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Screening Characterization and Stability of Biosurfactant Produced by *Pseudomonas aeruginosa* SCMU106 Isolated from Soil in Northern Thailand

[1]S. Techaoei, [2]S. Lumyong, [3]W. Prathumpai, [1]D. Santiarwarn and [1]P. Leelapornpisid
[1]Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, 50200, Thailand
[2]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand
[3]National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Klong 1, Klong Luang, Pathumthani, 12120, Thailand

Corresponding Author: P. Leelapornpisid, Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, 50200, Thailand

ABSTRACT

The aim of this research was to study isolation, characterization and stability of biosurfactant-producing bacteria. One hundred and ninety-seven bacterial strains (69 from hot springs and 128 from garage sites) were isolated from Northern Thailand and screened for biosurfactant production, determined for its activities by oil displacement area measurement, emulsification test and surface tension measurement on parafilm. Twenty-five strains of bacteria from garages site showed a promising biosurfactant production. Strain SCMU106 that presented the highest values of oil displacement area (143.20 cm^2), emulsification index (60%) and droplet formation on parafilm surface (8.0 mm) was identified as *Pseudomonas aeruginosa* by 16S rDNA sequence analysis. Combination of 1% glucose plus 0.1% corn oil and 0.5% $(NH_4)_2H_2PO_4$, as carbon and nitrogen source, respectively was the optimal biosurfactant producing condition. The effects of pH, salinity and temperature on the produced biosurfactant were also evaluated. The produced biosurfactant was non-cytotoxic and stable to high temperature, neutral to alkaline pH (pH 6-10) and moderate concentration to electrolyte (up to 8% NaCl).

Key words: Biosurfactant, surface tension, oil spreading, emulsification index, *Pseudomonas aeruginosa*

INTRODUCTION

Biosurfactants are compounds produced by microorganisms, which reduce surface tension at the air-water interface and reduce interfacial surface tension between immiscible liquids, or at the solid-liquid interface (Desai and Banat, 1997; Karanth *et al*., 1999). These biosurfactants are used in various industries such as food, pharmaceutical and cosmetic, as they are biodegradable and less toxic than the synthetic surfactants currently used (Karanth *et al*., 1999).

The most important surface-active properties evaluated in screening microorganisms for biosurfactants with potential industrial applications are surface tension reduction, emulsion forming and stabilizing capacity. The criterion used for selection of biosurfactant producers is the ability to reduce the surface tension below 40 mN m^{-1} (Tabatabaee *et al*., 2005; Bodour and Miller-Maier, 1998). Examples of biosurfactants include rhamnolipids produced by *Pseudomonas*

aeruginosa (Lang, 2002), surfactin from *Bacillus subtilis* (Bognolo, 1999), mannosylerythritol lipids from *Candida antarticas* (Kitamoto *et al*., 1993) and trehalose lipids from *Rodococcus erythropolis* (Singer *et al*., 1990). Sophorose lipids produced by certain strains of yeast have been formulated for anti-dandruff solutions, hair gels, deodorant sticks, after-shave lotions, and hair and body shampoos. Besides, some microorganisms capable to produce biosurfactant which applied to cosmetic application such rhamnolipid from *Pseudomonas aeruginosa*, mannosylerythriltol lipids from *Candida antratica*, have excellent antimicrobial activity (Williams, 2009; Arutchelvi *et al*., 2008; Kulakovskaya *et al*., 2003). However, a variety of raw materials, efficient production processes, and use of mutant or recombinant strains to enhance biosurfactant yield are all promising strategies that could lead to further large-scale application in the cosmetics industry (Mukherjee *et al*., 2006; Thavasi *et al*., 2009; Rahman *et al*., 2002).

The present study was carried out in order to search out for potent biosurfactant produced from microorganisms found naturally in Thailand such as soils collected from various hot springs and garages and to characterize the surface active molecules that may have potential applications in the cosmetic industry.

MATERIALS AND METHODS

Isolation and screening of biosurfactants production from culture media: Soil samples were collected from Sankamphaeng (Chiang Mai, Thailand) and Mae Ka Jan (Chiang Rai, Thailand) hot springs, some garages in Muang district (Chiang Mai, Thailand) and some bacterial strains were obtained from Laboratory of Applied Microbiology, Biology Department, Faculty of Science, Chiang Mai University, Thailand. All samples were collected on February 2008. Each one-gram of soil sample was incubated in 100 mL of enrichment culture medium, pH 7, containing 0.1% soybean oil as a carbon source. Culture medium was composed of (per liter) 20 g glucose, 5.0 g glutamic acid, 1.0 g K_2HPO_4, 1.02 g $MgSO_4$, 0.5 g KCl and 1 mL of trace elements solution containing (per liter distilled water) 0.5 g $MnSO_4.7H_2O$, 0.16 g $CuSO_4.5H_2O$ and 0.015 g $FeSO_4.7H_2O$. All the samples were incubated with shaking at 150 rpm at 45°C (hot spring samples) or 30°C (garage and culture collection samples) for 3 days. Bacterial suspension (in distilled water) was counted by serial dilution on spread plates. Isolated bacteria were maintained on Nutrient agar. The effects of various carbon sources on biosurfactant production were determined using medium supplemented with (0.1% concentration) corn oil, soy bean oil, palm oil, coconut oil, rice bran oil, or cotton seed oil, or with (1% concentration) sorbose, glucose, galactose or sucrose. The effects of nitrogen sources were evaluated using McKeen medium containing 1% glucose and 0.1% corn oil as C source supplemented with (0.5%) monosodium glutamate, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, NH_4NO_3, KNO_3, or $(NH_4)_2H_2PO_4$. The bacteria isolate that best produced biosurfactant was selected to identify and used for further study.

Characterization of the produced biosurfactant

Oil spreading test: The produced biosurfactants were separated from culture media by centrifuged to get culture supernatant and characterized by measuring diameters of clear zones caused when a drop of a biosurfactant-containing solution was placed on an oil-water surface. Fifty milli liter of distilled water was added to a large Petri dish (15 cm diameter) followed by addition of 20 μL of crude oil to the water surface and 10 μL of culture broth supernatant. A clear halo was visible under light. The area of this circle was measured and calculated for Oil Displacement Area (ODA) using the following equation:

$$ODA = 22/7 (radius)^2 cm^2$$

The triplicate assays from the same sample was measured (Sarubbo *et al*., 2007).

Emulsification index (E_{24}): Emulsifying capacity was evaluated as an emulsification index (E_{24}). E_{24} of culture sample was determined by mixing 2 mL of kerosene and 2 mL of cell-free broth for 2 min and allowing the mixture to stand for 24 h. E_{24} was calculated by dividing the height of the emulsion layer by the mixture total height and then multiplying by 100 (Tabatabaee *et al*., 2005; Sarubbo *et al*., 2007).

Parafilm test: A 25 μL aliquot of bacterial supernatant was added to the hydrophobic surface of a parafilm. The shape of the drop on the surface was inspected after 1 min and the diameter of droplet was measured. Sodium lauryl sulfate (1%) and 0.5 M phosphate buffer pH 7.0 was used as positive and negative control respectively (Hewald *et al*., 2005; Morita *et al*., 2007).

Stability study of biosurfactant: The stability of the biosurfactant produced from selected bacteria isolate to temperature, pH and electrolyte effects was studied as the following test procedures, then determined for oil spreading area, emulsification index and diameter of droplet on parafilm surface, respectively.

Temperature effect: About 4 mL of the culture supernatant were stored at 4 and 25°C and heated at 70, 100 and 121°C for 15 min and then cooled to room temperature.

Effect of pH change: The pH change effect was determined by adjusting the culture supernatant with acid (1 N HCl) or alkaline (1 N NaOH) to pH values ranging from 1 to 12.

Electrolyte effect: The culture supernatant was added 2-20% NaCl then the samples were subjected to oil spreading test, measurement of E_{24} and parafilm test.

Identification of biosurfactant-producing bacteria: 16S-rDNA sequencing identified biosurfactant-producing bacteria. DNA templates for PCR amplification were prepared by the method of Marmur (1961). DNA coding for 16S rRNA regions was amplified by means of PCR with *Taq* polymerase, as described by Kawasaki *et al*. (1993), Yamada *et al*. (2000) and Katsura *et al*. (2001). Genomic DNA was extracted and 16S rRNA gene was PCR amplified using primers 20 F (5′ GAG TTT GAT CCT GC TCA G 3′) and 1500R (5′ GTT ACC TTG TTA CGA CTT 3′) (Brosius *et al*., 1981). Amplicons were purified using commercially available kits (QIAGEN GmbH, Hilden, Germany), sequencing of the purified PCR products was carried out with an ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (version 3.0, Applied Biosystems, Foster City, California, USA. The primers 20F, 1500R, 520F (5'-CAG CAG CCG CGG TAA TAC-3'; positions 519-536), 520R (5'-GTA TTA CCG CGG CTG CTG-3'; positions 536-519), 920F (5'-AAA CTC AAA TGA ATT GAC GG-3'; positions 907-926) and 920R (5'-CCG TCA ATT CAT TTG AGT TT-3'; positions 926-907) were used for sequencing of 16S rDNA and subjected to phylogenetic analysis. The topology was analyzed by Neighbor-joining method.

Nucleotide sequence accession number: The nucleotide sequence of SCMU106 has been deposited with GenBank under accession No. GQ355592.

Cytotoxicity assay: The culture supernatant from selected bacteria isolate was tested for cytotoxicity against Vero-GFP cells (African green monkey kidney fibroblasts, ATCC CCL-81) in 384-well tissue culture plates (Costar 3701, Corning). One hundred and ninety micro liter of Vero cell suspension 1×10^5 cells mL^{-1}) and 10 μL of test compound were added to each well in triplicate. Ellipticine 8.0-0.25 mg mL^{-1} and 10% DMSO was used as positive and negative control respectively. Cells were incubated at 37°C for 72 h in an atmosphere of 5% CO_2. Cytotoxicity was determined by the colorimetric method of Skehan *et al.* (1990) and expressed as IC_{50}, i.e., concentration inhibiting 50% cell growth compared to untreated cell.

RESULTS AND DISCUSSION

Isolation and screening of biosurfactant-producing bacteria: One hundred and ninety-seven bacterial strains were isolated from soils collected at hot spring and garage sites in Chiang Mai and Chiang Rai province, Thailand and 31 strains were obtained from Division of Microbiology, Faculty of Science, Chiang Mai University, Thailand. Twenty-five bacterial strains from garage sites showed biosurfactant activity (Table 1), suggesting that these sites had a variety of hydrocarbon substrates. Ninety-six percent of the isolates were Gram-negative (Table 1) similar to the report of Batista *et al.* (2006) and this may be a specific that contributes to survival of these populations reflect the ability of these types of organisms to survive in such extreme

Table 1: Production of biosurfactant by bacterial strains isolated from garage sites

Isolate	Oil displacement (ODA)	$\%E_{24}$	Gram staining	Shape
SCMU6	+	0	P	cocci
SCMU22	++	57.8±1.9	N	rod
SCMU23	+++	7.8±1.9	N	rod
SCMU27	+	21.5±1.3	N	rod
SCMU28	++	0	N	rod
SCMU29	+	52.2±1.9	N	rod
SCMU37	+	56.7±0	N	rod
SCMU89	+++	14.4±3.9	N	rod
SCMU94	+	0	N	rod
SCMU98	+	8.9±3.8	N	rod
SCMU103	++	50.0±0	N	rod
SCMU106	++++	57.8±1.9	N	rod
SCMU108	++	14.4±3.9	N	rod
SCMU109	++	0	N	rod
SCMU110	++	0	N	rod
SCMU111	++	0	N	rod
SCMU112	++	0	N	rod
SCMU114	+	0	N	rod
SCMU115	++	0	N	rod
SCMU117	++	0	N	rod
SCMU119	++	0	N	rod
SCMU120	+	0	N	rod
SCMU121	+	0	N	rod
SCMU122	+	0	N	rod
SCMU123	+	0	N	rod

P = Gram-positive, N = Gram-negative, $\%E_{24}$ = Emulsification index; + = 0.1-3.14 cm^2, ++ = 3.14 - 12.57 cm^2, +++ = 12.57 – 28.28 cm^2, ++++ = >28.28 cm^2

environments (Bicca *et al*., 1999). This finding was in agreement with the experimental results of Ghojavand *et al*. (2008) which demonstrated that the surfactant producer were able to grow at high temperature and salinities to about 55°C and 10% w/v, respectively. However, There was a report showed that hydrocarbon compound inhibited the biosurfactant production of *Bacillus subtilis* (Cooper *et al*., 1981).

Bacteria strain SCMU106 exhibited the highest oil clearing zone and oil displacement area. The oil spreading test is a better predicator of biosurfactant production than the drop collapse method because it is very sensitive, requires a small sample volume (Youssef *et al*., 2004) rapid and easy to be carried out, and does not require any specialized equipment (Plaza *et al*., 2006). In addition, this method depends on the decrease in water-oil interfacial tension caused by the biosurfactant regardless of its structures (Morikawa *et al*., 2000).

One of the desirable properties of surfactant is emulsion formation. Although some strains showed positive oil displacement, they did not exhibit any emulsification capability, reflecting the presence of biosurfactants with different emulsification properties. The emulsifying capacity evaluated by the E_{24} emulsification index method, bacteria strain SCMU106 also gave the highest emulsion formed (57.8±1.9) therefore, was chosen for further studies. On the other hand, published data on E_{24} in the literature, reported a value of approximately 65% (Ilori *et al*., 2005).

Effects of culture media on biosurfactant production: Bacteria strain SCMU106 could grow in culture media containing of all the types of carbon sources used (data not shown). Maximum biosurfactant activity occurred with glucose and corn oil used as carbon sources (oil surface displacement of 38.5 and 2.93 cm^2, respectively). The bacteria had poor growth and produced minimal amounts of biosurfactants in media containing soybean oil, palm oil, coconut oil, rice bran oil, cotton seed oil, galactose, sucrose, and sorbose (data not shown). Results of oil displacement activity correlated with emulsification index and droplet formation on parafilm surface (Table 2). This finding was in agreement with that of Rendell *et al*. (1990) which showed that glucose is the best carbon source for producing biosurfactant by *Pseudomonas* sp. In addition, Mata-Sandoval *et al*. (1999) demonstrated that *Pseudomonas aeruginosa* UG2 is able to grow on corn oil as substrate for produce rhamnolipid. Thampayak *et al*. (2008) report that the biosurfactant from culture of *Streptomyces* sp. strain S71, S71 and S177 had 48, 66, 55% of emulsification index, respectively.

Table 2: Substrates for growth of bacteria strain SCMU106 with shaking at 150 rpm and incubated at 37°C for 3 days

Substrate	Growth	ODA (cm^2)	$\%E_{24}$	Parafilm (mm)
Oil (0.1%)				
Corn	+++	2.93±0.12	60±0	8.0±0
Soy bean	++	1.94±0.06	60±0	8.0±0
Palm	++	2.75±0.12	60±0	8.0±0
Coconut	+	0.23±0	56.67±0	8.0±0
Rice bran	+	0.35±0	56.67±0	7.0±0
Cotton seed	+	0.35±0	56.67±0	7.0±0
Sugar (1%)				
Galactose	+	2.75±0.15	33.33±0	5.0±0
Sucrose	++	0.22±0.06	45±0	7.0±0
Glucose	+++	38.5±1.32	60±0	8.0±0
Sorbose	+	0.68±0.16	33.33±0	7.0±0

Evaluation +++ = Very good growth, ++ = Good growth, + = Moderate growth

Table 3: Combination of glucose plus corn oil as carbon source for biosurfactant production in shake-flask condition at 150 rpm and incubated at 37°C for 3 days

%glucose + 0.1% corn oil	ODA (cm^2)	$\%E_{24}$	Parafilm (mm)
1.0	66.55±1.35	60	8
1.5	56.77±0.00	60	8
2.0	42.30±2.69	60	8
3.0	22.11±1.78	60	8
3.5	20.99±1.97	60	8
4.0	10.38±0.53	60	8

Table 4: Effect of nitrogen source on growth and biosurfactant production with shaking at 150 rpm and incubated at 37°C for 3 days

Nitrogen source (0.5%)	ODA (cm^2)	$\%E_{24}$	Parafilm (mm)
Monosodium glutamate	10.83±0.29	60	8
$NH_4H_2PO_4$	110.67±0.58	60	8
$(NH_4)_2SO_4$	10.83±0.29	60	8
NH_4NO_3	9.83±0.76	60	8
KNO_3	11.17±0.29	60	8
$(NH_4)_2H_2PO_4$	143.20±0.50	60	8

A combination of carbohydrate plus vegetative oil has been proposed for biosurfactant production as renewable resources for increased production and activity (Kim *et al*., 1999; Daniel *et al*., 1998; Hildebrand *et al*., 1998; Zhou and Kosaric, 1995; Hommel *et al*., 1994). Thus, biosurfactant production was studied in media containing glucose and corn oil as carbon sources. The bacteria strain SCMU106 was able to produced biosurfactant in media with 1% glucose plus 0.1% corn oil because, displaying maximum bisurfactant activity of 66.55±1.35 cm^2 of oil displacement (Table 3). However, when the concentration of glucose was increased over 1%, biosurfactant activity slightly decreased. Emulsification index and droplet formation on parafilm surface are not significantly increased with increase in glucose concentration. Similar results have been reported of surfactants produced by yeast using carbohydrate and vegetable oil as substrates (Daniel *et al*., 1999; Chen *et al*., 2006; Sarubbo *et al*., 2007) and fungi (Spoeckner *et al*., 1999).

The effects of nitrogen sources on biosurfactant production in Mckeen medium containing 1% glucose and 0.1% corn oil as carbon source under shake-flask condition are shown in Table 4. Addition of nitrogen sources, such as monosodium glutamate, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, NH_4NO_3, KNO_3 and $(NH_4)_2$ H_2PO_4 enhanced the biosurfactant activity. $(NH_4)_2H_2PO_4$ gave the highest oil displacement area (143.20±0.50 cm^2) but, E_{24} and droplet formation on parafilm surface did not show any significant differences from other nitrogen sources.

Stability of biosurfactant: The pH of culture cell-free broth was varied from 2-12 to study its effects on emulsification activity, oil displacement area and droplet formation on parafilm surface. Emulsification index was stable (60%) in pH rage of 6 to 12, but at lower pH, its activity decreased especially at pH 2-4 (Fig. 1). Whereas, the emulsification activity of biosurfactants produced by *Bacillus subtillis* has been shown to be pH stable (Makkar and Cameotra, 1998). For oil displacement area assay, the produced biosurfactant was also stable at pH range 4-11, but at higher and lower pH, it presented a decrease in oil displacement area. This presented the acidic condition affected its emulsification ability, may be due to its negatively charged molecules or other effects which will be further investigated. Similar results had been reported for biosurfactant production of rhamnolipid (Yin *et al*., 2009), surfactin (Cooper *et al*., 1981), lichenycin B

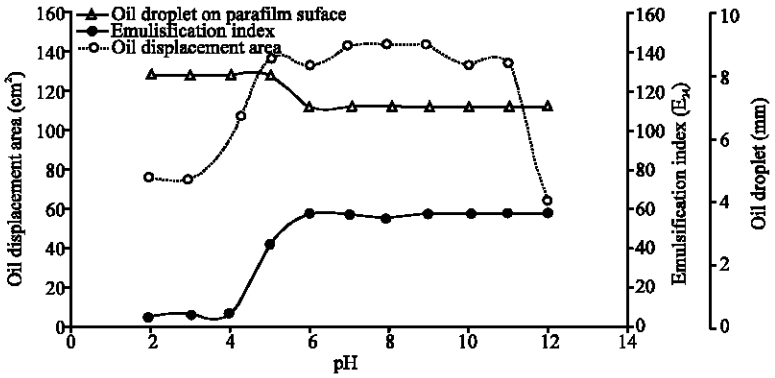

Fig. 1: Oil displacement area, emulsification index and oil droplet versus pH of biosurfactant produced from *Pseudomonas aeruginosa* SCMU106

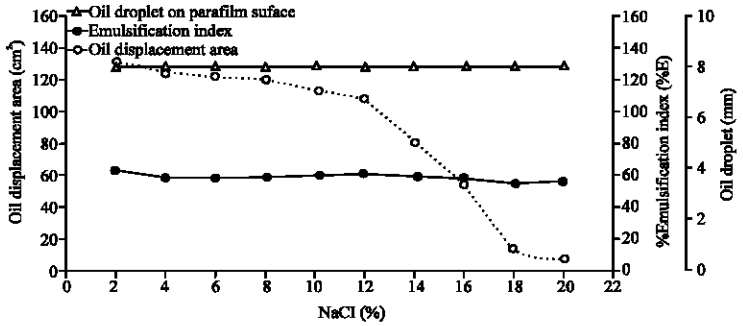

Fig. 2: Oil displacement area, emulsification index and oil droplet versus NaCl of biosurfactant produced from *Pseudomonas aeruginosa* SCMU106

(McInerney *et al*., 1985) and biosurfactant BF 2.2 (Sutthivanitchakul *et al*., 1999) were stable at a wide range of pH 6.0 to 12.0.

The emulsification index (60%) and droplet formation on parafilm surface (8 mm) of the produced biosurfactant were stable in the presence of NaCl in the concentration range of 2-20% w/v (Fig. 2). This corresponds to Sarubbo *et al*. (2007) reported that NaCl concentration did not affect biosurfactant emulsification from *Candina lipolytica*, which suggested that the emulsifier's activity depended on its affinity for hydrocarbon substrate, which involves a direct interaction with the hydrocarbon itself rather than an effect on the surface tension of the medium (Abu-Ruwaida *et al*., 1991). On the other hand, its oil displacement area decreased at high NaCl concentration (above 8% w/v).

The emulsification index of the produced biosurfactant from bacteria strain SCMU106 indicated that no appreciable changes with temperature. However, Sarubbo *et al*. (2007) reported loss of biosurfactant emulsification capacity after heating for 1 h at 70°C. Its oil displacement area and droplet formation on parafilm surface decreased when stored at higher temperature (Fig. 3).

Identification of biosurfactant-producing bacteria: For identified biosurfactant-producing bacteria was done by 16S rRNA gene sequence analysis. It is evident from 16S rRNA gene phylogenetic tree that isolate SCMU106 is member of genus *Pseudomonas aeruginosa* (Fig. 4). It

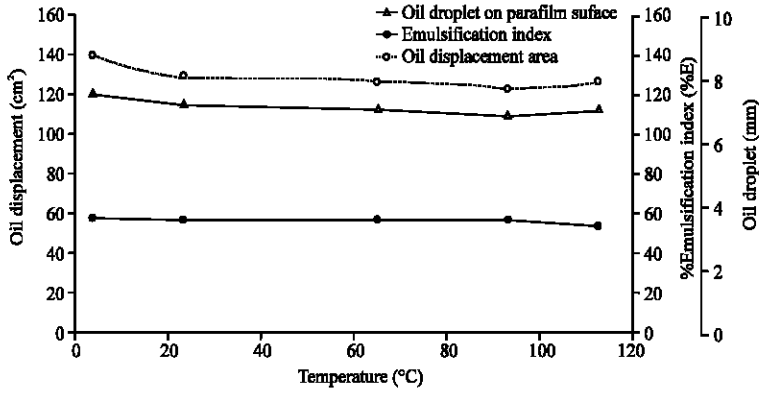

Fig. 3: Oil displacement area, emulsification index and oil droplet versus temperature of biosurfactant produced from *Pseudomonas aeruginosa* SCMU106

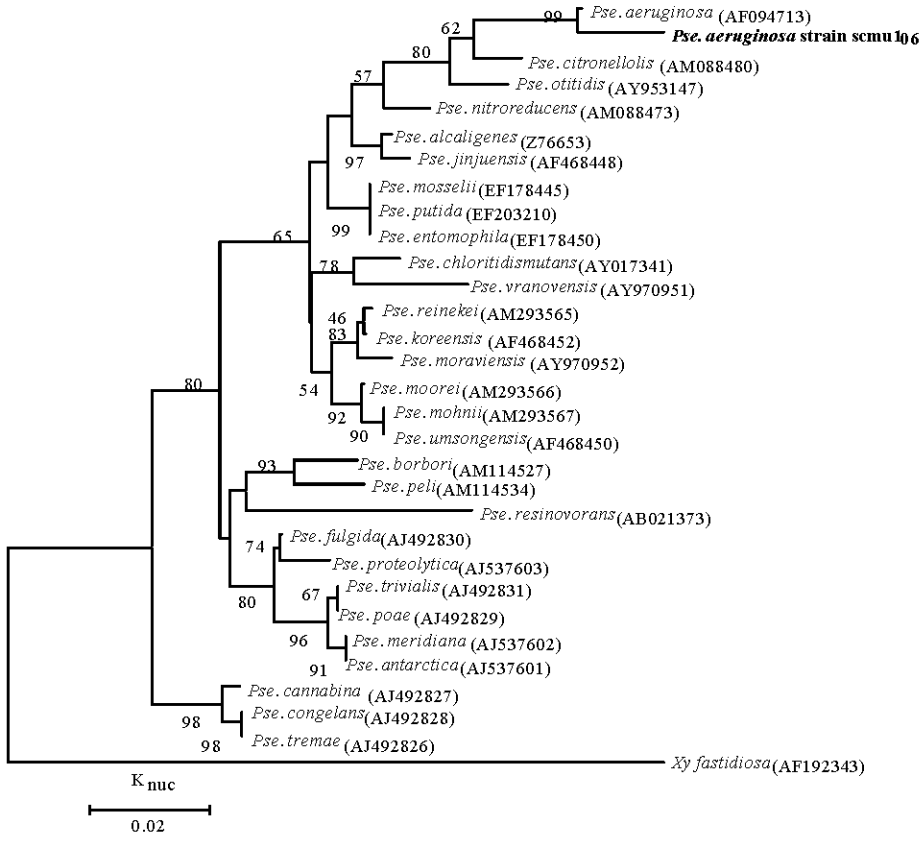

Fig. 4: Phylogenetic analysis based on of 16S rDNA sequences of isolate SCMU106 and related *Pseudomonas* species. Evolutionary distances were computed using the maximum composite likelihood model and tree reconstruction on the Neighbor-joining method. Bootstrap values (1,000 replicates run%) are included. GeneBank accession numbers are listed species names. *Xy. fastidiosa* was also used as outgroup

formed phyloletic line that was closely related to *Psudomonas aeruginosa* AF094713 sharing 16S rRNA gene similarities with 99%. The majority of bacteria isolates with biosurfactant production to the genus *Pseudomonas* (Widada *et al*., 2002) while, Bento *et al*. (2005) analyzed the 16S rRNA gene of bacteria from soils contaminated with diesel oil were indentified as genus *Bacillus, Acinetobacter* and *Pseudomonas*, respectively.

Determination of cytotoxicity assay: The supernatant of bacteria strain SCMU106 exhibited no cytotoxicity against Vero-GFP cells at concentration of 50 μg mL^{-1} (data not shown). This results is similar to the report of Thanomsub *et al*. (2007) showed that the biosurfactant, RL-a and RL-b from *Pseudomonas aeruginosa* B189 had no effect on the normal cell line (vero cell) at concentration up to 50 μg mL^{-1}.

CONCLUSION

The majority of biosurfactant-producing bacteria, determined using the qualitative oil displacement area technique, isolated from soils in northern Thailand was Gram-negative bacteria. The optimal carbon source was a combination of 1% glucose plus 0.1% corn oil, resulting in 66.55±1.35 cm^2 oil displacement area, 60% emulsification index and 8.0 mm droplet formation on parafilm surface. The optimal nitrogen content for biosurfactant production was 0.5% $(NH_4)_2H_2PO_4$. The biosurfactant produced was non-cytotoxic and stable to high temperature, neutral to alkaline pH (pH 6-10) and moderate concentration to electrolyte (up to 8% NaCl).

This study strongly indicated that the produced biosurfactant play a promising role in cosmetic industry due to its emulsification capability, stability and non toxicity. The optimization of biosurfactant production in pilot scale and its purification will be further investigated for the application in cosmetics.

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