

# Optimization of the Production of Biosurfactant by *Pseudomonas aeruginosa* HR Isolated from an Iranian Southern Oil Well

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**ABSTRACT:** In this study 152 bacterial strains were isolated from the contaminated oils in the southwest of Iran. Hemolysis was used as a criterion for the primary isolation of biosurfactant producing-bacteria. Fifty five strains had haemolytic activity, among them twelve strains were good biosurfactant producers by measuring surface tension and emulsification activity. Two microorganism showed the highest biosurfactant production when grown on paraffin and glycerol as the sole carbon source. Glycolipid production by the isolated bacterium using different carbon (*n*-hexadecane, paraffin oil, glycerol, molasses) and nitrogen sources ( $\text{NaNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{CH}_4\text{N}_2\text{O}$ ) was studied. Biosurfactant production was quantified by surface tension reduction, critical micelle dilution (CMD), emulsification capacity (EC), and thin layer chromatography. Best results were obtained when using glycerol at a C/N ratio of 55/1 and sodium nitrate as nitrogen source. Production of the rhamnolipid, expressed by rhamnose was 4.2 g/L and the yield in relation to biomass, was  $Y_{p/x} = 0.65$  g/g. Additionally, physical-chemical characteristics of the spent broth with and without cells showed a low critical micelle concentration of 19 mg/L and a decrease in surface tension to 20 mN/m (%).

**KEY WORDS:** Biosurfactants, Glycolipids, Rhamnolipids, *Pseudomonas aeruginosa*, Molasses, Nitrogen source, Carbon source, Surface tension, Optimization, Iranian oil.

## INTRODUCTION

There is a recent increase of interest in the production of biosurfactants because of their biodegradability, reduced toxicity compared to synthetic surfactants and

their application in enhanced oil recovery and food emulsification [1]. The industrial demand for surfactants has grown to about 300% within the U.S. chemical

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industry during the last decade. Rapid advances in biotechnology over the past decades have led to considerable interests in the development of biological methods for manufacturing surfactants on the industrial scale [1,2]. Almost all surfactants currently in use are chemically derived from petroleum. However interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendliness, possibility of their production through fermentation and their potential application in such areas as the environmental protection, surplus crude oil recovery, health care and the food –processing industries [3-5].

Various types of biosurfactants are synthesized by a number of microbes particularly during their growth on water-immiscible substrates. A majority of biosurfactants are produced by bacteria. Among the bacteria, the *Pseudomonas* species is well known for its capability to produce rhamnolipid biosurfactants with potential surface-active properties when grow on different carbon substrates. Rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*, in particular offer special advantages because of their potent emulsifying activity and low critical micelle concentration [3]. This particular bacteria (*Pseudomonas aeruginosa*) produces two types of glycolipids both containing rhamnose as the carbohydrate moiety. These glycolipids are produced after attaining the stationary phase when the nitrogen is depleted in the medium [3,4].

The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils, to produce rhamnolipid-type biosurfactants [3,8]. Several studies have been carried out to define the best ratio between carbon, nitrogen, phosphorus and iron needed to obtain high production yields [3,8].

Optimization of the carbon/nitrogen ratio in continuous cultures of *Pseudomonas aeruginosa* has been studied, indicating ratios between 15 and 23 as the optimum range for achieving high specific productivity of rhamnolipids, using glucose and vegetable oil as substrates, respectively [5,9]. After nitrogen has been fully consumed, cell metabolism is directed to producing rhamnolipids, whose production increases after the exponential growth phase [8,9,18].

The purpose of this work was to study the production

of a rhamnolipid-type biosurfactants by a strain isolated from oil, as well as to evaluate the tension-active properties and the toxicity of the spent broth and production on sugar beet molasses.

## MATERIALS AND METHODS

### *Identification of the Microorganism*

The microorganism was isolated from oil wells in the southern of IRAN. The method of serial dilutions of the sample and plate count in selective medium Cetrimide agar was used for isolation purposes. The plates were incubated at 30 °C for 48 hours. The strain was activated in a triptic soy agar medium (TSA), cultivated at 30 °C for 48 hours and transferred to a 250 mL flask, containing 50mL of TSA. The flask was incubated in 30 °C and 250 rpm for 20 hours, (shaker, Gallenkamp, England). Cells were harvested by centrifugation at 6000 rpm for 20 minutes, (centrifuge, Shimadzu, Japan.)

The centrifuged microbial mass was suspended in a culture medium (medium salt production - MSP) with the following composition (g/L): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0; KH<sub>2</sub>PO<sub>4</sub>, 3.0; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.2. The pH was adjusted to 7.0 with a solution of KOH (1N) plus 1% v/v of glycerol P.A. (Merck) in order to obtain the initial inoculum concentration of 0.005, 0.075 and 0.1g/L, in accordance with a calibration curve of dry weight versus absorbance [2,5,9,15].

The production of rhamnolipids was studied during a seven-day incubation period in flasks under agitation with the initial seeding material standardized in a culture medium, as mentioned previously, maintained at a temperature of 30 °C and stirred in a rotary shaker at 120 rpm. The carbon sources used were n-hexadecane paraffin oil collected at flowing wells in the Khark island, IRAN, (consisting of 32% saturated hydrocarbons, 23% aromatics, 36% of resins and 9.1% asphaltenes), glycerol (PA - Merck, Darmstadt) and molasses from Pak company, Tehran, IRAN. In addition to the carbon sources studied, the C/N ratio varied with the following concentrations of glycerol: 0.5, 1, 2, 3, 4, 5 and 6% v/v, corresponding to C/N ratios of 20, 40, 60, 80, 100 and 120. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, NaNO<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and CH<sub>4</sub>N<sub>2</sub>O were employed at the following concentrations: 1.45, 1.0, and 0.51 g/L and glycerol 3% v/v.

**Table 1: Rhamnolipids and surface tension measurements at the end of seven days of fermentation by *Pseudomonas aeruginosa* HR using carbon sources at C/N ratio of 20/1.**

Carbon Source	Rhamnose mg/l	Initial surface tension D/cm	Final surface tension D/cm	% Surface tension reduction
n-hexadecane	138	51.4	37.03	38.8
Paraffinic oil	260	54	51.6	4.4
Molasses	150	35	27.3	28
Glycerol	690	53	27.46	48.2

### **Biomass Concentration**

Bacterial growth was monitored by measurement of optical absorbance at a wave length of 610 nm. 50 mL samples were removed from the flasks at regular intervals and centrifuged at 6000 rpm for 15 minutes. The centrifuged cells were suspended in 5 mL of distilled water and the biomass, expressed in dry weight (g/L), was obtained from a calibration curve.

### **Quantification of Rhamnose and Glycerol**

The quantification of rhamnolipids expressed in rhamnose (g/L) was measured in the cell-free culture medium, using the phenol sulfuric acid method [13,16]. Glycerol was assessed by the enzymatic-colorimetric method for triglyceride content evaluation.

### **Determination of the Critical Micelle Concentration (CMC)**

The surface tension of the biosurfactant was measured by the ring method [12] using a CSC-Dunouy tensiometer (Cole-Parmer Instrument Co, Bunker, IL, U.S.A) at room temperature. The concentration at which micelles began to form was represented as the CMC. At the CMC, sudden changes in surface tension, electrical conductivity and detergency were observed [14,18].

The CMC was determined by plotting the surface tension as a function of the biosurfactant concentration, and surface tension at this point was designated as  $\gamma$  CMC [12,14].

## **RESULTS AND DISCUSSION**

### **Microbial Identification and Preservation**

This strain showed an ability to use carbon sources, such as fructose, glucose, mannitol, mannose, glycerol and lactic acid, which are known as good carbon sources

for rhamnolipid production [8,9,10].

### **Effect of the Carbon Source**

The production of rhamnolipids by the *Pseudomonas aeruginosa*, using substrates such as n-hexadecane, paraffin oil, molasses and glycerol, is displayed in Table 1. The strain was able to use n-hexadecane, producing 138 mg/L of rhamnose, with a 38.8 % drop in surface tension at the end of seven days of fermentation. The use of paraffinic oil, which is a very complex and heterogeneous carbon source, resulted in a considerable production of rhamnolipids (260 mg/L) however, practically no variation in surface tension was found at the end of fermentation (4.4%). This fact could probably be due to the formation of an emulsion during fermentation, which interfered in the quantification of the surface tension. The use of glycerol as carbon sources to produce rhamnolipids seems to be an interesting and low cost alternative [11,13]. The bacterium produced 150 mg/L of rhamnolipids at the end of the fermentation with a drop of 28% in the surface tension of the spent medium when molasses was used as carbon source. As reported elsewhere, Table 1 shows a low initial superficial tension in the medium with molasses (35 D/cm) due to the tenso-active properties of the fatty acids. Pimienta et al. (1997) who carried out fermentation studies with strains of *Pseudomonas aeruginosa* grown in glucose, glycerol for a C/N ratio of 20/1, reported production of 700 mg/L, 1300 mg/L and 1400 mg/L of rhamnolipids, respectively, in seven days, showing the greatest potential for rhamnolipid production. Nevertheless, it can be observed in Table 1 that the best rate of rhamnolipid production (690 mg/l) associated with the best surface-active characteristics (48.2% variation in surface tension drop) was achieved when glycerol was employed. This result

was expected since this carbon source is taken up more easily than compared to the others. An abundant formation of foam was observed in the culture medium containing glycerol. Our results are in agreement with those obtained by Itoh et al. (1971), who worked with the strain *Pseudomonas aeruginosa* CFTR-6, which produced glycolipids (620 mg/l) when glycerol (2% w/v) was used as carbon and energy source.

The microbial growth kinetics and rhamnolipid production in the fermentation with a 1% concentration of glycerol with a C/N ratio of 20/1 are represented in Fig. 1. The stationary phase was reached after 40 hours of fermentation at the same time rhamnolipid production was increased. The rhamnolipid and biomass concentrations after 168 hours (sevendays) were 1000 mg/L and 1470 mg/L, respectively. Glycerol was entirely consumed within 145 hours of fermentation and the rhamnolipid concentration peaked after another 100 hours. The production of this rhamnolipid is typical of a secondary metabolite and increased considerably in the stationary phase.

#### Effect of Carbon/Nitrogen Ratio

Aiming to increase the production of rhamnolipids by *Pseudomonas aeruginosa*, a study with increasing glycerol concentrations (1; 2; 3; 4; 5 and 6% v/v) was conducted and a standard inoculum of 0.1 g/L was employed. Fig. 2 shows the yield factors relating production to substrate consumption ( $Y_{p/s}$ ) and production to biomass ( $Y_{p/x}$ ). The best results ( $Y_{p/s} = 0.13$  g/g;  $Y_{p/x} = 0.70$  g/g) were obtained when glycerol was used in a concentration of 5% v/v, corresponding to a C/N ratio of 55/1. Additionally, it is possible to observe that the yield factor  $Y_{p/s}$ , decreased after this optimum glycerol concentration, reaching its lowest value ( $Y_{p/s} = 0.075$  g/g) for the highest glycerol concentrations (6% v/v) thereby indicating a possible inhibitory effect on the bacterium metabolism due to a likely nutrient transport deficiency [6,7,8].

#### Effect of the Nitrogen Source

Fig. 3 shows that sodium nitrate ( $Y_{p/x} = 0.7$  g/g) is more effective than ammonium sulfate ( $Y_{p/x} = 0.35$  g/g) and urea ( $Y_{p/x} = 0.5$  g/g). As shown in this figure, the use of nitrate at a C/N ratio of 55/1 implies better productivity than use of ammonium at the same C/N ratio, using 5% v/v of glycerol as carbon source. This

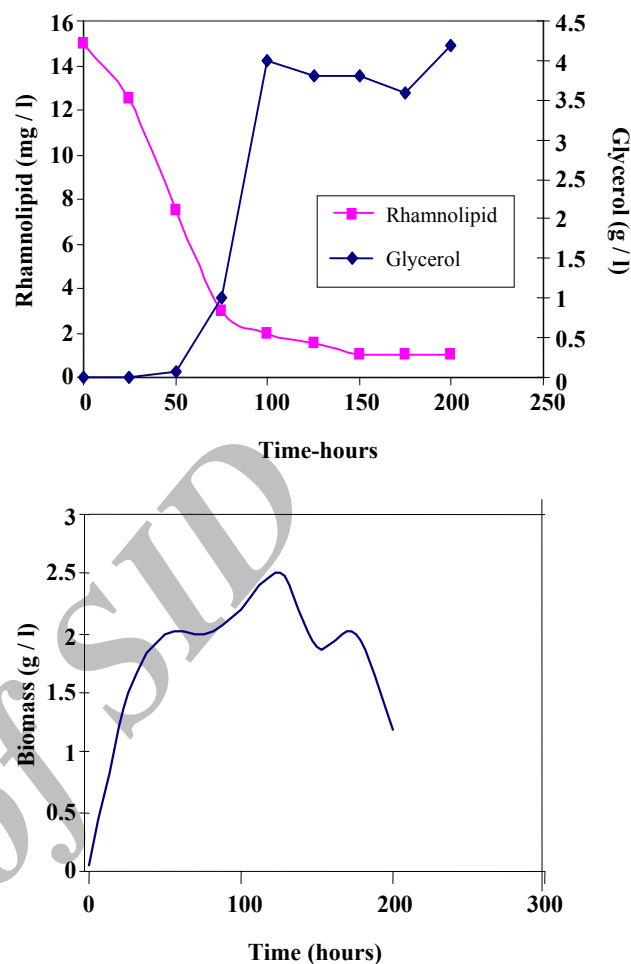


Fig. 1: Microbial growth curve, rhamnolipid production and consumption of glycerol by *Pseudomonas aeruginosa* during 200 hours, using a 1% glycerol concentration.

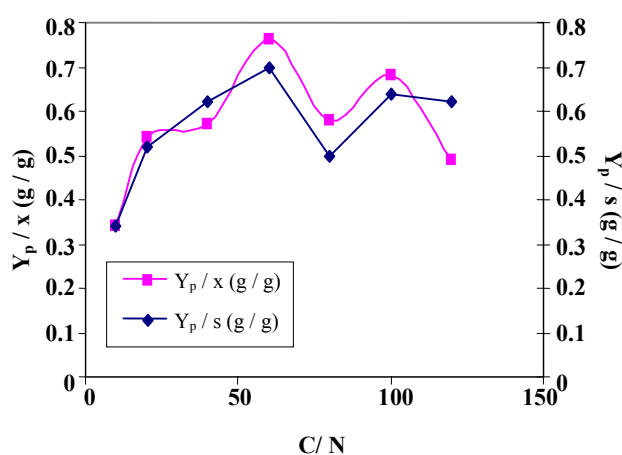


Fig. 2: Yields of rhamnolipids related to biomass ( $Y_{p/x}$ ) and glycerol consumption ( $Y_{p/s}$ ) for fermentations by *Pseudomonas aeruginosa* with different C/N ratios.

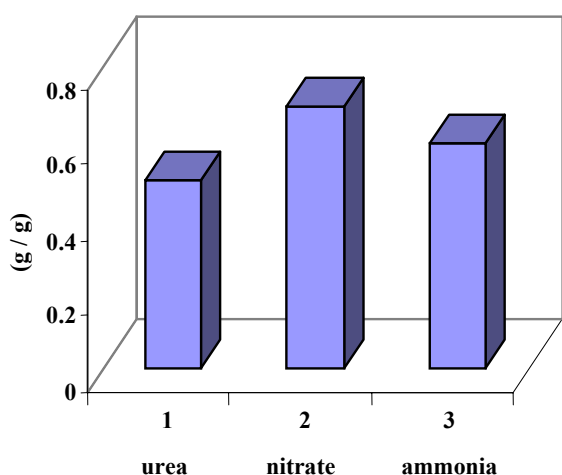


Fig. 3: Effect of nitrogen sources on the production of rhamnolipids by *Pseudomonas aeruginosa*.

result can be explained by the fact that nitrate first undergoes dissimilatory nitrate reduction to ammonium and the assimilation by glutamine-glutamate metabolism.

This means that assimilation of nitrate as nitrogen source is so slow that it would simulate a condition of limiting nitrogen [9,11,14].

*Pseudomonas aeruginosa* is able to use nitrogen sources such as ammonia or nitrate. However, in order to obtain high concentrations of rhamnolipids it is necessary to have restrained conditions of this macro-nutrient. Our studies showed that nitrate is more effective in the production of rhamnolipids than ammonia and urea, which is in agreement with other studies reported in the literature [5,7,9].

#### Determination of the Critical Micelle Concentration

The experiment was aimed at evaluating the tension-active properties of the rhamnolipids accumulated in the fermented medium, using 5% v/v glycerol and 1.45 g/l sodium nitrate as the carbon and nitrogen sources, respectively. Fig. 4 displays the results of superficial tension related to different concentrations of rhamnolipids present in cell-free fermented medium. The measurement for superficial tension of the medium at the end of fermentation was of 26.5 D/cm. At lower concentrations of rhamnolipids, high values of superficial tension were verified. It was also observed that the rhamnolipid concentration of 19 mg/L, corresponding to a superficial tension of 27 D/cm, was the point on the deflection curve;

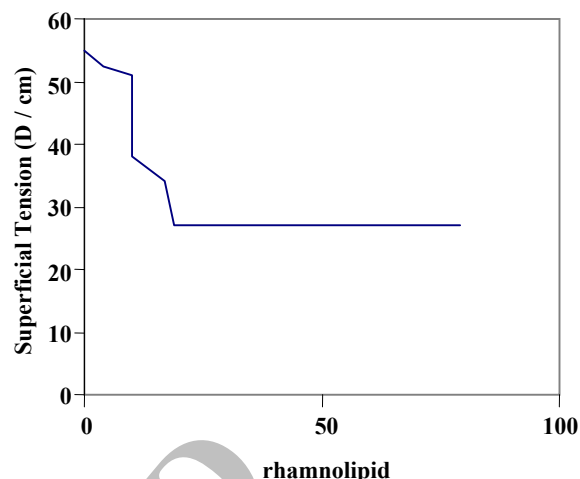


Fig. 4: superficial tension (D/cm) versus rhamnolipid concentration(mg/l)of fermented medium by *Pseudomonas aeruginosa*.

therefore it was assumed to be the critical micelle concentration of rhamnolipids that has satisfactory tension-active properties. Working with *Pseudomonas aeruginosa*, cultivated in 2% w/v of glycerol, Robert et al. (1989) observed a drop in the superficial tension of 30 D/cm in the cell-free fermented medium. The critical micelle concentration obtained by the authors was of 20 mg/L, very close to that obtained in our work.

#### CONCLUSIONS

The strain isolated from oil was identified as *Pseudomonas aeruginosa*. It has the capacity to use carbon sources such as fructose, lactic acid, glucose, mannitol, mannose and glycerol. This strain can produce rhamnolipid-type biosurfactants from substrates such as n-hexadecane, paraffin oil, molasses and glycerol. However, the use of glycerol as carbon source showed the best results.

The variation in concentration of glycerol as carbon source from 1 to 6% v/v showed that with 5%v/v glycerol, the highest biomass concentration (4.26 g/L) and the greatest production of rhamnolipids (2.8 g/L) were obtained, and that when the concentration of glycerol rose above 5%v/v there was an inhibitory effect on microbial growth and the production of biosurfactants. This inhibitory effect was ascribed to problems linked to the solubility of glycerol and the difficulty of the bacterium to gain access to the nutrients in the culture medium.

The use of sodium nitrate (C/N = 55/1) caused an increase in the production of rhamnolipids of 4.2g/L at the end of seven days of fermentation.

The critical micelle concentration of 19 mg/L was in agreement with other values reported in the literature, and the tension-active properties of these molecules indicate good prospects for application in industry, when compared to the values of the CMC of chemical anionic surfactants.

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