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Original Article

Production and characterization of bioemulsifier from a marine bacterium, *Acinetobacter calcoaceticus* subsp. *anitratus* SM7

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Abstract

Marine bacterium strain SM7 was isolated as a bioemulsifier-producing bacterium from oil-spilled seawater in Songkhla lagoon, Thailand. It was identified as *Acinetobacter calcoaceticus* subsp. *anitratus* based on morphology, biochemical characteristics and 16S rRNA sequence. *A. calcoaceticus* subsp. *anitratus* SM7 produced an extracellular emulsifying agent when grown in a minimal salt medium (pH 7.0) containing 0.3% (v/v) *n*-heptadecane and 0.1% (w/v) ammonium hydrogen carbonate as carbon source and nitrogen source, respectively, at 30°C with agitation rate of 200 rpm. Crude bioemulsifier was recovered from the culture supernatant by ethanol precipitation with a yield of 2.94 g/l and had a critical emulsifier concentration of 0.04 g/ml. The crude bioemulsifier was capable of emulsifying *n*-hexadecane in a broad pH range (6-12), temperatures (30-121°C) and in the presence of NaCl up to 12% (w/v). The bioemulsifier was stable in salt solution ranging from 0 to 0.1% (w/v) of $MgCl_2$ and $CaCl_2$. The broad range of pH stability, thermostability and salt tolerance suggested that the bioemulsifier from *A. calcoaceticus* subsp. *anitratus* SM7 could be useful in environmental application, especially bioremediation of oil-polluted seawater.

Keywords: bioemulsifier, marine bacteria, *Acinetobacter* sp., production, characterization

1. Introduction

Petroleum hydrocarbons are major pollutants of the marine environment as a result of terrestrial and freshwater run-off. Evaporation and photo-oxidation play an important role in oil detoxification. Ultimate and complete degradation is accomplished mainly by marine microflora (Yakimov *et al*., 1998). Past surveys have indicated that natural removal of the oil-spilled in marine environment is slow and oil deposits persist for many years. Biological processes involved in the clean-up of oil-spills in the marine environment have not been widely and successfully implemented. The addition of nitrogen and phosphorus nutrients has been shown to accelerate the speed of biodegradation (Harayama *et al*., 2004). The emulsification activity of hydrocarbons by biosurfactants or bioemulsifiers produced by microorganisms is considered an essential step in hydrocarbon biodegradation in the marine environment (Yakimov *et al*., 1998). The use of biosurfactants/bioemulsifiers to protect the marine environment seems possible since a number of marine bacterial strains can produce biosurfactants during growth on hydrocarbons (Bertrand *et al*., 1993). For the sake of the environment, the use of biosurfactants is preferable to those of synthetic surfactants. However, little information on either biosurfactants produced by marine microorganisms or biosurfactants active in marine environment has been reported so far.

Biosurfactants have unique amphipathic properties since their complex structures are composed of hydrophobic region and hydrophilic portion (Ron and Rosenberg, 2001). As a consequence, biosurfactants can partition preferentially at the interface (Desai and Banat, 1997). Many microorganisms can produce biosurfactant which adheres to cells or is excreted extracellularly in growth medium (Makkar and

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Cameotra, 1998). Biosurfactants have several important advantages over chemical surfactants, which should allow them to become prominent in industrial and environmental applications (Toren *et al*., 2001).

The majority of *Acinetobacter* strains produce high-molecular-weight biosurfactant. All biosurfactants were nondialyzable and contained polysaccharide, as their major component (Navon-venezia *et al*., 1995). Bioemulsifier produced by *Acinetoobacter* strains included emulsan from *A. calcoaceticus* RAG-1 and BD4 (Toren *et al*., 2001), alasan from *A. radioresistens* (Navon-venezia *et al*., 1995) and biodispersan from *A. calcoaceticus* A2 (Rosenberg *et al*., 1988). In this paper, production and characterization of bioemulsifier produced by the newly isolated marine bacterium, *A. calcoaceticus* subsp. *anitratus* SM7 was studied.

2. Materials and Methods

2.1 Chemical

Aliphatic hydrocarbons (*n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane and *n*-heptadecane) were purchased from Nacalai Tesque Inc. (Tokyo, Japan). Benzene, toluene and xylene were obtained from Lab-Scan (Bangkok, Thailand). Synthetic surfactants; sodium dodecyl sulfate (SDS) and Tween 80 were purchased from Bio-Rad (California, USA) and Ajax finechem Ltd (Auckland, New Zealand), respectively. All chemicals were of analytical grade.

2.2 Microorganism and cultivation

Isolate SM7 isolated from oil-spilled seawater in Songkhla lagoon, Thailand, was used throughout this study. Enrichment culture technique was used to isolate bioemulsifier producing bacteria (Batista *et al*., 2006). It was maintained in 25% (v/v) glycerol at -20°C. The bacterium was grown in Marine Broth 2216 (Difco, USA) at 30°C with shaking at 200 rpm for 24 h and was used as inoculum at 1% (v/v).

2.3 16S ribosomal RNA sequencing

The full length 16S ribosomal RNA sequence of strain SM7 was analyzed based on the methods of Rochelle *et al*. (1995). Nucleotide sequencing was carried out using an ABI Prism 377-18 DNA sequencer (Applied biosystems, Tokyo, Japan) and a Thermo sequence fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) according to their instruction manuals.

2.4 Production of bioemulsifier

Isolate SM7 was grown aerobically by shaking at 200 rpm in 250 ml flask with 100 ml working volume of minimal salt medium with the following composition (per liter): 2.2 g $K_2HPO{\cdot}3H_2O$, 0.73 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$ and 30 g NaCl (as found in seawater). The initial pH of mineral salt medium was about 7.0. The substrates used as carbon source included *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane and *n*-heptadecane. Different nitrogen sources involving NH_4NO_3, $(NH_4)_2SO_4$, NH_4HCO_3 were also tested. Further experiment was conducted to determine the optimal amount of carbon source and nitrogen source by varying their concentration from 0.1 to 0.5% (w/v). Effects of different initial pHs (5-8) for production bioemulsifier were determined at room temperature.

2.5 Recovery of bioemulsifier

Culture broth was centrifuged at 11,000 x g for 20 min at 4°C. Bioemulsifier was precipitated from the supernatant by using 4 methods; ammonium sulfate (Navon-venezia *et al*., 1995; Rosenberg *et al*., 1988; Rosenberg *et al*., 1979b), acetone (Cameotra and Singh, 1990), methanol and ethanol precipitation (Nohato *et al*., 1996). The method showing the highest yield and the lowest critical emulsifier concentration was used to recover bioemulsifier from *A. calcoaceticus* subsp. *anitratus* SM7.

2.5 Stability of bioemulsifier

The bioemulsifier from *A. calcoaceticus* subsp. *anitratus* SM7 (4%, w/v) in distilled water was prepared. To investigate the effects of pH, salts concentration (NaCl, $CaCl_2$ and $MgCl_2$) and temperature on emulsification activity of bioemulsifier, the bioemulsifier solution was adjusted with 1 N HCl or NaOH to obtain the pHs of 2-12. NaCl was added to the sample to obtain the final concentrations of 0-12% (w/v). $CaCl_2$ and $MgCl_2$ were also added to the samples to obtain the final concentrations of 0-0.1% (w/v). For thermal stability study, bioemulsifier solution was incubated for 1 h at different temperatures (30-121°C) and cooled to 30°C. Remaining activity was then determined. Chemically synthetic surfactants (sodium dodecyl sulfate (SDS) and Tween 80) at the same concentration were also subjected to stability study.

2.6 Analytical method

Bacterial growth was determined by total cell protein measurement using the Lowry method (Lowry *et al*., 1951). Prior to analysis, cells were collected by centrifugation at 11,000 x g for 15 min. Then, wet cells were resuspended in 0.1N NaOH and boiled at 100°C for 20 min (Shabtai, 1990). Emulsification activity was measured according to the method of Cooper and Goldenberg (1987) with a slight modification. To 1 ml of cultural supernatant or crude bioemulsifier, 1 ml of *n*-hexadecane was added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. Emulsification activity (%EA) is defined as the height of the emulsion layer divided by the total height and expressed as percentage. The experiments

were done in triplicate and results were reported as the average from triplicate determinations.

2.7 Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test. Statistical analysis was performed using Statistical Package for Science (SPSS 10.0 for Window, SPSS Inc., Chicago, IC).

3. Results

3.1 Isolation and identification of strain SM7

Strain SM7 was isolated as a crude oil-emulsifying producing bacterium and characterized as follows: Gram-negative, non-spore-forming, motile, catalase positive, oxidase negative, O/F glucose negative (-/-), citrate positive and negative for indole production. 16S rRNA analysis of the strain showed 99.38% homology with *A. calcoaceticus* subsp. *anitratus*. The 16S rRNA sequence (approx. 1.5 kb) was deposited in DDBJ/EMBL/GenBank as accession no. AB302132.

3.2 Production of bioemulsifier

The optimization of bioemulsifier production by *A. calcoaceticus* subsp. *anitratus* SM7 in a minimal salt medium (initial pH about 7.0) was studied by examining its ability to emulsify *n*-hexadecane of culture supernatant at 48 h of cultivation time. *A. calcoaceticus* subsp. *anitratus* exhibited emulsification activity toward *n*-hexadecane when *n*-hexadecane and *n*-heptadecane were used as carbon source (Table 1a). The emulsification activities about 30% and 27% were obtained, respectively, when *n*-heptadecane and *n*-hexadecane were used as carbon sources. As shown in Table 1, a decrease in pH value was also observed. *A. calcoaceticus* subsp. *anitratus* SM7 did not use *n*-tridecane, *n*-tetradecane and *n*-pentadecane as carbon sources. *n*-heptadecane was selected as carbon source to study growth and bioemulsifier production by the strain and the optimal concentration of *n*-heptadecane was 0.3% (v/v) (Table 1b).

The nitrogen source affected bioemulsifier production as depicted in Table 2a. Ammonium hydrogen carbonate was the best source of nitrogen when tested with the optimum concentration of 0.1% (w/v) (Table 2b). Emulsification activity of 58.33% was obtained when 0.1% (w/v) ammonium hydrogen carbonate was used as nitrogen source. Figure 1 presents emulsification activity obtained for culture supernatant at different initial pH values in minimal salt medium. For all the pH values tested except pH 5.0, an emulsification activity against *n*-hexadecane was noted by *A. calcoaceticus* subsp. *anitratus* SM7. These results indicate that the strain has ability to produce bioemulsifier at pH values ranging from 6.0 to 8.0. The optimum initial pH of minimal salt

Table 1. Effect of type and concentration carbon sources on bioemulsifier production by *A. calcoaceticus* subsp. anitratus SM7 in a minimal salt medium, initial pH about 7.0, at 30°C after 48 h cultivation.

(a) Effect of carbon source

C-source (0.1% (v/v))	Final pH*	Emulsification activity (%)*	Total cell protein (mg/ml)*
n-tridecane	6.96±0.01	0	ND
n-tetradecane	6.95±0.02	0	ND
n-pentadecane	6.96±0.02	0	ND
n-hexadecane	6.82±0.08	27.08±1.31[a]**	0.01±0.0
n-heptadecane	6.34±0.13	30.67±3.77[a]	0.22±0.1

(b) Effect of *n*-heptadecane concentration

n-heptadecane concentration (% (v/v))	Final pH*	Emulsification activity (%)*	Total cell protein (mg/ml)*
0.1	6.55±0.05	28.08±5.87[b]**	0.18±0.04
0.3	5.28±0.04	46.31±3.34[a]	0.36±0.08
0.5	5.24±0.02	29.25±4.78[b]	0.48±0.02

* Values are given as mean ± SD from triplicate determinations.

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

Table 2. Effect of type and concentration of nitrogen sources on bioemulsifier production after growth of *A. calcoaceticus* subsp. *anitratus* SM7 at 30°C after 48 h cultivation.

(a) Effect of nitrogen source

N-source (0.1% (w/v))	Initial pH	Final pH*	Emulsification activity (%)*	Total cell protein (mg/ml)*
NH_4NO_3	6.75±0.02	5.67±0.08	42.54±3.85[b]**	0.34±0.07
$(NH_4)_2SO_4$	6.55±0.01	5.55±0.12	38.55±3.38[b]	0.41±0.03
NH_4HCO_3	6.95±0.02	6.58±0.02	48.67±1.15[a]	0.47±0.04

(b) Effect of NH_4HCO_3 concentration

NH_4HCO_3 concentration (%)	Initial pH	Final pH*	Emulsification activity (%)*	Total cell protein (mg/ml)*
0.1	6.95±0.02	6.65±0.10	58.33±4.17[a]**	0.59±0.09
0.3	7.52±0.02	7.37±0.06	36.59±2.61[b]	0.40±0.13
0.5	9.03±0.04	9.05±0.07	0[c]	ND

* Values are given as mean ± SD from triplicate determinations.
** Different letters in the same column indicate significant differences ($p<0.05$).

medium for bioemulsifier production was found to be 7.0.

Finally, when *A. calcoaceticus* subsp. *anitratus* SM7 was grown in 0.3% (v/v) *n*-heptadecane as carbon source and ammonium hydrogen carbonate at 0.1% (w/v) as nitrogen source with initial pH 7.0, at 30°C and agitation rate of 200 rpm, it was observed that bioemulsifier production was growth-associated. It increased with cell mass up to 48 h (Figure 2). After 48 h there was a sharp decrease in emulsification activity. Maximal bioemulsifier production was achieved in the late logarithmic phase.

3.3 Recovery of bioemulsifier

Crude extract of the bioemulsifier was recovered from the culture supernatant of *A. calcoaceticus* subsp. *anitratus* SM7 by precipitation with salt and solvents. Among four precipitation methods, ethanol precipitation was the most efficient in bioemulsifier recovery from culture supernatant (Table 3). Recovery yields of 2.94 g/l, 1.04 g/l and 0.76 g/l were obtained when ethanol, acetone and methanol were used, respectively. In addition, critical emulsifier concentrations were 0.04 g/ml, 0.06 g/ml and 0.05 g/ml when precipitated by ethanol, acetone and methanol, respectively. Precipitation with ammonium sulfate could not recover bioemulsifier from culture supernatant.

3.4 Stability of bioemulsifier

Effect of pH on emulsification activity of crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 and chemically surfactants, Tween 80 and SDS, is shown in Table 4. The emulsification activity of bioemulsifier from

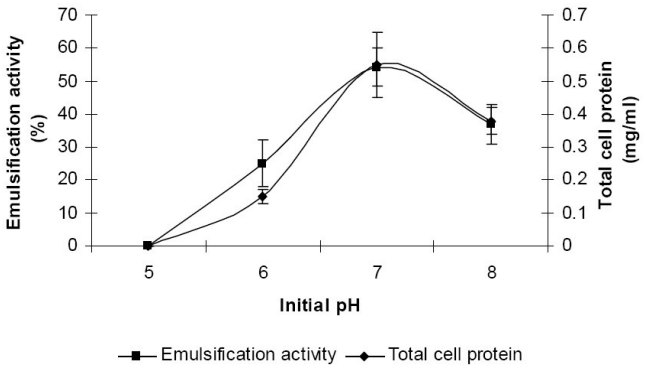

Figure 1. Effect of initial pH on bioemulsifier production by *A. calcoaceticus* subsp. *anitratus* SM7 at 30°C after 48 h cultivation.

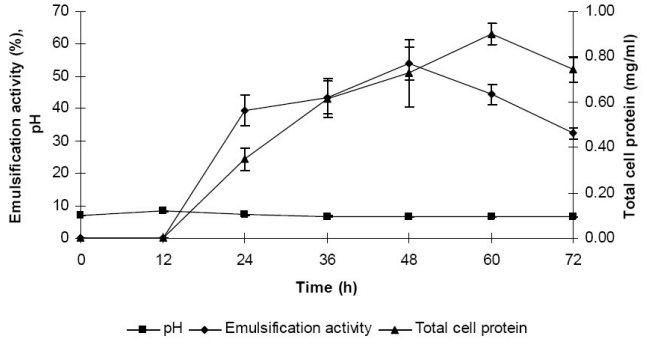

Figure 2. Time courses of growth (▲), culture pH (■) and emulsification activity (◆) by *A. calcoaceticus* subsp. *anitratus* SM7 grown in minimal salt medium (pH 7) with 0.3% (v/v) *n*-heptadecane and 0.1% (w/v) ammonium hydrogen carbonate at 30°C and agitation rate 200 rpm.

Table 3. Effect of recovery methods on yield and emulsifying activity of bioemulsifier produced by *A. calcoaceticus* subsp. *anitratus* SM7.

Precipitation method	Yield (g/l)* (Critical emulsifier concentration (g/ml))	Emulsification activity (%)*
Ammonium sulfate	0.00^{d}** (0^{c})	0^{b}
Acetone	1.04^{b} (0.06^{a})	64.86 ± 1.03^{a}
Methanol	0.76^{c} (0.05^{ab})	65.00 ± 1.41^{a}
Ethanol	2.94^{a} (0.04^{b})	65.58 ± 2.74^{a}

* Values are given as mean ± SD from triplicate determinations.
** Different letters in the same column indicate significant differences ($p<0.05$).

Table 4. Effect of pH on emulsification activity of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7.

pH	Emulsification activity (%)*		
	Crude extract	SDS	Tween 80
2	48.24 ± 3.36^{D}**^{b}***	50.30 ± 0.44^{Fb}	58.72 ± 0.86^{Da}
3	46.53 ± 4.34^{Db}	55.11 ± 0.70^{Ea}	60.33 ± 0.56^{Da}
4	49.31 ± 1.20^{Dc}	60.07 ± 0.80^{Db}	64.84 ± 0.61^{Ca}
5	53.08 ± 1.09^{Cc}	64.55 ± 1.35^{Cb}	68.46 ± 0.57^{Ba}
6	63.26 ± 0.75^{Bc}	67.47 ± 1.57^{ABb}	70.92 ± 1.27^{Aa}
7	66.22 ± 0.39^{ABc}	69.24 ± 0.92^{Ab}	72.39 ± 0.84^{Aa}
8	65.97 ± 1.21^{ABb}	67.98 ± 0.93^{ABb}	72.80 ± 0.92^{Aa}
9	67.37 ± 1.38^{Ab}	67.93 ± 0.43^{ABb}	71.53 ± 0.36^{Aa}
10	66.89 ± 2.12^{ABb}	67.47 ± 1.57^{ABb}	71.34 ± 1.89^{Aa}
11	67.31 ± 1.32^{Ab}	66.16 ± 0.44^{BCb}	71.04 ± 2.01^{Aa}
12	67.15 ± 1.06^{Ab}	66.42 ± 0.88^{Bb}	70.82 ± 2.17^{Aa}

* Values are given as mean ± SD from triplicate determinations.
** Different capital letters in the same column indicate significant differences ($p<0.05$).
*** Different letters in the same row indicate significant differences ($p<0.05$).

A. calcoaceticus subsp. *anitratus* SM7, Tween 80 and SDS decreased obviously with decreasing pH below 5. However, no changes in activity were noticeable in the pH range of pH 7-12. From the result, the crude extract of bioemulsifier from *A. calcoaceticus* subsp. *anitratus* SM7 showed slightly lower activity than Tween 80 at all pHs tested. However, the crude extract showed no significant differences in emulsification activity in comparison with SDS. Therefore, the bioemulsifier crude extract from strain SM7 may have potential in the bioremediation of hydrocarbons over a wide pH range.

After the bioemulsifier crude extract, SDS and Tween 80 were incubated at 30-100°C for 1 h and at 110 and 121°C for 15 min, the residual activity was determined (Table 5). Temperatures ranging from 30 to 121°C did not show any influence on the emulsification activity toward *n*-hexadecane of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 (Table 5). SDS showed thermal stability pattern similar to bioemulsifier crude extract. Tween 80 showed higher activity than the bioemulsifier crude extract from strain SM7 at all temperatures tested (Table 5).

The effect of salts on emulsification activity of bioemulsifier crude extract and commercial surfactants is illustrated in Figure 3. At very high NaCl concentration, emulsification activity of bioemulsifier crude extract tended to decrease (Figure 3a). Bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 exhibited no significant differences in emulsifying activity toward *n*-hexadecane as NaCl increased up to 2% (w/v) ($p<0.05$). At 12% (w/v) NaCl, bioemulsifier crude extract still exhibited emulsification activity of more than 60%. For the chemically synthetic surfactants, Tween 80 and SDS, their emulsification activities were also lowered with increasing NaCl concentration (Figure 3a). Tween 80 showed the highest activity, followed by SDS and bioemulsifier crude extract, respectively, in all NaCl concentrations tested. From the results, varying activity among all samples was observed with different NaCl concentrations.

The effect of $MgCl_2$ on emulsification activity is

Table 5. Effect of temperature on emulsification activity of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7.

Temperature (°C)	Emulsification activity (%)*		
	Crude extract	SDS	Tween 80
30	65.50±2.27^{AB**b***}	67.71±1.62^{Ab}	72.19±0.94^{Aa}
40	65.96±2.13^{ABb}	67.18±1.16^{ABb}	72.72±0.62^{Aa}
50	65.52±1.06^{ABb}	67.19±1.71^{ABb}	72.19±0.50^{Aa}
60	66.42±2.47^{Abb}	67.02±1.81^{ABb}	72.25±1.85^{Aa}
70	68.28±0.41^{Ac}	66.93±0.88^{ABb}	72.93±0.35^{Aa}
80	66.44±1.55^{ABb}	66.93±0.88^{Ab}	72.53±1.64^{Aa}
90	63.25±3.03^{Bb}	65.70±0.84^{ABb}	71.55±1.89^{ABa}
100	63.97±1.13^{Bb}	66.42±0.88^{ABb}	71.55±1.89^{ABa}
110	63.63±2.43^{Bb}	65.67±1.13^{ABb}	69.63±0.12^{Ba}
121	60.15±0.66^{Bc}	65.16±0.74^{Bb}	69.29±1.09^{Ba}

* Values are given as mean ± SD from triplicate determinations.

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

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

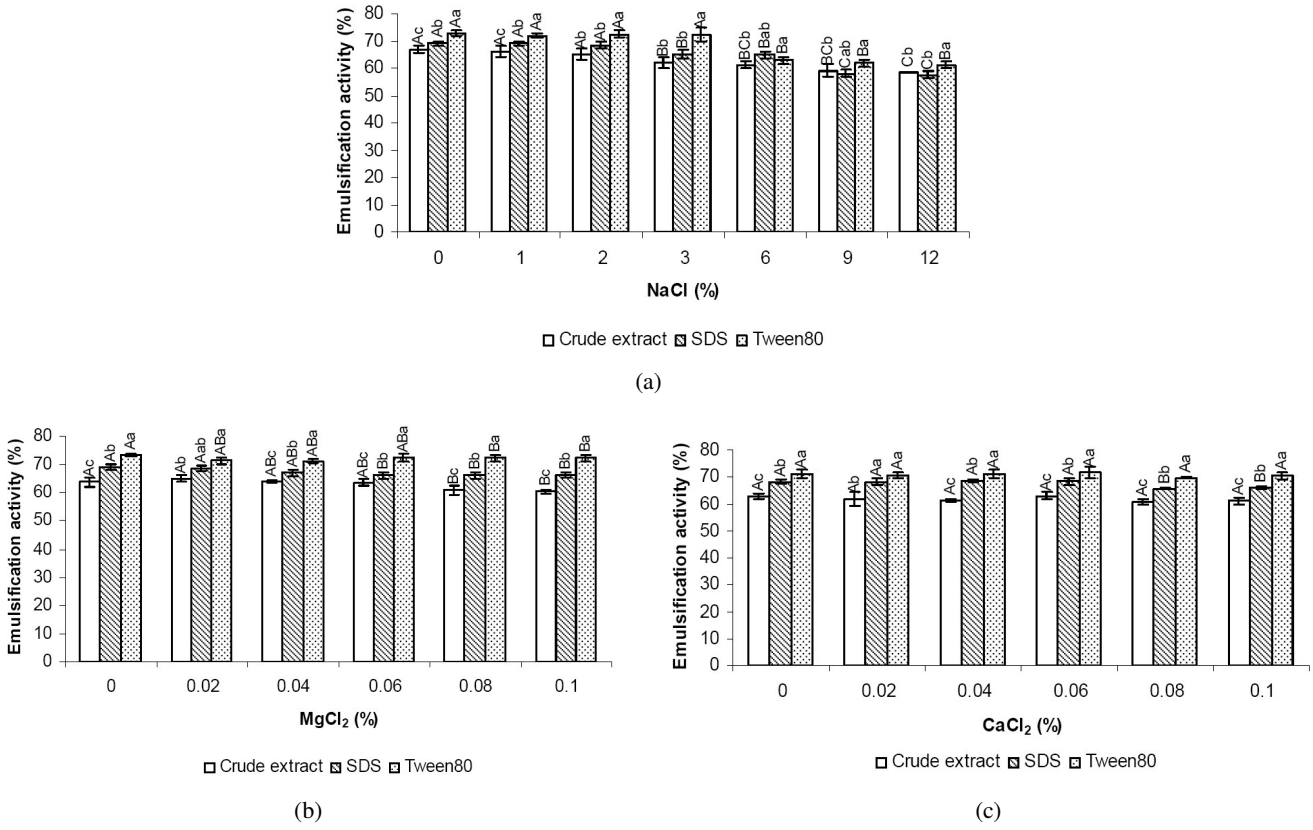

Figure 3. Effect of salts concentration on emulsification activity of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7, SDS and Tween 80 (a) NaCl (b) $MgCl_2$ (c) $CaCl_2$. The concentration of bioemulsifier solution was 0.04 g/ml. Bars represent the standard deviation from three determinations.

presented in Figure 3b. $MgCl_2$ ranging from 0 to 0.06 % (w/v) had no effect on emulsification activity of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 and Tween 80. Tween 80 showed higher emulsification activity toward *n*-hexadecane in the presence of $MgCl_2$ than bioemulsifier crude extract and SDS at all concentrations

tested (Figure 3b).

$CaCl_2$ ranging from 0 to 0.1% (w/v) had no effect on emulsification activity of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 and Tween 80 (Figure 3b). A slight decrease in activity of SDS was observed when 0.08% (w/v) $CaCl_2$ was added. However, chemically synthetic surfactants exhibited higher emulsification activity than bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 in all $CaCl_2$ concentrations tested.

3.5 Emulsification properties of bioemulsifier

Hydrocarbon specificity of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 is shown in Table 6. Aliphatic hydrocarbons ranging from *n*-tridecane to *n*-heptadecane and aromatic hydrocarbons were assayed for their ability to serve as substrates for emulsification. Bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 exhibited no significant differences in emulsification activity, compared with aliphatic hydrocarbons. Aromatic hydrocarbons were emulsified more effectively than aliphatic hydrocarbons by bioemulsifier crude extract. Emulsification activities of about 75%, 74% and 71% were obtained with toluene, xylene and benzene, respectively.

4. Discussion

Acinetobacter sp. strain cannot utilize sugars as a carbon source but grows on other various carbon sources such as hydrocarbons, alcohols, fatty acids and triglycerides (Shabtai, 1990). It is a widespread phenomenon that *Acinetobacter* species produces extracellular bioemulsifiers (Navon-venezia *et al*., 1995; Rosenberg *et al*., 1988). *Acinetobacter calcoaceticus* RAG-1 produced bioemulsifier when grown in *n*-hexadecane and ethanol-containing medium. The maximum amount of emulsifying activities was 14 U/ml and 25 U/ml of cell-free supernatant when grown in *n*-hexadecane medium and ethanol medium, respectively (Rosenberg *et al*., 1979b). Some experiments used vegetable oils or fatty acids as carbon source to produce bioemulsifier because of some important advantages regarding lower toxicity, higher productivity and lower commodity prices (Shabtai, 1990). In the present study, *Acinetobacter calcoaceticus* subsp. *anitratus* SM7 produced an extracelluar emulsifying agent when grown in minimal salt medium containing *n*-heptadeane as the carbon source. Production of bioemulsifier by *A. calcoaceticus* subsp. *anitratus* SM7 was growth-associated. This result was in agreement with Rosenberg *et al*. (1979b) who reported the production of bioemulsifier by *Acinetobacter* RAG-1 in culture medium containing *n*-hexadecane as a carbon source. The ratio of emulsifying activity to cell biomass during production of alasan by *A. radioresistens* KA53 increased from 5.3 to 7.3 and to 11.6 at 24, 64 and 87 h of cultivation time, respectively (Navon-venezia *et al*., 1995).

Table 6. Hydrocarbon substrates specificity of bioemulsifier from *A. calcoaceticus* subsp. *anitratus* SM7.

Hydrocarbon	Emulsification activity (%)*
n-tridecan	61.50±1.32^{c}**
n-tetradecane	64.66±2.40^{c}
n-pentadecane	61.07±1.01^{c}
n-hexadecane	61.60±1.64^{c}
n-neptadecane	62.86±2.68^{c}
benzene	71.42±3.06^{b}
toluene	75.51±2.84^{a}
xylene	74.82±0.84^{ab}

* Values are given as mean ± SD from triplicate determinations.

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

In general, ammonium sulfate precipitation was used for recovery bioemulsifier produced by *Acinetobacter* sp. (Navon-venezia *et al*., 1995; Rosenberg *et al*., 1988; Rosenberg *et al*., 1979b). In this paper, precipitation with ammonium sulfate did not recover bioemulsifier from culture supernatant. Factors such as pH, temperature and protein purity play important roles in determinating the salting out point of a particular protein (Scopes, 1981).

Precipitation of bioemulsifier crude extract occurred at pHs below 6, but no changes in activity were obtained in the pH range of 6-12. The stability of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7 to alkali pH indicates that ester linkages are not required for its emulsifying activity (Navon-venezia *et al*., 1995). At pH close to isoelectric point, there is no electrostatic repulsion between neighboring molecules, and the compounds tend to coalesce and precipitate (Milewski, 2001). Emulsifying activity of alasan from *A. radioresistens* KA53 was observed over the pH 3.3 to 9.2, with a maximum at pH 5.0 (Navon-venezia *et al*., 1995). Biodispersan from *A. calcoaceticus* A2 had optimum pH between pH 9 to 12 for limestone-dispersing activity (Rosenberg *et al*., 1988).

NaCl activated biosurfactant activity of many strains isolated from seawater or petroleum reservoirs (Yakimov *et al*., 1995). Nevertheless, bioemulsifier extracted from *A. calcoaceticus* subsp. *anitratus* SM7 still had the emulsification activity in the absence of NaCl. Calcium and magnesium salts are also present in seawater. Those divalent ions frequently break the oil/water emulsion (Kim *et al*., 1997). Magnesium and phosphate ions strongly inhibited activity of biodispersan A2. Fifty percent inhibition of biodispersan A2 activity occurred at 0.035% K_2HPO_4 and 0.076% $MgCl_2$ (Rosenberg *et al*., 1988). However, $MgCl_2$ and $CaCl_2$ had had no effect on emulsification activity of bioemulsifier crude extract from *A. calcoaceticus* subsp. *anitratus* SM7.

Bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1, BD4 and BD413 poorly emulsified pure aliphatic and aromatic hydrocarbons. They have an absolute

requirement for a mixture of an aliphatic and an aromatic hydrocarbon for efficient hydrocarbon emulsification in water (Kaplan and Rosenberg, 1982; Rosenberg *et al*., 1979a). In contrast, bioemulsifier produced from strain SM7 was able to emulsify pure aliphatic and aromatic hydrocarbons, which is similar with alasan obtained from *A. radioresistens* (Navon-venezia *et al*., 1995).

Stability to pH, temperature and salts and the ability to emulsify hydrocarbons are properties of bioemulsifier produced from *A. calcoaceticus* subsp. *anitratus* SM7 that could be useful in environmental application especially bioremediation of oil-polluted in seawater. Nonetheless, additional tests must be done prior to the practical use of bioemulsifier for eliminating hydrocarbons.

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References

Batista, S.B., Mounteer, A.H., Amorim, F.R. and Totola, M.R. 2006. Isolation and characterization of biosurfactant/ bioemulsifier-producing bacteria from petroleum contaminated sites. Bioresource Technology. 97, 868-875.

Bertrand, J.C., Bianchi, M., Mallah, M.A., Acquaviva, M. and Mille, G. 1993. Hydrocarbon biodegradation and hydrocarbonoclastic bacterial communities composition grown in seawater as a function of sodium chloride concentration. Journal of Experimental Marine Biology and Ecology. 168, 125-138.

Cameotra, S.S. and Singh, H.D. 1990. Purification and characterization of alkane solubilizing factor produced by pseudomonas PG-1. Journal of Fermentation and Bioengineering. 69, 341-344.

Cooper, D.G. and Goldberg, B.G. 1987. Surface active agents from *Bacillus* species. Appllied and Environmental Microbiology. 53, 224-229.

Desai, J.D. and Banat, I.M. 1997. Microbial production of surfactants and their commercial potential. Microbiology and Molecular Biology Reviews. 61, 47-64.

Harayama, S., Kasai, Y. and Hara, A. 2004. Microbial communities in oil-contaminated seawater. Current Opinion in Biotechnology. 15, 205-214.

Kaplan, N. and Rosenberg, E. 1982. Exopolysaccharide distribution of and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. Applied and Environmental Microbiology. 44, 1335-1341.

Kim, H.S., Yoon, B.D., Lee, C.H., Suh, H.H., Oh, H.M., Katsuraki, T. and Tani, Y. 1997. Production and properties of lipopeptide biosurfactant from *Bacillus subtilis* C9. Journal of Fermentation and Bioengineering. 84, 41-46.

Lowry, O.H., Rosebrough, J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry. 193, 265-275.

Makkar, R.S. and Cameotra, S.S. 1998. Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis*. Journal of Industrial Microbiology and Biotechnology. 20, 48-52.

Milewski, S. 2001. Protein structure and physicochemical properties. In Chemical and functional properties of food proteins, Z.E. Sikorski, editor. Technomic Publishing Company Inc, Pennsylvania, U.S.A., pp 35-55.

Navon-venezia, S., Zosim, Z., Gottlieb, A., Legmann, R., Carmell, S., Ron, E.Z. and Rosenberg, E. 1995. Alasan, a new bioemulsifier from *Acinetobacter radioresistens*. Applied and Environmental Microbiology. 61, 3240-3244.

Nohato, Y., Azuma, J. and Kurane, R. 1996. Structural studies of a neutral polysaccharide produced by *Alcaligenes latus.* Carbohydrate Research. 293, 213-222.

Rochelle, P.A., Will, J.A.K., Fry, J.C., Jenkins, G.J.S., Parkes, R.J., Turley, C.M. and Weightman, A.J. 1995. Extraction and amplification of 16S rRNA genes from deep marine sediments and seawater to assess bacterial community diversity. In Nucleic acids in the environment, J.T. Trevors, J.D. Elsas, editor. Springer, Berlin, Germany, pp 219-239.

Ron, E.Z. and Rosenberg, E. 2001. Natural roles of biosurfactants: Minireview. Environmental Microbiology. 3, 229-236.

Rosenberg, E., Perry, A., Gibson, D.T. and Gutnick, D.L. 1979a. Emulsifier *Arthrobacter* RAG-1: Specificity of hydrocarbon substrate. Applied and Environmental Microbiology. 37, 409-413.

Rosenberg, E., Rubinovitz, C., Gottlieb, A., Rosenhak, S. and Ron, E.Z. 1988. Production of biodispersan by *Acinetobacter calcoaceticus* A2. Applied and Environmental Microbiology. 54, 317-322.

Rosenberg, E., Zuckerberg, A., Rubinovitz, C. and Gutnick, D.L. 1979b. Emulsifier *Arthrobacter* RAG-1: Isolation and emulsifying properties. Applied and Environmental Microbiology. 37, 402-408.

Scopes, R.K. 1982. Protein Purification. Principles and Practice. Springer-Verlag, New York.

Shabtai, Y. 1990. Production of exopolysaccharides by *Acinetobacter* strains in a controlled fed-batch fermentation process using soap stock oil (SSO) as carbon source. International Journal of Biological Macromolecules. 12, 145-152.

Toren, A., Navon-venezia, S., Ron, E.Z. and Rosenberg, E. 2001. Emulsifying activities of purified alasan proteins from *Acinetobacter radioresistens* KA*53*. Applied and Environmental Microbiology. 67, 1102-1106.

Yakimov, M.M., Timmis, K.N., Wray, V. and Fredrickson, H.L. 1995. Characterization of new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. Applied and Environmental Microbiology. 61, 1706-1713.

Yakimov, M.M., Golyshin, P.N., Lang, S., Moore, E.R.B., Abraham, W.R., Lunsdorf, H. and Timmis, K.N. 1998. *Alkanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. International Journal of Systematic Bacteriology. 48, 339-348.