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FULL LENGTH ARTICLE

Selection of *Pseudomonas aeruginosa* for biosurfactant production and studies of its antimicrobial activity

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Abstract Biosurfactants are generally microbial metabolites with the typical amphiphilic structure of a surfactant. This study investigated potential biosurfactants production of *Pseudomonas aeruginosa* ATCC-10145 and *Bacillus subtilis* NCTC-1040 using glucose and *n*-hexadecane as substrates separately and compared it with the production in conventional medium. *Pseudomonas aeruginosa* growing in BHMS (Bushnell hass mineral salt) medium with glucose as substrate decreased the surface tension from 72 of distilled water to 32 mN/m, this strain had higher reduction than *Bacillus subtilis* among all the substrates tested. The selection of *Pseudomonas aeruginosa* for the separation of biosurfactant was determined. The crude biosurfactant was extracted from the supernatant and the yield of the crude biosurfactant was about 1 g/l. Some surface properties of rhamnolipids biosurfactant were evaluated. It also showed antimicrobial activity against different bacteria and fungi strains. The crude biosurfactant showed good action as antimicrobial activity against different bacterial and fungal species.

1. Introduction

Biosurfactants are biological amphiphatic compounds consisting of hydrophilic and hydrophobic moieties. where the hydrophobic moiety is either a long chain fatty acid, hydroxy fatty acid, or α-alkyl-β-hydroxy fatty acid and the hydrophilic moiety can be, a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid or alcohol, etc. [1].

In the past few decades, biosurfactants had gained attention because they exhibited some advantages such as biodegradability, low toxicity, ecological acceptability and ability to be produced from renewable and cheaper substrates [2,3].

The first microbiological biosurfactants on the market were sophorolipids. Of all currently known biosurfactants, rhamnolipids have the highest potential for becoming the next generation of biosurfactants introduced in the market [4].

Microorganisms have been reported to produce several classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants [5–7].

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Majority of known biosurfactants are synthesized by microorganisms grown on water immiscible hydrocarbons, but some are produced on water soluble substrates such as glucose, glycerol, and ethanol [8].

Up to now, the most commonly isolated and best studied groups of biosurfactants are those of glycolipid compounds and phospholipids [9]. Rhamnolipids are glycolipid compounds produced by *Pseudomonas* sp. which could reduce water surface tension and emulsify oil [10–12]. These compounds are biodegradable and have a potential industrial and environmental application.

Among the many classes of biosurfactants, lipopeptides from *Bacillus subtilis* were particularly interesting because of their high surface activity and therapeutic potential [13,14].

The present study aimed in:

1. Production of biosurfactant by *Pseudomonas aeruginosa* ATCC-10145 and *B. subtilis* NCTC-1040 grown on two carbon sources separately.
2. Selection of the best bacterial strain which has higher reduction of surface tension.
3. Separation and evaluation of some surface properties of the crude biosurfactant.
4. Tested the biosurfactant activity for antimicrobial activity against a broad spectrum of bacteria and fungi.

2. Experimental

2.1. Bacterial strains

Pseudomonas aeruginosa ATCC-10145 was supplied by the microbial resources center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. *Bacillus subtilis* NCTC-1040 was supplied from Application of Biotechnology in the Field of Petroleum Industry Lab, Department of Process, Design and Development, Egyptian Petroleum Research Institute (EPRI), Cairo, Egypt.

2.2. Inoculum and media preparation

The bacterial strains were streaked on a nutrient agar slant and incubated for 24 h at 30 °C. Two loops of culture were inoculated in 25 ml of nutrient broth in a 50 ml Erlenmeyer flask and incubated in a rotary shaker 150 rpm at 30 °C for 8–12 h until cell numbers reach 10^8 CFU/ml, an aliquot of 2 ml of inoculum was transferred to 100 ml of Bushnell haas mineral salt medium (BHMS) in a 250 ml Erlenmeyer flask and the cultures were incubated on a temperature controlled shaker incubator at 150 rpm and 30 °C for 96 h, the medium contained (g/l): dipotassium phosphate 1.0, magnesium sulfate 0.2, calcium chloride 0.02, potassium dihydrogen phosphate 1.0, ammonium nitrate 1.0, ferric chloride 0.05. The carbon sources carbohydrate (glucose) was added to make the final concentration 1% (w/v). The hydrocarbon (*n*-hexadecane) was added at 1% (v/v). Samples were collected at different time intervals (0, 24, 48, 72 and 96 h) and submitted to analysis. The experiments were conducted in three independent replicates.

2.3. Analytical measurements

a. Biomass determinationAliquots measuring 2 ml at different time intervals of culture were taken in Eppendorf tubes and centrifuged at 10,000×*g* for 10 min. Biomass obtained was dried overnight at 45 °C and weighed [15].

b. Separation of the crude biosurfactantRhamnolipids biosurfactant produced by *Pseudomonas aeruginosa* was recovered from the culture supernatant after the removal of cells by centrifugation at 10,000×*g* for 20 min. Rhamnolipids were then precipitated by acidification of the supernatant to pH 2.0 and allowing the precipitate to form at 4 °C overnight. The precipitate thus obtained was pelleted at 10,000×*g* for 15 min, the precipitate was dissolved in 0.05 M sodium biocarbonate (pH 8.6), reacidified, and recentrifugation at 12,000×*g* for 20 min, following centrifugation, the precipitate was extracted with chloroform/methanol (2/1) three times. The organic solvent was evaporated using a rotary evaporator and a yellowish oily residue was obtained [16].

2.4. Estimation of biosurfactant activity

The activity of the biosurfactant was determined by measuring:

a. Oil displacement test

The oil displacement test is a method used to determine the surface activity by measuring the diameter of the clear zone after 96 h of incubation period, which occurs after dropping a surfactant-containing solution on a thin layer of oil on water. The oil displacement test was done by adding 40 ml of distilled water to a petri dish with a diameter of 10 cm. After that, 15 µl of crude oil was dropped to form a thin oil layer on the surface of the water, and the 10 µl of a test solution was dropped on to the surface of oil. The test was conducted at room temperature. The maximum diameter of the clear zone was observed under light and measured [17].

b. Surface tension values

The surface tension values were measured on a ring tensiometer (krüss-tensiometer K6) using the cell free culture (50 ml) at 28 °C at different time intervals, while a solution of 0.1% by weight was tested at 28 °C when evaluating the crude biosurfactant [18].

c. Foam height

Foaming of biosurfactant in culture medium was determined by shaking vigorously the supernatant (10 ml) after 96 h of incubation period for 2 min and then foaming was calculated according to the following equation [19].

$$\text{Foaming} = \frac{\text{Height of foam}}{\text{Total height}} \times 100$$

d. Critical micelle concentration (CMC)

The critical micelle concentration values of the biosurfactant were determined using surface tension method. The CMC was determined from a semilog plot of surface tension versus rhamnolipid concentrations [16].

2.5. Antimicrobial tests

The cup assay method [20,21] was carried out. The rhamnolipid compound was tested for its in vitro antibacterial activity against *Bacillus pumilus*. *Micrococcus luteus* (Gram positive), and *Sarcina lutea* (Gram negative) and the antifungal activity were screened against *Penicillium chrysogenum*, yeast against *Candida albicans* using the agar diffusion technique.

Metronidazole and Erythromycin were used as standard drugs for antifungal and antibacterial activity, respectively. All compounds were dissolved in 5 mg/ml dimethylformamide (DMF).

The antimicrobial assay medium nutrient agar medium was sterilized and cooled to 40 °C then inoculated with different microorganisms, then poured in plates. Holes 1 cm in diameter were made using a sterile cork borer and then 0.1 ml of biosurfactant was added. The plate were incubated at 5 °C for 1–2 h then transferred into the incubator at 28–30 °C overnight and at 28 °C for 72 h for the fungal strains. The diameter of the inhibition zones caused by the effect of biosurfactant on the tested microorganisms was measured.

3. Results and discussion

The bacteria used for production of biosurfactants were selected, two different types of biosurfactants including lipopeptides, rhamnolipids produced by *Bacillus subtilis* and *Pseudomonas aeruginosa*, respectively [22,23]. In the present investigation, the ability of two bacterial strains (*Bacillus subtilis* and *Pseudomonas aeruginosa*) to grow on two different carbon sources is presented in Table 1. In the glucose supplemented medium, due to the heavy growth of the bacterial strain *P. aeruginosa* could be produced with higher biomass than *B. subtilis* at different time intervals until the end of incubation period (96 h). In *n*-hexadecane supplemented medium, also *P. aeruginosa* exhibited better growth with the biomass yield than *B. subtilis* for the entire growth period. The above results have been confirmed by that of the Bordoloi and Konwar [15] the bacterial isolate *P. aeruginosa* MICC7815 produced the highest biomass in media using glucose, *n*-hexadecane as substrates, respectively.

On the other hand, the production of biosurfactants by measurement of the surface tension in cell free culture at different time intervals is shown in Table 2, *P. aeruginosa* exhibited lower surface tension of the culture medium supplemented with glucose and *n*-hexadecane from 72 of distilled water into 32 and 46 mN/m at 96 h, respectively. The observation of glucose the best carbon source for the growth and production of the biosurfactant by the bacterial strain *P. aeruginosa* was found to be in agreement with other workers like [24–26]. Wu et al. [27] reported that glucose and glycerol were effective carbon substrates for rhamnolipid production. While, Tuleva et al. [28] investigated that, the best carbon source for the production of rhamnolipid by *P. putida* was hexadecane.

On the basis of biomass production and biosurfactant production was the strain *P. aeruginosa* was selected for subsequent works.

After the bacterial strain *P. aeruginosa* was grown under the optimum conditions, the recovery of biosurfactant from cell free culture was done by the classical techniques that are well suited for batch recovery. They include solvent extraction, precipitation and crystallization. The yield of the biosurfactant was relatively low (1 g/l). In fact, modification of the succeeding fermentation process is expected to raise the production rate. This is supported by the results of Rodrigues et al. [29] where they reported that the potential use of alternative fermentative medium instead of the synthetic medium for biosurfactant production by *Lactococcus lactis* 53 and *Streptococcus thermophilus* effectively proceeded with high yields and productivities of biosurfactant. An increase about 1.2–1.5 times the mass of the produced biosurfactant per gram cell dry weight was achieved. Tuleva et al. [28] reported that, *Pseudomonas putida* when grown on hexadecane as the sole carbon source showed biosurfactant values of 1.2 g/l. Pornsunthorntawee et al.[17] showed about 2.17 g of the biosurfactant was extracted per liter of *P. aeurginosa* culture medium. Dubey and Juwarkar [30] reported rhamnolipid production at 0.92 g/l using whey waste as the carbon source by *P. aeruginosa* BS2. Rhamnolipids production at a concentration of 1.3 and 0.709 g/l by *P. aeruginosa* J4 using diesel and kerosene as the sole carbon source has been reported [31]. From the literature review, the bacterial strain (*P. aeurginosa*) produced selectively rhamnolipids biosurfactant [10–12,24–27].

Microbial biosurfactants have an advantage over their chemical counterparts due to their better physical and chemical properties, e.g. foaming, environmental compatibility and higher biodegradability. Also, used at extreme temperatures, acidity and salt concentrations [32].

Surface activity of the obtained biosurfactant (Table 3) proved that the crude biosurfactant could reduce the surface

Table 1 Determination of bacterial biomass in media supplemented with different carbon sources using two different bacterial strains.

Cell free culture media of the bacterial strain in different carbon sources	Bacterial biomass (g) at different time intervals (h)				
	0	24	48	72	96
Carbon source: glucose					
Control*	0.01	0.03	0.03	0.02	0.02
Pseudomonas aeruginosa	0.01	0.09	1.0	1.5	2.0
Bacillus subtilis	0.02	0.08	0.9	1.0	1.5
Carbon source: n-hexadecane					
Control**	0.01	0.01	0.02	0.01	0.02
Pseudomonas aeruginosa	0.02	0.08	1.0	1.0	2.0
Bacillus subtilis	0.01	0.05	0.9	1.0	1.0

* Control: sample containing medium without microorganism using glucose as the carbon source.
** Control: sample containing medium without microorganism using *n*-hexadecane as the carbon source.

Table 2 Measurement of surface tension of culture supernatant with different carbon sources using two different bacterial strains at different time intervals.

Bacterial strain in different carbon sources	Surface tension (mN/m) of culture media at different time intervals (h)				
	0	24	48	72	96
Carbon source: glucose					
Control*	58	57	57	58	58
Pseudomonas aeruginosa	57	52	50	47	32
Bacillus subtilis	57	50	50	49	47
Carbon source: n-hexadecane					
Control**	55	56	56	55	56
Pseudomonas aeruginosa	54	53	50	49	46
Bacillus subtilis	55	54	53	54	52

* Control: sample containing medium without microorganism using glucose as the carbon source.
** Control: sample containing medium without microorganism using *n*-hexadecane as the carbon source.

Table 3 Valuation of some surface properties of the crude biosurfactant produced by *Pseudomonas aeruginosa*.

Surface tension (mN/m)	CMC (mg/l)	Foaming (%)	Oil displacement (cm)
32	50	70	8

tension to 32 mN/m, which was acceptable in terms of its purity. It was stated by Mulligan [33] that a good surfactant can lower the surface tension of water from 72 to 35 mN/m. Moreover, the production of biosurfactants by *Pseudomonas aeruginosa* MTCC7814 lowered the surface tension to 34 mN/m [15]. Also, trehalose lipids from *Rhodococcus erythropolis* and *Arthrobacter* sp. lowered the surface in the culture broth to 25–40 mN/m [34,35].

One of the most important properties of a surfactant is their spontaneous aggregation in water and the formation of well-known structures such as spherical micelles, cylinders, etc. the surface tension decreases gradually with increasing surfactant concentrations. At a certain concentration called critical micelle concentration (CMC), this decrease stops. Above the CMC, the surface tension remains almost constant [36].

In the present study the surface tension decreased from 60 to 32 mN/m with small increases in the rhamnolipids concentrations up to 50 mg/l. Further the addition of rhamnolipids concentration had no effect until 70 mg/l (Fig. 1). This result was found to be in agreement with other workers like [17,18].

Abbasi et al. [37] and Mohamed [38] demonstrated the surface tension of distilled water decreased gradually with increasing biosurfactant concentrations to 32.5 mN/m, with CMC values of 10.1 mg/l. Pornsunthorntawee et al. [17] reported that *Pseudomonas aeruginosa* sp., the extracted biosurfactant in the culture supernatant could decrease the surface tension of distilled water from 72 to 28.3 mN/m and the CMC was estimated to be 120 mg/l.

Biosurfactants produced by *P. aeruginosa* strains were found to reduce the surface tension of distilled water from 72 to 30 mN/m with CMCs in the range of 5–200 mg/l [39,40]. Molecular structural differences including ratio and composition of homologs, the presence of unsaturated bonds, the branching and length of aliphatic chains of chains of rhamnolipids can explain different CMC values of biosurfactants produced by *Pseudomonas* strains [41]. Several

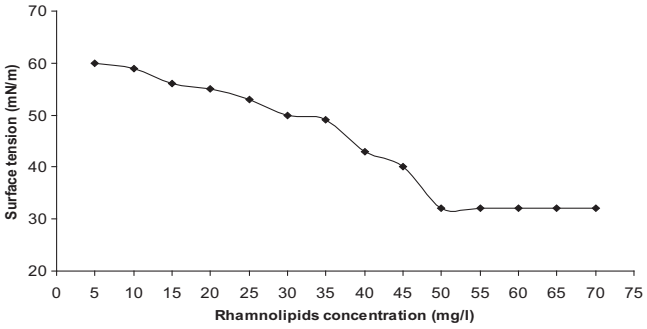

Figure 1 Determination of critical micelle concentration of rhamnolipids biosurfactants.

biosurfactants show low critical micelle concentrations (CMC) and high surface activities and are therefore, promising substitutes for environmental applications due to their biodegradability and relatively low CMC [42,43].

One of the most important properties, which should exist in biosurfactants, is the foaming power. The observed foaming due to the biosurfactant obtained in the supernatant of *P. aeruginosa* was found to be 70%. El-Sheshtawy [19] investigated that, the foaming height due to biosurfactant production by *B. subtilis* DSM 15029 was obtained at 51%. The stable foaming coupled with a reduction in surface tension of a medium is considered as a qualitative indication of biosurfactant production [44]. It is worth to note that the formation of foaming during enrichment of a culture in a mineral medium with glucose as the carbon source was potential for the application of biosurfactants in microbial enhanced oil recovery [15].

The surface active property of the rhamnolipids biosurfactant therefore was tested by the oil displacement test. This technique can be used in qualitative and quantitative assays. The oil displacement test has several advantages in requiring a small volume of sample, are rapid and easy to carry out, and do not require specialized equipment [45]. The bacterial strain was highly positive for biosurfactant production by given 8 cm of oil displacement test Table 3. Rismani et al. [46] reported that, the area of clearly formed oil displacement circle was measured as the activity of biosurfactants. It was a circle of 7 cm diameter. Although the mechanism of the oil displacement by surfactants has not yet been clarified on the molecular level this method was provided with a sensitive and easy

Table 4 Antimicrobial activity of crude rhamnolipids biosurfactant produced by *Pseudomonas aeruginosa* for 96 h at 30 °C and 150 rpm.

Compound	Zone of inhibition (in mm)				
	Bacteria			Fungi	
	Gram negative	Gram positive			
	Sarcina lutea	*Micrococcus luteus*	*Bacillus pumilus*	*Penicillium chrysogenum*	*Candida albicans*
Crude biosurfactant	18	21	20	25	20
Erythromycin	40	32	32	–	–
Metronidazole	–	–	–	25	20

system of biosurfactants. The drop collapsing test is not as sensitive as the oil displacement test in detecting low levels of biosurfactants production [47].

The crude biosurfactant compound was screened for its in vitro antibacterial and antifungal activities by zone of inhibition presented in Table 4. This compound showed good antimicrobial activity against all tested different species of bacteria and fungi strains. Mohammed, [38] reported that, nearly 45% of the tested strains was positively inhibited by rhamnolipids biosurfactant e.g. *Trichoderma viride* > *Bacillus* sp. > *Pseudomonas* sp. > *Cellulomonas flavigena* = *Rhodococcus erythropolis*. Also, in the present study the activity of antibacterial compound toward Gram positive and negative bacterial strains is known to have different effects. *Sarcina lutea* was found to be more resistant than Gram positive bacteria (*M. luteus* and *Bacillus pumilus*). Previous reports indicate that Gram negative bacteria are more resistant to rhamnolipid action than the Gram positive bacteria. These might be due to their unique outer membrane lipopolysaccharide (LPS). The LPS either acts as a barrier or provides protection to the inner sensitive membrane and cell wall from the toxic compounds [48]. The result suggests that the rhamnolipid molecule having both hydrophobic and hydrophilic groups could insert its fatty acid components into a cell membrane that caused considerable alteration in the ultrastructure of the cell such as ability of the cell to interiorize the plasma membrane. Alternately, it may also be possible that insertion of the shorter acyl tails of the rhamnolipid into the cell membrane causes disruption between cytoskeleton elements and the plasma membrane, allowing the membrane to lift away from the cytoplasmic constituents [49].

4. Conclusion

In this present study we can conclude that:

- The two types of biosurfactant-producing microorganisms, *P. aeruginosa* ATCC-10145 and *B. subtilis* NCTC-1040 were suitable for the biosurfactants production.
- The strain *P. aeruginosa* ATCC-10145 could grow in the culture medium containing glucose as the carbon source better than the strain *B. subtilis* NCTC-1040 after 96 h.
- The surface tension measurement indicated that the production of biosurfactant by *P. aeruginosa* had better surface activity than that produced by *B. subtilis*.
- The rhamnolipid biosurfactant produced by *P. aeruginosa* had antimicrobial activity against different species of microorganisms. Thus the produced antimicrobial compound can be further characterized and can be applied as a biocontrol agent.

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