0273-2289/1559–0291 (Online)/07/136–140/539–554/$30.00

Biosurfactant Production by Cultivation of *Bacillus atrophaeus* ATCC 9372 in Semidefined Glucose/Casein-Based Media

Luiz Carlos Martins das Neves,*[,1] Kátia Silva de Oliveira,[1] Márcio Junji Kobayashi,[1] Thereza Christina Vessoni Penna,[1] and Attilio Converti[2]

[1]University of São Paulo, School of Pharmaceutical Sciences, Biochemical and Pharmaceutical Technology Department, São Paulo, Brazil, E-mail: lucaneves@usp.br; and [2]Dipartimento di Ingegneria Chimica e di Processo, Università degli Studi di Genova, Genova, Italy, E-mail: converti@unige.it

Abstract

Biosurfactants are proteins with detergent, emulsifier, and antimicrobial actions that have potential application in environmental applications such as the treatment of organic pollutants and oil recovery. *Bacillus atrophaeus* strains are nonpathogenic and are suitable source of biosurfactants, among which is surfactin. The aim of this work is to establish a culture medium composition able to stimulate biosurfactants production by *B. atrophaeus* ATCC 9372. Batch cultivations were carried out in a rotary shaker at 150 rpm and 35°C for 24 h on glucose- and/or casein-based semidefined culture media also containing sodium chloride, dibasic sodium phosphate, and soy flour. The addition of 14.0 g/L glucose in a culture medium containing 10.0 g/L of casein resulted in 17 times higher biosurfactant production (B_{max} = 635.0 mg/L). Besides, the simultaneous presence of digested casein (10.0 g/L), digested soy flour (3.0 g/L), and glucose (18.0 g/L) in the medium was responsible for a diauxic effect during cell growth. Once the diauxie started, the average biosurfactants concentration was 16.8% less than that observed before this phenomenon. The capability of *B. atrophaeus* strain to adapt its own metabolism to use several nutrients as energy sources and to preserve high levels of biosurfactants in the medium during the stationary phase is a promising feature for its possible application in biological treatments.

Index Entries: *Bacillus atrophaeus;* batch cultivation; biosurfactant production; casein; glucose; kinetics.

Introduction

Biological surfactants or biosurfactants are a diverse group of natural surface-active chemical compounds *(1,2)* produced spontaneously by microorganisms, extracellularly or as part of the cellular membrane.

*Author to whom all correspondence and reprint requests should be addressed.

Biosurfactants are proteins with detergent, emulsifier, and surfactant power to lower the surface tension of water and other solvents, and have potential application in environmental uses such as organic pollutants treatment and oil recovery. The production of biosurfactants is related to the consumption of hydrocarbons, including oily residues, and occurs during exponential cellular growth.

Cyclic lipopeptide biosurfactants with antimicrobial activity (surfactin, iturin, and fengicin), also reduce surface tension, critical micelle concentration, and interfacial tension in both aqueous and hydrocarbon mixtures *(2)*. Surfactin, one of the most effective cyclic lipopeptide biosurfactants produced by *Bacillus subtilis (3–5)* can lower the surface tension of water from 72 to 50 mN/m and has a critical micelle concentration of 2.5×10^{-5} *M (6,7)*. When compared with chemically synthesized surfactants, biosurfactants exhibit a higher specific activity, lower toxicity, and environmental impact, higher biodegradability, and lower costs *(3,5,8)*.

B. subtilis is considered a suitable source for biosurfactant production owing to the absence of pathogenicity, which permits the use of its products in the food and pharmaceutical industries *(9,10)*. Cell viability is easily assayed and its spores have successfully been used as biological indicators *(11–13)*. Therefore, the development of improved strains and culturing methods of *B. subtilis* can provide a safe source of surfactants for waste treatment.

Biosurfactants can be produced in *B. subtilis* cultures using sugars (sucrose, glucose, and lactose), vegetable oils, or starch as carbon sources *(14–19)*. However, the high cost of the process and recuperation of these proteins and low productivity can limit their application for environmental purposes *(20)*. Alternative sources of culture media as sugar cane molasses, water of maize *(21)*, and industrial effluent wastes *(22,23)* have been considered to reduce the final cost of the process to obtain higher productivity of biosurfactants.

Among several industrial effluents, milk serum, a residue of the milk derivative industries, is distinguished as potential source of biosurfactants based on a rich composition in proteins (6%), fats (3.2%), sugars, and salts. In Brazil, for each 1000 L of milk used in the manufacture of cheese, approx 820 L of milk serum are produced and about 50% is poured directly in rivers, with no previous treatment, causing a serious environmental problem *(24)*. The use of milk serum as a culture medium to produce biosurfactants at low costs is an interesting alternative to reduce this environmental problem.

However, the use of this residue as substrate to obtain biosurfactants for industrial use, or as a nutritional source during the biological treatment of effluents, can be an interesting alternative to reduce environmental impact.

The use of casein in the culture of microorganisms is usually associated to the production of proteases or to the research of its properties *(25–27)*. However, few studies have developed a culture medium with casein associated with other nutrients to improve the production of valuable

biotechnological products. Thus, it is interesting to evaluate the productivity yield of biosurfactants by *B. subtilis* in an alternative culture medium with other sources of carbon, including casein, which is abundant in milk serum. The aim of this work is to develop a growth medium able to stimulate biosurfactants production by *B. atrophaeus* ATCC 9372. Batch cultivations are carried out at 150 rpm and 35°C for 24 h on glucose- and/or casein-based culture media to relate the biosurfactant production with cellular growth and nutrient consumption.

Materials and Methods

Maintenance of the Strains

A strain of *B. atrophaeus* ATCC 9372 was maintained according to the following procedure. All the materials and 500 mL of culture medium were sterilized at 121°C for 30 min. Spore suspensions stored at 4°C in 0.02 mol/L calcium acetate (pH 9.0) were directly utilized for these experiments. Five 7-mL glass tubes containing slants of plate count agar (Merck, Darmstadt, Germany) were inoculated with spores of *B. atrophaeus* ATCC 9372 and incubated for 24 h at 35°C. Cells were suspended in 2 mL of physiological solution (0.9% NaCl) and transferred to Roux flasks containing 200 mL of tryptone soy agar (Merck). After incubation at 35°C for 24 h, the flasks were washed with 100 mL of 0.02 mol/L calcium acetate solution, and their contents transferred to sterilized capped flasks containing 30.0 g of glass beads with 3-mm diameter. The pH of these suspensions was adjusted to 9.6 with 1 mol/L NaOH solution.

Cultivations

250-mL Erlenmeyers containing 100 mL of culture media, containing glucose and casein at variable concentrations, 5.0 g/L of sodium chloride, 2.5 g/L of dibasic sodium phosphate, and 3.0 g/L of soy flour, were inoculated with cell suspensions to give a starting biomass concentration (X_o) of 0.2 g_X/L ($OD_{600\ nm} = 0.014$). All batch runs were performed in triplicate at 35°C utilizing a rotary shaker at 150 rpm. Samples were collected every 1–2 h, transferred to Eppendorfs, and then centrifuged at 17,091*g* for 20 min. 10°C. Both liquid and precipitated fractions were stored at 4°C and subsequently analyzed for determinations of extracellular and intracellular concentrations of glucose, total proteins, and biosurfactant, according to circumstances.

Cell Disruption

Cell mass separated by centrifugation at 17,091*g* was resuspended in 1 mL of a buffer solution containing protease inhibitors consisting of (mmol/L) 50 Tris-HCl buffer (pH 7.5), 5 $MgCl_2$, 10 β-mercaptoethanol, 2 aminocaproic acid, and 0.2 ethylenediaminetetraacetic acid. Glass

spheres with 0.5-mm diameter were then added to the suspension as abrasive agent up to 1/300 w/w ratio. After mixing for 12 min in a water bath cooled at 4°C with ice, cell debris and glass spheres were removed by centrifugation at 4°C and 17,091*g*, and the supernatant was utilized for determinations of biosurfactant concentration and total protein content *(28)*.

Analytical Determinations

Biomass concentration (X [g_X/L]) was determined in cell suspension before centrifugation using a calibration curve ($OD_{600\ nm} = 0.5820\ X - 0.1022$) obtained relating optical density at 600 nm (Beckman DU-640, Fullerton, CA) to dry mass of *B. atrophaeus* ATCC 9372 cells in the exponential growth phase *(29–30)*. Average deviation of the experimental data from the fitting curve (σ) and determination coefficient (r^2) were 4.5% and 0.996, respectively. Glucose concentration (G [g/L]) in the liquid phase was determined by the glucose oxidase peroxidase (GOD-POD) enzymatic assay no. 11538 (Biosystem, São Paulo, Brazil), using an absorbance calibration curve ($OD_{500\ nm} = 0.3496\ G - 0.0011$; $r^2 = 0.999$; $\sigma = 0.06\%$) obtained from glucose solutions with variable known concentrations *(30)*.

Casein concentration was determined as total proteins (TP [g/L]) on aliquots of the liquid phase from centrifugation after cell disruption. This methodology was based on the direct absorbance of samples at 660 nm after a color-developing reaction *(31)*. The calibration curve, obtained using casein solutions with different concentrations, was described by the linear equation $OD_{660\ nm} = 0.663\ TP + 0.0133$ ($r^2 = 0.993$; $\sigma = 0.2\%$). Aliquots of samples of the supernatant were used to determine biosurfactant concentrations. According to Morikawa et al. *(6)*, the concentration of biosurfactant (B) was related to the diameter of the halos (D) formed by 10 μL samples on a thin layer of 10 mL oil dispersed in 40 mL water. Comparison of sample diameters with those obtained with standard water/surfactin dispersions allowed expressing the biosurfactant concentration as mg_B/L. The calibration curve, obtained using surfactin solutions with different concentrations, was described by the linear equation $D = 0.0133\ B - 0.0007$ ($r^2 = 0.999$; $\sigma = 1.8\%$).

Protease assay was determined according to Ahamed et al. *(32)* in aliquots of the supernatant. Samples (0.5 mL) were incubated with buffered casein (bovine milk, Sigma, St. Louis, MO) solution (2.5 mg/mL in 0.05 *M* sodium phosphate buffer) at pH 6.5 and 37°C in a final assay volume of 2 mL. The reaction was stopped, and residual protein was precipitated by addition of 4 mL trichloroacetic acid (10% [w/w]). After standing 1 h, supernatant was obtained by centrifugation (3000*g*, 6 min). To 1 mL of supernatant, 5 mL of 0.4 *M* sodium carbonate was added followed by 0.5 mL folin-phenol reagent. After 10 min, tyrosine liberated by the action of protease was measured at 660 nm. One unit of protease activity liberates 1.0 mg of tyrosine per minute under assay conditions. The calibration curve, obtained using subtilisin solutions, a characteristic protease

produced by *B. subtilis* with different concentrations, was described by the linear equation $C = 0.0133\ E - 0.0007$ ($r^2 = 0.999$; $\sigma = 2.0\%$) when C is the casein concentration and E is the enzymatic activity of subtilisin.

Calculation of Fermentation Parameters

The specific rates of cell growth (μ_X), biosurfactant formation (μ_B), and substrate consumption (μ_S) were expressed, respectively, as $1/h$, $mg_B/g_X \cdot h$, and $g_S/g_X \cdot h$ and defined as:

$$\mu_x = \frac{1}{X}\frac{dX}{dt} \tag{1}$$

$$\mu_B = \frac{1}{X}\frac{dB}{dt} \tag{2}$$

$$\mu_S = \frac{1}{X}\frac{dS}{dt} \tag{3}$$

where S is the substrate concentration and t the time. Maximum specific growth rate (μ_{Xmax}) was determined during the exponential growth phase according to the equation:

$$\mu_{X_{max}} = \frac{1}{t}\ln\frac{X}{X'} \tag{4}$$

where X' and X are cell concentrations at the start of the exponential phase and after a time (t), respectively.

Generation time was determined according to the equation:

$$t_g = \frac{\ln 2}{\mu_{max}} \tag{5}$$

Volumetric cell productivity (P_X), expressed as $g_X/L \cdot h$, was calculated according to the equation:

$$P_X = \frac{\Delta X}{\Delta t} = \frac{(X_{max} - X_0)}{t} \tag{6}$$

where X_{max} and X_0 are the maximum and initial values of biomass concentration, respectively, whereas t is the time needed to reach X_{max}. Volumetric biosurfactant productivity (P_B), expressed as $mg_B/L \cdot h$, was calculated according to the equation:

$$P_B = \frac{\Delta B}{\Delta t} = \frac{(B_f - B_0)}{t} \tag{7}$$

where B_0 and B_f are the initial and final biosurfactant concentrations attained after the time t. The yield of biosurfactant on cell mass, expressed as mg_B/g_X, was calculated as the ratio of the biosurfactant concentration (ΔB) to the corresponding biomass concentration after the same time (ΔX).

The average value of this yield ($Y_{B/X}$) was calculated as the ratio of the variation in biosurfactant concentration from the start to the maximum cell concentration to the corresponding variation in biomass concentration.

The yield of biosurfactant on total proteins, expressed as mg_B/g_{TP}, was calculated as the ratio of the biosurfactant concentration (ΔB) to the corresponding total proteins concentration (ΔTP) after the same time. The average value of this yield ($Y_{B/TP}$) was calculated as the ratio of the variation in biosurfactant concentration from the start to the maximum cell concentration to the corresponding variation in total proteins concentration. The yield of biomass on consumed substrate ($Y_{X/S}$), expressed as g_X/g_S, was calculated as the ratio of the difference between maximum and initial cell concentrations ($X_{max} - X_0$) to the corresponding substrate consumption (glucose, ΔG and casein, ΔC) after the same time interval.

The yield of biosurfactant concentration on consumed substrate ($Y_{B/S}$), expressed as mg_B/g_S, was calculated as the ratio of the difference between final and initial biosurfactant concentration ($B_f - B_0$) to the corresponding substrate consumption (glucose, ΔG and casein, ΔC) after the same interval.

Results and Discussion

Batch cultivations of *B. atrophaeus* ATCC 9372 were performed on medium containing variable levels of hydrolyzed casein (0.0–10.0 g/L) and glucose (0.0–18.0 g/L) to follow the production of biosurfactants associated with cell growth. The concentration ranges of these ingredients were selected on the basis of the values most widely accepted in the literature for the growth of different microorganisms in synthetic media *(20–21,33–34)*. Table 1 lists the values of the main fermentation parameters obtained under different nutritional conditions, i.e., either in the presence of both nutrients or in the presence of only one of them.

A look at the data of the volumetric cell productivity points out that the lowest values of this parameter in the presence of both glucose and casein were obtained in tests 1 ($P_X = 0.36\ g_X/L \cdot h$) and 2 ($P_X = 0.34\ g_X/L \cdot h$), i.e., cultivations in which the casein level was almost the same or higher than that of glucose. On the other hand, when glucose concentration was 80% higher than that of casein (test 3), this parameter exhibited an average increase by 50%.

The simultaneous presence of these metabolizable nutrients seemed to influence the relation between carbon source uptake and cell growth. Glucose behaved as the preferential carbon source in all cultivations performed in its presence (tests 1–5), being consumed since the beginning. However, when glucose concentration was increased from 2.5 g/L (test 1) to 14.0 g/L (test 2) in the medium containing casein as well, the yield of biomass on consumed substrate (glucose, ΔG) decreased by 67%, hence suggesting that glucose was likely present in excess with respect to the

Table 1
Main Kinetic Results of Batch Cultivations of *B. atrophaeus* ATCC 9372 Performed at Different Initial Glucose and Casein Concentrations

Test	G_0[a] (g_S/L)	C_0[b] (g/L)	pH[c]	$Y_{B/X}$[d] (mg_B/g_X)	$Y_{B/TP}$[e] (mg_B/g_{TP})	μ_{Xmax}[f] (h^{-1})	t_g[g] (h)	ΔG[h] (g_S/L)	ΔC[i] (g/L)	B_{max}[j] (mg_B/L)	P_X[k] (g_X/L·h)	P_B[l] (mg_B/L·h)	$Y_{X/S}$[m] (g_X/g_S)	$Y_{B/S}$[n] (mg_B/g_S)
1	2.5	10.0	6.7 ± 0.3	121	25.4	0.180	3.8	1.78	1.10	346	0.36	58	1.20	36.1
2	14.0	10.0	6.7 ± 0.3	162	53.6	0.084	8.2	13.8	0.80	635	0.34	29	0.40	43.9
3	18.0	10.0	5.8 ± 0.5	256	53.8	0.247	2.8	16.3	4.31	574	0.54	191	0.73	4.70
4	2.5	0.0	6.3 ± 0.03	30.1	22.8	0.104	6.7	2.4	–	53.9	0.15	3.4	1.38	7.90
5	5.0	0.0	6.6 ± 0.04	5.50	3.40	0.242	2.9	2.6	–	10.0	0.14	1.4	1.15	0.00
6	0.0	10	5.7 ± 0.5	70.1	15.2	0.117	5.9	–	1.70	537	0.39	23	3.71	68.0

The average kinetic parameters were calculated at the end of the cell growth phase.
[a]Initial glucose concentration.
[b]Initial casein concentration.
[c]pH variation during cultivation.
[d]Average yield of biosurfactant on cell mass.
[e]Average yield of biosurfactant on total proteins.
[f]Maximum specific growth rate.
[g]Generation time.
[h]Glucose consumption during the growth phase.
[i]Casein consumption during the growth phase.
[j]Maximum biosurfactant concentration.
[k]Volumetric biomass productivity.
[l]Volumetric biosurfactant productivity.
[m]Yield of biomass on consumed substrate during growth phase.
[n]Yield of biosurfactant on consumed substrate during growth phase.

uptake ability of the microorganism, and the exponential phase took twice longer (16 h). In both cases the occurrence of stationary growth phase was coincident with total glucose consumption.

Comparison of the results listed in Table 1 shows that casein was simultaneously consumed, although never completely, and that its consumption (ΔC) in test 1 was 27% larger than in test 2, likely because the low glucose concentration in the medium made this substrate limiting for the growth and forced the microorganism to metabolize other carbon sources. These results confirm that glucose was the primary carbon source and that the initial concentration of casein used in this cultivation (10.0 g/L) was higher than the needs of the microorganism.

The results of biosurfactants produced during cultivations performed at glucose levels comparable with that of casein (test 2) or less (test 1) point out that these compounds were metabolized insofar as they were released during cell growth. The maximum concentration of biosurfactants (B_{max} = 346.0 mg/L) detected at the end of the exponential growth phase of test 1 (t = 6 h) does in fact suggest that this production was associated to growth (Fig. 1A). The connection between curves of specific growth rate (μ_X), substrate consumption rate (μ_S), and biosurfactant formation rate (μ_B) suggests a primary metabolism to biosurfactant production in tests 1 (Fig. 1B) and 2 (Fig. 2B). When glucose concentration was increased (test 2), the production of biosurfactants exhibited almost the same behavior as that observed in test 1 (Fig. 2A), in that it increased during the exponential growth phase (8–10 h), achieved a maximum value of 635.0 mg/L, and then decreased (t = 14 h, B_{max} = 278.0 mg/L). Because the occurrence of the stationary phase was related in both cultivations to the depletion of the primary carbon source (glucose), it was likely that, under these nutritional stress conditions, not only the production of biosurfactants was strongly affected but also biosurfactants were utilized as the preferred carbon source. It is noteworthy that the concentration of total proteins, related to the presence of casein, did not show large variations during tests 1 (ΔC = 1.10.0 g/L) and 2 (ΔC = 0.80 g/L) (Table 1), which suggests that the microorganism preferred the biosurfactant to casein as a secondary carbon source. A possible cause of this behavior could be the simpler structure of biosurfactants, which are simple lipopeptides containing only 7–10 aminoacids, and then could have been hydrolyzed and metabolized more quickly than casein. The production of biosurfactants can alternatively be evaluated through the yield of biosurfactants on biomass ($Y_{B/X}$), a volume-independent parameter very useful in scale-up operations. Comparing the results of tests 1 and 3 (Table 1) shows that a sevenfold glucose concentration in the medium led to a >200% increase in this parameter.

When initial glucose level was higher (18.0 g/L) than that of casein (10.0 g/L) (test 3), *B. atrophaeus* growth curve exhibited a clear inflection after 10 h of cultivation. Such a sort of diauxic growth (Fig. 3A) could be ascribed to the activation of alternative routes to metabolize the new

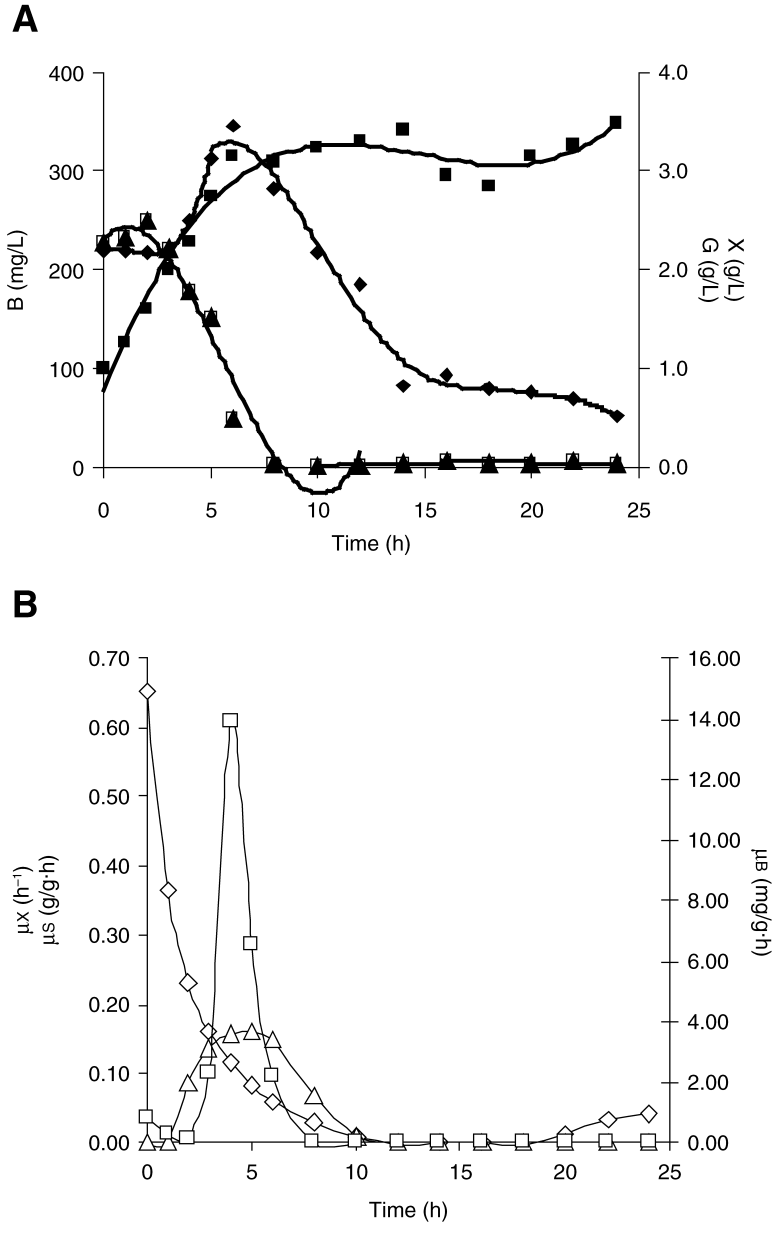

Fig. 1. Time behaviors of **(A)** the concentrations of (X-■) biomass, (G-▲) glucose, and (B-◆) biosurfactants and **(B)** the specific rates of growth (μ_X-□), substrate consumption (μ_S-△), and biosurfactant formation (μ_B-◇) during the cultivation of *B. atrophaeus* ATCC 9372 on 2.5 g/L glucose and 10.0 g/L casein.

carbon source, when the concentration of glucose, the less-energy consuming substrate, decreased to less than 10.0 g/L. Casein uptake started at the beginning of the stationary phase preceding the diauxic phenomenon, then went on simultaneously to that of glucose (Fig. 3A, Table 1), and was

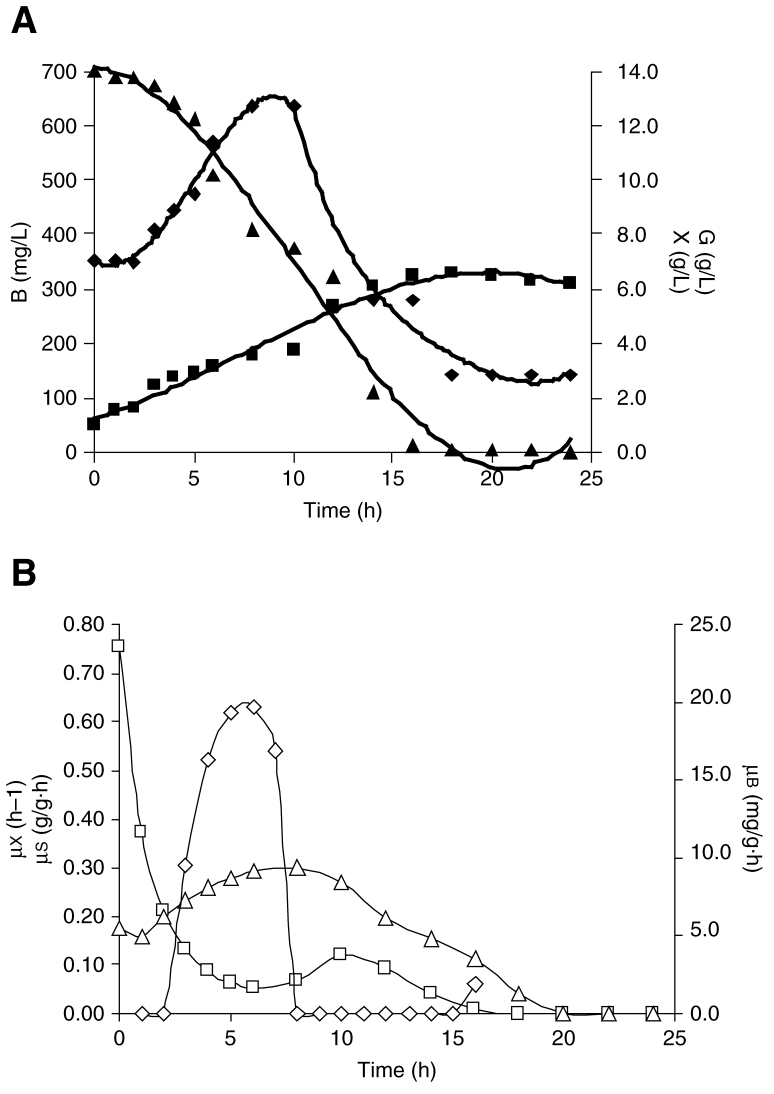

Fig. 2. Time behaviors of **(A)** the concentrations of (X-■) biomass, (G-▲) glucose, and (B-◆) biosurfactants and **(B)** the specific rates of growth (μ_X-□), substrate consumption (μ_S-△), and biosurfactant formation (μ_B-◇) during the cultivation of *B. atrophaeus* ATCC 9372 on 14.0 g/L glucose and 10.0 g/L casein.

about fourfold that obtained at lower initial glucose levels (tests 1 and 2). Therefore, we can believe in the existence of a relationship between the metabolic change associated to casein consumption and diauxic growth, which resulted in a new phase of cell growth up to 22 h. This behavior is confirmed by the curves of Fig. 3B showing two different phases of cell growth at G_0 = 18.0 g/L and an intermediate interval of quick increase in the specific growth rate (μ_X).

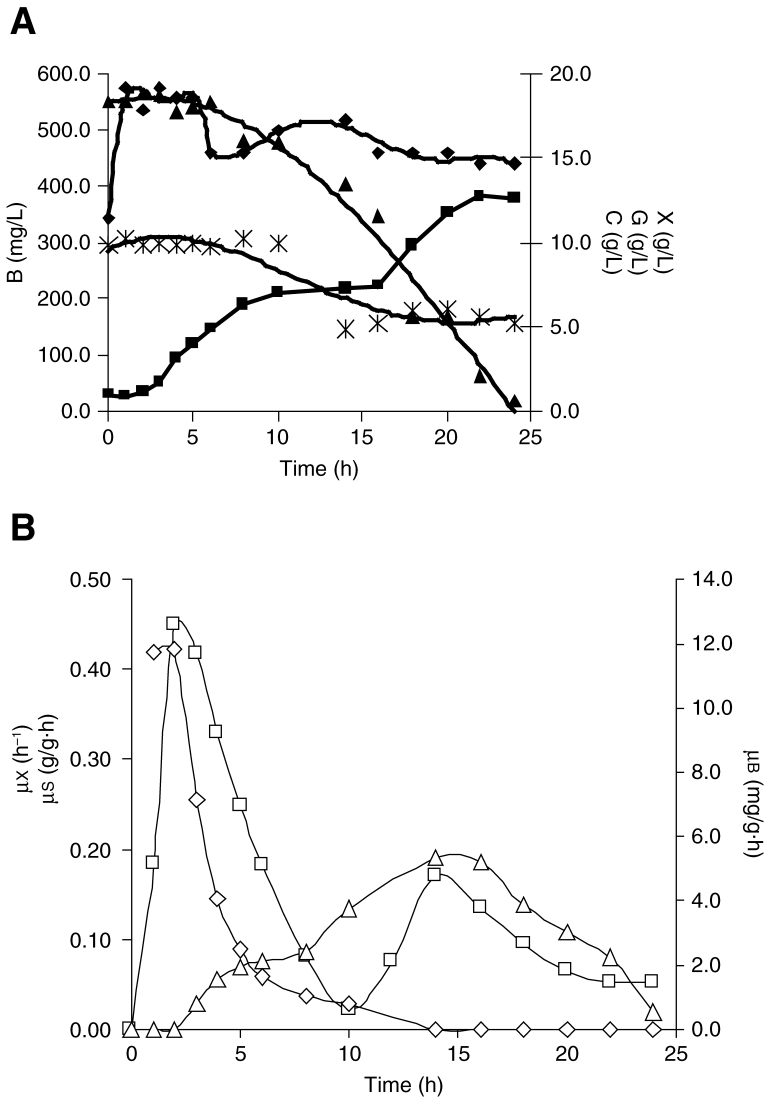

Fig. 3. Time behaviors of **(A)** the concentrations of (X-■) biomass, (G-▲) glucose, (B-◆) biosurfactants, and (C-∗) casein and **(B)** the specific rates of growth (μ_X-□), substrate consumption (μ_S-△), and biosurfactant formation (μ_B-◇) during the cultivation of *B. atrophaeus* ATCC 9372 on 18.0 g/L glucose and 10.0 g/L casein.

The simultaneous uptake of glucose and casein can explain the scarce consumption of biosurfactants, whose concentration in the extracellular medium decreased only by 135.0 mg/L from the beginning to the end of test 3 (Fig. 3A). This means that biosurfactants released by the cell almost stopped to be utilized as carbon and energy source, contrary to what it was observed at lower glucose levels (tests 1 and 2).

The significance of casein in the production of biosurfactants can be deduced from the results of tests 4 and 5, which were performed using only

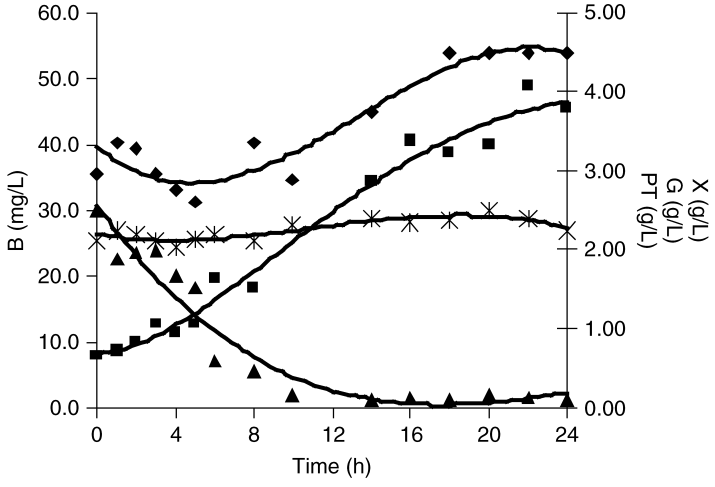

Fig. 4. Time behaviors of **(A)** the concentrations of (X-■) biomass, (G-▲) glucose, (B-◆) biosurfactants, and (PT-*) total extracellular proteins during the cultivation of *B. atrophaeus* ATCC 9372 on 2.5 g/L glucose without casein.

glucose as a carbon and energy source. As an example, comparing test 4 with test 1, the yield of biosurfactants on biomass did in fact dramatically decrease (by 75%) when using a medium lacking of casein. Figures 4 and 5, which deal with cultivations in the absence of casein performed at glucose concentrations of 2.5 and 5.0 g/L, respectively, show that the concentration of total extracellular proteins in the medium kept almost unvaried, hence demonstrating that soy flour was not utilized as energy source by the system. Moreover, at the higher glucose level a reduction of biosurfactant concentration was observed throughout the whole experiment. Although biosurfactants are products of primary metabolism, i.e., their formation is associated to cell growth, high-starting level of glucose as the only carbon and energy source did not favor their production. Therefore, the 25% increase in the concentration of total extracellular proteins, illustrated in Fig. 5, was likely the result of the synthesis of some other protein without surfactant activity.

Test 6 demonstrated the ability of the microorganism to adapt its metabolism to uptake casein as energy source. This change led to a lag phase period of 10 h before starting cell growth (Fig. 6A). Casein was consumed throughout the whole cultivation, bringing about a decoupling of the curves of the specific rates of growth (μ_x) and substrate consumption (μ_S) (Fig. 6B). Besides, the occurrence of diauxie is demonstrated by the presence of two separated exponential growth phases, i.e., two peaks of μ_x in Fig. 6B. Similar to the situation in which glucose and casein were simultaneously present in the medium, growth acceleration after diauxie was less marked with respect to the former peak at the beginning of the cultivation.

Comparison of the volumetric cell productivities (P_x) listed in Table 1 shows that, in cultivations performed with both nutrients at starting glucose

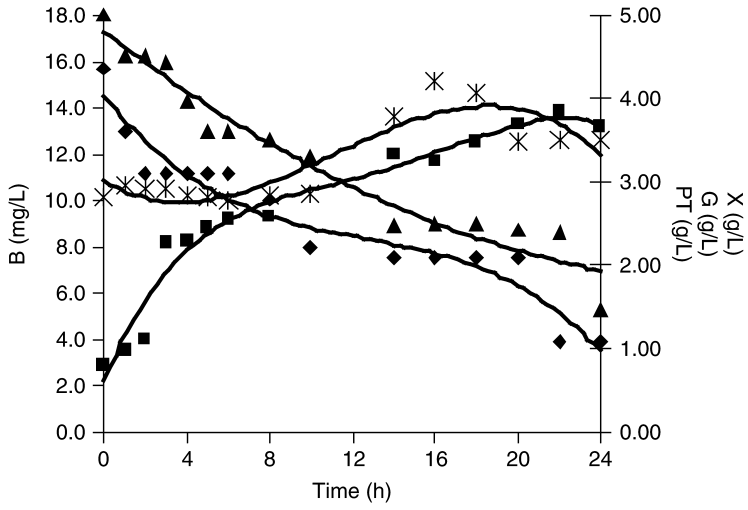

Fig. 5. Time behaviors of **(A)** the concentrations of (X-■) biomass, (G-▲) glucose, (B-◆) biosurfactants, and (PT-✳) total extracellular proteins during the cultivation of *B. atrophaeus* ATCC 9372 on 5.0 g/L glucose without casein.

level comparable with that of casein or less (tests 1–2), this parameter was almost the same as that obtained in the presence of casein alone (test 6), whereas a 27–37% increase was observed at the highest glucose level (test 3). Nevertheless, in the presence of casein as the only energy source, the microorganism exhibited higher yield of biomass on substrate ($Y_{X/S} = 3.71\ g_X/g_S$).

The release of biosurfactants started only after the lag phase (10 h), and deceleration of casein consumption (μ_s) simultaneously took place (Fig. 6B). The possibility to metabolize casein can only be associated to the capability of the microorganism to synthesize and release extracellular proteases, mainly subtilisin, which also exhibits biosurfactant properties *(35)*. Therefore, the behavior of Fig. 6A suggests coupling of subtilisin release and cell growth after diauxie. After 15 h of cultivation, the decrease in μ_S (Fig. 6B) did in fact occur together with certain maintenance of TP level (Fig. 6A), after the achievement of stationary growth phase. However, this condition appeared not to affect the synthesis of biosurfactants, as suggested by the continuous increase in their concentration along the whole cultivation. Resuming the addition of casein in a medium containing sugars and salts favored either the production or the stability of biosurfactants. The microorganism did in fact shift its metabolism to utilize the excess protein (casein) as a carbon source. The significance of the use of media containing both sugar and casein is associated to the possibility of maintaining high biosurfactant levels in environmental applications, even after the microorganism has entered the stationary growth phase.

As is well known, the major limitations in the application of biotechnologies to environmental applications are related to their high costs.

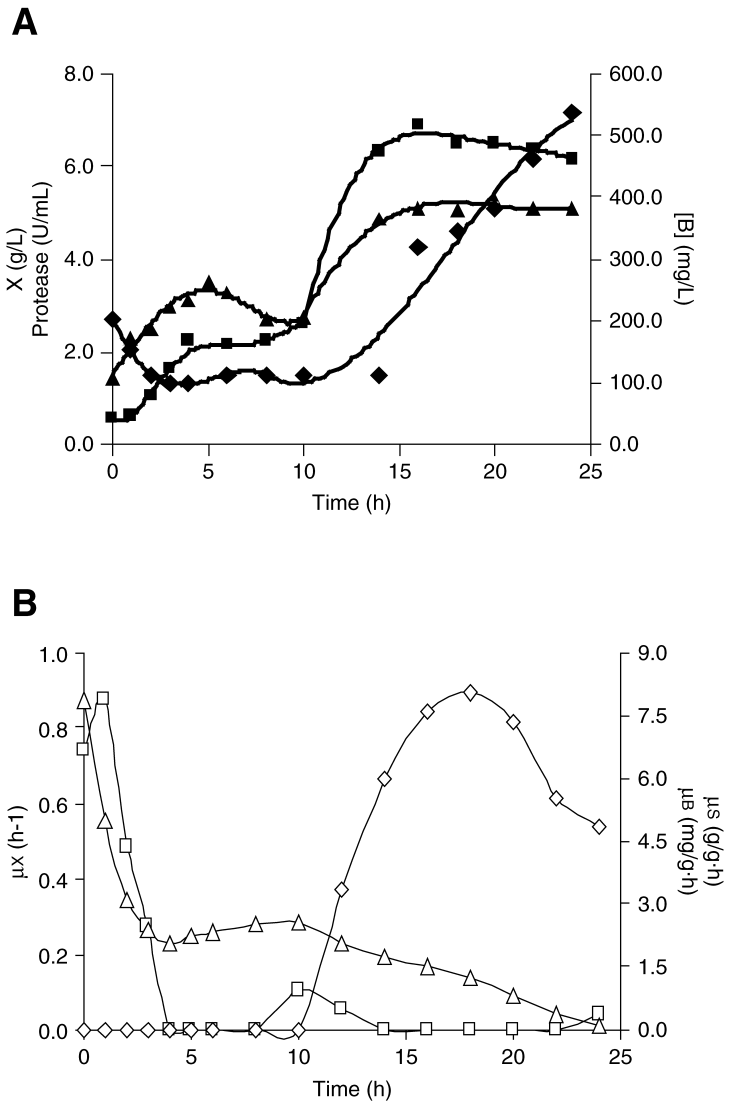

Fig. 6. Time behaviors of **(A)** the concentrations of (X-■) biomass, (▲) protease, and (B-◆) biosurfactants, and **(B)** the specific rates of growth (μ_X-□), substrate consumption (μ_S-△), and biosurfactant formation (μ_B-◇) during the cultivation of *B. atrophaeus* ATCC 9372 on 10.0 g/L casein without glucose.

Among the possibilities to reduce them there is the substitution of synthetic media with natural media having almost the same nutritional composition and less purity. To this purpose, industrial residues could be used as alternative media for the production of biosurfactants to be used in environmental applications. Mixtures of sugars, salts, and casein can in fact be obtained from residual sources such as whey, after adjustment of the concentrations of the main ingredients to the best values previously determined in

synthetic medium. The next attempt will deal with (a) the use of whey as alternative medium for biosurfactant production by *B. atrophaeus* ATCC9372 cultivations and (b) the application of such a system to biotreatments in which relatively stable levels of biosurfactants are required.

Conclusions

The inclusion of casein in a sugar-based medium for *B. atrophaeus* ATCC 9372 cultivation favored the synthesis and release of biosurfactants thanks to the ability of the microorganism to simultaneously metabolize them. The highest concentration of biosurfactants (B_{max}= 635.0 mg/L) was obtained in the presence of 14.0 g/L glucose and 10.0 g/L casein. Under these conditions, biosurfactants were released in the medium only during the exponential growth phase, after which they were metabolized. At the highest glucose level tested in this work (18.0 g/L), biosurfactants kept at high levels in the medium even after the microorganism entered its stationary growth phase. These results are promising for possible application of this biosystem to biological, enviromental applications.

References

1. Banat, I. M. (1993), *Biotechnol. Lett.* **15,** 591–594.
2. Banat, I. M. (1995), *Biores. Technol.* **51(1),** 1–12.
3. Rosengerg, E. (1986), *CRC Crit. Rev. Biotechnol.* **3(2),** 109–132.
4. Peypoux, F., Bonmatin, J. M., and Wallach, J. (1999), *Appl. Microbiol. Biotechnol.* **51,** 553–563.
5. Haferburg, D., Hommel, R., Claus, R., and Kleber, H. P. (1986), *Adv. Biochem. Eng. Biotechnol.* **33,** 53–93.
6. Morikawa, M., Hirata, Y., and Imanaka, T. (2000), *Biochem. Biophys. Acta* **1488,** 211–218.
7. Noah, C. W., Shaw, C. I., and Ikeda, J. S. (2005), *J. Food Prot.* **68(4),** 680–686.
8. Cooper, D. G. (1986), *Microbiol. Sci.* **3(5),** 145–149.
9. Neves, L. C. M., Miyamura, T. T. M. O., Moraes, D. A., Vessoni-Penna, T. C., and Converti, A. (2006), *Appl. Biochem. Biotechnol.* **129(1–3),** 130–152.
10. Singh, P. and Cameotra, S. S. (2004), *Trends Biotechnol.* **22(3),** 142–146.
11. Vessoni-Penna, T. C., Ishii, M., Machoshvili, I. A., and Marques, M. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 539–551.
12. Vessoni-Penna, T. C.., Ishii, M., Machoshvili, I. A., and Marques, M. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 525–538.
13. Vessoni-Penna, T. C., Chiarini, E., Machoshvili, I. A., Ishii, M., and Pessoa, A., Jr. (2002), *Appl. Biochem. Biotechol.* **98–100,** 791–802.
14. Kosaric, N. (1992), In: *Biotechnology.* Rehm, H. J., Reed, G., Puhler, A., and Stadler, P. (eds.), vol. 6, VCH, Weinheim, pp. 659–717.
15. Lang, S. and Wullbrandt, T. (1999), *Appl. Microbiol. Biotechnol.* **51,** 22–32.
16. Makkar, R. S. and Comeotra, S. S. (1998), *J. Ind. Microbiol. Biotechnol.* **20,** 48–52.
17. Adamczak, M. and Bednarski, W. (2000), *Biotechnol. Lett.* **22,** 313–316.
18. Ferraz, C., De Araújo, A. A., and Pastore, G. M. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 841–847.
19. Mukherjee, A. K. and Das, K. (2005), *FEMS Microbiol. Ecol.* **54,** 479–489.
20. Davis, D. A., Lynch, H. C., and Varley, J. (1999), *Enzyme Microb. Technol.* **25,** 322–329.
21. Patel, R. M. and Desai, A. J. (1997), *Lett. Appl. Microbiol.* **26,** 91–94.
22. Lang, S. and Wagner, F. (1987), *In: Biosurfaotants and Biotechnology.* Kosaric, N., Cairns, W. L., Gray, N. C. C. (Eds.), Surfactant Science Series, vol. 25, Marcel Dekker, NY, 247–331.

23. Nitschke, M. and Pastore, G. M. (2003), *Appl. Biochem. Biotech*, **105–108,** 295–301.
24. Petrus, J. C. C. Reutilização do soro de leite, CTC-UFSC, http://inventabrasilnet.t5.com.br/soro.htm (March 20, 2006).
25. Fall, R., Kinsinger, R. F., and Wheeler, K. A. (2004), *Syst. Appl. Microbiol.* **27,** 372–379.
26. Çalik, P., Çelik, E., Telli, I. E., Oktar, C., and Özdemir, E. (2003), *Enzyme Microb. Technol.* **33,** 975–986.
27. Kim, J. M., Lim, W. J., and Suh, H. J. (2001), *Process Biochem.* **37,** 287–291.
28. Neves, L. C. M., Pessoa, A. Jr., and Vitolo, M. (2003), *Braz. J. Pharm. Sci.* **39(3),** 160–163.
29. Neves, L. C. M., Pessoa, A. pJr., and Vitolo, M. (2005), *Biotechnol. Prog.* **21,** 1135–1139.
30. Rossi, F. G., Ribeiro, M. Z., Converti, A., Vitolo, M., and Pessoa, A., Jr. (2003), *Enzyme Microb. Technol.* **32,** 107–113.
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193(1),** 265–275.
32. Ahamed, A., Singh, A., and Ward, O. P. (2006), *Process Biochem.* **41,** 789–793.
33. Shimogaki, H., Takeuchi, K., Nishino, T., et al. (1991), *Agric. Biol. Chem.* **55(9),** 2251–2258.
34. Prakash, M., Banik, R. M., and Koch-Brandt, C. (2005), *Appl. Biochem. Biotechnol.* **127(3),** 143–155.
35. Bognolo, G. (1999), *Coll. Surf. A*: *Physicochem. Eng. Aspects* **152,** 41–52.