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Screening Three Strains of *Pseudomonas aeruginosa*: Prediction of Biosurfactant-Producer Strain

Gholamreza Dehghan-Noudeh, Mohammad Hasan Moshafi, Effat Behravan,
Saeed Torkzadeh and Masoud Ahmadi Afzadi
Pharmaceutics Research Center, Kerman University of Medical Sciences, Kerman, Iran

Abstract: Problem statement: The chemical surfactants have some disadvantages; especially, toxicity and no biodegradability. **Approach:** Biosurfactants were the structurally diverse group of surface-active molecules synthesize by micro-organisms. The microbial surfactants were interesting, because of the biodegradable and have many applications in industry, agriculture, medicine. **Results:** In the present study, the production of biosurfactant by three strains of *Pseudomonas aeruginosa* (PTCC 1074, 1310 and 1430) was investigated. The hemolytic and foam forming activity of different strains were studied and consequently, *P. aeruginosa* PTCC 1074 was selected as the suitable strain. *P. aeruginosa* PTCC 1074 was grown in the nutrient broth medium and biosurfactant production was evaluated every 24 h by emulsification index and surface tension for the best of production time. After that, in order to get maximum production of biosurfactant, the selected strain was grown with different additives in nutrient broth and the best culture medium was found. The biosurfactant was isolated from the supernatant and its amphipathic structure was confirmed by chemical methods. **Conclusion:** Biosurfactant produced by *Pseudomonas aeruginosa* PTCC 1074 would be considered as a suitable surfactant in industries due to its low toxicity.

Key words: *Pseudomonas aeruginosa*, biosurfactant, surface tention, emulsification index

INTRODUCTION

Microbial-derived surfactants are amphipatic molecules produced by a wide variety of bacteria, yeasts and filamentous fungi. Increasing environmental concern had led to consider biological surfactants as alternative to chemical manufactured compounds. The most important advantage of biosurfactants when compared to synthetic surfactants is their ecological acceptance, owing to their low toxicity and biodegradable nature[1]. Another advantage of biosurfactants is that they can be modified by biotransformation to generate new products for specific requirements[2]. Microbial surfactants are complex molecules, comprising a wide variety of chemical structures, such as glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, peptides, phospholipids and neutral lipids[3]. Potential applications of biosurfactants include emulsification, phase separation, wetting, foaming and surface activity that can be exploited in food, oil, cosmetic and pharmaceutical industries[4]. In the environmental sector, microbial surfactants show promising applications in bioremediation and waste treatment to remove hazardous materials[5]. The antimicrobial activity showed by rhamnolipids is another promising field of application for these molecules Bacteria of the genus *Pseudomonas* are known to produce a glycolipid surfactant containing rhamnose and 3-hydroxy fatty acids[6]. The rhamnolipids produced by *P. aeruginosa* have been widely studied and are reported as a mixture of homologous species RL1 (RhaC10C10), RL2 (RhaC10), RL3 (Rha2C10C10) and RL4 (Rha2C10)[7]. The properties showed by rhamnolipids depend on their homologues composition and distribution that are determined by the bacterial strain, culture conditions and medium composition[8]. Rhamnolipids are easily isolated from culture broth and can be produced using hydrophobic and hydrophilic substrates such as carbohydrates, hydrocarbons, vegetable oils or wastes from food industry[9-11]. In this study, the production of biosurfactant by three strains of *P. aeruginosa* is reported and investigated some physicochemical properties such as foam activity and emulsification index of produced biosurfactant.

MATERIALS AND METHODS

Test organisms: Three strains of *P. aeruginosa* (PTCC 1074, PTCC 1310 and PTCC 1430) were obtained from

Corresponding Author: Gholamreza Dehghan-Noudeh, Pharmaceutics Research Center, Kerman University of Medical Sciences, Kerman, Iran Tel: ++98-341-3205014 Fax: ++98-341-3220799

the Persian Culture Type Collection (PTCC), Tehran, Iran. The strains were streaked on the surface of nutrient agar plates (Merck, Germany). After incubation at 37°C for 24 h, distinct colonies were isolated. Nutrient Broth medium was used. The strains of *P. aeruginosa* was grown in 500 mL Erlenmeyer flasks, each containing 100 medium. The flask was incubated at 37°C on a shaker incubator (Pars Azma Co., type: IN07) at 200 rpm for 96 h[12].

Detection of biosurfactant producer strain: Hemolytic activity: Isolated strains were screened on blood agar plates (Merck) containing 5% (v/v) sheep blood and incubated at 37°C for 48 h. Hemolytic activity was detected as the presence of a definite clear zone around a colony[13].

Foam forming activity: All of the strains were grown separately in 500 mL Erlenmeyer flasks, each containing 100 mL of nutrient broth (Merck at pH 7.4) medium. The flasks were incubated at 37°C on a shaker incubator (200 rpm) for 96 h. Foam activity was detected as duration of foam stability, foam height and foam shape in the graduated cylinder[14].

Surface activity measurement: Surface tension and critical micelle dilution (CMD^{-1} and CMD^{-2}) were determined with a duNouy Tensiometer (modle-703, sigma). All measurements were made on supernatant. CMD^{-1} and CMD^{-2} measurements were performed by measuring the surface tension of 10 times and 100 times diluted supernatant. Negative control consisted of sterile culture medium plus *P. aeruginasa* PTCC 1074 (an inoculum), at zero time[10,15].

Emulsification test: For estimation of the emulsification index, 5 mL of liquid paraffin, olive oil, rashid oil and castor oil was added to 5 mL of supernatant in a graduated tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h. The E_{24} was calculated by measuring the emulsion layer formed[16].

Growth conditions: *P. aeruginasa* PTCC 1074 was initially grown in 500-mL Erlenmeyer flasks, each containing 100 mL nutrient broth medium. The flasks were incubated at 37°C in a shaker incubator (Pars Azma Co., type: IN07) at 200 rpm. In some experiments, the selected strain was grown in nutrient broth with additives such as some oils (paraffin oil, castor oil, almond oil and olive oil) and trace metals to the nutrient broth medium in order to get maximum production of biosurfactant. Samples were withdrawn every 24 h (three cultures for each time) to analyze the surface activity, emulsification index and biomass weight and therefore to select the best conditions and additives for biosurfactant production. Nutrient broth medium (Merck) was used. In some experiments, the selected strain was grown in nutrient broth with different additives such as paraffin oil, castor oil, (oil source)[17,18], starch (carbohydrate source)[7] and trace metal cations (Fe^{2+}, Fe^{3+}, Zn^{2+}, Mg^{2+} and Mn^{2+} respectively) (0.1, 0.2, 0.2, 0.1, 0.2 and 0.5 g L^{-1})[12,10,15,19] to the nutrient broth medium in order to get maximum production of biosurfactant. *P. aeruginosa* PTCC 1074 was initially grown in 500 mL Erlenmeyer flasks, each containing 100 mL nutrient broth medium. The flasks were incubated at 37°C in a shaker incubator (Pars Azma Co., type: IN07) at 200 rpm. Samples were withdrawn every 24 h to analyze the surface activity and therefore to select the best time of biosurfactant production.

Isolation of biosurfactant: After the bacterial cells were removed from the liquid culture by centrifugation (9000 rpm) in centrifuge (Vision, VS.35SMTi), the crude biosurfactant was isolated by adding H_2SO_4 (6 N) to the supernatant. A flocculated precipitate was formed at pH 2.0 that could be collected by centrifugation (12000 g, 20 min). The precipitate was dried under vacuum in dissolved 0.1 Tric-Hcl (pH = 8.0) and extracted three times with chloroform: ethanol (2:1). The organic solvent layer was evaporated under vacuum on a rotary evaporator to dryness and used as the crude extract for further analysis[20-22].

Identification of lipid moiety: The biosurfactant was hydrolyzed with 6 M HCl 110°C for 20 h and subsequently the lipid moiety was separated by extraction with chloroform. Then several drops of bromine water were added to the extract[23].

Identification of sugar moiety: Two drops of 20% L-napthol solution (in ethanol) was added in tube and mixed to 2 mL of a 0.1% solution of the sample. About 2 mL of concentrated H_2SO_4 was poured to the side of the tube (molish test)[23].

RESULTS

Three different strains of *P. aeruginosa*, from nutrient agar cultures were isolated and tested by hemolytic and foam forming methods[14]. The nutrient agar cultures of all strains tested and had hemolytic activity (Table 1).

The results of foam forming activity test for three different strains indicate that *P. aeruginosa* PTCC 1074 produces more foam than the other strains (Table 2).

Table 1: Hemolytic activity for different strains of *P. aeruginosa*

Microbial strain	Kind of hemolytic
P. aeruginosa PTCC 1310	++
P. aeruginosa PTCC 1430	+
P. aeruginosa PTCC 1074	++++

(+): The presence of unknown zone around a colony with red color; (++): The presence of an approximately clear zone around a colony with orange color; (++++): The presence of a definite clear zone around a colony with bright yellow color

Table 2: Foam properties at different strains of *P. aeruginosa*

Microbial strain	Foam properties	Foam stability course (min)	Foam height (mm)
P. aeruginosa PTCC 1310	++	125	13
P. aeruginosa PTCC 1430	+	8	11
P. aeruginosa PTCC 1074	++++	165	35
Negative control	-	2	4

(-): Bubbles with coarse sizes, very disperse and very low stability; (+): Bubbles with coarse sizes, very disperse and low stability; (++): Bubbles with medium sizes, concentrated and medium stability; (++++): Bubbles with fine sizes, concentrated and high stability

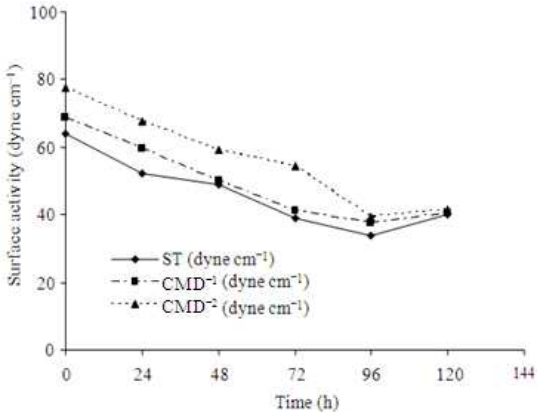

Fig. 1: Surface activity profile of *P. aeruginosa* PTCC 1074

P. aeruginosa PTCC 1074 was cultured in nutrient broth and biosurfactant production, as evident from surface tension lowering, started from first day and continued until 96 h of growth. CMD^{-1} and CMD^{-2} values (Table 3) followed a similar pattern as surface tension lowering. CMD^{-1} and CMD^{-2} measurements were performed by measuring the surface tension of 10 and 100 times diluted cell-free broth[14]. Maximum of biosurfactant production was achieved in 96 h of incubation and CMD values (Fig. 1). The production yield was improved by addition of iron, magnesium and manganese salts. The addition of hydrocarbons, such as castor and liquid paraffin oils to the culture medium reduced the biosurfactant production.

Table 3: Surface tension studies

Time (h)	Surface tension (mN m^{-1}) ± SD	CMD^{-1} (mN m^{-1}) ± SD	CMD^{-2} (mN m^{-1}) ± SD
0	64.112±0.019	68.730±0.092	77.637±0.187
24	52.100±0.031	59.671±0.112	67.482±0.420
48	48.829±0.401	49.986±0.018	59.348±0.311
72	39.018±0.132	41.331±0.441	54.360±0.527
96	33.832±0.179	37.487±0.292	39.518±0.345
120	40.205±0.209	40.386±0.071	41.641±0.011

CMD^{-1}: Critical Micelle $Dilution^{-1}$; CMD^{-2}: Critical Micelle $Dilution^{-2}$; Results for supernatant of *P. aeruginosa* PTCC 1074, grown in nutrient broth medium

Table 4: Emulsification index; E_{24}: Results for supernatant of *P. aeruginosa* PTCC 1074, grown in nutrient broth medium

Time (h)	Paraffin oil	Castor oil	Olive oil	Almond oil
0	0.00±0.000	0.000±0.00	0.000±0.00	0.000±0.00
24	3.30±0.100	36.60±0.17	43.30±0.23	43.30±0.13
48	3.30±0.150	56.60±0.12	53.30±0.11	53.30±0.30
72	10.00±0.12	56.60±0.14	53.30±0.18	53.30±0.17
96	13.30±0.17	60.00±0.19	60.00±0.10	66.60±0.11
120	6.60±0.110	53.30±0.30	56.60±0.90	50.00±0.19

DISCUSSION

Screening for biosurfactant producer strain: The screening of biosurfactant-producing microorganisms is generally carried out using monitoring parameters that estimate surface activity, such as surface tension, , the ability to emulsify oils' hemolytic capacity and foam activity. In present study, these were performed as potential predictor of surfactant-producing bacteria.

The best hemolytic action was observed for *P. aeruginosa* PTCC 1074. Reduction of surface tension is s as a selection criterion that biosurfactant-producing capacity of microorganisms in liquid medium[14]. Emulsification index values followed a similar pattern as surface tension lowering[9,11] (Table 4). These results suggest that *P. aeruginosa* PTCC 1074 is a better biosurfactant producer.

Biosurfactant production: In molecular terms, surface-active compounds are amphiphilic agents containing both hydrophilic and lipophilic parts. Their efficiency in foaming and emulsifying depends on their amphiphilic structure. Rhamnolipid is a detergent-like glycolipide with excellent foaming properties, which is produced by *P. aeruginosa*[9]. When *P. aeruginosa* PTCC 1074 was grown in the nutrient broth medium, the production of the biosurfactant was poorly.Iron, magnesium and manganese cations caused enhancement of the yield. While manganese and magnesium caused a larger surface tension lowering than the iron cation. Also, from surface activity studies, it can be concluded that when manganese, magnesium

and iron was added to nutrient broth medium the best yield of biosurfactant was obtained. The biosurfactant was extracted and its amphiphilic (sugar-lipide) structure was confirmed.

CONCLUSION

Biosurfactant produced by *Pseudomonas aeruginosa* PTCC 1074 would be considered as a suitable surfactant in industries due to its low toxicity.

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