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Production of emulsifier by a strain of *Pseudomonas aeruginosa* (C1 LBPVMA-UFAL) using lubricant oil as main carbon source

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ABSTRACT. The intention of this work was to evaluate the growth of *Pseudomonas aeruginosa* (C1 LBPVMA-UFAL) on lubricant oil and verify the production of rhamnolipid biosurfactant. Tests in solid medium containing lubricant oil supplemented with different sources of nitrogen were used to evaluate the growth of *P. aeruginosa*. The growth medium used for production of rhamnolipid was (g L^{-1}): yeast extract, 0.1; NaCl, 1.0; $MgSO_4$, 0.2; $MnSO_4.H_2O$, 0.02; 0.5% lubricant oil (w v^{-1}). The biosurfactant concentration was detected 24h after the inoculation, during the exponential phase, with the maximum value obtained up to 120h, although no drastic alteration of pH had been verified. The emulsifier activity was also first detected after 24h of incubation, with maximum production after 120h. The characterization of the produced biosurfactant was performed through thin layer chromatography, which showed the presence of two spots with R*f* values equal to 0.71 and 0.5, revealed by reagents specific to rhamnolipids. These results suggested that two types of rhamnolipidic biosurfactant are produced by the strain of *P. aeruginosa* in limited conditions of nutrients, able to use lubricant oil as main carbon source. This bacterium, isolated from agro-industrial effluent, showed potential to bioremediation assays of contamination with petrol and his derivates.

Key words: *Pseudomonas aeruginosa*, biosurfactant, lubricant oil, emulsifier activity, agro-industrial effluent.

RESUMO. Produção de emulsificante por linhagem de *Pseudomonas aeruginosa* (C1 LBPVMA-UFAL), usando óleo lubrificante como única fonte de carbono. O objetivo deste trabalho foi avaliar o crescimento de *Pseudomonas aeruginosa* (C1 LBPVMA-UFAL) sobre óleo lubrificante e verificar a produção de biosurfactante ramnolipídico. Testes em meio sólido com óleo lubrificante suplementado com diferentes fontes de nitrogênio foram usados para avaliar o crescimento de *P. aeruginosa*. O meio de crescimento utilizado para a produção de ramnolipídeo foi (g L^{-1}): extrato de levedura, 0,1; NaCl, 1,0; $MgSO_4$, 0,2; $MnSO_4.H_2O$, 0,02; 0,5% de óleo lubrificante (p v^{-1}). A concentração de biosurfactante foi detectada 24 h após a inoculação, durante a fase exponencial, com valor máximo obtido a 120h, sem verificar alteração drástica de pH. A atividade emulsificante foi também detectada após 24h de incubação, com máxima produção após 120h. A caracterização do biosurfactante produzido foi avaliada por cromatografia de camada delgada, revelando a presença de duas bandas com valores de R*f* iguais a 0,71 e 0,5, revelada por reagentes específicos para ramnolipídeos. Estes resultados sugerem que dois tipos de biosurfactantes ramnolipídicos são produzidos pela linhagem de *P. aeruginosa* em condições limitadas de nutrientes, sendo capaz de utilizar óleo lubrificante como única fonte de carbono. Esta bactéria, isolada de efluente agroindustrial, revelou potencial para ensaios de biorremediação de contaminação com petróleo e seus derivados.

Palavras-chave: *Pseudomonas aeruginosa*, biosurfactante, óleo lubrificante, atividade emulsificante, efluente agroindustrial.

Introduction

Petrol is a complex mixture of many compounds such as alkanes, aromatics, resins and asphaltenes, which could potentially be eliminated by microbial degradation (BARATHI; VASUDEVAN, 2001). In general, microorganisms may require some physiological adaptation to mineralize or grow on substrates with low solubility in water. Through evolution, bacteria have adapted themselves to feeding on water-immiscible materials by manufacturing and using a surface active product that helps them in the aqueous phase. So, they

adsorb, emulsify and disperse or solubilize the water immiscible material (HEALY et al., 1996).

The use of synthetic surfactants in treating hydrocarbon systems contaminating soil improves the restoration of these areas, increasing the surface for microbial attack. However, they may promote accumulation in the ecosystem causing serious environmental damage (CUNHA; LEITE, 2000). Biosurfactants in many cases have proved to be more effective than chemical surfactants, and have added benefit to be biodegradable (BANAT, 1995). The rate of hydrocarbon biodegradation is dependant of the biosurfactant effect, in two ways: by increasing solubilization and dispersion of the hydrocarbon, and by changing the affinity between microbial cells and hydrocarbons, by inducing increases in cell surface hydrophobicity (ZHANG; MILLER, 1995).

The best studied class of glycolipidic biosurfactants is the rhamnolipidc one, produced by several species of *Pseudomonas*. *P. aeruginosa* can produce them from substrates including alkanes, glycerol, olive oil and mannitol (LANG; WULLBRANDT, 1999). The great metabolic diversity of the *Pseudomonas* genus makes possible the use of these microorganisms for environmental treatment. The objective of this work was to evaluate the growth of different species of *Pseudomonas* on lubricant oil and verify the production of rhamnolipid biosurfactant.

Material and methods

Microorganism and screening of nitrogen source

The bacteria used in this work were isolated from agro-industrial effluent ('S.A. Usina Coruripe Açúcar e Álcool'), in the 'Laboratório de Bioquímica do Parasitismo Vegetal e Microbiologia Ambiental – LBPVMA', at the 'Instituto de Química e Biotecnologia' – 'Universidade Federal de Alagoas/Brazil – UFAL'. Later, this isolate was identified by morphological and biochemical analysis as *Pseudomonas aeruginosa*.

The influence of nitrogen source was studied in the medium containing 0.5% lubricant oil (w v^{-1}), 0.1% NaCl, 0.1% of one of the three different nitrogen sources [yeast extract, $NaNO_3$ or $(NH_4)_2SO_4$] and 15.0 g L^{-1} agar. The inoculum volume was estimated to obtain approximately 1 x 10^3 cells mL^{-1}. The plates were incubated at 30°C, in the dark, for 72h. The number of viable cells was measured in parcels of the liquid medium (cells mL^{-1}), using a Newbauer chamber and light microscope (640 X).

Growth conditions

The growth medium used for production of rhamnolipid was (g L^{-1}): yeast extract, 0.1; NaCl, 1.0; $MgSO_4$, 0.2; $MnSO_4.H_2O$, 0.02. The fermentation process was carried out in 150 mL Erlenmeyer flasks, containing 50 mL of medium, in triplicate. After that, 0.5 % of lubricant oil (w v^{-1}) was added. The final pH in the medium was adjusted to 7.0, using 0.1 N KOH. The inoculum volume was estimated to obtain approximately 1 x 10^5 cells mL^{-1}. Submerged microbial cultures were incubated in the dark, at 30°C, 200 rpm, for 120h. The growth was monitored each 24h, by cell counting as well as optical density at 600 nm.

Analytical methods

Rhamnolipid concentration was determined in triplicate by using orcinol assay (CHANDRASEKARAN; BEMILLER, 1980). To 0.1 mL of each sample from the cells culture supernatant, 0.9 mL of a solution was added containing 0.19% orcinol (in 53 % H_2SO_4), and the mixture was heated at 80°C for 30 min. Then, the samples were cooled for 15 min. at room temperature and the $A_{421\ nm}$ was measured. The rhamnolipid concentration was evaluated based on a standard curve prepared with different concentrations of L-rhamnose (equivalent mg rhamnose mL^{-1}).

The assay of emulsification activity was determined by the method of Cirigliano and Carman (1984) modified. To 2 mL of each sample from the cells culture supernatant, it was added 1 mL of cyclohexane, and then mixed using vortex for 2 min. The resulting uniform emulsion was allowed to sit for 10 min. After that, its absorbance was measured at 540 nm. The blank contained 2 mL of the filtered sterilized medium.

The different types of rhamnolipids were separated by thin layer chromatography (TLC). The cell culture supernatant was concentrated by liophylization. The concentrate was treated twice with $CHCl_3$:MeOH (2:1, v v^{-1}) and then solvents were removed by evaporation. The material was dissolved in $CHCl_3$:H_2O (1:1, v v^{-1}) and analyzed by TLC on silica gel plates (G60, $F_{254\ nm}$; Merck, Germany). Chromatograms were developed with the system $CHCl_3$:CH_3OH:H_2O (65:25:4, v v^{-1} v^{-1}) as eluent, and visualized with different reagents (PARRA et al., 1989).

Results and discussion

Screening of nitrogen source

The influences of the nitrogen source (yeast extract, ammonium and nitrate) on the growth of *P. aeruginosa* isolated was studied using the medium containing 0.5% lubricant oil expressed in log of the number of cells mL^{-1}, after 72h of incubation, in the dark, at 30°C. The maximum concentrations of cells in media supplemented with yeast extract, $NaNO_3$ or $(NH_4)_2SO_4$ were respectively 5.3 x 10^7, 5 x 10^6 and 3.8 x 10^6 mL^{-1} (Table 1). The choice of the nitrogen

sources was based on the results here obtained and on the observations reported by Guerra-Santos et al. (1984), which demonstrated the influences of nitrogen source (yeast extract) on biosurfactant production from *P. aeruginosa* DSM2659. According to these authors, in the absence of yeast extract, the biomass concentration of *P. aeruginosa* decreases, and a moderate accumulation of glucose occurs during the biosurfactant production.

Table 1. Screening of different nitrogen sources for growth of *Pseudomonas aeruginosa* (C1*) in medium containing 0.5% of lubricant oil.

Culture medium	*P. aeruginosa* (cells mL^{-1})	
	0h	72h
**Yeast extract	1×10^3	5.3×10^7
Sodium nitrate	1×10^3	5.0×10^6
Ammonium sulfate	1×10^3	3.8×10^6

**P. aeruginosa* C1 – isolate from agro-industrial effluent, identified at the LBPMA-UFAL.
***Yeast extract (0.5%; w v^{-1}) was chosen as the nitrogen source for the medium containing lubricant oil as the main source of carbon for growth and biosurfactant production. The study was performed in triplicate.

Production of biosurfactant in lubricant oil medium

The results of both turbidity and viable cell counting indicate the production of biosurfactant by *P. aeruginosa* C1 using lubricant oil as the main carbon source. The initial concentration of biosurfactant was 29.62 eq mg rhamnose L^{-1}, reaching the maximum value around of 36 eq mg rhamnose L^{-1} at 120h after the inoculation. The results of cellular growth and biosurfactant production are shown in Figure 1.

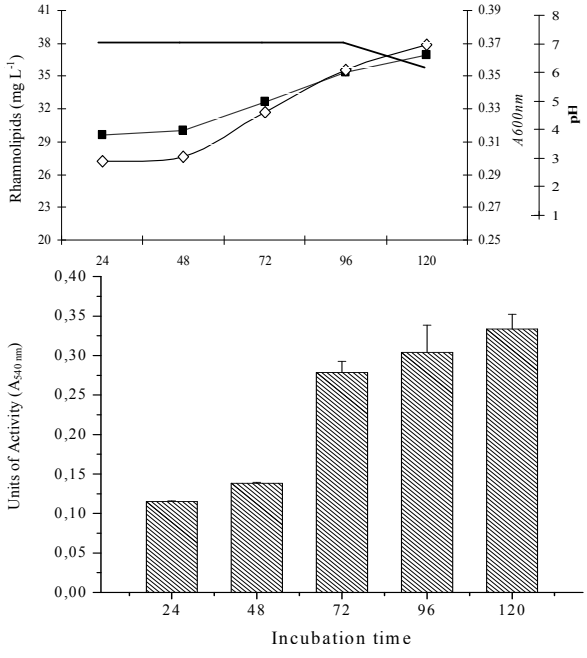

Figure 1. Production of rhamnolipid and bacterial growth of *Pseudomonas aeruginosa* C1 (LBPVMA) isolated from agro-industrial effluent on liquid media with lubricant oil as main carbon source, during 120h of incubation (in the dark, at 30ºC, 200 rpm). Equivalent mg rhamnolipids L^{-1} (■); Optical density (◊); pH (-); Emulsification activity (▒).The study was performed in triplicate.

In comparison with data obtained by Déziel et al. (1996), who investigated the capacity of biosurfactant production by *P. aeruginosa* 19SJ on salt medium supplemented with 2% of naphthalene or 2% mannitol, separately, the isolate of *P. aeruginosa* studied here was more efficient in the production of biosurfactant, since the 19SJ strain had its maximal productivity around 1.6 and 0.4 eq mg rhamnose L^{-1} using mannitol and naphthalene, respectively.

Zhang et al. (2005) suggested that the hydrocarbon biodegradation probably occurs with increase of cell surface hydrophobicity after extraction of lipopolysaccharides from the cellular envelope by rhamnolipids, which subsequently stimulates uptake via direct contact between cells and hydrocarbon droplets. This observation is supported by data of Ozdemir and Malayoglu (2004), which showed the role of R1 and R2 rhamnolipids produced by *P. aeruginosa* in the transport and assimilation of hydrocarbon. Abalos et al. (2004) verified that the consortium between *P. aeruginosa* and other microorganisms increased the biodegradation of crude oil, with subsequent rhamnolipid production.

Property emulsifier of biosurfactant produced in lubricant oil medium

The emulsification activity was first detected after 24h the incubation, with maximum production after 120h (Figure 1). Surface active agents assist degradation of hydrocarbon pollutants by facilitating desorption from the soil, and/or by dispersing small droplets that are more easily attacked by microorganisms. In previous work, Zhang and Miller (1995) demonstrated surfactant effects on hydrocarbon biodegradation, and it depends on the structure of the surfactant, on the physical state and amount of alkane dispersion and emulsification, and on the degrading isolate of microorganism. Although these factors are interdependent, each one can be considered separately in order to help interpret the system as whole.

Characterization of the biosurfactant obtained

The biosurfactant extracted from the concentrated culture supernatant was analyzed by TLC, and visualized with specific reagents, producing spots with different R*f* values (retention factor). The spots with R*f* 0.71 and R*f* 0.5 showed positive reactions for glycids (Molish reagent) and lipids (rhodamine B reagent), but negative reactions for amino groups (ninhydrin and UV), and can be seen in Table 2. These results suggested that *P. aeruginosa* C1 (LBPVMA-UFAL) produces two types of rhamnolipids in lubricant oil medium.

Table 2. Thin layer chromatography parameters of the different forms of rhamnolipids found in the growth medium of *Pseudomonas aeruginosa* C1 (LBPVMA-UFAL), after 120h of incubation (in the dark, at 30ºC, 200 rpm).

*Spots	Rf	[a] UV	[b]Ninhydrin	[c]Carboxylic acid	[d]Lipid	[e]I_2	[f]Sugar
1	0,71	-	-	+	+	+	+
2	0,50	-	-	+	+	+	+

*Solvent system CH_3Cl:CH_3OH:H_2O (65:25:4 v v^{-1} v^{-1}); [a]UV: Detection of compounds with native fluorescence; [b]Ninhydrin: reagent used for detection of free amino groups; [c]Bromocresol green: reagent used for detection of free carboxylic acid; [d]Rhodamine 6G: reagent used for detection of lipids; [e]Iodine vapours: reagent used for detection of organic compounds; [f]Molish: reagent used for detection of glycids.

In general, some authors reported monorhamnolipids as the predominant component of rhamnolipid surfactant mixture, whereas many reports described the dirhamnolipids as the main component present. In contrast, the predominance of mono or dirhamnolipid forms and the relationship with surfactant production characteristics are not well understood, but some attempts have been made (NITSCHKE et al., 2005).

Conclusion

These results suggest a correlation between growth of the isolate of *Pseudomonas aeruginosa* obtained from agro-industrial effluent (C1 LBPVMA-UFAL) in oil lubrificant medium and the production of biosurfactant with emulsifier activity. Because of its origin, this strain of *P. aeruginosa* has also potential application in consortium of microorganisms to remediation of environments polluted by hydrocarbon oil derivatives.

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