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Antifungal Activity of Rhamnolipid Biosurfactant Produced by *Pseudomonas aeruginosa* A4 against Plant Pathogenic Fungi

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Abstract

Biosurfactants have been shown to have a variety of other agricultural applications. Antimicrobial activity is a desirable property in a variety of biosurfactants. Several biosurfactants produced from bacteria have antibacterial activity against plant diseases, making them a promising biocontrol molecule for ensuring agricultural sustainability in the long run. The purpose of this research was to characterize biosurfactant produced at different carbon source by biosurfactant-producing bacterial strains isolated from contaminated soapstock sediment, as well as its antifungal properties. Biosurfactant-producing bacteria were isolated from soapstock-contaminated soil which bacteria were selected by using drop collapse and oil displacement tests. Twelve isolates reduced surface tension of culture broth from 50 to 31 - 43 mN/m. According to 16S rRNA sequence analysis, these isolates belong to 8 different genera (*Acinetobacter*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas* and *Xanthomonas*). The supernatant of *Pseudomonas aeruginosa* A4 grown in MSM supplemented with soapstock was the most effective biosurfactant against *Aspergillus flavus* F2, *Aspergillus niger* F14, *Cunninghamella bertholletiae* F1 and *Rhizopus oryzae* F5, inhibiting mycelium growth by 54, 61 59 and 50 %, respectively. The extract substance inhibited spore germination against *A. flavus* F2 and *R. oryzae* F5 with a minimum inhibitory concentration of 2.75 mg/mL. TLC, FT-IR, ESI-MS and GC-MS analysis demonstrated that the mono-rhamnolipids and di-rhamnolipids had the same 3-hydroxy fatty acid composition of C8, C10 and C12. Overall, the biosurfactant-producing strains identified in this investigation show promise for future development and application in cost-effective industrial-scale biotechnological processes.

Keywords: Rhamnolipid, Biosurfactant, Soapstock, Antifungal activity, Oil palm seed

Introduction

Biosurfactant-producing microorganisms can be found in a variety of habitats depending on their physiological roles. Biosurfactant is produced by microorganisms to aid in the solubilization of hydrophobic chemicals in their environment, allowing them to be used as substrates [1]. By generating micelles, biosurfactants aid in the emulsification of hydrocarbons in the aqueous phase, increasing their availability for microbial uptake and breakdown [2]. The vegetable oils industry generates a considerable amount of wastes, which is difficult to dispose of soapstock is a by-product of vegetable oil refining. It is a lipid emulsion with about 50 % water, free fatty acids, phosphatides, triglycerides, pigments, and other minor non-polar chemicals in a heavy alkaline aqueous emulsion [3]. Soapstock accounts for around 6 % of all crude oil processed [4]. Refined vegetable oil use will rise, leading in an increase in the generation of these by-products.

Microbes almost often exist in mixed populations in natural habitats, containing a variety of strains and species. A pure culture is required for assessing the properties of a defined organism from such a mixed population. Enrichment cultures on hydrophobic substrates, in addition to direct isolation of strains by diluting and plating, are particularly promising for the isolation of biosurfactant generating bacteria. Several methods for detecting biosurfactant-producing microorganisms and quantifying their production capacity have been developed. The majority of them are based on the culture supernatant's surface or interfacial activity. This feature also indicates on the synthesis of biosurfactants.

Microorganisms have been shown to create variety of biosurfactants, including glycolipids, lipopeptides, neutral lipids, phospholipids, fatty acids, and polymeric substances, based on their physicochemical properties [5]. Biosurfactants have been demonstrated to have a variety of additional uses

in agriculture. Antimicrobial activity is a valuable characteristic of several biosurfactants. Biosurfactant is thought to have a detergent-like impact on cell membrane permeability due to its structure. Several biosurfactants derived from bacteria exhibit antimicrobial activity against plant diseases, making them a prospective biocontrol molecule for achieving long-term agricultural sustainability.

The goal of this study was to characterize biosurfactant produced at different carbon source by biosurfactant-producing bacterial strains isolated from contaminated soapstock sediment, as well as to determine its antifungal uses.

Materials and methods

Microorganism

The fungi, *Aspergillus flavus* F2, *Aspergillus niger* F14, *Cunninghamella bertholletiae* F1 and *Rhizopus oryzae* F5, that cause root rot in palm seed, were obtained from the Faculty of Agro-Industry, Prince of Songkla University. The fungi employed in this study were isolated from palm oil seed infected with a fungal infection.

Materials

Kerosene was obtained from Fluka (Munich, Germany). Commercial sugar (CS) was purchased from Mitrphol, Thailand. Commercial monosodium glutamate (MSG > 99 % purity) was purchased from Ajinomoto, Thailand. All chemicals and solvents used in this study were analytical grade. Crude glycerol was obtained from biodiesel production pilot plant at Faculty of Engineering, Prince of Songkla University, Songkhla, Thailand. The solid raw glycerol (containing about 50 % w/w glycerol) was used as a carbon source in the medium without further purification. Palm oil soap stock was kindly provided from the Pikunthong Royal Development Study Centre, Narathiwat, Thailand. Soapstock is created during the biodiesel production process from crude palm oil. Used palm oil (UPO) which contains around 2.6% free fatty acid was obtained from canteen in Prince of Songkla University, Thailand. Molasses was purchased from the agency in Hat Yai, Songkhla, Thailand. The total sugar content of the molasses tested in this study was 41 % (w/w). The motorbike mechanical workshop at Prince of Songkla University in Songkhla, Thailand, provided the used lubricating oil (ULO).

Sampling and isolation of biosurfactant-producing bacteria

Twenty soils were collected from the Pikunthong Royal Development Study Centre at Narathivat province. These samples were obtained at random near the polluted palm oil refinery and soapstock locations. Soil samples were taken 1 - 10 cm below the surface and maintained in a sterile bag for biosurfactant-producing bacteria isolation. Biosurfactant-producing bacteria were isolated from soils using the enrichment culture technique. Biosurfactant-producing bacteria were isolated using minimal salt medium (MSM) which contained (per liter) K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; $CaCl_2$, 0.05 g; $MgCl_2$, 0.5 g; $FeCl_2$, 0.01 g; NaCl, 5.0 g. The pH of the medium was adjusted to 7.0±0.2 [6]. Five grams of soil were introduced to 250 mL Erlenmeyer flasks containing 50 mL MSM supplemented with palm oil soapstock as a carbon source, and the flasks were incubated at 30 °C for 3 days on a rotary shaker (Orbitek, Scigenics Biotech, India). Then, in a 250 mL flask, 2 mL of the culture broth was transferred to fresh 50 mL soapstock containing MSM and cultured under the same conditions as mentioned before. This technique was carried out 3 times. Following that, the 3rd time culture broth was streaked on nutrient agar plates (NA, HiMedia, India), and phenotypically distinct bacterial colonies were transferred to fresh NA plates several times to obtain pure isolates. For further investigation, all isolates were grown in nutrient broth (NB, HiMedia, India) and stored at –20 °C in NB containing 30 % glycerol.

Screening of biosurfactant-producing bacteria

All of the bacterial isolates in NB were used to make seed inoculums. Two milliliters seed inoculums were inoculated into 250 mL Erlenmeyer flasks containing 50 mL MSM enhanced with 1 % (w/v) soapstock as the carbon source and incubated for 48 h at 30 °C with 150 rpm shaking. The supernatant was collected by centrifugation (Legend XTP, Thermo, USA) at 12,000 ×g for 15 min. Biosurfactant production by each bacterial isolate was assessed using a drop collapse assay and a displacement test of the supernatant [17,18]. All assays were performed at least in triplicate.

Identification of biosurfactant-producing bacteria

Biosurfactant-producing bacterial strains were identified by using 16S rRNA sequence analysis. Chromosomal DNA from each strain was extracted using a Genomic DNA mini kit (Geneaid Biotech Ltd,

Taiwan) following the manufacture's instruction. The 16S rRNA gene was amplified using the PCR method with universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR reactions were carried out in the following mixture; 22 µL of 2x EmeraldAmp GT PCR MasterMix (Takara Bio, USA); 1 µL of extracted chromosomal DNA and 1 µL of 10 µM each of primers in a total volume of 50 µL. Thermal cycling was performed using a gradient thermal cycler (TC-512, USA) as follows: Initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s; primer annealing at 50 °C for 30 s; primer extension at 72 °C for 120 s and final extension at 72 °C for 5 min. The PCR products were visualized by agarose gel electrophoresis and purified using a HiYieldTM Gel/PCR DNA Fragments extraction kit (Real Biotech Co., Taiwan). The 16S rRNA gene sequences obtained about 1400 bps were aligned along with the sequences of type strains obtained from the GenBank by using the program ClustalW [7]. Sequence homologies were examined using BLAST version 2.2.12 of the National Center for Biotechnology Information and a consensus neighbor-joining tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. A neighbor-joining tree was constructed using MEGA 3.1 program.

The partial rRNA sequences were analyzed using a BLAST search algorithm to estimate the degree of similarity to other rRNA sequences obtained from the NCBI/GenBank. Phylogenetic tree was constructed by Neighour-Joining and the ClustalX program [8]. Sequence homologies were examined using BLAST version 2.2.12 of the National Center for Biotechnology Information, and the 16S rDNA gene sequences were submitted to GenBank.

Assessment of biosurfactant production

In 250 mL Erlenmeyer flasks containing 50 mL MSM with 1 % (w/v) carbon source, the chosen strains were examined for biosurfactant production. Soapstock, crude glycerol (CG), commercial sugar (CS), glucose, molasses, and used palm oil (UPO) were all investigated as carbon sources for biosurfactant production. The strains were activated by growing them on NB for 24 h at 30 °C. 2 mL seed inoculums were inoculated into 250 mL Erlenmeyer flasks containing 50 mL MSM and agitated for 48 h at 150 rpm at 30 °C. The supernatant and cell pellet were separated by centrifugation at 12,000×g for 15 min. Biosurfactant activities were measured by using emulsion test assay and surface tension measurement [19,20]. The MSM medium supplemented with the different carbon sources without inoculums was used as a negative control. The supernatant was extracted two times with an equal volume of ethyl acetate [9]. A rotary evaporator and vacuum drying were used to evaporate the solvent. The crude extract was weighed after drying. Each condition was measured in triplicate.

Suppression of mycelial growth

The ability of biosurfactant-producing bacteria strains to prevent fungal growth was investigated. The antifungal activity of the supernatant from previous studies was tested. The phytopathogenic fungi (*A. flavus* F2, *A. niger* F14, *C. bertholletiae* F1 and *R. oryzae* F5) were cultured for 2 days at 30 °C on a Potato Dextrose Agar plate (PDA; HiMedia, India). The antifungal activity was determined by inoculating a 5-mm fungal plug in the center of a Petri dish with PDA mixed with the supernatant (50 % v/v) of each of the 6 carbon sources that had been autoclaved [10]. Plates were incubated at 30 °C for 4 days, the diameter of mycelium growth was measured and compared to control growth, where the supernatant was replaced with sterile distilled water. Each experiment using a single pathogen isolate was run in triplicate. The percent inhibition was calculated according to the formula as below [11].

$$\text{Inhibition (\%)} = \frac{(G_c - G_t)}{G_c} \times 100$$

where G_c (growth control) represents the mean diameter of fungal grown in PDA, and G_t (growth treatment) represents the mean diameter of fungal in treated plates.

Spore germination assay

The supernatant exhibiting the highest antifungal activity was chosen to determine the inhibition fungal spore germination. Stock solutions of crude extract and cycloheximide were prepared in DMSO and the stocks concentration was 11 and 0.5 mg/mL, respectively. Then, by serial dilution in 100 µL of potato dextrose broth medium (PDB), 1:1 dilutions were generated in 96-well polystyrene microtiter plates. Spore suspension (100 µL at 2×10^5 spores/mL) was then added to the wells. Wells receiving 100 µL of PDB without crude extract and 100 µL of spore suspension served as controls. Plates were incubated in the dark

at 25 °C for 24 h [12]. Microscopic observations were used to compare the inhibition of spore germination to the control. Each experiment using a single pathogen isolate was run in triplicate.

Recovery of biosurfactant

To recover biosurfactant, the strain with the highest surface tension activity and antifungal activity was chosen. The selected strain was cultivated in MSM with the suitable carbon source. After 2 days of cultivation, the bacterial culture broth was centrifuged for 15 min at 12,000 ×g to remove the biomass. The cell free supernatant was acidified to pH 2.0 with 5N HCl and kept at 4 °C for precipitation. The resulting solution was extracted with the equal volume of chloroform: methanol (2:1 v/v ratio). The solvent was removed by rotary evaporator and vacuum drying. The crude biosurfactant was kept at 4 °C.

Characterization of biosurfactant

Thin layer chromatography

Crude biosurfactant exhibiting the highest antifungal activity was dissolved in methanol and 3 µL of this solution was spotted at a point of origin near the bottom of TLC plate (silica gel 60, Germany). The plate was developed in a solvent system of chloroform: methanol: acetic acid in a 81:17:2 v/v ratio [13]. After development, one of the plates was sprayed with copper sulfate reagent followed by heating at 105 °C for 5 min to detect lipid as brown spots [14]. Another plate was sprayed with anisaldehyde reagent followed by heating at 105 °C for 5 min to detect carbohydrate as purple spots [15]. R_f value was then calculated in comparison with a standard rhamnolipid (sigma-aldrich, Germany).

Fourier transform infrared spectroscopy

The chemical structure and components of crude biosurfactant can be determined using Fourier transform infrared spectroscopy (FTIR). For this experiment, 1 mg of crude biosurfactant with the highest antifungal activity was combined with 100 mg of KBr and pressed to form a pellet. The FTIR spectrum was performed in the 400 - 4,000 cm^{-1} on a Vertex70 series FTIR system (Bruker, Germany), with the 0.01 cm^{-1} for wave number and 4 for resolution of spectrum [2].

Electrospray mass spectrometry (ESI-MS) analysis

The electrospray ionization mass spectrometry (ESI–MS) analysis of the biosurfactant was carried out in a 6545 LC/Q-TOF mass spectrometer (Agilent, USA) using a direct insertion pump equipped with an ESI source (Thermo Scientific, USA). ESI conditions in the electrospray Q-TOF mass spectrometer were as follows: ionization source temperature 80 °C, electrolyte voltage 200 V, and spray inlet temperature 120 °C. The equipment was run in a negative ion mode.

Chromatography-mass spectrometry analyses of fatty acids

Crude biosurfactant was subjected to a gas chromatography-mass spectrometer. Fatty acid composition of crude biosurfactant was determined as fatty acid methyl esters (FAMEs) by gas chromatography-mass spectrometry (GC-MS). The method for fatty acid methyl esters (FAME) was prepared by acid-catalyzed esterification [16]. The GC-MS analyzes were performed using a 5977A series GC/MSD system (Agilent, USA). The upper hexane layer was injected directly onto the GC-MS system, and FAMEs were separated on a capillary column (30 m×0.25 mm×0.25 µm×7 inch cage; HP-5ms Ultra Inert, Agilent, USA). The GC oven program was held at 80 °C for 8 min, raised to 250 °C at a rate 5 °C min^{-1}, raised to 290 °C at 10 °C min^{-1} and then held at 290 °C for 2 min. Helium was used as a carrier gas (1 mL/min), and the injector and ion source temperatures were maintained at 290 and 280 °C, respectively. The mass spectrometry obtained on electro ionization (EI) mode at 70 eV. FAMEs were identified using a mass spectra library in National Institute of Standards and Technology (NIST) and Wiley 10 mass spectra library.

Analytical methods

Drop collapse method

The drop collapse method was performed with micro-titre plates coated with oil. Ten microliters of culture supernatant were added to the center of the well and observation carried out for 1 min. The drops remain stable if the sample does not contain biosurfactant. If biosurfactant is present in the sample, the drops will spread or possibly collapse [17].

Oil displacement test

Oil displacement test is a method used to measure diameter of clear zone, which occur biosurfactant activity. Fifty milliliters of distilled water were added to Petri dishes followed by addition of 100 µL of ULO to the surface of the water. Then, 10 µL of the culture supernatant was put on the crude oil surface [18]. The diameter of the clear zone on the oil surface was measured.

Surface tension measurement

A Model 20 Tensiometer (Fisher Science Instrument Co., PA, USA) was used to measure the surface tension of the supernatant after two days of incubation at 25 °C. Before each sample reading, the validity of these measurements was checked by measuring the surface tension of distilled water (72 mN/m). An un-inoculated medium served as the control. The platinum ring was cleaned 3 times with water, 3 times with acetone, and left to dry between each measurement [19]. The percentage of surface tension reduction was calculated according to the formula as below:

$$\text{Surface tension reduction (\%)} = \frac{(ST_i - ST_f)}{ST_i} \times 100$$

where ST_i is the initial surface tension of culture medium and ST_f is the surface tension of culture broth after cultivation.

Emulsion test assay

Emulsification index E24 was measured the emulsification capacity of biosurfactant with hydrocarbons. Two milliliters of cell free surfactant were placed in screw-top glass test tubes in triplicate and overlaid with 2 mL of kerosene to evaluate the emulsification index. To determine the emulsification index, a mixture was vortexed for 2 min and the height of the emulsion layer was measured after 24 h [20]. Culture broth was used as a negative control for emulsification test. The emulsification index E24 (%) was calculated by dividing the measured height of emulsion layer by the mixtures total height and multiplying by 100.

Hemolytic test

Hemolytic activity was carried out as described by Carrillo *et al*. [21]. The selected strains were tested on blood agar plates containing 5 % (v/v) blood and incubated for 24 h at 30 ºC. The formation of a clear zone around a colony was used to detect hemolytic activity.

Biomass estimation

Biomass was determined as dry cell weight (DCW). Ten milliliters of culture broth were transferred to 15 mL centrifuge test tube. The sample was centrifuged at 12,000×g for 15 min. In order to eliminate residual substrates, the pellet was mixed with 10 mL of 0.85 % NaCl and centrifuged at 12,000×g for 15 min. The biomass obtained was dried overnight at 105 °C for 24 h. The biomass placed in the desiccator for 24 h and weighed.

Statistical analysis

The data was calculated with mean values and standard deviations (means ± SD) were determined from triplicate determinations. Statistical significance of the results was evaluated by one way ANOVA (analytical of variance) and Duncan multiple tests ($p < 0.05$) using SPSS 10.

Results and discussion

Isolation and screening of biosurfactant-producing bacteria

Fifty-four bacterial isolates showing different colony morphologies were obtained from soapstock-contaminated soil samples by the enrichment culture technique. Ninety-five percent (51 of 54) of the bacterial isolates were Gram-negative. It has been reported that the majority of bacteria recovered from regions affected by oil or its byproducts are Gram-negative bacteria. This is because Gram-negative bacteria have outer membranes which act as biosurfactant [22].

Primary screening of biosurfactant-producing bacteria for biosurfactant production was performed. Twenty-one isolates showed positive results by the drop collapse method and the oil displacement test (data not shown). The positive result obtained in the drop collapse test revealed the wetting activity of the

biosurfactant and its potential to produce surface-active molecules [23]. The oil displacement test is an indirect evaluation of the surface activity of a surfactant sample that has been tested against oil; a greater diameter indicates that the testing solution has a higher surface activity [24]. The oil displacement area test and the drop collapse method are quick and straightforward ways to screen bacteria isolates for biosurfactant synthesis.

Based on secondary screening results, which included reducing surface tension in the range of 31 to 43 mN/m, 12 isolates were chosen (**Table 1**). Cooper [25] stated that if an isolate reduces the surface tension of a liquid medium to 40 mN/m or less, it can be a potential biosurfactant producer. The isolates of A4, TR2, TR3, TW2, and TW3 achieved high surface tension decrease of the culture supernatant in this investigation, with values of 37, 29, 28, and 25 %, respectively. The surface tension of the culture supernatant was reduced by 17 - 23 % in 7 isolates (TP1, TR1, TU1, TO2, TW1, TO1, and ABS). The surface tension of the control (uninoculated medium) and deionized water was 50±0.5 and 72±0.0 mN/m, respectively.

Table 1 The ability of the selected isolates to produce biosurfactants and their activities.

Isolate	DC test	ODA test (mm)***	Hemolytic activity**	ST (mN/m)***	STR (%)***
TP1	+	$17\pm1.2^{f****}$	-	$42\pm0.1^{h****}$	$16\pm1.3^{g****}$
TR1	++	20 ± 1.0^{e}	-	41 ± 1.7^{gh}	17 ± 3.1^{fg}
TW3	+++	40 ± 0.6^{c}	+	37 ± 0.0^{c}	25 ± 0.4^{c}
TR3	+++	26 ± 1.2^{d}	-	36 ± 0.6^{b}	29 ± 1.2^{b}
TR2	+++	58 ± 1.0^{b}	-	35 ± 0.0^{b}	29 ± 0.7^{b}
TU1	+	21 ± 0.6^{e}	-	41 ± 0.6^{fg}	19 ± 1.0^{ef}
TO2	+++	16 ± 0.6^{fg}	-	38 ± 0.6^{d}	23 ± 2.1^{cd}
TW1	++	15 ± 1.0^{gh}	-	40 ± 1.2^{ef}	21 ± 2.8^{de}
TW2	+++	16 ± 0.6^{fg}	-	36 ± 0.0^{bc}	28 ± 0.8^{b}
TO1	++	20 ± 0.6^{e}	-	39 ± 0.5^{de}	23 ± 2.0^{cd}
A4	+++	70 ± 0.6^{a}	+	31 ± 0.5^{a}	37 ± 1.3^{a}
ABS	+	15 ± 1.2^{h}	-	43 ± 0.0^{b}	28 ± 0.3^{b}

Abbreviations; DC test, drop-collapsing test; ODA test, oil displacement test; ST, surface tension; STR, surface tension reduction

*+, low flat, ++, middle flat, +++ complete flat

**+, positive, -, negative

*** Values are given as mean ± SD from triplicate determinations. **** Different letters in the same column within the same parameter studied indicate significant differences ($p < 0.05$).

16S rDNA gene sequence analysis

Amplification and sequencing of the 16S rRNA gene were used to identify the selected biosurfactant producing strains, which were then compared to a database of known 16S rRNA gene sequences. All sequences of 12 bacterial strains were assigned with the NCBI database and deposited in DDBJ/EMBL/GenBank with GenBank accession number AB905599-AB905610. The phylogenetic tree of these isolated strains is shown in **Figure 1**. The 16S rRNA gene sequence revealed the present of 8 different genera *Stenotrophomonas*, *Pantoea*, *Klebsiella*, *Enterobacter, Xanthomonas*, *Acinetobacter*, *Citrobacter* and *Pseudomonas*. Three strains belonged to the genus *Stenotrophomona*s; TW3 (AB905600), TR2 (AB905606) and TR3 (AB905601) were identified as *Stenotrophomonas acidaminiphila*, *S. acidaminiphila* and *Stenotrophomonas* sp., with 100, 100 and 99 % sequence identity, respectively. The strains TP1 (AB905599) and TR1 (AB905603) were affiliated to *Pantoea* sp., with the sequence homology ranged from 99 to 100 %. The isolated strains TU1 (AB905602) and TO2 (AB905609) were identified as *Klebsiella pneumoniae*, with 99 % sequence identity. The strains TW1 (AB905604), TW2 (AB905605), ABS (AB905607), TO1 (AB905608) and A4 (AB905610) were identified as *Enterobacter* sp., *Xanthomonas* sp., *Acinetobacter baumannii*, *Citrobacter koseri* and *Pseudomonas aeruginosa* with 99, 99, 100, 99 and 100 % sequence identity, respectively.

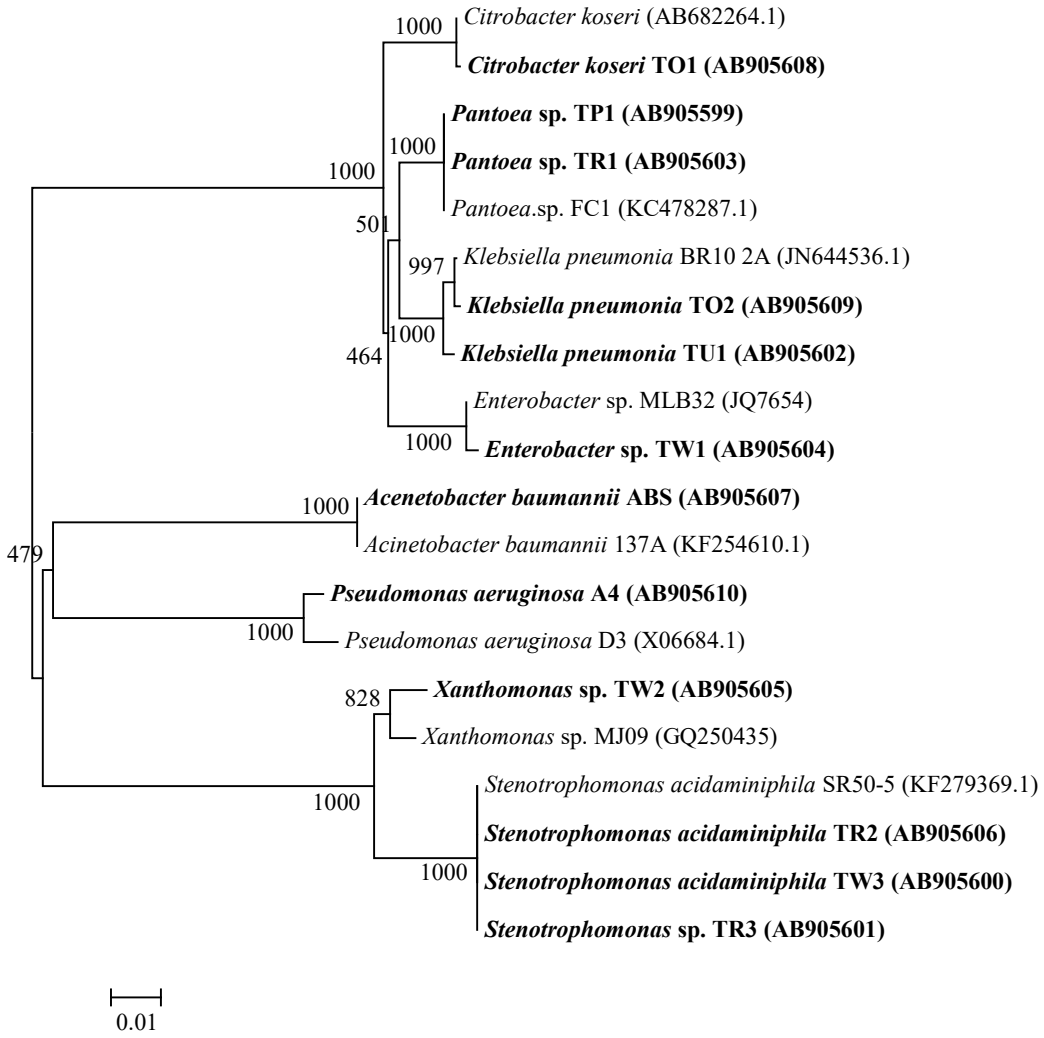

Figure 1 Phylogenetic tree based on the 16S rRNA gene sequence comparison of the selected biosurfactant producing strains featured in this study (bold) and the nearest relative in GenBank. The unrooted tree was created by using the neighbor-joining method. Scale bar 0.01 substitutions per nucleotide position.

Assessment of biosurfactant production

Surface tension and emulsification stabilization are all indicators of biosurfactant activity. When grown in MSM supplemented with soapstock, 12 strains were chosen as candidates for biosurfactant production and found to reduce surface tension to around 31 - 43 mN/m. These strains were tested for their ability to produce biosurfactant utilizing low-cost substrates. The strains were grown in a variety of carbon sources and incubated for 48 h (**Table 2**). Extracellular biosurfactant was measured at the end of the cultivation. The crude extract of TR1 (2.33 g/L) and TO2 (2.02 g/L) gives the maximum yield when crude glycerol (CG) is utilized as a carbon source. Most strains grew well on soapstock, which was one of the 6 carbon sources examined. When soapstock was used as a carbon source, the maximum dry cell weight of TW3 (4.78 g/L), TU1 (4.28 g/L), and TO2 (3.85 g/L) were obtained. Additionally, most strains grew slowly in the medium containing glucose and CS as carbon sources. The inhibition could be caused by a drop in the pH of the culture broth during cultivation. The generation of secondary acid metabolites such as uronic acid is thought to be the cause of the pH drop [26]. When the strains were grown on water-insoluble substrates, however, they grew faster. Most strains could produce biosurfactant/emulsifier to uptake immiscible substrate, according to these findings. The fact that the metabolism of substrates to synthetize biosurfactants is dependent on the intrinsic enzyme package of each strain could explain the variation in surface activity between strains and substrates [27].

Table 2 Dry cell weight, crude extract, emulsification activity and surface tension reduction of supernatants obtained from the selected biosurfactant- producing strains grown in shake flask in MSM medium supplemented with different carbon sources (1 %, w/v) at 30 °C and 150 rpm for 48 h.

Strain	Molasses				CS			
	DCW* (g/L)	CE* (g/L)	STR* (%)	E24*(%)	DCW*(g/L)	CE*(g/L)	STR*(%)	E24*(%)
TP1	1.82±0.1^{ab**}	0.33±0.2^{c}	0^{d}	62±3.8	3.65±0.0^{a}	0.25±0.0^{f}	15±1.4^{e}	51±3.9
TR1	1.13±0.0^{c}	0.82±0.0^{ab}	0^{d}	53±3.9	1.80±0.0^{c}	0.87±0.1^{bcd}	2±1.7^{gh}	56±3.9
TW3	1.17±0.2^{c}	1.10±0.1^{a}	0^{d}	65±4.0	0.77±0.4^{e}	0.62±0.1^{e}	1±0.9^{hi}	0
TR3	0.68±0.1^{e}	0.68±0.2^{b}	0^{d}	0	0.23±0.2^{f}	0.75±0.0^{de}	0^{i}	0
TR2	0.73±0.3^{de}	0.70±0.1^{b}	0^{d}	0	0.15±0.0^{f}	0.78±0.0^{d}	0^{i}	0
TU1	2.12±0.0^{a}	0.82±0.1^{ab}	0^{d}	42±7.7	2.50±0.1^{b}	1.16±0.0^{a}	52±0.6^{a}	58±3.9
TO2	1.05±0.0^{cd}	0.69±0.1^{b}	0^{d}	0	1.45±0.3^{d}	0.88±0.0^{bcd}	4±0.7^{g}	53±0.0
TW1	1.92±0.4^{a}	1.06±0.4^{a}	11±1.8^{b}	53±0.0	1.28 ±0.4^{d}	0.74±0.1^{de}	20±0.8^{d}	0
TW2	0.50±0.1^{ef}	1.66±0.1^{b}	10±1.0^{b}	0	0.15±0.0^{f}	1.00±0.1^{b}	8±0.8^{f}	0
ABS	0.27±0.1^{f}	1.02±0.1^{a}	0^{d}	0	0.35±0.1^{f}	0.83±0.0^{cd}	21±1.4^{d}	0
TO1	0.27±0.3^{f}	1.06±0.2^{a}	5±1.7^{c}	44±3.9	0.75±0.2e	0.94±0.1^{bc}	35±1.6^{b}	0
A4	1.57±0.2^{b}	0.22±0.1^{c}	33±2.0^{a}	0	0.25±0.1^{f}	0.19±0.1^{f}	25±2.4^{c}	58±3.9
Strain	**UPO**				**Crude glycerol**			
	DCW*(g/L)	CE*(g/L)	STR*(%)	E24*(%)	DCW*(g/L)	CE*(g/L)	STR*(%)	E24*(%)
TP1	3.75±0.1^{a}	1.51±0.1^{c}	40±1.3^{a}	0	3.73±0.1^{a}	0.58±0.2^{g}	6.6±1.5^{b}	14±11
TR1	1.05±0.0^{ef}	0.80±0.1^{ab}	3.5±0.5^{efg}	0	2.02±0.2^{c}	2.33±0.2^{a}	0^{d}	62±7.7
TW3	1.25±0.1^{def}	1.50±0.1^{a}	39±0.7^{a}	0	0.78±0.1^{e}	1.45±0.2^{de}	6.7±1.6^{b}	0
TR3	0.45±0.2^{f}	1.50±0.1^{b}	4.4±3.9^{def}	0	1.13±0.0^{e}	1.37±0.1^{def}	14±2.1^{a}	0
TR2	0.83±0.1^{ef}	0.90±0.1^{b}	4.8±2.9^{de}	0	0.92±0.1^{e}	1.75±0.1^{bcd}	13±3.3^{a}	0
TU1	2.15±0.7^{bcd}	0.90±0.1^{ab}	1.3±2.2^{fg}	0	2.88±0.1^{b}	1.89±0.2^{bc}	3.8±2.2^{c}	44±3.9
TO2	1.27±0.1^{def}	1.05±0.2^{b}	18.±0.8^{c}	0	1.63±0.1^{d}	2.02±0.1^{ab}	7.6±1.7^{b}	49±7.7
TW1	1.52±1.3^{cde}	1.00±0.1^{a}	7.6±1.9^{d}	0	2.30±0.6^{c}	1.59±0.6^{cde}	0^{d}	0
TW2	1.42±0.4^{cde}	1.20±0.3^{b}	19±2.0^{c}	0	1.03±0.0^{e}	1.62±0.1^{bcd}	5.7±0.0^{bc}	0
ABS	0.23±0.2^{bc}	1.00±0.1^{a}	6.1±1.9^{de}	0	2.30±0.2^{c}	0.98±0.1^{e}	0^{d}	0
TO1	1.13±0.5^{ef}	1.20±0.1^{a}	0^{g}	0	0.98±0.2^{e}	1.01±0.3^{e}	0^{d}	0
A4	2.62±0.3^{b}	1.16±0.2^{c}	35±0.4^{b}	0	3.13±0.2^{b}	1.18±0.0^{ef}	5.7±0.1^{bc}	58±3.9
Strain	**Glucose**				**Soapstock**			
	DCW*(g/L)	CE*(g/L)	STR*(%)	E24*(%)	DCW*(g/L)	CE*(g/L)	STR*(%)	E24*(%)
TP1	0.85±0.0^{b}	0.16±0.1^{e}	16±1.7^{d}	11±3.9	2.90±0.6^{d}	0.77±0.2^{cd}	16±1.3^{f}	30±0.0
TR1	1.80±0.2^{a}	0.46±0.2^{cd}	5±0.8^{g}	58±3.9	3.65±0.2^{c}	1.26±0.1^{c}	17±3.1^{f}	0
TW3	0.68±0.1^{bc}	0.49±0.1^{c}	0^{h}	0	4.78±0.3^{a}	1.91±0.1^{a}	25±0.8^{c}	0
TR3	0.43±0.2^{cd}	0.69±0.1^{ab}	0^{h}	0	1.83±0.2^{e}	1.06±0.1^{cde}	29±1.2^{b}	0
TR2	0.62±0.2^{bc}	0.68±0.0^{ab}	0^{h}	0	2.73±0.0^{d}	1.61±0.0^{b}	30±0.4^{b}	0
TU1	0.75±0.1^{bc}	0.68±0.1^{ab}	10±0.0^{f}	58±3.9	4.28±0.2^{ab}	1.02±0.2^{cde}	19±2.7^{ef}	0
TO2	0.73±0.0^{bc}	0.67±0.0^{ab}	13±1.3^{e}	60±0.0	3.85±0.1^{bc}	1.13±0.3^{cd}	23±2.1^{cd}	0
TW1	0.72±0.2^{bc}	0.54±0.1^{bc}	19±1.4^{c}	0	2.62±0.1^{d}	1.30±0.2^{c}	21±2.8^{de}	0
TW2	0.48±0.1^{cd}	0.72±0.1^{ab}	18±0.7^{c}	0	1.72±0.2^{e}	1.30±0.0^{c}	28±0.1^{b}	0
ABS	0.30±0.3^{d}	0.67±0.0^{ab}	18±1.1^{cd}	0	3.72±0.4^{c}	0.93±0.1^{de}	13±0.5^{g}	0
TO1	0.22±0.4^{d}	0.73±0.1^{a}	31±1.5^{a}	44±3.9	1.52±0.6^{e}	0.81±0.3^{e}	22±0.5^{cd}	0
A4	0.48±0.1^{cd}	0.32±0.1^{d}	27±1.6^{b}	0	3.07±0.2^{d}	1.28±0.4^{ab}	37±1.2^{a}	60±0.0

Abbreviations; DCW, dry cell weight; CE, crude extract, STR, surface tension reduction; E24, emulsification index.
*Values are given as mean ± SD from triplicate determinations, ** Different letters in the same column within the same parameter studied indicate significant differences ($p < 0.05$).

Surface tension and emulsification stabilization were used to quantify biosurfactant activity in this investigation. When CS, UPO, UPO, and soapstock were employed as carbon sources, the highest surface tension reduction was recorded in *K. pneumoniae* TU1 (52 %), followed by *Pantoea* sp. TP1 (40 %), *S. acidaminiphilla* TW3 (39 %), and *P. aeruginosa* A4 (37 %), respectively. Estimating the E24 is another method for identifying prospective biosurfactant-producing bacteria. With kerosene, some strains could produce emulsions, although this was dependent on the carbon source. Although some strains lowered surface tension, they were unable to emulsify kerosene. The results showed that the lowering of surface tension and the creation of emulsions were unrelated. This was similar to the results reported by Saimmai *et al*. [28]. According to Willumsen and Karlson [29], a good bioemulsifier had an E24 of more than 50 %. In the present study, *Pantoea* sp. TP1, *Pantoea* sp. TR1, *S. acidaminiphilla* TW3, *K. pneumoniae* TU1, *K.*

pneumoniae TO2, *Enterobacter* sp. TW1 and *P. aeruginosa* A4 exhibited an E24 higher than 50 %. When soapstock, glucose, and CG were utilized as carbon sources, the maximum emulsion index was obtained from *P. aeruginosa* A4, *K. pneumoniae* TO2, and *Pantoea* sp. TR1 with values of 60, 60, and 62 %, respectively. The stability of the emulsions has been reported to be important for both the performance and the effectiveness of the emulsifier. The stability and compactness of kerosene-supernatant emulsions were tested after 24 h and found to be stable for up to 48 h. These findings show that the bioemulsifier presented in this study could be effective in biodegradation of water-immiscible substrates or hydrocarbons, as well as improving oil recovery.

Biosurfactant produced by microorganisms also can be divided into low-molecular-mass and high-molecular-mass surfactant. Biosurfactants are low-molecular-mass surface-active agents, while emulsifiers are high-molecular-mass biosurfactants. Glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids, and polymeric biosurfactants are examples of low-molecular-mass biosurfactants, whereas high-molecular-mass biosurfactants are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers [30]. The majority of the strains in this study created low-molecular-mass emulsions, as evidenced by surface activity found greater than the emulsion.

According to the obtained results, *P. aeruginosa* A4 had the maximum ODA and surface tension reduction, but no hemolytic activity. In comparison to other strains, it also utilized soapstock as a carbon source to produce the highest biosurfactant (1.28 g/L). As a result, it was chosen for further studies.

Antifungal activity test

On the 4th day after inoculation, the effect of biosurfactant produced by biosurfactant producing bacteria on fungal growth (*A. flavus* F2, *A. niger* F14, *C. bertholletiae* F1, and *R. oryzae* F5) was calculated by comparing percentage inhibitions to the control. The antifungal activity of each supernatant was examined after the 12 biosurfactant-producing bacteria were grown in MSM medium with various carbon sources supplied. When soapstock and CG were utilized as carbon sources in this work, the supernatant of *P. aeruginosa* A4 demonstrated wide antifungal efficacy against all fungal tests. After 4 days of incubation, the supernatant of *P. aeruginosa* A4 cultured in soapstock as a carbon source revealed the highest reduced fungal growth of *A. flavus* F2 (53.7 %), *A. niger* F14 (60.8 %), *C. bertholletiae* F1 (58.8 %) and *R. oryzae* F5 (50.3 %) (**Figure 2**). Supernatants of all 12 strains exhibited to have no antifungal action against *C. bertholletiae* F1 and *R. oryzae* F5 when cultivated in MSM supplemented with MO, CS, and UPO as a carbon source, although utilizing glucose as a carbon source did not limit fungal growth of *R. oryzae* F5 (data not shown). Mold inhibition was shown to be linked to the amount of biosurfactant produced by bacteria. *P. aeruginosa* A4 grown in MSM with soapstock as a carbon source produced the highest biosurfactant content and had the best inhibitory effect against all 5 tested fungi (**Figure 3**). As a result, the crude extract obtained from *P. aeruginosa* A4 with soapstock as a carbon source was chosen for further investigation on fungal spore germination (**Table 3**). The crude extract from *P. aeruginosa* A4 inhibited *A. flavus* F2 and *R. oryzae* F5 spore germination at a minimum inhibitory concentration (MIC) of 2.75 mg/mL. Whereas, the inhibiting fungal spore germination of *A. niger* F14 and *C. bertholletiae* F1 with MIC were 5.5 and 11 mg/mL, respectively. It is therefore interesting that *P. aeruginosa* A4 produces a biosurfactant that has potent inhibitory effect against *A. flavus* F2 and *R. oryzae* F5. To investigate the preliminary structure, the biosurfactant from *P. aeruginosa* A4 was chosen.

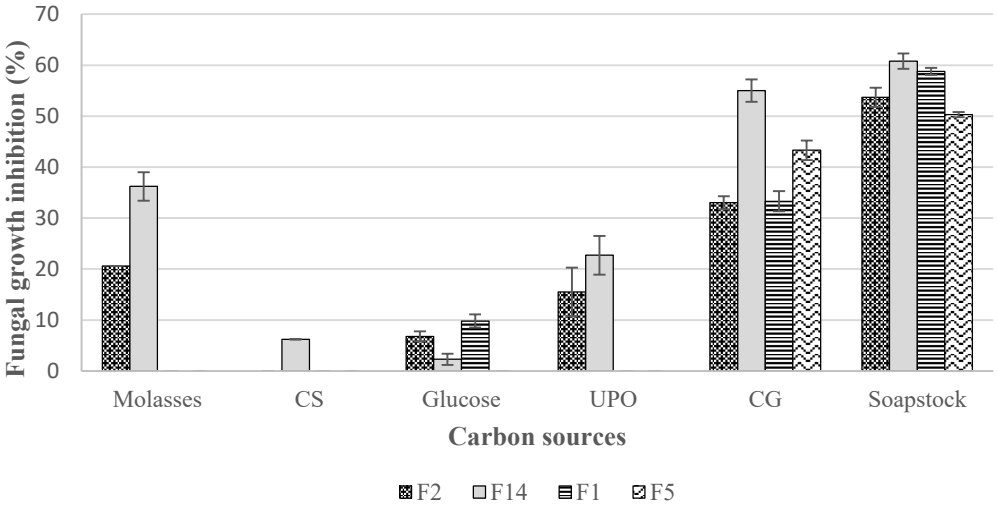

Figure 2 Antifungal activity of supernatants obtained from *P. aeruginosa* A4 cultures grown in shake flask using MSM medium supplemented with different carbon sources (1 %, w/v) at 30 °C and 150 rpm for 48 h. F2, *Aspergillus flavus* F2; F14, *Aspergillus niger* F14; F1, *Cunninghamella bertholletiae* F1 and F5, *Rhizopus oryzae* F5. CS: commercial sugar, UPO: used palm oil, CG: crude glycerol.

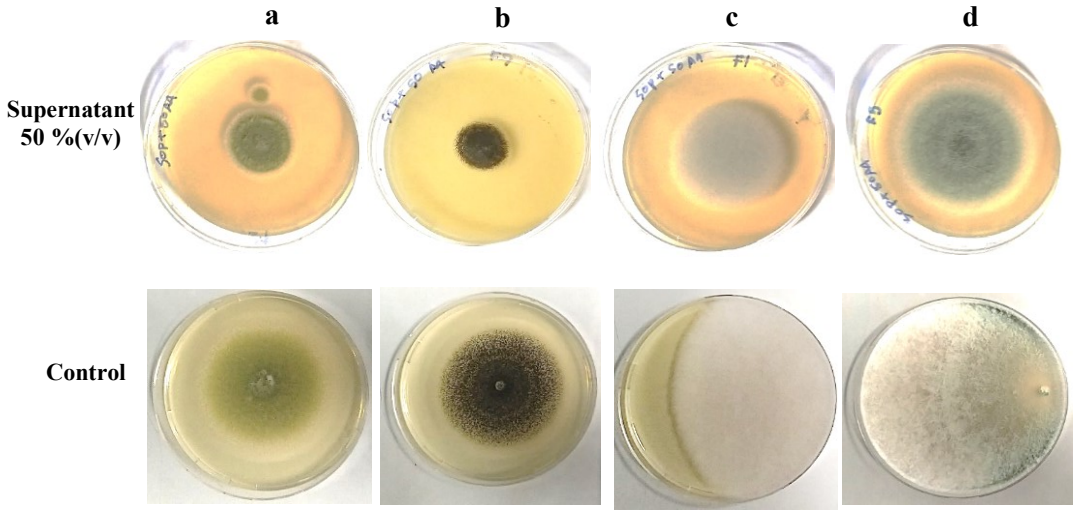

Figure 3 Photograph showing mycelial growths of fungal on potato dextrose agar (PDA) medium supplemented with the supernatant from *P. aeruginosa* A4 using sopstock as a carbon source and incubated in the dark at 25 °C for 4 days. (a), *Aspergillus flavus* F2; (b), *Aspergillus niger* F14; (c), *Cunninghamella bertholletiae* F1 and (d), *Rhizopus oryzae* F5.

Table 3 Inhibition of fungal spore germination by crude extracted from *P. aeruginosa* A4.

Fungal strains	Minimum inhibitory concentration (MIC, mg/mL)	
	Cycloheximide	A4
Aspergillus flavus F2	0.25	2.75
Aspergillus niger F14	0.13	5.5
Cunninghamella bertholletiae F1	0.5	11
Rhizopus oryzae F5	0.13	2.75

Abbreviations: A4, crude extracted from *P. aeruginosa* A4

The antifungal activity of *P. aeruginosa* has been studied in a number of investigations. *P. aeruginosa* produces rhamnolipids, a biosurfactant that affects the integrity of the fungal cell wall as well as the permeability of the cytoplasmic membrane of hyphae and spores [31]. Rhamnolipid biosurfactants produced by *P. aeruginosa* have been reported to exhibit strong antifungal activity against *Aspergillus flavus* [32] and *Aspergillus niger* [33]. The results obtained from the present study showed that *P. aeruginosa* A4 could inhibit *A. flavus* F2, *A. niger* F14, *C. bertholletiae* F1, and *R. oryzae* F5 from growing. This is the first time that a biosurfactant produced by *P. aeruginosa* A4 has been shown to be effective in controlling rot root disease in seed oil palms caused by phytopathogenic fungi.

Characterization of biosurfactant

Through thin layer chromatography, the partial characterization of biosurfactant obtained from fermentation media with soapstock as a carbon source to the bacterial strain *P. aeruginosa* A4 revealed the presence of 2 distinct areas with R_f values of 0.73 and 0.36 (**Figure 4**), which was close to the R_f values reported for similar rhamnolipid produced by *P. aeruginosa*. The R_f values of 0.74 and 0.36, according to Bhat *et al*. [13], are consistent and relate to mono-rhamnolipid and di-rhamnolipid, respectively.

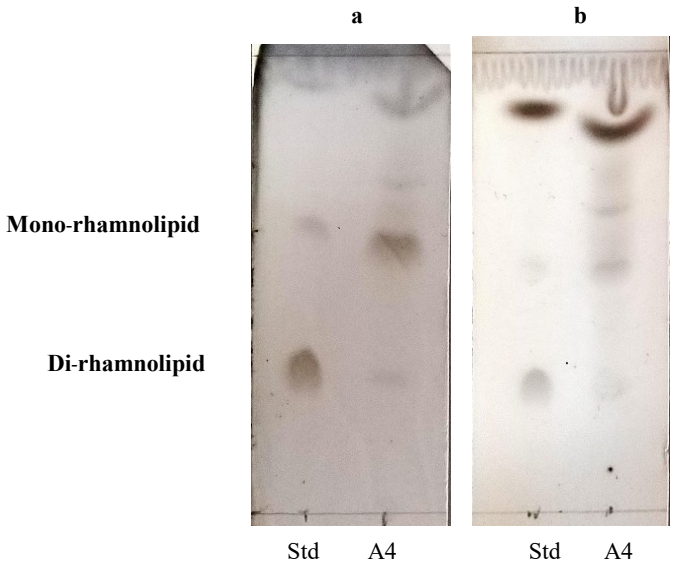

Figure 4 Thin layer chromatography analysis of rhamnolipid mixture produced by *P. aeruginosa* A4 utilizing soapstock as a carbon source, Std - Rhamnolipids standard; A4 - Crude biosurfactant. (a), Using anisaldehyde as a spraying reagent for sugar detection. (b), Using $Cu(SO_4)_2$ reagent as a spraying reagent for fatty acid detection.

FT-IR spectral analysis of the crude biosurfactant showed major peak at 3,393 cm^{-1} indicated the presence of O-H stretching vibrations (**Figure 5**). The 2 peaks in the difference spectra are at 2,853 and 2,923 cm^{-1}, which the peaks can be assigned to -CH stretching mode of CH_3 and CH_2 groups in alkyl chains. At 1707 cm^{-1} can be assigned to the C=O stretching mode of the carboxylic acid groups. The glycolipid composition of the biosurfactant was confirmed by the information from the respective wave numbers. The FT-IR spectra of rhamnolipid were similar to those of other glycolipid biosurfactants produced by *Pseudomonas aeruginosa* TMN [34] and *Pseudomonas aeruginosa* PAO1 [35].

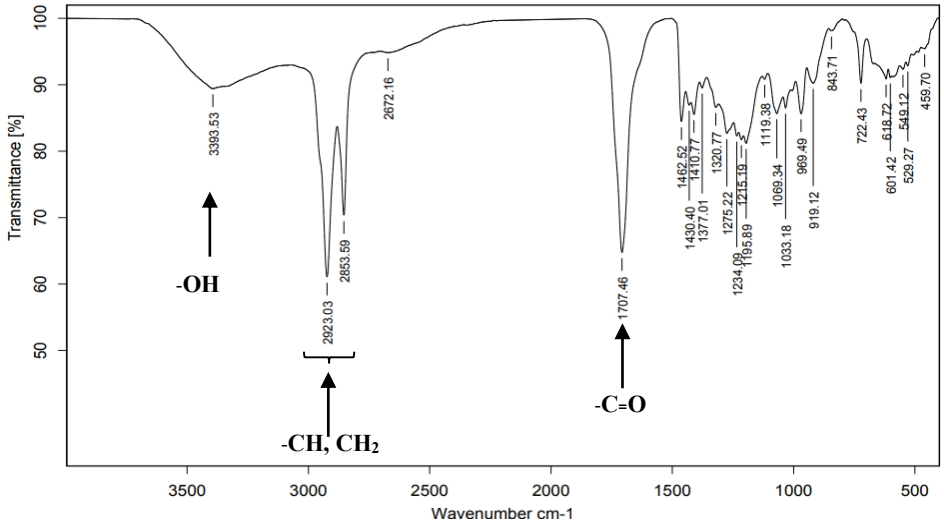

Figure 5 The FTIR spectrum of crude biosurfactant produced by *P. aeruginosa* A4.

Crude biosurfactant was subjected to ESI-MS analysis for molecular weight determination. **Figure 6** shown MS spectra in rang of 300 - 800 m/z. ESI-MS analysis also indicated the presence of both mono- and di-rhamnolipids peaks, which base on the results of Liu *et al*. [36] and Abdel-Mawgoud *et al*. [37]. The mono-rhamnolipid congener was obtained as Rha-C10-C10, Rha-C8-C10 and Rha-C10-C12. The di-rhamnolipid congener was obtained as Rha-Rha-C10-C10. The m/z values obtained for components Rha-C8-C10, Rha-C10-C10, Rha-C10–C12 and Rha-Rha-C10-C10 were 475.29, 503.4, 531.35 and 650.86, respectively.

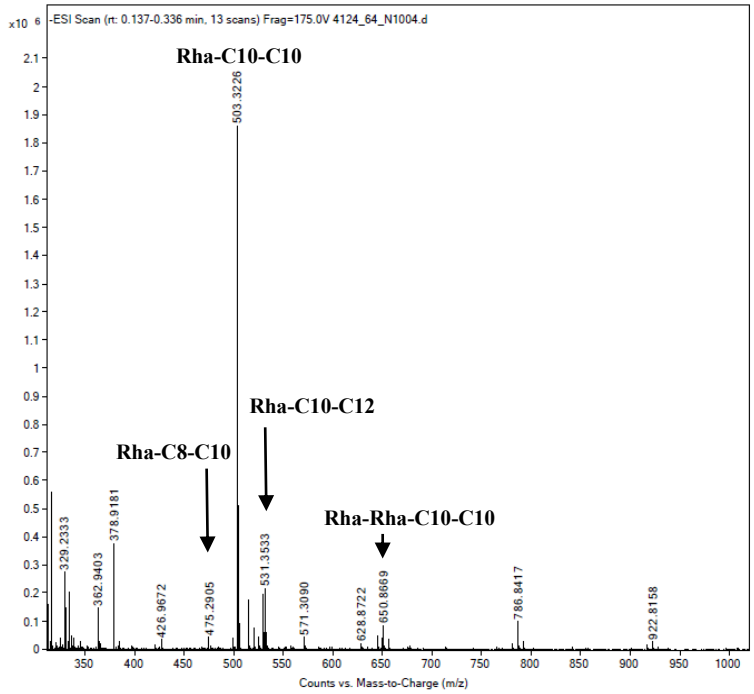

Figure 6 The ESI–MS spectrum in negative model of crude biosurfactant produced by *P. aeruginosa* A4.

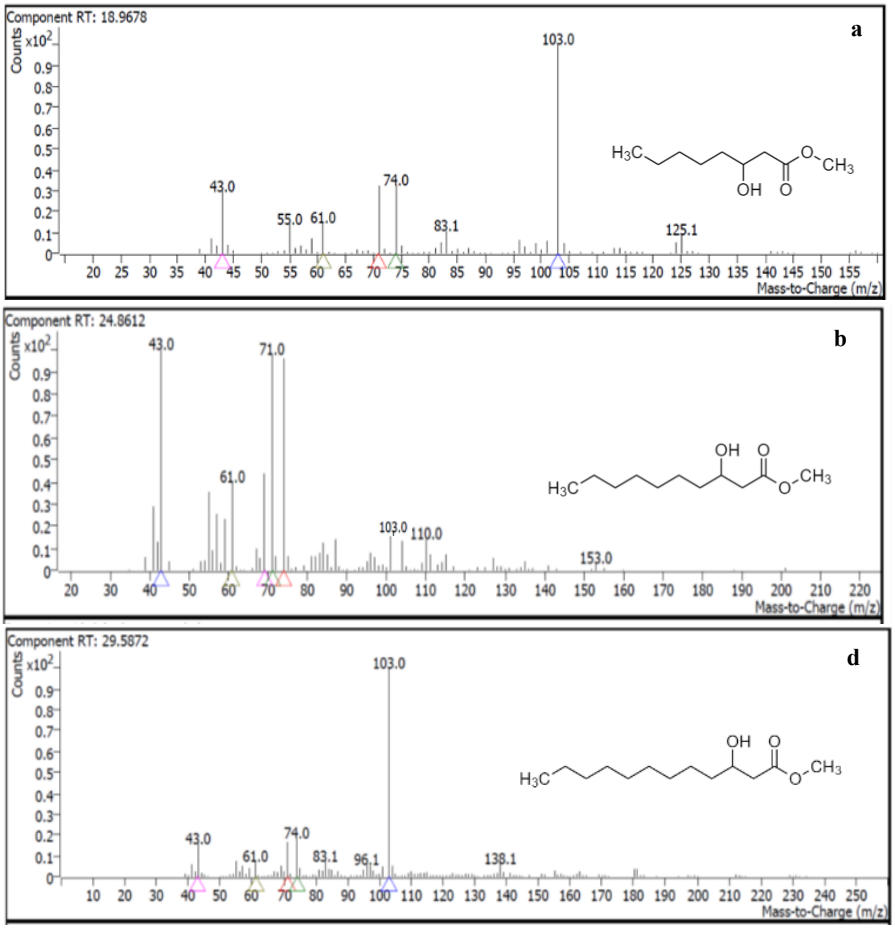

Figure 7 The MS spectra of methyl-esterified fatty acids of 3-hydroxy octanoic acid, methyl ester (a), 3-hydroxy decanoic acid, methyl ester (b) and 3-hydroxy dodecanoate, methyl ester (c).

The GC-MS, which has a greater resolution and sensitivity, is used to screen trace amounts of fatty acids for rhamnolipid congeners, which are present in low abundance. **Figure 7** depicts the results of the mass spectra. The mass spectrometry was performed at 70 eV in electro ionization (EI) mode. A mass spectra library at the National Institute of Standards and Technology (NIST) was used to identify FAMEs. According to the retention time and the appearance of the typical ions at m/z 103 of 3-hydroxy fatty acids, 5 3-hydroxy fatty acids (loss of a methyl group) were identified by MS spectra to be 3-hydroxyoctanoic acid (C8) with a retention time of 18.9678, 3-hydroxydecanoic acid (C10) with a retention time of 24.8612 and 3-hydroxydodecanoate (C12) with a retention time of 29.5872. Thus, there are rhamnolipids with 3-hydroxy fatty acids C8, C10 and C12. Due to their trace concentration, the whole structures of these rhamnolipids have yet to be determined.

Rhamnolipids are made up of one or 2 rhamnose molecules attached to up to 3 molecules of hydroxyl fatty acids with chain lengths ranging from 8 to 14, the most common of which is 3-hydroxydecanoic acid [38]. Under normal growth conditions, *Pseudomonas aeruginosa* produces two forms of rhamnolipids: mono-rhamnolipids and di-rhamnolipids, both of which have two hydrophobic chains. The presence of rhamnolipid congeners in both the mono-rhamnolipid and di-rhamnolipid was also discovered C8, C10 and C12 are examples of 3-hydroxy fatty acids. Mono-rhamnolipid and di-rhamnolipid have been linked to these components.

Conclusions

Biosurfactants are now unable to compete economically with chemical surfactants due to their low yield and higher production costs. The utilization of low-cost substrates such as industrial and domestic wastes, together with the selection of high-yield strains, provides an appropriate balance in the nutrient composition required for the microbial population. In the present study, biosurfactant-producing bacteria were isolated from soil contaminated with palm oil or soapstock from the palm oil industry and a biodiesel production plant, and biosurfactant production from agro-industrial by-products or wastes was evaluated. The use of low-cost raw materials and wastes will help to reduce production costs. *P. aeruginosa* A4 produced the highest biosurfactant (rhamnolipid) content and had the best antifungal activity when cultivated in MSM with soapstock as a carbon source. As a result, commercial applications of rhamnolipid biosurfactants should be encouraged. Furthermore, testing the obtained rhamnolipid in the field against various fungal plant diseases could be useful and could have agricultural applications in the future.

Acknowledgments

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