6th International Workshop

on

WATER DYNAMICS

Program & Abstracts

Sendai International Center, Sendai Japan
4-6, March 2009

Graduate School of Environmental Studies, Tohoku University

Global COE Program
"Global Education and Research Center for Earth and Planetary Dynamics"

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Field Excursion

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6th International Workshop on WATER DYNAMICS

Program

4-Mar			chair
13:30-13:40	opening address	E. Ohtani	N. Tsuchiya
13:40-14:10	keynote lecture	The role of water in the deep upper mantle and transition zone; Deep Dehydration and Big Mantle Wedge E. Ohtani	N. Tsuchiya
14:15-14:45	invited talk	N isotopes in Precambrian rocks: a fingerprint of past aquatic environments and biogeochemical cycles Pinti L Daniele	N. Tsuchiya
14:45-15:00	coffee break		N. Tsuchiya
15:00-15:20	poster preview	Category C	T. Kakegawa
15:20-16:00	poster session		
16:00-16:30	invited talk	Implications of Multiple Sulfur Isotope Characteristics during Thermochemical Sulfate Reduction by Solid Organic Compounds. Y. Watanabe	C. Inoue
16:35-17:05	invited talk	Potential Insights into Volatile Cycling in Earth's Mantle from Kimberlites John W Hernlund	C. Inoue
17:10-17:35	keynote lecture	Earliest ecosystem in early oceans of the Earth: report from Isua, Greenland T. Kakegawa	C. Inoue

5-Mar			chair
9:30-10:00	keynote lecture	Structured water on mineral surface revealed by Infrared spectroscopy under supercritical conditions N. Tsuchiya	C. Inoue
10:05-10:35	invited talk	Dissolution kinetics of feldspar-water interactions Roland Hellmann	C. Inoue
10:40-11:10	invited talk	The dynamics of very rapid fluid transfer from the mid-crust: fluidization, fragmentation, diatremes and mineralization Nicholas H. S. Oliver	C. Inoue
11:10-11:20	coffee break		K. Ioku
11:20-11:50	invited talk	Hydrogen incorporation into olivine coexisting with H_2O-CO_2-bearingfluid with implication to 410 km discontinuity Konstantin Litasov	K. Ioku
11:55-12:25	invited talk	Single crystal growth of mantle rock forming minerals in water-bearing systems Anton Shatskiy	K. Ioku
12:25-13:30	lunch		
13:30-14:20	poster preview	Category A &B	A. Okamoto
14:20-15:30	poster session		
15:30-16:00	invited talk	In situ Observation of Water in Hydroxyapatite by Neutron Diffraction H. Fujimori	K. Ioku
16:05-16:35	invited talk	Insights into vein formation from experimental systhesized quartz veins Brian Rusk	K. Ioku
16:40-17:10	invited talk	Morphology Control of Particles through Hydrothermal Reactions K. Yanagisawa	K. Ioku

6-Mar			chair
9:30-9:25	invited talk	Ground Water Crisis in Metropolitan Bandung and Its Possible Solution Alternatives Lilik Eko Widodo	C. Inoue
9:30-9:55	invited talk	The Optimation of Bioemulsifier Production from *Azotobacter vinelandii* Agus Jatnika Effendi	C. Inoue
10:00-10:25	invited talk	Present state of environmental problems in Mongolia and their solution Batkhishig Bayara	C. Inoue
10:30-10:55	invited talk	Not fixed Phan Van Minh	C. Inoue
10:55-11:10	coffee break		
11:10-11:35	invited talk	Selective Control of Toxic Cyanobacterial Water-Blooms in Eutrophic Freshwater K. Kaya	N. Tsuchiya
11:35-12:00	invited talk	Groundwater of arsenic in Asian countries T. Komai	N. Tsuchiya
12:00-12:30	poster preview	Category D	M. Kamitakahara
12:30-14:00	lunch & poster session		

The Optimation of Bioemulsifier Production from *Azotobacter vinelandii*

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Abstract
Azotobacter vinelandii was found to be able in producing an emulsifier in a cultivation medium supplemented with 2% glucose as carbon source. Based on the quantity of bioemulsifier produced and emulsification index, it was indicated that the bioemulsifier was optimally produced after 72 hour of fermentation. Various factors affecting on growth and bioemulsifier production by *Azotobacter vinelandii* was studied. High concentration of bioemulsifier was achieved when the growth of *Azotobacter vinelandii* was supplemented with 0.25g/L Ammonium nitrate, 0.2 g/L Magnesium sulfate and in the absence of Ferrous sulfate. Higher nitrogen concentration resulted in higher yield of cell growth. In contrary, the increase of cell growth was followed by the reduction of bioemulsifier produced by *Azotobacter vinelandii.* The production of bioemulsifier decreased when nitrogen concentration was increased. The addition of metal cations was found to affect the bioemulsifier production. The presence of metal ions in high concentration inhibited the production of bioemulsifier from *Azotobacter vinelandii*. Under these conditions it was found that the production of bioemulsifier was yield up to 68.8 g/g biomass. Emulsification properties of the bioemulsifier were stable at wide range of temperature, pH and salinity indicating advantages over its applications in the bioremediation of oil-contaminated soil and others purposes in oil industry. The addition of this bioemulsifier in a broth culture was able to reduce the surface tension from 72.67 to 45.85 dyne/cm.
Keywords: Bioemulsifier, *Azotobacter vinelandii*, Emulsification index, Surface tension

Introduction

Commercial interest in bioemulsifier has been generated by their applicability in wide spectrum application including environmental, pharmaceuticals, and food processing. The advantages of bioemulsifier over their chemical counterparts include biodegradability, production from cheap, renewable substrates, and functionality under extreme conditions. Interest in bioemulsifier is growing due to their applications in the protection of the environment and in the petrochemical industry. Their environmental uses are principally to the bioremediation of petroleum hydrocarbon contaminated sites (Pekdemir et al., 1999; Banat et al., 2000; Christofi and Ivshina, 2002). In the oil industry, bioemulsifier are used in microbial enhanced oil recovery (MEOR) and or bioemulsifier-mediated enhanced oil recovery, facilitating transportation of heavy crude oil through pipelines and in the cleaning of contaminated vessels (Kosaric, 1992; Olivera et al., 2000). Application of bioemulsifier in enhanced oil recovery process must meet any requirement involving the extreme condition of oil reservoir. These processes frequently involve exposure to extremes of temperatures, pressure, salinity, pH and organic solvents, hence there is a continuing need to isolate microbes that are able to function under extreme conditions. Such microbes are found naturally in a diverse range of environments which, whilst considered extreme by man, are optimal for their growth and development. Recently the term``extremophiles'' has been used to describe them. The group includes thermophiles, psychrophiles, acidophiles, alkaliphiles, halophiles, osmophiles, etc (Cameotra and Makkar, 1998; Makkar and Cameotra, 2002; Pruthi and Cameotra, 2003). This paper describe the bioemulsifier production by *Azotobacter vinelandii* as bioemulsifier-producing bacteria and

characterize its product in order to meet the requirement in petrochemical industry.

Materials and Methods

Reagents. All chemicals were of reagent grade, purchased from Merck, J.T. Baker and Sigma Chem Co. Growth media were purchased from Oxoid ltd.

Microorganism. *Azotobacter vinelandii* was obtained from the culture collection of Environmental Biotechnology Laboratory-Environmental Engineering Department, Institut Teknologi Bandung, Indonesia. The microorganism was maintained at 4^0 C on mannitol enrichment agar slants containing (g/L): 20 mannitol, 20 yeast extract, 20 tryptone, 15 agar. Sub-cultures were made to fresh agar slants every 2 month to maintain viability.

Cultivation Conditions. Cultures were grown on a minimal basal medium (MB) which composed the following components (g/L) of distilled water: 1.5 K_2HPO_4; 0.5 KH_2PO_4; 0.2 $MgSO_4$; 0.5 $(NH_4)_2\ SO_4$; 2% glucose as substrate. 10 ml Trace Element solution was added per liter of MB medium. The composition of this trace element (g/L) are 12 $Na_2EDTA_2.H_2O$; 2 $FeSO_4.7H_2O$; 1 $CaCl_2$; 0.4 $ZnSO_4.7H_2O$; 10 $NaSO_4$; 0.4 $MnSO_4.4H_2O$; 0.1 $CuSO_4.5H_2O$; 0.5 $Na_2MoO_4.2H_2O$. The medium was sterilized by autoclaving at 121^0C for 15 min.

The inoculum of *Azotobacter vinelandii* was prepared by transferring cells grown on a slant to 250 ml Erlenmeyer flasks containing 50 ml of MB broth. Culture was incubated in an orbital shaker at room temperature, 110 rpm for 2 days. The MB containing 10^6 cells/mL was used to initiate growth using 1% (v/v) inoculum. Cultivation was carried out in 250 mL Erlenmeyer flasks containing 100 mL minimal medium at room temperature with shaking at 110 rpm for 4 days in an orbital shaker. At regular intervals, samples were withdrawn for analyses. Growth of the culture was monitored by optical density (OD) method. The bioemulsifier concentration (g/L) and emulsification index (%) were also monitored during the fermentation. All analyses were performed in triplicate.

Effect of Nitrogen and Metal Cations. To study the effect of nitrogen on growth and bioemulsifier production, the MB was supplemented with different concentration of Ammonium nitrate (0, 0.25, 0.5, 1 g/L). The presence of metal cations influence the yield of bioemulsifier. Different metal supplements were added to the MB. The concentration of different metal cations used were: $MgSO_4$ 0, 0.1, 0.2, 0.4 g/L and $FeSO_4$ 0, 0.01, 0.02, 0.04 g/L respectively.

Emulsification Index (E24). To determine the emulsification index, Batista et al., (2006) method was applied. Centrifugation at 13000 rpm to separate bioemulsifier from microrganism cells yielding a *bioemulsifier cell free*. A mixture of 1:1 between bioemulsifier and crude oil is agitated for about 2 minute then stabilized for 24 hour. Emulsification index (%) determined by measuring the colom height of emulsified oil agains its total height multiplied by 100 times.

Bioemulsifier Isolation and Biomass Determination. A 30 ml sample of fermentation broth were centrifuged at 13.000 rpm for 30 minute to obtain a cell free broth. The biomass pellet was collected and measured with TSS meter. After centrifugation, the supernatant was then dissolved in a 4 N hydrochloric solution and allowed to stand overnight at 4^0C, followed by the bioemulsifier extraction step with a chloroform solvent at room temperature. The organic layer was transferred to a round-bottom flask and the aqueous layer was re-extracted two times for complete recovery of bioemulsifier. The organic phases were combined yielding a viscous brown-colored crude bioemulsifier product and then evaporated to remove the solvent, the residue was collected and weighted. Vermani et al. (1995) were used to determined the exopolysaccharide fraction of bioemulsifier.

A mixture of 1:2 (v/v) bioemulsifier and chilled acetone were agitated and stand overnight to precipitate. Formed precipitate were filtered and gravimetrically analyzed. The weight of bioemulsifier was determined and used to calculate the bioemulsifier yield (bioemulsifier weight/weight of cells).

Surface Tension Measurement. Surface tension was determined using a DuNouy Tensiometer at room temperature. A 30 ml of sample was put into a clean glass vessel that was placed on the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled through the liquid-air interface. Between each measurement, the platinum wire ring was rinsed three time with water and three time with acetone and allowed to dry (Sarubbo et al., 2007).

Emulsification Stability Test. Stability studies were done using the cell free broth obtained by centrifugation process as described above.

Effect of extreme temperature. The emulsification index of cell free broth also determined after heated at 70^0C, 120^0C for 30 min and cooled to room temperature, then the emulsification index was measured. The emulsification capacity of cells free broth was also determined after exposure at lower temperature (4^0C).

Effect of extreme ionic strength. To study the pH stability of bioemulsifier, five milliliter of cell free broth were exposured with various pH environment: pH value between 2-12.

*Effect of extreme salinity***.** The stability of bioemulsifier against various concentration of NaCl (1-10%) on the emulsification capacity was also determined.

Result and Discussion

Effect of Nitrogen Sources. To study the effect of nitrogen concentration on growth and bioemulsifier production, the minimal medium was supplemented with different nitrogen concentration. Many report showed that over-production of bioemulsifier occurred under limiting condition growth. This has been extensively demonstrated in *Pseudomonas aeruginosa* with an over-production of bioemulsifier in limitation of nitrogen. Generally, higher nitrogen concentration results in higher yield of cell growth. In contrary, the increase of cell growth is non linier with the production of bioemulsifier by *Azotobacter vinelandii.* The production of bioemulsifier decreased when nitrogen concentration was increased. The relative optimum production of bioemulsifier achieved when *Azotobacter vinelandii* supplemented with 0.25% nitrogen concentration (Figure 1).

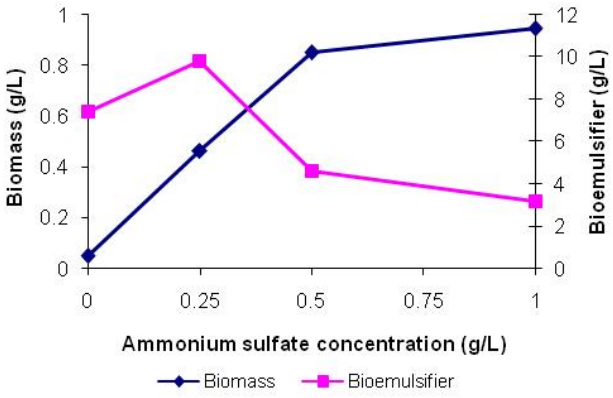

Figure 1. Effect of different nitrogen concentrations on growth and bioemulsifier production of *Azotobacter vinelandii* grown on 2% glucose after 48 hour incubation at room temperature.

There was evidences that nitrogen plays an important role in the production of surface active compounds by microorganisms. Makkar and Cameotra, (2002) reported that potassium nitrate was found to be the best nitrogen source for bioemulsifier production of *Bacillus subtilis* MTCC 2423 rather than sodium nitrate and urea. Nitrogen has been documented as a regulator of lipogenesis in most living organisms. Under such conditions, there are diminished nitrogen-dependent metabolic activities such as protein and nucleic acid biosyntheses. This nitrogen biosynthesis is essential for

metabolisms of most living organisms. Cell growth, fatty acid and EPS production by *Azotobacter vinelandii* supplemented with different nitrogen concentration is shown in Table 1.

Effects of metal cations on biosurfactant production. The addition of metal cations supplements affected the growth of and bioemulsifier production by *A. vinelandii*. Different concentrations of $MgSO_4$ added to the medium, and their effects on both growth and biosurfactant yield were examined. The best yield of bioemulsifier was obtained when Mg^{2+} was supplemented at a concentration of 0.2 g/L. A concentration of Mg^{2+} less or higher than 0.2 g/L inhibited the biosurfactant production as shown in Fig. 2.

Table 1. Effect of nitrogen concentration on bioemulsifier production from *Azotobacter vinelandii*

Nitrogen Concentration (g/L)	Time (day)	Cell Growth (A^0)	Fatty Acid (g/L)	EPS (g/L)
0	0	0.002	2.01	4.25
	1	0.045	3.58	3.71
	2	0.010	3.07	4.37
	3	0.110	1.49	3.85
	4	0.110	2.41	4.22
0.25	0	0.005	1.91	4.76
	1	0.613	3.87	4.62
	2	1.890	4.41	5.40
	3	2.610	3.77	4.03
	4	2.773	2.99	3.44
0.5	0	0.001	1.85	4.72
	1	0.663	3.93	4.03
	2	3.657	1.61	2.99
	3	4.370	1.33	0.99
	4	4.467	0.54	1.20
1	0	0.002	2.46	4.21
	1	0.710	3.06	4.54
	2	4.090	1.27	1.93
	3	4.487	0.91	1.01
	4	4.580	1.01	1.11

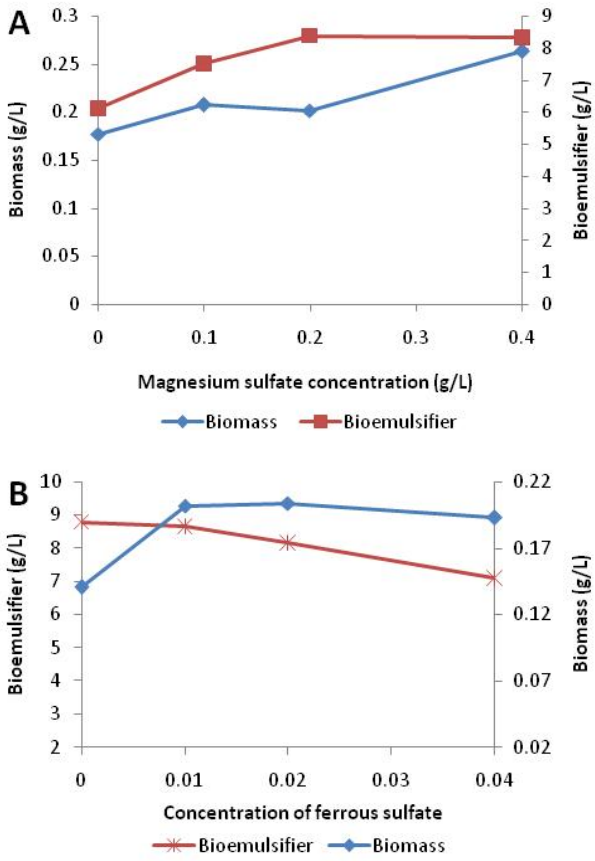

Figure 2. Effect of metal cations (A). Mg^{2+} and (B). Fe^{2+} on growth and biosurfactant production by *A. vinelandii* grown on 2% glucose after 48 hour incubation at room temperature.

The effect of $FeSO_4$ was quite different from that of Mg. There was a considerable decrease in biomass at higher concentrations of $FeSO_4$. However, the bioemulsifier yields were similar at higher concentrations. The best bioemulsifier production occurred with the absence of $FeSO_4$. $FeSO_4$ at a concentration higher than 0.01 g/L inhibited bioemulsifier production. It seem that Fe^{2+} did not induced the production of bioemulsifier although its promote the cells growth.

Emulsification Stability Test. Interest in bioemulsifier is growing due to their applications in the protection of the environment and in the petrochemical

industry. Their environmental uses are principally to the bioremediation of petroleum hydrocarbon contaminated sites. In the oil industry, they are used in microbial enhanced oil recovery (MEOR) and or bioemulsifier-mediated enhanced oil recovery, facilitate transportation of heavy crude oil through pipelines and in the cleaning of contaminated vessels. Application of bioemulsifier in enhanced oil recovery process must meet any requirement involving the extreme condition of oil reservoir. These processes frequently involve exposure to extremes of temperatures, pressure, salinity, pH and organic solvents, hence there is a continuing need to isolate microbes that are able to function under extreme conditions. Stability studies were performed using cell-free broth obtained by centrifugation the culture at 10.000 rpm for 30 minute as described in methods.

Effect of Extreme Temperature. The emulsification index of the culture broth free of cells was stable up to 48 hour either when stored at 4^0 C or at ambient/room temperature (27^0 C). It is interesting to be observed that the emulsification capacity of the bioemulsifier remain stable after heating for 30 minutes at 70^0 C and increased up to 120^0 C. The effect of thermal treatment (chilled/heated) on the activity of the bioemulsifier from *Azotobacter vinelandii* cultivated in minimum basal medium with 2% glucose as carbon source showed that no appreciable changes in emulsification capability occurred. The effect of extreme temperature on the emulsification activity of the cells free broth could be seen in Figure 3A.

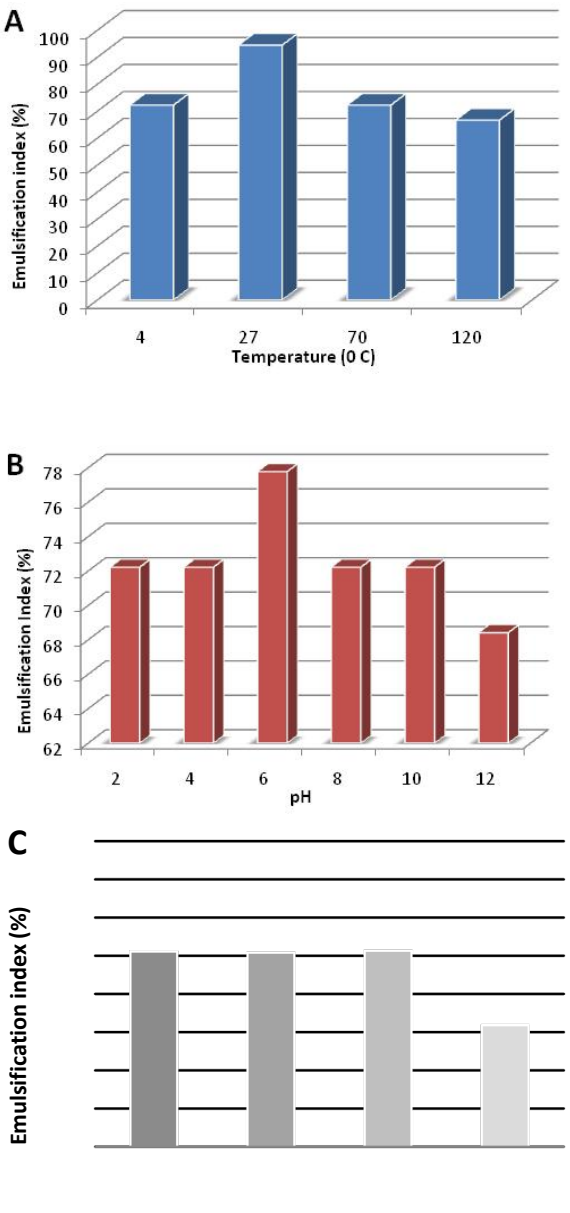

Figure 3. Effect of temperature (A), pH (B) and salt concentration (C) on the emulsifying activity of cell free broth of *Azotobacter vinelandii* grown in minimal basal medium with 2% glucose as carbon source.

The optimum temperature for emulsification activity was found at room temperature (27^0C). Heat treatment of some bioemulsifier caused no appreciable change in emulsifiers properties such as the lowering of surface tension and emulsification activity. Makkar and Cameotra, (2002) reported the biosurfactant produced by *B. subtilis* MTCC 2423 was thermostable and retained its

surface activity even after heating at 100^0C for 2 hour. Conversely, the effect of thermal treatment on the activity of the bioemulsifier from *Candida glabrata* cultivated in cotton seed oil plus glucose showed that no appreciable changes in emulsification capacity occurred if the cell free broth was heated only once, 10% of activity was lost at 80^0C (Sarubbo et al., 2006) and lost 60% of its activity after boiling for 1 hour (Cirigliano and Charman, 1984).

Effect of Extreme pH. The effect of pH on the emulsification activity of the cells free broth is shown in Figure 3B. Extreme of pH could possibly transform less surface-active species into more active emulsifiers by denaturation of proteinaceous components or by increasing ionization. The effectiveness of bioemulsifier produced from *Azotobacter vinelandii* was in the pH range between 2-10. Sarubbo et al, (2007) reported the effectiveness of bioemulsifier from *C. lipolytica* was limited from acid to neutral pH, whereas the emulsification activity of the bioemulsifier produced by Bacillus subtilis was pH stable and retained the surface activity from pH 4 to 12 (Makkar and Cameotra, 2002).

Effect of Extreme Salinity. The presence of percentual concentrations of salt practically did not affect bioemulsifier activity. The emulsification activity of the cell free broth containing the emulsifiers produced by *Azotobacter vinelandii* cultivated in minimal basal medium with 2% glucose as carbon source showed a relative tolerance over the salt concentrations. The effect of salinity on the emulsification activity of the cells free broth could be seen in Figure 3C. Rufino et al. (2006) reported a little changes were observed with the additions of up to 10% (w/v) salt on the emulsification index of *Candida lipolytica*. Most known synthetic surfacatants are less stable over such salt concentrations (10%), while salt concentrations of 2-3% are sufficient to inactivated synthetic surfactants (Desai and Banat, 1997).

Table 2. Surface activity of broth with several condition.

Parameter	Surface tension (dyne/cm)
Sterile broth/MB	72.67
Sterile aquadest	71.91
Water with addition of synthetic surfactant*	56.14
Cell free broth†	45.85

* 1 g/L sodium dodecil sulfate

† optimum fermentation condition for Ammonium nitrate, $MgSO_4$, $FeSO_4$ were 0.25, 0.2 and 0 g/L respectively.

The bioemulsifier isolated from *A. vinelandii* under the conditions studied in this work, in addition being a good emulsifier also has a good tensio-active properties (Table 2). The bioemulsifier has several properties that are desirable for the oil industries. It is stable in a wide range of temperature, ph and salt concentrations that usually found in many oil reservoirs. The result suggest it may constitute a future attractive choice for the oil industry to be considered in the processes of decontamination of polluted site, microbial enhanced oil recovery and any process involved emulsifier activities.

Acknowledgments

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