Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains

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Abstract

Screening of biosurfactant-producing ability of four *Lactobacillus* strains was performed, being shown that for all the tested strains biosurfactant production occurred mainly in the first 4 h. The *Lactobacillus* strains showed zones of clearing in the blood agar with a diameter <1 cm. The minimum surface tension value of the fermentation broth achieved was 39.5 mN/m for *Lactobacillus pentosus* CECT-4023 that represents a reduction in the surface tension of 10.5 mN/m comparing with the control.

Time courses of glucose, biomass and biosurfactant were modeled according to reported models. Using MRS broth (Man, Rogosa & Sharpe medium for *lactobacilli* strains) as culture medium, the values estimated by the modeling of biosurfactant were $P_{max} = 1.6$ g of biosurfactant/L and $r_p/X = 0.091$ g/(L h), for *Lactobacillus casei* CECT-5275, $P_{max} = 1.7$ g/L and $r_p/X = 0.217$ g/(L h) for *Lactobacillus rhamnosus* CECT-288, $P_{max} = 1.7$ g/L and $r_p/X = 0.069$ g/(L h) for *L. pentosus* CECT-4023 and $P_{max} = 1.8$ g/L and $r_p/X = 0.090$ g/(L h) for *Lactobacillus coryniformis* subsp. *torquens* CECT-25600. P_{max} is the maximum concentration of biosurfactant (g/L), and P_r is the ratio between the initial volumetric rate of product formation (r_p) and the initial product concentration P_0 (g/L). Using whey as production medium, the values estimated by the modeling of biosurfactant for *L. pentosus* CECT-4023 were $P_{max} = 1.4$ g of biosurfactant/L and $r_p/X = 0.093$ g/(L h).

In conclusion, the results obtained for *L. pentosus* CECT-4023 showed that this is a strong biosurfactant producer strain and that cheese whey can be used as an alternative medium for biosurfactant production.
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Keywords: Biosurfactant production; Surface tension; Fermentation; *Lactobacillus*

1. Introduction

Lactobacillus species are often together with *Streptococcus* being used as acid and flavor producers in the dairy industry [1]. In addition to their occurrence in plant material and food products, lactobacilli also inhabit the gastrointestinal tract of healthy mammals, and they are the most common members of indigenous microflora of the urogenital tract [2]. *Lactobacillus* and *Streptococcus* species have been shown to be able to displace adhering uropathogenic *Enterococcus faecalis* from hydrophobic and hydrophilic substrata in a parallel-plate flow chamber, possibly through biosurfactant production [3].

Biosurfactants are biological surface-active compounds released by microorganisms that can have some influence on interfaces. With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes [4]. Biosurfactants have also been reported to have strong antibacterial, antifungal and antiviral activity [5]. Interest in biosurfactants has been increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food

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Nomenclature

F value	F-test statistical parameter
MRS broth	medium introduced by De Man, Rogosa and Sharpe for cultivation of *Lactobacillus* species, purchased from OXOID, Basingstoke, UK
P	biosurfactant concentration (g/L)
P_0	initial biosurfactant concentration (g/L)
P_{max}	maximum concentration of biosurfactant (g/L)
P_r	ratio between initial volumetric rate of biosurfactant formation (r_p) and initial biosurfactant concentration P_0 (h^{-1})
PBS	phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0)
r^2	correlation coefficient
r_p	initial volumetric rate of biosurfactant formation (g/(L h))
S	substrate (glucose or lactose) concentration (g/L)
S_0	initial substrate (glucose or lactose) concentration (g/L)
X	biomass concentration (g/L)
X_0	initial biomass concentration (g/L)
X_{max}	maximum concentration of biomass (g/L)
$Y_{P/S}$	product yield (g/g)
$Y_{X/S}$	biomass yield (g/g)
μ_{max}	ratio between initial volumetric rate of biomass formation (r_p) and initial biomass concentration X_0 (h^{-1})

processing industries [4]. Dairy *Streptococcus thermophilus* strains, for example, can produce biosurfactants that cause their own desorption [6] and Rodrigues et al. [7] found that a biosurfactant obtained from *S. thermophilus* A showed a significant antimicrobial activity against several microorganisms that contribute to the premature failure of voice prostheses. In another study the use of a biosurfactant from *Lactococcus lactis* 53 as antimicrobial and/or anti-adhesive agent and its ability to inhibit adhesion in a parallel-plate flow chamber of various microorganisms isolated from explanted voice prostheses has been demonstrated [8].

Depending upon the nature of the biosurfactant and the producing microorganisms, several patterns of biosurfactant production by fermentation are possible. Velraeds et al. [3] showed that biosurfactant release by lactobacilli is maximum for cells in the stationary phase, and growth-associated biosurfactant production has been described for the release of biodispersan by *Acinetobacter calcoaceticus* [9]. In addition, biosurfactant production may occur or be stimulated by growing the microbial cells under growth-limiting conditions. *Pseudomonas aeruginosa* shows an overproduction of rhamnolipid when the culture reaches the stationary growth phase due to limitation of the nitrogen source [9].

The biosurfactant production by growing cells has been reported to be affected by environmental factors [1], for example, the carbon source plays an important role. A good substrate for biosurfactant production is lactic whey, as it is composed of high levels of lactose (75% dry matter), 12–14% protein, organic acids and vitamins. Whey is a waste product from cheese production that represents a major pollution problem for countries depending on dairy economics and is normally used as animal feed. Sophorolipids production using whey was reported by Otto et al. [10].

The aims of this study were to screen a number of *Lactobacillus* strains for biosurfactant production by blood agar method and surface tension determination, and to model the biosurfactant production as well as the time courses of glucose consumption and biomass growth. The relation between cellular growth and surface-activity of the biosurfactant in time (as a measure of its production) was determined for all the strains.

2. Materials and methods

2.1. Strains and culture conditions

Several *Lactobacillus* strains were investigated in this study. The bacterial strains *Lactobacillus casei* CECT-5275, *Lactobacillus rhamnosus* CECT-288, *Lactobacillus pentosus* CECT-4023 and *Lactobacillus coryniformis* subsp. *torquens* CECT-25600 obtained from the Spanish Collection of Type Cultures (Valencia, Spain) were stored at $-20\,°C$ in MRS broth (medium introduced by De Man, Rogosa and Sharpe for cultivation of *Lactobacillus* species, OXOID, Basingstoke, UK) containing 15% (v/v) glycerol solution until ready to use. From frozen stock, bacteria were streaked on MRS agar plates and incubated at the optimum temperature for each strain for further culturing.

2.2. Growth curves

Growth curves for the *Lactobacillus* strains were determined because biosurfactant production may be influenced by the growth phase of the organisms [9]. The bacterial strains were cultured in shake flasks without baffles with 100 ml MRS broth (OXOID, Basingstoke, UK) and growth was measured by determining the optical density at 600 nm during different time intervals up to 72 h. The biomass concentrations (g dry weight l^{-1}) were determined using a calibration curve. The calibration curve was calculated for each strain using dilutions of a biomass suspension with known optical density. A fixed volume of the dilutions was filtered (0.22 μm) and left to dry at $105\,°C$ for 24 h. All the filters were weighed before the filtration and after drying. Thus, a relationship between biomass concentration (g/l) and optical density (600 nm) can be determined ($C_{biomass}$ (g/L) = (OD$_{600\,nm}$ × 0.506) + 0.036; $r^2 = 0.9998$).

2.3. Blood agar screening method

The blood agar method is widely used to screen for biosurfactant production and several studies where this method was employed are reported in the literature [11–14]. Briefly, each strain was streaked onto blood agar plates and incubated for 48 h at 37 °C or 31 °C. The plates were visually inspected for zones of clearing around colonies. The diameter of the clear zones depends on the concentration of the crude biosurfactant. The zones of clearing were scored as follows: (−) no hemolysis; (+) incomplete hemolysis; (++) complete hemolysis with a diameter of lysis <1 cm; (+++) complete hemolysis with a diameter of lysis >1 cm but <3 cm; and (++++) complete hemolysis with a diameter of lysis >3 cm and green colonies.

2.4. Biosurfactant production

The bacterial strains were cultured in 100 ml MRS broth and grown for 72 h, in ambient air at 31 °C for *L. pentosus* and 37 °C for all the other *Lactobacillus* strains. For extracellular biosurfactant determination, at different time intervals samples were taken to assay the surface-activity of the media broth.

For intracellular biosurfactant determination, at the end of the experiments (72 h) cells were harvested by centrifugation (10,000 × g, 5 min, 10 °C), washed twice in demineralized water, and resuspended in 20 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) [2]. The bacteria were left at room temperature up to 24 h with gentle stirring for biosurfactant release. During extraction process samples were taken at different time intervals, bacteria were removed by centrifugation and the remaining supernatant liquid was tested for surface-activity.

For *L. pentosus* the biosurfactant production was also assayed growing the strain in whey at 31 °C. Commercial whey was prepared as follows: after adjusting the pH to 4.5 with 5 N HCl, it was heated at 121 °C for 15 min to denature the proteins. The precipitates were removed by centrifugation at 4 °C and 8000 × g for 10 min. The supernatants were adjusted to pH 6.3, sterilized at 121 °C for 15 min and used as culture media. The supernatant contained 56 g/L of lactose.

2.5. Analytical methods

Sugars were determined by high performance chromatography (Agilent, model 1100, Palo Alto, CA) using ION-300 column (Transgenomic Inc., San Jose, CA) with refractive index detector. The mobile phase was 0.01 N H_2SO_4 with a flow rate of 0.4 ml min^{-1}.

2.6. Surface-activity determination

The surface-activity of biosurfactants produced by the bacterial strains was determined by measuring the surface tension of the samples with the Ring method [5].

The surface tension of the culture broth samples of the PBS extraction samples and also of the whey fermentation with *L. pentosus* was measured by the Ring method [15] using a KRUSS Tensiometer equipped with a 1.9 cm De Noüy platinum ring at room temperature. Tensiometers determine the surface tension with the help of an optimally wettable ring suspended from a precision balance. In the Ring method the liquid is raised until contact with the surface is registered. The sample is then lowered again so that the liquid film produced beneath the ring is stretched. As the film is stretched a maximum force is experienced, the force is measured and used to calculate the surface tension. To increase the accuracy an average of triplicates was used for this study.

The biosurfactant concentrations (g/L) were determined for each *Lactobacillus* strain using a calibration curve (surface tension (mN/m) = −8.6465 concentration (g/L) + 76.984, $r^2 = 0.9729$). The calibration curve was calculated for a commercial biosurfactant produced by several *Bacilli* (surfactin) using different concentrations of biosurfactant solution, below the critical micelle concentration with known surface tension. In this biosurfactant concentration range the decrease of surface tension is linear and it is possible to establish a relationship between the biosurfactant concentration and the surface tension [15]. Nevertheless, to estimate biosurfactant concentration it was necessary sometimes to dilute the culture broth to reach the critical micelle concentration.

2.7. Glucose consumption and biosurfactant production—fitting of data

Experimental data were fitted to proposed models using commercial software (Solver of Microsoft Excel 2002) by nonlinear regression using the least-squares method. Biosurfactant production was mathematically modeled following the equation proposed by Mercier et al. [14] for lactic acid production

$$\frac{dP}{dt} = P_r P \left(1 - \frac{P}{P_{max}} \right) \tag{1}$$

where t is time (h), P is biosurfactant concentration (g/L), P_{max} is maximum concentration of biosurfactant (g/L), and P_r is the ratio between the initial volumetric rate of product formation (r_p) and the initial product concentration P_0 (g/L). Eq. (1) can be directly solved to give the Eq. (2)

$$P = \frac{P_0 P_{max} e^{P_r t}}{P_{max} - P_0 + P_0 e^{P_r t}} \tag{2}$$

From the series of experimental data biosurfactant concentration/time, the model parameters P_0, P_{max}, and P_r can be calculated for each *Lactobacillus* strain growing in MRS broth, and also for *L. pentosus* growing on whey.

Also biomass production was mathematically modeled and can be interpreted by Eq. (3)

$$X = \frac{X_0 X_{max} e^{\mu_{max} t}}{X_{max} - X_0 + X_0 e^{\mu_{max} t}} \tag{3}$$

where t is time (h), X is biomass concentration (g/L), X_{max} is maximum concentration of biomass (g/L), and μ_{max} (h^{-1}) is the ratio between the initial volumetric rate of biomass formation and the initial biomass concentration X_0 (g/L). The model parameters X_0, X_{max}, and μ_{max} can be calculated from the series of experimental data biomass concentration/time.

Glucose consumption by the *Lactobacillus* strains can be interpreted by the Eq. (4)

$$S = S_0 - \frac{1}{Y_{P/S}}(P - P_0) - \frac{1}{Y_{X/S}}(X - X_0) \tag{4}$$

where $Y_{P/S}$ (g/g) and $Y_{X/S}$ (g/g) are the product yield for biosurfactant and biomass, respectively, P and P_0 are the final and initial biosurfactant concentrations (g/L), X and X_0 are the final and initial biomass concentrations (g/L), and finally S_0 is the initial glucose concentration (g/L). The model parameters $Y_{P/S}$, $Y_{X/S}$ and S_0 (g/L) were calculated for each *Lactobacillus* strain from the series of experimental data glucose concentration/time and the Eqs. (2) and (3).

The mathematical model proposed by Mercier et al. [16] was chosen because it fairly describes biomass growth, substrate consumption and product accumulation kinetic pattern, and is reasonable to predict that this mathematical model will adjust the biosurfactant production results with statistical significance of the parameters determined.

3. Results

3.1. Blood agar screening method

All of the tested *Lactobacillus* strains showed zones of clearing in the blood agar with scores ranging between (++)
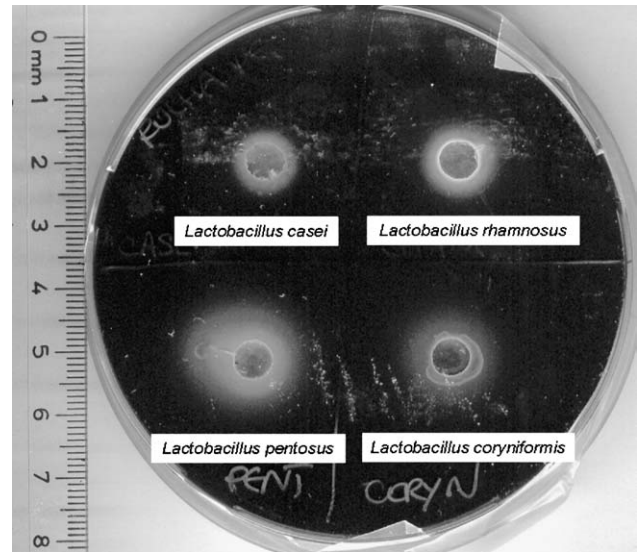

Fig. 1. Blood agar screening method results for *L. casei* CECT-5275 (up-left spot), *L. rhamnosus* CECT-288 (up-right spot), *L. pentosus* CECT-4023 (down-left spot) and *L. coryniformis* subsp. *torquens* CECT-25600 (down-right spot).

corresponding to complete hemolysis with a diameter <1 cm, as can be seen in Fig. 1. Table 1 shows that all these strains allowed for a surface tension reduction between 8 mN/m and 10.5 mN/m when compared with the control MRS broth (50 mN/m) consistent with their ability to produce biosurfactants.

3.2. Biosurfactant production

Growth curves were obtained for the four *Lactobacillus* strains in order to establish the relation between cell growth and surface-activity of the biosurfactant in time as can be seen in Fig. 2 and Table 1. For all the strains the biosurfactant production is occurring mainly in the first hours (4 h) where cell growth is almost inexistent and the substrate consumption is very low. However, the biosurfactant production continues

Table 1
Surface tension values (mN/m) obtained for the culture broth supernatants during the 72 h fermentation (the control surface tension (MRS) was 50 mN/m) and from the biosurfactant extraction with PBS during 24 h (the control surface tension (PBS) was 72 mN/m)

Lactobacillus strains	Surface tension of culture broth supernatants (mN/m)					
	0 h	4 h	10 h	24 h	48 h	72 h
L. casei	50.0 ± 0.0	44.0 ± 0.5	43.0 ± 0.1	43.0 ± 0.4	43.0 ± 0.3	42.0 ± 0.2
L. rhamnosus	50.0 ± 0.1	43.5 ± 1.0	42.3 ± 0.4	42.3 ± 0.1	42.0 ± 0.7	40.0 ± 0.9
L. pentosus	50.0 ± 0.0	45.0 ± 0.3	43.8 ± 0.8	43.3 ± 0.0	42.3 ± 0.1	39.5 ± 0.1
L. coryniformis	50.0 ± 0.1	43.0 ± 0.8	42.5 ± 0.2	41.5 ± 0.6	41.0 ± 0.0	40.5 ± 0.3

	Surface tension of biosurfactant extracted with PBS (mN/m)			
	0 h	0.5 h	8 h	24 h
L. casei	70.0 ± 0.1	55.0 ± 0.2	53.0 ± 0.3	53.0 ± 0.1
L. rhamnosus	70.0 ± 0.2	56.0 ± 0.0	52.0 ± 0.1	51.5 ± 0.2
L. pentosus	70.0 ± 0.3	56.0 ± 0.1	51.0 ± 0.2	50.5 ± 0.1
L. coryniformis	72.0 ± 0.1	60.0 ± 0.4	55.0 ± 0.5	55.0 ± 0.1

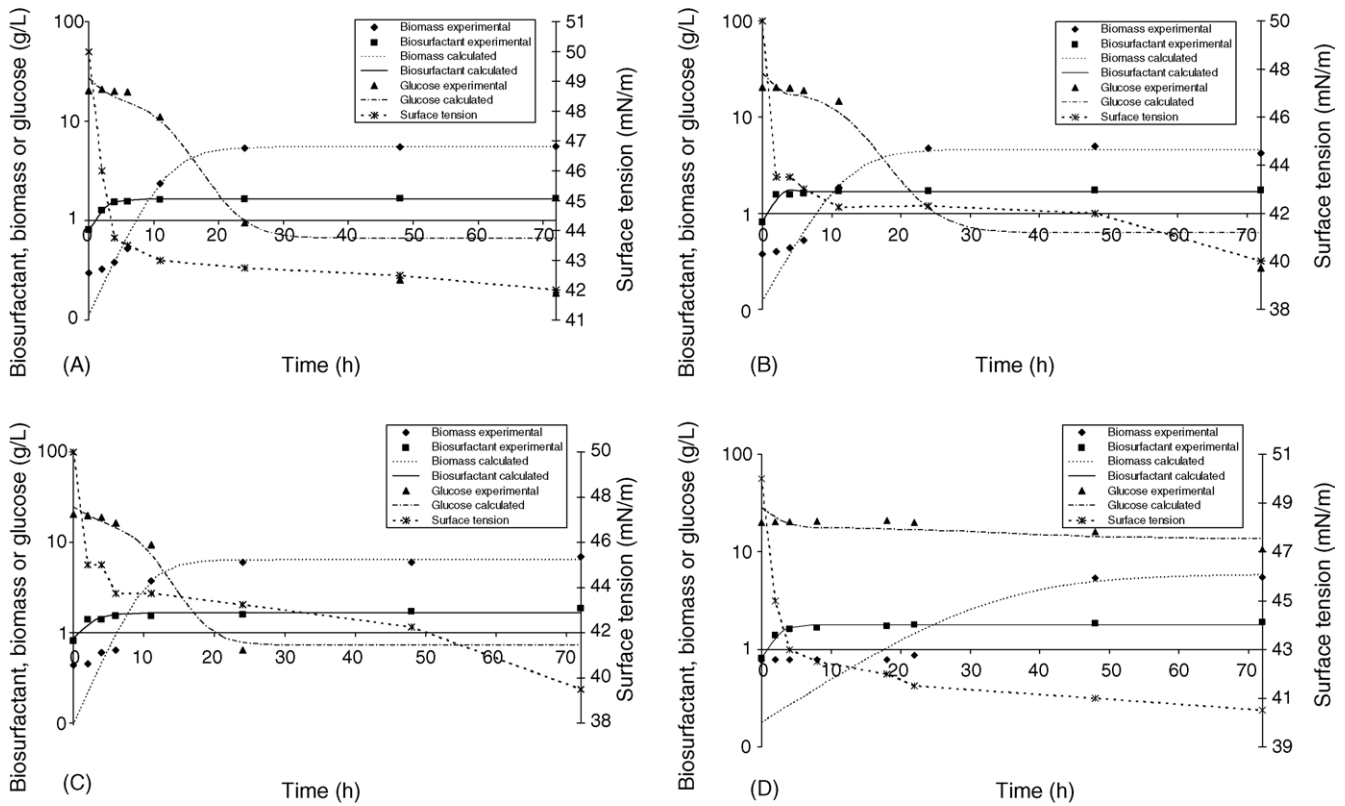Results are expressed as mean ± standard deviations of values from triplicate experiments.

Fig. 2. Representation of the surface tension variation (- -*- -), experimental data and calculated time courses of biomass (♦, - - -), glucose (▲, —·—) and biosurfactant concentrations (■, —) during fermentations carried out with MRS broth using (A) *L. casei* CECT-5275; (B) *L. rhamnosus* CECT-288; (C) *L. pentosus* CECT-4023; (D) *L. coryniformis* subsp. *torquens* CECT-25600. Results represent the average of three independent experiments.

during all 72 h of fermentation but at a very slow production rate. As the decrease in the surface tension exceeded 8 mN/m [2,6], all four strains were found to produce biosurfactants. The surface tension decreases were compared with the surface tension of MRS broth (control) to correct for lower initial surface tension values as a result of the medium ingredients that can have surface-active characteristics themselves.

3.3. Biosurfactant extraction with PBS—fitting of data

Reduction of surface tension during the PBS extraction of cells in stationary phase were fitted to proposed models using commercial software (Table Curve Windows v1.11). For all the *Lactobacillus* strains an exponential fit was possible according to the following equation:

$$y = a + be^{(-x/c)} \tag{5}$$

where y is surface tension (mN/m) and x is the extraction time (h). The equation parameters obtained were very similar for all the tested strains, with $a = 52 \pm 1.5$; $b = 18 \pm 1.2$; $c = 0.4 \pm 0.14$ and $r^2 = 0.991$. Table 1 compiles the surface tension values decrease along the 24 h extraction procedure with PBS and it was found that all the *Lactobacillus* strains released intracellular biosurfactants. These strains allowed a surface tension reduction between 17 mN/m and 21.5 mN/m

when compared with the control PBS (72 mN/m), being *L. pentosus* the best biosurfactant producer strain.

3.4. Fermentations in MRS broth

Fermentation runs were carried out using the fully supplemented medium MRS broth for all *Lactobacillus* strains. Fig. 2 shows the experimental data as well as the predicted values calculated by Eqs. (2)–(4) using the regression parameters listed in Table 2. All experiments show a kinetic pattern fairly described by the mathematical models with $r^2 > 0.891$, 0.907 and 0.907 for glucose consumption, biomass and biosurfactant production, respectively. It can be noted that *L. coryniformis* presents the highest P_{max} (1.8 g of biosurfactant/L) followed by *L. rhamnosus*, *L. pentosus* and *L. casei*. Regarding the $Y_{P/S}$ all the *Lactobacillus* strains present the values between 0.08 g/g and 0.09 g/g. With regard to the regression parameters listed in Table 2, the most remarkable finding was that the product yields calculated for all strains was roughly the same, which means that all the four strains showed a similar behavior concerning biosurfactant production. The r_p/X values listed in Table 2 reflect the activity of the microorganisms concerning biosurfactant production. It can be seen that *L. rhamnosus* presents the highest r_p/X value (0.217 g/(L h)) followed by *L. casei*, *L. coryniformis* and *L. pentosus*.

Table 2

Results obtained by regression of biosurfactant, biomass and glucose concentration data in MRS broth fermentations[a]

Lactobacillus strains	Biosurfactant production						Biomass growth					Glucose consumption				
	P_0 (g/L)	P_{max} (g/L)	P_r (h^{-1})	r_p/X (g/(L h))	r^2	F value	X_0 (g/L)	X_{max} (g/L)	μ_{max} (h^{-1})	r^2	F value	S_0 (g/L)	$Y_{P/S}$ (g/g)	$Y_{X/S}$ (g/g)	r^2	F value
L. casei	0.8	1.6	0.612	0.091	0.993	286[b]	0.11	5.5	0.324	0.998	858[b]	26.7	0.08	0.34	0.955	43[c]
L. rhamnosus	0.8	1.7	1.215	0.217	0.969	63[c]	0.12	4.6	0.299	0.984	145[d]	28.4	0.09	0.25	0.939	31[c]
L. pentosus	0.9	1.7	0.506	0.069	0.891	16[e]	0.10	6.4	0.409	0.990	204[b]	24.5	0.09	0.41	0.977	86[c]
L. coryniformis	0.8	1.8	0.637	0.090	0.974	76[c]	0.18	5.9	0.107	0.907	19[f]	28.1	0.09	1.38	0.956	43[c]

[a] Parameters defined in the nomenclature.
[b] Significance level >99.5%.
[c] Significance level >97.5%.
[d] Significance level >99%.
[e] Significance level >90%.
[f] Significance level >95%.

3.5. *L. pentosus* fermentations in whey

The lowest value of surface tension was achieved in the stationary phase (45 mN/m) and the reduction in the surface tension exceeded 8 mN/m [2,6]. The surface tension decreases were compared with the surface tension of whey broth control (54 mN/m).

Fermentation was carried out using whey as culture broth for *L. pentosus* strain because this was the strain that showed best results concerning simultaneously cell growth and biosurfactant production. Nothing else besides whey was used for media preparation. Fig. 3 shows the experimental data as well as the predicted values calculated by Eqs. (2)–(4) using the regression parameters listed in Table 3. The experiment show a kinetic pattern reasonably described by the mathematical model with 0.959 and 0.990 for biomass and biosurfactant production, respectively. The r^2 value obtained for lactose consumption was not so good ($r^2 = 0.698$) and this could be explained by the fact that not all the lactose was consumed during the cell growth. Moreover, the lactose consumption at certain time of fermentation becomes constant while biomass growth is still increasing maybe due to the fact that *L. pentosus* metabolizes other medium ingredients rather than lactose, thus very high residual sugar content was left at the end of fermentation. It was achieved $P_{max} = 1.4$ g of biosurfactant/L, $P_r = 0.353$ h^{-1} and $r_p/X = 0.093$ g/(L h). Regarding the $Y_{P/S}$ the value obtained was 0.09 g/g and $X_{max} = 1.5$ g/L with a μ_{max} of 0.05 h^{-1}. From Fig. 3 it is
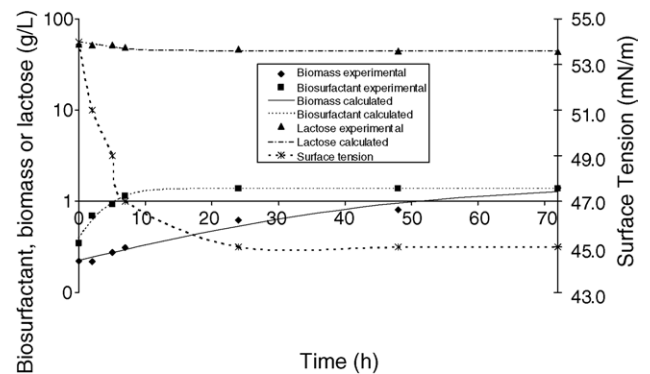

Fig. 3. Representation of the surface tension variation (- -*- -), experimental data and calculated time courses of biomass (♦, - - -), glucose (▲, — - —) and biosurfactant concentrations (■, —) during fermentations carried out with whey broth using *L. pentosus* CECT-4023. Results represent the average of three independent experiments.

possible to observe that this strain did not grow very well maybe because not all its nutritional requirements were fulfilled, although similar concentrations of biosurfactant were achieved if compared to those obtained with MRS medium. Comparing the kinetic parameters obtained with the two-tested medium, it was possible to notice that a lower μ_{max} (10% less than with synthetic medium) was obtained with whey medium, as well as a lower X_{max} (approximately one-third of the value obtained with synthetic medium).

Table 3

Results obtained by regression of biosurfactant, biomass and lactose concentration data for *L. pentosus* in whey broth fermentation[a]

Biosurfactant production						Biomass					Lactose consumption				
P_0 (g/L)	P_{max} (g/L)	P_r (h^{-1})	r_p/X (g/(L h))	r^2	F value	X_0 (g/L)	X_{max} (g/L)	μ_{max} (h^{-1})	r^2	F value	S_0 (g/L)	$Y_{P/S}$ (g/g)	$Y_{X/S}$ (g/g)	r^2	F value
0.4	1.4	0.353	0.093	0.990	195[b]	0.2	1.5	0.05	0.959	46[c]	55.6	0.09	3.1	0.698	5[d]

[a] Parameters defined in the nomenclature.
[b] Significance level >99%.
[c] Significance level >95%.
[d] Significance level >90%.

4. Discussion

The lactic acid bacteria *L. casei* CECT-5275, *L. rhamnosus* CECT-288, *L. pentosus* CECT-4023 and *L. coryniformis* subsp. *torquens* CECT-25600 were found to be biosurfactant-producing strains. Depending upon the nature of the biosurfactant and the producing microorganisms, several patterns of biosurfactant production by fermentation are possible [9]. In our study the biosurfactant production is occurring mainly in the first hours (4 h) where cell growth is almost inexistent and the substrate consumption is very low. However, for all strains the biosurfactant production continues during all 72 h of fermentation but at a very slow production rate. This slow production rate can be a consequence of product inhibition and pH reduction. The pH reduction results of simultaneous production of lactic acid that changes drastically the media conditions and can be responsible for the biosurfactant production inhibition. The lowest values of surface tension were obtained at the end of fermentation, therefore, our present observation that biosurfactant release by the selected lactobacilli strains is maximal for cells in the stationary cells is in accordance with the general notion on this point in the literature [2,3,9]. Four *Lactobacillus* strains were screened for biosurfactant production by surface tension determination, and the biosurfactant production as well as the time courses of glucose consumption and biomass growth modeled. The approach used in this study allowed the determination of the fermentation parameters as well as a way to deduce the biosurfactant extraction with PBS for the four strains studied. From the PBS extraction results it was found that the best biosurfactant production strain tested was *L. pentosus* allowing a surface tension reduction of 21.5 mN/m when compared to the PBS control (72 mN/m). Comparing results obtained in Table 1 is possible to conclude that with the accumulation of biosurfactant in the culture broth lower surface tensions are achieved and also for the extracellular biosurfactant results *L. pentosus* showed the higher reduction in the surface tension. The effectiveness of a surfactant is determined by its ability to lower the surface tension. For example, a good surfactant can lower the surface tension of water (air–water interface) from 72 mN/m to 35 mN/m [17]. In accordance with literature the biosurfactants produced by *Pseudomonas aeruginosa* and *Bacillus subtilis* lower the surface tension of water 29 mN/m and 27 mN/m, respectively [17].

For all four *Lactobacillus* strains, suitable models were found to describe the response of the experiments pertaining to glucose consumption, cell growth and biosurfactant production. The models were validated by comparing the observed and predicted values, and a deviation of about 5% was found. The modeling procedure allowed a better characterization of the biosurfactant production by the determination of the fermentation parameters and it was observed a reasonable fitting with a significance level over 90%. Additionally, the blood agar method was included in this study since it is widely used to screen for biosurfactant production,

and in some cases, it is the sole method used [14]. None of the studies reported in the literature [11–14] mention the possibility of biosurfactant production without a hemolytic activity. However, despite *Lactobacillus* species are not known to produce hemolysin, Andreu et al. [18] demonstrated that some species agglutinate blood cells. Furthermore, the biosurfactants obtained in this study were not purified, thus consist of a mixture of several compounds other than biosurfactants that may cause hemolysis. The hemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad [19] reported that the biosurfactant produced by *Bacillus subtilis*, surfactin, lysed red blood cells. Blood agar lysis has been used to quantify surfactin [11] and rhamnolipids [12] and has been used to screen for biosurfactant production by new isolates [13,14]. Carrillo et al. [13] found an association between hemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary method to screen for biosurfactant activity. However, only 13.5% of the hemolytic strains lowered the surface tension of water below 40 mN/m. In addition, other microbial products such as virulence factors lyse blood agar and biosurfactants that are poorly diffusible may not lyse blood cells. Thus, as not all biosurfactants have a hemolytic activity and compounds other than biosurfactants may cause hemolysis it is not clear whether blood agar lysis should be used exclusively to screen for biosurfactant production and surface tension can then be used to confirm the results if required.

Velraeds et al. [3] screened 15 *Lactobacillus* isolates for biosurfactant production and found that *Lactobacillus acidophilus* RC14 and *Lactobacillus fermentum* B54 were strongly biosurfactant-producing strains. Moreover, they found that biosurfactant layers of several *Lactobacillus* strains inhibited the adhesion of uropathogenic *Enterococcus faecalis* strain to glass in a parallel-plate flow chamber for 4 h, however the inhibition of the uropathogen by the several strains tested was not the same. This indicates that there are different aspects of adherence on the part of the pathogen and that it should not be expected that the products of different *Lactobacillus* strains would produce equivalent results for any given pathogen. Other biomedical applications of the biosurfactants were found in the literature, namely the use of biosurfactants obtained from *L. lactis* 53 and from *S. thermophilus* A to prevent the microbial colonization of silicone rubber voice prostheses [7,8]. In our study as the decrease in the surface tension exceeded 8 mN/m, all four strains were found to produce biosurfactants after reaching the stationary growth phase and from all the above can be used for further investigation. The reference surface tension value (8 mN/m) was previously described in the literature [6], and although it must be emphasized that the criterion of 8 mN/m is of course somewhat arbitrarily chosen and must not be considered as strict as suggested here, it represents twice the variation in surface tension of a suspension with a non-producing strain and is therefore "on the safe side".

Finally, *L. pentosus* was assayed for biosurfactant production using whey as the culture medium. Comparing with the

results obtained for this strain growing in MRS it was possible to see that *L. pentosus* did not grow well on whey medium probably due to some lack of nutrients, although similar biosurfactant concentrations were obtained, which means that with a culture medium optimization it could be possible to achieve higher biosurfactant concentrations. The lack of fit observed for the lactose consumption is probably caused by the first data point of experimental lactose that is the one which is worst fitted by the model, however the model has to be seen as a balance between biomass growth, lactose consumption and biosurfactant production, thus the model was considered adequate.

5. Conclusions

A model could be established to follow the biosurfactant production at any fermentation time for all the tested strains with a significance level over 90%. The results obtained for *L. pentosus* CECT-4023 showed that this is a strong biosurfactant producer strain and that whey can be used as an alternative medium for biosurfactant production.

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