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ORIGINAL PAPER

Utilization of palm oil decanter cake as a novel substrate for biosurfactant production from a new and promising strain of *Ochrobactrum anthropi* 2/3

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Abstract A biosurfactant-producing bacterium, isolate 2/3, was isolated from mangrove sediment in the south of Thailand. It was evaluated as a potential biosurfactant producer. The highest biosurfactant production (4.52 g/l) was obtained when the cells were grown on a minimal salt medium containing 25 % (v/v) palm oil decanter cake and 1 % (w/v) commercial monosodium glutamate as carbon and nitrogen sources, respectively. After microbial cultivation at 30 °C in an optimized medium for 96 h, the biosurfactant produced was found to reduce the surface tension of pure water to 25.0 mN/m with critical micelle concentrations of 8.0 mg/l. The stability of the biosurfactant at different salinities, pH and temperature and also its emulsifying activity was investigated. It is an effective surfactant at very low concentrations over a wide range of temperatures, pH and salt concentrations. The biosurfactant obtained was confirmed as a glycolipid type biosurfactant by using a biochemical test, fourier-transform infrared spectroscopy, MNR and mass spectrometry. The crude biosurfactant showed a broad spectrum of antimicrobial activity and also had the ability to emulsify oil and enhance polyaromatic hydrocarbons solubility.

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Introduction

Biosurfactants are amphiphilic biological compounds produced by microorganisms that can reduce the interfacial tension between difference fluid phases (Banat et al. 2010; Nawawi et al. 2010; Ramani et al. 2012). These compounds are a group of structurally diverse molecules and most of them are of a lipidic nature—glycolipids, lipoamino acids and lipopeptides (Sanchez et al. 2007). They constitute one of the most important classes of industrial bulk chemicals, with a total world production exceeding more than 13 million tons per year (Levinson 2009). About half of all surfactants are used in household and laundry detergents, and thus inevitably end up in aquatic systems (Tran et al. 2012). In the last few decade biosurfactants have gained increasing attention as environmental-friendly alternatives. This is because of growing environmental awareness, the bio-accumulation and eco-toxicity of synthetic surfactants has become an issue of major concern (Assadi and Tabatabaee 2010; Banat et al. 2010; Fracchia et al. 2012; Makkar and Rockne 2003; Pacwa-Plociniczak et al. 2011).

Biosurfactants are also have several advantages over the synthetic surfactants, such as they possess high superficial activity and effectiveness at extreme temperatures, pH and salinity, besides being biodegradable, less in toxicity and the ability to be synthesized from renewable substrate (Assadi and Tabatabaee 2010; Banat et al. 2010; Saimmai et al. 2012d, e). Despite the advantages of biosurfactants (Assadi and Tabatabaee 2010), they will not replace the synthetic ones unless there is a great improvement in

reducing the costs of the technology for producing biosurfactants. The raw materials such as carbon and nitrogen sources account for 10–30 % of the final product (Rodrigues et al. 2006).

The future promise of these compounds depends particularly on the establishment of fermentation conditions to maximize the yield and productivity of biosurfactants is essential for cost reduction and large-scale production (Reis et al. 2004). Some previous works have demonstrated some peculiarities about biosurfactants production. For instance, reports have appeared dealing with the use of locally available agricultural and food processing residuals for promoting biosurfactants synthesis by different microorganisms (Nawawi et al. 2010; Praveesh et al. 2011; Saimmai et al. 2011, 2012a, b, c, d, e, 2013). Palm oil decanter cake (PODC) (Abubakr et al. 2013) is the solid waste produced by palm oil milling companies after decanting the palm oil mill effluent, while spent bleaching clay is a solid waste from a palm oil refinery. Basically, this waste still contains 30–40 % of oil and this solid waste is currently disposed of directly in landfills without treatment, causing severe water and air pollution problems. A current problem in south of Thailand is to manage the wastes generated during the palm fruit processes. According to Chavalparit et al. (2006) the average waste generation rate per ton of fresh fruit bunches from palm oil mills in Thailand were 140 kg of fiber, 60 kg of shells, 240 kg of empty fruit bunches and 42 kg of PODC. PODC is known to be rich in N, P_2O_5, K_2O, CaO and MgO (Haron et al. 2008), and could further enhance the nutrient value of the growth and production of metabolite by microorganism. The utilization of PODC as a substrate for biosurfactant production will also improve the environment. This is because the disposal of sludge solid waste will increase the biochemical oxygen demand (BOD) of the land. Other than that, solid sludge disposal in landfills is expensive.

The aim of the present study was carried out to explore the feasibility of using PODC as a novel substrate for the production of biosurfactant by *Ochrobactrum anthropi* 2/3 and evaluate this process for prospective applications including antimicrobial activity and enhance solubilization of hydrophobic compounds.

Materials and methods

Biosurfactant producing strain

Ochrobactrum anthropi 2/3 (accession number AB542934) was isolated from mangrove sediment collected from Thungwa sediment, Satun Province in the south of Thailand, during a screening study of biosurfactant-producing bacteria in mangrove sediment (Saimmai et al. 2012e). The entire 16S rRNA gene sequence from *O. anthropi* 2/3 showed 100 % homology to *O. anthropi* (accession number GQ368700). *O. anthropi* 2/3 was maintained on NA plates and transferred monthly.

Media and cultivation conditions

Nutrient broth was used for the preparation of the inoculum. The composition of the nutrient broth used was as follows: beef extract 1.0 g; yeast extract 2.0 g; peptone 5.0 g; and NaCl 5.0 g in a liter of distilled water. To make nutrient agar, 15.0 g of agar was added to the nutrient broth. The culture was grown in this broth for 18–20 h at 30 °C. This was used as inoculum at the 2 % (v/v) level. For the biosurfactant synthesis a mineral salt medium (MSM) with the following composition (g/l) was used: K_2HPO_4, 0.8; KH_2PO_4, 0.2; $CaCl_2$, 0.05; $MgCl_2$, 0.5; $FeCl_2$, 0.01; and NaCl, 5.0 (Saimmai et al. 2012e). The pH of the medium was adjusted to 7.0. Carbon and nitrogen sources were added separately. Cultivation was performed in 250 ml flasks containing 50 ml medium at 30 °C, and shaken in a rotary shaker at 150 rpm for 4 days.

Medium optimization

The optimization of the medium was conducted in a series of experiments changing one variable at a time, and keeping the other factors fixed at a specific set of conditions. Three factors were chosen in aiming to obtain the higher productivity of the biosurfactant: the carbon source (C); the nitrogen source (N); and the C/N ratio. The carbon sources used were 1 % (w/v or v/v) of commercial sugar (CS; saccharose), crude glycerol (CG) a waste from biodiesel production, glucose, molasses, palm oil, PODC, soybean oil, stearic acid, or used lubricating oil (ULO), with $(NH_4)_2SO_4$ as a nitrogen source. To evaluate the most appropriate nitrogen sources for the production of biosurfactant, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, beef extract, yeast extract, peptone and commercial monosodium glutamate (CMSG) were employed at a concentration of 1 % (w/v) with the optimum carbon source. The C/N ratio (with the optimized carbon and nitrogen sources) was varied from 1 to 40 by keeping a constant nitrogen source concentration of 2 g/l.

Recovery of biosurfactant

Culture supernatant was obtained after centrifugation of the culture broth at 12,000 g for 15 min. Four solvent systems that were a mixture of chloroform–methanol (2:1), cold acetone, dichloromethane and ethyl acetate were examined for biosurfactant extraction to be able to employ the best method for efficient biosurfactant extraction (Saimmai

et al. 2012a, 2013). The culture supernatant was extracted three times with the equal volume of the solvent system and evaporated under vacuum. The crude biosurfactants were dissolved in distilled water and measured the biosurfactant activity. The solvent system showing the highest biosurfactant activity was used to recover biosurfactant from *O. anthropi* 2/3.

Study of biosurfactant stability

The effect of NaCl on the biosurfactant activity was also assayed at concentrations of 2–24 % of NaCl. To test the heat stability of the biosurfactant, the solution was heated at 45, 60, 80 and 100 for 1 h 15 min and at 121 °C for 15 min, cooled to room temperature (25 °C) and biosurfactant activity was determined. Biosurfactant activity was also determined after exposure to refrigeration (4 °C) for 1 h. The pH of the biosurfactant was adjusted to 2–12 using NaOH or HCl after which biosurfactant activity was determined.

Identification and properties of the biosurfactant

The carbohydrate content of the biosurfactant was determined by the phenol–sulfuric acid method (Dubois et al. 1956) using D-glucose as a standard. The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The lipid content was estimated by following the procedure of Folch et al. (1956).

The biosurfactant obtained was also evaluated by Fourier-transform infrared spectroscopy (FT-IR) with a Nexus-870 FT-IR spectrometer (Thermo Electron Co., Yokohama, Japan) by the KBr pellet method (Saimmai et al. 2012a). Further evaluation of the biosurfactant was carried out using ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR using $CDCl_3$ with a AMX 300 NMR spectrometer (Bruker, 500 MHz). The final evaluation of the compound was performed by liquid chromatography-mass spectroscopy (LC–MS) with a LCQ^{TM} quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) which utilizes electrospray ionization (Saimmai et al. 2012b).

Application of the biosurfactant in metal removal from aqueous solution

Application of the biosurfactant in metal removal from aqueous solution was performed by method as described by Das et al. (2009). Briefly, 50 ml of biosurfactant solutions at different concentrations i.e. 0.5 × CMC, 1.0 × CMC, 5.0 × CMC and 10 × CMC were incubated overnight with various concentrations of lead and cadmium i.e. 100, 200, 500 and 1,000 ppm in 250 ml Erlenmeyer flask at dark condition 30 °C and shaken (200 rpm). The resulting solution was centrifuged at 10,000 g to separate metal-biosurfactant co-precipitate and the supernatant was kept for atomic absorption spectroscopic quantification of the unbound metals. The analysis of metal concentrations in the supernatant was performed by an atomic absorption spectrophotometer (AAS) according to Das et al. (2009). The AAS utilized was a Model AAnalyst 100 by Perkin Elmer equipped with a flow spoiler (air-acetylene flame, wave length = 217 nm, slit width of 0.73 nm) equipped with a lamp ignited with acetylene. Software used to analyze the data was AAWinlab Analyst. The characteristic wavelengths utilized by AAS for cadmium and lead were 228.8 and 283.3 nm, respectively. The unbound metal concentration was represented as a function of biosurfactant concentration.

Application of the biosurfactant in ULO removal from contaminated sand

The suitability of biosurfactant for enhancing oil recovery was investigated using 1,000.0 g of acid washed sand impregnated with 100.0 ml of ULO. Fractions of 20.0 g of the contaminated sand were transferred to 250 ml flasks which were submitted to the following treatments: the addition of 60.0 ml distilled water (control); the addition of 60.0 ml aqueous solutions of the SDS; using Tween80; and assessing the biosurfactant at the critical micelle concentration (CMC) and above the CMC of each compound. The samples were incubated on a rotary shaker (200 rpm) for 24 h at 30 °C and centrifuged at 5,000 rpm for 20 min for the separation of the laundering solution and the sand. The amount of oil remaining in the sand after the application of the biosurfactant was gravimetrically determined as the amount of material extracted from the sand by hexane (Saimmai et al. 2011). The experiment was carried out at 25, 30, 45 and 60 °C to assess the influence of temperature on biosurfactant-induced oil recovery.

Laboratory experiment on biodegradation of ULO with biosurfactant

An experiment was conducted to study the impact of the biosurfactant derived from *O. anthropi* 2/3 on the biodegradation of ULO in natural seawater. Shake flask biodegradation experiments were carried out in 500 ml Erlenmeyer flasks with 100 ml of sterilized seawater. The experiment was conducted with four different sets: (1) bacterial cells alone; (2) bacterial cells with molasses and cells; (3) bacterial cells with cells and biosurfactant (0.1 %, w/v); and (4) bacterial cells with molasses and biosurfactant. Exactly 2.0 % (w/v) of ULO was added to the sterilized seawater. Inoculation was performed with 24 h old culture at the rate of 1 % (v/v, 10^2–10^3 CFU/ml) concentration. Flasks were

shaken at 150 rpm at 30 °C for a period of 7 days. The biodegradation of ULO was estimated fluorimeterically as described in the Intergovernmental Oceanographic Commission Manuals and Guide No.13 (1982). An uninoculated control was kept to assess the natural weathering of oil and degradation (abiotic control).

PAHs solubilization assay

A polyaromatic hydrocarbons (PAHs) solubilization assay was done as described by Barkay et al. (1999). Any of the following 0.6 µg of PAHs from 0.6 mg/ml stock in acetone, was distributed in a glass test tube (10 mm × 170 mm) and kept open inside an operating chemical fume hood to remove the solvent. Subsequently, 3.0 ml of assay buffer (20 mM Tris–HCl, pH 7.0) was added to the biosurfactant at increasing concentrations (0–50 mg/ml). Assay buffer containing the biosurfactant, but no PAH, was used as a blank. Tubes were capped with plastic closures and incubated overnight at 30 °C and shaken (200 rpm) in the dark. Samples were filtered through 1.2 µm filters (Whatman, Springfield Mill, United Kingdom) and 2.0 ml of this filtrate was extracted with an equal volume of hexane. This emulsion was centrifuged at 9,693 g for 10 min to separate the aqueous and hexane phases. The concentration of PAH was measured spectrophotometrically (Libra S22, Biochrom Ltd., Cambridge, England) at the specific wave lengths of each compounds (Barkay et al. 1999).

Antimicrobial activity of biosurfactant

The biosurfactant extracted was tested for antimicrobial activity using the agar well diffusion method and the area of the zone was measured (Saimmai et al. 2012a, b, 2013). The extracted biosurfactant was tested against pathogenic microorganisms obtained from Songklanakarin Hospital, Prince of Songkla University, Thailand. The extract was weighed and dissolved in distilled water, 10 mg/ml, and the extract was filter-sterilized using a 0.2 µm membrane filter. Each microorganism tested was suspended in Brain Heart Infusion (BHI, Hi-Media Laboratories, Mumbai, India) and diluted to obtain 10^6 CFU/ml. The agar plates were dried for 20 min at 25 °C. The wells were cut from the agar and 50 µl of the biosurfactant solution extracted was added to the wells, and incubated at 37 °C for 24 h and the clear zone was measured.

Analytical methods

The determination of the biomass was done in terms of dry cell weight. At different times of fermentation, samples were mixed in pre-weighted tubes with chilled distilled water and centrifuged at 9,693 g for 30 min. The biomass obtained was dried overnight at 105 °C and weighed.

An emulsification index (E24) was drawn up according to Cooper and Goldenberg (1987). Thus 4 ml of hydrocarbon was added to 4 ml of aqueous solution of culture supernatant in a screw cap tube, and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h, and the E24 was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

The surface tension was measured using a Model 20 Tensiometer (Fisher Science Instrument Co., PA, USA) at 25 °C. The CMC was determined by plotting the surface tension versus the concentration of biosurfactant in the solution.

The chemical characteristics of the biosurfactant were assessed by thin layer chromatography (TLC). The components of the chloroform–methanol extract were separated on silica gel 60 plates (Merck, Darmstadt, Germany) using $CHCl_3$:CH_3OH:H_2O (65:15:1) as the solvent system. Spots were revealed by spraying with: a) distilled water for detection of hydrophilic compounds; b) ninhydrin 0.05 % (w/v, in methanol/water, 1:1 v/v) for detection of compounds with free amino groups; c) and anisaldehyde for detection of the sugar moieties (Das et al. 2009). Treatments a, b and c were visualized after heating at 110 °C for 5 min. To detect the presence of lipids, the TLC plate was visualized under ultraviolet light after spraying with rhodamine B 0.25 % (w/v, in absolute ethanol).

All experiments were carried out in triplicate for the calculation of the mean value. Two well-defined synthetic surfactants, SDS and Tween80, were used as positive controls. Distilled water and an MSM medium were used as negative controls. All the chemicals used were of analytical grade. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 10.0, for Windows Inc., Chicago, IL).

Results and discussion

Effect of carbon source on growth and biosurfactant production by *O. anthropi* 2/3

The effect of different carbon sources, including water soluble and water immiscible substrates, on growth and biosurfactant production by the *O. anthropi* 2/3 was examined. The strain was grown in an MSM medium containing 1 % of carbon source for 48 h. The dry cell weight, surface tension reduction, biosurfactant yield and E24 toward kerosene and xylene were then determined. The strain was able to grow and produce biosurfactant

Table 1 Effect of carbon (C) concentrations, nitrogen (N) source and C: N ratio on growth and biosurfactant production by *O. anthropi* 2/3, cultivated in 250 ml flask containing 50 ml MSM at 30 °C in a shaking incubator at 150 rpm for 4 days

Parameters	Dry cell weight (g/l)*	Surface tension (mN/m)*	Biosurfactant (g/l)*	Emulsification index (E24, %)*	
Carbon source (1 g/l) [N-source, 1 g/l [$(NH_4)_2SO_4$)]				Kerosene	Xylene
No carbon source	0.17 ± 0.08^{h}	0^{h}	0^{i}	0^{g}	0^{h}
Commercial sugar	2.15 ± 0.25^{e}	13.1 ± 1.4^{c}	0.38 ± 0.12^{c}	49.76 ± 3.14^{b}	36.38 ± 5.01^{e}
Glucose	3.12 ± 0.09^{a}	16.1 ± 2.7^{b}	0.49 ± 0.20^{b}	50.63 ± 4.24^{b}	46.81 ± 5.45^{b}
Crude glycerol	1.04 ± 0.71^{g}	5.0 ± 0.3^{f}	0.10 ± 0.21^{g}	23.24 ± 2.88^{e}	20.41 ± 3.52^{f}
Molasses	2.04 ± 1.62^{f}	11.6 ± 0.6^{d}	0.34 ± 0.20^{cd}	46.61 ± 2.53^{c}	42.81 ± 0.45^{c}
Used lubricating oil	1.01 ± 0.16^{g}	5.3 ± 0.5^{f}	0.15 ± 0.17^{f}	25.16 ± 2.91^{e}	20.15 ± 4.33^{f}
Palm oil	2.49 ± 0.21^{d}	9.2 ± 1.5^{e}	0.32 ± 0.81^{de}	44.02 ± 1.65^{d}	39.52 ± 6.62^{d}
Soybean oil	2.01 ± 0.18^{f}	8.3 ± 0.8^{e}	0.30 ± 0.24^{e}	45.86 ± 2.53^{cd}	41.89 ± 5.33^{c}
Palm oil decanter cake	2.78 ± 0.35^{c}	18.2 ± 0.2^{a}	0.51 ± 0.09^{a}	56.32 ± 5.23^{a}	51.13 ± 6.16^{a}
Stearic acid	2.92 ± 0.82^{b}	3.3 ± 0.6^{g}	0.05 ± 0.13^{h}	9.83 ± 1.52^{f}	5.46 ± 1.28^{g}
Nitrogen sources, 1 g/l (C-source, 1 g/l palm oil decanter cake)					
No nitrogen source	0.51 ± 0.13^{h}	0^{g}	0^{g}	0^{g}	0^{h}
$(NH_4)_2SO_4$	2.78 ± 0.35^{g}	18.2 ± 0.2^{c}	0.51 ± 0.09^{b}	56.32 ± 5.23^{b}	51.13 ± 6.16^{b}
$NaNO_3$	3.82 ± 0.81^{d}	20.3 ± 2.3^{b}	0.45 ± 0.11^{c}	45.30 ± 5.20^{c}	37.05 ± 4.20^{d}
NH_4Cl	3.13 ± 1.22^{f}	12.0 ± 2.2^{e}	0.34 ± 0.03^{e}	39.15 ± 3.43^{e}	33.17 ± 4.54^{f}
NH_4NO_3	3.58 ± 1.80^{e}	19.1 ± 3.7^{c}	0.46 ± 0.84^{c}	46.57 ± 2.03^{c}	38.12 ± 3.59^{d}
Beef extract	4.92 ± 1.51^{a}	9.5 ± 2.6^{f}	0.25 ± 0.11^{f}	34.65 ± 2.54^{f}	30.72 ± 4.78^{g}
CMSG	3.18 ± 1.01^{f}	25.7 ± 1.8^{a}	2.03 ± 0.19^{a}	62.53 ± 2.51^{a}	57.42 ± 4.82^{a}
Peptone	4.47 ± 1.34^{c}	16.3 ± 2.3^{d}	0.39 ± 1.10^{d}	45.87 ± 3.58^{c}	41.86 ± 4.32^{c}
Yeast extract	4.62 ± 0.71^{b}	12.0 ± 2.0^{e}	0.35 ± 0.23^{e}	40.15 ± 2.41^{d}	35.01 ± 4.07^{e}
C: N ratio					
1	3.18 ± 1.01^{g}	25.7 ± 1.8^{h}	2.03 ± 0.19^{h}	62.53 ± 2.51^{c}	57.42 ± 4.82^{c}
5	3.63 ± 0.21^{h}	28.5 ± 3.0^{g}	3.04 ± 0.66^{g}	63.52 ± 2.07^{c}	58.16 ± 3.06^{c}
10	4.28 ± 1.35^{g}	30.9 ± 1.7^{f}	3.62 ± 0.93^{f}	63.52 ± 4.68^{c}	60.49 ± 2.81^{b}
15	5.37 ± 1.03^{f}	35.6 ± 2.7^{d}	4.01 ± 0.39^{d}	65.17 ± 5.04^{b}	61.60 ± 5.08^{b}
20	6.02 ± 1.42^{e}	39.5 ± 4.9^{c}	4.31 ± 0.12^{c}	68.54 ± 5.61^{a}	64.40 ± 5.52^{a}
25	6.59 ± 0.43^{d}	44.5 ± 2.6^{a}	4.52 ± 0.15^{b}	70.68 ± 4.52^{a}	65.24 ± 4.08^{a}
30	7.15 ± 1.38^{c}	41.7 ± 0.8^{b}	4.50 ± 0.02^{a}	69.52 ± 5.53^{a}	64.90 ± 2.32^{a}
35	7.95 ± 1.52^{b}	32.7 ± 3.2^{e}	3.95 ± 0.80^{e}	66.48 ± 3.22^{b}	62.28 ± 3.31^{b}
40	8.45 ± 1.13^{a}	27.0 ± 5.1^{g}	3.00 ± 0.15^{g}	60.61 ± 5.32^{d}	57.08 ± 3.00^{c}

Different superscript letters in the same column indicate significant differences ($p < 0.05$)

* All values are mean ± SD from triplicate determinations

using different types of carbon sources including sucrose and hydrocarbons, which indicates that various industrial wastes may be used as substrate for biosurfactant production. The highest biomass production, as dry cell weight, was obtained when the strain grown on glucose as a sole carbon source was used. The maximum biosurfactant activity (18.2 mN/m, 56.32 and 51.3 % for surface tension reduction, E24 toward kerosene and xylene, respectively) and biosurfactant yield (0.51 g/l) were obtained in the culture grown on PODC (Table 1).

From these results we can conclude that the optimum carbon source for *O. anthropi* 2/3 growth is different from the best carbon source for biosurfactant production. These findings are in accord with those of Wu et al. (2008) who observed that *Pseudomonas aeruginosa* EM1 isolated from an oil-contaminated site show the highest bacterial cell growth when glucose was used as the carbon source. However, the highest yield of biosurfactant production was obtained when glycerol was used as the carbon source. Shavandi et al. (2011) have also shown that the initiation of glycolipid production by *Rhodococcus* sp. TA6 is induced by the presence of poorly soluble hydrophobic substrates. The surface tension of the cultures grown on kerosene (29 mN/m) and paraffin (30 mN/m) was comparable. On the other hand, the higher biomass was found in the culture grown on sucrose.

Effect of nitrogen source on growth and biosurfactant production by *O. anthropi* 2/3

The type of nitrogen sources was strongly affected in biosurfactant production by *O. anthropi* 2/3 (Table 1). *O. anthropi* 2/3 was able to use nitrogen sources such as ammonia and urea for biosurfactant production. However, in order to obtain high biosurfactant activity it is necessary to set restrained conditions for these macro-nutrients. CMSG was the best source of nitrogen for growth and biosurfactant synthesis. Ammonium salts in the form of ammonium chloride were used for growth but not for biosurfactant production and caused a significant decrease in pH (4.03) (Prieto et al. 2008). The maximum E24 (62 %) and surface tension reduction (25.7 mN/m) were obtained in media with CMSG. No significant change in pH was observed in this case. A similar result was reported in biosurfactant derived from *Leucobacter komagatae* 183 by Saimmai et al. (2012c).

Effect of carbon to nitrogen (C/N) ratio on biosurfactant production by *O. anthropi* 2/3

The C/N ratio was also known as a vital factor influencing performance in biosurfactant production (Saimmai et al. 2012a, b, c, 2013). The effect of the C/N ratio on biosurfactant production was therefore investigated using PODC and CMSG as a carbon and nitrogen sources, respectively. As indicated in Table 1, the best biosurfactant yield (4.52 g/l) was obtained at a C/N ratio of 25. However, the biosurfactant yield tended to decrease as the C/N ratio increased from 30 to 50, especially for a C/N ratio higher than 45. Saimmai et al. (2012a, b, c, 2013) observed that biosurfactant production is more efficient under nitrogen-limited conditions. The results show that a possible inhibitory effect on the bacterial metabolism may occur due to a possible nutrient transport deficiency. That is, nitrate first undergoes dissimilatory nitrate reduction to ammonium and is then assimilated by glutamine–glutamate metabolism. It is likely that the assimilation of the nitrate as the nitrogen source is very low, leading to a simulated condition limiting the nitrogen (Barber and Stuckey 2000). *O. anthropi* 2/3 is able to use nitrogen sources such as ammonia or nitrate. However, to obtain a high concentration of biosurfactant, restrained conditions for this macro-nutrient is preferable.

Kinetics of biosurfactant production

A time course study of biosurfactant production by *O. anthropi* 2/3 was carried out using a production medium with 25 % PODC and 1 % CMSG as a carbon and nitrogen source respectively at 30 °C, 200 rpm. Bacterial growth (DCW), E24 (%) against xylene, surface tension, biosurfactant concentration (g/l) and the pH of the culture broth were monitored during the incubation time (Fig. 1). The bacterial growth was quite fast and a maximum dried cell mass of about 6 g/l was obtained after 90 h of cultivation; this then decreased during the remaining time of the incubation. Biosurfactant production started after 18 h of incubation (0.2 g/l) and progressively increased up to 4.5 g/l after 69 h (Fig. 1). The cell growth was gradually increased at 18 h of incubation, biosurfactant production continued and reached 4.52 g/l at 96 h of cultivation.

According to the data presented in Fig. 1, the production of *O. anthropi* 2/3 biosurfactant is a dynamic phenomenon and it is produced via an overflow metabolism, where the production continues after the stationary phase has been reached. A two stage production of biosurfactant has previously been reported (Abbasi et al. 2012; Saimmai et al. 2012a, b, c, 2013). The pH of culture medium was not changed significantly and it was around 7.0 during the incubation time. Its variation was from 6.7 to 7.4 (data not shown). E24 of *O. anthropi* 2/3 at the end of the incubation time was 70 %. Our results are consistent with previous reports that biosurfactants are secondary metabolites secreted by *subtilis* MUV4 (Suwansukho et al. 2008), *Oleomonas sagaranensis* AT18 (Saimmai et al. 2012b), *L. komagatae* 183 (Saimmai et al. 2012c), and *Selenomonas ruminantium* CT2 (Saimmai et al. 2013). On the other hand, this is in contrast with the finding of de Lima et al. (2009) who reported that biosurfactant production by *P. aeruginosa* PACL produced a biosurfactant that was a primary metabolite when it was grown on residual soybean oil.

Recovery of biosurfactant

Crude extract of the biosurfactant was recovered from the culture supernatant of *O. anthropi* 2/3 by extraction with several organic solvents. Among four solvent systems, ethyl acetate extraction was the most efficient in biosurfactant recovery from culture supernatant of this strain (data not shown). A recovery yield of 4.52 g/l was obtained from *O. anthropi* 2/3 after 96 h of cultivation. It was also reported previously that the extraction of bioproducts with considerably high polarity by ethyl acetate solvent is quite efficient (Saimmai et al. 2013). Because the recovery and concentration of biosurfactants from fermentation broth largely determines the production costs, ethyl acetate is a better choice than the highly toxic chloro-organic compounds such as chloroform–methanol or dichloromethane solvent systems.

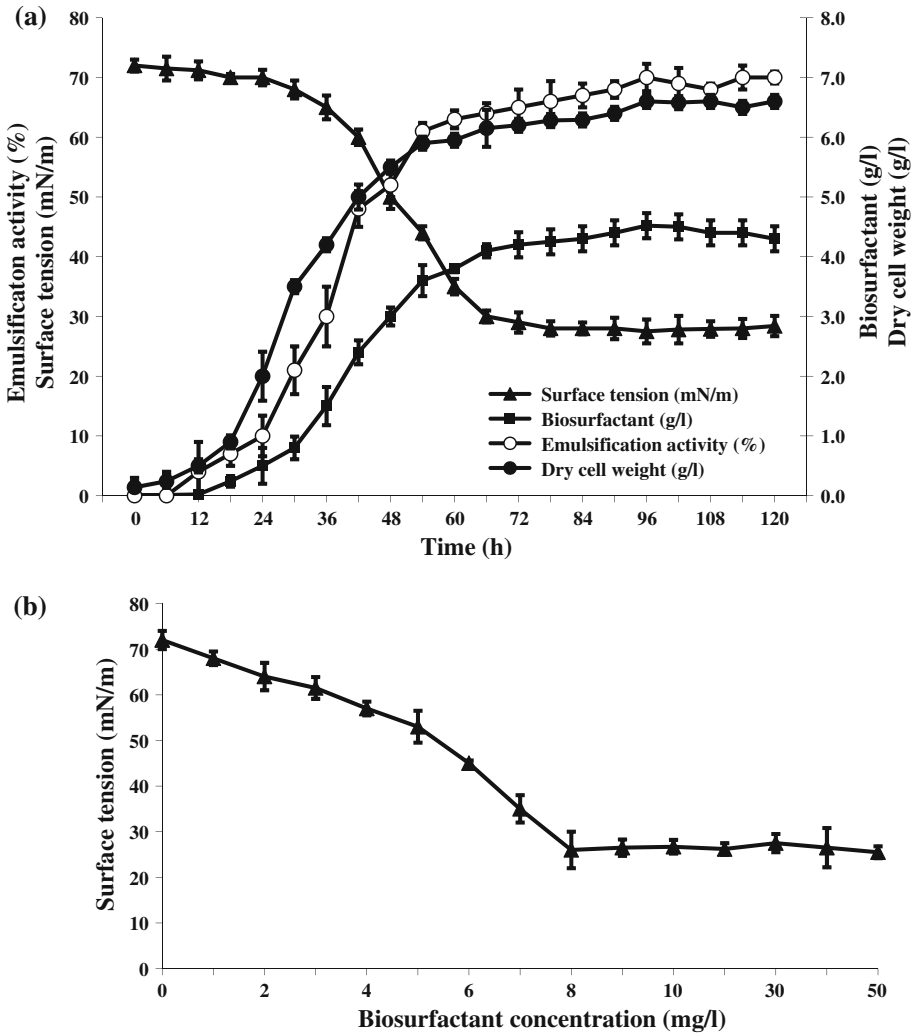

Fig. 1 Time course of growth and biosurfactant production in optimal medium (**a**) and surface tension *versus* biosurfactant concentration of biosurfactant produced (**b**) by *O. anthropi* 2/3

Surface tension and critical micelle concentration (CMC)

The relationship between surface tension and concentration of the extracted biosurfactant solution was determined by a du Nouy'a ring tensiometer (Fig. 1b). The biosurfactant produced exhibited excellent surface tension reduction activity. The surface tension of water (72.2 mN/m) decreased to 25.0 mN/m by increasing the concentration of the solution up to 8.0 mg/l. Further increases in the concentration of the biosurfactant solution did not reduce the surface tension of water, indicating that the CMC had been reached at this concentration. The biosurfactant from *O. anthropi* 2/3 showed a lower minimum surface tension and CMC value than those of the biosurfactant from *Lactobacillus paracasei* A20 (41.8 mN/m, 2.5 mg/ml) (Gudina et al. 2010), *P. aeruginosa* MA01 (32.5 mN/m, 10.1 mg/l) (Abbasi et al. 2012), *P. gessardii* (31.0 mN/m, 65.4 mg/l) (Ramani et al. 2012), *S. ruminantium* CT2 (25.5 mN/m, 8.0 mg/l) (Saimmai et al. 2013), and *L. komagatae* 183 (26.5 mN/m, 9.0 mg/l) (Saimmai et al. 2012c).

Effect of temperature, pH and salinity on biosurfactant stability

Heating of the biosurfactant solution up to 100 °C (or autoclaving it at 121 °C) caused no effect on the biosurfactant's performance and its E24 (data not shown). The surface tension reduction and E24 were relatively stable at the temperatures used. The activity of the biosurfactant solution and its E24 were also affected by the pH (data not shown). When the pH was acidic and set to 2.0, 3.0 and 4.0 the biosurfactant activities were 70, 67 and 66 mN/m, respectively. Correspondingly, the emulsification ability of biosurfactant was limited to an acid to neutral pH and E24 up to 15, 49 and 59 %, respectively was obtained. The

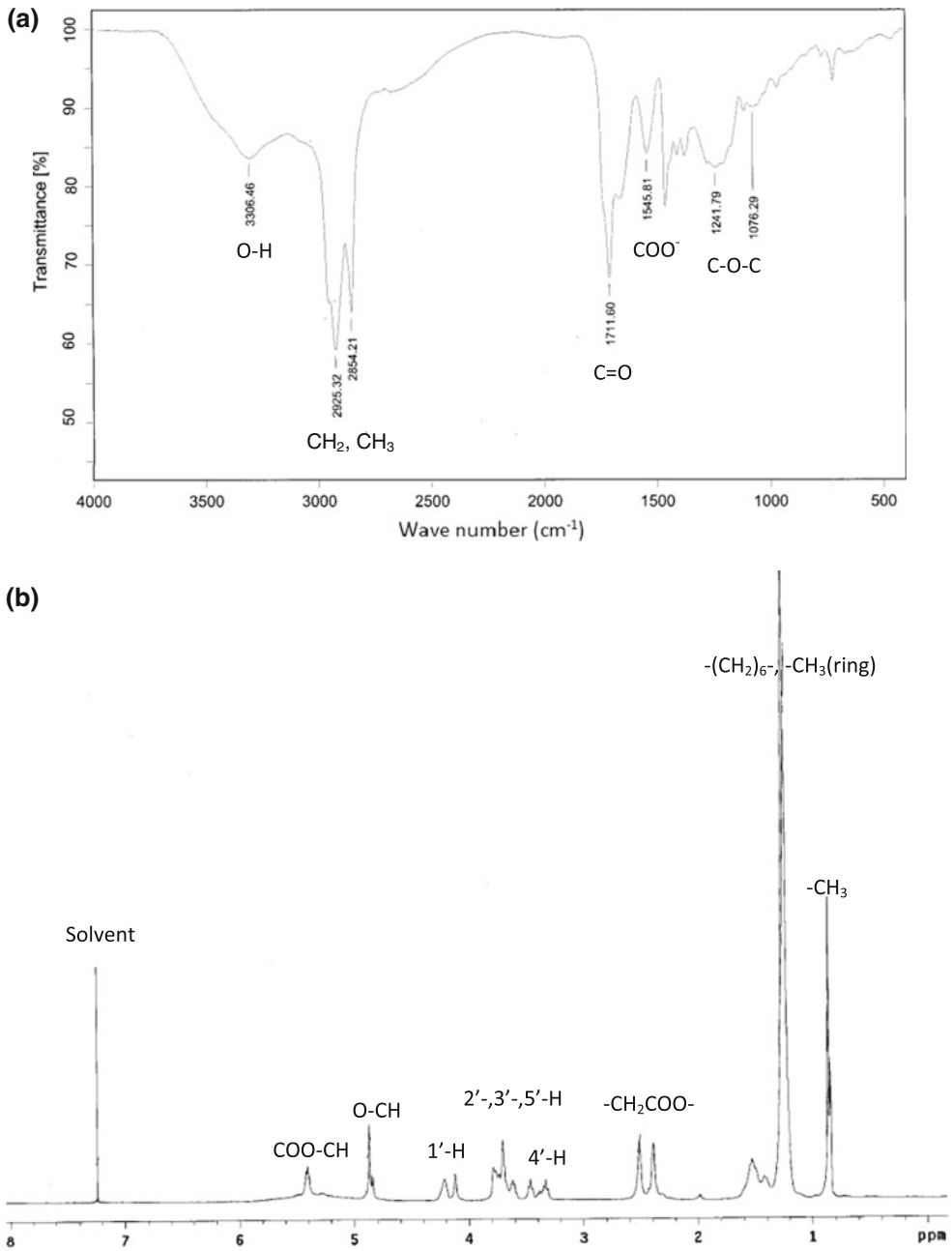

Fig. 2 Fourier transform infrared spectrum (**a**) and ^{1}H-nuclear magnetic resonance spectrum (**b**) of biosurfactant produced by *O. anthropi* 2/3

results showed that negligible changes were observed in the biosurfactant activity with an increase in the NaCl concentration up to 18 %. Likewise, an increase in the NaCl concentration up to 16 % did not cause a significant effect on E24 (data not shown).

Chemical properties of the biosurfactant

The chemical nature of the biosurfactant from *O. anthropi* 2/3 was seen as a single spot on TLC. This fraction showed a positive reaction with anisaldehyde and rhodamine B reagent, indicating the presence of sugar and lipid moieties in the molecule (data not shown). The biochemical composition of the biosurfactant revealed that it is a mixture of carbohydrate and lipid in a combination of 65:28 %, respectively. These results indicated the existence of a glycolipid biosurfactant. Infrared analysis of the *O. anthropi* 2/3 biosurfactant revealed a pattern similar to that of monorhamnolipid, indicating the presence of a rhamnose component, as can be seen in Fig. 2a. Based on the information of the band characteristics, the glycolipid nature of this compound was confirmed: wave number 3,306 cm^{-1}

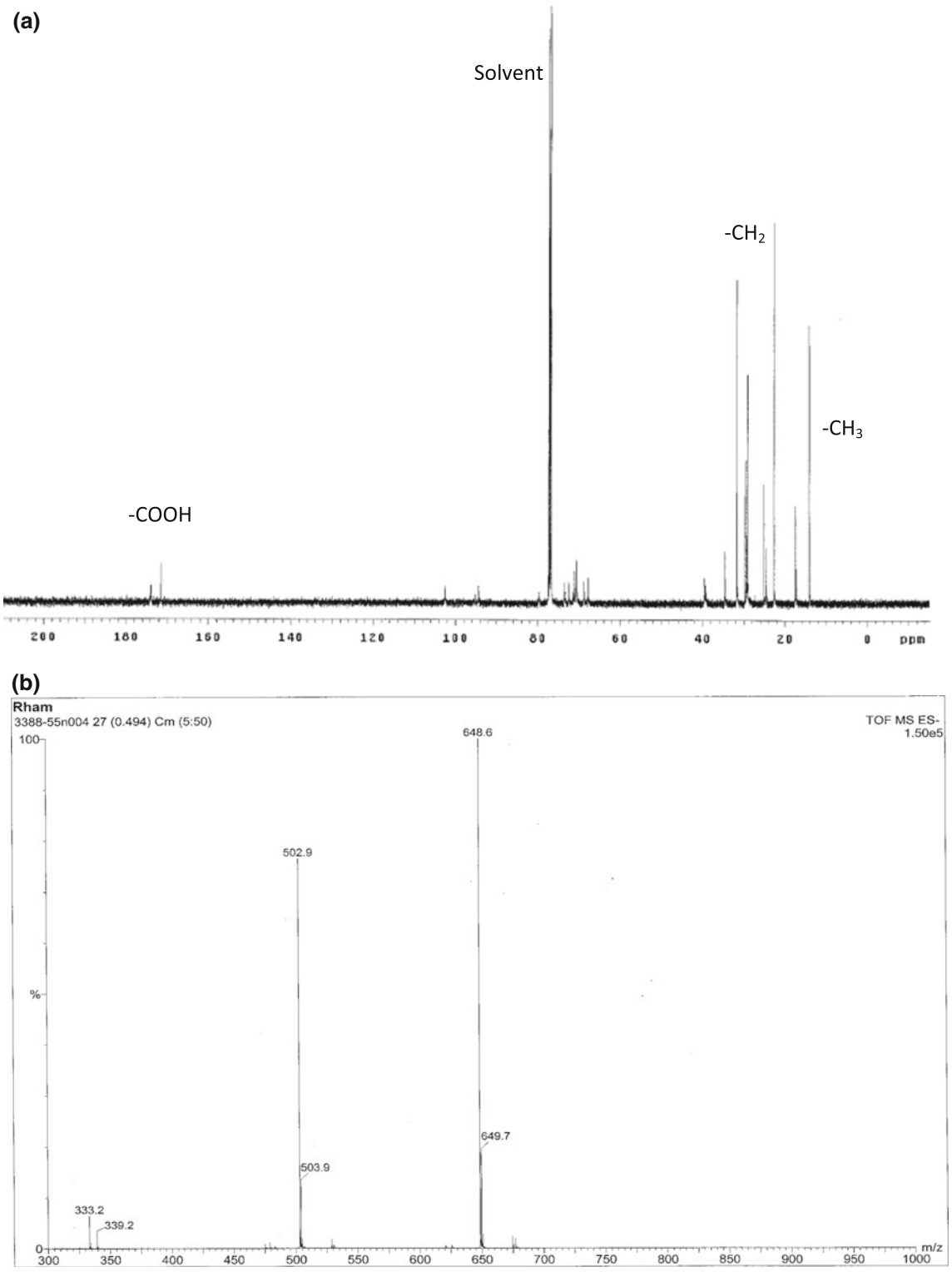

Fig. 3 ^{13}C-nuclear magnetic resonance spectrum (**a**) and mass spectrum (**b**) of the biosurfactant produced by *O. anthropi* 2/3

shows the O–H stretching (free hydroxyl groups of rhamnose rings), the stretching bands of the methylene and terminal methyl groups of the acyl chains between 2,854 and 2,925 cm^{-1}, the stretching band of the ester C=O groups at approximately 1,711 cm^{-1}, the free $–COO^-$ band (free carboxyl group of the second fatty acid) around 15.645 cm^{-1}, and the C–O–C vibrations (rhamnose rings) between 1,076 and 1,241 cm^{-1}. This analysis confirmed

the glycolipid nature of our biosurfactant which could, in fact, correspond to rhamnolipids (Abbasi et al. 2012).

For further confirming the results of this study, an NMR analysis was performed. The characteristic chemical shifts observed from ^{1}H-NMR analysis were 0.855 ppm (for-CH_3), 1.250 ppm (for $-(CH_2)_6$) or CH_3 (ring), 2.512 ppm (for $-CH_2-COO-$), 4.851 ppm (for –O–CH–), and 5.405 ppm (for–COO–CH–) (Fig. 2b). The ^{13}C NMR also displayed lipid signals present in the rhamnolipid consisting of CH_3 at $\delta = 14.1$ ppm, CH_2 from $\delta = 22.6$ to 34.5 ppm, and ester and carboxylic groups signals at $\delta = 171.4$ and $\delta = 173.8$ ppm (Fig. 3a). All these results indicate the molecular structure of L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL1) and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL2), which are common rhamnolipids types produced by many strains of *P. aeruginosa* (Wu et al. 2008). In addition, mass spectrometry analysis of the biosurfactant obtained shows major signals at m/z 502 and 648 (Fig. 3b). This corresponds to the mono-rhamno-di-lipidic congeners (Rha–C_{10}–C_1) and di-rhamno-di-lipidic congeners (Rha–Rha–C_8–$C_{12:1}$) reported by Abdel-Mawgoud et al. (2010). Moreover, based on the best of our knowledge, this is the first report of the production of rhamnolipid from genus *Ochrobactrum*.

Application of the biosurfactant in metal removal from an aqueous solution

The biosurfactant-mediated metal removal was assessed through study using AAS. The AAS results showed that the metal content in the solution, obtained after biosurfactant treatment, was significantly lower compared to the starting concentration of the metal as the metal co-precipitated with the biosurfactant. The percentage removal for both the metals varied with the different concentrations of metals and biosurfactant. As a general trend, higher concentrations of biosurfactant removed metals in greater amounts. The biosurfactant-mediated remediation was also evident at concentrations below the CMC value. The percentage removal at 0.5 × CMC was 22.54, 18.84, 15.37, 13.21 and 35.40, 30.28, 27.98, 22.07 for 100, 200, 500 and 1,000 ppm of cadmium and lead, respectively. In addition, the percentage removal was increased to 73.25, 68.75, 61.69, 58.58 and 95.68, 88.37, 80.50, 78.25 for cadmium and lead, respectively, at 10 × CMC of biosurfactant (Fig. 4a, b). This is in accord with reports in which biosurfactant below CMC were found to be less efficient in metal remediation (Mulligan et al. 2001). At a higher concentration of biosurfactant (10 × CMC) almost complete removal of the metals was obtained. This trend of enhanced metal remediation capacity with increasing amounts of biosurfactant had also been reported earlier. However, the concentration

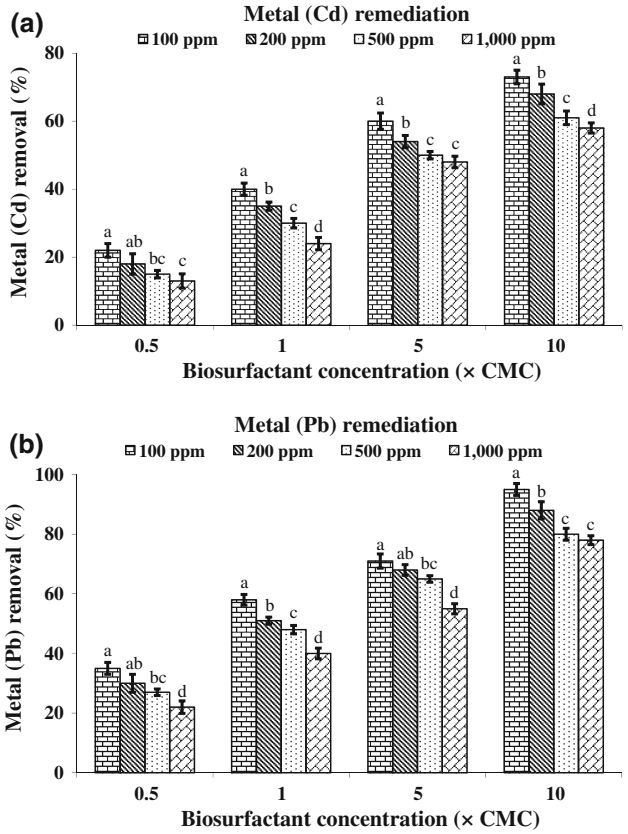

Fig. 4 Percentage removal of cadmium (**a**) and lead (**b**) by different concentrations of biosurfactant produced by *O. anthropi* 2/3

of biosurfactant used for remediation was higher (Kim and Vipulanandan 2006) as compared to that used in the present study.

Application of the biosurfactant in motor oil removal from contaminated sand

The capacity of aqueous biosurfactant and synthetic surfactant (Tween80 and SDS) solutions to remove oil from contaminated sand at different temperatures was investigated (Fig. 5a). The biosurfactant of *O. anthropi* 2/3 and Tween80 could recover 22–25 % of motor oil from contaminated sand at 25 °C: 39–42 % at 30 °C: 54–55 % at 45 °C and 65–68 % at 60 °C. The synthetic surfactant SDS was found to be less efficient. In the case of the control (distilled water), very little recovery (7–22 %) could be obtained in the temperature range used (Fig. 5a). The pattern of oil removal from sand by biosurfactant was similar to Tween80. The reason for this result could come from the characteristics of nonionic surfactants. These results indicated the superior performance of biosurfactant over the synthetic surfactant SDS in terms of the mobilization of oil pollutants from the contaminated soil.

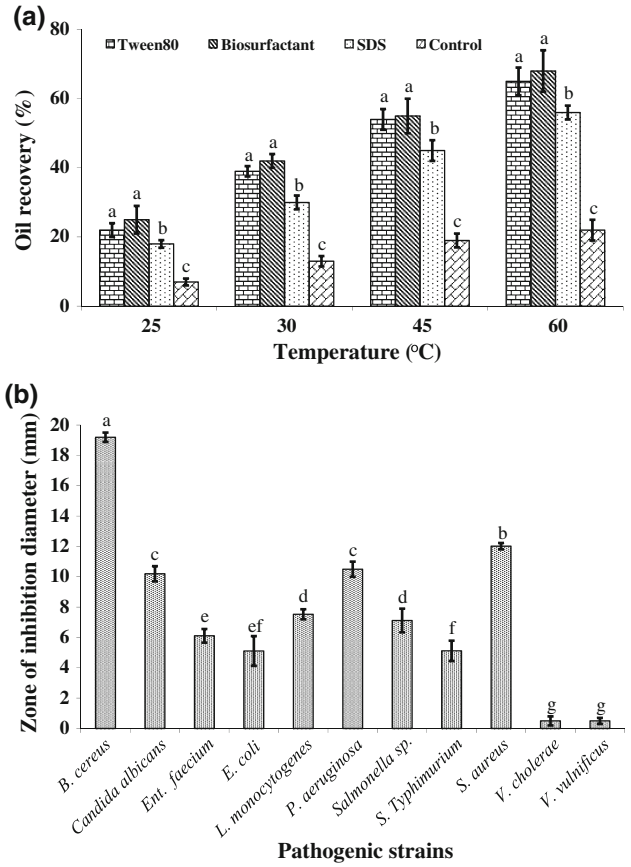

Fig. 5 Microbial enhanced oil removal (**a**) and antimicrobial activity (**b**) of the biosurfactant produced by *O. anthropi* 2/3

Laboratory experiment on biodegradation of ULO with biosurfactant

The biodegradation of ULO in the laboratory scale experiment suggested that maximum biodegradation was found with biosurfactant and molasses-added mix (70.2 %) followed by biosurfactant (64.9 %), molasses (41.8 %) and 28.5 % in a control mix. The improved biodegradation levels obtained with biosurfactant indicated that it is the most efficient accelerators for hydrocarbon biodegradation through improved oil-bacteria accessibility (Maneerat 2009). The addition of nutrients, such as molasses, in combination with biosurfactant could enhance the hydrocarbon degradation rate through stimulating the growth rate of microorganisms (Saimmai et al. 2012f).

Effect of biosurfactant on PAH solubilization

Table 2 shows the apparent aqueous solubility of PAHs in the presence of biosurfactants. It is clear that the apparent solubility increased linearly with increased biosurfactant concentration. The solubility of fluorine, naphthalene or phenanthrene was found to improve by about 2–3 times in the presence of 30 mg/ml of biosurfactant. However, the solubility of anthracene, fluoranthene and pyrene was found to improve by about 18–20 times at the same biosurfactant concentration. The data presented suggests that interactions with hydrophobic regions in biosurfactants are the most plausible explanation for the mechanism whereby biosurfactants solubilize compounds with limited aqueous solubility. The mechanisms proposed for the enhancement of the aqueous solubility of hydrophobic substances by surfactants include solubilization in the hydrophobic core of the multimolecular surfactant structures formed at above-aggregation concentrations, such as in micelles (Assadi and Tabatabaee 2010) and liposomes (Miller and Bartha 1989). There is decreased surface tension of the solvent and interaction with hydrophobic tails of surfactant monomers (Barkay et al. 1999).

Antimicrobial activity of biosurfactant

The results of the antimicrobial activity of the biosurfactant from *O. anthropi* 2/3 against the various Gram-positive, Gram-negative bacteria and yeasts had been shown in Fig. 5b. A maximum clear zone diameter of 19.2 mm was observed against *Bacillus cereus* and for the other Gram-positive bacteria the diameter was 14–17 mm. The diameter of 8–12 mm appeared for the Gram-negative bacteria. This compound exhibited higher activity against the Gram-positive bacteria when compared with its action over the Gram-negative bacteria. Similar results were also reported by Costa et al. (2010) for antimicrobial activity of the rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* L2-1 against several pathogens.

Conclusions

This present study reports the production of the biosurfactant from *O. anthropi* 2/3 which was isolated from mangrove sediment. The growth characteristics were identified and the study of the properties of the biosurfactant indicate the possibility of its industrial application. The spectra obtained from FT-IR spectroscopy, NMR, and LC–MS confirmed the presence of lipopeptides in the sample. The potential of this biosurfactant for industrial use was shown by studying its physical properties, such as the surface tension, critical micelle concentration and E24, and its stability in relation to environmental stresses such as salinity, pH and temperature. The surface tension of an aqueous solution of this biosurfactant at a CMC value of 8.0 mg/l, reached 25.0 mN/m. These values are very low compared with other surfactants. The properties of the biosurfactant obtained have potential application, especially for microbial enhanced oil recovery and/or reducing the intensity of environmental contamination. Finally, the

Table 2 Dose dependent solubilization of PAHs by crude biosurfactant isolated from *O. anthropi* 2/3

Concentration of biosurfactant (mg/ml)	Solubility of PAHs* (mg/l)					
	Anthracene	Fluoranthene	Fluorene	Naphtalene	Phenantrene	Pyrene
0	0.07 ± 0.01^{g}	0.25 ± 0.03^{f}	1.98 ± 0.21^{g}	30.31 ± 1.20^{g}	1.40 ± 0.17^{g}	0.15 ± 0.02^{g}
5	0.21 ± 0.2^{f}	0.87 ± 0.07^{e}	2.07 ± 0.09^{f}	39.0 ± 3.09^{f}	1.69 ± 0.12^{f}	0.82 ± 0.10^{f}
10	$0.48 \pm .01^{e}$	1.69 ± 0.14^{d}	2.91 ± 0.42^{e}	46.3 ± 2.54^{e}	1.92 ± 0.14^{e}	1.37 ± 0.09^{e}
15	0.80 ± 0.05^{d}	2.77 ± 0.15^{c}	3.88 ± 0.32^{d}	51.87 ± 4.22^{d}	2.56 ± 0.21^{d}	1.69 ± 0.12^{d}
20	0.97 ± 0.06^{c}	3.20 ± 0.21^{b}	4.51 ± 0.14^{c}	57.50 ± 2.73^{c}	2.90 ± 0.34^{c}	2.08 ± 0.07^{c}
25	1.18 ± 0.07^{b}	4.69 ± 0.11^{a}	5.32 ± 0.12^{b}	61.78 ± 3.57^{b}	3.40 ± 0.21^{b}	2.60 ± 0.21^{b}
30	1.48 ± 0.04^{a}	4.90 ± 0.35^{a}	6.12 ± 0.28^{a}	72.45 ± 6.51^{a}	3.68 ± 0.31^{a}	2.98 ± 0.45^{a}

Different superscript letters in the same column indicate significant differences ($p < 0.05$)

*Values are given as mean ± SD from triplicate determinations

biosurfactant is a suitable alternative to synthetic medicines and antimicrobial agents and may be used as a safe and effective therapeutic agent.

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