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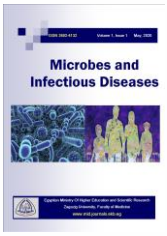

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Original article

Biosurfactants production and antimicrobial effects in environmental isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Background: Surfactants are a group of polar molecules with two parts, hydrophilic and hydrophobic that usually stay between two phases with different polarity. This property enables the reduction of surface and interfacial tension of liquids and leads to the formation of microemulsions. **Methods**: In this study, the production of rhamnolipid from *Pseudomonas aeruginosa* was investigated using different amounts of carbon including crude oil, engine oil, burnt oil, and coconut oil. **Result**: In the screening results of 29 isolates, 15 isolates in crude oil, 8 isolates in engine oil, 7 isolates in brake fluid, 7 isolates in burnt oil, and 7 isolates in coconut oil showed positive results. The best result was obtained using crude oil and engine oil, brake fluid, burnt oil, and coconut oil all producing the same amount of rhamnolipid from *Pseudomonas aeruginosa*. **Conclusion**: *Pseudomonas aeruginosa* uses the majority of oils, especially crude oil, to produce rhamnolipid, and this feature significantly increases the decomposition of hydrocarbons.

Introduction

Surfactants are a group of polar molecules with two hydrophilic and hydrophobic parts that usually remain between two phases with different polarities. This feature reduces the interfacial and interfacial tension of liquids and leads to the formation of microemulsions. There is increasing concern about environmental pollution from the use of chemical surfactants due to their active microbial surface compounds due to their low toxicity and biodegradability. Many types of biosurfactants produced by microorganisms such as glycolipids (rhamnolipids, sulfurolipids, terhalolipids), lipoproteins, and lipopeptides have been identified (Nayarisseri et al., 2018; Meliani and Bensoltane, 201). These amphiphilic compounds on living surfaces are produced by different microorganisms. These molecules sit on the cell surface or are secreted into the extracellular environment (Desai et al. 1997).

Biosurfactants were first considered as hydrocarbon solubilizers in the late 1960s. The use of these materials has expanded significantly in the last five decades due to their good biodegradability compared to chemical surfactants (Cameotra et al. 2004). Biosurfactants play an important role in cleaning soil pollution. These compounds can be used to control the density of fat cells, stabilize aeration systems, improve the shelf life of food emulsions and products containing starch, improve the rheological properties of fat-based products, and control consistency in the baking and ice cream

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industries. , is used to delay aging and improve the volume and shelf life of bakery products (Nitschke. 2007).

Biosurfactants can change the enzyme structure and thus its activity by reacting with microbial proteins. These compounds have a lethal effect on some microorganisms, so they can be antimicrobial agents to kill human, animal, and plant pathogenic microbes (Heyd et al. 2008). In 2014, Rashidi et al. investigated the production of rhamnolipid from *Pseudomonas aeruginosa* (*P. aeruginosa*) using different ratios of molasses and showed that production depends on bacterial growth (Rashdi et al. 2005). In the present study, the antimicrobial effects of *P. aeruginosa* rhamnolipid on the microbial pathogens *Escherichia coli (E. coli)* and *Staphylococcus* were investigated.

Materials and methods

Sampling

Fifty (50) g of soil sample was collected from different parts of Aliabad Katul city and placed in a plastic bag and stored in the refrigerator before being transferred to the laboratory. During serial dilution, 50 g of soil sample was isolated and suspended in 10 ml of physiological saline. By adding 1 ml of the above suspension to 9 ml of physiological saline, initial dilution was prepared and finally, serial dilution was performed: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} was prepared.

Cultivation

From the prepared suspensions, the third and fourth tubes of each were cultured in the amount of 0.1 ml in king B culture medium and incubated at 37 ° C for 24 hours. After bacterial growth, warm staining, oxidase test, and TSI test, the production of green pyocyanine pigment and production of fluorescence pigment under UV were performed.

Culture medium for rhamnolipid production

Raminolipid culture medium is one of the selected medium isolates (MSM). Mineral salt medium contains Na2HPO4(4 g), KH2PO4(1.5g), NH4Cl(1g), MgSO4 .7H2O (0.2g), Ferric ammonium citrate(5mg), Modified Hoagland Trace element solution (1ml) and distilled water (1l)

Screening and evaluation of rhamnolipid production

Oil spreading test

Ten (10) microliters of crude oil, engine oil, brake oil, burnt oil and coconut oil were placed on the surface of 40 ml of distilled water in 5 plates and 10 microliters of the surface solution of each culture was poured on the surface of the (crude) oil layer. The oils used included engine oil, brake oil, burnt oil and coconut oil. If the supernatant contains biosurfactant, the used oil motor will separate and form a clear zone (Kurniati et al. 2019: Nisha et al. 2023).

Hemolytic activity

Pure culture of bacterial isolates on fresh blood agar medium cultured for 48-72 hours at 37 ° incubated hemolytic activity was characterized by a clear halo of colony appearance (Kurniati et al. 2019).

Blue agar plate method

Mineral salt agar medium enriched with 2% glucose, 0.5-gram citriemide, and 800 µl methylene blue was used to detect rhamnolipid. wells with 4 mm diameter were created in the culture medium and then filled with 30 μl of a fresh culture of each isolate. The plates were incubated at 37 ° C for 48-72 hours. A clear dark blue halo around the crop is tested as the production of anionic biosurfactant (Kurniati et al. 2019).

Investigation of antimicrobial effects against selected pathogens

The antimicrobial activity of biosurfactant was tested against two pathogenic microbial strains, *Staphylococcus aureus*, and *E. coli* by well diffusion method. This test was carried out by broth dilution techniques and agar well diffusion method. In this method, Mueller Hinton agar medium was used for the evaluation of the antimicrobial activity of rhamnolipid against the test pathogens. A 24 h fresh culture of two test pathogens in LB culture and then on the nutrient medium plates was prepared.

A Suspension is equivalent to 0.5 McFarland's turbidity was prepared from overnight grown cultures of these bacterial strains and from this turbidity made a lawn culture using a sterile swab on Mueller Hinton agar medium. Four wells with 6 mm diameter were made in the medium and the wells were filled with 100 μl culture filtrate. The plates were incubated at 37°C for 24 h and then the zone of inhibition of microbial growth was measured. All tests were performed in triplicate and the inhibition zone diameter values represented the mean value ± SD.

Results

The present study is an explanatory observational study In this study, soil samples were taken from different soil areas of the city Aliabad Katul

Agricultural Land (Gholam Block, Pajak Mahalla, Farm, Zarrin Gol, Mohammad Abad), Mountain (Malavir, Chili, Rig Cheshmeh, Maple board, GNU) and forest (Zarrin Gol, Kaboudwal, Mohammadabad, Qarq) were transferred to the laboratory. And the necessary tests were performed on them and the results were reported.

Identification of isolates

Conventional *P. aeruginosa* detection methods are based on the biological characteristics of the bacterium under certain culture conditions, such as Gram staining, Non-fermentative activity in TSI medium and growth at 42°C, oxidase, pyocyanin and fluorescence pigment production.

In this study, the production of rhamnolipid from *P. aeruginosa* was investigated using different amounts of carbon including crude oil, engine oil, burnt oil, and coconut oil. In the screening results of 29 isolates, 15 isolates in crude oil, 8 isolates in engine oil, 7 isolates in brake fluid, 7 isolates in burnt oil, and 7 isolates in coconut oil showed positive results. And the best result was obtained using crude oil and engine oil, brake fluid, burnt oil, and coconut oil all producing the same amount of rhamnolipid from *P. aeruginosa*. The reason for the production of rhamnolipid by *P. aeruginosa* with crude oil is the existence of water-in-oil emulsions (oil emulsions). And this feature significantly increases the decomposition of hydrocarbons.

Hemolytic activity

All isolates of *P. aeruginosa* showed beta hemolysis on blood agar and all RBCs are completely lysed around the colony and a clear halo was visible around the colony.

Blue agar plate method

Pseudomonas bacteria use glucose in the environment to grow in this test, the mineral environment of salt agar is the carbon source for the production of biosorfactan, the result of which appears as a halo.

Antimicrobial effects against selected pathogens

In this investigation antimicrobial effects, the inhibitory effect of rhamnolipid-producing isolates on the 3- and 6-day culture of *Staphylococcus aureus* and *E. coli*, and the results were recorded.

Isolate numbers 3, 13, 15, 20, 23, 24 had the highest inhibition zone of growth in 3-day-old *Staphylococcus* bacteria and isolate 26 had the highest growth in 6-day-old *Staphylococcus* bacteria. comparing the growth of 3 and 6-day -old *Staphylococcus* bacteria shows that the highest Inhibition zone diameter occurred in *Staphylococcus* bacteria in 3-day-old.

Isolate numbers 8, 17, 22, 24, 28, 32 had the highest inhibition zone of growth in 3-day-old *E. coli* and isolates 1 and 4 have the highest growth in 6-day-old *E. coli*. comparing the growth of 3 and 6-day -old *E. coli* shows that the highest Inhibition zone diameter occurred in *E. coli* in 3-day-old.

The reason for the increase in growth in 3-day-old *Staphylococcus* and 3-day *E. coli* was the effect of various factors such as carbon source, temperature and duration of incubation and proper rotation in the centrifuge on the production of rhamnolipids from *P. aeruginosa*.

Table 1. Results of biochemical tests of *Pseudomonas aeruginosa*

Bacterium	**Gram staining**	**Oxidase**	**TSI**	**growth at 42°C**	**Fluorescence pigment**	**pyocyanin pigment**
Pseudomonas aeruginosa	-	+	ALK/ALK	+	+	+

Table 2. Results of oil spreading test for rhamnolipid production

Isolated number	Crude oil	engine oil	brake oil	Burnt oil	coconut oil
1	+	-	-	+	+
3	-	-	+	-	-
4	+	-	-	-	-
8	+	-	-	-	-
13	+	+	+	-	-
15	+	-	-	-	+
17	-	-	+	-	-
20	+	+	-	+	-
23	+	-	-	+	+
24	+	-	-	+	+
26	+	-	-	+	+
28	+	+	-	+	+
32	-	-	-	+	-

Table 3. Results of Romanolipid antimicrobial effect against *Staphylococcus aureus* 3 and 6 –day-old isolates

***Staphylococcus* 6 –day-old (Inhibition zone diameter)**	**Staphylococcus 3 –day-old (Inhibition zone diameter)**	**Number**
20	24	1
14	26	3
16	26	4
16	20	7
5	21	8
5	18	9
26	20	10
7	20	11
8	19	12
14	26	13
18	19	14
7	25	15
17	19	16
16	20	17
19	20	18
22	27	19
18	31	20
14	20	22
20	28	23
19	26	24
7	25	25
24	20	26
12	21	27
21	15	28
16	20	29
22	27	30
18	27	31
7	22	32
30	24	33

Table 4. Results of Romanolipid antimicrobial effect against *E. coli* 3 and 6 –day-old isolates

***E. coli* 6 –day-old (Inhibition zone diameter)**	***E. coli* 3 –day-old (Inhibition zone diameter)**	**Number**
25	10	1
15	21	3
24	11	4
18	16	7
17	26	8
17	21	9
7	22	10
15	14	11
8	17	12
7	7	13
14	19	14
16	7	15
14	20	16
14	23	17
17	19	18
16	20	19
15	19	20
16	27	22
7	17	23
8	22	24
10	20	25
10	20	26
7	21	27
7	25	28
17	17	29
17	18	30
20	21	31
17	23	32
8	18	33

Discussion

Ramenolipids are glycolipids composed of one or two molecules of rhamnose. Which bind to one or two alkyl fatty acid chains they are composed of homologs Composed primarily of di-rhamnolipids and mono-rhamnolipids, rhamnolipid biosurfactants show many beneficial properties for the food industry, such as activity - low toxicity levels In addition, this biosurfactant has shown antimicrobial activity against various small organisms such as Gram-positive bacteria, *Staphylococus aureus*, Bacillus subtilis and Gram-negative bacteria. The mechanism of action of rhamnolipids is not fully understood, but there is a hypothetical possibility of their activity in cell membranes. Because they have an amphipathic nature that allows them to be exchanged with phospholipids(magalhaes 2013). A group of microorganisms (bacteria, yeasts, and fungi) can produce biosurfactants. , Which have a variety of industrial applications. Due to their biodegradability, low toxicity, high potential activities, and stability at absolute temperature, pH, and salinity. According to the mentioned properties, biosurfactants can be used for the following:

Control of agglomeration of fat cells, stabilization of aerated systems, improvement of shelf life of food emulsions and starch-containing products, improvement of rheological properties of fat-based detergent products, control of consistency in baking and ice cream industries, delaying staleness, Improving the volume and shelf life of bakery products (Nitschke,2007).

Rhamnolipids produced by *P. aeruginosa* inhibit the growth of *Listeria monocytogenes*. In this study, Pseudomonas rhamnolipid had an inhibitory effect on the Gram-positive bacteria *Staphylococcus* aureus (Luna Magalhaes, Marc Nitschke, 2012).

The effect of rhamnolipid biosurfactants extracted from *P. aeruginosa* on cell

growth/survival, biofilm formation, and membrane permeability of methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial cells was investigated. The results showed that by increasing the concentration of rhamnolipid from 30 to 120 mg/ml, the cell viability decreased by about 70% and the permeability of the cell membrane increased by approximately 20%. In the present study, an inhibitory effect on *Staphylococcus aureus* was also observed (Saddati M et al.,2022)

In the research, the amount of rhamnolipid production from *P. aeruginosa* was investigated using different amounts of carbon, including olive oil, palm oil, and coconut oil, and the best results were obtained using olive oil. In this research, most of the bacteria produced rhamnolipid with crude oil, burnt oil, and coconut oil, which is somewhat consistent with the mentioned research (Taniavaren et al., 2006). (Taniavaren et al., 2006).

In another study, the production of rhamnolipid from *P. aeruginosa* was investigated using different amounts of carbon, including crude oil, and inorganic glycerol was introduced as a nutrient source for the production of rhamnolipid biosurfactant. In the current research, different oils were used to produce this compound, and crude oil, coconut oil, and burnt oil accounted for the most production. (Ravish Bath dayamani k.j et al, 2015)

Other studies have shown that vegetable oils are the best carbon source for *P. aeruginosa* to produce rhamnolipids. Because these substances are in the presence of hydrophilic compounds (such as glucose and fructose), due to the proper solubility of these compounds in water, the cell does not need to produce biosurfactant and release it. It does not have to improve its solubility in the environment. In the present research, vegetable oils such as coconut oil were introduced as suitable oils (Abouseud et al., 2008; Wei et al., 2005)

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