

Microbial enhanced oil recovery using biosurfactant produced by *Alcaligenes faecalis*

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Abstract

A bacterial strain (designated as *Alcaligenes* sp. MS-103) isolated from oil sample of the Aghajari oilfield in the south of Iran, was able to produce an effective extracellular lipopolysaccharide biosurfactant (1.2±0.05 g/l) on molasses as a sole carbon source. The highest surface tension reduction to level 20 mN/m was achieved by biosurfactant produced by cells grown on molasses under optimum conditions. The optimum values of carbon to nitrogen ratio (C/N), salinity, pH and temperature for biosurfactant production were determined as 60:1, 7.5%, 7.0 and 50°C, respectively. Biosurfactant flooding experiments were carried out on both fractured and unfractured carbonate cores. The highest recovery of residual oil among different experiments was about 10.7% in the unfractured cores. Oil displacement indicates that recovery of crude oil can be increased by 9.2% from fractured core with a permeability of 12 mD. The results showed that the biosurfactant produced by *Alcaligenes* sp. MS-103 has the potential for industrial applications and may be used in microbial enhanced oil recovery (MEOR).

Keywords: *Alcaligenes*; Biosurfactant; Carbonate reservoir; Core flooding; Crude oil; MEOR; Surface tension

INTRODUCTION

Carbonate rocks include more than 50% of the world's proven hydrocarbon reserves (Halley and Schmoker, 2004). 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 30-45% 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 has been established by Beckman since 1926 (Bryant *et al.*, 1987; Beckman *et al.*, 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 *et al.*, 1987).

This current research investigates biosurfactant production by *Alcaligenes* sp. MS-103, optimization of certain factors affecting biosurfactant production

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and evaluates the potential use of the isolate for MEOR using core flooding tests under simulated reservoir conditions.

MATERIALS AND METHODS

Screening of microorganisms: Samples of crude oil and oily sludge were collected in sterile bottles from the Aghajari (well#121) oilfield located in the southern part of Iran. A one ml sample of oil was added to 99 ml of synthetic medium (SM) in a 250 ml glass flask. The SM medium was comprised of nutrient broth and mineral salts solution (see belows). This mixture was then shaken at 160 rpm for 5 h at 37°C. The screening was followed by transferring serial dilutions of the samples onto nutrient agar (Merck, Germany) plates. The plates were then incubated at 37°C for 48 h. Twenty three isolates were identified and maintained on nutrient agar slants. The ability of biosurfactant producing colonies was evaluated using the hemolysis test and emulsification Index (E24) (Cooper and Goldenberg, 1987; Mulligan *et al.*, 1984).

Identification of biosurfactant producing microorganism: The selected isolate was identified in the National Laboratory of Industrial Microbiology (NLIM) at Alzahra University according to Kersters and De Ley (1984) and Mac-Faddin (2000).

Medium and culture conditions: The production medium consists of mineral salts solution (g/l): NaNO₃, 7; KH₂PO₄, 0.5; K₂HPO₄, 1.0; KCl, 0.1; MgSO₄·7H₂O, 0.5; CaCl₂·2H₂O, 0.01; FeSO₄·7H₂O, 0.01 supplemented with yeast extract (0.1 g/l) and glucose (2%, w/v), which were used for biosurfactant production. Cultures were inoculated with a 2% (v/v) inoculum (OD₆₀₀ = 0.9 ± 0.05) and incubated at 37°C, pH 7.0, with shaking at 160 rpm in a rotary shaker incubator.

To evaluate the effect of various carbon sources on biosurfactant production, the respective carbohydrates (glucose, molasses) and hydrocarbon substrates (crude oil, kerosene, and hexane) were added separately at the final concentration of 20 g/l or 2% (w/v). Hydrocarbon substrates were sterilized using filtration through a Millipore membrane with a pore size of 0.22 μm. All the chemicals used were of analytical grade and purchased from Merck, Germany. Crude oil used in this research was obtained directly from the Aghajari reservoir and the kerosene was prepared at the Isfahan Oil Refinery, Isfahan, Iran.

Extraction and biochemical analysis of biosurfactant: The cell free supernatant was acidified to pH 2 using HCl (6 M) and left overnight at 4°C for complete precipitation of biosurfactant. The acidified precipitate was then separated and distilled water added. The biosurfactant was extracted by adding chloroform/methanol (2:1, v/v) mixture at room temperature, then concentrated using a rotary evaporator 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).

Emulsification index (E24): The emulsification index was determined by adding 2 ml of crude oil to the same amount of culture supernatant. The resulting mixture was vortexed for 2 min and left to stand for 24 h. The E24 is determined as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) (Cooper and Goldenberg, 1987).

Hemolysis test: Evaluation of hemolytic activity was used for rapid isolation of biosurfactant producing microorganisms by plating cells onto blood agar and incubating at 37°C for 48 h. The formation of clearing zones around the colonies showed those that were positive for hemolysis and potential biosurfactant producers (Ghojavand *et al.*, 2008; Pornsunthorntawee *et al.*, 2008; Mulligan *et al.*, 1984).

Drop-collapse test: The modified drop-collapse test was used for rapid detection of biosurfactant producers. Five microliters of crude oil from Aghajari oil samples were placed in a sterilized microwell plate (Biolog, Hayward, CA), then 10 μl of culture medium was added to the crude oil and observed after 5 min. Potential biosurfactant producers resulted in flat drops and were scored as positive (Bodour and Miller-Maier, 1998).

Surface tension measurement: The surface tension (SF) of the cell free medium was determined using a tensiometer (Kruss model, Germany) at Sharif University of Technology, Tehran.

Optimization of biosurfactant production using the Taguchi method: A standard orthogonal array (L₁₆) was used to examine 4 factors and 4 levels (Table 1). Analysis of data was carried out using the analysis of variance (ANOVA) method. The results were then

Table 1. Variables and their levels used in the Taguchi experimental design method.

Factors	Level 1	Level 2	Level 3	Level 4
Carbon to nitrogen ratio(C/N)	20:1	40:1	60:1	80:1
pH	5	6	7	8
NaCl (mg/l)	2.5	5	7.5	10
Temperature (°C)	30	40	50	60

Table 2. Characteristics of the studied cores.

Core Sample	Diameter (cm)	Length (cm)	Porosity (%)	Permeability (mD)	Pore volume (ml)	SWi* (%)
1	5.8	35	18	54	129.7	22
2	3.2	35	12.5	1	28.48	19
3(Fractured)	3.2	35	14	12	30.33	24

*Initial water saturation

examined to determine the main effects of all factors. The ANOVA was carried out to determine which factors are statistically significant. All calculations were performed using the Taguchi experimental design (Qualitek4, Demo version) software.

Core preparation and characteristics: Carbonate core models obtained from outcrops of the Aghajari mountains were cut on average into 3.2×35 cm or 5.8×35 cm cylinders using a core cutter. In order to conduct the flooding experiments, the cores were cleaned with toluene and then dried at 110°C and a pressure of 50 mbar for 24 h. The Core dimensions, porosity, permeabilities, pore volume (PV) and capillary pressure were determined and the critical injection rate was calculated. In order to study the effect of core permeabil-

ity on oil recovery, three core systems (two unfractured and one fractured with an angle of 180°C) were used. Figure 1 shows a schematic diagram of a fractured core. The characteristics of the studied cores were obtained using pressure-volume-temperature (PVT) experiments (Table 2). The properties of the crude oil samples are presented in Table 3.

Core flooding apparatus: Figure 2 shows the schematic diagram of the core flooding apparatus located in the oil laboratory at Sharif University. Two accumulators were connected to a variable rate injection pump for feeding of water, cell free medium containing biosurfactant, and crude oil, respectively. Pressure and temperature transducers were connected at both ends of the cores. A chart recorder and a digi-

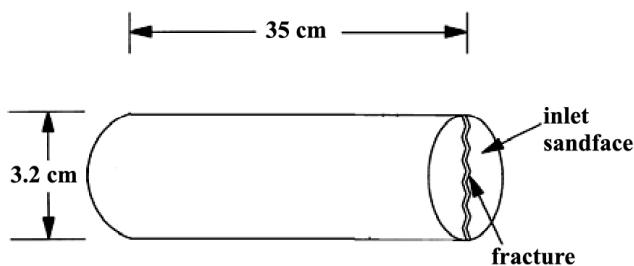


Figure 1. A schematic representation of fractured core (angle=180°C).

Table 3. Characteristics of crude oil.

Property	Value
API*	34
Viscosity of residual oil at 100 F, (cP)	0.7
Bubble point pressure, MPa	11.5
Temperature (°C)	82.8
Sulfur content (w/w)	1.4
Solution gas oil ratio at bp. (v/v)	160.3
Oil formation volume coefficient (v/v)	1.30

*The American Petroleum Institute.

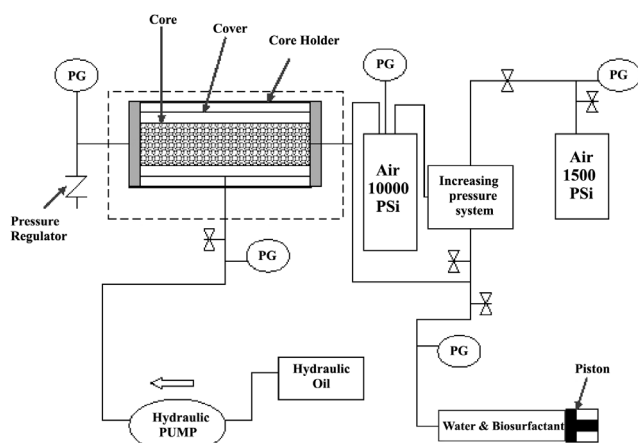


Figure 2. Flowsheet of core flooding apparatus. PG: Pressure gauge

tal pressure recorder were connected to the temperature and pressure transducers, respectively.

Core flooding experiment: The core sample placed in the core holder was first saturated by water flooding using an ionic brine solution with the following composition (g/l): Na^+ , 12.4; K^+ , 0.25; Ca^{2+} , 3.43; Mg^{2+} , 0.93; Cl^- , 26.54; SO_4^{2-} , 1.56; HCO_3^- , 0.09 (Standness and Austad, 2000). The brine saturated core was flooded with crude oil until no water was observed in the output and an initial water saturation (S_{wi}) was obtained. Depending on the type of cores, water or oil flooding was carried out with 120 ml of brine solution or crude oil (about 2-3 pore volumes). Then brine flooding followed until irreducible residual oil saturation (S_{oc}) was achieved. Subsequently, the core was flooded with cell free culture containing biosurfactant. The injected flow rates were approximately 20-40 ml/h for 48 h. All core flooding experiments were conducted at 50°C, under a pressure of 1100 psi. Finally, the cores were cleaned and dried for further use.

RESULTS

Screening of microorganisms: Out of 23 different isolates separated from crude oil and oily sludge samples of the Aghajari oilfield, nine strains appeared with clear zones on blood agar, showing considerable emulsification activity and grew in liquid medium containing glucose. Because blood agar may cause a large number of errors due to insufficient amount of secreted biosurfactant or other microbial products such as virulence factors (Youssef *et al.*, 2004), the drop collapse test was also carried out. The K5 strain which

showed both the fastest collapse time on the oil surface and the highest E24 within 7 days was selected for further studies.

Identification of biosurfactant producing microorganism: The isolate was aerobic, motile, rod-shaped, Gram-negative, oxidase and catalase positive. According to the morphological and biochemical characteristics in the NLIM's report (Table 4), the selected isolate may belong to *Alcaligenes faecalis*, which was designated as *Alcaligenes* sp. MS-103.

Effect of carbon and nitrogen sources on biosurfactant activity: The emulsification activity of the biosurfactant produced by *Alcaligenes* sp. MS-103 using carbon sources such as glucose, molasses, crude oil, kerosene, and hexane (2%, w/v) are displayed in Figure 3. Based on these data, molasses was selected as an economic carbon source for further experiments. Sodium nitrate was used as the only inorganic nitrogen source. The emulsification index was enhanced in the presence of sodium nitrate (7 g/l) (Fig. 4).

Optimization of biosurfactant production: Production of the biosurfactant by *Alcaligenes* sp. MS-103 was optimized using the Taguchi experimental design statistical method for carbon to nitrogen ratio (C/N), pH, NaCl and temperature. The influence of each variable was obtained by ANOVA. The contribution of C/N, pH, NaCl and temperature to growth of the strain was determined as 38.79, 34.92, 6.63 and 14.45%, respectively (Table 5). Carbon to nitrogen

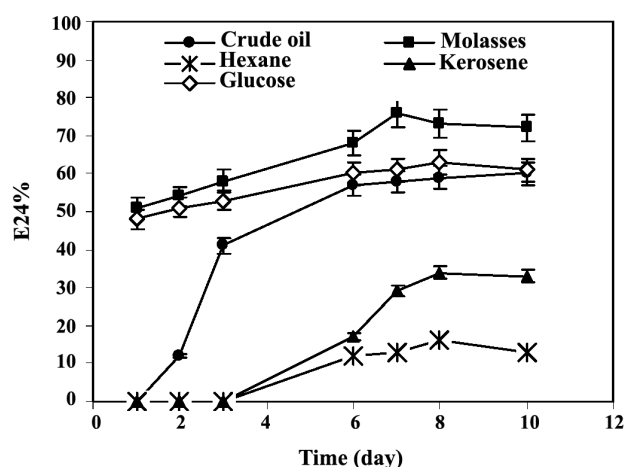


Figure 3. Effect of carbon source on the emulsification index (E24) under optimum conditions. Initial nitrogen contents in medium include NaNO_3 , 7 g/l; Yeast extract, 0.1 g/l.

Table 4. Some morphological and biochemical characteristics of the isolated strain.

Characteristics	<i>Alcaligenes faecalis</i>	Isolate K5
Gram stain	-	-
Shape	Rods, coccil rods or cocci	Rods, coccil rods or cocci
MacConkey agar	G	G
Catalase	+	+
Oxidase	+	+
Motility	+	+
KIA	ALK/ ALK	ALK/ ALK
TSI	ALK/ ALK	ALK/ ALK
Nitrate reduced to nitrite	-	-
Nitrate to gas	-	-
Indole	-	-
H ₂ S production	-1	-1
Utilization of Citrate	+	+
DNase	-	-
Esculin hydrolysis	-	+
Gelatin liquefaction	-	-
Urease	- (2% +)	+
Acid from Glucose	-	-
O/F glucose	O	O
SS agar	+	+
Hydrolysis of Starch	-	-
Cetrimide agar	V	G
Voges-Proskauer test	-	-
Methyl red	-	-

ratio and pH were the most significant factors in production of biosurfactant. The optimum values of C/N, pH, NaCl and temperature obtained were 60:1, 7, 7.5% and 50°C, respectively. The value of error calculated was 5.198. From the obtained F-ratio, it can be concluded that the selected factors considered in the experimental design are significant at more than 95%

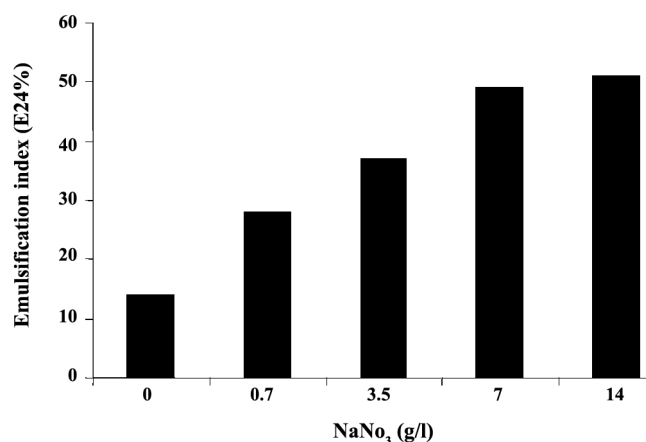


Figure 4. Effect of NaNO₃ concentration on the E24 of biosurfactant produced by *Alcaligenes* sp. MS-103 in the presence of molasses (20 g/l) and yeast extract (0.1 g/l).

of the confidence limit. To validate the proposed optimum conditions, experiments on biosurfactant production were repeatedly performed under optimum conditions. The experimental surface tension obtained was close to the expected surface tension estimated by the Taguchi software in the range of 18.813±1.43321 with a confidence level of 95% and a confidence interval of 1.43321. The best obtained E24, SF and interfacial tension (IFT), by cell free culture using molasses (2%, w/v) as a sole carbon source, were 76%, 20 mN/m and < 1 mN/m, respectively under optimum conditions (Fig. 5).

Biochemical characterization of biosurfactant: The phenol sulfuric acid assay was positive and confirmed the presence of a considerable sugar content of approximately 84% in the biosurfactant. Lipid content of the biosurfactant was approximately 7%.

Effect of water and biosurfactant flooding on oil recovery: Matrix permeability affects the performance of oil recovery from carbonate reservoirs. Figure 6 shows a plot of oil recovery versus pore volume injected. The enhanced oil recovery from core models with

Table 5. Analysis of variance (ANOVA).

No.	Factors	DOF	Sums of Square	Variance	F-ratio	Pure sum	Contribution (%)
1	C/N	3	455.995	151.998	78.160	450.161	38.793
2	pH	3	411.137	137.045	70.471	405.303	34.927
3	NaCl Conc. (mg/l)	3	82.785	27.595	14.189	76.950	6.631
	Temperature (°C)	3	173.534	57.844	29.744	167.700	14.451
Other/Error		19	36.948				5.198
Total		31	1160.401	1.944			100.00

*DOF: Degree of freedom. **F-value shows that the selected factors are significant.

permeabilities of 1 and 54 mD using biosurfactant flooding were 5.6 and 10.7%, respectively. The effects of water flooding followed by biosurfactant flooding at different core permeabilities are presented in Table 6. In laboratory investigations for permeabilities of 1 and 54 mD, in which the reservoir conditions were simulated, the recovery values of crude oil after water flooding were 44 and 41% of the original oil in place. Although, these enhancements in oil recovery with biosurfactant produced by *Alcaligenes* sp. MS-103 do not seem very large, perhaps it could be sufficient to be economical in large scale oil extraction processes from oil wells (Zekri and El-Mehaideb, 2002). Figure 7 shows a significant comparison between fractured and unfractured cores. In the fractured core (case 3) with a permeability of 12 mD, 47.5% of the original oil in

place was obtained by water flooding compared to 56.7% using biosurfactant flooding after water flooding.

DISCUSSION

In this study, an indigenous strain belonging to the *Alcaligenes* sp. was isolated from oil sample of well 121 of Aghajari oilfield in the southern part of Iran. Results of this study showed that *Alcaligenes* sp. MS-103 was able to grow on molasses and produce exocellular biosurfactant with high emulsification activity. The *Alcaligenes* sp. MS-103 considerably reduced the surface tension of the medium from 63 to 20 mN/m and was capable of producing biosurfactant at a level

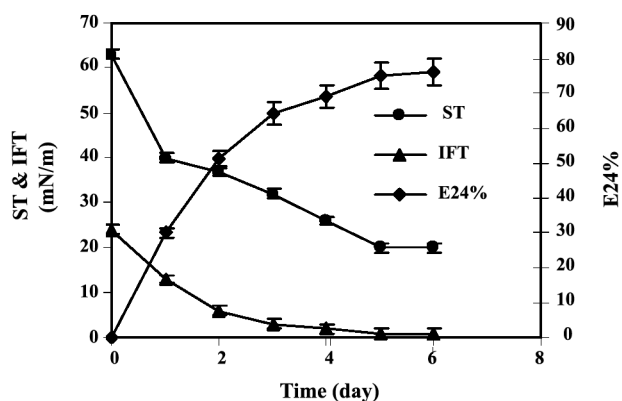


Figure 5. Time course of the emulsification characteristics of cell free medium containing 1.2 ± 0.05 g/l of crude biosurfactant produced by *Alcaligenes* sp. MS-103 using molasses (2% (w/v)) as a sole carbon source under optimum production conditions. Emulsification index (E24), surface tension (ST), and interfacial tension (IFT).

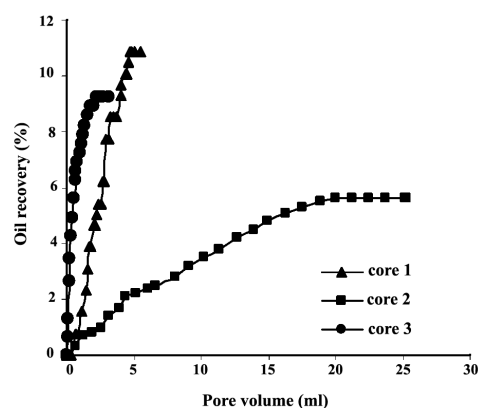


Figure 6. Relationship between oil recovery and pore volume in core flooding experiments using cell free medium containing 1.2 ± 0.05 g/l of crude biosurfactant (BF) produced by *Alcaligenes* sp. MS-103. The injected flow rates were approximately 20-40 ml/h for 48 h.

Table 6. Effect of water flooding and biosurfactant flooding on oil recovery.

Core	Water flooding		Biosurfactant flooding after water flooding							
	Permeability (mD)	OOIP	Recovery		OIPWF (ml)	PVi	Recovery			
			(ml)	(%)			(ml)	%OOIP	%OIPWF	%WF+BF
1	54	129.7	53	41	76.7	5.55	14	10.7	18	51.7
2	1	28.4	12.5	44	15.98	25.28	1.6	5.6	10	49.6
3	12	30.33	14.4	47.5	15.93	3.17	2.8	9.2	15.7	56.7

PVi: injected pore volume. OOIP: original oil in place. OIP: oil in place after water flooding. WF: water flooding. BF: biosurfactant flooding.

of 1.2 ± 0.05 g/l under optimum conditions. Although, there are many reports on the production of biosurfactants by different strains (Joshi *et al.*, 2008; Jinfeng *et al.*, 2005; Rashedi *et al.*, 2005; Mei *et al.*, 2003; Desai and Banat, 1997; Cooper and Goldenberg, 1987), such surface tension reduction has not been reported by other biosurfactant producers belonging to the *Alcaligenes* species (Toledo *et al.*, 2008; Poremba *et al.*, 1991).

In this study, the process for enhancing oil recovery by means of MEOR using fractured and unfractured carbonate core models was evaluated. The highest recovery of residual oil among different experiments was approximately 10.7% using biosurfactant produced by *Alcaligenes* sp. MS-103. Abtahi *et al.* (2003) earlier reported an oil recovery efficiency of 14.3% for

unfractured core models by a different indigenous microorganism isolated from oil samples from the Payedar oilfield. In this study 2% (w/v) sucrose was used as the main carbon source, together with 0.4% (w/v) $(\text{NH}_4)_2\text{HPO}_4$ and 1% (w/v) NaCl (Abtahi, *et al.*, 2003). Mei and his colleagues (2003) have reported that oil recovery can be improved 5% by microbial flooding solely, 7% of oil recovery can be enhanced by adding polymer buffer after injection of the microbial solution. Considerable oil recovery has been obtained at a fractured angle of 180° with a permeability of 12 mD. Although the data obtained by other researchers (Abtahi *et al.*, 2003; Mei *et al.*, 2003; Zekri and El-Mehaideb, 2002) are related to specific environmental conditions, oil characteristics and reservoir properties, some case by case comparisons are feasible. Zekri and co-worker have also reported that fracture angle has a significant effect on performance of the process and have indicated that microbial mediated oil recovery is capable of increasing the recovery by 6-10% of initial oil in place, depending on oil saturation at the start of the flooding (Zekri and El-Mehaideb, 2002).

In conclusion, the bacteria *Alcaligenes* sp. MS-103 produces a highly effective lipopolysaccharide biosurfactant using molasses as a cheap carbon source. Biosurfactant mediated-oil recovery experiments in model carbonate cores promises potential biotechnological applications for use in environmental remediation and microbial enhanced oil recovery.

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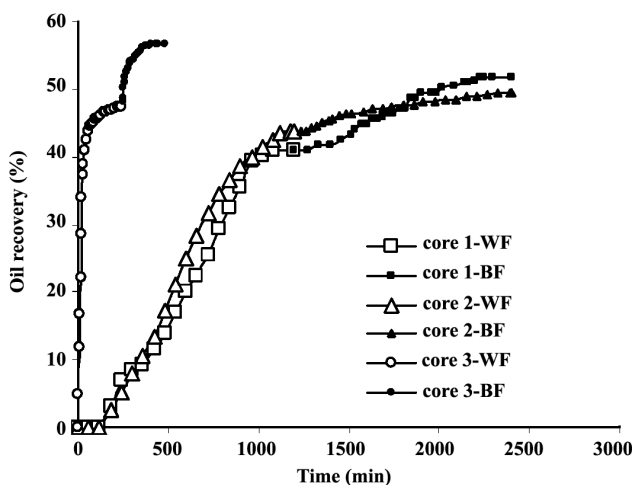


Figure 7. Relationship between oil recovery and pore volume in core flooding experiments using cell free medium containing 1.2 ± 0.05 g/l of crude biosurfactant (BF) produced by *Alcaligenes* sp. MS-103. The injected flow rates were approximately 20-40 ml/h for 48 h.

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