrates and reducing their polluting effect at the same time (Kosaric, 2001). The biosurfactants accumulate at the interface between two immiscible fluids or between a fluid and a solid. By reducing surface (liquid-air) and interfacial (liquid-liquid) tension, they reduce the repulsive forces between two dissimilar phases and allow these two phases to mix and interact more easily (Soberón-Chávez and Maier, 2011). The most active biosurfactants can lower the surface tension of water from 72 to 30 $mN \cdot m^{-1}$ and the interfacial tension between water and *n*-hexadecane from 40 to 1 $mN \cdot m^{-1}$ (Soberón-Chávez and Maier, 2011). Biosurfactant activities depend on the concentration of the surface-active compounds until the critical micelle concentration (CMC) is obtained. At concentrations above the CMC, biosurfactant molecules associate to form micelles, bilayers and vesicles. Micelle formation enables biosurfactants to reduce the surface and interfacial tension and increase the solubility and bioavailability of hydrophobic organic compounds (Whang et al., 2008). The CMC is commonly used to measure the efficiency of surfactant. Efficient biosurfactants have a low CMC, which means that less biosurfactant is required to decrease the surface tension (Desai and Banat, 1997). Biosurfactant have special advantage over their commercially manufactured counterparts because of their lower toxicity, biodegradable nature, and effective at extreme temperature, pH, salinity and ease of synthesis (Banat, 1995; Desai and Banat, 1997; Makker and Cameotra, 1998). Oil production using the natural inherent energy in the reservoir may yield 20 to 30% of the original oil in place. Using general primary and secondary water flooding methods, over half of the original oil in place remains unrecoverable. Conventional technologies used in oil recovery extract only about 30 to 45% of the original oil in place (Ramirez, 1987). Today, successful enhancement of oil recovery depends on the right combination of technologies. The use of microorganisms to improve oil recovery was established by Beckman since 1926 (Bryant, 1987; Beckman, 1926). Zobell (1946) reported a process for secondary oil recovery using anaerobic, hydrocarbon utilizing, sulfate reducing bacteria. Microbial methods for oil recovery have many advantages such as economical, low toxicity, biodegradability and biocompatibility, selectivity, specificity at extreme temperatures, pH and salinity (Desai and Banat, 1997). Therefore, microbial enhanced oil recovery (MEOR) is a good alternative in improving the recovery of crude oil from reservoir rocks by using microorganisms and their metabolic by products. 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; Mei et al., 2003; Zekri and El-Mehaideb, 2002). There are three mechanisms by which microorganisms can contribute to increased oil production: 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). They are potential candidate for many commercial applications in the oil recovery industries (Banat, 1995; Desai and Banat, 1997; Makker and Cameotra, 1998). This paper describes the isolation of a potent biosurfactant producing bacteria from contaminated water of Arabian Sea for use in Marine Enhance Oil Recovery.

MATERIALS AND METHODS

Microbial culture

Isolation of biosurfactant producing bacteria was performed according to Francy et al. (1991) but with these modifications: 20 bacteria strains from oil spilled areas of Arabian Sea [marine sediment (MS - 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10)] and [marine water (MW-1, 2, 3, 4, 5, 6, 7, 8, 9 and 10)] were isolated by adding 1 ml of oil samples to 99 ml of standard nutrient broth (SNB) medium in 250 ml flasks. The SNB medium used in this study contained 1:1 ratio of NB and MSS. It contained per liter distilled water, KH_2PO_4, 20 g; K_2HPO_4, 5.0 g; $(NH_4)2SO_4$, 30 g; NaCl, 0.1 g; $FeSo_4.7H_2O$, 0.01 g; $MgSO_4.7H_2O$, 0.2g; $MnSO_4.7H_2O$, 0.2 g; $Cacl_2.2H_2O$, 0.01 g; glucose, 0.03%; yeast extract, 0.03% g; pH was adjusted to 7.2 (Francy et al., 1991). The (oil sample + SNB) was placed on a shaker at 200 rpm for 2 h at 30°C to produce a well-dispersed suspension and for isolation of biosurfactant producing bacteria. The suspension was diluted serially in SNB and plated in triplicate on SNB with 2% Bacto agar to isolate heterotrophs and on ENDO (peptone, 1.0%; K_2HPO_4, 0.25%; lactose, 1.0%; Na_2SO_4, 0.33%; fuchsine, 0.03%; agar, 1.25%), EMB (peptone, 10.0 g/l; lactose, 10.0 g/l; dipotassiummonohydrogenphosphate, 2.0 g/l; methylene blue, 0.065 g/l; eosine, 0.4 g/l; agar, 15.0 g/l; final pH 7.1 ± 0.2 at 25°C), Mackonkey agar (peptone, 20.0 g; trehalose, 10.0 g; bile salts (Difco), 1.5 g; sodium chloride, 5.0 g; neutral red, 0.05 g; crystal violet, 0.001 g; agar, 15.0 g; distilled water, 1.0 liter; pH 7.4 ± 0.2°C) to characterize the Gram negative from Gram positive bacteria. The plates were kept for overnight incubation at 30°C. After incubation, viable cell counts of all strains were determined. Streaking method was done to isolate and purify the isolates. 20 out of 25 strains were isolated and maintained on nutrient agar (NA) slants (Himedia) at 4°C, for one month by repeatedly sub culturing the isolated strains (Francy et al., 1991).

Identification of bacteria

The isolated colonies were identified by following microbiological and biochemical tests; Gram staining, carbohydrate fermentation test, H_2S production test, indole production test, methyl red test, Voges-proskauer test, citrate utilization test, urease test, catalase

test, oxidase test, litmus milk reaction, starch hydrolysis test, gelatin hydrolysis test, lipid hydrolysis test, spore staining, motility, fluorescent pigment production and penicillin sensitivity test (Mahesh et al., 2006).

Extraction of biosurfactant

For studying the biosurfactant activity, the selected isolates were inoculated in nutrient broth (peptone, 10.0 g/l; meat extract, 10.0g/l; sodium chloride, 05.0 g/l; distilled water, 1000 ml; pH 7.2 ± 0.2) containing mixtures of oils (petrol + kerosene + diesel) in 1:1:1 ratio and incubated overnight for 10 days at 30°C. All the bacterial cells were removed by centrifugation (12,000 x *g*, 4°C, 30 min). Cultural supernatant was acidified with 6N HCl to obtain the pH of 2.0. The extraction was performed twice with an equal volume of ethyl acetate. Pooled solvent extracts were concentrated using an evaporator under reduced pressure. White precipitate formed culture was used for further experimentation.

Analytical methods

Biosurfactant activity assay tests

Haemolytic activity: Isolated strains were screened on blood agar plates containing 5% (v/v) goat blood and incubated at room temperature for 24 h. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carrillo et al., 1996).

Drop collapsing test: 2 µl of mineral oil were added to each well of a 96-well micro titer plate lid. The lid was equilibrated for 1 h at room temperature, and then 5 µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant-producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production (Youssef et al., 2004).

Emulsification measurement: Emulsification activity was measured according to the method of Cooper and Goldenberg (1987) with a slight modification. To 4 ml of culture supernatant or biosurfactant crude extract (0.5%, w/v), 4 ml of n-hexadecane were added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed as percentage.

$$\text{Emulsification activity} = \frac{\text{Height of emulsion layer}}{\text{Total height}}$$

Oil displacement test: 15 µl of weathered crude oil were placed on the surface of distilled water (300 µl) in a Petri dish (150 mm in diameter). Then, 20 µl of the culture supernatant were gently put on the center of the center of the oil film. The diameter and area of clear halo visualized under visible light were measured and calculated after 30 s as described by Morikawa et al. (1993).

Measurement of surface tension

Pre cultures of bacteria strains were prepared in NB. 1.1 ml of inocula were added to 100 ml MSS and 1% oil (P+K+D in 1:1:1 ratio) as hydrocarbon source. The mixtures with control samples (100 ml MSS and 1% P+K+D oil without bacterial strains) were incubated at 30°C on shaker at 150 rpm for three days. The surface tension was measured by using a F6 tensiometer (Abu-Ruwaida et al., 1991).

Investigating the effect of salinity on surface tension

The effect of salinity on surface tension was determined by adding different concentrations (1 to 13%) of NaCl on cultures growing on MSS (Himedia). The mixtures were incubated at 30°C on shaker at 150 rpm for three days (Prommachan et al., 2001).

Investigating the effect of different pH on surface tension

The effect of pH on surface tension were carried out by changing pH (4.0 to 8.0) of cultures growing on MSS (pH=7.0). The cultures were incubated at 30°C on shaker at 150 rpm for three days (Yakimov et al., 1995).

Investigating the effect of different temperatures on surface tension

The effects of temperatures on surface tension were carried out by placing cultures growing on MSS at different temperatures (20 to 50°C) are investigated. The cultures with control samples were placed on shaker at 150 rpm for three days in selected temperature (Yakimov et al., 1995).

Biosurfactant extraction

The biosurfactant produced by MW2 were extracted by culturing the MW2 strain in the presence of 2% glucose in NB. The cultures were centrifuged for 15 min at 10,000 *g* to obtain cell-free supernatant with the help of chloroform - methanol (1:3 ratio). After the extraction of biosurfactant, chloroform - methanol was removed. The extracted biosurfactant was analyzed by thin layer chromatography (TLC). The TLC tank was filled with solvent mixture (chloroform – methanol acetic acid - water, 25:15:4:2). The lipid components were detected as brown spots on the plate after spraying with chromo sulfuric acid and carbohydrate compounds were detected as red spots on the plate after spraying with naphthol and sulfuric acid (David, 1987; Abu-Ruwaida et al., 1991).

RESULTS

Microbial isolates

This study reveals that six strains, MW1 (*Acinetobacter* sp.), MW2 (*Pseudomonas* sp.), MW4 (*Bacillus* sp.), MS2 (*Arthrobacter* sp), MS5 (*Gluconobacter* sp.) and MS6 (*Pseudomonas* sp.) out of 20 isolated strains had shown the higher biosurfactant activity, in which MW2 (*Pseudomonas* sp.) strain showed the highest emulsification activity that is, 70.5 ± 0.55% and oil displacement test was found 3.14 ± 0.02 cm^2 as shown in Table 1. These strains were selected for further studies.

Identification of the best biosurfactant producers

Out of the isolated strains, six strains showed the ability

Table 1. Oil displacement activity, emulsification activity and drop collapsing test of cultural supernatant from bacteria strains.

Strain	Emulsification activity (%)	Oil displacement test (cm^2)	Drop collapsing test	Haemolytic activity
MW1	62.0 ± 0.40^c	2.45 ± 0.02^c	+	+
MW2	70.5 ± 0.55^a	3.14 ± 0.02^a	+	+
MW4	65.0 ± 0.50^b	2.55 ± 0.03^b	+	+
MS5	65.0 ± 0.36^b	2.20 ± 0.03^e	+	+
MS2	60.0 ± 0.30^d	2.30 ± 0.03^d	+	+
MS6	50.0 ± 0.30^d	1.14 ± 0.02^f	+	+

Different letters in the same column indicate significant differences ($p<0.05$).

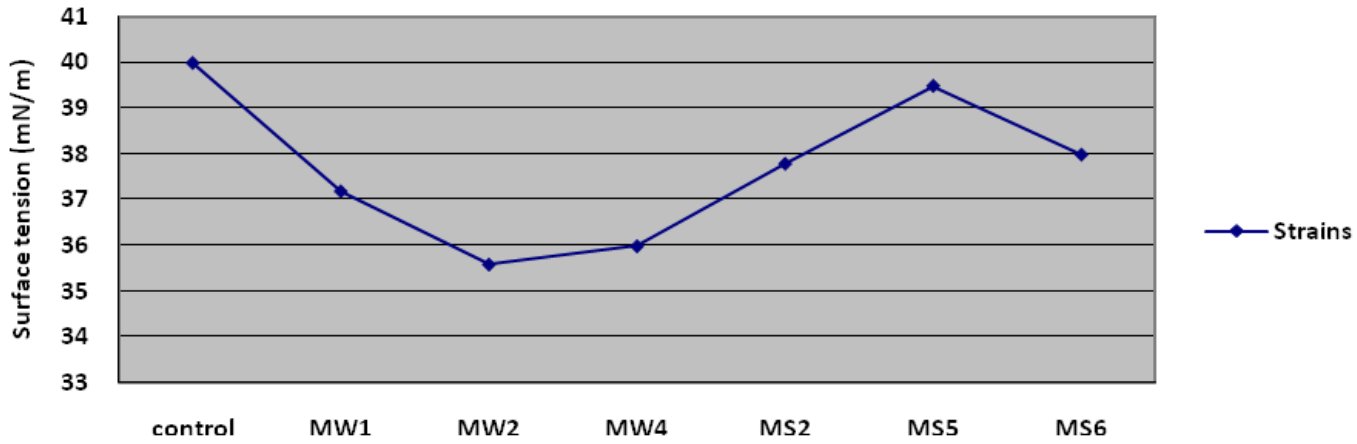

Figure 1. The best biosurfactant producers in the presence of the mixtures of oils (P+K+D). The control was the medium that had100 ml MSS and 1% oil (P+K+D in 1:1:1 ratio) as hydrocarbon source without inocula.

to reduce culture-broth surface tension to values below 40 mN/m (Figure 1). The isolated cultures were designated as MW2 (*Pseudomonas.* sp.), and MW4 (*Bacillus* sp.).

Effect of salinity

Surface tension of culture broth of selected strains was reduced at all NaCl concentrations tested (1 to 13% w/v) as shown in (Figure 2).

Effect of pH on surface tension

The surface tension of whole broth of selected strains was maintained nearly constant at all tested pH (4.0 to 8.0) at 30°C, indicating that pH variation has no appreciable effect on surface tension. But the maximum of surface tension reduction was at pH range from 6.0 to 7.0 (Figure 3).

Effect of temperature on surface tension

All of the strains reduced surface tension in the tested temperatures but the best range of temperature for selected strains was between 30 to 40°C (Figure 4).

Biosurfactant nature

Preliminary analysis of the biosurfactant using TLC produced by the MW2 strain indicated the presence of glycolipids and or neutral lipids on the basis of Rf-value (0.6). An alpha-naphthol/sulfuric acid were also detected indicating that the lipid extract contains a carbohydrate substance.

DISCUSSION

In this study, the initial isolation of suspected biosurfactant producers was done on blood agar plates, utilizing the ability of many biosurfactants to lyse erythrocytes, which results in a band of beta hemolysis surrounding biosurfactant-producing bacterial colonies (Bernheimer and Avigad, 1970; Banat, 1995a; Banat, 1995b; Lin, 1996). After haemolysis test, stabilization of an oil and water emulsion is commonly used as a surface activity indicator. Several studies focused on high

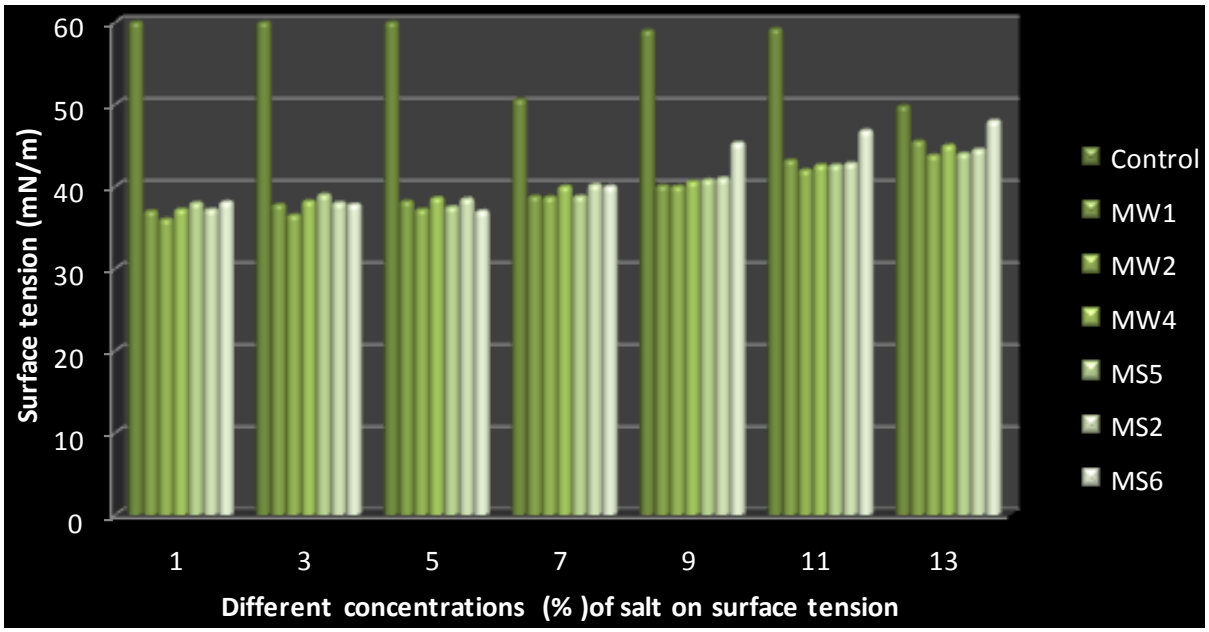

Figure 2. Effect of salinity on surface tension (mN/m). The control was the medium with 100 ml MSS, 1% oil (P+K+D in 1:1:1 ratio) as hydrocarbon source without inocula and also had different concentrations of salt.

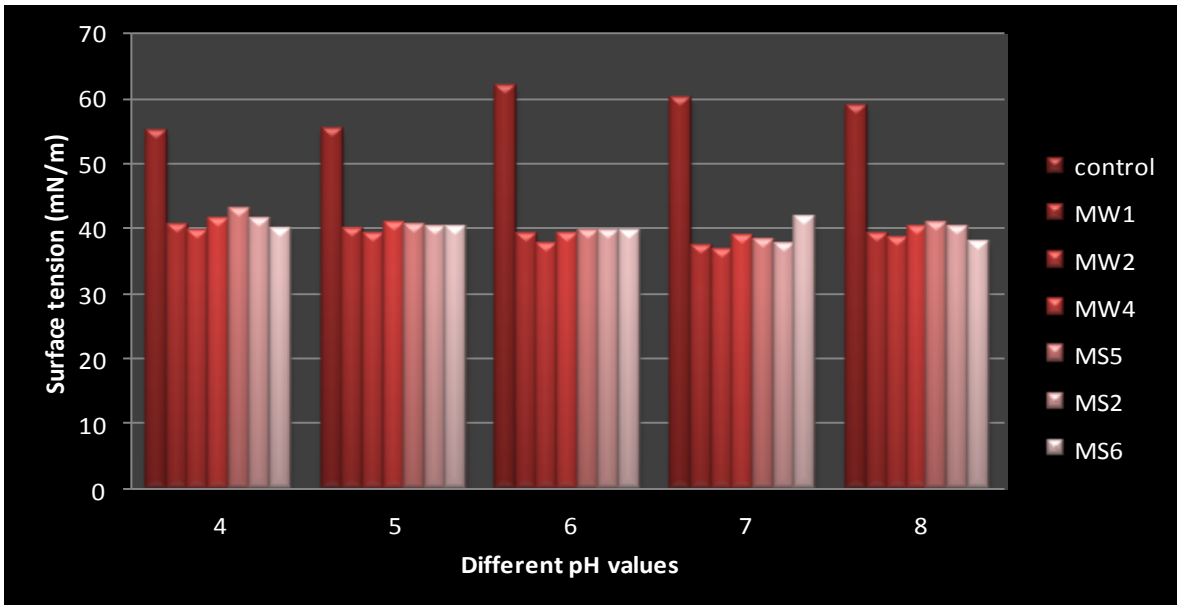

Figure 3. Effect of different pH on surface tension (mN/m). The control was the medium having 100 ml MSS and 1% oil (P+K+D in 1:1:1 ratio) as hydrocarbon source without inocula on different pH.

emulsifying abilities (Francy et al., 1991; Bicca et al., 1999; Bodour et al., 2004). Identification of biosurfactant activity of bacteria can be further confirmed by values of surface tension. Direct measurement of the interfacial or surface activity of the culture supernatant is the most straightforward screening method and very appropriate for a preliminary screening of biosurfactant producing microbes (Lin, 1996). Reduction of surface tension measurements by isolated bacteria from Arabian Sea (Mumbai) indicates the production of surface-active compounds. Similar results were obtained by Banat et al. (1991). They isolated several bacteria which showed the

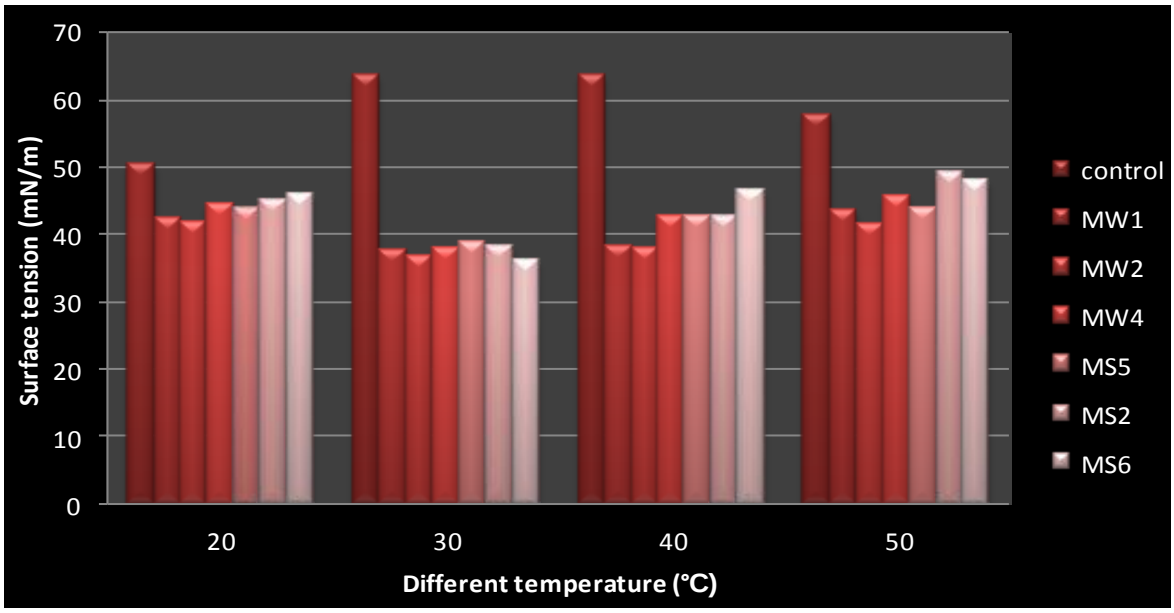

Figure 4. Effect of different temperature (°C) on surface tension. The control was the medium having 100 ml MSS and 1% oil (P+K+D in 1:1:1 ratio) as hydrocarbon source without inocula on different temperature.

ability to reduce culture-broth surface tension to values below 40 Mn/m. Salt concentration also affected biosurfactant production depending on its effect of cellular activity which is very near to the results obtained by Yakimov et al. (1995). He isolated *Bacillus Licheniformis* BAS50 which grew and produced a lipopeptide surfactant when cultured on a variety of substrate at different salt concentration of 13% NaCl. Depicted results show that the biosurfactant production was optimal at 5% NaCl. In the presence of 3 to 9% NaCl, micellization could be enhanced and emulsification was maximized. Micellization of biosurfactant or modification of the molecular area at the air-water interface can be improved with monovalent cation (Thimon et al., 1992). This phenomenon might be a common feature of active compounds produced by marine bacteria. The concentration of salts in aquatic environments ranges from less than 0.05% (w/v dissolved salts) to saturated salt up to 30% and above and NaCl is a major component of seawater (Cameotra and Makkar, 1998); NaCl activated biosurfactant activity of many strains, which were isolated from sea water or petroleum reservoirs (Yakinov et al., 1995). Various environmental factors and growth conditions such as pH, temperature, and O_2 availability also affect biosurfactant production through their effects on cellular growth or activity. Comparable results were obtained by Kim et al. (1997). They found that the surface tension reducing activity of *Bacillus subtillis* C9 was stable to pH over the range of pH 5.0 to 9.5. Also, Abu-Ruwida et al. (1991), observed that the biosurfactant production of *Rhodococcus* was at pH 6.5 to 7.2 that determined by surface tension. In this study 30 to 40°C was identified as the best range of temperature for the surface tension reduction of selected strains. Also Abu-Ruwaida et al. (1991) found the optimum biosurfactant production of *Rhodococcus* sp. at 37°C. According to surface tension reducing and emulsification characteristics of (MW2) strain and its stability over a wide range of pH, temperatures and high salt concentrations suggest that (MW2) strain is suitable for use as MEOR and removal of oil pollutions from different oil polluted areas. However, there are several reports showing the ability to degrade aliphatic and aromatic hydrocarbons by *Pseudomonas* sp. Whyte et al. (1997) isolated a hydrocarbon-degrading *Pseudomonas* sp. which degraded both alkanes and naphthalene. *Mycobacterium* sp. strain CH1 which can degrade pyrene was also capable of using a wide range of branched alkanes and n-alkanes as sole carbon and energy sources (Churchill et al., 1999). In China, Wang (1991) came with documented results concerning the production and application in China oil fields of biopolymers produced by *Leuconostoc mesen- teroides* and *Pseudomonas aeruginosa* strains, as well as by *Brevibacterium viscogenes, Corynebacterium gumiform,* and *Xanthomonas campestris;* the last three species were obtained using hydrocarbons for biopolymer production. During the last 15 to 20 years, China was very active in MEOR method and today is still active in this field and could be considered one of the leaders in this field (Wang, 1999; Wang, 1999; He et al., 2000). The main advantages of microbiological method of bioremediation of hydrocarbon polluted sites are use of biosurfactant producing bacteria without necessarily

characterizing the chemical structure of the surface active compounds. The cell free culture broth containing the biosurfactants can be applied directly or by diluting it appropriately to the contaminated site. The other benefit of this approach is that the biosurfactants are very stable and effective in the culture medium that was used for their synthesis (Płociniczak et al., 2011).

REFERENCES

ABU-Ruwaida AS, Banat M, Haditirto, Salem S, Kadri A (1991). Isolation of biosurfactant producing bacteria product characterization and evaluation. Acta Biotechnol. 11(4):315-324.

Banat IM (1995a). Biosurfactants Production and Possible Uses in Microbial-Enhanced Oil Recovery and Oil Pollution Remediation: A Review. Bioresour. Technol. 51:1-12.

Banat IM (1995b). Characterization of Biosurfactants and Their Use in Pollution Removal – State of the Art (Review). Acta Biotechnol. 3:251-267.

Banat IM, Samarah N, Murad M, Horne R, Benerjee S (1991). Biosurfactant production and use in oil tank clean-up. World J. Microbiol. Biotechnol. 7:80-84.

Beckman JW (1926). The action of bacteria on mineral oil. Ind. Eng. Chem. 4:23-26.

Bernheimer AW, Avigad LS (1970). Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. J. Gen. Microbiol. 61:361-369.

Bicca FC, Fleck LC, Zachio MA (1999). Production of biosurfactant by hydrocarbon degrading *Rhodococcus rubber* and *Rhodococcus erythropolis*. Rev. Microbiol. 30(3).

Bodour AA, Gerrero-Barajas C, Maier M (2004). Structure and characterization of Flavolipids, a novel class of Biosurfactants produced by Flavolipid sp. Strain MTN11. Appl. Environ. Microbiol. 10(6):1114-1120.

Bryant RS (1987). Potential uses of microorganisms in petroleum recovery technology. Proc. Okla. Acad. Sci. 67:97-104.

Cameotra SS, Makkar RS (1998). Synthesis of biosurfactants in extreme conditions. Appl. Microbiol. Biotechnol. 50:520-529.

Carrillo PG, Mardaraz C, Pitta – Alvarez S I, Giulietti AM (1996). Isolation and selection biosurfactant – producing bacteria. World J. Microbiol. Biotechnol. 12:82-84.

Churchill SA, Harper JP, Churchill PF (1999). Isolation and characterization of a *Mycobacterium* species capable of degrading three and four-ring aromatic and aliphatic hydrocarbons. Appl. Environ. Microbiol. 65:549-552.

Cooper DG, Goldenberg BG (1987). Surface-active agents from two *Bacillus* species. Appl. Environ. pp. 224-229.

Desai JD, Banat IM (1997). Microbial production of biosurfactants and their commercial potential. Microb. Mol. Biol. Rev. 61(1):47-64.

Fietcher A (1992). Biosurfactant moving toward industrial application. Tibtech 10:208-217.

Francy DS, Thomas JM, Raymond RL, Ward CH (1991). Emulsification of hydrocarbon by surface bacteria. J Ind. Microbiol. 8:237-246.

He Z, She Y, Xiang T, Xue F, Mei B, Li Y, Ju B, Mei H, Yen T F (2000). MEOR pilot sees encouraging results in Chinese oil field. J. Oil Gas 98:46-52.

Jinfeng L, Lijun M, Bozhong M, Rulin L, Fangtian N, Jiaxi Z (2005). The field pilot of microbial enhanced oil recovery in a high temperature petroleum reservoir. J. Pet Sci. Eng. 48:265-271.

Joshi S, Bharucha C, Jha S, Yadav S, Nerurkar A, Desai AJ (2008). Biosurfactant production using molasses and whey under thermophilic conditions. Biores. Technol. 99:195-199.

Kim HS, Yoon BD, Lee CH, Oh HM, Katsuragi T, Tani Y (1997). "Production and properties of a lipopeptide biosurfactant from *Bacillus subtilis* C9. J. Ferment. Bioeng. 84(1):41-46.

Kosaric N (2001). Biosurfactants and their application for soil bioremediation. Food Technol. Biotechnol. 39:295-304.

Lin S (1996). Biosurfactants: Recent advances. J. Chem. Tech. Biotechnol. 66(2):109-120.

Mahesh N, Murugesh S, Mohana V (2006). Determination of the presence of biosurfactant produced by the bacteria present in the soil samples. Res. J. Microbiol. 1(4):339-345.

Makkar RS, Cameotra SS (1998). Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis*. J. Ind. Microbiol. Biotechnol. 20:48-52.

Mei S, Wei L, Guangzhi L, Peihui H, Zhaowei H, Xinghong C, Ying W (2003). Laboratory study on MEOR after polymer flooding. SPE84865. In: Proceedings of the SPE International Improved Oil Recovery Conference in Asia Pacific, October 20-21, Kuala Lumpur.

Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y and Imanaka T (1993). A new lipopeptide biosurfactant produced by *Arthobacter* sp. Strain MIS 38. J. Bacteriol. 175:6459-6466.

Płociniczak MP, Płaza GA, Seget ZP, Cameotra SS (2011). Environmental Applications of Biosurfactants: Recent Advances. Int. J. Mol. Sci. 12:633-654.

Prommachan, H-Kittikun, Kawai F (2001). Production of biosurfactant from *Bacillus* MUV4. The Annual Meeting of the Thai Society for Biotechnology, Bio Thailand: From Research to Market, 7-10, Bangkok, Thailand. Chapter 1.

Ramirez WF (1987). Application of optimal control theory to enhanced oil recovery. Elsevier Scientific Publisher, Amsterdam, NL. First edition.

Rashedi H, Jamshidi E, Mazaheri Assadi M, Bonakdarpour B (2005). Isolation and production of biosurfactant from *Pseudomonas aeruginosa* isolated from Iranian southern wells oil. Environ. Sci. Technol. 2:121-127.

Soberón-Chávez G, Maier RM (2011). Biosurfactants: a General Overview. In: Soberón-Chávez G, editor. Biosurfactants. Springer - Verlag. Berlin, Germany. pp. 1-11.

Thimon L, Peypoux F, Michel G (1992). Interaction of surfactin, a biosurfactant from *Bacillus subtilis*, with inorganic cations. Biotechnol. Lett. 14:713-718.

Toledo FL, Gonzalez J, Calvo C (2008). Production of bioemulsifier by *Bacillus subtilis*, *Alcaligenes faecalis* and *Enterobacter* species in liquid culture. Biores. Technol. 99:8470-8475.

Wang WD (1999). MEOR Center Shangli Oil Field, Dong Yong City, Shan Dong Province. Personal communication.

Wang XY (1991). Advances in research, production and application of biopolymers used for MEOR in China. In: Microbial Enhancement of Oil Recovery, Recent Advances. Development in Petroleum Science Series, 31, Donaldson, E. C. (Ed.). Amsterdam: Elsevier.

Wang YX (1999). Institute of Microbiology, Chinese Academy of Sciences, Beijing. Personal communication.

Whang LM, Liu PWG, Ma CC, Cheng SS (2008). Application of biosurfactant, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. J. Hazard. Mater. 151:155-163.

Whyte LG, Bourbonniere L, Greer CW (1997). Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (alk) and naphthalene (nah) catabolic pathways. Appl. Environ. Microbiol. 63:3719-3723.

Yakimov MM, Kenneth, Timmis, Wray V, Fredrickson L (1995). Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. Appl. Environ. Microbiol. pp. 1706-1713.

Youssef nh, Dunacn KE, Nagle, Savage DP, Knapp RM, McInerney MJ (2004). Comparison of methods to detect biosurfactant production by diverse microorganism. J. Microbiol. Meth. 56:339-347.

Zajic JE, Stiffens W (1994). Biosurfactants. CRC Rev. Biotechnol. 1:87-106.

ZoBell CE (1946). Bacteriological process for treatment of fluid-bearing earth formations. United States Patent Office, No. 2,413,278.