

Bioemulsan Production by Iranian Oil Reservoirs Microorganisms

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ABSTRACT

The biosurfactants are believed to be surface active components that are shed into the surrounding medium during the growth of the microorganisms. The oil degrading microorganism *Acinetobacter calcoaceticus* RAG-1 produces a poly-anionic biosurfactant, hetero-polysaccharide bioemulsifier termed as emulsan which forms and stabilizes oil-water emulsions with a variety of hydrophobic substrates. In the present paper results of the possibility of biosurfactant (Emulsan) production by microorganisms isolated from Iranian oil reservoirs is presented. Fourty three gram negative and gram positive, non fermentative, rod bacilli and coccobacilli shaped bacteria were isolated from the oil wells of Bibi Hakimeh, Siri, Maroon, Ilam, East Paydar and West Paydar. Out of the isolated strains, 39 bacterial strains showed beta haemolytic activity, further screening revealed the emulsifying activity and surface tension. 11 out of 43 tested emulsifiers were identified as possible biosurfactant producers and two isolates produced large surface tension reduction, indicating the high probability of biosurfactant production. Further investigation revealed that, two gram negative, oxidase negative, aerobic and coccoid rods isolates were the best producers and hence designated as IL-1, PAY-4. Whole culture broth of isolates reduced surface tension from 68 mN/m to 30 and 29.1mN/m, respectively, and were stable during exposure to high salinity (10%NaCl) and elevated temperatures(120°C for 15 min).

Keywords: *Biosurfactant, Bioemulsan, Surface tension, Iranian oil reservoir*

INTRODUCTION

Biosurfactants have been used in a variety of industrial and environmental applications (Fiechter, 1992; Desai, 1994; Gorkovenko, 1997; Horacio, 2003). These bioemulsifiers are believed to be capsular polymers that are shed into surrounding medium during the growth of the strains (Gutnick, 1989).

Surfactants are amphipatic molecules with both hydrophilic and hydrophobic moieties that par-

tition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. These properties render surfactants capable of reducing surface and interfacial tension and forming microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons (Jitendra, 1997).

Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which makes surfactants some of the most versatile process chemicals (Greek, 1990, 1991). Emulsan is a complex extracellular acy-

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lated polysaccharide by the gram-negative bacterium *Acinetobacter calcoaceticus* with an average molecular weight of about 1000 KD (Pines, 1985; Kim, 1997), which is ubiquitous in nature and which is considered to be part of the normal human commensal load (Bouvet, 1986, 1989; Bruce, 2002). Emulsan has been extensively researched for its industrial applications as an emulsifier (Gorkovenko, 1999). This molecule is composed of an unbranched polysaccharide backbone with O-acyl and N-acyl bound fatty acid side chains. The polysaccharide backbone consists of three aminosugars, D-galactosamine, D-galactosaminouronic acid and a dideoxydiaminohexose in the ratio of 1:1:1 (Zhang, 1997; Bruce, 2002). The fatty acid side chains range in length from 10 to 22 carbon atoms and can represent from 5 to 23% (wt/wt) of the polymer. The emulsan amino groups are either acetylated or covalently linked by an amide bound to 3-hydroxybutyric acid. The combination of hydrophilic anionic sugar main chain repeated units and the hydrophobic side groups leads to the amphipathic behavior of emulsan and, therefore, its ability to form stable oil-in-water emulsions (Gorkovenko, 1997). This study demonstrates production of Emulsan from oil reservoirs microorganisms in Iran.

MATERIALS AND METHODS

Twenty four oil samples were collected from Iran oil reservoirs including wells coded: Bibi (three samples), Siri (seven samples), Maroon (three samples), Ilam (five samples) and East Paydar (five samples), West Paydar (one samples). 1 ml of each oil sample was added to 9 ml of 0.9% sodium chloride solution. Mixture was placed on a reciprocal skaker for 1 h to produce a well-dispersed suspension (Francy, 1991).

The suspension was serially diluted in phosphate-buffered saline solution (1.24g Na_2HPO_4 , 0.180g NaH_2PO_4 , H_2O and 8.5g NaCl per liter of deionized water) and was in triplicate on one-

half strength Nutrient Broth containing 1.5% Agar-Agar.

After the stabilization of number of colonies, viable cell counts in 24 oil samples were determined. Three successive pour plate isolations were performed on isolates selected for emulsification tests to ensure that pure cultures were obtained.

Isolation Medium The medium used for isolation and cultivation of biosurfactant-producing bacteria had the following components (per liter) 2 g KH_2PO_4 , 5 g K_2HPO_4 , 3g $(\text{NH}_4)_2\text{SO}_4$, 0.1 g NaCl , 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.02 g $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$. It also contained 1 ml/l trace element solution containing (per liter) 0.29 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.25 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.17g $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$. The pH was adjusted at 7 (Francy, 1991).

Crude oil samples were screened for biosurfactant-producing microorganisms by using the following procedure.

Screening and Isolation of Biosurfactant-producing Microorganisms For screening of biosurfactant-producing microorganisms haemolytic and emulsification activity were studied (Jain, 1991).

Haemolytic Activity Haemolytic activity of bacterial strains was determined by inoculating sheep blood agar (Jain, 1991). The plates were incubated at 30° C for 48-72h. Plates were examined for clearing zones around the colonies. All experiments were repeated two times.

Emulsification Activity Emulsification activity was measured using the method described by Cooper and Goldenberg (Abu-Ruwaida, 1991), whereby (with a modification) 0.5 ml of crude oil or other suitable hydrocarbon was added to 5 ml of the culture broth in a graduated screw cap test tube and vortexed at high speed for 2 min. using a vortex-GENIE. The emulsion stability was determined after 24h and the emulsification index (E24) was calculated by dividing the measured height of the emulsion layer by the mixture's total and multiplying by 100.

Measurement of Surface Tension Surface tension reduction was measured by the application of du Nouy ring method (Adria, 2003). Cell suspension were centrifuged ($10,000 \times g$, Beckman model J2-21 centrifuge), and the cell-free supernatant was placed into a clean glass 50-ml beaker.

A surface tensiometer was used to measure the surface tension. Between each pair of measurements, the platinum wire ring used to measure surface tension was rinsed three times with water, three times with acetone, and then allowed to be dried (Cooper, 1987; Adria, 2003).

RESULTS

The screening program resulted in the isolation of more than 43 isolates from twenty four oil samples. The initial screening on blood agar yielded a total of 39 isolates showing beta-haemolytic activity. Isolated strains were grown in MSM-crude oil for measurement of emulsification activity.

11 bacterial strains showed over 60% emulsification activity and the ability to reduce culture-broth surface tension to values of below 23-33 mN/m that indicates the production of surface-active compounds. Two isolates which reduced culture-broth surface tension to values of below 23 mN/m were selected for further studies and named IL-1 and PAY-4.

Identification of isolates IL-1 and PAY-4

Two isolates were found to be aerobic, gram – negative, coccoid rod shaped bacterium exhibiting some morphological variations; one of them was tentatively assigned to the *Acinetobacter* sp, other one assigned to the *Pseudomonas* sp.

Dynamics of Surfactant Production Figs .1, 2 show the dependence of growth and bioemulsan production surface tension, CMD*. Emulsi-

fication activity by two isolates (IL-1 and PAY-4) compared with *A. calcoaceticus* (PTCC) ** was obtained from Iranian type culture collection (Bio technology center) as standard strain. All the three bacteria were cultivated in MSM containing 2% crude oil. The surface tension of culture broth of RAG-1, IL-1, and PAY-4 dropped rapidly after inoculation reaching their lowest values of 28, 30 and 29.1, respectively, during the exponential phase after about 24h growth.

The CMD plot (a measure of bioemulsan concentration) showed that insufficient surfactant was initially present to form micelles.

At about 24h of growth, the surfactant concentration started to increase, reaching its maximum after about 32-36h of growth.

A. calcoaceticus PTCC, IL-1 and PAY-4 had a doubling time of 0.5, 0.4 and 0.38h and were reached to stationary phase after 17, 16.5 and 16h, respectively. After 72h of growth, total cell numbers and cell dry weights were determined and the values obtained were 3.2×10^8 cells per ml and 3.6 g per liter for *A. calcoaceticus*, 2.9×10^8 and 4.1 g per liter for IL-1 and 1.7×10^9 cells per ml and 4.8 g per liter for PAY-4, respectively.

pH of the growth medium decreased from an initial value of 7.0 to a minimum of 6.5 at 10-20h and then increased to 6.9 at the end of fermentation.

E24 values increased with increasing cell growth, reaching their optimum at about 32h and remaining constant until the end of fermentation.

These results indicate that the biosurfactant biosynthesis from crude oil microorganisms occurred predominantly during the exponential growth phase, suggesting that the biosurfactant is produced as a primary metabolite accompanying cellular biomass formation.

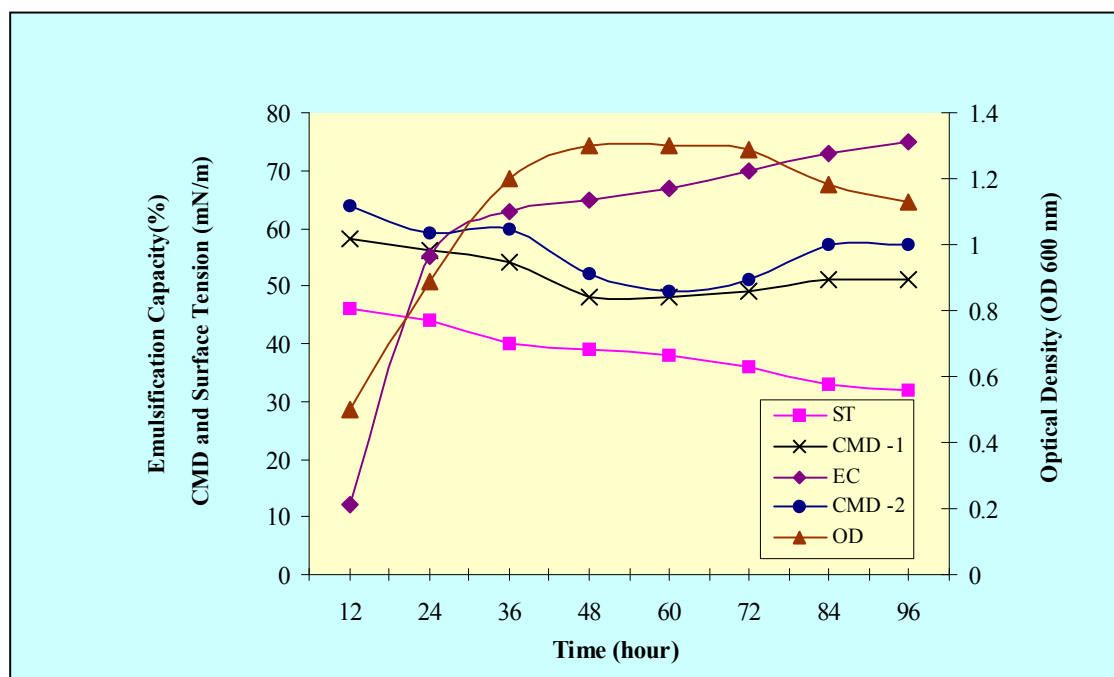


Fig. 1: Typical batch fermentation of crude oil by IL-1 bacterial culture at 30°C and pH 7. CMD⁻¹ and CMD⁻² are critical micelle dilutions (broth was diluted 1:10 and 1:100, respectively, and surface tension was measured)

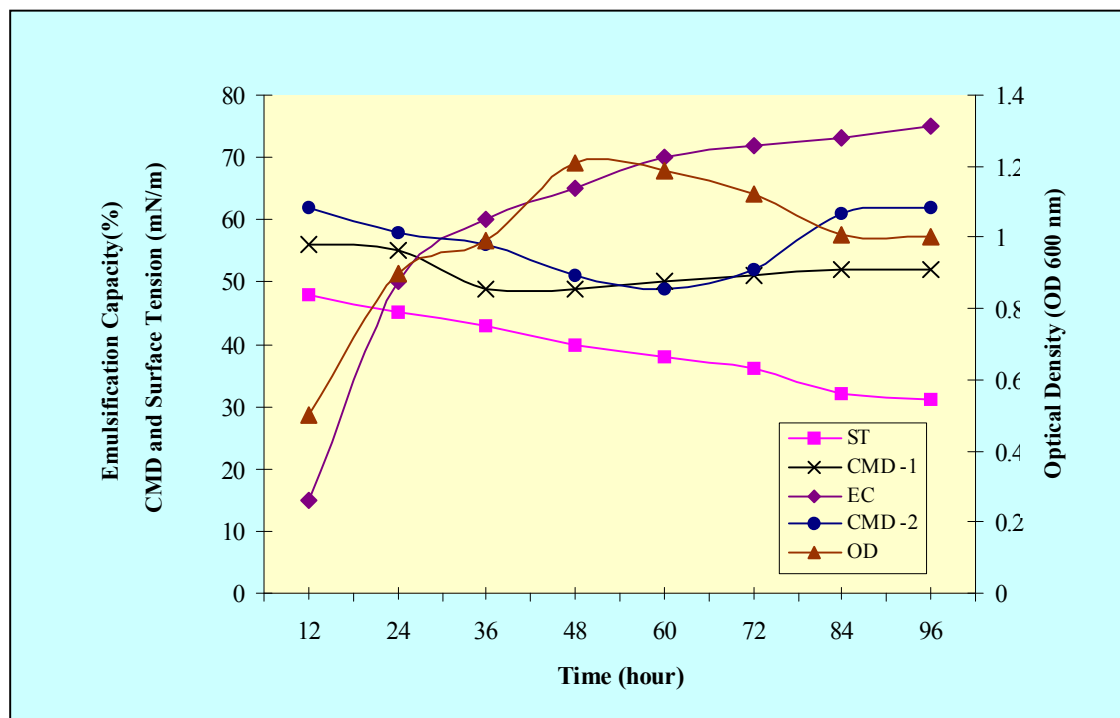


Fig. 2: Typical batch fermentation of crude oil by PAY-4 bacterial culture at 30°C and pH 7. CMD⁻¹ and CMD⁻² are critical micelle dilutions

In addition to surface tension, stabilization of an oil and water emulsion is commonly used as a surface activity indicator (Abu-Ruwaida, 1991). Table 1 presents experimental results on the emulsifying activities for the whole broth of two isolates and *A. calcoaceticus* RAG-1 as standard with various short and long-chain hydrocarbon substrates. The highest emulsion values (water-in-oil) of about 78% for *A. calcoaceticus*, 70 % for IL-1 and 69% for PAY-4 were obtained using crude oil. Hexadecanemethylnaphthalene produced emulsions approxi-

mately lower than crude oil. No emulsifying activity was observed when gasoline and diesel were used.

These results indicate that the biosurfactant produced by the isolates had high emulsification specificity toward crude oil and a rather low efficiency with hexadecane- methylnaphthalene. These findings suggest that the emulsifier's activity depends on its affinity for hydrocarbon substrates which involves a direct interaction with the hydrocarbon itself rather than an effect on the surface tension of the medium.

Table 1: Emulsification of various hydrocarbons by whole broth cultures of three bacteria

Hydrocarbon	E.C ^T in RAG-1 [%]	E.C in IL-1 [%]	E.C in PAY-4 [%]
Crude oil	78	70	69
Gasoline	10	11	19
Disel	15	20	18
Hexadecane-methylnaphthalene	42	48	52

Effect of Sodium Chloride Figs 3, 4. show the effect of sodium chloride addition on surface tension and surfactant concentration (CMD) of IL-1 and PAY-4 biosurfactants. Little changes were observed in either parameter with the addition of 0 to 10% [w/v] sodium chloride, although CMD values decreased slightly with increasing of salt concentration, indicating increased biosurfactant activity in the presence of sodium chloride.

Effect of temperature Table 2. shows the

effect of heat treatment on the biosurfactant activity of IL-1 and PAY-4 culture, demonstrating that no significant changes in biosurfactant properties occurred, in the case that culture broth and cells suspended in saline solution were heated.

The biosurfactant properties, measured as surface tension, CMD and E₂₄, remained stable after exposure to high temperatures of (100, 120) °C for 15 min. There was also no change in biosurfactant activity at lower temperature (0-4) °C.

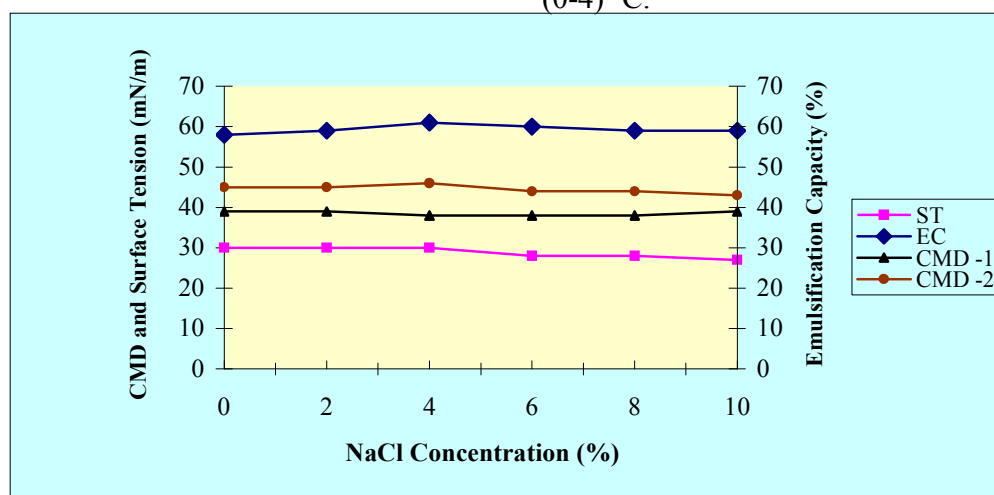


Fig. 3: Effect of Sodium Chloride on IL-1

Table 2: Heat stability of the biosurfactant from bacterial cultures

Isolate	Biosurfactant Properties	Before heat treatment	After heat treatment temp/time, °C/min	
			100/15	120/15
IL-1	Culture broth			
	S.T. [mN/m]			
	CMD ⁻¹ [mN/m]	30	30.7	29.4
	CMD ⁻² [mN/m]	31.6	31.5	31.3
	CMD ⁻² [mN/m]	39.7	41.8	40.2
	E ₂₄ [%]	44.0	44.1	44.0
	Cell suspension			
	S.T. [mN/m]			
	CMD ⁻¹ [mN/m]	28.5	28.4	29.5
	CMD ⁻² [mN/m]	35.1	33.0	33.3
PAY-4	CMD ⁻² [mN/m]	63.5	63.2	64.0
	E ₂₄ [%]	35	41.0	37.1
	Culture broth			
	S.T. [mN/m]	29.1		
	CMD ⁻¹ [mN/m]	30.5	30.1	31.2
	CMD ⁻¹ [mN/m]	38.6	30.3	30.9
	CMD ⁻² [mN/m]	43.0	39.0	37.0
	E ₂₄ [%]		43.2	44.0
	Cell suspension			
	S.T. [mN/m]	28.4	29.1	29.9
	CMD ⁻¹ [mN/m]	36.4	34.9	34.2
	CMD ⁻² [mN/m]	62.6	62.0	64.1
	CMD ⁻² [mN/m]	34.0	38.0	38.0
	E ₂₄ [%]			

DISCUSSION

In this study two isolates were screened with a potential of the highest biosurfactant production. These strains produced a bioemulsan like biosurfactant.

Acinetobacter calcoaceticus cells accumulated capsular material on the cell surface during logarithmic phase and then released this polymeric material in the form of an active emulsifier in stationary phase or during conditions of unbalanced growth (Kaplan, 1982).

Although biomulsifier production by microorganisms is generally associated with growth on hydrocarbons (Rubinovitz, 1982), *A. calcoaceticus* RAG-1 (standard strain) produces at least much emulsan when grown on crude oil and

ethanol rather than on hexadecane medium (Rubinovitz, 1982).

IL-1 and PAY-4 showed the ability to reduce culture broth surface to values below 30 and 29.1 [mN/m], respectively, if grown with water-immisible hydrocarbons (crude oil) as their sole carbon source. This reduction of surface tension measurements indicated the production of surface-active compounds, by the microbial culture, which has been shown to aid metabolism of the substrate and stimulate microbial growth (Abu-Ruwaida, 1991).

Maximum biomass concentration (about 0.41 g/l dry weights for IL-1 and 0.58 g/l for PAY-4) was achieved after 32-36h of growth. Growth rate (0.24h⁻¹) and maximum biomass obtained were much higher for this culture than for other biosurfactant-producing microorganisms on hydrocarbons reported in the literature (Rosenberg, 1979).

The CMD plot, a measure of biosurfactant concentration, showed that insufficient surfactant was initially present to form micelles. At about 24h of growth, the surfactant concentration started to increase, reaching its maximum after about 36h.

These results indicate that the biosurfactant biosynthesis occurred predominately during the exponential growth phase, suggesting that the biosurfactant is produced as a primary metabolite accompanying cellular biomass formation. Similar observations have been made for other biosurfactant-producing microorganisms. (Abu-Ruwaida, 1991).

The effect of sodium chloride addition on surface tension and CMD, indicated increased biosurfactant activity in the presence of sodium chloride. Brown et al. (1985) reported a similar effect on efficiency and effectiveness upon the addition of %5 sodium chloride, with a biosurfactant produced by the aerobic bacterium designated isolate 1165 (Abu-Ruwaida, 1991).

Results of emulsification activity indicated that

produced biosurfactant by two isolates had a high emulsification specificity with crude oil and this result differ to that of Kaplan and Rosenberg (1982) and Abu and Banat (1991). These findings suggest that the emulsifier's activity depends on its affinity for hydrocarbon substrates which involves a direct interaction with the hydrocarbon itself rather than an effect on the surface tension of the medium.

The surface tension of biosurfactant IL-1 and PAY-4 were maintained uniformly at all pHs ranging from 2 to 10, indicating that variation in pH has no appreciable effect on the surface tension. Most known biosurfactants are less stable over such an extreme pH range (Kaplan, 1982).

Both types of the biosurfactants remained stable, after exposure to high temperatures of 100°C and 120°C for 15 min. (Kim, 2000).

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REFERENCES

- Abu-Ruwaida A S, Banat I M, Haditirto S, Salem A, Kadri M (1991). Isolation of biosurfactant-producing bacteria product characterization, and evaluation. *Acta Biotechnologica*, 4: 315-24.
- Adria A, Bodour K, Drees P, Raina M M (2003). Distribution of biosurfactant-producing bacteria in undisturbed and contaminated Arid South Western soils. *Appl Environ Microbiol*, 69: 3280-87.
- Bouvet P J M, Jeanjean S (1989). Delineation of new proteolytic genomic species in the genus *Acinetobacter* *Res Microbiol*, 140: 291-99.
- Bouvet P J M, Grimout P A D (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., *Acinetobacter junii* sp. nov., and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int J Syst Bacteriol*, 36:228-40.
- Brown M J, Foster M, Moses V, Robinson J P, Shales S W, Spingham, D G (1985), In: Proceeding of the 3rd European Meeting on Improved Oil Recovery, Rome 241.
- Panilaitis B, Johri A, Blank W, Kaplan D, Fuhrman J (2002). Adjuvant activity of emulsan, a secreted lipopolysaccharide from *Acinetobacter calcoaceticus*. *Appl Environ Microbiol*, 9: 1240-47.
- Cooper D G, Goldenberg B G (1987). Surface-active agents from two *Bacillus* species. *Appl Environ Microbiol*. 53:224-29.
- Desai A J, Patel R M, Desai J D (1994). Advances in production of biosurfactants and their commercial applications. *J Sci Ind Res*, 53: 619-29.
- Fiechte A (1992). Biosurfactants: Moving towards industrial application. *Trends Biotechnol*, 10: 208-217.
- Francy D S, Ttomas V, Raymond RL, Ward C H (1991). Emulsification of hydrocarbons by subsurface bacteria. *Journal of Industrial Microbiology*, 8: 237-46.
- Gorkovenko A, Zhang V, Gross V, Allen V, Kaplan D L (1997). Bioengineering of emulsifier structure: Emulsan analogs. *Can J Microbiology*, 43: 384-90.
- Gorkovenko A, Zhang J, Gross R A, Kaplan DL, Allen AL (1999). Control of unsaturated fatty acid substitutes in emulsans. *Carbohydr Polym*, 39:79-84.
- Greek B F (1990). Detergent industry ponders products for new decade. *Chem Eng News*, 68: 37-8.

- Greek B F (1991). Sales of detergents growing despite recession. *Chem. News*. 69: 25-52.
- Gutnick H (1989). Bioemulsifier production by *Acinetobacter calcoaceticus*. United states patent. 4,883,757:1-8.
- Bach H, Berdichevsky Y, Gutnick D (2003). An exocellular protein from the oil degrading microbe *Acinetobacter venetianus* RAG-1 enhances the emulsifying activity of the polymeric bioemulsifier emulsan. *Appl Environ Microbiol*, 69:2608-2615.
- Jain D K, Collins-Thompson D L, Lee H, Trevors JT (1991). A drop-collapsing test for screening surfactant-producing microorganisms. *J Microbiol methods*, 13: 271-279.
- Jitendta D D, Banat I M (1997). Microbial production of surfactants and their commercial potential. 47-67.
- Kaplan N, Rosenberg E (1982). Exopolysaccharide distribution of and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. *Appl Environ Microbiol*, 44: 1335-1341.
- Kim S H, Lim EJ, Lee S O, Lee J D, Lee T H (2000). Purification and characterization of biosurfactant from *Nocardia* sp. L-417. *Biotechnol Appl Biochem*, 31:249-253.
- Kim S Y, Oh D K, Kim J H (1997). Biological modification of hydrophobic group in *Acinetobacter calcoaceticus* RAG-1 emulsan. *Journal of Fermentation and Bioengineering*, 84: 162-164.
- Pines O, Gutnick D (1985). Role for emulsan in growth of *Acinetobacter calcoaceticus* RAG-1 on crude oil. *Appl Environ Microbiol*, 51: 661-663.
- Rosenberg E, Zuckerberg A, Rubinovitz C, Gutnick DL (1979). Emulsifier of *Arthrobacter* RAG-1: Isolation and emulsifying properties. *Appl Environ Microbiol*, 37: 402-408.
- Rubinovitz C, Gutnick D L, Rosenberg E (1982). Emulsan production by *Acinetobacter calcoaceticus* in the presence of chloramphenicol. *Journal of Bacteriology*, 126-132.
- Zhang J, Gorkovenko A, Gross R A, Allen A L, Kaplan D L (1997). Incorporation of 2-hydroxyl fatty acids by *Acinetobacter calcoaceticus* RAG-1 to tailor emulsan structure. *Int J Biol Macromol*, 20: 9-21.