March 2014, Vol. 8, No. 3, pp. 201-210
Journal of Life Sciences, ISSN 1934-7391, USA

Biosurfactant Production in Aerobic and Anaerobic Conditions by Different Species of the Genus *Pseudomonas*

Mayri Díaz De Rienzo[1], Isora Urdaneta De Ranson[2] and Blas Dorta[2]

1. School of Chemical Engineering and Analytical Science, University of Manchester, Manchester M13 9PL, UK

2. Department of Cell Biology, Venezuela Central University, Caracas 1010A, Venezuela

Received: December 06, 2012 / Accepted: March 21, 2013 / Published: March 30, 2014.

Abstract: In a time where surface active agents are capable of reducing the energy of the bonds between water molecules by interacting with them to reduce surface tension, it would be unwise not to be able to generate these in masses. Different *Pseudomonas* species were grown in MSP (minimal sulphate phosphate) media containing salts, glycerol and glucose. *P. aeruginosa* grown aerobically in the presence of glycerol as carbon source showed the highest emulsion percentage (81.48%), most significant decrease in surface tension (20 mN/m) and rhamnose production of 2.86 mg/mL. However, in anaerobic conditions there was no emulsion, rhamnolipid production or decrease in surface tension. The rhamnolipids were molecularly characterized using ESI-MS (electrospray ionization-mass spectrometry), *P. aeruginosa* CVCM 411 is able to produce mono-rhamnolipids and di-rhamnolipids, being rhamnolipid $RhC_{10}C_{12:1}$ the predominant monomer. The specific growth rate for isolates of *P. aeruginosa* and *P. fluorescens* in MSP are 0.6732 h^{-1} and 0.2181 h^{-1}, respectively. In conclusion, the formation of rhamnolipids by *P. aeruginosa* is linked to its growth (depending on μ), and its ability to generate about 35% of the μ_{max}, in the presence of glucose (carbon source) and glycerol (applied as pulses).

Key words: Surfactants, fermentation, rhamnolipids, *Pseudomonas*.

1. Introduction

Surface active agents commonly known as surfactants are molecules capable of reducing surface tension force, as energy between water molecules. Surfactants are either chemically produced (synthetic) or based on biological materials (biosurfactants). However, synthetic surfactants can destabilize toxic organic compounds which can enter the environment and have negative effects. Water soluble surfactants can wash out of dried paints and enter the environment. Some of these surfactants are directly toxic to animals and the environment as well as increase the ability of other toxic contaminants present to enter the environment. Therefore, recent studies have been geared towards finding alternative products that are compatible with the environment [1-3].

While synthetic surfactant is flawed, on the other hand, biosurfactants offer an advantage of production from six classes of microorganisms. Bognolo [1] states that biosurfactant are biodegradable, biocompatible, posses stable activity in extreme environments, are less expensive and toxic, when comapred to chemical surfactants. The most important types of biosurfactants are glycolipids, phospholipids, fatty acids, lipopolysaccharide/lipopeptides, polymers and particulates. Glycolipid-type biosurfactants as rhamnolipids are of great interest, because they can be obtained from *Pseudomonas* bacteria which can be isolated from environmental samples [4].

Rhamnolipids have multiple commercial applications, especially in oil, pharmaceutical and

Corresponding author: Mayri Díaz De Rienzo, Ph.D., research associate, research fields: biotechnology and microbiology. E-mail: mayri.diazderienzo@manchester.ac.uk.

chemical industries [5, 6]. Biosurfactants produced by *Pseudomonas aeruginosa* (*P. aeruginosa*) have been characterized and studied as agents capable of removing hydrophobic compounds from soil [7]. Although this microorganism has been studied extensively in the production of biosurfactant, it is not the only species belonging to this genus capable of producing biosufactants. Dubeau et al. [8] demonstrated the feasibility of the production of rhamnolipid biosurfactants by *Burkholderia thailandensis* microorganism. Rhamnolipid produced by *P. aeruginosa* have been reported to contain two molecules of rhamnose (mono-rhamnolipid, $RhC_{10}C_{10}$ and di-rhamnolipids, $Rh_2C_{10}C_{10}$) and two molecules of acid (ß-hidroxidecanoic) [7, 9].

Rhamnolipid have a wide range of applications, especially in the oil industry, where they are used to treat interfacial-related problems associated with drilling operations to the packaging of finished products. Therefore, the aim of this study is to produce biosurfactants from different *Pseudomonas* species, both under aerobic and anaerobic conditions. Also, different carbon sources were investigated in order to select the best hydrocarbon source that will encourage biosurfactant production and increase biomass performance.

2. Materials and Methods

2.1 Microorganisms and Growth Conditions

The different *Pseudomonas* species used in this study were supplied by Venezuelan Center Collection of Microorganisms including: *P. putida* (CVCM 629), *P. aeruginosa* (CVCM 1500, 411, and 625) and *Pseudomonas fluorescens* (CVCM 1100). *Pseudomonas* species were grown in MSP (minimal sulphate phosphate media) and supplemented with different concentrations of glucose or glycerol, mono-potassium phosphate (KH_2PO_4, 3 $g \cdot L^{-1}$), di-potassium phosphate (K_2HPO_4, 7 $g \cdot L^{-1}$), magnesium sulphate ($MgSO_4 \cdot 7H_2O$, 0.1 $g \cdot L^{-1}$), ammonium sulphate $(NH_4)_2SO_4$, 1 $g \cdot L^{-1}$) and chloramphenicol (50 $\mu g \cdot mL^{-1}$), at pH 7. Precultures of *Pseudomonas* species were inoculated for 36 h in the growth media, aerobically at 30 °C, and 110 rpm with an eccentricity of 2.5 cm.

Pseudomonas species were grown anaearobically in MSP medium (plus salts and glucose as mentioned above), in the presence of sodium nitrate ($NaNO_3$, 5 $g \cdot L^{-1}$) as nitrogen source. Planktonic cells were grown for 36 h in serum bottles with a rubber stopper under nitrogen atmospheric pressure, which contained degassed 100 mL of growth medium.

2.2 Continuous Flow Experiment

Fermentation unit (New Brunswick BioFlo C30) was employed to perform continuous flow of *P. aeruginosa* (CVCM 411) and *P. fluorescens* (CVCM 1100). Microorganisms used in this study were aerobically (air supply is at 0.5 VVM) incubated in MSP medium, at 37 °C, and 400 rpm speed for 36 h. Peristaltic pump was used to control the flow to achieve different dilution rates. At a corresponding stationary state of the microorganism the growth medium (MSP) in the bioreactor was supplemented with either nitrogen ($NH_4(SO_4)_2$, 10 $g \cdot L^{-1}$) or carbon (glycerol, 2.5 $g \cdot L^{-1}$) source, as alternatives to applied pulses.

2.3 Cell Biomass Analysis

Microorganism cultures of approximately 6,000 g were centrifuged for 10 min. Supernatant are stored at −20 °C, until further use for each analytical determination. Cell pellets were washed three times with distilled water under the same conditions of centrifugation and dried at 60 °C to a constant weight. Cell growth was monitored by measuring the weight (g) of the dry pellets.

2.4 Determination of Glycerol Concentration

The concentration of glycerol in the culture medium was determined by colorimetric method [10], 1 mL of 0.05 M periodic acid was added to 2 mL of sample,

stirred and allowed to stand for 5 min, followed by an addition of 1 mL of NaOH (1 N) and 1 mL of 0.2% phloroglucinol and the absorbance was read at 480 nm after 45 min (against air). The glycerol concentration was estimated using a standard curve prepared with glycerol in the concentration range of 0 to 1 $g \cdot L^{-1}$.

2.5 Determination of Glucose Concentration

Glucose concentration was determined via glucose oxidase enzyme colorimetric method (nzytech®) [11]. The amount of coloured compound (quinoneimine dye) formed through the combined action of GOD (glucose oxidase) and POD (peroxidase), measured at 510 nm, is stoichiometric with the amount of D-glucose in sample volume.

2.6 Measuring of Surface Tension

Surface tension was evaluated in 10 mL aliquots of fermented cultures in the presence or absence of biomass, using an inexpensive Wilhelmy balance based on a fibre optic sensor capable of sensitive surface tension measurements [12]. Distilled water was used to calibrate the instrument and measurements were performed in triplicate.

2.7 Emulsifying Capacity Determination

Emulsifying capacity was measured [13]. For measuring, 5 mL of kerosene is added to 5 mL of aqueous sample. The mixture is vortex at high speed for 2 min. After 24 h, the height of the stable emulsion layer is measured. The emulsion index *E* is calculated as the ratio of the height of the emulsion layer and the total height of liquid:

$$\boldsymbol{E} = \frac{h\ emulsion}{h\ total} \times 100\%$$

2.8 Quantification of Biosurfactants

This was determined indirectly in the cell-free broth by measuring rhamnose concentration [14]. For this purpose a standard curve was prepared with glucose from 0 to 100 $mg \cdot L^{-1}$, control and samples were treated with 2.5 mL of anthrone reagent prepared in concentrated sulfuric acid at 2 mg/mL. Samples were boiled for 15 min to stabilize the color, and the concentration of rhamnose was determined at 625 nm.

2.9 Rhamnolipids Extraction

Rhamnolipid mixtures (6,000 g) were extracted from growth media after pellet cells were removed after centrifugation for 15 min. The pH of the supernatant was adjusted to 2.0, using sulphuric acid (H_2SO_4) and an equal volume of chloroform/methanol at 2:1 ratio. The mixture was vigorously shaken for 5 min and allowed to set until phase separation. The organic phase was removed and the extraction process was further repeated twice. The obtained rhamnolipid product was made concentrated from the pooled organic phases, by using a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45 °C.

2.10 ESI-MS Analysis

For mass analyses partially purified rhamnolipid preparations were dissolved in 50% acetonitrile-water and characterized by ESI-MS (electrospray ionization-mass spectrometry) in the negative ionization modus. The analyses were performed with a Quadrupole time-of-flight hybrid mass spectrometer (Q-TOF™II, Waters Micromass, Milford, Massachusetts) equipped with an orthogonal electrospray source (Z-spray).

3. Results and Discusion

3.1 Production of Rhamnolipids: Batch Cultivations

The results in the present study showed that all *Pseudomonas* species used can grow under aerobic conditions. After 72 h all the species evaluated were able to produce rhamnolipids in growth medium containing 1% glucose and in the presence of 0.5% glycerol, accompanied by a slight increase in pH (Table 1). Here, lactose or sucrose substrates could not be fermented. Therefore, there considerations in

Table 1 Growth of the different species of the genus *Pseudomonas* on different carbon sources under aerobic conditions.

Microorganisms CVCM	Lactose (1%)		Lactose/Glycerol (1%)		Glycerol (1%)		Glucose (1%)		Sucrose (1%)	
	Growth	pH	Growth	pH	Growth	pH	Growth	pH	Growth	pH
411	-	/	+	7.4	+	7.2	+	7	-	/
625	-	/	+	7.5	+	7.1	+	7	-	/
629	-	/	+	7.5	+	7.3	+	7.2	-	/
1500	-	/	+	7.4	+	7.2	+	7.2	-	/
1100	-	/	+	7.5	+	7.4	+	7.3	-	/

(+) There was growth; (-) There was not growth; (/) Parameter not measured.

Table 2 Growth of the different species of the genus *Pseudomonas* on different carbon sources under anaerobic conditions.

Microorganisms CVCM	Glycerol (0.5%)		Glycerol (1%)		Glucose (1%)	
	Growth	pH	Growth	pH	Growth	pH
411	+	7.3	+	7.2	+	7.1
625	-	7.1	-	7.1	-	7.1
629	-	7	-	7	-	7.1
1500	-	7.1	-	7	-	7.1
1499	+	7.4	+	7.3	+	7.2

(+) There was growth; (-) There was not growth.

scaling studies are limited. The application of glycerol by all microorganisms shows the same potential for rhamnolipids production, under aerobic conditions. Glycerol has been described as a promoter for the synthesis of biosurfactants [15].

Under anaerobic conditions, metabolic energy is directed to biomass production, which may require the presence of hydrocarbon inducers to observe measurable production of extracellular surfactant [16]. Also, *P. aeruginosa* strains (CVCM 411) were able to grow in denitrifying conditions, as seen in micro-organisms grown in aerobic conditions with a slight increase in pH [16, 17] (Table 2). The duration of these processes was 168 h, in contrast to the aerobic condition where the stationary growth phase is reached before 72 h.

Biomass production of rhamnolipids was based on the weight of dry and partially purified strains, both in aerobic and anaerobic conditions at the expense of glucose and glycerol. Of all the tested substrates, 1% glycerol showed the highest biomass concentration. Also, highest rhamnolipid yields were obtained when glucose was supplemented with the growth media. In the absence of nitrogen, rhamnolipid biomass concentration obtained when growth media were supplemented with glycerol (between 0.5% and 1.0%) are equal. However, rhamnolipid biomass production increased with the initial concentration of glycerol. Therefore, glycerol consumption was high (between 70% and 80%) in most cases.

Excess glycerol consumption above the ceiling, imposed by absence of nitrogen, may arise due to the formation of extracellular products that do not contain nitrogen in their structure. These products could be structural polysaccharides, which once produced are still associated with cells and are quantified in conjunction with them as dry biomass. In fact, it is known that in the absence of nitrogen, *Pseudomonas* pieces are capable of producing structural polysaccharides containing glucose, galactose, mannose and rhamnose as monosaccharide units [18, 19].

Glycerol is metabolized by *Pseudomonas* through the glycolytic pathway, as opposed to glucose which is mainly metabolized in these microorganisms through the Entner-Doudoroff pathway. Glucose is phosphorylated to glucose-6-phosphate to produce gluconic acid. Comparison of these two methabolic pathways shows that glycerol is metabolized quicker.

In analyzing such differences in stoichiometric, it is assumed that the energy content of glycerol carbon-mol is higher than glucose, which is evident when comparing the degrees of reduction of the two molecules and *P.* species. Thus, glycerol $CH_{2.66}O$ (formula for a c-mol of glycerol) has a degree of reduction of $\gamma_{glycerol} = 4.66$, the biomass of *P.* species is represented by $CH_{2.00}O_{0.52}N_{0.23}$ with a $\gamma_{biomass} = 4.37$ and glucose (CH_2O) $\gamma_{glucose} = 4.00$. This implies that if the degree of reduction of glycerol is higher than *P.* species, biomass production is limited by carbon source.

In contrary if the degree of reduction of glucose is lower than *P.* species, biomass production is limited by energy. Therefore, the maximum allowable stoichiometric yield for the production of *P. species* at the expense of glycerol is higher than that for glucose. Stoichiometric analysis allows the calculation of the above bioconversion of biomass substrates. These calculations are possible when you have the experimental yields, which are presented in Table 3.

All tested *P. aeruginosa* strains used in the present study were capable of producing surfactants at the expense of glycerol, under aerobic conditions (Table 4). This fact was evidenced by a marked reduction in surface tension of the respective cell-free broth, with values ranging between 26 mN/m and 31 mN/m. These values are similar to those previous reported [20] for *P. putida* 21 BN (31 mN/m). Results in Ref. [21] showed that *P.* species have surface tension values between 25 and 30 mN/m and the studies in [22] reported a decrease of 28 mN/m for *Bacillus licheniformis* (JF-2), grown under similar conditions. However, in this study under anaerobic conditions rhmanolipids were not detected.

The different microorganism were assessed for their ability to form stable emulsion from their cell-free broth, and the results showed that the highest percentage of emulsion (81.48%) is obtained when all strain were grown under aerobic condition, in the presence of glycerol.

Likewise, the authors see that the emulsifying capacity correlates with changes reflected in the surface tension, which confirms the presence of a surface active agent. Under anaerobic condition the emulsifying capacity of *P. aeruginosa* (CVCM 411) was evaluated, and was reported to be approximately 5%. In aerobic conditions the highest concentrations of rhamnose (2.86 $mg \cdot L^{-1}$) were obtained when strains were grown in 0.5% glycerol. In contrast, under anaerobic conditions rhamnose were not produced, but surfactant activity was present.

3.2 Production of Rhamnolipids: Chemical Analysis

Spectroscopic analysis confirmed the presence of rhamnolipid-type surfactants for *P. aeruginosa* (CVCM 411), with both mono (Rh_a) and di-ramnolipid (Rh_aRh_a) coupled with several lipid chains substitutions (C_8-$C_{12:1}$). Twenty one corresponding isomers have been previously identified in *P. aeruginosa*-produced rhamnolipids [23, 24], *P. aeruginosa* strain (CVCM 411) is able to produce four rhamnose isomers at the expense of glycerol as carbon source in MSP Rh_aC_{12}, $Rh_aRh_aC_{10}C_{12:1}$, Rh_aC_8 and $Rh_aC_{10}C_{12:1}$ (Fig. 1).

Table 3 Biomass and yields production of cultures from different microorganisms under aerobic and anaerobic conditions.

Microorganisms	Glycerol (1%)		Glycerol (0.5%)		Glucose (1%)	
CVCM	X ($g \cdot L^{-1}$)	$Y_{x/s}$	X ($g \cdot L^{-1}$)	$Y_{x/s}$	X ($g \cdot L^{-1}$)	$Y_{x/s}$
411 aerobic conditions	4.29	0.55	3.64	0.63	1.05	0.22
411 anaerobic conditions	1.32	0.37	0.83	0.33	0.59	0.12
625 aerobic conditions	6.26	0.82	3.99	0.98	1.43	0.28
629 aerobic conditions	5.81	0.77	2.89	0.78	1.59	0.32
1500 aerobic conditions	4.37	0.67	2.59	0.722	1.62	0.32
1100 aerobic conditions	3.09	0.54	1.71	0.52	1.31	0.26

Table 4 Changes on surface tension of fermented cultures by different microorganisms.

Microorganisms CVCM	Surface tension γ (mN/m)		
	Glycerol (1%)	Glycerol (0.5%)	Glucose (1%)
411 aerobic conditions	23	20	6
411 anaerobic conditions	0	0	0
625 aerobic conditions	25	17	0
629 aerobic conditions	1	5	3
1500 aerobic conditions	19	19	0
1100 aerobic conditions	5	6	0

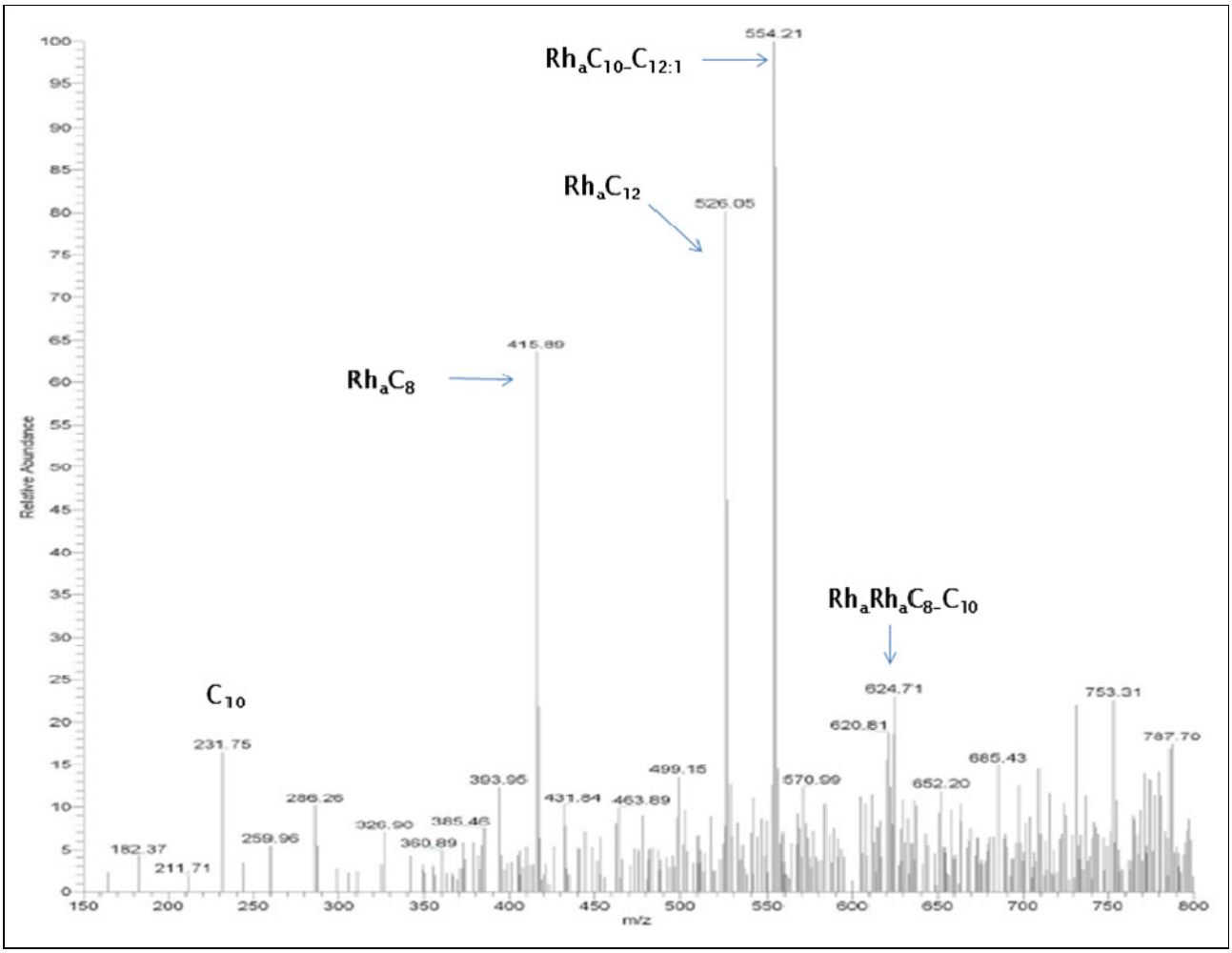


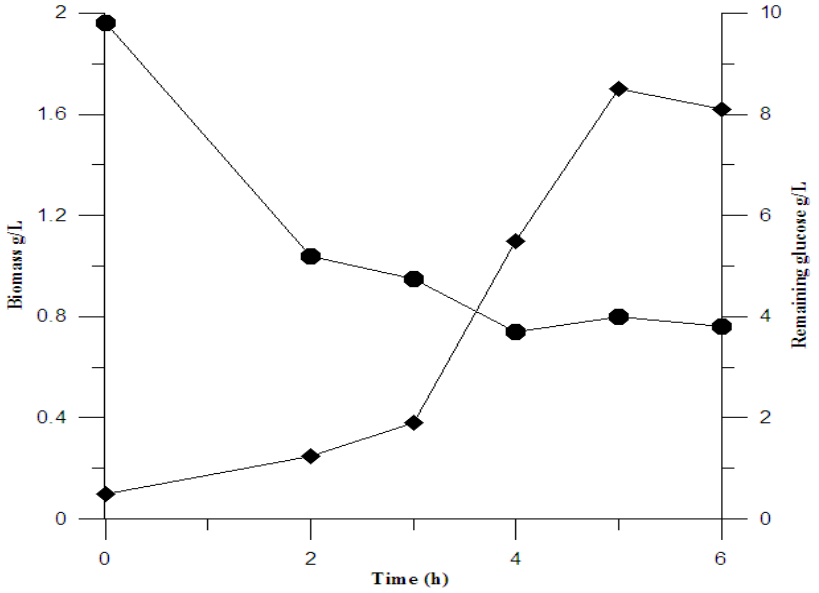
Fig. 1 ESI-MS analysis. Spectrum of partially purified extracts from fermented cells of *Pseudomonas aeruginosa* CVCM 411 (Rh_a: rhamnose molecules).

These results are comparable with those obtained before [24], where scientists identified six types of rhamnolipids in *P. aeruginosa* (57RP) extracts of which four corresponds to those shown in this study, but with different relative abundances (peak area).

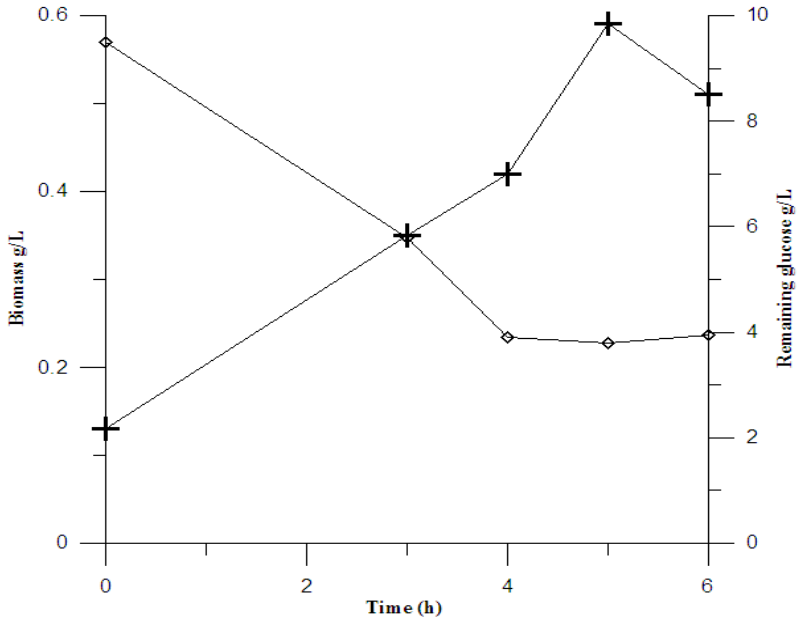
It is showed that the majority of isomer produced by *P. aeruginosa* (LB1) was $Rh_aC_{10}C_{10}$, with a 52.83% relative abundance [25]. However, in this work the rhamnolipid produced in greater proportion by *P. aeruginosa* (CVCM 411) is the mono-rhamnolipid $Rh_aC_{10}C_{12:1}$ with a relative abundance of 100%, which is different to previous results [23] where only 7.9%, when it was grown in the presence of mannitol and naphthalene as carbon and energy sources.

3.3 Production of Rhamnolipids: Continuous Flow Cultivations

Continuous flow culture of *P. aeruginosa* (CVCM 411) and *P. fluorescens* (CVCM 1100) were initiated in mineral medium under nitrogen limitation using glucose as carbon source, in the absence of fresh medium. In such conditions the microbial growth occurred according to the kinetics described in Fig. 2. *P. aeruginosa* (CVCM 411) reaches stationary growth phase at about 4 h, and it was fed with fresh media at a dilution rates of 0.2-0.6 hr^{-1}. Here, concentrations of biomass limiting substrate (in this case nitrogen source) and any extracellular component remained constant over time.



(a)



(b)

Fig. 2 (a) Growth curve of *Pseudomonas aeruginosa* CVCM 411 in the presence of glucose as a carbon and energy source (♦) and glucose consumption ($g \cdot L^{-1}$) (●); (b) growth curve of *Pseudomonas fluorescens* CVCM 1100 in the presence of glucose as a carbon and energy source (◊) and glucose consumption ($g \cdot L^{-1}$) (+).

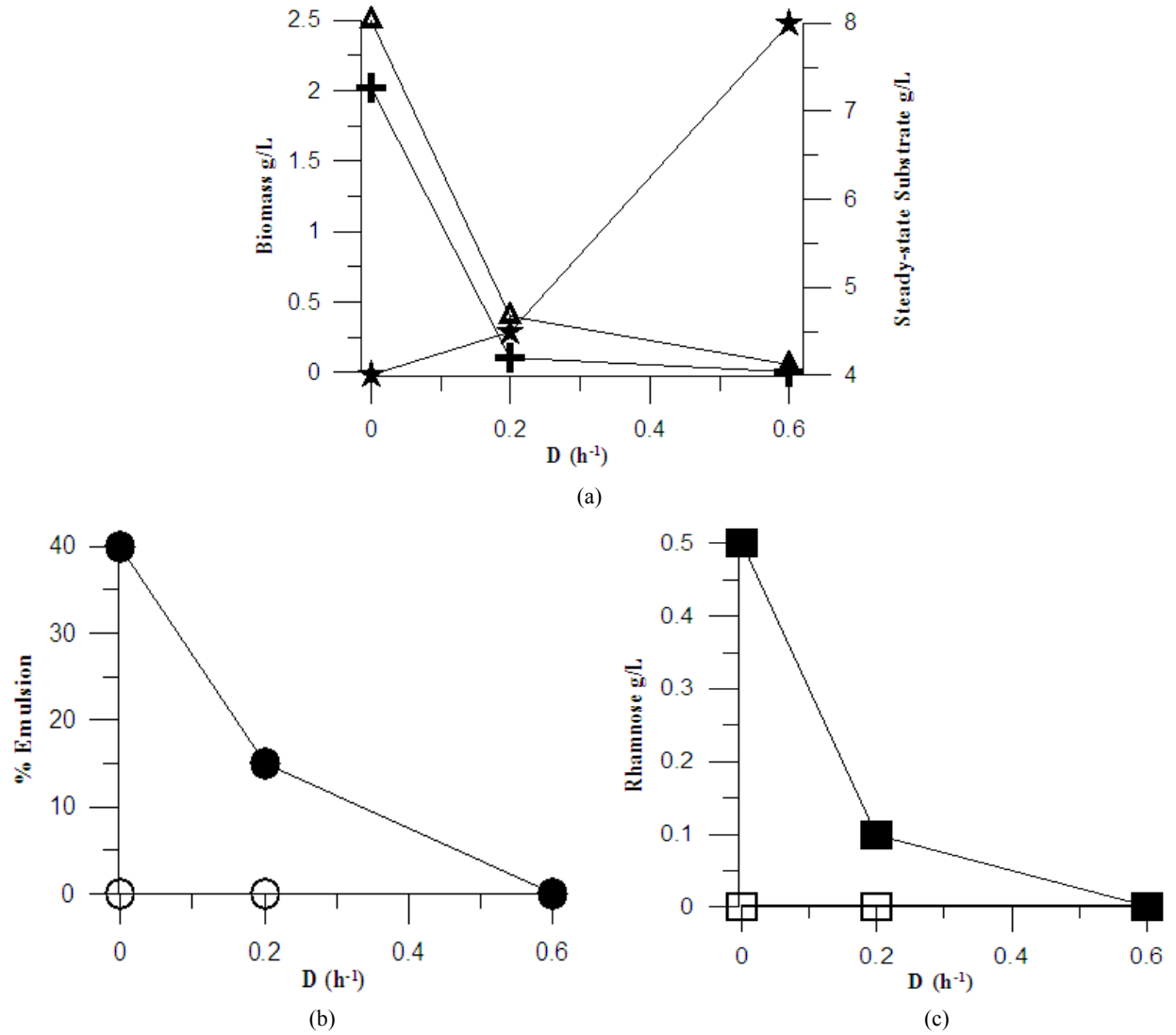


Fig. 3 Continuous culture of *Pseudomonas aeruginosa* CVCM 411. (a) Biomass (g·L^{-1}) of *Pseudomonas aeruginosa* CVCM 411 obtained in each steady state depending on the dilution rate under nitrogen limitation. Glucose 10 g·L^{-1} as carbon and energy source (+). Glycerol 2.5 g·L^{-1}, applied in the form of pulses once the respective stationary states were reached (Δ). Substrate concentration at steady state (¾). (b) Emulsion Index in the presence of glycerol 2.5 g·L^{-1} (●) and glucose 10 g·L^{-1} (○). (c) Rhamnose Concentration in presence of glycerol 2.5 g·L^{-1} (■) and glucose 10 g·L^{-1} (□).

Under conditions of continuous flow cultivation of microorganisms peak concentrations of percentage emulsion and rhamnose concentration were obtained, before and after feeding the medium with glycerol supplements. Maximum reduction of surface tension (23 mN/m), highest percentage of emulsion (40%, Fig. 3b) and the highest concentration of rhamnose (1 g·L^{-1}) were attained (Fig. 3c). Formation of rhamnolipid by *P. aeruginosa* (CVCM 411) is linked to its growth, due to its ability to yield the highest production value below 35% of μ_{max}. Although during the exponential growth stage biosurfactants were not detected. *P. fluorescens* (CVCM 1100) is unable to produce surface active agents, under the conditions showed in this present work.

4. Conclusions

All species of *Pseudomonas aeruginosa* tested in the present study were able to grow and produce rhamnolipid biosurfactants aerobically under nitrogen

limitation at the expense of glycerol as sole carbon source in MSP culture medium. The highest yield of biomass (0.735) as well as the efficiency of bioconversion (0.875) of glycerol was obtained with *Pseudomonas aeruginosa* CVCM 411 and positive signals of dirhamnolipids and monorhamnolipids were detected through ESI-MS analysis. The production of rhamnolipids in medium MSP by *Pseudomonas aeruginosa* CVCM is linked to its growth (depending on μ) giving a maximum output values close to 35% of the μ_{max}.

References

[1] G. Bognolo, Biosurfactants as emulsifying agents for hydrocarbons, Colloids and Surfaces A: Physicochemical and Engineering Aspects 152 (1-2) (1999) 41-52.

[2] Y. Lee, S.Y. Lee, J.W. Yang, Production of rhamnolipid biosurfactant by fed-batch culture of *Pseudomonas aeruginosa* using glucose as a sole carbon source, Bioscience, Biotechnology, and Biochemistry 63 (5) (1999) 946-947.

[3] A. Abalos, A. Pines, M. Infante, M. Casals, F. Garcia, A. Manresa, Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes, Langmuir 17 (5) (2001) 1361-1371.

[4] K.H. Shin, K.W. Kim, E.A. Seagren, Combined effects of pH and biosurfactant addition on solubilization and biodegradation of phenanthrene, Applied Microbiology and Biotechnology 65 (3) (2004) 336-343.

[5] K. Pattanathu, E. Gakpe, Production, characterization and applications of biosurfactants—review, Biotechnology 7 (2) (2008) 360-370.

[6] J.D. Desai, I.M. Banat, Microbial production of surfactants and their commercial potential, Microbiology and Molecular Biology Reviews: MMBR 61 (1) (1997) 47-64.

[7] M.I. Van Dyke, P. Couture, M. Brauer, H. Lee, J.T. Trevors, *Pseudomonas aeruginosa* UG2 rhamnolipid biosurfactants: Structural characterization and their use in removing hydrophobic compounds from soil, Canadian Journal of Microbiology 39 (11) (1993) 1071-1078.

[8] D. Dubeau, E. Deziel, D.E. Woods, F. Lepine, *Burkholderia thailandensis* harbors two identical rhl gene clusters responsible for the biosynthesis of rhamnolipids, BMC Microbiology 9 (2009) 263.

[9] J.C. Mata-Sandoval, J. Karns, A. Torrents, Effect of nutritional and environmental conditions on the production and composition of rhamnolipids by *P. aeruginosa* UG2, Microbiological Research 155 (4) (2001) 249-256.

[10] M. Lambert, A. Neish, Rapid method for estimation of glycerol in fermentation solutions, Canadian Journal of Research 3 (1950) 83-89.

[11] P. Trinder, Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor, Annals of Clinical Biochemistry 6 (1969) 24-25.

[12] M. Gutierrez, J. Castillo, J. Chirinos, M. Caetano, Inexpensive Wilhelmy balance based in a fiber optic sensor for the study of Langmuir films, Review of Scientific Instruments 76 (2005) 045112-1.

[13] D.G. Cooper, B.G. Goldenberg, Surface-active agents from two bacillus species, Applied and Environmental Microbiology 53 (2) (1987) 224-229.

[14] K. Mopper, E.M. Gindler, A new noncorrosive dye reagent for automatic sugar chromatography, Analytical Biochemistry 56 (2) (1973) 440-442.

[15] L.M. Prieto, M. Michelon, J.F. Burkert, S.J. Kalil, C.A. Burkert, The production of rhamnolipid by a *Pseudomonas aeruginosa* strain isolated from a southern coastal zone in Brazil, Chemosphere 71 (9) (2008) 1781-1785.

[16] C. Chayabutra, J. Wu, L.K. Ju, Rhamnolipid production by *Pseudomonas aeruginosa* under denitrification: Effects of limiting nutrients and carbon substrates, Biotechnology and Bioengineering 72 (1) (2001) 25-33.

[17] T. Nozawa, T. Tanikawa, H. Hasegawa, C. Takahashi, Y. Ando, M. Matsushita, et al., Rhamnolipid-dependent spreading growth of *Pseudomonas aeruginosa* on a high-agar medium: Marked enhancement under CO_2-rich anaerobic conditions, Microbiology and Immunology 51 (8) (2007) 703-712.

[18] P. Calcott (Ed.), Continuos Culture of Cells, I ed., CRC Press, Florida, USA, 1981.

[19] N. Manesh, S. Murugesh, V. Mohan, Determination of the presence of biosurfactant produced by the bacteria present in the soil samples, Research Journal of Microbiology 1 (4) (2006) 339-345.

[20] B.K. Tuleva, G.R. Ivanov, N.E. Christova, Biosurfactant production by a new *Pseudomonas putida* strain, Zeitschrift fur Naturforschung. C, Journal of Biosciences 57 (3-4) (2002) 356-360.

[21] J. Desai, A. Desai, Production of biosurfactants, in: N. Kosaric (Ed.), Biosurfactants, Production Properties, Applications, Volume 48, Marcel Dekker, INC, New York, USA, 1993, pp. 65-97.

[22] M. Javaheri, G.E. Jenneman, M.J. McInerney, R.M. Knapp, Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2, Applied and Environmental Microbiology 50 (3) (1985) 698-700.

[23] E. Dèziel, F. Lepine, D. Dennie, F. Boismenu, O. Mamer,

R. Villemur, Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* 57RP grown on mannitol or naphthalene, Biochimica et Biophysica Acta 1440 (1999) 244-252.

[24] S.A. Monteiro, G.L. Sassaki, L.M. de Souza, J.A. Meira, J.M. de Araujo, D.A. Mitchell, et al., Molecular and structural characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614, Chemistry and Physics of Lipids 147 (1) (2007) 1-13.

[25] S. Costa, M. Nitsche, R. Haddad, R. Wberlin, J. Contiero, Production of *Pseudomonas aeruginosa* LB1 rhamnolipids following growth on Brazilian native oils, Process Biochemistry 41 (2) (2006) 483-488.