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Oxygen-controlled Biosurfactant Production in a Bench Scale Bioreactor

Frederico de Araujo Kronemberger · Lidia Maria Melo Santa Anna · Ana Carolina Loureiro Brito Fernandes · Reginaldo Ramos de Menezes · Cristiano Piacsek Borges · Denise Maria Guimarães Freire

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Abstract Rhamnolipids have been pointed out as promising biosurfactants. The most studied microorganisms for the aerobic production of these molecules are the bacteria of the genus *Pseudomonas*. The aim of this work was to produce a rhamnolipid-type biosurfactant in a bench-scale bioreactor by one strain of *Pseudomonas aeruginosa* isolated from oil environments. To study the microorganism growth and production dependency on oxygen, a nondispersive oxygenation device was developed, and a programmable logic controller (PLC) was used to set the dissolved oxygen (DO) concentration. Using the data stored in a computer and the predetermined characteristics of the oxygenation device, it was possible to evaluate the oxygen uptake rate (OUR) and the specific OUR (SOUR) of this microorganism. These rates, obtained for some different DO concentrations, were then compared to the bacterial growth, to the carbon source consumption, and to the rhamnolipid and other virulence factors production. The SOUR presented an initial value of about 60.0 mgO_2/g_{DW} h. Then, when the exponential growth phase begins, there is a rise in this rate. After that, the SOUR reduces to about 20.0 mgO_2/g_{DW} h. The carbon source consumption is linear during the whole process.

Keywords *Pseudomonas aeruginosa* · Biosurfactant · Oxygenation · Rhamnolipid · Bioreactor

F. A. Kronemberger (✉) · C. P. Borges
Instituto Alberto Luiz Coimbra de Pós Graduação e Pesquisa de Engenharia, Programa de Engenharia Química, Universidade Federal do Rio de Janeiro, Centro de Tecnologia, bloco G, sala 115—Cidade Universitária, P.O. Box 68502, 21945-970 Rio de Janeiro, RJ, Brazil
e-mail: frederico@peq.coppe.ufrj.br

L. M. M. Santa Anna
Gerência de Biotecnologia e Tratamentos Ambientais, Centro de Pesquisa e Desenvolvimento Leopoldo Américo Miguêz de Mello, Cidade Universitária, Rio de Janeiro, RJ, Brazil

A. C. L. B. Fernandes · R. R. Menezes · D. M. G. Freire
Departamento de Bioquímica, Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

Introduction and Objectives

The progresses in science and technology since the industrial revolution have been constantly raising the possibility of the exploration of natural resources. That exploration has been generating disturbances in the global elementary cycles. The introduction of exogenous chemical products to certain area can always surpass its capacity of self-remediation, resulting in the harmful accumulation of these products. Hydrocarbon contaminations in oil spills have been causing serious environmental problems, and extra consideration is being shown to the development and implementation of new technologies for the proper cleaning. Several methods of soil remediation are being developed as alternatives environmentally correct.

The main factor that limits the biodegradation of these pollutants is their limited availability to the native microorganisms. Hydrocarbons usually aggregate to soil components, which make the removal or degradation very difficult. Surface-active compounds reduce the superficial and interfacial tensions through the accumulation in the interface of immiscible fluids or of a fluid and a solid, increasing the availability and subsequent biodegradation. These compounds can be produced in a chemical or biochemical way and are denominated surfactants.

These substances are used industrially with several objectives, but for many years, they have been synthesized chemically. The biological surface-active compounds, biosurfactants, which began to be used in the last decades, are produced by some bacterial strains that degrade or transform petroleum components [1, 2]. These biosurfactants are getting notoriety because they can be used in several industrial applications, as a result of their biodegradability advantages, production from renewable sources, and functionality under extreme conditions [3].

Microorganisms are capable to produce a great variety of products with excellent surface-active properties. However, its use in certain applications depends on the production and purification costs for specific activities, if compared to the corresponding synthetic surfactants. Thus, the last works are concentrated in the identification of potentials surfactants, in the evaluation of their properties, and in the optimization of the fermentative processes for their production [4].

In the present moment, the biosurfactants are still not capable to compete economically with the chemically synthesized surfactants at the market because of the high production costs. That is result of the inefficient methodology of the bioprocessing, the low productivity of the microbial strains, and the need of using expensive substrates. Consequently, for the biosurfactants to reach a significant portion of the market, it is necessary a greater knowledge and ability for the manipulation of the producing strains metabolism, for the possibility of the use of cheaper substrates and the technological improvement of the production process.

Many biosurfactants and their productive processes were patented, but only some of them were commercialized. Nowadays, some products based in biosurfactants can be found at the international market, like PD5, produced by Pendragon Holdings, sold as an addictive for fuels based on a mixture of rhamnolipid biosurfactants and enzymes, EC-601, produced by EcoChem Organics, sold as a dispersive agent of water-insoluble hydrocarbons (rhamnolipids) and the products JBR, of Jeneil Biosurfactant, rhamnolipids in aqueous solutions with different purity degrees or in a semisolid form.

The cost of biosurfactants production is approximately from three to ten times larger than the one of chemical surfactants. Usually, the biosurfactants are produced during the growth of the microorganisms in hydrocarbons, which are usually expensive, increasing the

total cost of the process. However, other cheaper and water-soluble substrates, as glucose, glycerol, and ethanol, have been used. In this search for more economical raw materials for the biosurfactant production, the industrial effluents and the agriculture residues are shown as an excellent option [5].

In agreement with Gruber et al. [6], the prerequisites for the competitive production of biosurfactants are the attainment of products with great activity, produced from cheap substrates through economical viable processes and with high yields. A favorable economical aspect for the biosurfactants production in Brazil, from low-cost substrates, was the recent sanction of the Law 11.097 of January 13, 2005, which authorizes the introduction of the biodiesel in the Brazilian energetic matrix. The law establishes that in the next 3 years, the addition of 2% of biodiesel to the petroleum diesel will be authorized. In 2008, the mixture will be obligatory in this percentage, and it will be raised to 5% in 2013. Thus, some private projects are already in process for the new fuel production, with total installed capacity above 100 million liters per year. Because glycerol is the main byproduct of the biodiesel production, it can be used at a very low cost for the biosurfactant production [7]. The greater challenge then relapses in the technological development of an efficient productive process, with high yields, and in the reduction in the subsequent stages of recovery and purification of the product.

Most of the known biosurfactant-producing microorganisms need aerobic conditions for the production in an efficient way. However, the use of the conventional submerged aeration can lead to the formation of very stable foams, causing serious operational problems, and that is particularly valid for the rhamnolipid-type biosurfactant production. The high foam formation is still increased by the presence of extracellular proteins, resulting in great expenses for the process control, making the productive process impracticable. Mechanic foam breakers are not very efficient, and chemical antifoamy agents can alter the quality of the product and the pollutant potential of the final effluent of the bioreactor [6]. To overcome that difficulty, a different oxygenation process was used. It was similar to the one described by Gruber et al. [6] and to the other, used in alcoholic fermentations, patented by L'Air Liquide [8].

This oxygenation process is promising for the use in bioreactors, reaching good productivities. However, the oxygenation manual control was shown difficult and even inefficient because of the fact that the fermentations last approximately 7 days, demanding night operation of the unit.

Thus, an automated control system of that oxygenation was developed, using a Programmable Logical Controller (PLC) coupled to control equipment with electrical impulses. That system made possible the control and the attendance of the level of dissolved oxygen (DO) in the bioreactor and of the amount of oxygen transferred to the same.

It was also possible to evaluate the oxygen uptake rate (OUR) and the Specific OUR (SOUR) in all the phases of the *Pseudomonas aeruginosa* species bacterial growth, which are still not reported in the scientific literature, even when these bacteria, a pathogenic species involved in many diseases, are an important objective of study in the medical area. The expression of many virulence factors of *P. aeruginosa* is dependent of particular environmental conditions as the concentration of iron, readiness of nitrogen, temperature, and oxygen. Enzymes (proteases and elastases), just like the rhamnolipids and some toxins, are virulence factors of *P. aeruginosa*.

In this context, the main objective of this work was the evaluation of the DO concentration influence along fermentation in the production of biosurfactants (rhamnolipid type) using glycerol (byproduct of the biodiesel production) as the carbon source. The

microbial growth, the productivity, the consumption of the carbon source, and the production of proteins and of some extracellular enzymes related as virulence factors, as the proteases and, specifically, the elastase, were also evaluated.

The effectiveness of a cell-free rhamnolipid biosurfactant, produced by the same *P. aeruginosa* strain, in the removal of two different kinds of oil from impacted sandy soils was investigated by Santa Anna et al. [9]. The study indicates that the use of this biosurfactant was effective in reducing oil concentrations in impacted soil.

Biosurfactants: Definition and Classification

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. These properties render surfactants capable of reducing surface and interfacial tension and forming microemulsions where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing properties, which make surfactants some of the most versatile process chemicals.

A biosurfactant is defined as a surface-active molecule produced by living organisms, most of them being microorganisms. The main physiological function of biosurfactants is to allow the microorganisms growth in substrates immiscible in water, through the reduction in the interfacial tension between the phases, making the substrate available for the assimilation and metabolization [3].

Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions, and hydrophilic–lipophilic balance. Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. The surface tension of distilled water is 72 mN/m, and the addition of surfactants lowers this value to about 30 mN/m [5].

Although there are a number of reports on the synthesis of biosurfactants by hydrocarbon-degrading microorganisms, some biosurfactants have been reported to be produced on water-soluble compounds such as glucose, sucrose, glycerol, or ethanol. The biosurfactant-producing microbes are distributed among a wide variety of genera.

Most of the known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the most recognized are the rhamnolipids, the trehalolipids, and the sophorolipids [5].

Rhamnolipids

Rhamnolipids, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid, are the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described by Jarvis and Johnson [10]. The main glycolipids produced by *P. aeruginosa* are rhamnolipids of the types 1 and 2, L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate. Their structures can be seen in Fig. 1. The formation of rhamnolipid types 3 and 4 containing one β-hydroxydecanoic acid with one and two rhamnose units, methyl ester derivatives of rhamnolipids 1 and 2, and rhamnolipids with alternative fatty acid chains have also been reported [5].

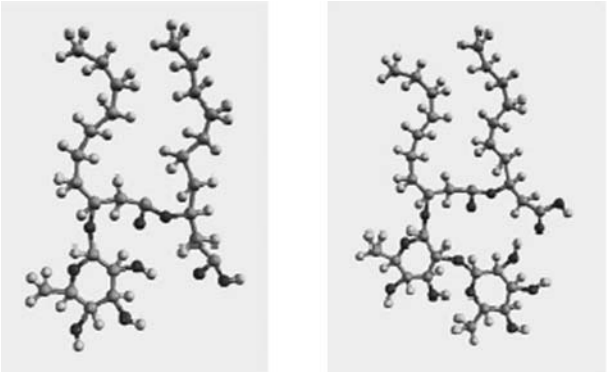

Fig. 1 The hard sphere model of rhamnolipids types 1 and 2 in their minimum energy positions: oxygen (*dark gray*), hydrogen (*light gray*), and carbon [11]

Materials and Methods

Maintenance of Microorganism and Preinoculum Preparation

The *P. aeruginosa* PA1 strain, previously selected from a petroleum environment as a good biosurfactant producer [12, 13], was preserved in glycerol 10% in an ultrafreezer at −80 °C. The preinoculum was cultivated in a plate with YPDA (yeast extract 0.3%, peptone 1.5%, dextrose 0.1%, and agar 1.2%) at 30 °C for 48 h and transferred to 1,000-ml flasks with 300 ml of a culture medium. After 24 h of cultivation, the fermentation medium containing the cells was stocked in cryotubes with 25% of glycerol and preserved at −18 °C to serve as a preinoculum pattern for all the fermentations.

Inoculum Preparation

The content of a cryotube was inoculated in 300 ml of a fermentation medium. The flasks were incubated in a rotatory shaker at 30 °C and 170 rpm for 40 h. At the end of that period, the cells of each flask were recovered by centrifugation (6,000 rpm for 15 min) and used as inoculum in the bioreactors.

Sterilization

The bioreactor, with the culture medium, was sterilized in an autoclave at 121 °C for 15 min before each production. The oxygenation system was sterilized with the circulation of a solution of sodium hypochlorite at 1% for 1 h. After this procedure, distilled sterilized water was circulated in the system for the elimination of chlorine residues. Only after this procedure, the inoculation of the microorganisms was accomplished.

Rhamnolipid production from *Pseudomonas aeruginosa*

The fermentations were accomplished in a BioFlo IIc bioreactor (Batch/Continuous Fermentor; New Brunswick Scientific; USA) with nominal capacity of 5.0 l of volume. The useful volume used in the fermentations was 3.0 l, and the temperature was maintained at 30 °C and the agitation in 100 rpm, with a Rushton 6-blade impeller. The oxygenation was nondispersive with the use of pure oxygen [14]. The culture medium was composed by

glycerol (3.0%) as the sole carbon source, $NaNO_3$ (0.1%), K_2HPO_4 and KH_2PO_4 (1.0%), and traces of iron (II) and magnesium sulfates [12].

Determination of the Biomass Concentration

The cellular concentration of the *P. aeruginosa* suspensions was determined through the light absorbance at 600 nm (MultiSpec-1501; Shimadzu, Japan). The absorbance value was converted in cell dry weight concentration (g/l) through a calibration curve.

Glycerol Quantification

The glycerol, the source of carbon used in the fermentations, concentration in the cell-free samples—the cells were removed by centrifugation at 6,000 rpm for 15 min—was evaluated by the enzymatic/colorimetric method for triglycerides determination (Enzymatic Triglyceride-Bioclin; Quibasa Basic Chemistry, MG-Brazil).

Rhamnolipid Quantification

The quantification of the rhamnolipids was carried through an indirect way, using the rhamnose as reference—the rhamnose is a byproduct of the acid hydrolysis of the rhamnolipids. A modified method described by Pham et al. [15] was used, where the rhamnolipids extraction phase was suppressed.

Nitrate Concentration

The nitrate concentration was determined through the colorimetric reaction of that ion with brucine sulfate. The absorbance values were measured (410 nm) and converted to milligram per liter values with a calibration curve [16].

Total Proteins Determination

The method used for the quantification of the extracellular proteins was described by Lowry et al. [17].

Protease Activity Determination

The protease activity was determined by the method described by Charney and Tomarelli [18] that is based on the formation of colored protein-derived compounds by the digestion of an azocasein solution with the proteolitic enzymes present in the enzymatic extract.

Elastase Activity Determination

A test based on the degradation of an elastin linked to Congo red was used for the elastase activity determination. The degradation of this substrate liberates that pigment, allowing the evaluation of the activity. The procedure was described by Braga et al. [19].

Results and Discussion

The first and clearest observation in the rhamnolipid production with the use of the nondispersive oxygenation is the foam absence in the vase containing the culture medium during the fermentative process. In this nondispersive oxygenation device, the interface between the liquid and the gaseous phase is well defined and fixed. The oxygen is transferred to the medium through this interface instead of being transferred through the bubble interface. As there is no bubbling in this system, the foam is not formed. The comparison between the production carried out in this work and a production with the conventional aeration can be observed in Fig. 2.

The foam absence is clearly noticed in Fig. 2a, corresponding to the fermentation with nondispersive oxygenation. To discard the possibility that the foam absence could be related to the absence of the biosurfactant in the medium, air was injected directly by a disperser, with low flow, simulating the conventional oxygenation. In a few seconds, the condition seen in the Fig. 2b was achieved. This fact once again proves the inefficiency of this fermentative process for the rhamnolipid production from *P. aeruginosa* with the conventional oxygenation by the means of bubbling.

The oxygen supply is a very important parameter in the rhamnolipid production. The largest modification in the productive process was the medium oxygenation, and special consideration was given to the DO concentration. The values were registered continuously with the use of a computer coupled initially to the DO sensor connected to the bioreactor and, later, to the PLC that controlled the necessary equipment for the oxygenation of the medium in the bioreactor.

The DO concentration control along the fermentations was initially accomplished manually. However, the difficulty of that control resulted in great variations in the DO concentration value during the fermentations. That variation was not desired, as the objective was to evaluate the influence of the amount of oxygen transferred and also of its concentration in the medium during the biosurfactant production. This fact justified the implementation of a control system with more efficient equipment connected to a PLC. The difference in the DO profile during fermentation with the manual-controlled system and with the use of the new system can be observed in the Fig. 3. It was noticed that the

Fig. 2 Comparison between the rhamnolipid production with the conventional oxygenation and the one used in this work. **a** Nondispersive oxygenation. **b** Conventional oxygenation

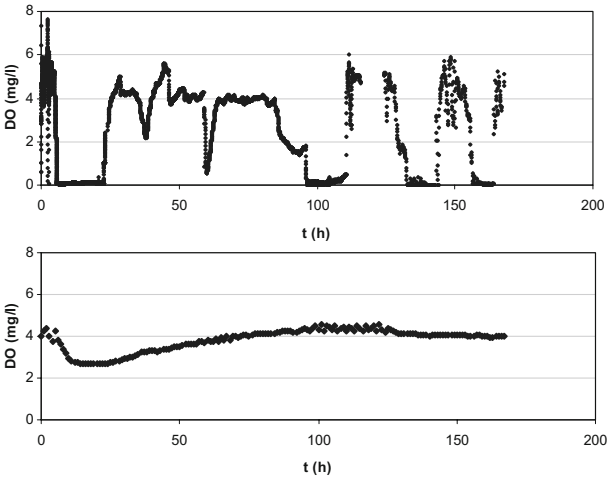

Fig. 3 Dissolved oxygen profile (mg/l) with time (h)—*t*—in fermentations with different controls for the nondispersive oxygenation system. *Above*, there is the manual control DO profile. In both fermentations, the DO set point was 4.0 mg/l

extreme DO variation during the fermentation process was over and that the desired investigation could continue.

Thus, the influence of the medium DO concentration in the fermentation was investigated. Fermentations were carried out with DO concentration set points at 1.0, 4.0, and 6.0 mg/l. Those values correspond, respectively, to 14, 57, and 86% of the saturation concentration of oxygen that was equal to 7.0 mg/l in the defined fermentation conditions. This value was calculated using an expression stated by Blanch and Clark [20] for the temperature and medium composition used in the experiments. The control system (with the PLC) actuated in the oxygenation device to raise the oxygenation rate (for example, raising the oxygen gas pressure) any time that the DO concentration in the medium got below the set point.

As all the oxygen was supplied to the medium through the nondispersive system and as there was no bubbling and the loss of that nutrient to the atmosphere was negligible, it was possible to affirm that the amount of the gas supplied, known by the previous characterization of the system, was exactly equal to the amount consumed by the bacteria. That made possible the analysis of the OUR, expressed in milligram of oxygen by liter per hour, and the SOUR, expressed in milligram of oxygen per gram of biomass in dry weight per hour. The SOUR was obtained by dividing the OUR by the biomass concentration during the fermentation.

An interesting fact was that the oxygen consumption profile was not affected by the variation of the DO concentration. The values found for these rates were also not affected by the change in the DO concentration, adjusted to 1.0, 4.0, or 6.0 mg/l. The profiles of the oxygen consumption during fermentation are presented in Fig. 4.

The variation in the oxygen consumption along the different phases of the cellular metabolism is clearly observed. After the adaptation phase, which lasts 4 h at the most, there is a significant increase in the OUR, which varies from 40.0 mg/l h to values above 100.0 mg/l h. Then, that value diminishes slowly until the stabilization in a value close to the initial. That behavior is also observed for the specific rate, SOUR, which confirms that the variation is not only related to the raise in the cellular concentration. The SOUR presents an increase from an initial value of 60.0 up to 85.0 mg/g h, diminishing later to a value around 20.0 mg/g h.

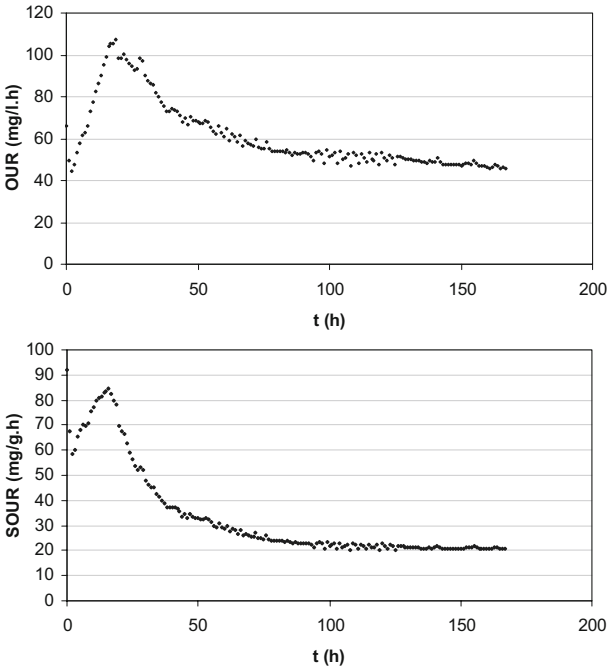

Fig. 4 Oxygen consumption in a fermentation (DO set point equal to 4.0 mg/l). *OUR* Oxygen uptake rate, in milligrams of oxygen per liter per hour, *SOUR* specific oxygen uptake rate, in milligrams of oxygen per cell dry weight in gram per hour

This raise in the oxygen consumption begins at 4 h of fermentation and lasts about 12 h, when the fermentation reaches 16 h. It is interesting to notice that this increase coincides with the exponential phase of the microbial growth, and it can be observed in Fig. 5. Thus, it can be concluded that the oxygen is an essential nutrient for the microbial growth, as it is consumed with a very high rate in that phase. It is still important to point out that the SOUR stays above 20.0 mg/g h along all the fermentations, being the oxygen also important to the cellular maintenance.

In Fig. 6, the biomass, glycerol, and rhamnolipid concentration are presented along fermentation. It is observed that the cellular behavior is simple, with the initial adaptation phase and the exponential growth phase, followed by the stationary one. Besides, the

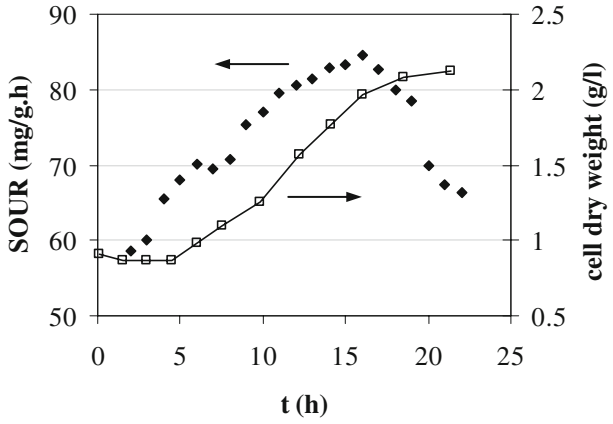

Fig. 5 Correlation between cell growth and SOUR in a rhamnolipid production by *Pseudomonas aeruginosa*. Biomass concentration in dry weight (g/l)×time (h) and SOUR in milligrams of oxygen per cell dry weight in gram per hour

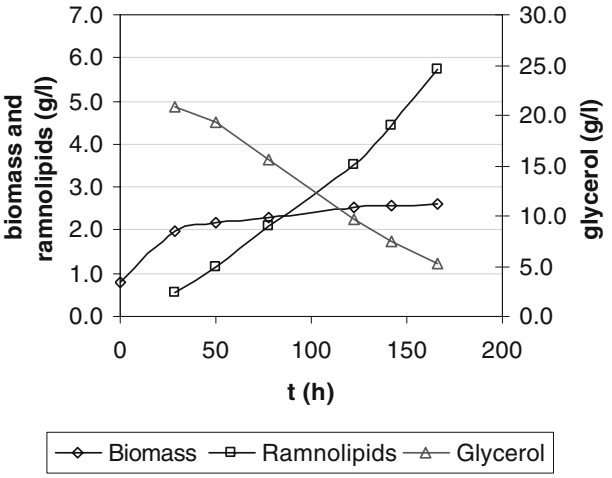

Fig. 6 Time variation of growth, rhamnolipid production, and glycerol consumption (in g/l) during *Pseudomonas aeruginosa* cultivation (DO set point equal to 4.0 mg/l)

consumption of the carbon source remains constant along the fermentation. That general behavior is not influenced by the DO concentration in the medium.

For the best understanding of the influence of the DO concentration in the fermentation, all results were compared with the values obtained in a conventional fermentation, carried out in an agitated flask. The biomass concentration was not influenced by the variation of the DO concentration. Besides, the values were similar to the one of the conventional fermentation, indicating that the cells have adapted to the oxygenation system. The values of the rhamnolipids and glycerol concentration along the fermentations are presented in Fig. 7.

The experiments with a DO set point of 4.0 mg/l are duplicate experiments. The experimental error is high in almost all biochemical reaction, and the rhamnolipid determination assay is not infallible. That is why there are two experiments in this middle point. In addition, all the discussion is made based in the global productivity instead of only in one single point.

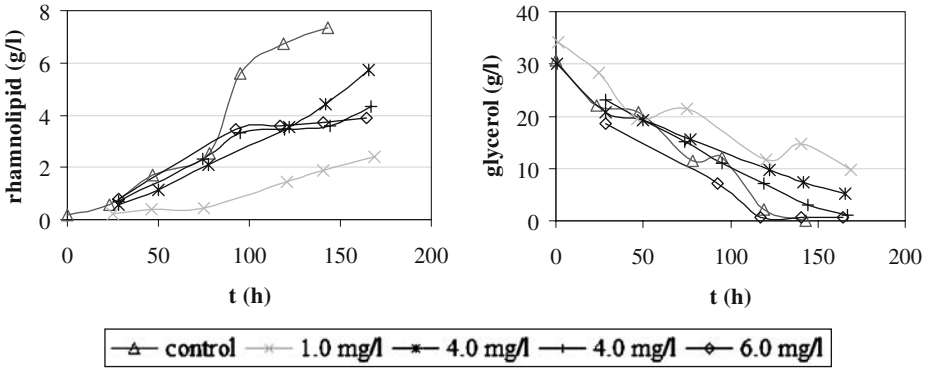

Fig. 7 Rhamnolipid and glycerol concentration (g/l) in fermentations with different DO set points—1.0, 4.0, and 6.0 mg/l—and the control conventional shake flask fermentation

It is noticed that both the rhamnolipid production and the glycerol consumption are reduced in the fermentation carried out with the lowest DO concentration, 1.0 mg/l. That amount of oxygen should be insufficient for the cells access to that nutrient that is shown to be necessary to the rhamnolipid production. The biosurfactant productivity under that condition is 15.0 mg/l h, and the rhamnolipid concentration reaches a value of about 2.0 g/l after 7 days of fermentation. It is also observed that until the third day of fermentation, the biosurfactant production is the same under all the other investigated conditions. The productivity values stay around 30.0 mg/l h of rhamnolipids for the bioreactor fermentations carried out with 4.0 and 6.0 mg/l of DO, while the productivity in agitated flasks equals to 60.0 mg/l h. For the fermentations carried out in the bioreactor, the reduction in the glycerol consumption with the decrease in the biosurfactant production can be observed, indicating a substrate in the product yield approximately constant with the variation of the DO concentration. Thus, it is noticed that the oxygenation system used in this work is efficient and makes possible the scale-up of the process. The great difficulty in scaling up biochemical processes is the oxygenation condition. In a conventional oxygenation system, the bubble dispersion in a big fermentation vessel is the greatest problem to maintain the oxygenation rate. With the proposed system, the oxygenation rate could be doubled simply by doubling the interface area. Therefore, when increasing the fermentation medium volume by a factor of two, the interfacial area of the oxygenation device should be doubled to keep the oxygenation conditions unchanged.

However, this process can still be improved, as the cells can reach greater productivities in other growth conditions. Furthermore, these productivity and yield values obtained for the bioreactor fermentation are comparable to the majority of the ones recently reported in the scientific literature for the rhamnolipid production by *P. aeruginosa* from low-cost materials, as seen in Table 1.

To complete the evaluation of the cellular metabolism behavior with the variation in the DO concentration, the nitrate and total protein concentrations and the protease and elastase activities were determined along the fermentations. The nitrogen source was consumed before 24 h of fermentation in all experiments. The results, normalized by the cellular concentration, are exhibited in Fig. 8. It can be observed that along most of the bioreactor fermentation, the protease and elastase activities are lower to the ones in the agitated flasks fermentation. This result is coherent with the fact that the proteases, including the elastase, and the rhamnolipids are virulence factors, and both are produced under a certain condition. The total proteins concentration values are similar along the 7 days of fermentation. This indicates that besides the virulence factors that are produced in a lower amount in the new system, other proteins could be secreted. These proteins could be somehow inhibiting the

Table 1 Rhamnolipid production by *Pseudomonas aeruginosa* from low-cost materials.

Reference	Carbon source	Rhamnolipid productivity (mg/l h)	Yield—$Y_{P/S}$ (%)
Present work	Glycerol	30	20
Jeong et al. [21]	Fish oil	31	37
Benincasa et al. [22]	Soapstock	210[a]	33
Santa Anna et al. [13]	Glycerol	19	13
Haba et al. [23]	Waste frying oil	27	–

[a] Considering only the stationary phase of cell growth

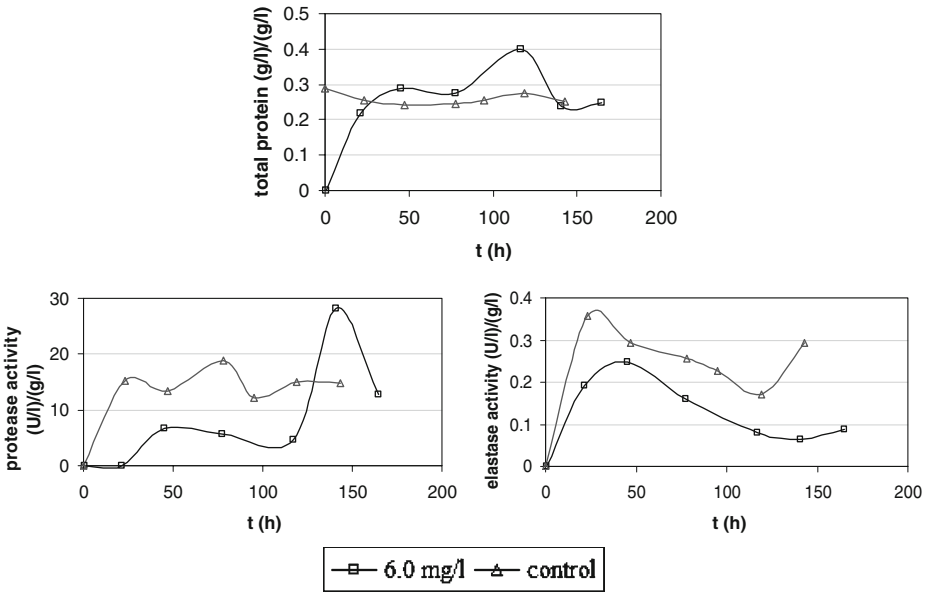

Fig. 8 Time variation of total proteins (g/l) and protease and elastase activities (U/l) during a rhamnolipid production in the bioreactor with the DO set point equal to 6.0 mg/l and in the conventional shake flask fermentation. All presented values are normalized by the biomass content

rhamnolipid production, or at least, they can be identified and used hereafter as indicators of the biosurfactant production inhibition.

Conclusions

The control system for the DO concentration developed was shown to be very efficient for the maintenance of a constant value for that parameter. An interesting observed fact was that the profile of oxygen consumption remained unaffected by the variation of the oxygen concentration. It is clearly observed the variation in the oxygen consumption oxygen along the different phases of the cellular metabolism. After the adaptation phase, which lasts 4 h at the most, there is a significant increase in the OUR. That behavior is also observed for the specific rate, SOUR, which confirms that the variation is not only related to the change in the values of cellular concentration. It is interesting to notice that the increase in the SOUR coincides with the microbial exponential growth phase. Thus, it can be concluded that the oxygen is an essential nutrient for the microbial growth, as it is consumed at a very high rate in that phase. It is still important to point out that the SOUR values remain above 20.0 mg/g h along all of the fermentations, being also important for the cellular maintenance.

The results presented in this work indicate that the proposed process is viable and promising, being a very good alternative for the rhamnolipids production, favoring the control and the scale-up of the production process.

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