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Preliminary evaluation of a new low-cost substrate (amurca) in production of biosurfactant by *Pseudomonas aeruginosa* isolated from fuel-contaminated soil

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Abstract

Biosurfactant producing bacterium, designated strain MA-1, was isolated from gas station soil contaminated with fuel and was identified as *Pseudomonas aeruginosa* based on morphological, physiological and biochemical tests. In this study, amurca (olive oil lees) was used as a carbon source to produce biosurfactant. The results showed that the growth of the isolate on medium containing amurca as a sole carbon source gave an emulsification index (E_{24}) of 37.5 % after 104 h of incubation and a cleaning activity of 30% after 112h. On the other hand, biosurfactant produced by the isolate showed antibacterial activity against pathogenic bacteria: *Staphylococcus aureus* ATCC 43300 and *Staphylococcus aureus*. The FTIR analysis showed that Pseudomonas aeruginosa strain tested in this study produced glycolipids in the medium. Besides, amurca medium yielded 12.4 g/L of biosurfactant in 112h

1. Introduction

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity. These molecules reduce the surface tension and interfacial tension and create microemulsions [1]. Several studies have examined the production of biosurfactants by microbes [2-5]. Biosurfactants are produced by bacteria or yeast from various substrates including sugars, glycerol, oils, hydrocarbons and agricultural wastes [6]. Biosurfactants are classified as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, and polymeric or particulate compounds [7]. Generally their structure includes both hydrophobic group (unsaturated or saturated fatty acid) and hydrophilic group (amino acids or peptides anions or cations; mono-, di-, or polysaccharides) [8]. Biosurfactants are of interest because of their chemical diversity, their large-scale production [9], and their use in environmental protection [10]. Their properties have greatly extended their applications in the food, pharmaceutical and oil industries, especially as improved alternatives to chemical surfactantssuch as1-tetradecyl-2-(tetradecylthio)-1H-benzimidazole[11] andnon ionic surfactants (Tween 20 and Tween 80) [12]. Advantageous properties of biosurfactants for commercialization are their specific action, lower toxicity, higher biodegradability, effectiveness at extremes of temperatures, pH and salinity, widespread applicability and their unique structures, which provided them with newer applications [13]. However, biosurfactantsare characterized by a high production costs as compared to synthetic surfactants [14].

The choice of inexpensive raw materials is important to overall economy of the process because often, the amount and type of a raw material can contribute considerably to the production cost[7]. There are few reports on biosurfactants production using inexpensive raw materials as substrates.

The purpose of this work was to study the biosurfactant production by *Pseudomonas aeruginosa* using amurca; which is known as waste product from olive oil industry; as carbon and nitrogen source. The produced biosurfactant was also testeda for its antibacterial activity against some pathogenic bacteria.

2. Materials and methods

2.1. Microorganism and culture conditions

The bacterial strain capable of producing biosurfactants was isolated from gas station soil samples contaminated with fuel located in Boumerdès, Algeria, by selective enrichment culture technique. This kind of soil was selected because it may contain indigenous bacterial strains, which should be capable of growing in oil using it as sole carbon source. They could, therefore, present a high emulsifying activity by producing biosurfactants.Briefly, soil sample (2.5 g) was introduced in an Erlenmeyer flask containing 250 mL mineral medium described below, amended with 2% (v/v) mixture of two fuels (petrol and diesel) (1:1, v/v). After 7 days of incubation at room temperature under magnetic stirrer agitation, 5 mL of the culture were used to inoculate a new 250 mL Erlenmeyer flask containing fresh culture medium and then incubated under the same conditions. This operation was repeated four times. Serial decimal dilution technique was used to isolate the bacterial strains capable of producing biosurfactants.Briefly, a sample of 0.1 mL of each decimal dilution (10^{-1}-10^{-5}) was spread on nutrient agar (Institut Pasteur d'Algérie, Algeria) plates. After incubation for 48h at 35°C, morphologically different individual bacterial colonies were isolated from the agar plates and separately plated on nutrient agar.

The bacterial strain, which exhibited the highest biosurfactant production,was identifiedusing API 10 S test kit (API system, BioMérieux, Marcy, France) which is a standardized system used for the identification of non-fastidious, non-enteric Gram-negative rod-shaped bacteria such as *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, etc. This test kit consists of 11 miniaturized biochemical tests. The strains are identified according to the API identification manual. On the basis of its morphological, physiological, biochemical tests, the strain tested in this study was Gram negative, obligatorily aerobic, motile, straight rods. The culture has the ability to reduce nitrate and was oxidase and catalase positive. The numerical profile of the strain (6422) supported its affiliation to *Pseudomonas aeruginosa* species and it was tentatively named*Pseudomonas aeruginosa* MA-1.

2.2. Medium and culture conditions

Amurcawas obtained from an olive oil producer located in Bouzoulem, Béjaïa, Algeria. Before being used as source of nutrient, amurca was amended with the mineral medium described below to make a final concentration of 4% (v/v) amurca: K_2HPO_4 (1.6g/L), KH_2PO_4 (0.4g/L), $MgSO_4$ (0.09g/L), NaCl (15g/L), NH_4NO_3 (0.1g/L), $CaCl_2$ (0.02g/L), $ZnSO_4$ (0.01g/L), $FeSO_4{\cdot}7\ H_2O$ (0.05g/L), $MnSO_4{\cdot}H_2O$ (0.008g/L), $CuSO_4{\cdot}5\ H_2O$ (0.004g/L), $Co(NO_3)$ (0.0026g/L), 1 L of distilled water.

The obtained growth medium was autoclaved at 120°C for 20 min and stored at 6°C for further use.

2.3. Kinetic study

The subculture of *Pseudomonas aeruginosa*, was conducted at room temperature (22.7°C, max. deviation ± 1°C) for 48 h at 50 rpm in 100 ml Erlenmeyer flask containing 50 ml of amurca medium and inoculated with 1 ml of the cell suspension corresponding to an inoculum of 1.5×10^7 colony-forming units (CFU)/ml. The pH of the media was 7.0. The cultivation was conducted in 500 ml shaking (150 rpm) Erlenmeyer flask containing 200 ml of fresh amurca medium and incubated at room temperature and pH 7. The flask was subsequently inoculated with 50 ml subculture. Samples were taken every 8 h during almost 5 days (112h). The samples were first centrifuged at 5000 rpm for 30 min and the cell-free supernatants were checked for E_{24}. Besides, the $E_{24}$of fresh amurca medium used as a negative control was also determined. All cultures were conducted under aerobic conditions.

2.4. Analytical procedures

2.4.1. Physicochemical analysis of amurca

Amurcawas subjected to the following analyses:

The content of reducing sugars was determined by dinitrosalicylic acid method [15]. The total concentration of protein was determined by Bradford method [16]. The amount of nitrogen was determined using N:P conversion factor [17]. It was calculated by dividing the protein content by a factor of 5.74. All reagents and solvents used in the experiments were of analytical grade.

2.4.2. Biosurfactant estimation

Biosurfactant production was estimated by determining the emulsification index (E_{24}) which reflects the emulsifying capacity of the produced biosurfactant. The E_{24} was determined by introducing 2 ml of kerosene and 3 ml of the spent culture medium after centrifugation at 5000 rpm for 10 min to remove cells, in 15 ml tube test, and vortexing at high speed (2500rpm) for exactely two minutes [18]. The height of emulsion layer was measured using a graduated ruler after 24h. The equation used to determine the emulsification index (E_{24}) is as follows [18]:

$$E_{24} = \frac{The\ height\ of\ emulsion\ layer\ (cm)}{The\ height\ of\ total\ solution\ (cm)} \times 100$$

All experimental measurements were carried out in duplicate and average values were used.

2.4.3. Cleaning test

The cleaning ability of the produced biosurfactant was determined according to Pruthi and Cameotra (2000) [19] procedure. The test was performed by coating the inner walls of 10 ml beaker with motor oil (CHABA HD 40, RebexOil, Algeria). Exactly 10 ml of cell-free culture medium were then added into the beaker, and vortexed for 1 min. The mixture was left for 6 h, and the removal of adhered oil was determined according tothe following Equation:

$$Oil\ removal\ (\%) = (H_c/H_t)\times 100$$

Where H_c and $H_t$indicate the cleaned height and the total height, respectively.
Control tests were also done by adding Sodium Dodecyl Sulfate (SDS) solution at 0.2% (w/v) (positive control) and uninoculated culture medium (control).

2.4.4. Antibacterial effect study

Antibacterial activity of the produced biosurfactant was assessed against two standard pathogenic bacterial strains (*Escherichia coli* ATCC 25922 and *Staphylococcusaureus* ATCC 43300) and against three clinical isolates of pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*) obtained from the Laboratory of Microbiology of Nedir Mohamed Hospital (Tizi-Ouzou, Algeria). All strains were stored in Mueller-Hinton agar (Institut Pasteur of Algiers, Algeria) until the moment of testing.
The antibacterial activity of the crude biosurfactant was determined by using agar diffusion method on Mueller-Hinton agar [20]. Briefly, discs (5 mm diameter filter paper, Whatman, no. 1) were soaked with 100 µL of four different concentrations of crude biosurfactants: 0.5%, 0.25%, 0.1% and 0.05% (w/v), and with sterilized distilled water (control). The discs were dried and then placed on the surface of Mueller-Hinton agar plates previously inoculated with 1 mL of each pathogenic bacterial suspension (1.5×10^7 CFU/mL). Following incubation for 24 h at 37°C, the diameter of the zone of inhibition around the disc was measured and recorded. These experiments were carried out in duplicate.

2.4.5. Biosurfactant characterization

2.4.5.1. Biosurfactant extraction

Crude biosurfactant wasobtained using a solvent extraction method described by Silva et al. (2010) [21] with modification of the centrifugation speed. After 5 days of cultivation at room temperature (22.7°C, max. deviation ± 1°C), the bacterial cells were removed by centrifugation at 5000 rpm for 30 min. In order to remove the protein fraction, the cell free supernatant was acidified with 1N HCl to pH 2 prior to biosurfactant extraction using chloroform/methanol (2:1) mixture. The mixture was shaken vigorously for 15 min then the organic phase was carefully separated from the aqueous phase. The procedure was repeated with the organic phase. The solvent was then removed on a rotary evaporator. The obtained crude biosurfactantwas solubilized in methanol before being concentrated at 45°C and weighted.

2.4.5.2. Ninhydrin test

This test was carried out for detection of peptide moiety in the biosurfactant molecule, and was done as follows: 3 drops of 0.5% (w/v) ninhydrin solution were added in 2 ml of 0.75% (w/v) crude biosurfactant solution. The mixture was heated in boiling water bath for 4 min and then observed for change in color [22].

2.4.5.3. Phenol-sulfuric acid assay

The phenol-sulfuric acid test is commonly used for detection of neutral sugars in different biomolecules such as glycolipids and glycoproteins. In this study, the test was performed according to Zhang et al. (2012) [22] with some modifications. One ml (0.5% (w/v)) phenol, 5 ml (98%) sulfuric acid and 2 ml of 0.075% (w/v) crude biosurfactant solution were introduced into a test tube. The mixture was vortexed and heated in boiling water bath for 15 min. After cooling to room temperature, the absorbance was measured at 480 nm with a UV-Visible spectrophotometer (RAYLEIGH UV-9200, China).

2.4.5.4. Structural analysis of biosurfactant

The crude biosurfactant extract obtained was analyzed using Fourier transform infrared**(**FTIR) technique, which is used to identify unknown compounds. The FTIR instrument used in this study was ALPHA spectrometer (Bruker Corporation, USA) operating in the wavenumber range of 380 to 4000 cm^{-1}.

3. Results and discussion

3.1.Kinetic of biosurfactant production

Biosurfactant production was studied using basal mineral salt medium with amurca as carbon source and ammonium nitrate (NH_4NO_3)as nitrogen source. As reported in Figure 1, the results showed that *Pseudomonas aeruginosa* was able to produce biosurfactants from amurca. The biosurfactant production profile of the isolated strain started with a latency stage, which lasted around 40h. This phase was immediately followed by two exponential phases separated by a stationary stage, which lasted 60h. Biosurfactant production reached a maximal rate of 37.5% after 104h culture. After this period, production slightly decreased probably due to enzymatic degradation of the produced biosurfactants. Different investigations stated that *Pseudomonas* sp. produced biosurfactantsas primary metabolites [5,21,23]. However, other studies such as the work of Abbasi et al. (2012) [3] reported that biosurfactant produced by*Pseudomonas aeruginosa* MA01 was a secondary metabolite.

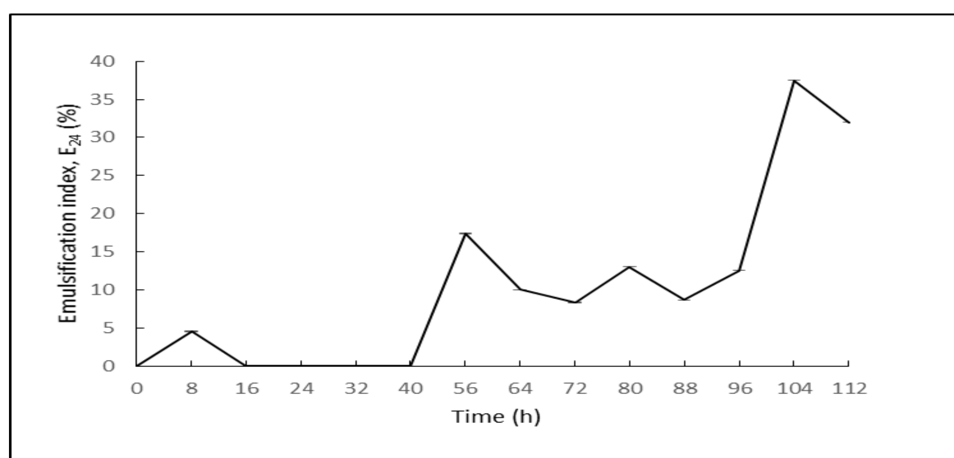

Figure 1: Kinetic of biosurfactant production on amurca medium. The culture was incubated at 22.7°C ± 1°C for 112h at 150 rpm and pH 7.0.

In literature, several carbon sources were used for biosurfactant production by *Pseudomonas aeruginosa*. Abbasi et al. (2012) [3] have tested the effect of different carbon sources on biosurfactant production by *Pseudomonas aeruginosa* MA01. They showed that among all examined carbon substrates (mannose, sucrose, corn oil, canola oil, sorbitol, etc.), vegetable oils were the most effective on biosurfactant production. In the present study, the yield of biosurfactants obtained by using amurca as carbon source is substantial (12.4 g/L) when compared with other studies which used different carbon sources: pyruvate, citrate, fructose, glycerol, and olive oil [23], glucose, olive oil, hexadecane [24] , glycerol [25], glucose [26], crude oil, nonane, decane, dodecane, n- paraffins, kerosene, diesel, xylene [27], corn steep liquor, sugarcane molasses (Gudiña et al. 2016) [28]. However, in another study, a high yield of biosurfactant (13.86 g/L) was obtained from a culture of *Candida Antarctica* isolate using soybean oil as a carbon source [29].

The nature of carbon source is essential to achieve significant biosurfactant production [3]. The biosynthetic link between conventional carbon sources such as glucose or fructose and glycolipids-type biosurfactant is well established. Nevertheless, the exact metabolic pathways of the biosynthesis of this biosurfactant using more efficient complex carbon sources such as vegetable oils, are not yet elucidated [30].

In the present study, amurca addition seemed to promote biosurfactant production, this could be explained by the fact that in addition to phenolic compounds (oleuropen, gallic acid, 3-hydroxyphenol, etc.), amurca contains carbohydrates, proteins and fats [31], which could stimulate both cell growth and anabolic pathways of biosurfactants.

Others medium constituents such as nitrogen source also affect the production of biosurfactants. Two pathways of ammonium assimilation are known in *Pseudomonas aeruginosa*. In ammonium-poor environments, ammonium is incorporated into L-glutamate by NADP-dependent glutamate dehydrogenase (high ammonium pathway). In the presence of low concentrations of ammonium, two enzymes are involved: glutamine synthetase and glutamate synthase (low ammonium pathway) [32-34]. In this study, ammonium nitrate seems to be a good nitrogen source used by *Pseudomonas aeruginosa* strain selected. This could be explained by the fact that ammonium nitrate uptake (transport and metabolism) activity in the strain tested would be high.

The culture medium which is formed by the basal mineral salt medium supplemented with 4% (v/v) amurca and 0.1 g/L ammonium nitrate (C/N ratio of 1.16) yielded high amount of biosurfactant (12.4 g/L). This observation

indicates that biosurfactant production is favored by high nitrogen concentration (low C/N ratio). It follows that the cellular metabolism was directed towards product formation, which is not linked to nitrogen limitation. This result is not in agreement with that of Guerra-Santos et al. (1984) [35], who showed maximum biosurfactant production in *Pseudomonas aeruginosa* after nitrogen limitation with a C/N ratio of 18, and no surfactant production when the culture was not nitrogen limited. In similar manner, biosurfactant production was reported to be increased by nitrogen limitation in *Pseudomonas aeruginosa* strain. Sodium nitrate was found to be the best nitrogen source and C/N ratio of 100 gave the highest biosurfactant production yield [36]. In another study, Patil et al. (2014) [37] who studied the production of rhamnolipid by *Pseudomonas aeruginosa* F23 strain reported that the best rhamnolipid yield occurred at C/N ratio of 7 when Coconut oil and potassium nitrate were used as carbon source and nitrogen source, respectively.

It is well known that there is a direct relationship between enhanced biosurfactantbiosynthesis and increased glutamine synthetase activity in *Pseudomonas aeruginosa* [38]. Biosynthesis of glutamine synthetase and urease, which are upregulated under nitrogen limiting conditions, are under the control of the RpoN (σ^{54}) factor, which is involved in nitrogen assimilation in *Pseudomonas aeruginosa* [39]. Furthermore, this factor has been found to exert a negative control on quorum sensing systems [40], which are known to regulate the rhamnolipid production in *Pseudomonas aeruginosa*.

3.2. Cleaning test

The result of cleaning test revealed that the biosurfactant produced from amurca medium gave a cleaning activity value of 30%, which is less than that of the positive control using SDS (100%). On the other hand, no cleaning activity was recorded with fresh culture medium.

3.3.Antibacterial activity study

The results of diameters of the zones of inhibition of crude biosurfactantare depicted in Table 1. The crude biosurfactant was found effective only against *Staphylococcus aureus* ATCC 43300 (inhibition zone of 9.5±0.5) and *Staphylococcus aureus* (inhibition zone of 8.5±0.5), and the highest inhibition effect was recorded with the concentration of 0.5% for both strains.

Table 1: Antibacterial activity profile of crude biosurfactant against some pathogenic bacterial strains.

Concentration of crude biosurfactant (% (w/v)	Bacterialstrains	Zone of inhibition (mm)
0.5	*Staphylococcus aureus* ATCC 43300	9.5±0.5
	Escherichia coli ATCC 25922	00
	Staphylococcus aureus	8.5±0.5
	Escherichia coli	00
	Klebsiellapneumoniae	00
0.25	*Staphylococcus aureus* ATCC 43300	9±1
	Escherichia coli ATCC 25922	00
	Staphylococcus aureus	8.5±0.5
	Escherichia coli	00
	Klebsiellapneumoniae	00
0.1	*Staphylococcus aureus* ATCC 43300	8.5±5
	Escherichia coli ATCC 25922	00
	Staphylococcus aureus	8±0.00
	Escherichia coli	-
	Klebsiellapneumoniae	-
0.05	*Staphylococcus aureus* ATCC 43300	7±1
	Escherichia coli ATCC 25922	00
	Staphylococcus aureus	8±1
	Escherichia coli	00
	Klebsiellapneumoniae	00

There are few reports focusing on the antimicrobial properties of biosurfactants. For example, Ferhat et al. (2011) [24] demonstrated that glycolipids produced by *Ochrobactrum*sp. 1C and *Brevibacterium*sp. 7G exhibited antibacterial effects against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Gomaa (2013) [41] reported antimicrobial activities of lipopeptide produced by *Bacillus licheniformis*M104 against *Candida*

albicans. De Rienzo et al. (2015) [42] reported disruption effect of sophorolipidbiosurfactants on biofilm of *Bacillus subtilis* BBK006 inducing leakage of cytoplasmic constituents. More recently, Tedesco et al. (2016) [43] described monorhamnolipids produced by *Pseudomonas* sp. BTN1, isolated from Antarctic sediments that exhibited an inhibitory effect against *Burkholderiacepacia*.

3.4.Ninhydrin and phenol-sulfuric acid tests

Ninhydrin test was negative indicating the absence of peptide moiety in the molecule. However, phenol-sulfuric acid test was positive, suggesting the presence of glucidicmoiety in the structure of the purified biosurfactant. These results suggest that *Pseudomonas aeruginosa* strain tested in this study probably produced glycolipids in medium supplemented with amurca as the sole carbon source.

3.5. *Biosurfactant characterization by FTIR*

The chemical characterization of the biosurfactant produced was done using FTIR technique.The FTIR spectrum of crude biosurfactant was on 557-3381 cm^{-1} wavenumber range (Figure 2). Free hydroxyl group was detected on 3418.09 cm^{-1} wavenumber (stretching peak), while the absorption peak around 1418.25 cm^{-1} (bending vibration) indicates the presence of hydroxyl group bonded to carbonyl group. On the other hand, absorption at 2933.19 cm^{-1} is assigned to CH (stretching vibration), and absorption at 1638.67, 1450, 1106.09 and 996.69 cm^{-1} correspond to C=C (stretching vibration), CH_2 (bending vibration), C-O (stretching vibration) and CH=CH (bending vibration), respectively. These results indicate that the isolated *Pseudomonas aeruginosa* strain very likely produces glycolipid-type biosurfactantin liquid culture. This class of biosurfactantis known to be produced by the species *Pseudomonas aeruginosa* [44]. Multiple studies have reported that *Pseudomonas aeruginosa* strains produced glycolipids using different substrates such as glycerol [22], glucose [45], sugarcane molasses [28].

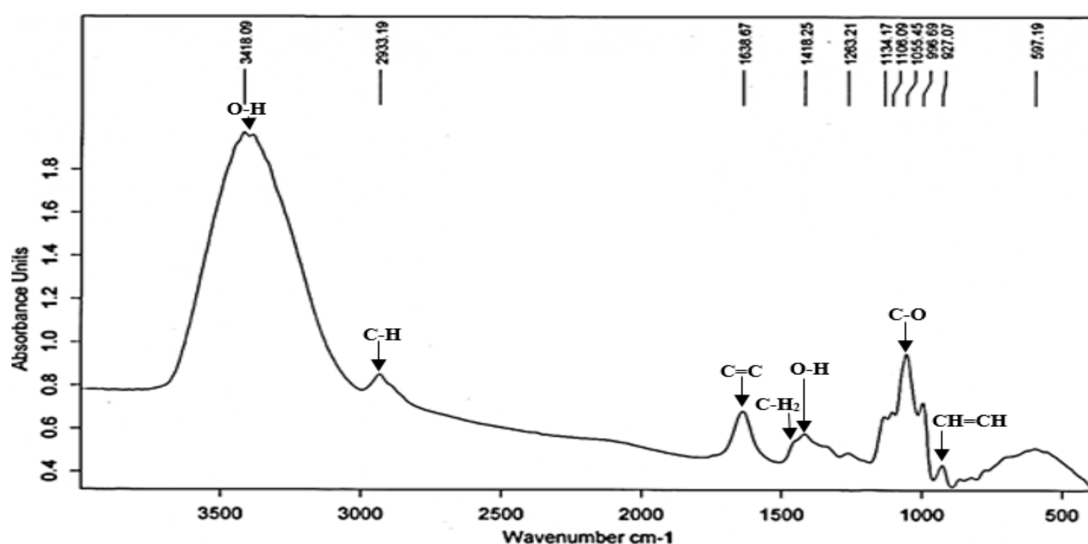

Figure 2: FTIR spectrum of crude biosurfactant.

Conclusions

Pseudomonas aeruginosa strain MA-1 used in this study seems to produce biosurfactants (glycolipids) when grown in batch culture. Results from our studies show performances of amurca for biosurfactant production. With this substrate, a substantial amount of biosurfactants was generated (12.4 g/L). Nevertheless, further investigation is needed to prove the appropriateness of this available low cost substrate in industrial-level biosurfactant production process. Besides, the biosurfactants produced by the indigenous isolated strain showed an antimicrobial property against Gram-positive pathogenic bacteria (*Staphylococcus aureus* ATCC 43300 and *Staphylococcus aureus*).

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