Isolation and production of biosurfactant from *Pseudomonas aeruginosa* isolated from Iranian southern wells oil

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**Abstract**

In this study one hundred and fifty two bacterial strains were isolated from oil contaminated. 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 microorganisms showed the highest biosurfactant production when grown on paraffin and glycerol as sole carbon source. As a result of biosurfactant synthesis the surface tension of the medium were reduced from 73 mN/m to values below 32 mN/m. A rhamnolipid producing bacterium, *P.aeruginosa* isolate from oil wells in the southern of Iran. Isolated strain was identified by morphological, biochemical, physiological. The identified *Pseudomonas aeruginosa* confirmed by Persian type culture collection. Glycolipid production by isolated bacterium using different carbon (gasolin, paraffin oil, glycerol, whey) and nitrogen sources (NaNO₃, (NH₄)₂SO₄ and CH₄N₂O) was studied. Biosurfactant production was quantified by surface tension reduction, critical micelle dilution (CMD), emulsification capacity (EC), and ThinLayer Chromatography. The best result were obtained when using glycerol as a C/N ratio of 55/1 and use of sodium nitrate as nitrogen source resulted in higher production of the rhamnolipid, expressed by rhamnose (4.2 g/l) and by the yield in relation to biomass (Y_p/x = 0.65 g/g). Additionally, physical-chemical characteristics of the spent broth with and without cells were studied, providing a low critical micelle concentration of 19 mg/l and surface tension was reduced to 20 mN/m (%).

**Key words:** production of biosurfactants, wild type, glycolipids, rhamnolipids, *Pseudomonas aeruginosa*, surface-active substances

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**Introduction**

Surfactants and emulsifiers are widely used in the petroleum, pharmaceutical, cosmetic and food industries. Most of these compounds are chemically synthesized and it is only in the past few decades that surface-active molecules of biological origin have been described. At present biosurfactants are readily bio-degradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counterparts. Among the heterogeneous group of biosurfacntants, the rhamnose-containing glycolipids produced by *Pseudomonas* (Arima, *et al.*, 1968 and Chandrasekaran and Bemiller, 1980). Almost all surfactant currently in use are chemically derived from petroleum. However interest in microbial surfactant has been steadily increasing in recent years due to their diversity, environmentally friendly characteristics, the possibility of their production through fermentation and their potential application in such areas as the environmental protection, surfactants and emulsifiers are widely used in the petroleum, pharmaceutical, cosmetic and food industries. Most of these compounds are chemically synthesized and it is only in the past few decades that surface-active molecules of biological origin have been described. At present biosurfactants are readily bio-degradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counterparts. Among the heterogeneous group of biosurfactants, the rhamnose-containing glycolipids produced by *Pseudomonas* (Arima, *et al.*, 1968 and Chandrasekaran and Bemiller, 1980). Almost all surfactant currently in use are chemically derived from petroleum. However interest in microbial surfactant has been steadily increasing in recent years due to their diversity, environmentally friendly characteristics, the possibility of their production through fermentation and their potential application in such areas as the environmental protection, surfactants and emulsifiers are widely used in the petroleum, pharmaceutical, cosmetic and food industries. Most of these compounds are chemically synthesized and it is only in the past few decades that surface-active molecules of biological origin have been described. At present biosurfactants are readily bio-degradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counterparts. Among the heterogeneous group of biosurfactants, the rhamnose-containing glycolipids produced by *Pseudomonas* (Arima, *et al.*, 1968 and Chandrasekaran and Bemiller, 1980). Almost all surfactant currently in use are chemically derived from petroleum. However interest in microbial surfactant has been steadily increasing in recent years due to their diversity, environmentally friendly characteristics, the possibility of their production through fermentation and their potential application in such areas as the environmental protection...
23 as the optimum range for achieving high specific productivity of rhamnolipids, using glucose and vegetable oil as substrates, respectively (Cooper and Zajic, 1980 and Guerra-Santos, et al., 1983). After nitrogen has been fully consumed, cell metabolism is directed to producing rhamnolipids, whose production increases after the exponential growth phase (Finnerty and Singer, 1983 and Guerra-Santos, et al., 1983). The purpose of this work was to study the production of a rhamnolipid-type biosurfactant by a strain isolated from oil, as well as to evaluate the tension-active proprieties and the toxicity of the spent broth and production on sugar beet molasses. This research has done in Biotech center in Iranian Research Organization for science and technology during years of 2004-2005

**Materials and Methods**

**Isolation, identification and preservation 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.

**Inoculum**

The strain was activated in a triptic soyer agar medium (TSA), cultivated at 30 ºC for 48 hours and transferred to a 250 ml. flask, containing 50 ml. of TSA. The flask was incubated at 30 ºC and 250 rpm. during 20 hours. Cells were harvested by centrifugation at 6000 rpm. during 20 minutes. The centrifuged microbial mass was suspended in a culture medium (medium salt production - MSP) with the following composition (g/l): (NH₄)₂SO₄, 1.0; KH₂PO₄, 3.0; MgSO₄·7H₂O, 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 0.1 g/l, in accordance with a calibration curve of dry weight versus absorbance (Chandrasekaran and Bemiller, 1980; Cooper and Zajic, 1980; Guerra-Santos, et al., 1983 and Jarvis and Johnson, 1949).

**Fermentations**

The production of rhamnolipids was studied during a seven-day fermentation 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 gasoline paraffin oil collected at flowing wells in the khark island of Iran, consisting of 32% saturated hydrocarbons, 23% aromatics, 36% of resins and 9.1% asphaltenes), glycerol (PA - Merck, Darmstadt) and whey from pak company. 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, NaNO₃, (NH₄)₂SO₄ and CH₄N₂O were employed at the following concentrations: 1.45, 1.0, and 0.51 g/l and glycerol 3% v/v.

**Biomass concentration**

Bacterial growth was monitored by measurement of absorbance at a wave length of 610 nm. Samples of 50 ml 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 (Itoh, et al., 1971 and Kappeli and Finnerty, 1980). 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-Dunoy tensiometer (cole-parmer instrument co, Bunker, IL, U.S.A) at room temperature. The concentration at which micelles began to from was represented as the CMC. At the CMC, sudden changes in surface tension, electrical conductivity and detergency were observed (14). 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 (Holdom and Turner 1969 and Itoh and Suzuki, 1972).

**Results**

**Microbial isolation, identification and preservation**

This strain showed an ability to use carbon sources, such as fructose, glucose, mannitol, mannose, glycerol and lactic acid, which are knowns good carbon sources for rhamnolipid prod-
Isolation and production of...

Effect of the carbon source

The production of rhamnolipids by the *Pseudomonas aeruginosa*, using substrates such as gasoline, paraffin oil, whey 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 (Hisatsuka, et al., 1971; Itoh, et al., 1971). 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 whey was used as carbon source.

As reported elsewhere, Table 1 shows a low initial superficial tension in the medium with whey (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 Figure 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 at increasing 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 standardized inoculum of 0.1 g/l was employed. Figure 2, 3 shows the yield factors relating substrate consumption to production (YP/S) and production to biomass (YP/X). The best results (YP/S = 0.13 g/g; YP/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 YP/S decreased after this.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Rhamnose mg/l</th>
<th>Initial surface tension D/cm</th>
<th>Final surface tension D/cm</th>
<th>% variation in surface tension</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexadecane</td>
<td>138</td>
<td>51.4</td>
<td>37.03</td>
<td>38.8</td>
</tr>
<tr>
<td>Paraffinic oil</td>
<td>260</td>
<td>54</td>
<td>51.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Babassu oil</td>
<td>150</td>
<td>35</td>
<td>27.3</td>
<td>28</td>
</tr>
<tr>
<td>Glycerol</td>
<td>690</td>
<td>53</td>
<td>27.46</td>
<td>48.2</td>
</tr>
</tbody>
</table>

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optimum glycerol concentration, reaching its lowest value ($Y_{PS} = 0.075 \text{ g/g}$) 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 (Department of Energy, 1979; Edwards and Hayashi 1965; Finnerty and Singer 1983).

Figure 1: Microbial growth curve, rhamnolipid production and consumption of glycerol from the fermentation of Pseudomonas aeruginosa during 200 hours, using a 1% glycerol concentration.
Effect of the nitrogen source

Figure 4 shows that sodium nitrate ($Y_{p,X} = 0.7 \text{ g/g}$) is more effective than ammonium sulfate ($Y_{p,X} = 0.35 \text{ g/g}$) and urea ($Y_{p,X} = 0.5 \text{ g/g}$). 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 result can be explained by the fact that nitrate first undergoes dissimilatory nitrate reduction to ammonium and then 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 (Guerra-Santos, 1983; Hisatsuka, et al., 1971; Itoh and Suzuki, 1972). *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. The 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 (Cooper and Zajic, 1980; Edwards and Hayashi, 1965; Guerra-Santos, et al., 1983).

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. Figure 5 displays the results of superficial tension related to different concentrations of rhamnolipids present in free-cell fermented medium. The measurement for superficial...
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Figure 4: Effect of nitrogen sources on the production of rhamnolipids by *Psuedomonas aeruginosa*

Figure 5: Superficial tension (D/cm) versus rhamnolipid concentration (mg/l) of fermented medium by *Psuedomonas aeruginosa*

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; therefore it was assumed to be the critical micelle concentration of rhamnolipids that has satisfactory tension-active properties. Working with *Psuedomonas 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 free-cell fermented medium. The critical micelle concentration obtained by the authors was of 20 mg/l, very close to that obtained in the present work.

**Discussion and Conclusion**

The strain isolated from oil was identified as *Psuedomonas 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 gasoline, paraffin oil, whey 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 4.2 g/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.

References


