ORIGINAL ARTICLE

Characterization of cell-associated bioemulsifier from *Myroides* sp. SM1, a marine bacterium

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Abstract

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Emulsification activity of bioemulsifier derived from *Myroides* sp. SM1, a marine bacterium, isolated from oil-spilled seawater in Songkhla Lake, Thailand, was investigated. Cell suspension and culture supernatant were able to emulsify weathered crude oil effectively, especially with increasing incubation time as evidenced by the smaller droplet size of weathered crude oil. Weathered crude oil in marine broth inoculated with *Myroides* sp. SM1 was completely emulsified within 6 h with the coincidental attachment of cells around the oil droplet. When mixing the cells with various hydrocarbons, cells migrated to hydrocarbon phase differently. *Myroides* sp. SM1 adhered to weathered crude oil to the highest extent, indicating that those cells used had the high affinity to weathered crude oil. However, weathered crude oil and other hydrocarbons were not used by *Myroides* sp. SM1 as sole carbon source in a minimal salt medium. *Myroides* sp. SM1 cultivated in marine broth reached stationary phase at 24 h; however, no differences in cell density were observed from 30 h to 48 h of cultivation time. Emulsifying activity toward weathered crude oil was found in cell suspension cultivated for 12 h and no differences in activities were noticeable in those cultivated for 12-48 h. Chloroform-methanol mixture at the ratio of 1:1 (v/v) was the most effective solvent to extract cell-associated

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bioemulsifier from *Myroides* sp. SM1. The crude bioemulsifier was capable of emulsifying weathered crude oil in a broad pH range (5-12) and in the presence of NaCl up to 1.54 M and $MgCl_2$ up to 0.1 M. The bioemulsifier was stable when heated at a temperature ranging from 30 to 121°C.

Key words : cell-associated bioemulsifier, *Myroides* sp. SM1, marine bacteria, weathered crude oil, oil-spilled seawater

บทคัดย่อ

ศุภศิลป์ มณีรัตน์ และ ปวีณา ดิกิจ

คุณลักษณะของไบโออิมัลซิไฟด์เออร์ที่ติดกับตัวเซลล์จากเชื้อ *Myroides* sp. SM1 ที่แยกจากน้ำทะเล

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จากการศึกษาความสามารถในการอิมัลซิไฟด์น้ำมันดิบของเซลล์แขวนลอยและส่วนใสจากการเลี้ยง *Myroides* sp. SM1 ซึ่งเป็นแบคทีเรียที่แยกได้จากน้ำทะเลบริเวณทะเลสาบสงขลาซึ่งมีคราบน้ำมันลอยปกคลุมอยู่ พบว่าความสามารถในการอิมัลซิไฟด์น้ำมันดิบเพิ่มขึ้นเมื่อเลี้ยงเชื้อไว้เป็นระยะเวลานานขึ้นซึ่งจะสังเกตได้จากอนุภาคน้ำมันดิบที่มีขนาดเล็กลง เมื่อเลี้ยงเชื้อใน Marine broth ที่มีการเติมน้ำมันดิบพบว่าน้ำมันดิบถูกอิมัลซิไฟด์อย่างสมบูรณ์ภายใน 6 ชั่วโมงของการเลี้ยงเชื้อซึ่งจะเห็นได้จากการเกาะติดของเชื้อกับอนุภาคน้ำมันดิบ เมื่อเขย่าเซลล์แขวนลอยกับน้ำมันดิบหรือไฮโดรคาร์บอนชนิดต่าง ๆ เซลล์เคลื่อนที่เข้าไปในชั้นของไฮโดรคาร์บอนได้แตกต่างกัน โดยเซลล์เข้าไปเกาะติดกับน้ำมันดิบได้ดีที่สุดซึ่งแสดงว่าเซลล์มีแอฟฟินิตี้สูงสุดกับน้ำมันดิบ อย่างไรก็ตาม *Myroides* sp. SM1 ไม่สามารถใช้น้ำมันดิบและไฮโดรคาร์บอนชนิดต่าง ๆ เป็นแหล่งคาร์บอนได้ เมื่อเลี้ยง *Myroides* sp. SM1 ใน Marine broth เชื้อเข้าสู่ระยะการเจริญคงที่ที่เวลา 24 ชั่วโมง และไม่มีความแตกต่างของจำนวนเซลล์เมื่อเลี้ยงเชื้อเป็นเวลา 30-48 ชั่วโมง ความสามารถในการอิมัลซิไฟด์น้ำมันดิบของเซลล์แขวนลอยเกิดขึ้นเมื่อใช้เซลล์ที่เลี้ยงเป็นเวลา 12 ชั่วโมงและความสามารถดังกล่าวไม่แตกต่างกันเมื่อใช้เซลล์ที่เลี้ยงเป็นเวลา 12-48 ชั่วโมง พบว่าคลอโรฟอร์มและเมธานอลในอัตราส่วน 1:1 (ปริมาตร/ปริมาตร) เป็นตัวทำละลายที่ดีที่สุดในการสกัดไบโออิมัลซิไฟด์เออร์ที่ติดกับตัวเซลล์ของ *Myroides* sp. SM1 ไบโออิมัลซิไฟด์เออร์สกัดหยาบสามารถอิมัลซิไฟด์น้ำมันดิบได้ในช่วงพีเอชกว้าง (5-12) และในสภาวะที่มีเกลือโซเดียมคลอไรด์ 1.54 โมลาร์ หรือแมกนีเซียมคลอไรด์ 0.1 โมลาร์ ไบโออิมัลซิไฟด์เออร์ยังคงมีกิจกรรมหลังจากการผ่านความร้อนในช่วงอุณหภูมิ 30-121°C

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Oil pollution on land and in the sea presents a serious problem to public health, commercial fisheries and water resources (Sanders *et al*., 1980; Widbom and Oviatt, 1994; Hester and Mendelssohn, 1999; de la Huz *et al*., 2005). Petroleum is a complex mixture of many compounds such as alkanes, aromatics, resins and asphaltenes which could be potentially eliminated by microbial degradation (Huy *et al*., 1999; Rouviere and Chen, 2003; Chen *et al*., 2005). However, intrinsic biodegradation often takes a long time to complete because of the low water solubility of hydrocarbons, limitation of nutrients and inappropriate environment (van Dyke *et al*., 1993; Snape *et al*., 2006). Biodegradation of hydrocarbons depends on its dispersion state and is maximized when the substrate is dissolved or emulsified (Kanga *et al*., 1997; Schippers *et al*., 2000). Biosurfactants or bioemulsifiers are either extracellular compounds or localized on the cell

surface (Desai and Banat, 1997; Beal and Betts, 2000). In the latter case, the microbial cell itself is a biosurfactant and adheres to hydrocarbon (Kappeli *et al*., 1984; Southam *et al*., 2001). Recently, *Myroides* sp. SM1 was isolated from oil-spilled seawater in Songkhla Lake, Thailand. This strain produced biosurfactants, ornithine lipids, which were excreted into culture supernatant. Ornithine lipids were composed of ornithine and the different couples of iso-3-hydroxy fatty acid (C_{15}-C_{17}) and iso-fatty acid (C_{15} or C_{16}) (Maneerat *et al*., 2006). Ornithine lipids are known to spread mainly in outer cell membranes of various Gram-negative bacteria (Wilkinson, 1972; Wilkinson *et al*., 1982; Okuyama and Monde, 1996). However, no information regarding the bioemulsifier particularly those associated with the cell from marine bacteria isolated in Thailand has been reported. The objective of this study was to characterize cell-associated bioemulsifier from *Myroides* sp. SM1 isolated from Songkhla Lake.

Materials and Methods

Chemicals

Hydrocarbons (*n*-tridecane, *n*-tetradecane, *n*-hexadecane, *n*-octadecane, toluene, xylene, kerosene, paraffin and mineral oil) were purchased from Nacalai Tesque Inc. (Tokyo, Japan). Weathered crude oil was a gift from Prof. Dr. Fusako Kawai, Research Institute for Bioresources, Okayama University, Japan. All other chemicals used were analytical grade.

Microorganism and cultivation

Myroides sp. SM1 used throughout this work was isolated from oil-spilled seawater in Songkhla Lake, Thailand (Maneerat *et al*., 2005). The bacterium was grown in Marine broth 2216 (Difco, USA). All experiments were carried out in 500 ml flasks containing 100 ml of medium. Culture broth of *Myroides* sp. SM1 was incubated at 30°C with continuous shaking at 200 rpm for up to 48 h (GFL, Burgwedel, Germany). Growth during incubation was monitored. For medium without hydrocarbon, growth was determined absorbance at 600 nm (A_{600}) using a spectrophotometer (Genesys 10-S, Madison, USA). For hydrocarbons-containing minimal salt medium (Olivera *et al*., 2003), the concentration of cells in the culture was determined by total cell protein measurement using the Lowry method (Lowry *et al*., 1951). Prior to analysis, cells were collected by centrifugation at 12,000g for 30 min (Hitachi SCR 20B, Tokyo, Japan). Then, wet cells were resuspended in 0.1 N NaOH, 30 min at 100°C (Shabtai, 1990).

Preparation of bioemulsifier from *Myroides* sp. SM1

Culture broth of *Myroides* sp. SM1 at 24 h of cultivation was centrifuged (12,000g, 4°C, 30 min). The pellet was washed twice in 50 mM K-phosphate buffer (pH 7.0). Then washed cells were finally resuspended in the same buffer with the equal volumes to supernatant. Culture supernatant was subjected to membrane filtration (0.45 μm cut off) and the filtrate was collected in a sterile flask.

Determination of emulsifying activity of bioemulsifiers

Emulsifying activity of cell suspension and supernatant filtrate were tested as described by Maneerat *et al*. (2006). Weathered crude oil was added into both cell suspension and culture supernatant to obtain a final concentration of 1% (v/v). The mixtures were shaken at 30°C at 200 rpm and emulsifying activity toward weathered crude oil was determined.

Microscopic study of emulsion and cell attachment

Culture broth obtained after 6 and 24 h was subjected to light microscopy (Olympus BX50, Tokyo, Japan) and the emulsion was observed. Cell attachment to weathered crude oil was checked using scanning electron microscope (JEOL JSM-5800LV, Tokyo, Japan) (Southam *et al*., 2001; Zinjard and pant, 2002).

Cell-surface hydrophobicity test

Collected cells were subjected to cell-surface hydrophobicity test. Cell-surface hydrophobicity

test was performed according to the methods of Rosenberg *et al*. (1980) and Pan *et al*. (2006). The cells were harvested by centrifugation at 12,000g for 30 min at 4°C, washed twice with 50mM K-phosphate buffer (pH 7.0) and resuspended in the same buffer. A_{600} of cell suspension was adjusted to 0.5 with the same prior to analysis. To 3 ml of cell suspensions, 0.5 ml of hydrocarbons were added and vortexed for 120 s. After allowing the hydrocarbon phase to rise completely for 10 min, the aqueous phase was carefully removed with a Pasteur pipette and transferred to a 1-ml cuvette A600 was then measured. The decrease in A_{600} of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated as follows:

$$H\% = [(A_0\text{-}A)]/A_0 \times 100$$

where A_0 and A were A_{600} before and after mixing with hydrocarbon, respectively.

Study on emulsification activity of bioemulsifier toward different hydrocarbons

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

To determine the emulsifying activity toward weathered crude oil, two drops of weathered crude oil were added into 2 ml of biosurfactant dissolved in 50 mM K-potassium phosphate buffer (pH 7.0) in an 8×15-mm test tube. The test tube at a tilt of 45° was reciprocally shaken at 90 strokes per min at room temperature overnight (Komukai-Nakamura *et al*., 1996).

Bioemulsifier extraction

Three extraction methods were performed as follows:

Method 1: Cell suspension was acidified with 10% H_2SO_4 to pH 3 and extracted twice with ethyl acetate. Solvent phase was dried by vacuum rotary evaporator (EYELA SB-651, Tokyo, Japan) (Passeri *et al*., 1992).

Method 2: Cells were suspended in 100 ml methanol/dichloromethane/phosphate buffer 20: 10:8 (v/v/v), treated for 15 min with an ultrasonic probe and kept overnight at room temperature. Additional 150 ml methanol/dichloromethane/ phosphate buffer (1:2:2, v/v/v) was added, followed by 5 min of ultrasonic treatment. The samples were transferred to a separating funnel to separate the solvent phase. The dichloromethane phase was added with Na_2SO_4 to remove water. The methanol/ phosphate buffer phase was re-extracted by addition of 25 ml dichloromethane, and the water separated and eliminated. The total dichloromethane phase was dried by vacuum rotary evaporator (Abraham *et al*., 1998).

Method 3: The culture supernatant obtained was extracted twice with an equal volume of chloroform/methanol (1:1). The combined extracts were concentrated to dryness using a vacuum rotary evaporator (Maneerat *et al*., 2006).

Characterization of biosurfactant crude extract

The biosurfactant crude extract (0.5%, w/v) in 50 mM K-phosphate buffer (pH 7.0) was characterized. To investigate the effects of pHs, salts concentrations (NaCl, $CaCl_2$ and $MgCl_2$) on emulsification activity of crude extract, the extract was adjusted with 1 M HCl or NaOH to obtain the pHs of 2-12. NaCl was added to the sample to obtain the final concentrations of 0-3.59 M. $CaCl_2$ and $MgCl_2$ were also added to the sample to obtain the final concentrations of 0-18 mM and 0-0.1 M, respectively. For thermal stability study, crude extract was incubated for 1 h at different temperatures (30-121°C) and cooled to 30°C and remaining activity was then determined.

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 Windows, SPSS Inc., Chicago, IL).

Results and Discussion

Emulsifying activity of bioemulsifiers toward weathered crude oil

The supernatant and cell suspension of *Myroides* sp. SM1 emulsified weathered crude oil similarly as shown by the continuous dispersion of oil droplet in the aqueous phase (data not shown). However, emulsification activity toward *n*-hexadecane was different. The emulsification of *n*-hexadecane by cell suspension was more pronounced, compared with that emulsified by supernatant ($p<0.05$) (Table 1). These results indicated that *Myroides* sp. SM1 produced biosurfactants and were either excreted into the culture broth or attached to the cell surface. *Myroides* sp. SM1 produced the extracellular, ornithine lipids, which could emulsify weathered crude oil (Maneerat *et al*., 2006). Generally, extracellular membrane vesicles partition hydrocarbons to form a micro-emulsion which plays an important role in alkane uptake by microbial cells (Desai and Banat, 1997). Bento *et al*. (2005) reported that cell culture broth of biosurfactant-producing microorganisms (*Acinetobacter junii, Actinomyces* sp., *Bacillus cereus, Bacillus fusiformis, Bacillus pumilus, Bacillus sphaericus, Pseudomonas* sp.) isolated from soil contaminated with diesel oil could emulsify diesel oil. No substantial emulsification was achieved with the cell-free broth, indicating that the emulsifying activity was not extracellular. Cell suspension of *Gordonia alkanivorans* CC-JG39 had an emulsification index (E_{24}) against kerosene and diesel of 20% and 17%, respectively. On the other hand, supernatant exhibited E_{24} toward kerosene and diesel of only 7% and 6%, respectively (Lin *et al*., 2005).

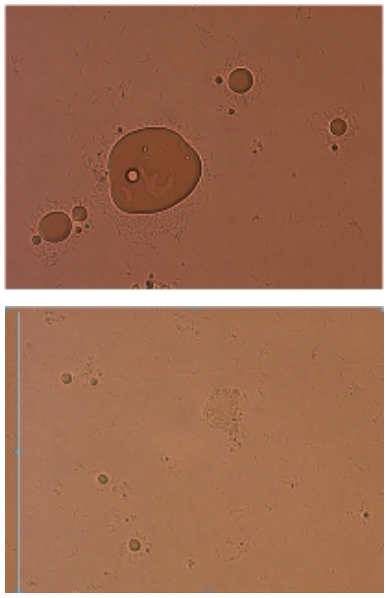
Table 1. Emulsification activity toward *n*-hexadecane of *Myroides* sp. SM1

Sample	Emulsification activity (%)
Supernatant	62.60 ± 2.66^{b}
Cell suspension	98.53 ± 1.33^{a}

Different superscripts indicate significant differences ($p<0.05$).

Emulsion and association of cell to oil droplet

Weathered crude oil was completely emulsified within 6 h of cultivation. Microscopic observation revealed that the longer incubation time led to the increased emulsification as evidenced by the appearance of smaller droplet size of weathered crude oil (Figure 1). In addition, *Myroides* sp. SM1 congregated and adhered to the surface of an emulsified oil droplet (Figure 2). Scanning electron microscopic observations showed that droplets of weathered crude oils were embedded with cells of

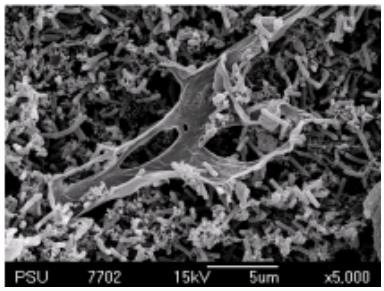


(a)

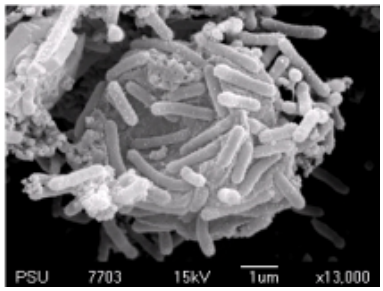
(b)

Figure 1. Microscopic observation (at 400 × magnification) of oil droplets and *Myroides* sp. SM1 attached oil droplets in weathered crude oil-containing marine broth at 6 h (a) and 12 h (b) of cultivation.

(Color figure can be viewed in the electronic version)



(a)



(b)

Figure 2. 5000 × magnification (a) and 13,000 × magnification (b) of colonization of *Myroides* sp. SM1 around the emulsified oil droplet at 24 h of cultivation.

Table 2. Cell-surface hydrophobicity of *Myroides* sp. SM1 toward different hydrocarbons

Hydrocarbon	Cell-surface hydrophobicity (%)
weather crude oil	85.48±5.13[a]
toluene	48.40±2.30[b]
xylene	28.07±3.07[c]
kerosene	17.42±2.35[d]
n-tridecane	17.13±0.62[d]
n-hexadecane	14.62±0.98[d]
n-tetradecane	14.62±1.40[d]
paraffin	5.23±1.04[e]
mineral oil (light)	3.78±1.36[ef]
n-octadecane	0.13±0.03[f]
mineral oil (heavy)	0.00±0.00[f]

Different superscripts indicate significant differences ($p<0.05$).

Myroides sp. SM1 (Figure 2). This result was in accordance with Southam *et al*. (2001) who found that hydrocarbon degrading bacteria emulsified and colonized the surface of waste engine oil. Bacterial adsorption to oil occurred in association with a highly charged, amphipathic bacterial surfactant interface with the thickness of 25-50 μm (Southam *et al*., 2001). Tropical marine yeast, *Yarrowia lipolytica* NCIM 3589, produced an emulsifier (lipid-carbohydrate-protein) complex associated with the cell wall when cultivated in medium containing alkanes or crude oil (Zinjarde and Pant, 2002). Kirschner *et al*. (1980) proposed two types of hydrocarbon interactions during biodegradation: adhesion to oil and pseudosolubilization. As a consequence the hydrocarbon degrading bacteria assimilate small droplets of emulsified oil.

Cell-surface hydrophobicity and hydrocarbon assimilation

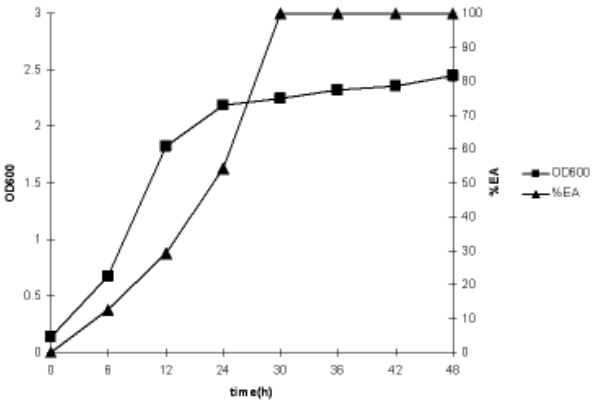
The microbial cell affinity to hydrocarbons was investigated and expressed as cell-surface hydrophobicity (Table 2). It shows that *Myroides* sp. SM1 possessed a higher tendency to adhere to weathered crude oil (85.48%) than toluene (48.40%) and xylene (28.07%) and other hydrocarbons. *Myroides* sp. SM1 tended to migrate to aromatic hydrocarbons (toluene and xylene) rather than aliphatic ones (*n*-tridecane, *n*-tetradecane, *n*-hexadecane and *n*-octadecane). This indicated that the high surface hydrophobicity of cells probably partitioned into higher non-polar hydrocarbon phase. Cell-surface hydrophobicity value depends on bacterial strain, hydrocarbons type and temperature (El-Sayed *et al*., 1996; Meylheuc *et al*., 2001). *Gordonia alkanivorans* CC-JG39 had cell-surface hydrophobicity value toward kerosene and diesel of 88% and 93%, respectively (Lin *et al*., 2005).

Hydrocarbon assimilation of *Myroides* sp. SM1 was performed in minimal salt media containing different hydrocarbons and cell growth was monitored by total cell protein. *Myroides* sp. SM1 did not use weathered crude oil, aliphatic hydrocarbons (*n*-tridecane, *n*-tetradecane, *n*-hexadecane and *n*-octadecane) or aromatic hydrocarbons (toluene and xylene) as sole carbon sources (data not shown) although this strain had high affinity to weathered crude oil, toluene and xylene. Adsorption to hydrocarbons may not necessarily indicate hydrocarbon assimilation of the strain and substrate emulsification is not a prerequisite for growth of

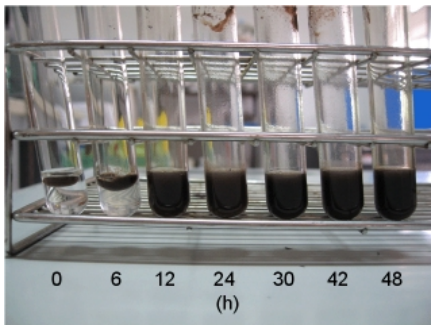
this organism on those hydrocarbons (Zinjarde and Pant, 2002).

Effect of cultivation time on the production of cell-associated bioemulsifier and microbial growth

Growth characteristics and emulsification activity toward *n*-hexadecane and weathered crude oil of cell-associated bioemulsifier of *Myroides* sp. SM1 are illustrated in Figure 3. *Myroides* sp. SM1 reached stationary phase at 24 h; however, no differences in cell density were observed after 30 h of cultivation time (Figure 3a). Stable emulsion of n-hexadecane emulsified by cell suspension was observed after 6 h of cultivation and the maximal emulsification (100%) was obtained at 30 h of cultivation. Emulsion of weathered crude oil emulsified by cell suspension was noted at 12 h of cultivation and no differences in emulsifying activities were noticeable in those cultivated for 12-48 h (Figure 3b). From the result, emulsifying activity of cell-associated bioemulsifier from *Myroides* sp. SM1 was concomitant with growth. The result was in accordance with Lee *et al*.(2006) who reported that lipopeptide biosurfactant from *Bacillus subtilis* A8-8 was produced at the exponential phase and reached a maximum at the stationary phase. In some cases, it is possible that prolonged cultivation possibly led to partial degradation of the surface active metabolites (Lin *et al*., 1998; Batista *et al*., 2006).



(a)



(b)

Figure 3. Growth of *Myroides* sp. SM1 in marine broth (A_{600}) and emulsification activity toward *n*-hexadecane during the cultivation of 48 h.

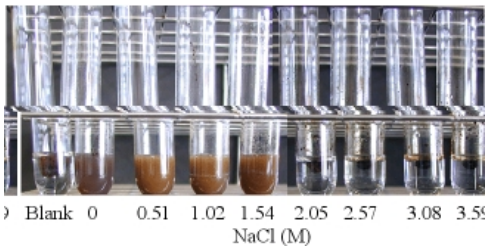
(Color figure can be viewed in the electronic version)

Effect of extraction methods on the recovery of cell-associated bioemulsifer

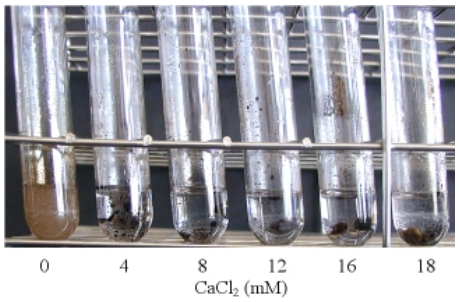
Among three extraction methods, the mixture of chloroform-methanol (1:1, v/v) was the most efficient in extracting the cell-associated bioemulsifier from cell suspension of *Myroides* sp. SM1 (data not shown). Extraction with ethyl acetate under acidic condition (pH 2.0) or with dichloromethane associated with ultrasonication did not extract weathered crude oil-emulsifying compound from cell suspension. With the extraction using chloroform/methanol mixture, the yield of 264 mg dried matter/1L culture broth was obtained. The common techniques for bioemulsifier extraction are using chloroform-methanol, dichloromethane-methanol, ethyl acetate, tetrahydrofuran as the extracting solvents (Morikawa *et al*., 1992; Yakimov *et al*., 1995; Mercade *et al*., 1996). However, the choice depends on microbial strain and biosurfactant type.

Characterization of biosurfactant crude extract

The crude bioemulsifier was able to emulsify weathered crude oil in the presence of NaCl from 0.51 M up to 1.54 M, but a loss in activity was found when NaCl concentration was above 1.54 M. The highest emulsification activity was observed in the presence of 1.02-1.54 M of NaCl. However, the activity was found in the absence of



(a)



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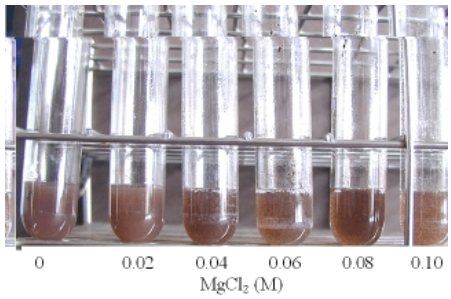

(c)

Figure 4. Effect of NaCl (M) (a), $CaCl_2$ (mM) (b) and $MgCl_2$ (M) (c) on emulsification activity of cell-associated bioemulsifier against weathered crude oil.

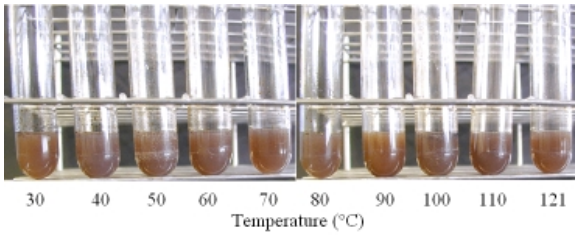

Figure 5. Thermal stability (°C) of cell-associated bioemulsifier. Weathered crude oil was used as hydrocarbons.

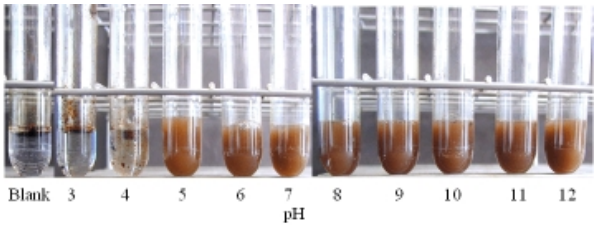

Figure 6. Effect of pH on emulsification activity of cell-associated bioemulsifier. Weathered crude oil was used as hydrocarbons.

(Color figure can be viewed in the electronic version)

NaCl (Figure 4). $CaCl_2$ (3 mM, the same as in Marine Broth 2216) inhibited the emulsification of weathered crude oil by crude extract (Figure 4). The activity was not inhibited in the presence of $MgCl_2$ up to 0.1 M, but was enhanced at 0.02 M $MgCl_2$ (Figure 4). NaCl activated biosurfactant activity of various strains isolated from seawater or petroleum reservoirs (Yakinov *et al*., 1995). Nevertheless, bioemulsifier extracted from *Myroides* sp. SM1 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). Biosurfactant from Bacillis licheniformis JF-2 showed the emulsifying activity when calcium was present at concentration up to 25 g/L (McInerney *et al*., 1900).

After crude extract was kept at 30-100°C for 1 h and at 110 and 121°C for 15 min, the residual activity was compared with the control (no treatment). Temperatures ranging from 30 to 121°C did not affect the emulsification activity of

crude extract toward weathered crude oil (Figure 5). This result was in agreement with Horowitz *et al*. (1990) who found that biosurfactant from *Bacillus licheniformis* 86 was stable in the temperature range of 25-120°C. The surface-active compound from *Bacillus subtilis* retained its properties after exposure to elevated temperature (100°C) for 2 h and to low temperature (-18°C) for 6 months (Nitschke and Pastore, 2006).

Effect of pHs on emulsification activity of crude extract toward weathered crude oil was studied (Figure 6). Precipitation of crude extract occurred at pHs below 5, but no changes in activity were obtained in the pH ranges of 5-12 (Figure 6). At pH close to the isoelectric point, there is no electrostatic repulsion between neighboring molecules, and the compounds tend to coalesce and precipitate (Milewski, 2001). Biosurfactant KP-2 (surfactin) from *Bacillus* sp. strain KP-2 retained its activity at pHs ranging from 6 to 10 and exhibited optimal activity at pH 6 (Roongsawang *et al*., 1999).

Conclusion

Weathered crude oil-emulsifying compound from *Myroides* sp. SM1 was attached to the cell surface. However, this strain did not use weathered crude oil or hydrocarbons as sole carbon source. Cells cultivated for 12 h was capable of emulsifying weathered crude oil. Chloroform/methanol mixture at the ratio of 1:1 (v/v) was the most effective solvent to extract the bioemulsifier from cells of *Myroides* sp. SM1. Cell-associated bioemulsifier crude extract produced from *Myroides* sp. SM1 was stable in a broad temperature ranges. Nevertheless, a loss in emulsifying activity was noted at very acidic condition and in high salt concentration.

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