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Short Communication

Purification and characterization of a biosurfactant produced by *Issatchenkia orientalis* SR4

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Biosurfactants are produced by a variety of microbes, secreted either extracellularly or attached to parts of cells, predominantly during growth on water-immiscible substrates. They are categorized mainly by their chemical compositions and their microbial origins. The major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids, fatty acids, polymeric biosurfactants and particulate biosurfactants (Desai and Banat, 1997). These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures (Khire and Khan, 1994). Biosurfactants are important biotechnological products with a wide range of applications in many industries such as food, cosmetic and pharmaceutical industries (Kitamoto et al., 2002). Furthermore, biodegradability, ecological safety, production from renewable resources, and functionality under extreme conditions are significant advantages of biosurfactants over chemical surfactants (Banat et al., 2000). Minimal studies have been reported on biosurfactants synthesized by yeasts (Hua et al., 2003) and most of them are related to bacteria (Koma et al., 2001). In the present work, we attempted to find new biosurfactants by the use of a mixture of xylene isomers as the sole carbon source for the cultivation of a newly isolated yeast strain.

A total of 237 yeast strains were isolated from oil-contaminated soils in Thailand and cultured in a basal salt medium (3 g/L NH_4NO_3, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L yeast extract) supplemented with 1 g/L weathered crude oil (kindly provided by Department of Chemical Engineering, Faculty of Engineering, Prince of Songkla University) as the sole carbon source. Among 81 yeast strains showing growth, only 5 strains showed high biosurfactant activity. These strains were further screened for higher activity in basal salt medium supplemented with different carbon sources. As a result, the highest activity was exhibited by strain SR4 grown in the optimized medium (3 g/L urea, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$) supplemented with 1 g/L of a mixture of xylene isomers (Lab-Scan Asia, Co., Ltd., Bangkok, Thailand). The characteristics of strain SR4 grown on YM agar plate (5 g/L

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peptone, 3 g/L yeast extract, 3 g/L malt extract, 10 g/L glucose, 20 g/L agar) at 30°C were off-white, oval shape, dull, and smooth with margins ranging from smooth to lobed. It was teleomorphic (reproducing sexually). Sequence analysis (approximately 1 kb, Applied Biosystems Japan, Tokyo, Japan) of 18S rDNA indicated that the SR4 strain belongs to *Issatchenkia orientalis* (99% identity). The 18S rDNA sequence was deposited in DDBJ (Japan) under the accession number AB300775.

Biosurfactant production by *Issatchenkia orientalis* SR4 was performed in an optimized medium 500 ml (3 g/L urea, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$) supplemented with 1 g/L of a mixture of xylene isomers (initial pH 5.0) in a Sakaguchi flask (3 L) at 28°C and agitated at 100 rpm for 48 h. The culture supernatant (10 L) obtained was extracted twice with an equal volume of mixtures of chloroform/methanol (2 : 1) (modified from Mercade et al., 1996). The combined extracts were concentrated to dryness *in vacuo*. The crude extract was separated by chromatography on a silica gel column (1.0×30 cm, Wako gel C-100, Wako Pure Chem. Ind., Ltd., Osaka, Japan) by sequential elution with hexane, ethyl acetate and methanol. All fractions were collected, dried and tested for their biosurfactant activities. The pooled active fraction C5 (20.56 mg) was fractionated by chromatography on a silica gel (C-200) column as above, again. The fraction with the highest surfactant activity C5 (18.52 mg) was purified further using preparative HPLC on Inertsil ODS-3 (20×250 mm, GL Sciences Inc. Japan) eluting with 95% aqueous acetonitrile containing 0.05% trifluoroacetic acid (TFA). Finally, 6 mg of the surface-active compound was obtained as an oily material.

Thin-layer chromatography of the purified compound was performed on silica gel 60 F_{254}-coated aluminium sheets (Merck, Darmstadt, Germany) with the solvent system hexane/ethyl acetate (7 : 3, v/v). Chromatograms were viewed in visible and UV light (254 and 365 nm) prior to treatment with either *p*-anisaldehyde-sulfuric acid reagent (mixture of 0.5 ml of *p*-anisaldehyde, 0.5 ml of concentrated sulfuric acid, and 9 ml of ethanol) or bromocresol green reagent (0.04% bromocresol green in methanol adjusted to a blue color (pH 7.5) with 0.1 M NaOH). The purified compound was detected as a blue-green spot with an R_f value of 0.34 on TLC by *p*-anisaldehyde sulfuric acid reagent. The compound was also detected as a yellow spot on a blue background by spraying with bromocresol green reagent, suggesting that the compound was acidic. From the R_f value and acidic character, the compound was assumed to be a fatty acid. Therefore, we attempted to identify the compound as a methyl ester.

The active compound was dissolved in anhydrous methanolic 5% HCl (GC, Tokyo Kasei Kogyo Co., Ltd. Japan) and heated at 100°C for 2 h. The reaction mixture was neutralized with 0.5 M $NaHCO_3$ and extracted with $CHCl_3$. The $CHCl_3$ layer was analyzed with a GC-MS system (Automass 20, JEOL, Tokyo, Japan) under the following conditions: GC, Hewlett Packard HP5890 Series II; column, DB-1 (30 m×0.25 mm, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA); carrier gas, He; injection temperature, 250°C; column temperature program, 150°C (1 min), 150–280°C at 10°C/min, 280°C (20 min); MS, electron ionization positive mode; ionization voltage, 70 eV; ion source temperature, 210°C. The compounds corresponding to the peaks in total ion chromatogram were tentatively identified by library search of the JEOL data system (Lucy version 5.0 software, JEOL). GC-MS total ion chromatogram of the derivative obtained by treatment of the compound with HCl-methanol showed a single peak with a retention time 10:11 min. The mass spectrum of this peak showed a weak molecular ion at *m*/*z* 296 and prominent ions at *m*/*z* 265, 223 and 181. The fragmentation pattern and a mass spectral library-search suggested that the compound corresponding to this peak was methyl oleate. This preliminary identification was confirmed by co-injection of authentic methyl oleate.

Finally, the active compound was analyzed by ODS-HPLC and compared with an authentic oleic acid (Tokyo Kasei Kogyo Co., Tokyo, Japan). HPLC analysis was performed on an Inertsil ODS-3 HPLC column (4.6×250 mm, GL Sciences Inc., Tokyo, Japan). The isocratic mobile phase was 95% aqueous acetonitrile with 0.05% TFA employed at a flow rate of 0.53 ml/min at 30°C and the effluent was monitored at 210 nm. The active compound showed the same retention time of 8:29 min as authentic oleic acid, and the identity was confirmed by co-injection. The origin of oleic acid was examined. The culture medium (without the inoculation of strain SR4) was extracted using the same protocol as the biosurfactant extraction. No spot or peak corresponding to oleic acid was detected in the crude extract of the culture broth by TLC or analytical HPLC, respectively. This result proved that the biosurfactant

oleic acid was originated from the yeast strain SR4.

All the crude biosurfactant fractions and the purified active compound were tested for biosurfactant activity by an oil displacement area (ODA) test (Youssef et al., 2004). Fifty milliliters of distilled water was added to a large Petri dish (15 cm diameter) followed by the addition of 20 µl of weathered crude oil to the surface of water to make an oil layer. Ten microliters of sample solution in methanol was then added to the surface of oil, resulting in a clear zone, according to its oil-displacing ability (surface activity). Each test was performed in triplicate. Methanol was used as a negative control (no clear zone was found). The minimum active dose (MAD) of the biosurfactant was defined as the amount necessary for giving a clear zone detectable by the naked eye on an oil layer. The biosurfactant property was also confirmed by emulsification activity (modified from Cooper and Goldenberg, 1987). The mixture of xylene isomers (1 ml) was added to 1 ml sample solution in a vial, which was vortexed at high speed for 2 min and allowed to stand for 10 min until the solvent and water layers separated and an emulsified layer appeared between them. The percent emulsification activity (%EA) was calculated by using the height of emulsion formed to divide by total height of solution, then multiply by 100.

The purified compound showed almost the same surface activity as an authentic oleic acid, and the activity was higher than that of synthetic surfactants such as SDS, triton X-100 and triton X-114, as shown in Table 1. Table 2 shows the emulsification activity of the purified compound in comparison with authentic oleic and synthetic surfactants. The %EA of purified compound was equal to %EA of authentic oleic acid. However, it was lower than those of the synthetic surfactants.

Many fatty acid biosurfactants produced by various bacteria have been reported such as corynomycolic acid (β-hydroxy α-branched fatty acids) by *Corynebacterium lepus* (Cooper et al., 1979), caprylic acids and its unsaturated analogues by *Pseudomonas* and *Burkholderia* strains (Puntus et al., 2005). Fatty acid biosurfactants were also produced by molds such as spiculisporic acid (4,5-dicarboxy-4-pentadecanolide) by *Penicillium spiculisporum* (Ishigami et al., 1982). However, our report is the first report to show that the yeast *Issatchenkia orientalis* SR4 produced oleic acid as a biosurfactant when grown in the medium with xylene as a carbon source.

Table 1. The minimum active dose (MAD) of oleic acid and synthetic surfactants.

Compound	MAD (µg)
Oleic acid (purified from the strain SR4 culture broth)	0.625
Oleic acid (guaranteed reagent)	0.500
Sodium dodecyl sulfate (SDS)	6.25
Triton X-100	1.25
Triton X-114	1.25

Table 2. Emulsification activity of oleic acid and synthetic surfactants.

Compound	%EA	
	Xylene	*n*-hexadecane
Oleic acid (purified from the strain SR4 culture broth)	25.0	30.0
Oleic acid (guaranteed reagent)	25.0	30.0
Sodium dodecyl sulfate (SDS)	76.9	76.9
Triton X-100	40.5	55.0
Triton X-114	40.5	55.0

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