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Improved Production of Live Cells of *Lactobacillus rhamnosus* by Continuous Cultivation using Glucose-yeast Extract Medium

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In this study, the growth kinetics of *Lactobacillus rhamnosus* and lactic acid production in continuous culture were assessed at a range of dilution rates (0.05 h^{-1} to 0.40 h^{-1}) using a 2 L stirred tank fermenter with a working volume of 600 ml. Unstructured models, predicated on the Monod and Luedeking-Piret equations, were employed to simulate the growth of the bacterium, glucose consumption, and lactic acid production at different dilution rates in continuous cultures. The maximum specific growth rate of *L. rhamnosus*, μ_{max}, was estimated at 0.40 h^{-1}, and the Monod cell growth saturation constant, K_s, at approximately 0.25 g/L. Maximum cell viability (1.3 × 10^{10} CFU/ml) was achieved in the dilution rate range of D = 0.28 h^{-1} to 0.35 h^{-1}. Both maximum viable cell yield and productivity were achieved at D = 0.35 h^{-1}. The continuous cultivation of *L. rhamnosus* at D = 0.35 h^{-1} resulted in substantial improvements in cell productivity, of 267% (viable cell count) that achieved via batch cultivation.

Keywords: probiotic, *Lactobacillus rhamnosus*, continuous culture, lactic acid, modeling

Several strains of *Lactobacillus rhamnosus* have been determined to exhibit probiotic values, and are commercially available. These lactic acid bacterial strains evidence protective activity against *Escherichia coli* infection (Saito *et al.*, 1980) and uropathogen growth on silicone rubber after incubation in urine (Velraeds *et al.*, 2000). Strains of *L. rhamnosus* are being increasingly applied in novel-type yoghurts (Schillinger, 1999). With the increasing popularity of probiotic products among consumers, large-scale techniques for the fermentation of lactic acid bacteria, including *Lactobacillus*, are gaining importance. Although traditional batch fermentation currently dominates the lactic acid bacterial fermentation industry (McCaskey *et al.*, 1994), a great deal of interest has been focused on the development of more rapid methods of using live cells to generate probiotics or lactic acid, most notably continuous culture methods. Continuous culture techniques allow for the provision of a steady state, and ensure that specific growth rate, as well as cell, product, and nutrient concentrations, remain constant over time. Continuous fermentation has been associated with reductions in processing time, the possibility of reducing equipment size, more uniformity in the products, and higher productivities, especially in cases in which the desired material generated is associated with biomass or growth. As such, a continuous process may appear potentially more efficient than the batch process, provided that difficulties in contamination and mutation, two primary problems inherent to the continuous cultivation of many microorganisms, can be circumvented (Maxon, 1955). Continuous cultivation has also proven quite useful for the purposes of kinetics studies, as steady state conditions are the optimal conditions for the determination of certain kinetic parameters (Williamson, 1975; Monroy and de la Torre, 1996).

In our previous work, efforts were made to improve the production of *L. rhamnosus* cells in batch cultivation, by optimizing the fermentation medium and parameters (Liew *et al.*, 2005). Such batch cultures proved capable of achieving a final cell concentration of 1.6 × 10^{10} CFU/ml within 12 h of fermentation. In this study, we have attempted to determine whether cell production could be further improved via continuous cultures, as continuous cultivation has been frequently associated with good productivity.

The primary objectives of this work were to

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evaluate the effects of different dilution rates on the growth and lactic acid production of *L. rhamnosus* cells, and to estimate growth kinetic parameter values, including the maximum specific growth rate, cell yield, and productivity, so as to establish a better understanding of the kinetic behavior of the bacterium when grown in continuous cultures. Such information may prove useful in evaluations of the potential use of continuous cultures for the production of *L. rhamnosus* cells.

Materials and Methods

Microorganism

The bacterium, *L. rhamnosus* (ATCC 7469), was employed throughout this study. The strain was stored at –80°C in 10% (v/v) glycerol (BDH Laboratory Supplies, England). For the preparation of inocula, the stock culture was subcultured for 16 h in a 1 L Erlenmeyer flask containing 200 ml de Mann, Rogosa, Sharpe (MRS) broth (Merck, Germany) at 37°C, in order to obtain an initial cell concentration of approximately 10^7 CFU/ml. In all fermentations, the inoculum consisted of 5% (v/v) of the culture.

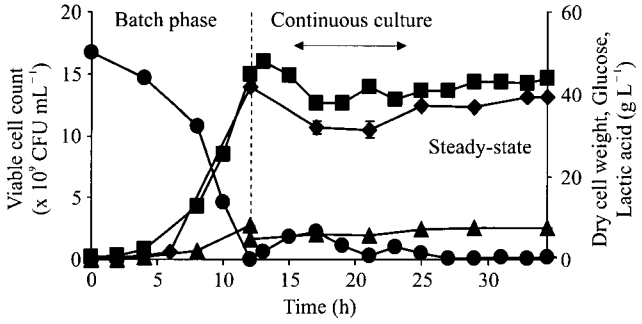

Fig. 1. Continuous culture of *L. rhamnosus* at D = 0.28 h^{-1}. Viable cell count (◆), dry cell weight (▲), glucose (●) and lactic acid (■). The vertical dotted line indicates the initiation of the continuous cultivation phase. For data points without error bars, the errors were smaller than the size of the symbols. Error bars indicate the mean ± standard deviation of two experiments.

Medium composition

The initial batch medium and fermentation conditions maintained during operation were identical to those previously optimized and reported (Liew *et al.*, 2005). The feed was similar to the initial medium in all aspects, except that the feed contained only 40 g/L glucose. The compositions of the initial batch and feed media are shown in Table 1.

Continuous fermentation procedures

Continuous fermentations were conducted using a 2 L stirred tank fermenter (Biostat, B. Braun Biotech International, Germany). The initial batch culture was started with a working volume (V) of 600 ml of optimized medium. The fermenter was sparged with nitrogen at 0.1 vvm prior to the commencement of fermentation, in order to promote an oxygen-free environment. Once the fermentation began, the gas flow was halted, and the fermentation was allowed to proceed without aeration. Temperature and pH were maintained at 37°C and 6.9, respectively. The pH was maintained via the automatic addition of 10 M NaOH. The fermenter was agitated at an impeller tip speed of 0.69 ms^{-1}. The fermenter was operated batchwise for 12 h, once the glucose had been depleted, and the feed medium was fed at an appropriate flow rate (F) into the fermenter in order to initiate the continuous culture. Continuous cultures were operated at a variety of dilution rates (0.05 to 0.4 h^{-1}). The dilution rate was calculated as D = F/V.

Each continuous culture was started with a new initial batch culture, then operated at the required dilution rate and halted only after a long-standing steady state had been achieved (continuous cultures were operated for at least 6 residence times = 6/D). During continuous fermentation, the volume of the culture was maintained at 600 ml, via a large diameter overflow tube connected to a peristaltic pump. This large tube diameter was necessary to prevent blockage.

Samples for analyses were withdrawn at two points, i.e. directly from the fermenter (at the sampling port) and also at the outflow tube located prior to the out-

Table 1. Composition of media used for continuous cultivation of *L. rhamnosus*

Medium	Ingredient (g/L)
Initial batch medium	Glucose, 50.1; yeast extract, 60; KH_2PO_4, 2.7; $MgSO_4.7H_2O$, 0.2; $MnSO_4.H_2O$, 0.05; Tween-80, 1 ml/L; vitamin solution, 12.8 ml/L. Vitamin solution (g/L): pyridoxine.HCl, 2.0; pantothenic acid, 1.0; niacin, 1.0; riboflavin, 1.0 and folic acid, 1.0.
Feed medium	Glucose, 40; yeast extract, 60; KH_2PO_4, 2.7; $MgSO_4.7H_2O$, 0.2; $MnSO_4.H_2O$, 0.05; Tween-80, 1 ml/L; vitamin solution, 12.8 ml/L. Vitamin solution (g/L): similar to that used in the initial medium described above.

flow reservoir. The results of our analyses of samples obtained from both the sampling port of the fermenter and the outflow tube were similar, suggesting that well-mixed conditions had been achieved in the fermenter.

Analytical procedure

The numbers of viable cells were expressed in colony-forming units (CFU). Serial decimal dilutions of each of the samples were plated in duplicate onto MRS agar plates. The plates were incubated for 48 h at 37°C prior to enumeration. Results of plate counting were expressed as colony forming units per ml. Residual glucose, lactic acid, and ethanol were determined with a glucose analyzer (Yellow Spring Instruments, USA), based on the enzymatic method with glucose oxidase, L-lactate oxidase, and alcohol oxidase, respectively.

Continuous fermenter model

A steady-state well-mixed continuous fermenter is operated at a constant inflow and outflow, and is assumed to be well-mixed, such that the concentrations of cells, substrate, and product are uniform throughout the medium volume. The volume, V, does not vary over time, such that $dV/dt = 0$. In such an operation, all environmental variables, such as temperature and pH, must remain constant. The model equations for continuous fermentation as suggested by Boonmee *et al.* (2003) were solved for steady state conditions when $dX/dt = 0$, $dS/dt = 0$, and $dP/dt = 0$. The models were predicated on the Luedeking-Piret model for growth-associated and non-growth-associated lactic acid production, which include terms for substrate (glucose) limitation, end-product (lactic acid) inhibition, and substrate (glucose) inhibition, as shown below:

$$dX/dt = \mu_{max}[S/(K_s + S)][1 - (P - P_i)/(P_m - P_{ix})][(K_i/(K_i + S)][X - DX] \quad (1)$$

$$dP/dt = \alpha(dX/dt) + q_{p.max}[S/(K_s + S)][1 - (P - P_i)/(P_m - P_i)] \times [(K_i/(K_i + S)][X - DP] \quad (2)$$

$$dS/dt = D(S_o - S) - q_{s.max}[S/(K_s + S)][1 - (P - P_i)/(P_m - P_i)] \times [K_i/(K_i + S)]X \quad (3)$$

Terms for all these model equations are fully defined in the Nomenclature.

Model fitting and statistical analysis

Analyses of variances for data were conducted using SAS (SAS Institute Inc., 1990). Duncan's multiple range tests were employed in order to determine significance among the treatment means. Maximum specific growth rate (μ_{max}) and the Monod cell growth saturation coefficient (K_s) were determined using Lineweaver-Burk, Langmuir, and Eadie-Hofstee plots.

Continuous fermenter models for cell production, substrate consumption, and lactic acid formation were proposed and tested via comparisons of the experimental data to the data calculated from those models. The kinetic models (Eqs. 1 to 3) were fitted to the experimental data via non-linear regression with a Marquadt algorithm, using MATLAB computer software. The model parameter values were initially evaluated by solving Eqs. 1 to 3, and the computer program was then employed as a search method to minimize the sum of squares of the differences between the predicted and measured values. The predicted values were then utilized to simulate the profiles of cell, substrate, and product concentrations during fermentation for the different dilution rates. The steady-state values determined for the cell, substrate, and product from the simulation exercise were then plotted against the dilution rate. The square of the correlation coefficient, r^2, and the F and P values were calculated, and the equations determined from the respective regressions were also considered in order to assess the goodness-of-fit. For all performed statistical analyses, significance was set at $P < 0.05$.

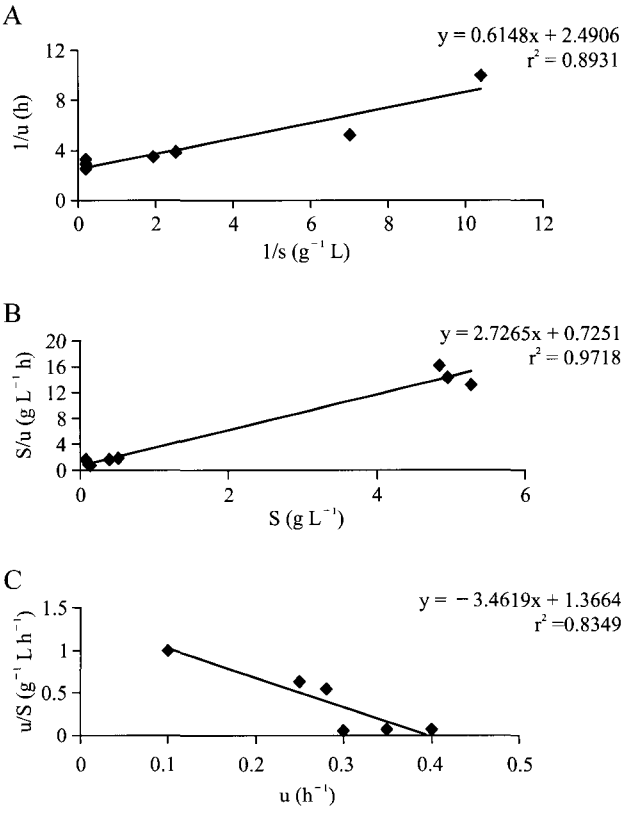

Fig. 2. Determination of μ_{max} and K_s for *L. rhamnosus* using (A) Lineweaver-Burk, (B) Langmuir and (C) Eadie-Hofstee plots.

Table 2. Comparison of cell and lactic acid production by *L. rhamnosus* in continuous cultures operated at different dilution rates

Kinetic parameter	Dilution rate, D (h^{-1})							
	0.05	0.10	0.19	0.25	0.28	0.30	0.35	0.40
Cell concentration	1.1^{c}	$1.2^{b,c}$	$1.2^{b,c}$	$1.2^{b,c}$	1.3^{a}	1.3^{a}	1.3^{a}	1.0^{d}
(× 10^{10} CFU/ml) (g/L)	5.86^{b}	7.10^{a}	7.11^{a}	7.58^{a}	7.51^{a}	7.61^{a}	7.49^{a}	6.95^{a}
Cell yield (× 10^{11}CFU/$g_{glucose}$)	2.9^{d}	$3.0^{c,d}$	2.8^{d}	$3.0^{c,d}$	$3.3^{b,c}$	3.4^{b}	3.7^{a}	3.0^{d}
(g_{DCW}/$g_{glucose}$)	0.15^{d}	0.18^{c}	0.18^{c}	$0.19^{b,c}$	$0.19^{b,c}$	0.22^{a}	0.22^{a}	$0.20^{a,b}$
Cell productivity (× 10^{11} CFU/L/h)	5.7^{h}	12.0^{g}	22.8^{f}	29.9^{e}	36.0^{d}	38.0^{c}	45.1^{a}	41.0^{b}
(g_{DCW}/L/h)	0.28^{h}	0.71^{g}	1.35^{f}	1.89^{e}	2.10^{d}	2.28^{c}	2.62^{b}	2.78^{a}
Lactic acid concentration (g/L)	26.38^{d}	31.44^{c}	37.73^{b}	42.16^{a}	43.09^{a}	43.73^{a}	44.98^{a}	31.85^{c}
Lactic acid yield ($g/g_{glucose}$)	0.66^{e}	0.79^{d}	0.95^{c}	1.07^{b}	1.08^{b}	1.24^{a}	1.28^{a}	0.92^{c}
Lactic acid productivity (g/L/h)	1.32^{g}	3.14^{f}	7.17^{e}	10.54^{d}	11.99^{c}	13.12^{b}	15.74^{a}	$12.74^{b,c}$
Fermentation efficiency ($g_{glucose\ utilized}$/(100g)$_{initial\ glucose}$)	99.84^{a}	99.81^{a}	99.72^{a}	99.17^{a}	98.96^{a}	90.29^{b}	90.08^{b}	89.44^{b}

* Mean values in the same row not followed by the same letter are significantly different ($P < 0.05$)

Results and Discussion

Kinetic analysis of growth under various dilution rates

Steady-states were achieved after three to six residence times at all the dilution rates investigated. Fig. 2 shows a typical time course of cell and lactic acid production, as well as glucose consumption, in a continuous culture. The homofermentative character of *L. rhamnosus* under the conditions established in this study was confirmed by the absence of ethanol and other organic acids other than lactic acid.

The steady-state values for biomass, lactic acid, and total glucose concentration for each dilution rate are shown in Table 2. By scanning the entire range of dilution rates tested, we noticed that maximum viable cell production (1.3×10^{10} CFU/ml) was achieved at D = 0.28 to 0.35 h^{-1}. The lowest cell viability was obtained at D = 0.40 h^{-1}. Maximum lactic acid concentration (approximately 42 to 45 g/L) was achieved at dilutions ranging from D = 0.25 to 0.35 h^{-1}. Residual glucose was observed only in cultures with dilution rates of 0.30 h^{-1} and above. The presences of excess sugars in higher dilution rate cultures, as well as their complete utilization at lower dilution rates, have been reported in the cultivation of lactobacilli on glucose (Major and Bull, 1989). Reductions in cell viability and lactic acid concentration were detected when the dilution rate was increased from D = 0.35 to D = 0.40 h^{-1}, thereby indicating that the maximum specific growth rate may be in the vicinity of 0.40 h^{-1}. Therefore, another continuous culture was conducted at D = 0.50 h^{-1} in which cell and lactic acid washout was observed (data not shown). Thus, no further attempts were made to conduct continuous cultures at dilution rates in excess of D = 0.40 h^{-1}.

Table 3. The values of μ_{max} and K_s as calculated from Lineweaver-Burk, Langmuir and Eadie-Hofstee plots

Parameter value	Lineweaver-Burk	Langmuir	Eadie-Hofstee
μ_{max} (h^{-1})	0.401	0.367	0.395
K_s (g/L)	0.247	0.266	0.289

The effects of dilution rate on yield and productivity are also shown in Table 2. Maximum viable cell yield was achieved at D = 0.35 h^{-1}. Viable cell productivity increased directly with dilution rate until D = 0.35 h^{-1}, after which productivity decreased when the dilution rate increased to D = 0.40 h^{-1}. Cell productivity (dry cell weight) increased with increasing dilution rates until D = 0.40 h^{-1}. Maximum lactic acid yield was achieved at D = 0.30 h^{-1} and 0.35 h^{-1}, while lactic acid productivity increased with increasing dilution rates until D = 0.35 h^{-1}. However, at this dilution rate, the residual glucose concentration exceeded 4 g/L. This result was not considered to be unusual, as there have been several reports of high productivity coupled with incomplete use of substrate (Goksungur and Guvenc, 1997).

Determination of K_s and μ_{max} values

Fig. 2 shows the Lineweaver-Burk, Langmuir, and

Table 4. Comparison of experimental and calculated data for continuous cultivation of *L. rhamnosus* using linear regression

Kinetic parameter	Equation[a]	Linear regression analysis		
		r^2	F-value	P-value
Viable cell count	$X_{exp} = -2.22\ X_{calc} + 4 \times 10^{13}$	0.52	5.37	5.37
Dry cell weight	$X_{exp} = -3.04\ X_{calc} + 29.88$	0.28	1.95	0.068
Lactic acid production	$P_{exp} = -5.37\ P_{calc} + 216.32$	0.93	71.31	0.0004
Glucose consumption	$S_{exp} = 0.86\ S_{calc} + 0.02$	0.96	112.92	0.0001

* X_{exp} and X_{calc}, experimental and calculated values, respectively.
Linear regression analyses conducted on data from continuous cultures at dilution less than 0.30 h^{-1}.

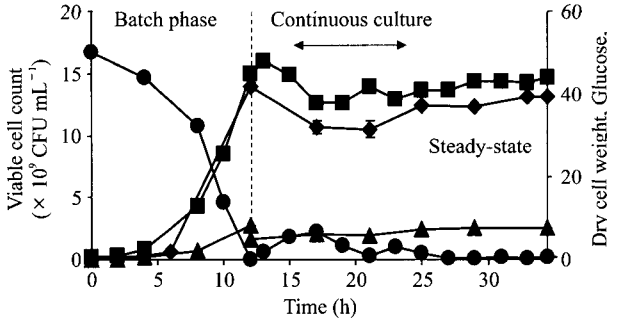

Fig. 3. Comparison of calculated and experimental cell growth data for continuous cultures of *L. rhamnosus*. Viable cell count (◆), dry cell weight (▲). Dotted (viable cell count) and solid (dry cell weight) lines represent data according to Equation 1.

Eadie-Hosftee plots used to determine the maximum specific growth rate (μ_{max}) and Monod cell growth saturation constant (K_s). Table 3 shows the μ_{max} and K_s values obtained from these three plots. The values obtained from these different plots are in good agreement with one another. Finally, the maximum specific growth rate, μ_{max}, was estimated as 0.40 h^{-1} and K_s as 0.25 g/L. The μ_{max} value obtained in this study is in fairly good agreement with that obtained by Berry *et al.* (1999), who determined the μ_{max} value for *L. rhamnosus* cultivation in batch cultures as 0.49 h^{-1}.

Testing of proposed models

Table 4 and Fig. 3 compare the experimental and calculated biomass production data from *L. rhamnosus* continuous fermentation, via linear regression. The simulation curve for the viable cell count (Equation 1) did not fit well with the experimental data at D = 0.05 h^{-1} and dilution rates above D = 0.30 h^{-1}. The simulation curve for dry cell weight showed unfavorable fits of the experimental and calculated data at dilution rates lower than D = 0.25 h^{-1} and higher than D = 0.35 h^{-1}.

Thus, the equations obtained from the linear regression analysis of the experimental and calculated cell viability and dry cell weight production data (Table 4) showed that the proposed model for cell growth was not sufficient to describe the growth of *L. rhamnosus* in continuous cultures at low and high dilution rates. At lower dilution rates (below D = 0.10 h^{-1} and D = 0.25 h^{-1} for viable cell count and dry cell weight, respectively), lower cell viabilities and biomass were observed, as compared to the predicted values. This discrepancy showed that, in a long-running continuous culture of bacteria operated at a low dilution rate, a considerable proportion of cells might be either non-viable or dead. The parameter values for the kinetic model of continuous cultivation of *L. rhamnosus* at different dilution rates are shown in Table 5. The μ_{max} values estimated from these models were found to be consistent with the values calculated using the Lineweaver-Burk, Langmuir and Eadiee-Hofstee plots; however, the values of the Monod saturation constant (K_s) were slightly higher. The values of the growth-associated constant (α) and non-growth-associated constant (β) were not significantly different at different dilution rates.

Several researchers have reported lower cell viabilities at low dilution rates (Sinclair and Topiwala, 1970; Baretto *et al.*, 1991). Barreto *et al.* (1991)

Table 5. Predicted parameter values for kinetic model of continuous cultivation of *L. rhamnosus* operated at different dilution rates

Parameter values	D (h^{-1})			
	0.10	0.25	0.30	0.40
μ_{max} (h^{-1})	0.42	0.41	0.39	0.45
K_s (g/L)	0.30	0.42	0.35	0.28
K_i (g/L)	296	103	138	200
P_i (g/L)	1.65	1.70	1.82	1.66
P_m (g/L)	32	35	40	35
$q_{s.max}$ (g/g/h)	3.6	3.7	2.9	2.8
α (g/g)	0.35	0.34	0.33	0.35
$q_{p.max}$ (=β) (g/g/h)	3.64	3.79	3.59	3.89

explained that although at lower dilution rates, cells are not exposed to sudden changes in the environment, including heat, pH changes or radiation, some cells may still die. Death and loss of viability can be caused by other processes such as age, lack of essential nutrients, or even involuntary autolysin activity, as was suggested by Mason *et al.* (1986). Therefore, other components such as cell age and cell death rate at low dilution rates might also require consideration in the model, in order to avoid such discrepancies in prediction.

During continuous cultivation, lactic acid was the main product formed, as high performance liquid chromatographic analysis of the culture broth detected the presence of no ethanol or any organic acids other than lactic acid. This observation indicates that *L. rhamnosus* did not alter its metabolic pathway in continuous cultures. As can be seen in Fig. 4, the proposed model for lactic acid production in continuous cultures was not in good agreement with the experimental values. Table 4 summarizes the relationships between the experimental and calculated data for lactic acid production and glucose consumption. From the r^2 and the F and P values obtained, it could be concluded that a relationship existed between the calculated and the experimental data, but the slope and intercept values obtained from linear regression analysis indicated that the model was not able to represent the experimental data accurately. This is because the slope had a large negative value, and the intercept value was also high. As can be observed from Equation 3, the calculated lactic acid values are associated with the predicted biomass concentration. Therefore, if the predicted growth curve did not perfectly match the experimental growth data, as was unfortunately observed at lower and higher dilution rates in this study, a less reliable predicted lactic acid concentration curve would be obtained. As only lactic acid was generated during the continuous cultures, the Luedeking-Piret model was an expected kinetic representation of product formation (Luedeking and Piret, 1959). However, Equation 3, despite its association with the Luedeking-Piret model, was not a suitable model for the representation of lactic acid production in continuous cultures. This indicated that greater model complexity would be required in order to reflect lactic acid production accurately. Variables that influenced cell growth and product formation, such as cell age, might require inclusion in the model, in order to achieve better predictive capabilities.

The calculated glucose concentration values were almost identical to the experimental values in continuous cultures operated at dilution rates below 0.30 (Fig. 4). In this dilution range, the equation describing the relationship between the calculated and experimental glucose concentrations evidenced a slope approximating 1 and a small intercept value of 0.02. Moreover, the square of the correlation coefficient, r^2, and the F value indicated good fits of the data (Table 4). However, once the dilution rate exceeded 0.30 h^{-1}, the proposed model no longer accurately predicted the experimental data.

This limitation of the model may be attributable to the fact that at dilution rates in the close vicinity of μ_{max}, glucose accumulation was higher than was predicted by the model. Goncalves *et al.* (1991) have also reported a good fit between the theoretical and experimental glucose consumption data from *L. delbrueckii* continuous cultures in the range of dilution rates tested here; nevertheless, as the washout dilution rate approached, the model no longer correlated well with the experimental data. The presence of excessive residual glucose at higher dilution rate cultures has also been reported by Major and Bull (1989) from the cultivation of *L. delbrueckii* on glucose. At the higher dilution rates used in this study, lactic acid and biomass productivities were generally in excess of those obtained at lower dilution rates. Goksungur and Guvenc (1997) have reported that it is not unusual for high productivity to be coupled with incomplete use of substrates.

Comparison of continuous and batch cultivations

Table 6 lists the data from batch and continuous fermentations of *L. rhamnosus,* as well as the results obtained by Goksungur and Guvenc (1997) in their study of *L. delbrueckii* IFO 3202 fermentation using pretreated beet molasses. In the present study (continuous cultures), slightly lower cell concentration was obtained as compared to the batch cultures, but this weakness was offset by the good cell productivity. A substantial improvement of 267% (viable cell count) and 341% (dry cell weight) in cell productivity was observed in continuous cultures operated at D =

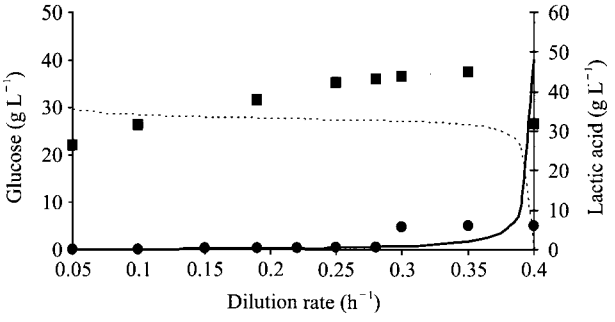

Fig. 4. Comparison of calculated and experimental data for continuous cultures of *L. rhamnosus*. Glucose (●) and lactic acid (■). Dotted (lactic acid) and solid (glucose) lines represent data according to Equations 2 and 3, respectively.

Table 6. Comparison of batch and continuous cultures for cell and lactic acid productions by *L. rhamnosus* and *L. delbrueckii*

Kinetic parameters	*L. rhamnosus* (data from this study)		*L. delbrueckii* (Goksungur and Guvenc, 1997)	
	Batch culture	Continuous culture	Batch culture	Continuous culture
Maximum cell concentration ($\times 10^{10}$ CFU/ml) (g/L)	1.5[a] 8.98[a]	1.3[b,A] 7.61[b,B]	7.61	5.65
Maximum cell yield ($\times 10^{11}$ CFU/$g_{glucose}$) ($g_{DCW}/g_{glucose}$)	2.9[b] 0.18[a]	3.7[a,C] 0.22[a,D]	–	–
Maximum cell productivity ($\times 10^{12}$ CFU/L/h) (g_{DCW}/L/h)	1.3[b] 0.63[b]	4.5[a,C] 2.78[a,E]	0.63	1.76
Maximum lactic acid concentration (g/L)	39.45[b]	44.98[a,C]	60.3	41.5
Maximum lactic acid yield ($g/g_{glucose}$)	0.78[b]	1.28[a,C]	0.77	–
Maximum lactic acid productivity (g/L/h)	3.25[b]	15.74[a,C]	4.83	11.20
Maximum fermentation efficiency ($g_{glucose\ utilized}/(100g)_{initial\ glucose}$)	100[a]	100[a,F]	–	–

Mean values in the same row not followed by the same letter in lower case are significantly different ($P < 0.05$).
Superscripts in capital letters refer to continuous cultures operated :
[A] At dilution rate 0.28, 0.30 and 0.35 h^{-1}
[B] At dilution rate 0.30 h^{-1}
[C] At dilution rate 0.35 h^{-1}
[D] At dilution rate 0.30 and 0.35 h^{-1}
[E] At dilution rate 0.40 h^{-1}
[F] At dilution rate 0.05, 0.10 and 0.19 h^{-1}
Batch cultures data are obtained from *L. rhamnosus* fermentation using 10 L of the optimized medium as shown in Table 1.

0.35 h^{-1} and 0.40 h^{-1}, respectively, over the batch cultures. Parallel with this improvement in cell productivity, continuous cultures of *L. rhamnosus* also exhibited higher lactic acid productivity as compared to the batch cultures. The maximum cell viability and dry cell weight from the continuous cultures were obtained in the dilution rate range of = 0.28 h^{-1} to 0.35 h^{-1} and these values were lower than those obtained in the batch cultures. This could be explained by the slightly higher concentrations of lactic acid in the continuous cultures, which may have contributed to a stronger inhibitory effect. The yield of viable cells relative to glucose observed in continuous cultures operated at D = 0.35 h^{-1} was superior to that attained in the batch cultures. It was noted that a stable continuous culture could be maintained at dilution rates close to the maximum specific growth rate of *L. rhamnosus* (0.40 h^{-1}), as steady-states in cell and lactic acid concentrations could be achieved. The only drawback was a slightly lower cell viability (1.3×10^{10} CFU/ml) and incomplete utilization of glucose, but this weakness was offset by the vast improvement in cell productivity.

Generally, the results obtained in this study are in good agreement with those of Goksungur and Guvenc (1997) who studied batch and continuous cultures of *L. delbrueckii* IFO 3202. Their batch cultures were operated at 45°C, pH 6.0 using pretreated beet molasses (equivalent to 78.20 g/L initial sugar concentration), while continuous cultures were operated at dilution rates between 0.10 and 0.70 h^{-1} using 52.10 g/L sugar from pretreated beet molasses. Similar to that observed in the present study, their cell concentration as well as biomass and lactic acid productivities in continuous cultures were lower when compared to batch cultures, while sugar accumulation was also observed at higher dilution rates.

The dilution rate clearly affected all important fermentation parameters in the continuous cultivation of *L. rhamnosus*. Maximum cell viability (1.3×10^{10} CFU/ml) was achieved at a dilution rate ranging from D = 0.28 h^{-1} to 0.35 h^{-1}. Both maximum viable cell yield and productivity were achieved at D = 0.35 h^{-1}. Continuous cultivation of *L. rhamnosus* at D = 0.35 h^{-1} yielded a substantial improvement in cell productivity, of 267% (viable cell count) as compared to the batch cultivations. From the continuous culture data, the maximum specific growth rate (μ_{max}) of *L.*

rhamnosus was estimated at 0.40 h^{-1} and the K_s at approximately 0.25 g/L.

Nomenclature

D dilution rate (flow rate/volume) (h^{-1})
K_i lactic acid inhibition constant (g/L)
K_s Monod cell growth saturation constant (g/L)
P lactic acid concentration (g/L)
P_i residual lactic acid concentration (g/L)
P_m maximum lactic acid concentration (g/L)
$q_{p.max}$ maximum specific lactic acid production rate (g/g/h)
$q_{s.max}$ maximum specific glucose utilization rate (g/g/h)
S glucose concentration (g/L)
S_o initial glucose concentration (g/L)
X cell concentration (g/L)
α growth associated constant (g/g)
B non-growth associated constant (g/g/h)
μ specific growth rate (h^{-1})
μ_{max} maximum specific growth rate (h^{-1})

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