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Original Article

Characterization and phylogenetic analysis of biosurfactant-producing bacteria isolated from palm oil contaminated soils

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Abstract

Biosurfactant-producing bacteria were isolated from 89 different soil samples contaminated with palm oil in 35 palm oil industry sites in the south of Thailand. The phylogenetic diversity of the isolates was evaluated by 16S rRNA gene analysis. Among 1,324 colonies obtained, 134 isolates released extracellular biosurfactant when grown on low-cost substrates by a drop collapsing test. Among these, the 53 isolates that showed the highest biosurfactant production on different substrates were found to belong to 42 different bacterial genera. Among these sixteen (*Caryophanon; Castellaniella; Filibacter; Geminicoccus; Georgenia; Luteimonas; Mesorhizobium; Mucilaginibacter; Nubsella; Paracoccus; Pedobacter; Psychrobacter; Rahnella; Sphingobium; Sphingopyxis* and *Sporosarcina*) were first reported as biosurfactant-producing strains. By using low-cost, agro-industrial by-products or wastes, *Azorhizobium doebereinerae* AS54 and *Geminicoccus roseus* AS73 produced extracellular biosurfactant, which exhibited the lowest surface tension reduction (25.5 mN/m) and highest emulsification activity (69.0%) when palm oil decanter cake and used palm oil was used as a carbon sources, respectively. Overall, this is the first study of a phylogenetic analysis of biosurfactant-producing bacteria from palm oil refinery industry site and their ability to produce biosurfactant on renewable substrates.

Keywords: characterization, biosurfactant, palm oil contaminated soils, surface tension, renewable substrate

1.Introduction

Oil palm is an important crop in the south of Thailand, approximately 1.7 million tons of crude palm oil was produced in the year 2008 that amounted to USD 34 million (Subkaree, 2008). The process to extract the oil generates a wide variety of wastes in large quantities especially residual oil that can contaminate soil and water (Singh *et al.,* 2011). Some microorganisms living in soil and water and producing biosurfactants for survival can adsorb, emulsify, wet, and disperse or solubilize the oil residue (Nerurkar *et al.,* 2009). The presence of biosurfactants can also increase the solubility of oil and hence potentially increase their bioavailability to be used as carbon and energy sources (Mulligan, 2009). Biosurfactants are amphiphilic compounds containing both hydrophilic and hydrophobic moieties in the molecules that produced by

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several microorganisms. These compounds can reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluids such as oil and water (Nayak *et al.,* 2009; Makkar *et al.,* 2011). Biosurfactants have gained attention because they exhibit some advantages such as biodegradability, low toxicity, ecological acceptability, and ability to be produced from renewable cheaper substrates, the possibility of their production through fermentation and specific activity at extreme temperature, pH level and salinity (Pansiripat *et al.,* 2010). Microbial surfactants exhibit high specificity and are consequently suited to new applications as evidenced by numerous reports published during the last decade on the application of biosurfactants in various industrial sectors (Perfumo *et al.,* 2010) and in environment protection (Das and Mukherjee, 2007).

Despite the advantages, fermentation must be competitive in terms of cost with chemical synthesis, as many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically or not. The choice of inexpensive and renewable substrates is important to the overall economy of the process because they account for 30-50% of the final product cost and also reduce the expenses of waste treatment (Bento *et al.,* 2005). In addition, only a few studies have been concerned with the diversity of biosurfactant-producing microorganisms (Saimmai *et al.,* 2012a,b). Accordingly, the aims of this study were to isolate and characterize of novel biosurfactant-producing bacteria isolated from palm oil contaminated soils in palm oil industry. In addition, low-cost substrates for biosurfactant production were evaluated.

2.Materials and Methods

2.1 Isolation of biosurfactant-producing bacteria

Biosurfactants-producing bacteria were isolated from soil and water contaminated with palm oil in palm oil refinery factories in the south of Thailand. The samples were collected in zipper bags and transported to the laboratory for screening and isolation. The method for screening was done by using serial dilutions of the samples and plated on minimal salt medium (MSM) (g/l): K_2HPO_4, 0.8; KH_2PO_4, 0.2; $CaCl_2$, 0.05; $MgCl_2$, 0.5; $FeCl_2$, 0.01; $(NH_4)_2SO_4$, 1.0; NaCl, 5.0 (Saimmai *et al.,* 2012a,b). MSM agar using 1% (w/v) of used palm oil or glucose as the carbon source was used for the isolation of bacteria. Morphologically distinct colonies were re-isolated by transfer onto fresh glucose- or used palm oil-containing agar plates at least three times to obtain pure cultures and subsequently Gram-staining. Pure cultures were stored at -20°C in nutrient broth (NB, Difco, MI, USA) mixed with sterile glycerol at a final concentration of 30%.

2.2 Screening of biosurfactant-producing bacterial strains

One loop of each isolate was then transferred to test tubes containing 5 ml of NB and shaken (150 rpm) at 30°C for 24 hrs. A 100 µl sample of each cell culture was transferred to 5 ml of MSM supplemented with 1% (w/v) of the following different carbon sources: acid oil; crude glycerol; crude palm oil; commercial sugar; glucose; molasses; palm oil decanter; palm oil mill effluent; rubber serum; used lubricating oil; or used palm oiland cultivated at 30°C, 200 rpm for seven days. Bacterial growth was monitored by measuring the optical density (OD) of the culture broth at 600 nm. The culture supernatant of each isolate after centrifugation at 9,693 g and 4°C for 10 min was tested for the presence of biosurfactant by using the drop-collapse test (Bodour and Maier, 1998), surface tension measurement (Jachimska *et al.,* 1995) and emulsification activity (De Acevedo and McInerney, 1996). The activity of the synthetic surfactants, sodium dodecyl sulfate (SDS; Sigma Chemicals, St. Louis, MO), Tween 80 (Sigma Chemicals, St. Louis, USA) and commercially available biosurfactant (Surfactin) (Sigma-Aldrich, St Louis, MO), was tested at concentrations higher than their critical micelle concentrations (2.0, 0.16 and 0.02 g/l, respectively). MSM supplemented with the different carbon sources without inocula was used as a negative control.

2.3 Strain identification and construction of phylogenetic tree

The selected bacterial isolates were identified by using 16S rRNA sequence analysis with Colony PCR technique. Colony PCR was carried out using Com primers (Schwieger and Tebbe, 1998) as previously reported (Gandolfi *et al.,* 2010). Briefly, colonies approximately 1 mm in diameter on NA were picked up with asterilized toothpick and directly transferred to the PCR tube as DNA templates. The thermal cycle programmed, run on a GeneAmp PCR system 9700 (Perkin Elmer) consisted of one cycle of 94°C for 10 min, 51°C for 2 min, 72°C for 2 min, and 35 cycles of 94°C for 20 s, 57°C for 45 s (decreased by 1 s per cycle), 72°C for 1 min, and then incubation at 72°C for 5 min, and a final incubation at 4°C. Then the DNA fingerprints were visualized on electrophoresed for 24 min at 100 V on 1% TAE agarose gel. The PCR product was purified using purification kit (QIAGEN, Inc.) following the manufacture's instruction. Then the purified PCR was electrophoresed for 24 min at 100 V on 1% TAE agarose gel. The sequencing reaction was performed by BigDye terminator sequencing kit (Perkin-Elmer Applied Biosystems, Massachusetts, U.S.A.) and analyzed by an automated sequencer ABI 310 Genetic Analyzer (Applied Biosystems, Massachusetts, U.S.A.). The nearest relative sequences in GenBank were retrieved using BLAST (Zhang *et al.,* 2000). A phylogenetic tree was drawn using the software program MEGA, version 4, by the neighbor-joining method (Tamura *et al.,* 2007).

2.4 Evaluation of biosurfactants production on renewable substrates

Fifty-three bacterial isolates were evaluated for the

biosurfactant production in 250 ml Erlenmeyer flasks containing 50 ml of MSM with 1% (w/v) of the carbon source. The tested carbon source for the biosurfactant production were as follows: acid oil, crude glycerol, crude palm oil, commercial sugar, glucose, molasses, palm oil decanter, palm oil mill effluent, rubber serum, used lubricating oil, or used palm oil. The isolates were activated by growing them on NB for 48 hours at 30°C. Subsequently they were transferred to test tubes containing 5 ml of NB and shaken (150 rpm) at 30°C for 24 hrs. Cell suspensions were adjusted to an optical density (OD) at 600 nm of 0.10, and 1 ml of these suspensions were used as starters. The flasks were incubated at 30°C and growths were monitored by reading OD_{600} by spectrophotometer (Libra S22, Biochrom Ltd., Cambridge, England). Biosurfactant activities were measured by using emulsification activity and surface tension by the duNouy Method using a ring tensiometer. The MSM medium supplemented with the different carbon sources without inoculums was used as a negative control.

2.5 Analytical methods

2.5.1 Growth

Growth was monitored by measuring the optical density (OD) of the culture broth at 600 nm.

2.5.2 Drop collapsing test

Two microliters of ULO was added to each well of a 96-well microtiter plate lid (Biolog, California, U.S.A.) and these were left to equilibrate for 1 hr at room temperature. Five microliters of the culture supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min with the aid of a magnifying glass. Biosurfactants-producing cultures giving flat drops were scored as positive (+). Those cultures that gave rounded drops were scored as negative (–), indicating no biosurfactants production (Bodour and Maier, 1998).

2.5.3 Emulsification activity assay

The emulsification activity of biosurfactants was measured using a method as described by De Acevedo and McInerney (1996). This was carried out using 1 ml of culture supernatant, after centrifugation at 9,693 g and 4°C for 10 min, with 0.5 ml xylene placed in an Eppendorf tube and vortexed for 30 seconds. The supernatants which produced a stable cloudy appearance in the emulsion layer were chosen to take part in a larger emulsification test using a final volume of 2 ml culture supernatant and 2 ml xylene. This suspension was then vortexed in a test tube for 2 min and left to stand for 24 hrs. The emulsification activity (E24) was calculated by dividing the measured height of the emulsion layer by the total height of the mixtures and multiplying by 100.

2.5.4 Surface tension measurement

Surface tension of culture supernatant, after centrifugation at 9,693 g and 4°C for 10 min and removed the oil residue by hexane extraction when oil was used as a carbon source, was measured using a Model 20 Tensiometer (Fisher Science Instrument Co., PA, U.S.A.) at 25°C (Jachimska *et al.,* 1995).

2.5.5 Extraction of biosurfactant

The gravimetric method described by Nitschke and Pastore (2004) was used for the determination of biosurfactant concentration with minor modification. The cell culture was centrifuged at 9,363 g for 10 min to prepare the culture supernatant and removed the oil residue by hexane extraction when oil was used as a carbon source. The culture supernatant was acidified with 1 N HCl to pH 2 and for 12 h at 4°C. The cloudy culture supernatant was twice extracted with an equal volume of ethyl acetate in a separating funnel. The pooled organic phase was evaporated under vacuum (N-1000; Eyela, Tokyo, Japan) at 40°C. The biosurfactant obtained was dried in an incubator at 37°C for 24 to 48 hrs (till reaching a constant weight). The net weight of the crude precipitate was determined, and the crude biosurfactant concentration (in g/l) was calculated.

2.6 Statistical analysis

All experiments were carried out at least in triplicate. Statistical analysis was performed using *Statistical Package for Social Science*, ver. 10.0 for Windows (SPSS, Chicago, IL, U.S.A.).

3.Results and Discussion

3.1 Isolation and screening of biosurfactants-producing bacteria

A total of 1,324 bacterial isolates were isolated including 235 strains from 6 factory samples in Chumphon Province, 365 strains from 10 factory samples in Krabi Province, 137 strains from two factory samples in Satun Province, 74 strains from one factory samples in Songkhla Province, 368 strains from 13 factory samples in Surat Thani Province, and 145 strains from three factory samples in Trang Province. Sixty-three percent of the bacterial isolates (827 of 1,324) were Gram-negative (data not shown). It has previously been reported that most bacteria isolated from sites with a history of contamination by oil or its byproducts are Gram-negative. This may be a characteristic that contributes to the survival of these populations in such harsh environments (Bicca *et al.,* 1999).

Fifty-three isolates were identified as biosurfactant-producing bacteria using the drop-collapse test and emulsifi-

cation activity test when low-cost, by-product or waste from agro-industry was used as substrates. Among them 12 isolates showed positive results in one assay (drop-collapsing test or emulsification activity) and 41 of them gave positive results for both assays (Table 1). The chosen substrates have distinct chemical compositions and have been extensively used for biosurfactant production (Makkar *et al.,* 2011). Among them, palm oil decanter cake and rubber serum emerged as two of the most important potential feed stocks, available in large quantities as a by-product of the palm oil purification process and latex processing factory found in southern Thailand, respectively (Johnson and Taconi, 2007). Among the tested carbon sources, palm oil decanter cake, palm oil mill effluent and used palm oil were the best carbon sources for biosurfactant production. Forty-nine isolated strains grew and produced biosurfactants when they were used as a carbon source. Acid oil and used lubricating oil supported the growth of a small number of strains after 48 hrs of incubation (12 and 15 of 53, respectively). The reasons for these results were probably its poor biodegradability (Chayabutra *et al.,* 2001) or toxicity to bacterial cells (Li and Chen, 2009). Base on the biosurfactant production screening by the drop-collapse test or emulsification activity, 5 types of carbon source that supported the highest biosurfactant production (crude palm oil, palm oil decanter cake, palm oil mill effluent, rubber serum and used palm oil) were selected for biosurfactant production in flasks.

3.2 Identification, taxonomy and phylogeny of the biosurfactants-producing bacteria

The identification of selected isolates was evaluated by 16S rRNA sequence analysis. All the isolates were assigned at genus level comparing their sequences with the RDP database. Their sequences were assigned with the NCBI database and deposited in DDBJ/EMBL/GenBank with an accession number (Table 2). The similarity values were obtained after pair-wise alignment of 16S rRNA sequences of studied strains and EMBL database sequences, and the sequences giving the highest scores were retrieved to construct the phylogenetic tree (Figure 1).

The 16S rRNA identification showed that the affiliation of the selected strains obtained from soil contaminated with palm oil in palm oil industry, southern of Thailand can be divided into five bacterial phylogenetic groups: Archae; Bacteroidetes; Firmicutes; Proteobacteria; and the Gram-positive branch (high G+C content Actinobacteria) (Table 2). The direct isolation method is often used to isolate the dominant members in a microbial community. The presence of 42 genera from the 53 isolates suggests that there is a wide biodiversity of biosurfactant-producing bacteria in soil contaminated with palm oil (Table 2 and Figure 1).

Acinetobacter, Bacillus, *Brevibacillus*, *Pseudomonas*, *Serratia* and *Rhodococcus* are the best known bacterial groups for biosurfactant-producing genera and they were also found in our screening. The majority of the isolated strains belonged to the genus *Bacillus* (6 isolates), *Acinetobacter* (3 isolates) and *Pseudomonas* (3 isolates). These are frequently isolated from hydrocarbon-contaminated environments and many strains belonging to these genera have been demonstrated to be efficient hydrocarbon degraders and biosurfactant-producing bacteria (Suwansukho *et al.,* 2008; Ruggeri *et al.,* 2009; Saimmai *et al.,* 2012a,b). To the best of our knowledge, we are the first to describe here the following sixteen genera as biosurfactant-producing bacteria: *Caryophanon; Castellaniella; Filibacter; Geminicoccus; Georgenia; Luteimonas; Mesorhizobium; Mucilaginibacter; Nubsella; Paracoccus; Pedobacter; Psychrobacter; Rahnella; Sphingobium; Sphingopyxis* and *Sporosarcina.*

The isolates AS56 and AS58 exhibited high homology (99%) with *Castellaniella caeni* NBRC 101664 and *Psychrobacter adeliensis* DSM 15333T, respectively (Table 2). *Buttiauxella* sp., *Castellaniella* sp., *Comamonas* sp. and *Psychrobacter* sp. were all capable of degrading turbine oil, which consisted mainly of recalcitrant cycloalkanes and isoalkanes (Ito *et al.,* 2008) and nonylphenol ethoxylates (NPnEO) with a low ethoxylationdegree and *n*-hexadecane, which is particularly resistant to biodegradation (Peng *et al.,* 2007; Di Gioia *et al.,* 2008). But no reports so far have been

Table 1. Growth and biosurfactant production of the isolates on different carbon sources, AO: acid oil; CG: crude glycerol; CP: crude palm oil; CS: commercial sugar; GL: glucose; MO: molasses; PD: palm oil decanter; PO: palm oil mill effluent; RS: rubber serum; UL: used lubricating oil, and UP: used palm oil.

Strain	Gram stain[a]	Growth[b]											DCT[c]	E24[c]
		AO	CG	CP	CS	GL	MO	PD	PO	RS	UL	UP		
AS4	+	-	+	+	+	+	+	-	+	+	-	+	+	+
AS6	+	+	-	-	+	-	+	+	-	+	+	+	+	+
AS9	+	+	+	+	+	+	+	+	+	-	-	+	+	+
AS11	+	-	+	-	-	+	+	+	+	+	+	-	+	+
AS13	+	-	-	+	+	+	+	+	+	+	-	+	+	+
AS18	-	+	+	+	+	-	+	+	+	+	-	+	+	-
AS20	-	-	+	+	+	+	+	-	-	-	+	+	+	+

Table 1. (Continued)

Strain	Gram stain[a]	Growth[b]											DCT[c]	E24[c]
		AO	CG	CP	CS	GL	MO	PD	PO	RS	UL	UP		
AS21	-	-	-	+	-	+	+	+	+	-	-	-	+	+
AS25	-	+	+	+	+	+	+	+	+	-	-	+	+	+
AS26	-	-	+	-	+	+	+	+	+	+	+	-	+	+
AS32	-	-	-	+	+	-	+	+	+	+	-	+	+	+
AS35	-	-	+	+	+	+	+	+	+	+	-	+	+	-
AS40	+	+	+	+	-	+	+	+	+	+	+	+	+	-
AS41	-	-	+	+	+	+	+	+	-	-	-	+	+	+
AS42	-	-	+	+	+	-	+	+	+	+	-	+	+	+
AS44	-	+	-	-	+	+	+	+	+	+	+	-	+	+
AS47	-	-	+	+	+	+	+	+	+	+	-	-	+	+
AS48	-	-	+	+	+	+	+	+	+	+	+	+	+	-
AS50	-	+	+	+	-	-	+	+	+	+	-	+	+	+
AS52	+	-	-	+	-	+	+	+	+	+	-	+	+	+
AS54	-	-	+	+	+	+	+	+	+	+	-	+	+	-
AS55	-	+	+	+	-	-	+	+	+	+	+	+	+	+
AS56	-	-	+	+	+	+	+	+	+	-	-	+	+	+
AS58	-	+	+	+	+	+	+	+	+	-	-	+	+	+
AS60	-	-	-	+	+	+	+	+	+	+	-	-	+	-
AS61	-	-	+	+	-	+	+	+	+	+	+	+	+	+
AS62	-	-	+	+	+	-	+	+	+	+	-	+	+	+
AS63	-	-	+	+	+	-	+	+	+	+	-	+	+	+
AS64	-	-	+	+	+	+	+	+	+	+	-	+	-	+
AS65	-	-	-	+	+	+	+	+	+	+	+	+	+	+
AS66	-	+	+	+	-	+	+	+	+	+	-	+	+	+
AS67	-	-	+	+	+	+	+	+	+	+	-	+	+	+
AS68	-	-	-	+	+	+	+	+	+	+	+	+	-	+
AS69	+	+	+	+	+	-	+	+	+	+	-	+	+	+
AS70	+	-	+	+	-	-	+	+	+	+	-	+	-	+
AS71	+	-	+	+	+	+	+	+	+	+	+	+	+	-
AS72	-	+	-	+	+	+	+	-	+	+	-	+	-	+
AS73	-	-	+	+	+	+	+	+	+	+	+	+	-	+
AS74	+	-	+	+	+	+	+	+	+	+	+	+	+	+
AS75	-	-	+	+	+	+	+	+	-	+	-	+	+	+
AS76	+	-	+	+	-	+	+	+	+	+	-	+	+	+
AS77	+	-	-	+	+	+	+	+	+	+	-	+	+	+
AS78	-	-	+	+	+	-	+	+	+	+	-	+	-	+
AS79	-	-	+	+	+	+	+	+	+	+	-	+	+	+
AS80	-	-	+	-	+	+	+	-	+	+	-	+	+	+
AS81	-	-	+	+	+	+	+	+	+	+	+	+	+	+
AS82	-	-	-	+	+	+	+	+	+	+	-	+	+	+
AS83	+	-	+	+	+	+	+	+	+	+	-	+	+	+
AS84	-	-	-	+	+	+	+	+	+	+	-	+	+	+
AS85	-	-	+	+	+	+	+	+	+	+	-	+	+	+
AS86	-	-	+	+	+	+	+	+	+	+	-	+	+	+
AS87	+	-	-	+	+	+	+	+	+	+	-	+	+	+
AS88	-	-	+	-	+	+	+	+	+	+	-	+	+	+

[a] Gram stain : +, Gram-positive; - Gram-negative; [b] +: Biomass increase more than 10-fold compared to the inoculum. –: non growth; [c] +: Positive test at least with one carbon source. –: negative test with the five tested carbon sources. E24: emulsification activity; DCT: Drop collapsing test

Table 2. Phylogenetic analysis of the bacterial strains isolated from palm oil contaminated soils in palm oil industry.

Taxonomic position	Strain code	Accession no.	16S rRNA gene sequence	Sequence identity (%)	RDP Classification (Confidence 80%)
			Nearest relative in GenBank		
Actinobacteria	AS40	AB720160	*Rhodococcus ruber* AM (JQ819733)	99	*Rhodococcus*
	AS52	AB721295	*Corynebacterium falsenii* 223 (JQ800469)	100	*Corynebacterium*
	AS68	AB727945	*Dietzia* sp. S-XJ-2 (FJ529035)	98	*Dietzia*
	AS71	AB727948	*Dietzia natronolimnaea* LL 51 (DQ821754)	99	*Dietzia*
	AS74	AB727950	*Georgenia muralis* KOPS24 (GQ497926)	100	*Georgenia*
	AS77	AB727954	*Kocuria flavus* CMG28 (GQ255646)	100	*Kocuria*
	AS83	AB727960	*Pimelobacter simplex* DSB7 (JQ342871)	99	*Pimelobacter*
Archaea	AS64	AB725661	*Haloplanus* sp. RO5-8 (EU931578)	98	*Haloplanus*
	AS65	AB725662	*Halobacteriaceae archaeon* EB21 (JF293279)	98	*Halobacteriaceae*
Bacteroidetes	AS44	AB721287	*Sphingobacterium multivorum* TND27 793 (JQ660535)	99	*Sphingobacterium*
	AS61	AB725658	*Mucilaginibacter* sp. SMS-12 (JQ739458)	99	*Mucilaginibacter*
	AS80	AB727957	*Nubsella zeaxanthinifaciens* NBRC102579 (AB681863)	99	*Nubsella*
	AS82	AB727959	*Pedobacter* sp. 148 (GU213382)	99	*Pedobacter*
Firmicutes	AS4	AB720124	*Bacillus subtilis* BCA26 (HE716895)	100	*Bacillus*
	AS6	AB720126	*Bacillus subtilis* BCA31 (HE716900)	100	*Bacillus*
	AS9	AB720129	*Bacillus licheniformis* PUFSTFMPi03 (JQ677088)	100	*Bacillus*
	AS11	AB720131	*Bacillus licheniformis* RBA08 (JQ780329)	100	*Bacillus*
	AS13	AB720133	*Bacillus mycoides* FKS9-207 (AB677940)	100	*Bacillus*
	AS69	AB727946	*Brevibacillus agri* NCHU1002 (AY319301)	100	*Brevibacillus*
	AS70	AB727947	*Caryophanon* sp. N36 (GU086435)	98	*Caryophanon*
	AS72	AB727949	*Filibacter limicola* DSM 13886 (NR042024)	99	*Filibacter*
	AS87	AB727964	*Sporosarcina globispora* NBRC16082 (AB681045)	99	*Sporosarcina*
	AS88	AB727965	*Bacillus subtilis* BCA26 (HE716895)	98	*Bacillus*
Proteobacteria	AS18	AB720138	*Acinetobacter parvus* CHCH:C:1:4#21 (HQ424463)	98	*Acinetobacter*
	AS20	AB720140	*Acinetobacter gyllenbergii* LUH1737 (AJ293692)	99	*Acinetobacter*
	AS21	AB720141	*Pseudomonas aeruginosa* AV2 (JQ839149)	99	*Pseudomonas*
	AS25	AB720145	*Pseudomonas oleovorans* NBRC13583 (AB680450)	99	*Pseudomonas*
	AS26	AB720146	*Pseudomonas fluorescens* BCA20 (HE716889)	100	*Pseudomonas*
	AS32	AB720152	*Serratia marcescens* R9-8A (HQ154570)	100	*Serratia*
	AS35	AB720155	*Acinetobacter junii* NW123 (JF915345)	100	*Acinetobacter*
	AS41	AB721284	*Sinorhizobium meliloti* T2c (AB539807)	99	*Sinorhizobium*
Proteobacteria	AS42	AB721285	*Stenotrophomonas rhizophila* T2j (AB539813)	98	*Stenotrophomonas*
	AS47	AB721290	*Comamonas terrigena* BR42 (FJ482015)	99	*Comamonas*
	AS48	AB721291	*Buttiauxella izardii* NEHU.FNSRJ.94 (JQ292906)	99	*Buttiauxella*
	AS50	AB721293	*Marinobacter pelagius* KJ-W14 (JQ799111)	99	*Marinobacter*
	AS54	AB725651	*Azorhizobium doebereinerae* BR5401 (NR041839)	98	*Azorhizobium*
	AS55	AB725652	*Mesorhizobium* sp. N3 (HM590823)	98	*Mesorhizobium*
	AS56	AB725653	*Castellaniella caeni* NBRC 101664 (AB681517)	99	*Castellaniella*
	AS58	AB725655	*Psychrobacter adeliensis* DSM 15333T (HE654010)	99	*Psychrobacter*
	AS60	AB725657	*Rahnella aquatilis* (AB682274)	100	*Rahnella*
	AS62	AB725659	*Ochrobactrum anthropi* Nf22SsD (HQ406750)	100	*Ochrobactrum*
	AS63	AB725660	*Sphingobium indicum* B90A (NR042943)	99	*Sphingobium*
	AS66	AB727943	*Achromobacter* sp. EP17 (AM398226)	98	*Achromobacter*
	AS67	AB727944	*Acidovorax* sp. NF1078 (JQ782387)	99	*Acidovorax*
	AS73	AB727950	*Geminicoccus roseus* D2-3 (NR042567)	99	*Geminicoccus*
	AS75	AB727952	*Gluconobacter oxydans* DR1 (JN004201)	100	*Gluconobacter*
	AS76	AB727953	*Achromobacter* sp. W-SL-1 (FJ529041)	100	*Achromobacter*
	AS78	AB727955	*Luteimonas* sp. Gr-4 (JQ349045)	100	*Luteimonas*
	AS79	AB727956	*Methylobacterium populi* 49B (HQ285774)	100	*Methylobacterium*
	AS81	AB727958	*Paracoccus marinus* Kongs-33 (HE800839)	99	*Paracoccus*
	AS84	AB727961	*Pseudoxanthomonas* sp. DBTS3 (GU122958)	99	*Pseudoxanthomonas*
	AS85	AB727962	*Pusillimonas terrae* (DQ466075)	98	*Pusillimonas*
	AS86	AB727963	*Sphingopyxis granuli* Kw07 (AY563034)	98	*Sphingopyxis*

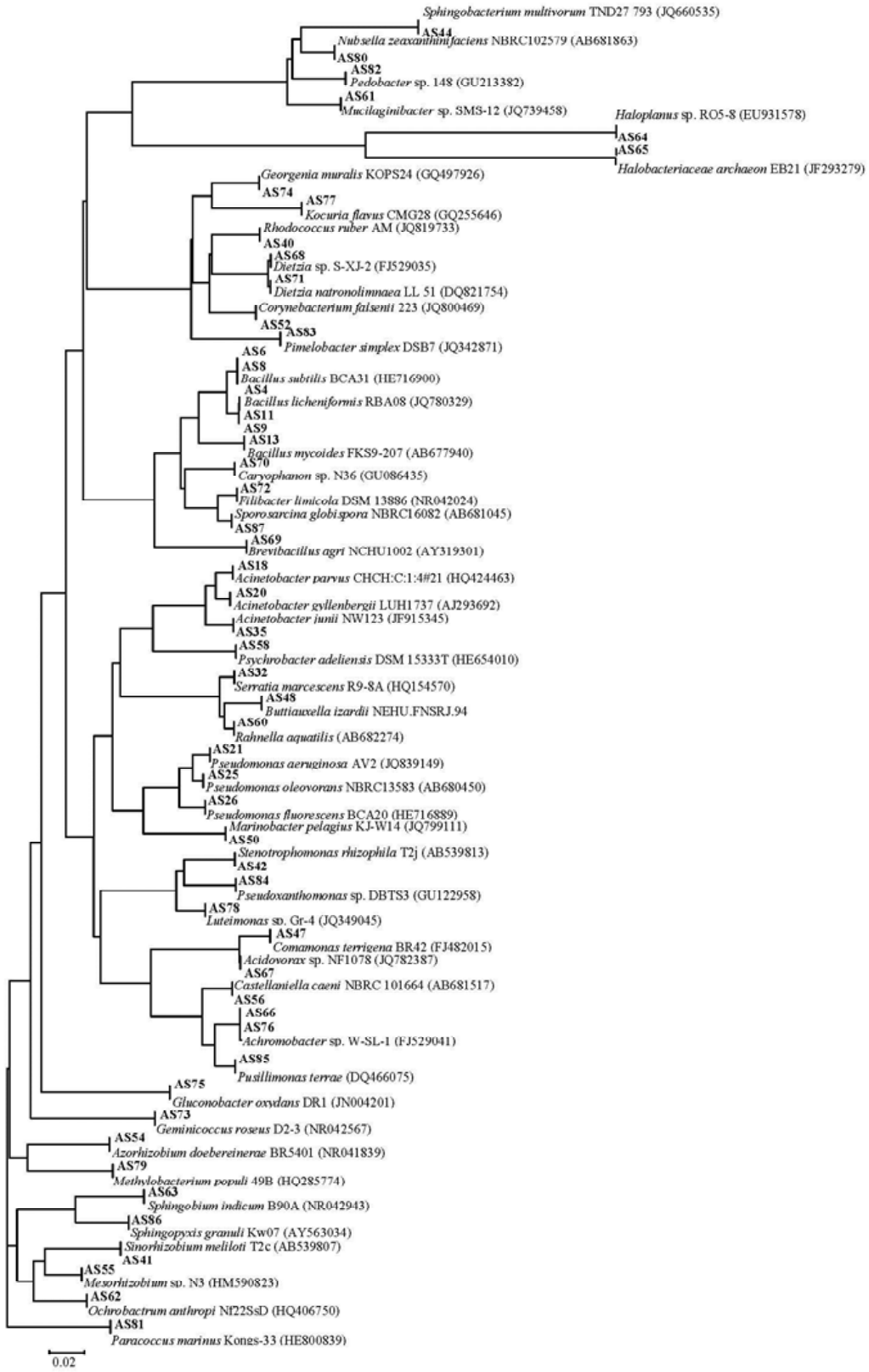

Figure 1. Unrooted phylogenetic tree based on 16S rRNA gene comparison of the bacterial isolates featured in this study (bold) and the nearest relative in GenBank. The branching pattern was generated by neighbor-joining methods and the bootstrap values, shown at the nodes, were calculated from 1000 replicates. Bootstrap probability values of <50% were omitted from the figure. The scale bar indicates substitutions per nucleotide position. GenBank accession numbers are given in parentheses.

found on the biosurfactant production capability of these genera.

Some of the genera that we described in this work as novel biosurfactant producers (*Mesorhizobium, Mucilaginibacter, Paracoccus, Pedobacter, Rahnella,* and *Sphingobium*) have been previously reported to produce extracellular polymers (EPSs). However, the surface properties of these compounds have not yet been reported. (Ozdemir *et al.,* 2003; Johnsen and Karlson, 2004; Diggle *et al.,* 2008; Han *et al.,* 2009; 2012). EPSs are important in the microbial interaction and emulsification of various hydrophobic substrates (Perfumo *et al.,* 2010). They are known to increase the viscosity of solutions at low pH value and to emulsify several hydrocarbon compounds (Calvo *et al.,* 1998). They are attracting and intriguing many researchers trying to harness their extraordinary properties and considerable potential applications in various fields (Desai and Banat, 1997).

The genus *Acidovorax*, *Pimelobacter* and *Stenotrophomonas* have already been described by Golubev *et al.* (2009) and Ruggeri *et al*. (2009) as emulsifier producing strains. However, the reduction of interfacial tension of these compounds in the present study suggested that they were low-molecular-weight biosurfactants rather than high-molecular-weight bioemulsifiers. To the best of our knowledge, we are the first to add here the following six genera to the list of already described bioemulsifier-producing bacteria: *Acidovorax; Gluconobacter; Kocuria; Methylobacterium; Pseudoxanthomonas;* and *Pimelobacter* (Nazina *et al.,* 2003; Mulligan, 2005; Golubev *et al.,* 2009; Nayak *et al.,* 2009).

3.3 Evaluation of biosurfactants production on renewable substrates

The 53 strains which gave positive results for biosurfactant production were further examined for their capability to use the low-cost, byproduct or waste from agro-industry factory and to produce biosurfactants in shake flasks. Table 3 shows the surface tension of the culture supernatants and emulsification activity (E24) of 53 strains that gave positive results for biosurfactant production in the preliminary screening. They grew on crude palm oil, palm oil decanter cake, palm oil mill effluent, rubber serum or used palm oil as carbon sources.

Biosurfactant activity can be measured by emulsification/emulsion stabilization and the changes in surface and interfacial tensions. Microbial candidates for bioemulsifier production are expected to have a stable hydrophobic-water emulsion of more than 50% (Willumsen and Karlson, 1997). In our work 23 strains exhibited the E24 equal or higher than that threshold (Table 3). Among of them, *B. subtilis* AS4, *B. mycoides* AS13, *Mucilaginibacter* sp. AS61, *Acidovorax* sp. AS67, *Geminicoccus roseus* AS73 and *Pimelobacter simplex* AS83 produced stable xylene-supernatant emulsions comparable to those of the synthetic surfactants SDS (63%) and Tween 80 (61%) when palm oil mill effluent, palm oil decantercake, palm oil mill effluent, crude palm oil, used palm oil and palm oil mill effluent were used as carbon sources, respectively (Table 3). The highest E24 (69.0%) was obtained from *Geminicoccus roseus* AS73 when used palm oil was used as a carbon source. To the best of our knowledge, we are the first to describe here bioemulsifier production from the genus *Geminicoccus.* The stability of the emulsions has been reported to be important for both the performance and the effectiveness of the emulsifier (Willumsen and Karlson, 1997). In this study, stable and compact emulsions of xylene-supernatant were observed after 1 hr and they were found stable up to 48 hrs (*B. mycoides* AS13, *A. gyllenbergii* AS20*, S. marcescens* AS32, *M. Pelagius* AS50, *R. aquatilis* AS60 and *P. simplex* AS83) (data not shown). It is interesting to note that the strain which had the highest emulsification activity (69% of E24 from *Geminicoccus roseus* AS73 when used palm oil was used as carbon source) was not able to show surface tension reduction ability. This result was in accordance with the report by Willumsen and Karlson (1997), Plaza *et al.* (2006) and Saimmai *et al.* (2012a,b), who reported uncorrelated between surface tension reduction and emulsification activity of the obtained biosurfactant. In general, the polymeric biosurfactants produced from the bacteria, Archaea and yeast show high emulsification activity but does not lower the surface tension significantly (Bodour and Maier, 2002).

Another approach for screening potential biosurfactant-producing microorganisms is to measure the surface tension of culture supernatant. In the present study all of the isolates were able to reduce the surface tension of culture supernatant after of 48 hr of cultivation when suitable carbon source was used. According to Pansiripat *et al.* (2010), a good candidate for biosurfactant production should reduce the surface tension of the medium to lower than 40 mN/m. In the present study, we obtained a surface tension of culture medium to a level equal or lower than the threshold from all tested strains except *Comamonas terrigena* AS47. The lowest surface tension was obtained from *A. doebereinerae* AS54 (26.0 mN/m) when palm oil decanter cakewas used as a carbon sources (Table 3). Palm oil decanter cake is a by-product from the palm oil milling decanting process. It contains about 3-5% (w/v) oil and is rich in the nutrients (N, P_2O_5, K_2O, CaO, and MgO) that are suitable for microbial growth (Haron *et al.,* 2008). From these results, it is possible to suggest that the biosurfactant from this study would be useful in applications for the biodegradation of hydrocarbon or other water-immiscible substrates and enhancing oil recovery. These properties are important to reduce the capillary forces that entrap oil within the pores of rocks. They are also a mobility control agent that could improve the sweep efficiency of a water flood in the petroleum industry (De Acevedo and McInerney, 1996). The use of the alternative substrates such as agro-based industrial wastes or by-products is one of the attractive strategies for economical biosurfactants production.

Table 3. Emulsification activity (E24) and surface tension of supernatants (ST) obtained from bacterial cultures grown in MSM medium supplemented with indicated carbon sources (1%, w/v) for 48 hrs at 30 °C, CP: crude palm oil, PD: palm oil decanter, PO: palm oil mill effluent, RS: rubber serum, and UP: used palm oil.

Strain	CP		PD		PO		RS		UP	
	ST*	E24**	ST*	E24**	ST*	E24**	ST*	E24**	ST*	E24**
Bacillus subtilis AS4	28.2±1.0	50.2±1.0	nd	nd	26.2±1.5	63.5±4.8	31.5±0.5	51.2±5.0	30.5±0.7	54.2±4.0
Bacillus subtilis AS6	nd	nd	29.5±0.2	52.8±2.1	nd	nd	30.9±0.5	35.5±4.0	32.5±0.0	45.8±5.2
Bacillus licheniformis AS9	30.0±1.5	55.4±1.0	31.0±1.7	54.0±2.0	32.8±1.4	52.2±4.1	nd	nd	34.0±2.0	51.4±1.0
Bacillus licheniformis AS11	nd	nd	26.5±0.7	51.4±1.0	28.0±1.5	58.5±1.2	29.8±0.7	50.5±2.0	nd	nd
Bacillus mycoides AS13	32.0±1.4	54.8±1.2	30.5±0.1	65.8±9.1	31.0±0.5	54.2±1.2	30.5±0.0	54.7±2.1	29.0±0.9	54.1±1.2
Acinetobacter parvus AS18	34.0±1.0	0	37.5±0.4	0	32.6±1.5	0	35.4±0.7	0	34.0±0.4	0
Acinetobacter gyllenbergii AS20	35.0±1.5	59.4±5.0	nd	nd	nd	nd	nd	nd	26.0±0.4	54.1±2.0
Pseudomonas aeruginosa AS21	40.5±1.7	55.0±4.0	61.7±1.2	25.8±2.4	32.0±1.5	14.5±1.7	nd	nd	nd	nd
Pseudomonas oleovorans AS25	35.0±1.7	46.5±2.1	34.4±0.8	40.1±2.0	35.0±4.1	45.0±2.0	nd	nd	36.0±1.4	40.5±2.0
Pseudomonas fluorescens AS26	nd	nd	31.0±0.5	30.2±2.9	52.0±1.3	41.0±2.4	60.7±2.1	40.0±2.2	nd	nd
Serratia marcescens AS32	55.0±1.4	41.2±4.1	45.1±2.4	34.8±6.0	39.8±2.0	43.8±4.1	31.0±1.7	58.7±2.0	34.2±1.7	58.7±3.0
Acinetobacter junii AS35	55.6±1.0	0	50.0±0.0	0	32.0±1.0	0	34.0±1.1	0	44.8±0.9	0
Rhodococcus ruber AS40	31.2±0.8	0	48.5±2.8	0	34.0±1.1	0	47.8±0.7	0	54.7±0.8	0
Sinorhizobium meliloti AS41	47.5±0.2	35.8±4.5	65.4±1.7	40.2±2.8	nd	nd	nd	nd	31.5±2.4	40.1±3.7
Stenotrophomonas rhizophila AS42	30.0±1.7	24.8±2.4	37.0±2.5	55.8±1.7	32.0±0.7	49.0±4.0	32.8±0.7	46.5±2.0	60.8±7.5	40.2±2.0
Sphingobacterium multivorum AS44	nd	nd	39.9±2.3	34.5±4.0	59.7±1.0	38.0±3.0	65.0±0.2	41.0±6.1	nd	nd
Comamonas terrigena AS47	50.2±1.0	29.5±4.0	56.0±0.4	31.0±8.0	50.5±0.4	25.0±5.0	57.4±1.6	34.0±1.0	nd	nd
Buttiauxella izardii AS48	60.2±1.5	0	56.8±0.9	0	26.0±0.6	0	44.5±2.9	0	50.4±0.8	0
Marinobacter pelagius AS50	59.2±2.0	40.2±2.0	60.5±0.7	38.0±3.0	62.5±1.8	40.3±2.1	31.0±0.4	40.7±2.1	60.5±0.2	60.7±2.4
Corynebacterium falsenii AS52	60.5±2.0	31.5±4.0	66.4±1.2	45.3±5.8	29.0±0.1	30.5±4.0	70.4±1.6	35.0±2.0	50.4±0.6	40.2±2.0
Azorhizobium doebereinerae AS54	55.4±3.5	0	25.5±0.5	0	51.0±0.8	0	71.0±0.7	0	41.0±0.9	0
Mesorhizobium sp. AS55	46.8±1.0	29.4±2.4	32.0±1.6	35.7±3.4	58.0±1.5	32.5±5.0	30.8±1.0	20.5±2.0	54.8±2.7	23.8±4.1
Castellaniella caeni AS56	55.1±1.5	44.0±6.1	60.8±2.7	15.5±0.9	62.0±4.3	38.4±2.8	nd	nd	30.0±0.4	25.9±2.0
Psychrobacter adeliensis AS58	60.4±1.4	40.3±4.0	51.7±2.6	27.0±1.7	29.5±0.8	30.2±1.0	nd	nd	60.4±1.9	35.4±1.8
Rahnella aquatilis AS60	55.0±4.0	52.0±5.0	28.2±0.2	45.0±4.0	66.7±1.2	40.1±1.8	70.8±0.9	40.2±1.8	nd	nd
Mucilaginibacter sp. AS61	46.2±1.1	0	65.4±1.8	0	31.0±1.8	68.7±4.0	50.0±1.5	0	55.2±2.0	15.3±1.1
Ochrobactrum anthropi AS62	60.2±1.0	30.1±1.8	49.5±2.0	38.2±5.0	70.2±1.0	34.5±2.8	31.8±4.0	48.5±2.7	29.0±1.0	40.3±5.8
Sphingobium indicum AS63	60.8±0.1	35.3±5.0	57.4±1.0	32.1±4.1	30.4±2.0	39.3±4.0	57.4±0.6	51.5±4.7	65.4±2.6	34.0±5.5
Haloplanus sp. AS64	35.5±0.5	34.7±2.0	32.0±1.4	40.8±2.8	71.0±2.0	32.0±4.0	71.0±1.5	40.5±4.0	65.8±1.5	54.3±4.2
Halobacteriaceae archaeon AS65	55.0±1.1	25.4±2.4	48.9±0.7	35.3±4.0	30.1±2.1	49.2±4.2	65.5±2.0	32.3±3.8	63.9±2.1	45.3±3.0
Achromobacter sp. AS66	48.2±1.0	0	44.7±2.6	0	51.2±0.9	0	32.4±1.0	0	29.8±0.7	0
Acidovorax sp. AS67	45.9±2.2	65.0±1.7	47.4±1.0	28.1±5.1	60.5±0.9	40.7±2.8	62.7±0.2	24.5±7.0	29.0±1.4	40.3±6.8
Dietzia sp. AS68	66.0±0.5	33.0±1.0	72.6±2.0	35.7±4.8	71.0±0.0	41.3±8.0	31.0±0.9	35.3±2.4	65.5±0.8	39.3±3.7
Brevibacillus agri AS69	30.7±1.2	14.8±2.4	51.4±0.9	24.3±1.8	49.0±0.1	41.8±5.0	71.4±0.6	34.2±4.0	65.0±2.1	35.3±5.6

Table 3. (Continued)

Strain	CP		PD		PO		RS		UP	
	ST*	E24**	ST*	E24**	ST*	E24**	ST*	E24**	ST*	E24**
Caryophanon sp. AS70	67.0±2.1	35.3±4.0	31.0±2.4	31.7±2.0	71.0±1.4	48.7±2.3	72.5±5.0	34.7±6.4	66.0±1.0	42.3±3.4
Dietzia natronolimnaea AS71	30.8±0.4	0	67.8±2.1	0	31.4±2.1	0	66.8±0.9	0	64.0±0.5	0
Filibacter limicola AS72	48.9±1.2	24.7±2.0	nd	nd	29.5±2.0	35.8±5.0	34.2±1.4	33.2±4.2	52.7±0.8	47.3±6.7
Geminicoccus roseus AS73	65.0±1.0	14.5±2.1	70.0±3.0	15.8±4.0	31.0±0.0	33.7±1.0	71.4±0.2	18.7±6.0	65.0±0.8	69.0±4.0
Georgenia muralis AS74	60.5±2.4	25.6±2.0	32.4±5.1	48.5±5.0	47.4±2.4	36.5±5.0	55.4±2.1	40.3±2.4	56.0±1.4	40.7±2.8
Gluconobacter oxydans AS75	35.2±1.0	24.3±3.8	48.9±1.7	36.5±2.0	nd	nd	32.5±0.8	46.0±7.0	60.5±2.4	32.4±4.8
Achromobacter sp. AS76	51.0±0.5	32.5±4.0	33.6±0.6	34.5±5.0	61.5±0.7	32.5±4.0	52.0±2.0	12.4±1.8	54.0±2.6	41.7±5.0
Kocuria flavus AS77	55.0±1.4	40.7±2.0	40.0±1.8	40.0±8.1	36.0±4.7	42.3±4.7	61.5±0.5	41.3±5.0	60.5±1.8	40.3±4.8
Luteimonas sp. AS78	66.7±0.2	46.0±3.7	30.0±0.5	40.7±2.9	72.0±5.5	45.0±2.8	72.0±0.5	40.2±1.4	66.8±4.1	32.5±2.0
Methylobacterium populi AS79	46.7±1.8	32.0±2.0	66.7±0.9	42.8±2.8	32.4±1.4	40.8±2.7	58.5±0.5	25.8±2.5	44.0±2.6	15.0±5.0
Nubsella zeaxanthinifaciens AS80	nd	nd	nd	nd	49.5±0.4	40.0±4.2	34.4±1.7	44.5±2.8	44.0±2.7	21.0±2.1
Paracoccus marinus AS81	55.2±1.0	25.3±1.8	36.7±0.8	32.5±5.0	32.5±0.9	41.7±2.0	47.6±0.1	24.3±5.1	42.8±0.4	26.7±4.0
Pedobacter sp. AS82	32.8±0.7	40.0±5.0	57.2±1.9	42.1±2.0	60.0±0.0	30.5±1.1	51.8±2.1	35.5±2.0	54.0±2.6	35.3±2.8
Pimelobacter simplex AS83	60.7±1.0	24.7±2.1	49.4±1.0	32.1±5.1	30.5±2.0	65.7±2.7	39.8±2.1	40.2±2.1	60.5±2.1	34.7±5.7
Pseudoxanthomonas sp. AS84	45.2±1.2	25.3±2.8	55.8±2.0	33.5±4.0	62.0±0.6	38.3±2.0	34.8±1.7	35.3±5.1	48.4±0.6	24.6±2.1
Pusillimonas terrae AS85	42.2±0.7	35.7±6.0	36.4±2.0	40.5±2.7	32.5±0.2	40.5±2.1	61.4±0.6	40.7±3.8	54.8±2.0	40.3±4.0
Sphingopyxis granuli AS86	54.6±0.8	48.1±7.4	47.0±1.7	33.2±5.0	65.4±0.7	24.4±1.8	35.4±2.1	36.5±7.0	44.5±2.0	51.8±4.1
Sporosarcina globispora AS87	60.5±1.9	40.2±1.0	37.4±1.0	29.1±1.1	32.0±1.1	15.3±5.0	65.0±1.0	44.2±1.5	60.8±5.4	41.7±3.0
Bacillus subtilis AS88	nd	nd	61.4±0.8	40.4±4.8	35.5±1.2	38.3±4.8	61.4±2.0	40.0±4.4	56.9±2.6	40.8±6.0
MSM with carbon source	65.2±1.2	0	70.0±0.8	0	71.0±0.5	0	71.2±0.2	0	64.4±0.6	0

nd: The value was not determined because the biomass increase of the tested strain was less than 10-fold (OD_{600}<1.00). *ST, Surface tension (mN/m): SDS (2.0 g/l) 41.0±0.5; Tween 80 (0.16 g/l) 40.2±0.7; Surfactin (0.02 g/l) 38.5±1.0. **E24, Emulsification activity (%): SDS (2.0 g/l; 63.0±2.9); Tween 80 (0.16 g/l; 65.4±4.0); Surfactin (0.02 g/l) 64.7± 3.4.

Table 4. Biosurfactant yield from bacterial cultures grown in MSM medium supplemented with indicated carbon sources (1%, w/v) for 48 h at 30°C, CP: crude palm oil; PD: palm oil decanter; PO: palm oil mill effluent; RS: rubber serum; UP: used palm oil.

Strain	Type of carbon	Biosurfactant (g/l)*	Strain	Type of carbon	Biosurfactant (g/l) *
Bacillus subtilis AS4	PO	1.02±0.21	*Sphingobium indicum* AS63	PO	2.02±0.28
Bacillus subtilis AS6	PD	0.85±0.14	*Haloplanus* sp. AS64	PD	1.57±0.65
Bacillus licheniformis AS9	CP	1.14±0.47	*Halobacteriaceae archaeon* AS65	PO	1.45±0.41
Bacillus licheniformis AS11	PD	0.78±0.08	*Achromobacter* sp. AS66	UP	1.68±0.32
Bacillus mycoides AS13	UP	1.20±0.34	*Acidovorax* sp. AS67	UP	1.78±0.14
Acinetobacter parvus AS18	PO	1.54±0.42	*Dietzia* sp. AS68	RS	0.65±0.12
Acinetobacter gyllenbergii AS20	UP	1.23±0.21	*Brevibacillus agri* AS69	CP	1.42±0.17
Pseudomonas aeruginosa AS21	PO	0.67±0.09	*Caryophanon* sp. AS70	PD	2.54±0.32
Pseudomonas oleovorans AS25	PD	0.51±0.14	*Dietzia natronolimnaea* AS71	CP	1.85±0.25
Pseudomonas fluorescens AS26	PD	0.49±0.07	*Filibacter limicola* AS72	PO	1.01±0.41
Serratia marcescens AS32	RS	1.89±0.54	*Geminicoccus roseus* AS73	PO	2.35±0.14
Acinetobacter junii AS35	PO	2.02±0.32	*Georgenia muralis* AS74	PD	1.85±0.24
Rhodococcus ruber AS40	CP	1.65±0.65	*Gluconobacter oxydans* AS75	RS	1.65±0.32
Sinorhizobium meliloti AS41	UP	1.17±0.47	*Achromobacter* sp. AS76	PD	1.32±0.12
Stenotrophomonas rhizophila AS42	CP	0.63±0.05	*Kocuria flavus* AS77	PO	1.45±0.09
Sphingobacterium multivorum AS44	PD	0.47±0.11	*Luteimonas* sp. AS78	PD	1.75±0.15
Buttiauxella izardii AS48	PO	1.36±0.20	*Methylobacterium populi* AS79	PO	2.21±0.21
Marinobacter pelagius AS50	RS	1.75±0.12	*Nubsella zeaxanthinifaciens* AS80	RS	0.85±0.07
Corynebacterium falsenii AS52	PO	1.14±0.23	*Paracoccus marinus* AS81	PO	1.43±0.14
Azorhizobium doebereinerae AS54	PD	2.81±0.08	*Pedobacter* sp. AS82	CP	1.64±0.12
Mesorhizobium sp. AS55	RS	1.78±0.20	*Pimelobacter simplex* AS83	PO	1.74±0.21
Castellaniella caeni AS56	UP	1.52±0.31	*Pseudoxanthomonas* sp. AS84	RS	1.98±0.24
Psychrobacter adeliensis AS58	PO	1.36±0.12	*Pusillimonas terrae* AS85	PO	1.52±0.36
Rahnella aquatilis AS60	PD	1.64±0.54	*Sphingopyxis granuli* AS86	RS	0.84±0.12
Mucilaginibacter sp. AS61	PO	1.82±0.24	*Sporosarcina globispora* AS87	PO	1.52±0.32
Ochrobactrum anthropi AS62	UP	1.35±0.31	*Bacillus subtilis* AS88	PO	1.63±0.25

*Values are given as means ± SD from triplicate determinations.

Table 4 demonstrates the yield of biosurfactant with reference of the carbon source for all of the fifty-two isolates. Biosurfactant concentration of all the isolates ranged between 0.47 to 2.81 g/l and can be categorized into three groups on the basis of their biosurfactant concentration (in g/l) obtained from culture supernatant. These are Group I with production range of <1.0 g/l (10 isolates); Group II with production range of 1.0-2.0 g/l (36 isolates) and Group III with production range of >2 g/l (6 isolates). Out of these 52 biosurfactant producing isolates, 10 isolates were found to be the prominent producers of biosurfactant whose production more than 2.0 g/l with isolate *Azorhizobium doebereinerae* SA45 as the most potential isolate with biosurfactant concentration of 2.81 g/l when palm oil decanter was used as a carbon source (Table 4).

Overall, the new biosurfactant-producing strains featured in this work display important characteristics for the future development of economically efficient industrial-scale biotechnological processes. They produced and released extracellular biosurfactants into the culture medium, which should simplify recovery procedures. In addition, bacterial growth and biosurfactant production are supported by low cost fermentative substrates, such as palm oil decanter and palm oil mill effluent that are wastes from the oil palm mill. The use of cheap raw materials and wastes will contribute to the reduction of the costs of processing. Finally, our data suggests future evaluation be undertaken of the potential application of biosurfactants synthesized by the new strains.

4.Conclusions

In this study, 53 biosurfactant-producing isolates were isolated from palm oil contaminated soil from the palm oil industry in the south of Thailand by using low cost by-products or waste from industry as a sole carbon source. The production of biosurfactants was determined on strains representative of 42 different bacterial genera distributed among Proteobacteria, Firmicutes and Actinobacteria division in Eubacteria and Archaea. The findings of this study added 23 new genera to biosurfactants-producing bacteria. Among them, *A. doebereinerae* AS54, newly isolated for biosurfactant production, produced extracellular biosurfactants which reduced the surface tension of the culture supernatant from 70.0 to 25.5 m/Nm when palm oil decanter cake was used as a carbon source. Forty-six isolates belonging to 38 genera can stabilize xylene-supernatant emulsion. The isolate *G.*

roseus AS73 exhibited the highest E24 (69%) when used palm oil was used as the carbon source. Overall, the new biosurfactant-producing strains obtained in this work show promising features for the future development of economically efficient industrial-scale biotechnological processes.

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