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Isolation of biosurfactant-producing marine bacteria and characteristics of selected biosurfactant

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Abstract

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Biosurfactant-producing marine bacteria were isolated from oil-spilled seawater collected from harbors and docks in Songkhla Province, Thailand. Haemolytic activity, emulsification activity toward *n*-hexadecane, emulsion of weathered crude oil, drop collapsing test as well as oil displacement test were used to determine biosurfactant producing activity of marine bacteria. Among two-hundred different strains, 40 strains exhibited clear zone on blood agar plates. Only eight strains had haemolytic activity and were able to emulsify weathered crude oil in marine broth during cultivation. Eight strains named SM1-SM8 were identified by 16S rRNA as *Myroides* sp. (SM1); *Vibrio paraheamolyticus* (SM2); *Bacillus subtilis* (SM3); *Micrococcus luteus* (SM4); *Acinetobacter anitratus* (SM6); *Vibrio paraheamolyticus* (SM7) and *Bacillus pumilus* (SM8). However, SM5 could not be identified. Strain SM1 showed the highest emulsification activity against weathered crude oil, by which the oil was emulsified within 24 h of cultivation. In addition, strain SM1 exhibited the highest activity for oil displacement test and emulsification test toward *n*-hexadecane. The emulsification activity against *n*-hexadecane of crude extract of strain SM1 was stable over a broad range of temperature (30-121°C), pH (5-12) and salt concentration (0-9% NaCl), whereas $CaCl_2$ showed an adverse effect on emulsifying activity.

Key words : weathered crude oil, marine bacteria, oil-spilled seawater, biosurfactant

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บทคัดย่อ

ศุภศิลป์ มณีรัตน์ และ กุลนรี เพชรรงค์

การแยกแบคทีเรียจากทะเลที่ผลิตสารลดแรงตึงผิวและคุณลักษณะของสารลดแรงตึงผิวที่คัดเลือกได้

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จากการแยกเชื้อแบคทีเรียที่สามารถผลิตสารลดแรงตึงผิวชีวภาพจากตัวอย่างน้ำทะเลที่ปนเปื้อนคราบน้ำมันบริเวณท่าเรือน้ำลึกและท่าเรือจังหวัดสงขลา อำเภอเมือง จังหวัดสงขลา โดยใช้การตรวจสอบความสามารถในการอิมัลซิไฟด์ weathered crude oil การย่อยเม็ดเลือดแดง และ drop collapsing test พบว่าสามารถแยกเชื้อได้ 200 สายพันธุ์จากน้ำทะเลที่ปนเปื้อนคราบน้ำมัน โดยมี 40 สายพันธุ์ที่สามารถทำให้เกิดวงใสบนอาหาร blood agar มีเพียง 8 สายพันธุ์ที่เกิดวงใสบนอาหาร blood agar และสามารถอิมัลซิไฟด์ weathered crude oil เมื่อเลี้ยงในอาหาร Marine broth เมื่อใช้เทคนิค 16S rRNA ในการจัดจำแนกสายพันธุ์ของเชื้อแบคทีเรีย 8 สายพันธุ์ พบว่า สายพันธุ์ SM1 คือ *Myroides* sp. สายพันธุ์ SM2 คือ *Vibrio paraheamolyticus* สายพันธุ์ SM3 คือ *Bacillus subtilis* สายพันธุ์ SM4 คือ *Micrococcus luteus* สายพันธุ์ SM6 คือ *Acinetobacter anitratus* สายพันธุ์ SM7 คือ *Vibrio paraheamolyticus* สายพันธุ์ SM8 คือ *Bacillus pumilus* ส่วนสายพันธุ์ SM5 ไม่สามารถจัดจำแนกได้ เมื่อพิจารณากิจกรรมในการอิมัลซิไฟด์ weathered crude oil พบว่าสายพันธุ์ SM1 ให้กิจกรรมสูงสุดโดยสามารถอิมัลซิไฟด์ weathered crude oil ได้สมบูรณ์ภายใน 24 ชั่วโมง นอกจากนี้สารสกัดจากสายพันธุ์ SM1 มีกิจกรรม oil displacement และสามารถอิมัลซิไฟด์ *n*-hexadecane ได้ในช่วงกว้างของอุณหภูมิ (30-121°C) พีเอช (5-12) และเกลือโซเดียมคลอไรด์ (0-9%) ส่วนแคลเซียมคลอไรด์มีผลลบต่อกิจกรรมการอิมัลซิไฟด์น้ำมันของสารสกัด

ภาควิชาเทคโนโลยีชีวภาพอุตสาหกรรม คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Pollution of the sea by crude oil, mostly caused by stranding of tankers, is one of the urgent and serious environmental issues over the world. Ship operations also produce wastes that are collected in the lowest part of the hull, called the bilge area. This oil-containing bilge waste must be managed properly to avoid environmental pollution (Olivera *et al*., 2003). The ordinary self-cleaning of the sea involving evaporation, photochemical oxidation or sedimentation of certain oil components, as well as biodegradation by marine microorganisms is overburdened due to the additional hydrocarbons, especially large oil spills (Harayama *et al*., 1999). Biodegradation of a given hydrocarbon depends on its dispersion state. The biodegradation is generally maximized when the water-insoluble substrate is solubilized or emulsified (Mattei *et al*., 1986). Synthetic detergents used to clean up these spillages have often led to more destruction of the environment. From an environmental viewpoint, it is important that all substances released into the environment should be degradable. Their potential for causing environmental damage should be assessed and the possibility of future harm due to build-up in the environment should be taken into consideration.

Biosurfactants are found as extracellular compounds or localized on the cell surface of microorganisms. For the latter case, the microbial cell itself is a biosurfactant and adheres to hydrocarbon. Those biosurfactants are capable of increasing the bioavailability of poorly soluble polycyclic aromatic hydrocarbons such as phenanthrene (Gilewicz *et al*., 1997; Olivera *et al*., 2003) and resins (Venkateswaran *et al*., 1995). Therefore, the use of biosurfactants should be a promising means to emulsify the polluted oils prior to biodegradation. Carrillo *et al*. (1996) and Fiebig

et al. (1997) screened biosurfactant-producing microorganisms using hemolysis of red blood cell. A direct thin-layer chromatography technique was used for a rapid characterization of biosurfactant-producing bacterial colonies (Schulz *et al*., 1991). Colony surrounded by an emulsified halo on L-agar plate coated with oil was classified as biosurfactant producer as described by Morikawa *et al*. (1992). A sensitive rapid method, a drop-collapsing test, was advised for screening bacterial colonies that produce surfactants. Drops of cell suspensions of surfactant-producing colonies collapsed an oil-coated surface (Jain *et al*., 1991). Bento *et al*. (2005) used the reduction of surface tension and the emulsify capacity to screen biosurfactant-producing microorganisms. Due to the long coasts along both Indian Ocean and the Gulf of Thailand, the importance biodiversity in the sea has been recognized. However, no information regarding the biosurfactant-producing marine bacteria has been reported. Our objectives were to screen and identify weathered crude oil-emulsifying marine bacteria from oil-spilled seawater in Songkhla Province.

Materials and methods

Isolation of biosurfactant-producing marine bacteria

Oil-spilled seawater samples were collected from harbors and docks in Songkhla Province, Thailand. The samples were spread on Nutrient Agar (Difco, USA) dissolved in seawater and incubated at room temperature for 1-2 days. After incubation, plates were enumerated and morphologically different bacteria were selected for biosurfactant screening (approximately 15 to 30 isolates per plate) and purified by re-streaking twice. Isolated colonies were inoculated into 100 ml of Marine Broth 2216 (Difco, USA) containing 2 drops of weathered crude oil (Dutta and Harayama, 2000) and incubated with continuous shaking (200 rpm) for 24-48 h at room temperature using a shaker (GFL, Burgwedel, Germany). Colonies possessing biosurfactant producing activity, as evidenced by emulsification of weathered crude oil, were chosen. In addition, the cell suspensions of isolated strains, cultivated without weathered crude oil, were then tested for the presence of surfactant by using haemolytic activity, the qualitative drop collapsing test, and quantitative oil displacement test and emulsification activity.

Biosurfactant activity assays

Haemolytic activity

Isolated strains were screened on blood agar plates containing 5% (v/v) human blood and incubated at room temperature for 24 h. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carrillo *et al*., 1996).

Drop collapsing test

Two microlitters of mineral oil was added to each well of a 96-well microtiter plate lid (Nunc, Roskilde, Denmark). The lid was equilibrated for 1 h at room temperature, and then 5 µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant-producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production (Youssef *et al*., 2004).

Emulsification measurement

Emulsification activity was measured according to the method of Cooper and Goldenberg (1987) with a slight modification. To 4 ml of culture 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.

Oil displacement test

Fifteen µl of weathered crude oil were placed on the surface of distilled water (40 µl) in a petri dish (150 mm in diameter). Then, 10 µl of the culture supernatant were gently put on the center

of the oil film. The diameter and area of clear halo visualized under visible light were measured and calculated after 30 second as described by Morikawa *et al*. (1993).

Identification of strains

Strain identification was carried out using the sequence of the gene encoding 16S rRNA according to the methods of Rochelle *et al*. (1995). The 16S rRNA gene of the bacterial isolates was amplified with a set of universal primers (Invitrogen, USA). The primers 8f (5'-AGAGTTTGATC CTGGCTCAG-3') and 1492r (5'-GGTTACCTT GTTACGACTT-3') were used. After amplification, 12 µl of each reaction mixture were separated on 0.7% (w/v) agarose gel to confirm the size and purity of PCR products. The DNA was then purified using a Quiaex II agarose gel extraction kit (Quiagen, Germany) following the protocol provided by the supplier. The PCR product was sequenced using the primers 8f and 1492r and internal primers derived from these sequences to obtain double stranded coverage of the product. The nucleotide sequence of the purified bands was determined by the dideoxy chain terminator method, using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Japan) and Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) according to their instruction manuals. Sequence data were compared to those in EMBL bank using FASTA3.

Extraction of the biosurfactant

Bacterial cells were removed by centrifugation (12,000 x g, 4°C, 30 min). Cultural supernatant was acidified with 6 N HCl to obtain the pH of 2.0. The extraction was performed twice with an equal volume of ethyl acetate. Pooled solvent extracts were concentrated using an evaporator (EYELA SB-651, Tokyo, Japan) under reduced pressure.

Characterization of biosurfactant crude extract

The biosurfactant crude extract (0.5%, w/v) in 50 mM potassium phosphate buffer, pH 7.0 was characterized. To investigate the effects of pHs, salts concentrations (NaCl, $CaCl_2$ and $MgCl_2$) and temperatures on emulsification activity of crude extract, the extract 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 0-21% (w/v). $CaCl_2$ 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. Remaining activity was then determined. Chemically synthetic surfactants (sodium dodecylsulfate (SDS) and Tween 80) were used to compare the activity with biosurfactant crude extract from strain SM1.

Results and discussion

Screening and identification of biosurfactant-producing marine bacteria

From the nutrient agar, 200 bacterial strains were isolated and tested. Among all strains, 40 strains exhibited the clear haloes on blood agar plates. Nevertheless, only 8 strains named SM1-SM8 had haemolytic activity and emulsified weathered crude oil in marine broth during cultivation (Figure 1). Strains SM1, SM3, SM6 and SM8 completely emulsified weathered crude oil in marine broth within 24 h of cultivation, while strain SM5 emulsified weathered crude oil within 48 h. Strains SM2, SM4 and SM7 could emulsify weathered crude oil to some extent. The strains with haemolytic activity and emulsifying activity against weathered crude oil and 5 non-haemolytic strains (named as SM9-SM13) were cultivated in liquid media and biosurfactant production was confirmed. Only strains exhibiting haemolytic activity and emulsifying activity showed the positive result with drop collapsing test, emulsification activity test and oil displacement test (Table 1). From the result, strain SM1 exhibited the highest activity for both oil displacement test toward weathered crude oil (3.14 cm^2) and emulsification activity against *n*-hexadecane (70.5%).

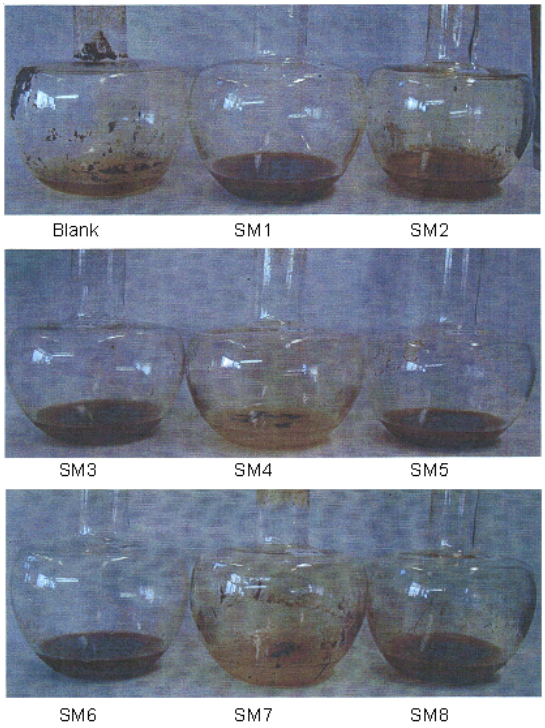

Figure 1. Emulsion of weathered crude oil by strain SM1-SM8 in marine broth after 2 days of cultivation at room temperature.

Table 1. Oil displacement activity and emulsification activity and drop collapsing test of culture supernatant from strains SM1-SM8.

Strains	Testing method		
	Oil displacement test (cm^2)	Emulsification activity (%)	Drop collapsing test
SM1	3.14±0.02a*	70.5±0.55a	+
SM2	1.13±0.05d	45.0±0.15d	+
SM3	2.55±0.03b	65.0±0.35b	+
SM4	1.13±0.03d	50.0±0.31c	+
SM5	2.01±0.05c	50.0±0.25c	+
SM6	2.55±0.03b	65.0±0.36b	+
SM7	1.13±0.04d	45.0±0.20d	+
SM8	2.55±0.02b	65.0±0.26b	+

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

It was noted that the strain with higher emulsifying activity toward weathered crude oil showed the greater oil displacement activity and emulsification activity. Haemolytic activity has been used for the isolation of lipopeptide biosurfactants (Mulligan *et al.*, 1989; Lin *et al.*, 1998) and rhamnolipids (Iqbal *et al.*, 1995). The hydrophilic part of biosurfactant -the cationic part- is proposed to initiate electrostatic interaction with the negatively charged components of the membrane

of microbes; the hydrophobic portion is supposed to permit the peptides to insert into and permeate the membrane (Pag *et al*., 2004). Biosurfactant-producing capacity in liquid medium was found to be associated with haemolytic activity (Carrillo *et al*., 1996; Fiebig *et al*., 1997). Haemolytic activity therefore appears to be a good screening criterion for surfactant-producing strains (Carrillo *et al*., 1996). However, only 13.5% of the haemolytic strains lowered the surface tension below 40 mN/m and not all biosurfactants had a haemolytic activity (Youssef *et al*., 2004). In addition, other microbial products such as virulence factors lyses blood agar and biosurfactants that are poorly diffusible may not lyse blood cells (Iqbal *et al*., 1995; Lin *et al*.,1998). Thus, it is not clear whether blood agar lysis should be used to screen for biosurfactant production. However, such screening can be used as a rapid method, in which samples with the positive result are subsequently subjected to biosurfactant-activity tests in liquid media.

When the 16S rRNA gene sequence of the isolates SM1, SM2, SM3, SM4, SM5, SM6, SM7 and SM8 were compared to previously published sequences on the EMBL database, the high homology was found with *Myroides* sp. (94.00% homology); a *Vibrio paraheamolyticus* (99.54% homology); a *Bacillus subtilis* (99.14% homology); a *Micrococcus luteus* (99.12% homology); an unidentified bacterium; a *Acinetobacter anitratus* (99.39% homology); a *Vibrio paraheamolyticus* (99.67% homology) and a *Bacillus pumilus* (99.34% homology) strain, respectively. Spiteller *et al*. (2000) reported that *Myroides odoratus*, isolated from insect gut, produced *N*-acylglutamine surfactants as alicitors of plant volatiles. Also, biosurfactant-producing bacteria from contaminated and uncontaminated soils were isolated (Bodour *et al*., 2003). Gram-positive biosurfactant-producing isolates were found in heavy metal-contaminated or uncontaminated soils, while gram-negative isolates were isolated from hydrocarbon-contaminated or co-contaminated soils (Bodour *et al*., 2003). The type of biosurfactant is generally governed by the types of microorganism (Desai and Banat, 1997). Different biosurfactants have been isolated from various marine bacteria: glucose lipid produced by *Alcaligenes* sp. (Poremba *et al*., 1991) and *Alcanivorax borkumensis* (Abraham *et al*., 1998); trehalose tetraester and trehalose diester produced by *Arthrobacter* sp. SI 1 (Schulz *et al*., 1991); and polymeric biosurfactants produced by *Pseudomonas nautica* (Husain *et al*., 1997) and an yeast, *Yarrowia lipolytica* (Zinjarde and Pant, 2002). Since strain SM1 emulsified weathered crude oil within 24 h and showed the highest emulsification activity and largest area with oil displacement test, it was selected for biosurfactant production and characterization.

Characterization of biosurfactant from strain SM1

Biosurfactant crude extract from strain SM1 exhibited the increasing emulsifying activity toward *n*-hexadecane as NaCl increased up to 6% ($p < 0.05$), but no activity was observed when NaCl was greater than 9% (Table 2). In the presence of 3-9% NaCl, micellization could be enhanced and emulsification was maximized. Micellization of biosurfactant or modification of the molecular area at the air-water interface can be improved with monovalent cation (Thimon *et al*., 1992). This phenomenon might be a common feature of active compounds produced by marine bacteria. The concentration of salts in aquatic environments ranges from less than 0.05% (w/v dissolved salts) to saturated salt up to 30% and above and NaCl is a major component of seawater (Cameotra and Makkar, 1998). NaCl activated biosurfactant activity of many strains, which were isolated from seawater or petroleum reservoirs (Yakinov *et al*., 1995). However, biosurfactant extract from strain SM1 still had emulsification activity in the absence of NaCl. For Tween 80 and SDS, chemically synthetic surfactants, their emulsification activities were lowered with increasing NaCl concentration (Table 2). In the presence of NaCl above 12%, no activity was obtained. However, Tween 80 still had activity even at 21% NaCl. NaCl, a monovalent cation, might affect the charge of Tween 80 (nonionic) and SDS (anionic) differently, leading to varying emulsification activity. When compar-

Table 2. Effect of salts (NaCl, $CaCl_2$ and $MgCl_2$) on emulsification activity of biosurfactant crude extract from strain SM1, SDS and Tween 80.

NaCl (%)	Emulsification activity (%)		
	SM1	SDS	Tween 80
0	50.73±0.25C**c*	85..51±2.51Aa	75.36±2.51Ab
3	65.17±0.21Bb	76.81±2.51Ba	60.87±0.00ABc
6	70.40±0.36Aa	69.57±4.35Ca	60.87±0.00ABc
9	70.20±0.20Aa	66.67±2.51CCa	42.87±31.18Ba
12	0Dc	59.42±2.51Db	63.77±2.51Aa
15	0Db	0Eb	65.22±0.00Aa
18	0Db	0Eb	65.22±0.00Aa
21	0Db	0Eb	65.22±0.00Aa
$CaCl_2$ (mM)	Emulsification activity (%)		
	SM1	SDS	Tween 80
0	50.47±0.50Ab	66.78±1.17Aa	64.17±3.06ABCa
4	0Bb	62.18±1.60Ba	62.35±0.774Ca
8	0Bb	62.51±1.31Ba	65.52±3.13ABCa
12	0Bb	67.33±1.15Aa	63.50±0.87BCb
16	0Bb	56.78±1.34Cb	66.24±0.74ABa
18	0Bb	58.44±1.50Cb	67.52±1.48Aa
$CaCl_2$ (mM)	Emulsification activity (%)		
	SM1	SDS	Tween 80
0	50.33±0.31Bc	74.67±2.31Aa	62.67±2.31Bb
0.02	60.57±0.40Ab	66.67±2.31Ba	65.22±1.25ABa
0.04	50.57±0.21Bc	58.67±2.31Cb	65.78±1.54AAa
0.06	50.50±0.40Bc	57.33±2.31Cb	64.46±0.80ABa
0.08	50.43±0.40Bc	56.00±0.00Cb	64.00±0.00ABa
0.1	50.33±0.35Bc	52.00±0.00Db	64.92±0.80ABa

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

ing the activity of surfactant from strain SM1 with SDS and Tween 80, SDS showed the highest activity, followed by Tween 80 and SM1, respectively, in the presence of 0-3% NaCl. From the result, varying activity among all samples was observed with different NaCl concentrations.

When the effect of $CaCl_2$ on emulsification activity was determined, the complete loss in activity of biosurfactant crude extract from strain SM1 was found in the presence of $CaCl_2$ ranging from 4 to 18 mM (Table 2). However, $MgCl_2$ ranging from 0 to 0.1 M had no effect on emulsifying activity of biosurfactant crude extract from SM1 and Tween 80 (Table 2). However, a slight increase in activity of crude extract was observed when 0.02 M $MgCl_2$ was added. Calcium and magnesium salt are also present in seawater. Those divalent ions frequently break the oil/water emulsion (Kim *et al.*, 1997). From the result, Tween 80 and SDS showed emulsification activity

toward *n*-hexadecane in the presence of $CaCl_2$ and $MgCl_2$ (Table 2). Hexane solubility in rhamnolipid solution was significantly increased by the addition of Na^+ and Mg^{2+}, but Ca^{2+} over 0.2 mM was found to cause precipitation of rhamnolipid (Bai *et al*., 1998).

After the biosurfactant 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 (Figure 2). Temperatures ranging from 30 to 121°C did not show any

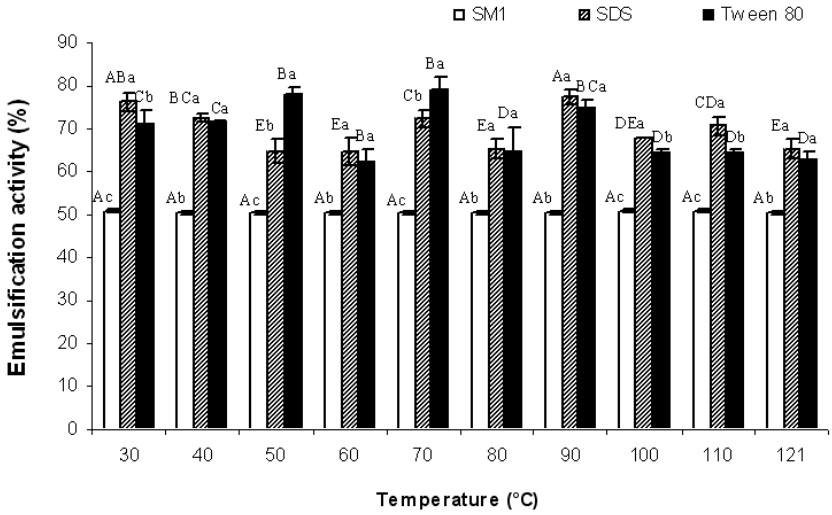

Figure 2. Thermal stability of biosurfactant crude extract from strain SM1, SDS and Tween 80. Samples were incubated at 30-100°C for 1 h, while incubated at 110°C or 121°C for 15 min. Bars represent the standard deviation from three determinations. Different capital letters indicate significant differences (p<0.05). Different letters within the same temperature tested indicate significant differences (p<0.05).

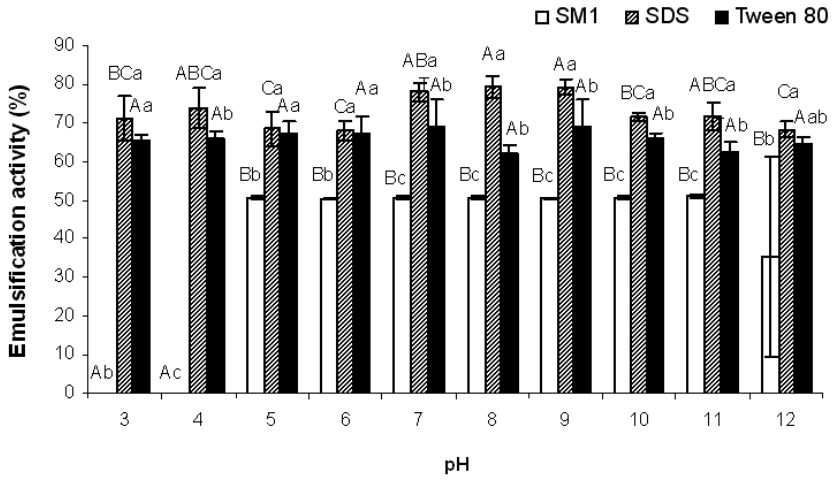

Figure 3. Effect of pH on emulsification activity of biosurfactant crude extract from strain SM1, SDS and Tween 80. Different capital letters indicate significant differences (p<0.05). Different letters within the same pH tested indicate significant differences (p<0.05).

influence on the emulsification toward *n*-hexadecane of biosurfactant crude extract from strain SM1 (Figure 2). The result was in accordance with Horowitz *et al*. (1990) who reported that biosurfactant from *Bacillus licheniformis* 86 was stable in the temperatures range of 25-120°C. With increasing temperatures, a decrease in emulsification activity was observed for both Tween 80 and SDS. However, both synthetic surfactants showed higher activity than the biosurfactant crude extract from strain SM1 at all temperatures tested (Figure 2).

Effect of pHs on emulsification activity of biosurfactant crude extract from strain SM1, Tween 80 and SDS is shown in Figure 3. At pH below 5, the precipitation of the biosurfactant from strain SM1 took place and was associated with the decrease in activity. At pH equal with isoelectric point, there is no electrostatic repulsion between neighboring molecules, and they tend to coalesce and precipitate (Milewski, 2001). However, no changes in activity were noticeable in the pH range of pH 5-12 (Figure 3). Protonation of carboxylic side chains of glutamic or aspartic acids in peptide portion alters the electrostatic properties of molecule (Lin et al., 1998). Biosurfactant KP-2 (surfactin) retained activity at pHs from 6 to 10 and exhibited optimal activity at pH 6 (Roongsawang *et al*., 1999). No influence of pHs on emulsification activity toward n-hexadecane was found with Tween 80. Nevertheless, some losses in activities were observed at very acidic and alkaline pHs for SDS. From the result, biosurfactant crude extract from strain SM1 showed slightly lower activity than Tween 80 and SDS at all pHs tested. Therefore, the biosurfactant crude extract from strain SM1 may have a potential in bioremediation of hydrocarbons in a wide pH range.

Conclusion

Eight biosurfactant-producing marine bacteria isolated from oil-spilled seawater were able to emulsify weathered crude oil. Biosurfactant crude extract produced from strain SM1 was stable in a broad temperature ranges. Nevertheless, the loss in emulsifying activity was noticeable at very acidic condition as well as at high salt concentration.

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