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Emulsification Properties of Biosurfactant Produced from *Pseudomonas aeruginosa* RB 28

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Abstract: Biosurfactant produced from *P. aeruginosa* RB 28 was extracted, purified and characterized. Thin layer chromatography results showed that the extract contained two different compounds. The identification of the nature of the two compounds showed that they were glycolipids and rhamnose was the sugar moiety in these glycolipids. It was concluded that these compounds were rhamnolipids. The production of biosurfactant was started at late log phase and reached its maximal level (2.7 g L^{-1}) at the stationary phase. Study of some rhamnolipid properties showed that sunflower oil, heptadecane and paraffin were efficiently emulsified and emulsions formed with vegetable oils (olive oil, corn oil and sunflower oil) were more stable than emulsions formed with hydrocarbons.

Key words: Biosurfactant, rhamnolipids, *Pseudomonas*, emulsification activity, emulsion stability

INTRODUCTION

Biosurfactants (bioemulsifiers) are microbial surface-active compounds have the ability to emulsify water immiscible compounds through the reduction of the surface tension of the culture media (Fiechter, 1992; Deziel *et al*., 1999). They acquired their importance from their use in several industrial fields such as pharmaceutical, petroleum and food industries. They are widely used in industrial and environmental fields such as Microbially Enhanced Oil Recovery (MEOR), oil tanks cleaning and bioremediation of oil polluted water and soil (Ochsner *et al*. 1995; Deziel *et al*., 1999; Okpokwasili and Ibiene, 2006).

Pseudomonas is widely used in the production of biosurfactant especially *P. aeruginosa* which produce rhamnolipid, the most studied biosurfactant (Fiechter, 1992; Deziel *et al*., 1999). *P. aeruginosa* produce two types of glycolipid (R1 and R2) using submerged culture (Ochsner *et al*., 1995), two other types (R3 and R4) when using different carbon sources or using resting cells (Syldatk *et al*., 1985b; Robert *et al*., 1989). Many studies reported the presence of different rhamnolipids. The absence of one type is due to cultural conditions especially the composition of media (Ochsner *et al*., 1995; Deziel *et al*., 1999; Deziel *et al*., 2000).

Rhamnolipid enhance the degradation and the dispersion of hydrocarbons and alkanes by the decreasing of the surface tension of the culture media (Herman *et al*., 1997). It was found that rhamnolipid caused an increase in Lipopolysaccharide (LPS) release which leads to an increase in cell surface hydrophobicity; giving rise to a high degradation rate (Al-Tahhan *et al*., 2000).

We report here the production of biosurfactant by a newly isolated *Pseudomonas aeruginosa* strain and the emulsification properties of this product.

MATERIALS AND METHODS

Bacterial isolates: Twenty bacterial isolates were isolated from oil contaminated-soil at the Department of Biotechnology University of Baghdad; Iraq. These isolates have the ability to degrade crude oil and several hydrocarbons. The isolates were maintained at 4°C on nutrient agar slants, nutrient broth was used for the activation of the isolates.

Screening media: Screening for biosurfactant production was carried out in 250 mL Erlenmeyer flasks containing 50 mL of the medium composed of (g L^{-1}): KH_2PO_4: 1, K_2HPO_4: 1, NH_4NO_3: 1, $MgSO_4.7H_2O$: 0.2, $FeCl_3.6H_2O$: 0.05, $CaCl_2.2H_2O$: 0.02 pH was adjusted to 7.0 (Patel and Desai, 1997) and 2% crude oil was added as sole carbon source. The medium was inoculated with 1% of the 16 h bacterial culture grown on nutrient broth. Incubation was carried out at 30°C in a shaker incubator at 180 rpm for 72 h.

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Determination of emulsification activity: Samples (0.5 mL) of cell free supernatant obtained after removal of cells by centrifugation (7500 rpm for 5 min) were added to a screw-capped tube containing 7.5 mL of Tris-Mg buffer [20 mM Tris HCl (pH 7.0) and 10 mM $MgSO_4$] and 0.1 mL of dodecane. After vigorous agitation for 2 min, tubes were allowed to sit for 1 h. Absorbance was measured at 540 nm. Emulsification Activity (E.A) was defined as the measured optical density. Assays were carried out in triplicates (Patel and Desai, 1997).

Surface tension: The surface tension of the culture supernatant was measured by the Tensiometer.

Biomass determination: A 5 mL sample was centrifuged at 9000 rpm for 15 min, the pellet was dried at 100°C and weight was determined.

Production medium and culture conditions: Production of emulsifier was carried out in 500 mL Erlenmeyer flasks containing 100 mL of the medium composed of (g L^{-1}): KH_2PO_4: 0.5, K_2HPO_4: 1, KCl: 0.1, $MgSO_4.H_2O$: 0.5, $FeSO_4.7H_2O$: 0.008, $CaCl_2$: 0.05, Urea: 6, yeast extract: 0.1 and 0.05 mL of trace elements solution (Br: 0.026%, Cu: 0.05%, Mn: 0.05% and Zn: 0.07%), 4% sunflower oil was added as sole carbon source, pH was adjusted to 7.0. The medium was inoculated with 1% of the 16 h bacterial culture grown on nutrient broth. Incubation was carried out at 30°C in a shaker incubator at 180 rpm for 60 h.

Extraction of biosurfactant: After the appropriate incubation time, the cell free supernatant was adjusted to pH 2.0 and allowed to stand overnight at 4°C, then extracted with chloroform-methanol (2:1 vol/vol). The solvents were removed by rotary evaporation (Patel and Desai, 1997).

Purification on silica gel column: Silica Gel 60 column was used. The sample dissolved in 10 mL chloroform was added to the column. Chloroform-methanol (9:1 vol/vol) was used as solvent system (Robert *et al*., 1989).

Characterization of the glycolipids: The isolated glycolipids were identified by analytical Thin-Layer Chromatography (TLC). TLC was conducted on silica gel plates (20×20 cm) (Merck) with chloroform-methanol-acetic acid (65: 15: 2 [vol/vol/vol]) as solvent system (Syldatk *et al*., 1985a). Molish reagent and 4-Methoxy-benzaldehyde in acetic acid and H_2SO_4 (0.5:50:1 [vol/vol/vol]) (Li *et al*., 1984) were served as spray reagent.

Acid hydrolysis: HCl 1N was added to an amount of the produced compound (glycolipid) and incubated for 3 h at 90°C. The sugar moiety was identified by thin-layer chromatography with the solvent system demonstrated above. Molish reagent served as spray reagent. Rhamnose was used as standard (Syldatk *et al*., 1985a).

Rhamnolipid estimation: The extracted rhamnolipids were estimated by the method of Dubois *et al.* (1956) and expressed in terms of mg mL^{-1} of rhamnose sugar (Dubois *et al*., 1956).

Growth and biosurfactant monitoring: Production was monitored by aseptically removing samples (2 mL) every 6 h during the experiment from the inoculated production medium. Biomass and rhamnolipid concentration were determined.

Emulsification properties of the produced compounds: The emulsification activity and the emulsion stability of the produced compounds from *P. aeruginosa* RB28 using different hydrocarbons (kerosene, hexane, octane, heptadecane, dodecane, paraffin, olive oil, sunflower oil, corn oil and crude oil) were tested. Emulsification activity was measured by adding 0.1 mg of rhamnolipid crude extract to a screw-capped tube containing 7.5 mL of Tris-Mg buffer and 0.1 mL of desired hydrocarbon. The activity was determined as described above (Patel and Desai, 1997). Emulsification activity was measured after 24 h to study the stability of the formed emulsions.

Role of biosurfactant on the crude oil utilization: Ten microgram per milliliter of purified biosurfactant was added to 50 mL of the production medium supplemented with 2% of crude oil as sole carbon source in 250 mL Erlenmeyer flasks. Media without biosurfactant were used as control. All flasks were inoculated with 1% of the 16 h bacterial culture grown on nutrient broth. Incubation was carried out at 30°C in a shaker incubator at 180 rpm for 60 h. Biomass and rhamnolipid concentration were determined.

RESULTS

The ability of twenty bacterial isolates of the genus *Pseudomonas* to produce biosurfactant, using crude oil as carbon source was tested (Table 1). From the twenty isolates, three of them decreased the culture medium surface tension below 40 mN m^{-1}. *P. aeruginosa* RB28 was selected because of its ability to utilize crude oil and to produce biosurfactant (surface tension 36 mN m^{-1} and E.A. 0.28).

Figure 1 showed the time course of biosurfactant production from *P. aeruginosa* RB28 during growth on sunflower oil as sole carbon source. The production of

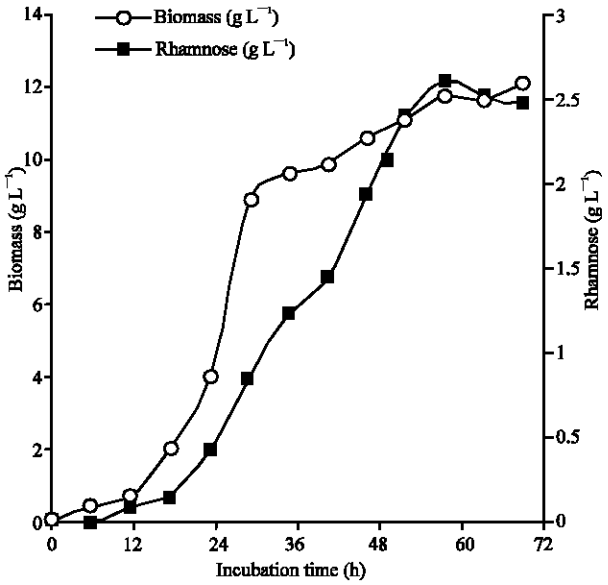

Fig. 1: Monitoring biosurfactant production by *P. aeruginosa* RB 28

Table1: Screening of the ability of several *Pseudomonas* strains to produce biosurfactant

Isolate	Biomass (g L^{-1})	Surface* tension (mN m^{-1})	Emulsification activity
Pseudomonas sp. RB2	0.27	48.8	0.10
Pseudomonas sp. RB3	0.11	44.1	0.03
Pseudomonas sp. RB4	0.07	50.5	0.03
Pseudomonas sp. RB5	0.05	48.5	0.03
Pseudomonas sp. RB7	0.07	44	0.03
P. aeruginosa RB11	0.12	49	0.04
P. aeruginosa RB 12	0.30	49.5	0.10
P. aeruginosa RB14	0.48	51	0.10
P. fluorescens RB16	0.64	47	0.10
P. aeruginosa RB17	0.53	46	0.06
P. aeruginosa RB18	0.89	44.9	0.06
P. aeruginosa RB19	0.71	46.4	0.09
Pseudomonas sp. RB24	0.36	53	0.03
P. aeruginosa RB25	0.42	43.8	0.10
P. aeruginosa RB26	0.26	51	0.10
P. aeruginosa RB27	0.46	36	0.17
P. aeruginosa RB28	0.48	36	0.28
Pseudomonas sp. RB29	0.10	45.5	0.04
Pseudomonas sp. RB30	0.10	46.5	0.04
P. aeruginosa RB31	0.54	38	0.21

*Surface tension of the screening medium + crude oil was 56 mN m^{-1}

biosurfactant was started at late log phase of the bacterial growth, about 12 h after inoculation and increased gradually with bacterial growth till reached its maximal level (2.7 g L^{-1}) after 60 h of incubation (stationary phase).

The produced compounds were partially purified with chloroform-methanol (2:1 vol/vol) extraction. Thin layer chromatography on silica gel plate showed that the produced compounds were composed of two moieties; lipid and sugar moiety. The compounds gave positive

Table 2: Emulsification activity of the produced biosurfactants and the stability of the formed emulsions

Hydrocarbons	Emulsification activity After 1 h	Emulsification activity After 24 h	The loss in E.A. (%)
Crude oil	0.75	0.51	32.0
Kerosene	0.75	0.50	33.33
Paraffin	0.90	0.70	22.22
Sunflower oil	1.05	0.90	14.28
Corn oil	0.78	0.76	2.56
Olive oil	0.43	0.39	2.50
Hexane (C6)	0.27	0.13	51.85
Octane (C8)	0.35	0.20	42.88
Dodecane (C12)	0.66	0.44	33.33
Heptadecane (17)	1.01	0.77	23.76

Table 3: Effect of biosurfactant on the growth of *P. aeruginosa* RB28 on crude oil

Medium	Biomass (g L^{-1})	Rhamnose (g L^{-1})
Medium + crude oil (control)	3.18	0.33
Medium + crude oil + biosurfactant	5.41	0.43

results with Molish reagent (for sugar detection) and with 4-Methoxy-benzaldehyde reagent (green color was appear with glycolipid). The RF of the spots (0.30 and 0.82) were near to the Rf reported by Syldatk *et al.* (1985a) (0.29 and 0.82) using the same solvent system: chloroform-m ethanol-acetic acid (65:15:2 [vol/vol/vol]). It is possible that the compounds produced in this study are rhamnolipids R1 and R2. Further identification of the sugar moiety after acid hydrolysis confirmed it as rhamnose. This result suggests that *P. aeruginosa* RB28 produces rhamnolipids.

The emulsification activity of the produced biosurfactants was tested with different hydrocarbons. Table 2 showed that sunflower oil, heptadecane and paraffin were efficiently emulsified. In addition crude oil, kerosene and corn oil were considerably emulsified. Interestingly, E.A. with n-alkanes increased with the increase of the number of carbon atoms. Emulsions formed with vegetable oils (olive oil, corn oil and sunflower oil) were more stable than emulsions formed with hydrocarbons (Table 2). About 32% loss in the emulsification activity was showed when using crude oil or kerosene.

Result of Table 3 showed that the addition of rhamnolipid biosurfactant to the medium enhanced the ability of *P. aeruginosa* RB28 to utilize crude oil (the sole carbon source in the medium). The increased biomass (about 1.7 fold) is an indicator for the utilization of crude oil by bacterial cells. A slight increase was shown in the rhamnolipid concentration.

DISCUSSION

Biosurfactant-producing bacteria are found in higher concentrations in hydrocarbon contaminated areas. These

strains represent a valuable source of new compounds with surface-active properties and potential application for bioremediation. Biosurfactants can improve the bioavailability of hydrocarbons to the microbial cells by increasing the area at the aqueous-hydrocarbon interface. This increases the rate of hydrocarbon dissolution and their utilization by microorganisms (Tuleva *et al*., 2002). In this work we describe some characteristics and properties of biosurfactant produce from a *P. aeruginosa* strain isolated form an oil contaminated-soil.

The twenty isolates tested in this study differ in their ability to degrade crude oil and produced biosurfactants. *P. aeruginosa* RB 28 strain which displayed the highest biosurfactant production was selected for more analysis. The production of biosurfactant with high emulsification activity from *P. aeruginosa* was reported (Patel and Desai, 1997; Herman *et al*., 1997; Deziel *et al*., 2000).

The biosurfactant compounds isolated from *P. aeruginosa* RB 28 were rhamnolipids, the surface-active glycolipids usually secreted by *Pseudomonas* sp. were responsible for the decreasing of surface tension. Furthermore, rhamnolipid has a role in the growth of microorganisms by degrading hydrocarbons to less complex compounds that can be easily utilized by bacterial cells (Guerra-Santos *et al*., 1986; Ochsner *et al*., 1995; Deziel *et al*., 1999).

Biosurfactant production by *P. aeruginosa* RB 28 (expressed as rhamnose equivalents) reached the maximum level at the stationary phase after 60 h of incubation. Production of biosurfactant from *P. aeruginosa* 44T1 started after 14 h of incubation and reached its maximal level after 58 h (Robert *et al*., 1989). Rhamnolipid production from *P. aeruginosa* GS3 started by 12 h and reached its maximum at 72 h (Patel and Desai, 1997). Since biosurfactants are secondary metabolites maximal glycolipid production was reached in the stationary growth phase (Tuleva *et al*., 2002).

Biosurfactant produced from *P. aeruginosa* RB 28 emulsified hydrocarbons, hydrocarbon mixtures and vegetable oils and formed stable emulsions. Emulsions formed with vegetable oils are more stable. It was reported that rhamnolipids had the ability to emulsify efficiently hydrocarbons and vegetable oils (Navon-venesia *et al*., 1995; Patel and Desai, 1997; Sifour *et al*., 2005). The ability of the produced biosurfactants to emulsify kerosene and crude oil is an important feature for bioremediation applications. Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil (Kosaric, 2001).

Addition of biosurfactant facilitated the growth of *P. aeruginosa* RB 28 on a medium containing crude oil as sole carbon source. Microorganisms facilitated the diffusion of insoluble substrate like hydrocarbons into the cell by producing biosurfactants. Biosurfactant reduced the surface tension and the interfacial tension in both aqueous solutions and hydrocarbon mixtures (Patel and Desai, 1997). Several reports showed that, the addition of rhamnolipid enhance the solubility and the biodegradability of hydrocarbons (Zhang and Miller, 1994; Zhang and Miller, 1995).

The ability of *P. aeruginosa* RB 28 to produce biosurfactant with efficient emulsification properties, could suggest potential use of this product in industrial and environmental applications.

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