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Improved growth model for two-stage continuous cultures of *Lactobacillus helveticus*

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Abstract An unstructured model for growth and lactic acid production during two-stage continuous cultures of *Lactobacillus helveticus* was previously developed. The Verlhust model was considered to describe growth kinetics. Production models was based on modified Luedeking-Piret expressions involving an inhibitory effect for the first stage (seed culture) and a nutritional limitation effect for the second stage (culture). To account for the decrease of the biomass concentration observed in the second stage, the dilution rate D_c was replaced by an exponential term of the dilution rate $\alpha.\exp\left(D_c/\beta\right)$ in the growth and product relations. Contrarily to the previous model, the important decrease of the biomass concentration observed at steady state in the second stage at high dilution rates, namely close to wash out, was correctly described by the new model. It also proved to satisfactory describes production data and volumetric productivity.

Keywords: continuous culture, growth, lactic acid fermentation, substrate limitation, unstructured models

INTRODUCTION

A great interest was reserved for the lactic acid production in these last years owing to its wide range of applications (John et al. 2007). Nowadays, the fermentative production of lactic acid is the world's leading technology (about 90% of world production). To increase the efficiency of the lactic acid fermentation processes, various modes of culture have been investigated (Lin and Wang, 2007; Nandasana and Kumar, 2008). Among them, batch culture remains the most commonly used approach in industrial lactic acid production. However, volumetric productivities are low due to the end-product inhibition (Amrane and Prigent, 1996; Kwon et al. 2001). Continuous processes appear therefore as a useful alternative (Amrane and Prigent, 1996; Lin and Wang, 2007). However, volumetric productivities reported for usual

continuous cultures remain low (Lin and Wang, 2007); while the efficiency of continuous two-stage bioreactors was demonstrated (Amrane and Prigent, 1996; Schepers et al. 2006). Several structured and unstructured (Nielsen et al. 1991; Burgos-Rubio et al. 2000; Boonmee et al. 2003; Bâati et al. 2004) models are available. Owing to their simplicity and their accuracy in the description of lactic acid fermentation, simple unstructured models were preferred in this work. Several ones are available in the literature, dealing with batch or continuous cultures of lactic acid bacteria (Luedeking and Piret, 1959; Richter and Nottelmann, 2004; Schepers et al. 2006; Balannec et al. 2007). However, there is a lack of models dealing with two-stage reactors for lactic acid fermentation (Kwon et al. 2001; Lin and Wang, 2007; Bouguettoucha et al. 2008).

Some unstructured models were previously developed in the laboratory. To account for the effect of the undissociated lactic acid (and pH), the main inhibitory specie (Kashket, 1987; Gätje and Gottschalk, 1991), an inhibitory term was introduced in the Luedeking and Piret expression (Balannec et al. 2007); while in the case of substrate limitations, an additional term was added to the Luedeking and Piret expression to account for cessation of lactic acid production when carbon became limiting (Amrane, 2001; Bouguettoucha et al. 2007). Both expressions were also merged to develop a generalised model involving a unique expression taking into account both effects (Bouguettoucha et al. 2007; Bouguettoucha et al. 2008). It was successfully applied to two-stage continuous cultures, which involves an inhibitory effect for the first stage (seed culture) and a nutritional limitation effect for the second stage (culture) (Bouguettoucha et al. 2009). The Verlhust model which proved to be relevant to describe growth kinetics (Pandey et al. 2000; Lan et al. 2006; Vázquez and Murado, 2008) was considered (Balannec et al. 2007; Bouguettoucha et al. 2007). These models showed interesting predictive potential to describe continuous two-stage culture of *L. helveticus* (Bouguettoucha et al. 2009), since rather reliable predictive calculated values were recorded for both stages by considering the parameter values obtained from the fitting of batch culture data carried out in similar conditions. However, the model did not account for the important decrease of the biomass concentration recorded at steady state in the second stage at high dilution rate, namely close to wash out (Amrane and Prigent, 1996). In the expression for the mass balance in the second stage, an additional term was therefore added to the dilution rate in the second stage. Validation of this modified model was the aim of the present work.

MATERIALS AND METHODS

Microorganism

Lactobacillus helveticus strain *milano* used throughout this work was kindly supplied by Dr. A. Fur (Even Ltd, Ploudaniel, France). Stock cultures were maintained on 10% (w v^{-1}) skim milk and deep-frozen at -16ºC. As required, these cultures were thawed and reactivated by two transfers in 10% (w v^{-1}) skim milk (42ºC, 24 hrs).

Media

Whey based media contained 48 g L^{-1} lactose, reconstituted from 67 g L^{-1} sweet cheese whey powder (Even Ltd). For culture medium preparation and just before use, whey proteins were hydrolysed by means of 0.8 g L^{-1} *Bacillus subtilis* endroprotease

B500 (Gist-Brocades, Séclin, France) at 50ºC and pH = 7.20 for 7 hrs (Amrane and Prigent, 1996). No supplementation was added to the culture medium.

The following supplementation was added to reconstituted whey for the preparation of seed culture medium (gr L^{-1}): yeast extract, 20; trypsic casein peptone, 5; pancreatic casein peptone, 5 (all from Biokar, Pantin, France); Tween 80, 1 (Merk, Darmstadt, Germany).

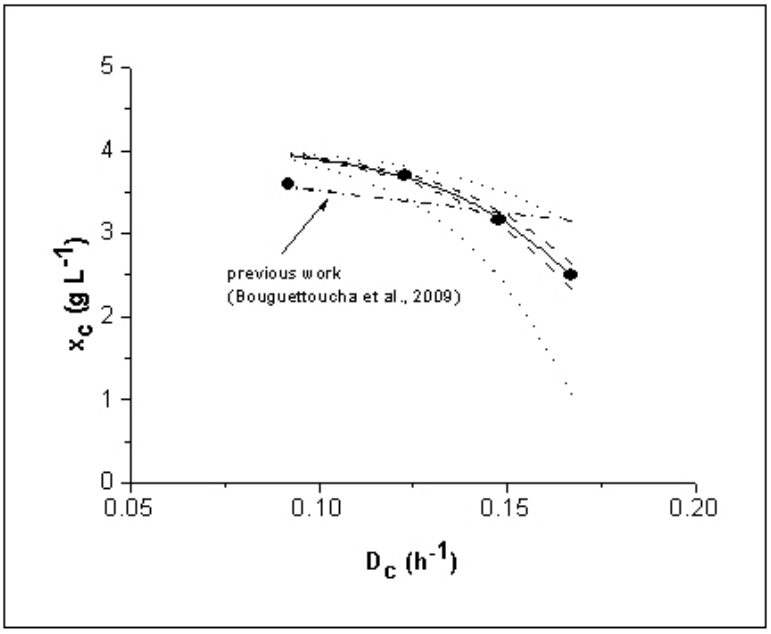

Fig. 1 Biomass concentrations at steady-state in the second stage of the system: experimental (symbols) and calculated (continuous lines) by means of the previous growth model (Bouguettoucha et al. 2009) (Equation 8) (dash dot) and by means of the modified growth model (Equation 9) (solid line). Parametric sensitivity of the modified growth model (Equation 9) upon parameters α (a) and β (b). Experimental (symbols) and calculated biomass data at steady-state in the second stage of the system by considering the optimal parameter values (continuous line), and by considering +10 (dash line) and -10 (dot line) % variation of the considered parameter. Values collected in Table 1 were considered for the other parameters.

Culture conditions

A schematic description of the system can be found in a previous paper (Bouguettoucha et al. 2009). Cultures were carried out in a 2 L reactor (Set 2M, SGI, Toulouse, France, magnetically stirred (300 rpm) at 42ºC. pH was controlled at 5.9 by automatic addition of 10 mol L^{-1} NaOH. In addition, the system was equipped with an aseptic recirculation loop (Watson-Marlow 101 FD/R peristaltic pump, Volumax, Montlouis, France) involving a turbidimetric measurement at 600 nm. The amount of NaOH added for pH control and turbidity were continuously recorded, allowing on-line calculation of lactic acid and total biomass concentrations, after suitable calibrations.

Reaction mixture overflowing the first stage and sterile culture medium were fed to the second stage through a peristaltic pump (Watson-Marlow 502U, Volumax, and PAP, SGI, respectively). The second stage was maintained at constant total mass by means of electronic weighing system (382MP8, Sartorius, Palaiseau, France) acting on a solenoid pinch valve (EG2, Sirai, Bioblock, Illkirch, France) in the bleed pipe.

200 mL of sterile seed culture medium were fed in the first-stage reactor (250 mL glass reactor) and inoculated with 1% (v v^{-1}) reactivated skim milk culture. At the end of the exponential growth phase, 120 mL of seed culture were aseptically transferred into the culture reactor containing 680 mL sterile culture medium. The first stage was continuously fed (F_i = 10 mL h^{-1}) with sterile seed culture medium and operated at a constant volume V_i = 120 mL. The mean residence time in the first stage was therefore set to 12 hrs (D_i = 0.083 h^{-1}), allowing to avoid large fluctuations of biomass concentrations, due to seed culture conditions close to wash out conditions (Amrane and Prigent, 1996). After 4-5 hrs, exponential growth took place in the second-stage reactor; then it was continuously fed at constant flow rate with both reaction mixture overflowing the first stage and sterile culture medium, at constant volume (V_c = 800 mL). Steady state for the second-stage reactor was achieved when both turbidity and NaOH addition rate (pH control) remained constant over a period of at least three mean residence times.

As required, the mean residence time in the second-stage was modified by varying the sole feed flow rate of sterile culture medium F_0 at constant culture volume V_c = 800 mL.

Table 1. Growth and production parameter values considered in both stages for calculations.

s_0 [a]	V_i [a]	D_i [a]	$\overline{x_i}$ [b]	x_{max_i} [c]	μ_{max_i} [c]	$\overline{p_i}$ [b]	p_0 [d]	V_c [a]	x_{max_c} [c]	μ_{max_c} [c]	pH_c [a]	$Y_{P/S}$ [d]	s_{lim} [d]	$[HL]_{inh}$ [e]
(g L^{-1})	(L)	(h^{-1})	(g L^{-1})	(g L^{-1})	(h^{-1})	(g L^{-1})	(g L^{-1})	(L)	(g L^{-1})	(h^{-1})	(-)	(-)	(g L^{-1})	(g L^{-1})
48	**0.12**	**0.083**	**1.4**	**1.54**	**0.68**	**8.0**	**0**	**0.80**	**4.7**	**0.72**	**5.9**	**0.9**	**3**	**8.5**

a) the parameter values are given in Materials and Methods.
b) calculated values for biomass and product concentrations at steady state in the first stage (Bouguettoucha et al. 2009).
c) the parameter values were taken from previous batch cultures of *L. helveticus* carried out on the same medium in similar physico-chemical conditions (Bouguettoucha et al. 2009).
d) the initial lactic acid concentration was neglected.
e) the inhibitory concentration of undissociated lactic acid (Bouguettoucha et al. 2008).

Analytical methods

Just after inoculation and at steady state sterile media and reaction mixtures were assayed for total biomass, lactose and lactic acid as previously described (Amrane, 2005). Four samples aseptically harvested during each run were also tested in order to check the lactic acid and total biomass concentration calculated on-line: the observed standard deviations were ± 1 and ± 0.2 gr L^{-1}, respectively.

Numerical methods

The Excel solver was used for the resolution of the considered equations and the parameters optimisation.

The following definition has been used for the determination of the residual standard deviation *RSD*:

$$RSD = \sqrt{\frac{\sum_{i=1}^{N}\left[Y_{i,\exp} - Y_{i,calc}\right]^2}{n-q}}$$

[Equation 1]

Y corresponding to the cellular *x* or the lactic acid *p* concentrations, *n* the number of experimental data points and *q* the number of parameters.

Theoretical aspects

Only the second stage was considered in this work, since the dilution rate in the first stage was maintained at a constant value.

Overall mass balance

The following assumptions were assumed:

(i) The fermentation process was carried out in continuous stirred-tank reactors (CSTR),
(ii) Sterile feeding for each stage ($x_0 = 0$),
(iii) At steady state conditions, there was no variation with time of biomass and product concentrations ($\frac{dx}{dt} = 0 ; \frac{dp}{dt} = 0$).

Mass balance can be expressed as follows:

For growth

$$\frac{dx_c}{dt} = D_i x_i \frac{V_i}{V_c} + \left(\mu_c - D_c\right)x_c$$

[Equation 2]

Where $D_c = \frac{F_c}{V_c}$ was the dilution rate in the second stage (h^{-1}).

At steady state conditions $\left(\frac{dx_C}{dt} = 0\right)$, Equation 2 became:

$$\frac{V_i}{V_c} D_i \bar{x}_i + \left(\mu_c - D_c\right)\bar{x}_c = 0$$

[Equation 3]

For production

$$\frac{dp}{dt} = \frac{V_i}{V_c} D_i \bar{p}_i - D_c \bar{p}_c + q_{p_c} \bar{x}_c$$

[Equation 4]

At steady state conditions $\frac{dp}{dt} = 0$, and Equation 4 can be therefore written as follows:

$$\frac{V_i}{V_c} D_i \bar{p}_i - D_c \bar{p}_c + q_{p_c} \bar{x}_c = 0$$

[Equation 5]

Where V_i and V_c were the volumes of the seed culture and the culture reactors, respectively. $\bar{x}_i, \bar{p}_i, \bar{x}_c, \bar{p}_c$ were biomass and lactic acid concentrations at steady state in the seed culture and the culture reactors, respectively; and q_{p_c} was the specific production rate in the second stage.

The above equation (Equation 5) can be rearranged as follows:

$$\bar{p}_c = \left[\frac{V_i}{V_c} D_i \bar{p}_i + q_{p_c} \bar{x}_c\right] \frac{1}{D_c}$$

[Equation 6]

Model development

The Verlhust model which proved to describe satisfactory growth kinetics (Pandey et al. 2000; Vázquez and Murado, 2008) was considered (Balannec et al. 2007; Bouguettoucha et al. 2007; Bouguettoucha et al. 2008):

$$\mu = \mu_{max}\left(1 - \frac{x}{x_{max}}\right)$$

[Equation 7]

Where x_{max} was the maximum biomass concentration and μ_{max} was the maximum specific growth rate.

Growth model

Introduction of the mass balance for biomass in the second stage (Equation 3) into the Verlhust model (Equation 7) led to the following implicit equation of $\bar{x}_c$:

$$\frac{V_i}{V_c}\bar{x}_i D_i + \left[\frac{\mu_{max_i}}{x_{max_i}}\left(x_{\max_i} - \bar{x}_c\right) - D_c\right]\bar{x}_c = 0$$

[Equation 8]

As previously observed (Bouguettoucha et al. 2009), the growth model (Equation 8) did not account for the important decrease of the biomass concentration at high dilution rate, namely close to wash out (Amrane and Prigent, 1996). From this, Equation 8 was modified by replacing the dilution rate in the second stage D_c by an exponential expression involving two additional parameters α and β, $\alpha.\exp\left(D_c/\beta\right)$. Hence, it came for growth:

$$\frac{V_i}{V_c}\bar{x}_i D_i + \left[\frac{\mu_{max_i}}{x_{max_i}}\left(x_{max_i} - \bar{x}_c\right) - \alpha exp^{D_c/\beta}\right]\bar{x}_c = 0$$

[Equation 9]

Production models

Substrate limitation model (SLM)

In order to take into account the carbon limitation recorded during culture at controlled pH, the Luedeking-Piret expression was previously modified (Bouguettoucha et al. 2007):

$$q_p = A\mu + B\left(1 - \frac{s_{lim}}{s}\right)$$

[Equation 10]

s_{lim} corresponded to the limiting lactose concentration 3 g L^{-1}.

By introducing the Verlhust expression (Equation 7) into the above modified Luedeking-Piret relation (Equation 10) and by considering a constant product on substrate yield $Y_{P/S}$, the specific production rate in the second stage q_{p_c} can be deduced (Bouguettoucha et al. 2009):

$$q_{p_c} = A_c \frac{\mu_{max_c}}{x_{max_c}}(x_{max_c} - \bar{x}_c) + B_c\left(1 - \frac{s_{lim}\, Y_{P/S}}{s_0\, Y_{P/S} - \bar{p}_c + p_0}\right)$$

[Equation 11]

Then, from the mass balance for the product in the second stage (Equation 7), the lactic acid concentration at steady state in the second stage $\bar{p}_c$ can be drawn (Bouguettoucha et al. 2009):

$$\bar{p}_c = \left[\frac{V_i}{V_c} D_i \bar{p}_i + \left(A_c \frac{\mu_{max_c}}{x_{max_c}}(x_{max_c} - \bar{x}_c) + B_c\left(1 - \frac{s_{lim}\, Y_{P/S}}{s_0\, Y_{P/S} - \bar{p}_c + p_0}\right)\right)\bar{x}_c\right] \frac{1}{\alpha \exp\left(D_c/\beta\right)}$$

[Equation 12]

Generalised model (GM)

To avoid the use of two expressions to describe production rate, depending on culture conditions, an unique expression taking into account both an inhibitory effect and a nutritional limitation effect was considered (Bouguettoucha et al. 2007 and Bouguettoucha et al. 2008):

$$q_p = A * \mu + B * \left(1 - \frac{s_{lim}}{s}\right) * \left(1 - \frac{[HL]}{[HL]_{inh}}\right)$$

[Equation 13]

Where $[HL]$ and $[HL]_{inh}$ were the undissociated lactic acid concentration and its inhibitory concentration, namely 8.5 g L^{-1} (Bouguettoucha et al. 2008). The concentration of the undissociated form of lactic acid [*HL*] was given by the Henderson-Hasselbach equation:

$$[HL] = \frac{p}{1 + 10^{\mathrm{pH} - \mathrm{pK_A}}}$$

[Equation 14]

Similarly to the substrate limitation model Equation 11 and by considering the Henderson-Hasselbach equation Equation 14, the specific production rate in the second stage q_{p_c} can be expressed as a function of the biomass and the lactic acid concentrations at steady state in the second stage $\bar{x}_c$ and q_{p_c}:

$$q_{p_c} = A_c \frac{\mu_{max_c}}{x_{max_c}} (x_{max_c} - \bar{x}_c) + B_c \left(1 - \frac{s_{lim}\, Y_{P/S}}{s_0\, Y_{P/S} - \bar{p}_c + p_0}\right)\left(1 - \frac{\bar{p}_c}{[1 + 10\text{^}(\mathrm{pH}_c - \mathrm{pK_A})][HL]_{inh}}\right)$$

[Equation 15]

Introduction of the above specific production rate Equation 15 into the mass balance for the product in the second stage Equation 6 led to the lactic acid concentration at steady state in the second stage $\bar{p}_c$

$$\bar{p}_c = \left[\frac{V_i}{V_c} D_i \bar{p}_i + \left(A_c \frac{\mu_{max_c}}{x_{max_c}} (x_{max_c} - \bar{x}_c) + B_c \left(1 - \frac{s_{lim}\, Y_{P/S}}{s_0\, Y_{P/S} - \bar{p}_c + p_0}\right)\right)\left(1 - \frac{\bar{p}_c}{[1 + 10\text{^}(\mathrm{pH}_c - \mathrm{pK_A})][HL]_{inh}}\right)\bar{x}_c\right] \frac{1}{\alpha \exp\left(D_c / \beta\right)}$$

[Equation 16]

RESULTS AND DISCUSSION

As indicated above (*c.f.* Theoretical aspects), since the dilution rate was maintained constant in the first stage, only the second stage was concerned by possible wash out, and was therefore considered in this work.

The calculated data displayed in Figure 1 resulted from the optimization of parameters α and β only, and the optimized values were 0.0008 and 0.028 for α and β respectively. Indeed, calculated values for biomass and product concentrations at steady state in the first stage were taken from a previous work and are given in Table 1, as well as the other parameter values also collected in Table 1 and deduced from previous batch cultures of *L. helveticus* carried out on the same medium in similar physico-chemical conditions (Bouguettoucha et al. 2009). As shows in Figure 1, the modified Verlhust model (Equation 9) improved significantly the fitting of experimental data, confirmed by the lower residual standard deviation (RSD) value obtained (0.26), if compared to that given by the previous growth model, 0.52 (Equation 8) (Bouguettoucha et al. 2009). The positive effect of the exponential expression of D_c was especially significant at high dilution rate, namely close to wash out (Figure 1), illustrated by the values of the least square recorded for the higher dilution rate (0.167 h^{-1}) which decreased from 0.43 for the previous model (Equation 8) (Bouguettoucha et al. 2009) to a negligible value (8 x 10^{-5}) for the modified model (Equation 9).

Table 2. Lactic acid concentration at steady state $\overline{p}_c$ in the culture stage obtained after optimization of the parameters for growth- (A_c) and non-growth- associated (B_c) production.

		Model	
		SLM	***GM***
		(Equation12)	**(Equation16)**
A_c		4.25	4.33
B_c	(h)	1.77	2.06
RSD	$\overline{p}_c$	2.60	2.51
	$D_c\,\overline{p_c}$	0.33	0.30

The parametric sensitivity of the two additional parameters α and β involved in this expression showed that as required the effect of both parameters increased with the dilution rate. A weak effect of the parameter α was recorded, since 10% variation of α from its optimal value led to less than 6% variation of the biomass concentration at stationary state (Figure 1). Contrarily, biomass concentration at steady state was significantly affected by the parameter β, involved in the exponential term, since at the higher dilution rate (0.167 h^{-1}), 25.9% increase and 56.9% decrease of $\overline{x}_c$ was recorded for 10% increase and decrease of the parameter β (Figure 1).

As previously shown, the Luedeking-Piret Model did not account for the slowing down of production recorded at the end of batch culture (Bouguettoucha et al. 2007) and appeared to fail in the description of experimental data recorded during two stages

continuous cultures (Bouguettoucha et al. 2009). From this, only the modified substrate limitation model (SLM) and the generalised model (GM) were considered in this work.

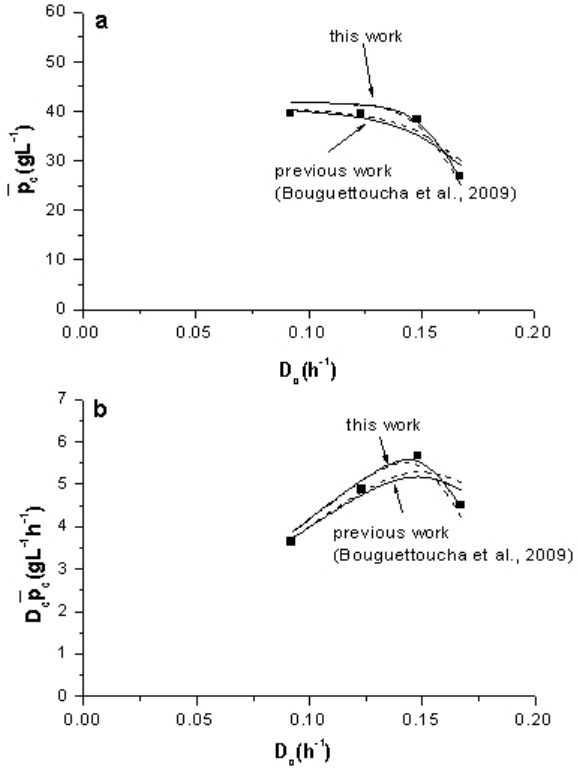

Fig. 2 Experimental (symbols) and calculated (continuous lines) lactic acid concentrations $\bar{P}_c$ (a) and volumetric productivity $D_c\bar{P}_c$, (b) at steady-state in the second stage of the system after optimization of growth- (A_c) and non-growth-associated (B_c) production parameters by means of the substrate limitation (Equation 12) and the generalized (Equation 16) models, as well as the previous model (Bouguettoucha et al. 2009).

The calculated data displayed in Figures 2a and 2b were the result of an optimization of the parameters A_c and B_c, which are summarized in Table 2. Similarly to growth modelization, the other parameter values were considered as indicated in Table 1. As observed, both modified SLM and GM models led to nearly similar values of the parameters A_c and B_c, similarly to the behaviour previously recorded (Bouguettoucha et al. 2009). Indeed, at controlled pH (5.9), the undissociated lactic acid concentration (approximately 0.3 g L^{-1}) is below the inhibitory threshold (Gätje and Gottschalk, 1991), namely almost negligible compared to the inhibitory undissociated lactic acid concentration, 8.5 g L^{-1} (Bouguettoucha et al. 2008). Consequently, the inhibition term of Equation 16 had no effect, since it was close to unit; and the main term was therefore the substrate limitation term. Both models led therefore to fairly similar

calculated data, which matched experimental data (RSD = 2.6 and 2.5, respectively; Table 2). If compared to the fitting given by the previous models (Bouguettoucha et al. 2009), and similarly to growth modelization (Figure 1), the improvement was significant at high dilution rates (0.167 h^{-1}; Figure 2a), illustrated by the lower least square values (between calculated and experimental data) recorded, 2.5 and 0.02 for SLM (Equation 12) and GM (Equation 16) models, versus 10.2 and 5.2 for the previous SLM and GM models (Bouguettoucha et al. 2009).

Calculated volumetric productivity in the second stage corresponded to the product of the lactic acid concentration at steady state $\overline{P}_c$ and the dilution rate D_c. The volumetric productivities displayed in Figure 2b were calculated using the optimised parameters A_c and B_c (Table 2). As observed, and similarly to product concentrations at steady state, both modified SLM and GM models matched experimental data; the residual standard deviation value was nearly 0.3 for both models, versus about 0.4 given by the previous SLM and GM models (Bouguettoucha et al. 2009). As expected from the above results, the improvement was significant at high dilution rate (0.167 h^{-1}), since the least square values decreased from 0.29 and 0.15 for the previous SLM and GM models (Bouguettoucha et al. 2009) to 0.07 and 0.0006 for the modified SLM (Equation 12) and GM (Equation 16) models.

CONCLUDING REMARKS

Since the previous models did not satisfactorily describe biomass data recorded at steady state in the second stage for high dilution rates, namely close to wash out (Bouguettoucha et al. 2009), the dilution rate in the second stage D_c was therefore replaced by an exponential expression involving two additional parameters α and β: $\alpha.\exp\left(D_c/\beta\right)$. As expected, the positive effect of the modified model was especially significant at high dilution rates; it was observed for all considered culture parameters, namely biomass concentration, lactic acid production and volumetric productivity, illustrated by the lower least square values (between calculated and experimental data) recorded, if compared to the previous model (Bouguettoucha et al. 2009).

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