ETA-11

PRODUCTION AND ANTIMICROBIAL ACTIVITY OF BIOSURFACTANT FROM MANGROVE ISOLATED RUBRIMONAS CLIFTONENSIS NA1

Paweena DIKIT[1], Wiboon RIANSA-NGAWONG[2], Teera CHOOKAEW[3], Suppasil MANEERAT[4], Noraphat HWANHLEM[5] Agarat KAMCHAROEN[6] and Atipan SAIMMAI[7, 8]*

[1]*Faculty of Science and Technology, Songkla Rajabhat University 160 Kanjanvanich Road, Songkhla 90000, Thailand*

[2]*Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok 129 Tambon Noenhom, Amphoe Mueang, Prachinburi 25230, Thailand*

[3]*Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang, 1 Chalongkrung Rd. Ladkrabang, Bangkok Thailand 10520 Bangkok 10520, Thailand*

[4]*Faculty of Agro-Industry, Prince of Songkla University 15 Karnjanavanich Road, Hat Yai, Songkhla 90112, Thailand*

[5]*Faculty of Agriculture Natural Resources and Environment, Naresuan University 99, Mueang, 65000, Phitsanulok, Thailand*

[6]*Faculty of Agricultural Technology, Burapha University Sakaeo Campus 254, Watthananakhon, 27160 Sakaeo, Thailand*

[7]*Faculty of Agricultural Technology, Phuket Rajabhat University 21 Thepkasattri Road, Phuket 83000, Thailand*

[8]*Andaman Halal Science Center, Phuket Rajabhat University 21 Thepkasattri Road, Phuket 83000, Thailand*

Corresponding author: Atipan SAIMMAI. E-mail: s4680108@hotmail.com

ABSTRACT

A mangrove bacterial isolated, Rubrimonas cliftonensis NA1, was able to grow and produce biosurfactant on minimal salts medium supplemented with molasses and commercial monosodium glutamate with a reduction in the surface tension up to 25.5 mN/m. R. cliftonensis NA1 produced 5.02 g/L of crude biosurfactant after 51 h of cultivation. For recovery of biosurfactant from the culture supernatant, various recovery methods either precipitation by pH, ammonium sulphate or solvent extraction were compared. Solvent extraction using ethyl acetate showed the best results in terms of biosurfactant recovery. Further purification was carried out by column chromatography including normal and reverse phase chromatography. The obtained biosurfactant displayed a significant antimicrobial against several tested bacterial and fungal strains suggesting its role as biocontrol applications.

Keywords: Antimicrobial activity; Biosurfactant; Rubrimonas cliftonensis; Mangrove

INTRODUCTION

Biosurfactants are microbial derived surface-active compounds and amphipathic molecules produced by various microorganisms [1]. In recent years, these molecules have attracted great scientific attention due to their superior properties like high surface activities, less toxicity and easy biodegrada-bility [2]. As biosurfactants are produced through biological processes, their medical use is advantageous owing to their structural diversity, low irritancy, and compatibility with human skin [1-2]. They have also been used for many other purposes such as food additives (emulsifiers) in the food industry, herbicides and pesticides in the agricultural industry, and even for bioremediation, cosmetics, and pharmaceuticals [3]. In their pharmaceutical applications, there have been reports claiming that biosurfactants exhibit biological activities in the form of anti-adhesive, antibiotic, anticancer, antifungal and antiviral effects [1-3].

In the last years, biosurfactants produced by bacteria (both Gram-negative and Gram-positive bacteria) are getting more attention for their antifungal activity, owing their lower toxicity for plants and animals, high biodegradability [1, 3]. Also, this caused bioavailability insoluble in water substratum, to bind heavy metals, to interfere to sensing quorum process specific microorganisms and to form biofilms [2]. The present study reports production and antimicrobial activity of biosurfactant from mangrove isolated *Rubrimonas cliftonensis* NA1. *R. cliftonensis* NA1 (AB68-5267) used in this study was isolated and preliminarily characterized as a biosurfactant-producing strain as reported earlier [4].

MATERIALS AND METHODS

Culture conditions and biosurfactant production

Biosurfactant production was carried out in 250 Erlenmeyer flask with a working volume of 100 mL. *R. cliftonensis* NA1 was cultivated in mineral salts medium (MSM) containing the following (g/L): 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 [4]. The pH of the medium was adjusted to 7.0±0.2, molasses (15g/L) and commercial monosodium glutamate (CMSG,1g/L) were used as the carbon and nitrogen sources, respectively. The sterilized culture medium was inoculated with 5% (v/v) inoculum containing 10^6 colony-forming units (CFU)/mL, and the culture was cultivated at 30°C, at 150 rpm for 48 h [4]

Recovery of biosurfactant

Acid precipitation, ammonium sulphate precipitation and four solvent systems, namely, chloroform: methanol (2:1), cold acetone, dichloromethane, ethanol and ethyl acetate [4-5], were examined for the biosurfactant extraction. The recovery method showing the highest biosurfactant activity was used to recover biosurfactant from *R. cliftonensis* NA1.

Purification of biosurfactant

After 54 h of fermentation, culture broth was centrifuged at 4,830 g for 20 min at 4°C to remove the cells. The biosurfactant was recovered from the culture supernatant by ethyl acetate extraction. The crude biosurfactant was extracted with methanol for further use. Further purification of the biosurfactant was performed by an adsorption chromatography on a silica gel column (1.0 30.0 cm, Wako gel C-100, Wako Pure Chem. Ind., Ltd., Osaka, Japan) by sequential elution with dichloromethane:chloroform (1:1), ethyl acetate and methanol. The 20 mL fractions were collected and assayed for surface activity by drop-collapsing test [6]. The fractions containing surface active agents were pooled and concentrated in vacuum. The residue was dissolved in a minimum volume of deionized water and further purified by a Sep-Pak C_{18} cartridges (Waters Ass-ociates, Milford, MA, USA). The pooled highest active fraction was chosen for further chemical characterization.

Chemical characterization of biosurfactant

The carbohydrate content of the biosurfactant was determined by the phenol-sulfuric acid method [7]. Protein content was determined by the method of Lowry et al. [8]. The lipid content was estimated by following the procedure of Folch et al. [9]. The chemical nature of the biosurfactants obtained was determined with thin layer chromato-graphy (TLC) as previously described by Chooklin et al. [4]. Further characterization of the biosurfactant was carried out using Fourier transform infrared spectroscopy (FT-IR) by the KBr pellet method [4].

Antimicrobial activity of biosurfactant

The crude biosurfactant from *R. cliftonensis* NA1 was individually tested against a panel of microorganisms including bacteria and fungi by using agar well-diffusion method described by Candan et al [10]. Briefly, each microorganism was suspended in sterile saline and diluted at ca. 10^6 CFU/mL for bacteria and 1×1 cm^2 for 5 days cultivated fungus (10^8 spore/mL). They were "flood-inoculated" onto the surface of Mueller-Hinton agar. The wells (5 mm in diameter) were cut from the agar and 100 µl of biosurfactant solution was delivered into them. After appropriated incubation time (1 and 5-7 days for bacterial and fungus, respectively), all plates were examined for any zones of growth inhibition, and the diameters of these zones were measured in millimeters [10].

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 activity was drawn up according to Cooper and Goldenberg [11]. 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 activity was determined after 24 h, and 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 Instru-

ment Co., PA, USA) at 25°C. The CMC was deter-mined by plotting the surface tension versus the concentration of biosurfactant in the solution.

The chemical characteristics of the biosurfactant were assessed by 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) anisaldehyde for detection of the sugar moieties [12]. 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 Statistical Package for Social Science (SPSS 10.0, for Windows Inc., Chicago, IL).

RESULTS AND DISCUSSION

Production of biosurfactant by *Rubrimonas cliftonensis* NA1

In order to reduce the cost of biosurfactant production, the cheaper carbon source molasses was used. Maximum biomass of 4.98 g/L was obtained at 48 h (Figure 1). However, maximum biosurfactant activity (25.5 mN/m and 66% of emulsification activity) and biosurfactant concentration (5.02 g/L) were achieved at 54 h of incuba-tion, when the cells reached their mid-stationary phase. Higher biosurfactant activity was found after the growth was offset (Figure 1). The reason for these results may be because of the release of cell-bound biosurfactant at the early stationary phase (54 h), which leads to an increase in biosurfactant activity in the culture medium. A similar observation was reported by Saimmai et al. [13-14] and Suwansukho et al. [15] while culturing *Oleomonas sagaranensis* AT18, *Selenomonas ruminantium* CT2, and *Bacillus subtilis* MUV4 by using molasses and glucose as the sole carbon source for biosur-factant production, respectively. The biosurfactant production using molasses as a carbon source (5.02 g/L which reduced surface tension to 25.5 mN/m) in the present study was higher than the earlier reports [13-14, 16-17]. This indicates the potential value of molasses as a renewable cheaper carbon source for biosurfactant production. There are several reports on biosurfactant production kinetics by Archaea and bacteria in the last few decades [12-18]. However, to our best knowledge, this is the first report on biosur-factant kinetics from the genus *Rubrimonas*.

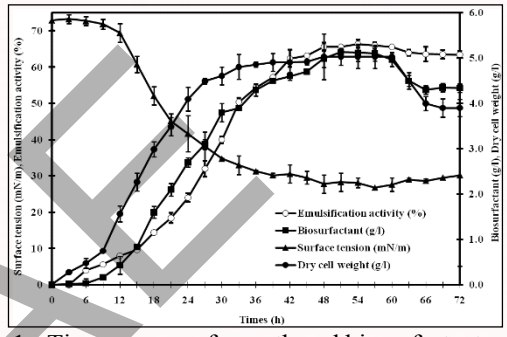

Fig.1. Times course of growth and biosurfactant production by Rubrimonas cliftonensis NA1 in minimal salts medium supplemented with 15 g/L of molasses and 1 g/L of commercial monosodium glutamate as a carbon and nitrogen sources, respectively. Bars indicate the standard deviation from triplicate determinations.

Recovery of biosurfactant

For industrial applications of biosur-factant, the efficient recovery from the culture broth is desired. Table 1 shows the recovery of biosurfactant from the culture supernatant of mangrove isolated *R. cliftonensis* NA1 by monitoring the reduction of surface tension and critical micelle concentration (CMC).

Table 1. Effect of extraction methods on yield and critical micelle concentration (CMC) of biosurfactant produced by *Rubrimonas cliftonensis* NA1.

Recovery method	Yield (g/L)	CMC (mg/L)	ST (mN/m)*
Acetone precipitation	6.65±0.85[b*]	16	61.5±2.32
Acid precipitation	7.34±1.24[a]	30	50.0±3.54
(NH4)2SO4 precipitation	3.58±0.23[e]	25	31.5±0.50
CH3Cl:MeOH extraction	4.21±0.55[d]	12	32.5±0.47

CH_2Cl_2 extraction	5.22±0.31[c]	25	29.0±0.70
Ethanol precipitation	3.27±0.27[e]	24	33.5±0.45
Ethyl acetate extraction	5.02±0.53[c]	8	25.5±0.55

* Different letters in the same row indicate significant differences ($p<0.05$)

The highest yield (7.34 g/mL) was obtained from acid precipitation however, the CMC and surface tension at CMC of this method was high (30 mg/L and 50 mN/m, respectively) when compared with other tested methods. Most of biosurfactants recovered by acid precipitation tend to exhibit emulsification activity rather than the reduction of surface tension. In addition, normally it is a high molecular weight biosurfactant which consist of protein [19]. Among seven methods, ethyl acetate extrac-tion was the most efficient in biosurfactant recovery with 5.02 g/L, 8 mg/L and 25.5 mN/m for yield, CMC and surface tension at CMC, respectively. It was also reported previously that the extraction of bioproducts with considerably high polarity by ethyl acetate solvent is quite efficient [20]. Moreo-ver, because the recovery and concentration of biosurfactants from fermentation broth largely determines the production cost, ethyl acetate is a better choice than the highly toxic chloro-organic compounds as chloroform-methanol or dichloromethane solvent systems.

Purification and identification of biosurfactant

The crude biosurfactant was determined to be lipopeptide in nature using ninhydrin reagent and iodine vapor (data not shown). Ninhydrin dye bound to the amino acid portions and the spots were detected after heated at 100°C for 5 min. Three blue spots were observed after ninhydrin reagent staining indicating amino acid containing compounds. From Table 2, the fraction B (Rf= 0.45) showed the highest biosurfactant activity and was further purified by Sep-Pak C_{18} column chromatography and gave three purified fraction (data not shown). After reverse-phase chromatography, the fraction D (Rf=0.43) showed the highest activity. Accor-dingly, this fraction was further used for antimicrobial activity study.

Table 2. Biosurfactant activity of the pooled fractions obtained from the normal- and reverse-phase column chromatography

Solvents used as eluent (proportion)	R_f (No.)	Drop-collapsing test (mm)*
Normal-phase		
CH_2Cl_2:$CHCl_3$ (1:1)	0.70 (A)	1.52±0.1[b***]
$C_4H_8O_2$:$CHCl_3$ (1:1)	0.45 (B)	3.87±0.2[a]
	0.21 (C)	1.20±0.0[b]
Reverse-phase**		
H_2O:$CHCl_3$ (9:1)	0.43 (D)	3.53±0.2[a]
H_2O:$CHCl_3$ (9:2)	0.40 (E)	1.07±0.3[b]
H_2O:$CHCl_3$ (9:4)	0.36 (F)	0[c]

* Values are given as means ± SD from triplicate determinations.
**Reverse-phase chromatography was performed by using fraction B (R_f = 0.45) from first step.
*** Different letters in the same row indicate significant differences ($p<0.05$)

Surface tension and critical micelle concentration

The relationship between surface tension and concentration of the extracted biosurfactant solution was determined by a du Nouy'a ring tensiometer (Figure 2). The biosurfactant produced exhibited excellent surface tension reduction activity. The surface tension of water (72.2 mN/m) decreased to 25.5 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 *R. cliftonensis* NA1 showed a lower minimum surface tension and CMC value than those of the biosurfactants from *Seleomonas ruminantium* CT2 (25.5 mN/m, 8.0 mg/L) [14], *Lactobacillus paracasei* A20 (41.8 mN/m, 2.5 mg/mL) [21], *Pseudomonas aeruginosa* MA01 (32.5 mN/m, 10.1 mg/L) [22], *Pseu-domonas gessardii* (31.0 mN/m, 65.4 mg/L) [23], and *Leucobacter komagatae* 183 (26.5 mN/m, 9.0 mg/L) [24].

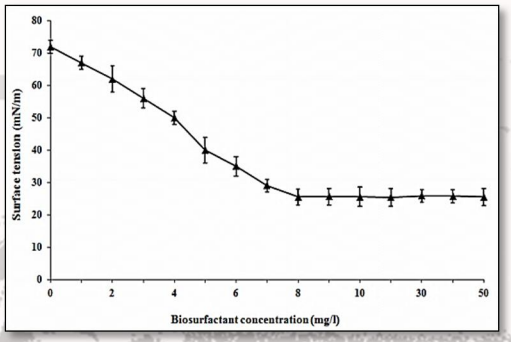

Fig.2 Surface tension *versus* biosurfactant concentration of biosurfactant produced by *Rubrimonas cliftonensis* NA1.

Chemical characterization of the biosurfactant

The biochemical composition of the biosurfactant revealed that it is a mixture of protein and lipid in a combination of 67 and 28%, respectively and the FT-IR spectra of the obtained is shown in Figure 2.

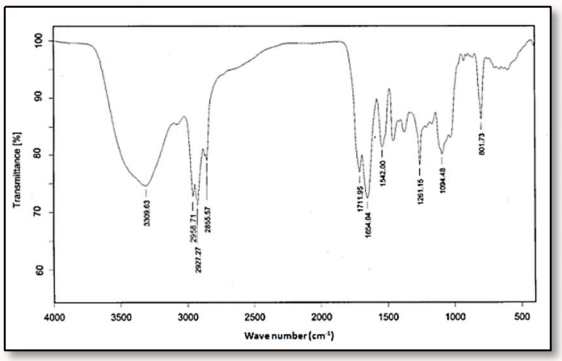

Fig.3. Fourier transform infrared spectrum of biosurfactant produced by *Rubrimonas cliftonensis* NA1.

FT-IR spectral analysis of the biosurfa-ctant showed a band at 3309 cm^{-1} assigned to the OH stretching band presence of carboxylic acids and another band at 2958 cm^{-1} assigned to N-H/C-H bonds of protein. C-H_2/C-H asymmetric vibrations were found at 2927 cm^{-1} and 2855 cm^{-1} which confirmed the presence of alkanes (C-H) (Figure 3). CH and CH_2 deformation was found at 801, 1094 and 1261 cm^{-1}. The presence of a C=O, C=C and C-O bond was found at 1711, 1654 and 1542 cm^{-1}, respectively. These data confirmed the lipopeptide nature of the biosurfactant [25]. To the best of our knowledge, this is the first report describing the lipopeptide type biosurfactant produced by the genus *Rubrimonas*.

Antibacterial activity of biosurfactant

The obtained biosurfactant of mangrove isolated *R. cliftonensis* NA1 was found to be an antibacterial agent however, depending on the microorganism as shown in Table 3 and Figure 4.

Table 3. In vitro antimicrobial activity of crude biosurfactant with different concentrations against a panel of microorganisms by agar well diffusion method.

Microorganisms	Antimicrobial zone diameter (mm) at concentration (mg/ml)*				
	200	100	50	25	12.5
Gram-positive bacteria					
Bacillus cereus	26.31±2.10	23.38±1.65	15.75±3.50	7.81±1.15	0
Staphylococcus aureus	21.78±3.52	17.65±3.55	12.96±3.22	5.66±2.05	0
Gram-negative bacteria					
Escherichia coli	13.15±2.04	9.35±1.65	7.70±2.10	0	0
Salmonella Thyphi	11.47±3.51	6.78±2.05	5.35±1.15	0	0
Vibrio vulnificus	18.89±2.06	15.33±2.36	10.72±3.23	0	0
Vibrio cholerae	10.73±1.50	7.72±1.68	5.39±2.50	0	0
Fungi					
Aspergillus niger	32.16±3.45	25.22±3.10	20.31±3.05	10.35±2.10	0
Aspergillus oryzae	30.63±3.93	27.76±5.41	21.44±4.71	7.36±2.21	0
Candida albicans	11.48±2.26	8.21±2.36	7.05±2.32	4.92±1.27	0
Mucor sp.	20.30±4.20	15.36±1.77	8.56±2.49	0	0
Penicillium sp.	8.41±2.74	5.39±4.25	0	0	0
Rhizopus oryzae	15.83±3.25	11.57±2.08	5.63±1.01	4.01±1.18	0

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

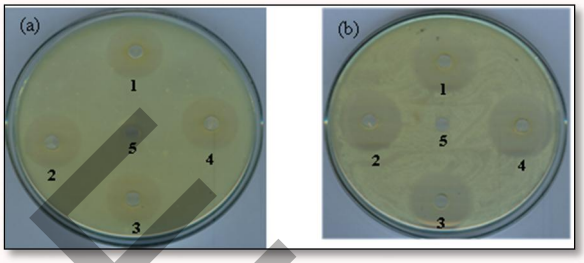

Fig.4. Antibacterial activity of the crude biosurfactant from Rubrimonas cliftonensis NA1 against Staphylococcus aureus (a) and Bacillus cereus (b). The numbers (1-4) denote the 50 mg/mL of crude biosurfactant dissolved in distilled water. Distilled water (5) was taken as a control in one of the wells.

Figure 4 shows the high antibacterial activity of the biosurfactant against *S. aureus and B. cereus*. The biosurfactant was able to inhibit *B. cereus* and *S. aureus* in concentrations as low as 25 mg/mL (Table 3). However, the MIC values for most of the tested Gram-negative bacteria (*E. coli, S.* Typhimurium*, V. vulnifus* and *V. choleare*) were 50 mg/mL. High MIC-values correlating with higher resistance were often seen when Gram-negative microorganisms were tested [26]. Higher resistance of Gram-negative bacteria to external substances had been reported [27]. It is attributed to the presence of lipopolysaccarides, making them naturally resistant to certain antibacterial agents, in their outer membrane [28]. On the other hand, Gram-positive tested strains showed higher sensitivity against the biosurfactant than the Gram-negative bacteria. The reason could be attributed to the differences between their cell wall compositions [29].

Antifungal activity of biosurfactant

Figure 5 shows the antifungal activity of the biosurfactant from mangrove isolated *R. cliftonensis* NA1 against *A. niger* and *Mucor*

sp. The results showed that the biosurfactant significantly inhibited the growth of all tested fungi (Table 3). The antifungal activity of the crude biosurfactant was dosage-dependent, at the high concentration tested (100 mg/mL) bio-surfactant was active against all tested fungal strains. Of the tested fungal, *A. niger*, *A. oryzae* and *R. oryzae* were more sensitive species than the rest. The obtained biosurfactant exhibited low activity against *Penicillium* sp.. Many compounds with antifungal activity have been isolated from different microorganisms. Among bacteria, the genus *Bacillus* sp. can produce more than 24 antifungal substances with a wide variety of structures, such as bioactive peptides and volatiles [30-31]. Surfactin produced by *B. subtilis* are a prominent group of cyclic peptidolipids, is constituted by a heptapeptide (ELLVDLL) with the chiral sequence LLDLLDL interlinked with *β*-hydroxy fatty acid of the chain lengths 12 to 16 carbon atoms to form a cyclic lactone ring structure [32]. Surfactins possess strong antifungal activities against various pathogenic yeasts and molds. The antifungal activity of surfactin lipopeptides is related to their interaction with the cytoplasmic membrane of target cells leading to an increase in K^+ permeability [33]. However, this is the first report describing the antifungal activity of biosurfactant obtained from *Rubrimonas cliftonensis*.

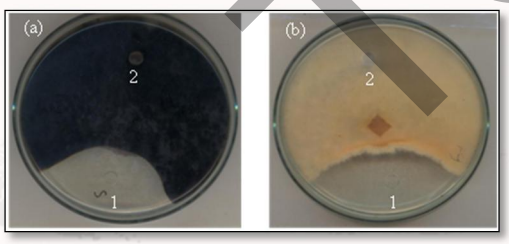

Fig.5. Antifungal activity of the crude biosurfactant from Rubrimonas cliftonensis NA1 against Aspergillus niger (a) and Mucor sp. (b) after incubation on Sabouraud Dextrose Agar for 6-7 days. The numbers 1 denote the 50 mg/mL of crude biosurfactant dissolved in distilled water. Distilled water (2) was taken as a control in one of the wells.

CONCLUSIONS

In the present study, the production of the biosurfactant from a *Rubrimonas cliftonensis* NA1 which was isolated from mangrove sediment is reported. The growth characteristics were obtained and studies on the properties of the biosurfactant indicate the possibility of its industrial application. The recovery method of biosurfactant from fermentation broth with *R. cliftonensis* NA1 culture was examined. Among tested methods, solvent extraction using ethyl acetate was the best biosurfactant recovery with 5.02 and 0.008 g/L for yield and CMC, respectively. At the CMC it can reduce surface tension of pure water to 25.5 mN/m. The obtained biosurfactant from column chromatography displayed a significant antibacterial and antifungal activity against several tested microorganisms suggesting its role in biocontrol as well as therapeutic applications.

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