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Optimization of the lactic acid production via fermentation means: Description of the process using structured and unstructured models

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NOMENCLATURE

		Eq.N°
A	coefficient for growth-associated production (dimensionless)	
B	coefficient for non-growth-associated production (h^{-1})	
c, d	constants	
C_P*	maximum concentration of inhibitory product(g L^{-1})	I-31
D	dilution rate (h^{-1})	
e	specific level of the enzyme	I-174
$\frac{e_i}{e_{\max,i}}$	specific relative growth enzyme levels inside the cell	I-61
F	an additional term (dimensionless)	I-91
$F_{e,}$	flow rate of aqueous phase ml. min^{-1}	I-145
$F_{ef,}$	removal rate of filtrate ml. min^{-1}	I-145
F_{if}	recycle rate of cell concentrated broth ml. min^{-1}	I-145
K_i	substrate concentration at which the substrate inhibition factor was: $e^{-(S/K_i)^{n_1}} = 0.368$	I-59
K_{ip}	lactic acid inhibition concentration at which the product inhibition factor was: $e^{-(P/K_{ip})^{n_2}} = 0.368$	I-59
K_α	substrate catabolic constant of affinity of the non-proliferating cells	I-68
$\overline{K}_p^{gc}$	lactic acid inhibition constant	I-69
K_s^{gc}	affinity constant of the growing cells for glucose	I-69
$K_{p\mu}$	inhibition constant for growth	I-171
$K_{p\pi}$	inhibition constants for lactate production	I-171
K	empirical equation constant depending on the maximum specific growth rate	
k_d	coefficient of inhibition by the death cells	
K_i	substrate inhibition parameter	I-34
$Ki_{,L}$,	lactate inhibition parameter	I-57
$Ki_{,,HL}$	undissociated lactic acid inhibition parameters respectively	I-57
Kp	parameter representing the pH dependence of product	I-57

	inhibition	
K_μ	kinetic parameter which describe the effect of the pH on μ_{max} and Kp.	I-35
Kp_m	kinetic parameter which describe the effect of the pH on μ_{max} and Kp.	I-36
k_p	kinetic parameter which describe the effect of the pH on μ_{max} and Kp.	I-36
$K_{\mu L,}$	dissociated lactic acid inhibition constant	I-38
$K_{\mu LH}$	undissociated lactic acid inhibition constant	I-38
K_{PI}	product inhibition constant (g L^{-1})	I-43
K_{pr}	saturation constant of 'usable proteins'	I-17
$(K_m)_\mu, (K_m)_S$	Michaelis constants	I-23
$(K_i)_\mu,$	the inhibitor (lactate) constant for cell growth	I-23
$(K_i)_S$	the specific glucose consumption rate (h^{-1})	I-24
K_p	product inhibition constant	
K_S	Monod constant (mol/m^3)	
h	constant	
f, h	toxic power for both biomass and lactic acid inhibition	
[HL]	undissociated lactic acid concentration (g L^{-1})	I-28
[HLc]	undissociated acetic acid concentration(g L^{-1})	I-29
$[HL]_C$	constant	I-105
L	Lactate concentration (g L^{-1})	
$[L^-]$	dissociated lactic acid concentration (g L^{-1})	
L_m^{-1} ,HL_m	constants	I-30
$[La_{t,s}]$	lactic acid concentration from hexose fermentation (g L^{-1})	I-92
$[Ma_t]$	total, total malic acid concentration (g L^{-1})	I-93
$[La_{t,Ma}]$	total lactic acid concentration from molate utilization (g L^{-1})	I-94
$[La_t]$	total lactic acid concentration (g L^{-1})	I-95
n	parameter used to describe product inhibition	
p	total lactic acid concentration (g L^{-1})	

pHc	Coded pH ((pHc = (pH -5.5))/0.8)	
pH_{opt}	pH optimal	I-51
p_r	concentration of 'usable proteins' (g L^{-1})	I-17
p_c^{gc}	critical lactic acid concentration (g L^{-1})	
q_p	specific production rate (h^{-1})	
RSD	residual standard deviation	
Q_1	volumetric bleed flow-rate	
Q_2	volumetric dilution rate	
r_P''', r_S'''	consumption or production rates of substrate and product	
s	carbon substrate concentration (g L^{-1})	
SD^2	the sum of the residual squares	
SIG	abbreviation for sigmoid	I-170
t	time (h)	
V	volume (L)	
x	biomass concentration (g L^{-1})	
$x_{g,,}$ x_{ng}	biomass in state of growth and in one of non-growth(g L^{-1})	I-178
x_m , p_m	inhibitory biomass and lactic acid concentrations (g L^{-1})	I-72
YE	yeast extract(g L^{-1})	
$Y_{P/S}$	product on substrate yield	I-102
$Y_{x/s}$	the biomass on substrate yield	
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WP	whey permeate concentration (g L^{-1})	I-57
WP_C	Coded whey permeate concentration (WP_C = (WP -60))/30)	I-58
μ	specific growth rate (h^{-1})	
μ_m	kinetic parameters which describe the effect of the pH on μ_{max} and Kp.	I-35
α_1, α_2	control coefficients corresponding to the genetic and metabolic regulations inside the cell	
β	biomass productivity coefficient [mol $g^{-1}s^{-1}$]	
Θ_m	maximum temperature beyond which there was no more growth	I-63

α, β , δ	constants	
σ , n	parameters in the Gaussian equation	I-51
υ_{mX} , λ_X	maximum growth rate (h^{-1}) and growth lag phase (h)	I-80
υ_{mp} , λ_p	maximum production rate (h^{-1}) and product lag phase (h)	I-116
$\gamma_{[s]}$	Monod equation	
$\gamma_{[HL]}$	The inhibition action of lactic acid production	
$\gamma_{[CNS]}$	The remaining self-inhibition coefficient	

Subscripts

ass	growth-associated
c	culture (second stage)
calc	calculated
exp	experimental
f	final
inh	inhibitory
i	seed culture (first stage)
inh	inhibitory
lim	limiting
non-ass	non-growth-associated
max	maximum
0	initial

Nomenclature

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INTRODUCTION

A great interest was reserved for lactic acid production by several authors in these last years. This product plays an important role in various applications, mainly in the food industry, but also in the production of pharmaceuticals, cosmetics and textiles industries (Rojan et al., 2007). It is also used as medical sutures, and green solvents (Dutta and Henry, 2006). The continuous increase in its demand has recently received much attention due to increasing applications in the preparation of new bioengineering materials such as biodegradable polymers like polylactic acid (PLA) and the more recent rise in the cost of petroleum which is usually used as feed stock for the production of lactic acid in the conventional chemical processes. The industrial production of lactic acid can be carried out by two alternative technologies: chemical synthesis from fossil fuels and biotechnological processes. 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 cell culture methods have been investigated (Nandasana and Kumar 2007, Lin and Wang, 2007). Batch fermentation remains the most commonly used approach in industrial lactic acid production.

On the other hand, an indispensable tool for the optimization, control, design and analysis of the production of lactic acid at industrial scale, is the development of mathematically robust models, formulated with parameters of clear biological significance and statistically consistent, and which can be easily implemented in bioreactor software (Gadgil and Venkatesh, 1997; Amrane and Prigent, 1994a and 1999a). Among these models, are those denominated as 'structured' that consider some basic aspects of cell structure, function and composition or intracellular metabolic pathways (with difficulties for the knowledge *in vivo* of the reaction rates of the implied enzymes) (Nielsen 1991; Gadgil and Venkatesh, 1997), but can appear complex, and the simplest, but equally useful and

descriptive in terms of experimental reality, denominated ‘unstructured’ that describe the production of biomass mediating one global variable (Luedeking and Piret, 1959a; Amrane and Prigent, 1999a; Amrane, 2001).

Some unstructured models were previously developed in our laboratory based on the partial linking between growth and lactic acid production (Luedeking and Piret, 1959a). Additional terms were introduced in the Luedeking and Piret expression to account for cessation of lactic acid production when carbon became limiting (Amrane and Prigent, 1994a and 1994b; Amrane 2001), observed during experiments carried out at constant pH (5.9) on one hand; and to account for the inhibitory effect of the undissociated lactic acid (and pH) (Amrane and Couriol, 2002) occurring during cultures without pH control, which is the case during seed cultures, on the other hand. The aim of this work is to improve the previously proposed models.

Since fermentative lactic acid production has been widely studied after the Second World War owing to an increasing cost of fossil resources, the first part of this manuscript (Chapter I) concerns a general exhaustive literature review about lactic acid modelling. The materials and the methods used are described in the Chapter II. Chapter III is divided into four sections. The inhibitory effect of the undissociated lactic acid part on lactic acid production in order to develop an unstructured model for batch cultures without pH control is examined in the first section. The second section is divided in two parts, the first one concerns the improvement of a substrate limitation model to account for nutritional limitation effect recorded during cultures at pH controlled at 5.9. The second one deals with the development of a generalized unstructured model where the above expressions are merged, leading to a unique expression taking into account both effects, a nutritional limitation effect and an inhibitory effect. A modified generalized model was investigated in the third section. The

objective of the last section was the development and the validation of the above models in the case of a two stage continuous culture. Finally we present the concluding remarks and prospects about this study on lactic production from whey permeate in batch and continuous culture using Lactobacillus *helveticus*.

I. LITERATURE REVIEW

I-1. INTRODUCTION:

Mathematical models may be useful for understanding the fermentation process and its optimization (modelling experimental data and studying the effects of experimental conditions on cultures kinetics) (Gadgil and Venkatesh, (1997); Amrane and Prigent, (1994a and 1999a). Lactic acid kinetics can be modelized par either structured or unstructured models. Structured models have been reported to accurately describe lactic acid fermentation, but are complicated for many normal uses (Nielsen, (1991); Gadgil and Venkatesh, (1997)). Unstructured models can be therefore preferred and have proven to accurately describe lactic acid fermentation in a wide range of experimental conditions and media (Luedeking and Piert (1959b); Rogers et al., (1978); Leh and Charles (1989); Amrane and Prigent (1994a and b); Kumar Dutta et al., (1996); Amrane and Prigent, (1997); Boonmee et al., (2003) etc.) We present below an exhaustive study for structured and unstructured models.

I-2. Unstructured Models:

I-2.a Batch fermentation:

I-2.a.1. Growth Kinetics

The traditional diagram of the growth of a bacterial population in a not renewed medium is presented on figure I-1. The kinetics of growth can be divided into five phases:

1- The latency phase, during which the mass remains identical to the initial bacterial mass. This phase is characterized by a nil specific growth rate μ value. The duration of the lag phase is variable and depends firstly on the nature of the medium, and secondly on size and nature of the bacterial inoculum.

2- The quasi-exponential growth phase, known also as the maximum phase of growth. At first glance, this phase is purely exponential. The slope of the right-hand side (when the bacterial

concentration is expressed in semi-logarithmic co-ordinates) corresponds to the specific maximum growth rate, μ_{max} (h^{-1}).

3- The deceleration growth phase which is the consequence of an increasing lack of nutrients or an accumulation of toxic products.

4- The stationary phase, starts at the end of the deceleration phase, when the net growth rate is zero (no cell division, or growth rate is equal to death rate)

5- And the phase of exponential decay, which appears when the medium becomes strongly unfavourable with the multiplication of dead cells.

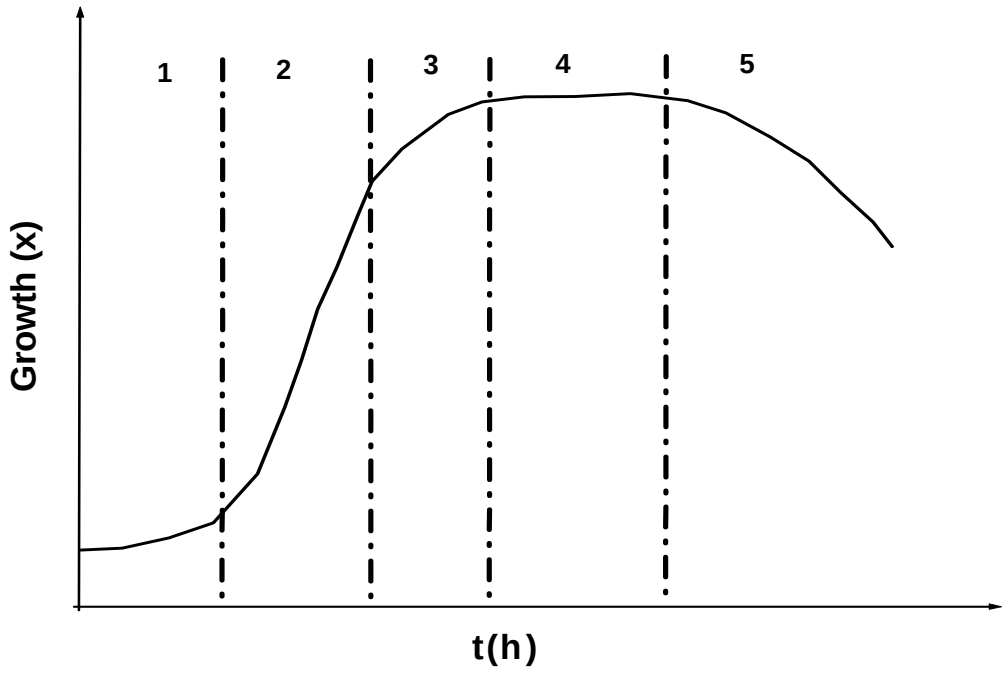


Figure I-1: Growth of bacterial population

Growth kinetics observed in practice were much diversified and have great variability compared to the simple and traditional diagram of figureI-1. Several contributions were however made to model growth kinetics. The complexity of this biological phenomenon

requires the use of nonlinear mathematical models to identify the growth parameters. Bacterial growth can be put in the differential following form:

$$\frac{dx}{dt} = \mu.x \qquad \text{(I-1)}$$

where x is the cellular concentration (g/l) and μ the specific growth rate (h^{-1})

$\frac{dx}{dt}$ represent the increase in the concentration of micro-organisms per unit of time g l^{-1} h^{-1}.

During the exponential growth phase (phase 2 – Fig.I-1), μ which represents the slope of the right-hand side $Lnx = \mu_{max} t + x_0$ is equal to its maximum value μ_{max}. During this phase, the biomass concentration is given by the following equation:

$$x = x_0 e^{\mu_{max}(t-t_0)} \qquad \text{(I-2)}$$

The limitation by the carbon substrate (absence of inhibition by the product) is often described by the Monod, (1942) model:

$$\mu = \mu_{max} \frac{s}{s + K_s} \qquad \text{(I-3)}$$

where s and K_S were the substrate concentration and the substrate saturation constant, respectively.

Piret, (1975) integrated the system formed by equations (I-1) and (I-3):

$$\mu_{max}.t = (1 + \frac{K_s.Y_{x/s}}{x_f}.\ln\frac{x}{x_0} + \frac{K_s.Y_{x/s}}{x_f}.\ln\frac{x_f - x_0}{x_f - x} \qquad \text{(I-4)}$$

with $Y_{x/s}$, the biomass on substrate yield, x_0 and x_f were the initial and finale biomass concentrations, respectively.

Many authors took into account in their growth model an inhibition by the formed product. If this production is non-competitive, the specific growth rate becomes:

$$\mu = \mu_{max} \frac{s}{s + K_s} \frac{K_p}{p + K_p} \qquad \text{(I-5)}$$

Where *p* and K_p are the concentration and the product inhibition constant, respectively.

In the case of a growth-associated production, Powell, (1984) integrated the system formed by equations (I-1) and (I-5):

$$\mu_m.t = \frac{Y_{P/X}}{K_P}(x - x_0) + (1 + \frac{K_S.Y_{X/S}}{x_f})\ln\frac{x}{x_0} + K_S.Y_{X/S}(\frac{1}{x_f} + \frac{Y_{P/X}}{K_P})\ln(\frac{x_f - x_0}{x_f - x}) \qquad \text{(I-6)}$$

with $Y_{p/x}$, the product on biomass yield.

Other authors chose for their modelling, an easy expression to describe the inhibition. Amongst them, Tayeb et al., (1984) considered a non-competitive inhibition, but without limitation by the carbon substrate:

$$\mu = \mu_{max}.\frac{K_P}{p + K_P} \qquad \text{(I-7)}$$

Luedeking and Piert (1959b) proposed an inhibitory law proportional to the product concentration:

$$\mu = \mu_{max} - h.p \qquad \text{(I-8)}$$

This relation, where h is a constant matched very well their results obtained for *L. delbrueckii* growing on glucose.

Theis model was very often considered by other authors, showing its relevance to describe LAB growth. Belhocine, (1987) showed that the results obtained in continuous culture of *L. helveticus* growing on lactose obeyed to a proportional inhibition (Eq.I-8) rather than a substrate limitation (Monod – Eq.I-3) and/or a non-competitive inhibition by the product (Eq.I-5 or Eq.I-7).

Moreover, a substrate limitation cannot describe the growth kinetics of lactic acid bacteria, owing to the low K_S values, which is negligible in comparison to the residual lactose concentration. From this, the substrate limitation model (Monod, (1942) – Eq.I-3) led to the following simplified expression:

$$\mu = \mu_{max} \frac{s}{s + K_s} \approx \mu_{max} \qquad \text{(I-9)}$$

Namely, the specific growth rate remains constant throughout culture, which is the case only during the exponential growth phase (see figure1).

Rogers et al., (1978) tested to describe experimental data on batch culture using *Streptococcus cremoris* HP[1] various growth models, Kendall, (1949 – Eq.I-10); Monod, (1942 – Eq.I-11); Ierusalimsky, (1967 – Eq.I-12) and Edwards, (1970 – Eq I-15):

$$\frac{dx}{dt} = k_1\left(1 - \frac{x}{k_2}\right)x \qquad \text{(I-10)}$$

$$\frac{dx}{dt} = k_1\left(\frac{s}{K_S + s}\right)x \qquad \text{(I-11)}$$

$$\frac{dx}{dt} = k_1\left(\frac{K_P}{K_P + p}\right)x \qquad \text{(I-12)}$$

$$\frac{dx}{dt} = k_1\left(\frac{s}{K_S + s}\right)\left(\frac{K_P}{K_P + p}\right)x \qquad \text{(I-13)}$$

$$\frac{dx}{dt} = k_1\left(\frac{s}{K_S + s}\right)\left(\frac{K_P}{K_P + p}\right)x - k_3 x \qquad \text{(I-14)}$$

$$\frac{dx}{dt} = k_1\left(\frac{s}{K_S + s}\right)\left(\frac{K_P}{K_P + p}\right)\left(\frac{K_1}{K_1 + s}\right)x \qquad \text{(I-15)}$$

According to Rogers et al., (1978), compared to equation (I-13), models (I-14) and (I-15) did not improve growth fitting, so the simplest (Eq.I-13) was preferred. Theses authors reported that lactose limitation and lactic acid inhibition had a significant effect on growth, while lactose substrate and cell mortality were negligible.

Roy et al., (1987) used the Verlhust model (Moraine et al., 1996, Pandey et al., 2000) to describe the growth of *Lactobacillus delbrueckii* on glucose, only growth parameters are involved in this logistic expression:

$$\mu = \mu_{max}(1 - \frac{x}{x_{max}}) \qquad \text{(I-16)}$$

where x_{max} is the maximum biomass concentration (g/l).

As we will subsequently see, this equation describes with accuracy the results obtained by Roy and co-workers, (1987).

Belhocine, (1987) also highlighted a nitrogen limitation during lactic acid fermentation. However, due to the fastidious nutritional requirements of lactic acid bacteria (especially nitrogen), it appears difficult to include these limitations in a growth model.

Leh and Charles, (1989) tried to solve this difficulty. Indeed, these authors considered two substrate limitations in their models, by the sugar and by nitrogen. To account for both limitations, the following modification of the Monod law (Eq.I-3) was considered:

$$\mu = \mu_{max} \cdot \frac{1}{1 + \frac{K_{pr}}{pr} + \frac{K_S}{s} + \frac{K_S}{s} \cdot \frac{K_{pr}}{pr}} \qquad \text{(I-17)}$$

In this relation, pr and K_{pr} were the concentration and the saturation constant of 'usable proteins', respectively. The difficulties encountered in the use of this model come from the definition of the 'usable proteins'.

By considering that during growth K_S is negligible compared to the carbon substrate concentration *s* (Leh, 1987); the above equation (Eq.I-17) can be simplified, leading to the MONOD equation modified to account for the nitrogen substrate limitation:

$$\mu = \mu_m \cdot \frac{pr}{pr + Kpr} \qquad \text{(I-18)}$$

By adding in the equation I-18 a product inhibition term, the specific growth rate becomes:

$$\mu = \mu_{max} \cdot \frac{pr}{pr + (\frac{P}{K_P + 1})^2 \cdot K_{pr}} \qquad \text{(I-19)}$$

Leh and Charles, (1989) are the first authors who account in their model for the fastidious problem of the nitrogen limitations during LAB growth, which was also (only) evoked by Belhocine, (1987). Amrane, (1991) reported that the above model (Eq. I-18) can only be usable if a clear definition and a relevant method for the determination of the really 'usable nitrogen' by the bacteria is given.

Consequently and on the basis of the results obtained by Belhocine, (1987) and his results obtained for *Lactobacillus Helveticus* cultivated at *pH* controlled at the optimal value, 5.9 (Hanson and Tsao, 1972; Venkatesh et al., 1993**;** Amrane and Prigent, (1994a and b) and Amrane 2001) to overcome the inhibition by the undissociated lactic acid concentration and the acidic pH, Amrane, (1991) and Amrane and Prigent, (1994a and b) proposed in the laboratory a logistic function (Eq. I- 20) to describe experimental data:

$$\mu = \mu_{max} \frac{1}{1 + \frac{c * e^{d*t}}{\mu_{max} - c}} \qquad \text{(I-20)}$$

Where c and d are constants.

$$\mu = \frac{dx}{xdt} = \frac{\mu_m}{1 + \frac{c.e^{d.t}}{\mu_{max} - c}} \qquad \text{(I-21)}$$

After integration, one obtains for x:

$$x = \exp\left\{\mu_{max}.t - \frac{\mu_{max}}{d}\left[\ln\left(\frac{\mu_{max} - c + c.e^{d.t}}{\mu_{max}}\right)\right]\right. \qquad \text{(I-22)}$$

The expression obtained for the biomass concentration x, with obvious biological meaning for the constants c and d, is a little complicated but it describes well the kinetics of growth until the end of the stationary phase.

Ishizaki and. Ohta, (1989a) studied the fermentation of L-lactate in batch culture employing *Streptococcus sp*. IO-1 at various carbon substrate concentrations. They observed

that the type of inhibition in this fermentation was uncompetitive, and hence proposed the following relationship:

$$\mu = \frac{dx}{xdt} \qquad \text{(I-1)}$$

$$\mu = \frac{\mu_{max} s}{(Km)_{\mu} + \{1 + L/(K_i)_{\mu}\} s} \qquad \text{(I-23)}$$

$$\mu_S = \frac{(\mu_S)_{max} s}{(Km)_S + \{1 + L/(K_i)_S\} s} \qquad \text{(I-24)}$$

where $(K_m)_{\mu}$, $(K_m)_S$ $(K_i)_{\mu}$ and $(K_i)_S$ were the Michaelis constants, the inhibitor (lactate) constant for cell growth and the specific glucose consumption rate, respectively. s and L are the glucose and lactate concentrations in the broth.

When the value of $(K_m)_{\mu}$ was very small, the above equation (I-24) can be approximated by the following equation (Ishizaki and al.,1989b):

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{(K_i)_{\mu}}{\mu_{max}}(L) \qquad \text{(I-25)}$$

And after rearrangement it come:

$$\frac{1}{\mu} = \frac{1}{(\mu_S)_{max}} \left\{ 1 + \frac{(K_m)_S}{s} \right\} + \frac{(K_i)_S}{(\mu_S)_{max}}(L) \qquad \text{(I-26)}$$

According to these authors, the proposed model gave a fairly good approximation of the observed results.

Fredereco and co-workers, (1994) found during their experiments dealing with batch cultures of *L. plantarum* without *pH* control, that 51% of lactic acid was produced after growth ceased when NaCl was not present in the medium, whereas no more than 18% of lactic acid was produced after growth ceased in the presence of NaCl, most likely due to an increase in the cell death rate. Consequently, Fredereco and co-workers, (1994) developed the

following model which takes into account in addition to the pH, the temperature, the NaCl concentration and the specific death rate:

$$r_x = \frac{d[x]_t}{dt} = \mu[x]_v \qquad \text{(I-1)}$$

$$r_x = \frac{d[x]v}{dt} = (\mu - k_d)[x]_v \qquad \text{(I-27)}$$

$$\mu = \mu_0 \left(\frac{[s]}{0.056 + [s]}\right)\left(1 - \frac{[H^+]}{[H^+]_{max}}\right)^{2.6}\left(1 - \frac{[HL]}{69}\right)^{2.0} \qquad \text{(I-28)}$$

where:

$$\mu_0 = 0.35\left(1 + \frac{1.5[HLc]}{5.8 + [HLc]}\right)\left(1 - \frac{[HLc]}{150}\right)^{1.7}\left(1 + \frac{1.6[NaCl]}{4.47 + [NaCl]}\right)\left(1 - \frac{[NaCl]}{11.8}\right) \qquad \text{(I-29)}$$

where [HLc] is undissociated acetic acid concentration

Experimental data concerning *L. plantarum* growth on malate and hexose as carbon sources were well described by the proposed model.

During their experiments (on freezing) dealing with immobilized cell of *L. delbruckii* IFO 3534 entrapped in calcium alginate and growing on glucose, Wang et al., (1995) showed that the cell density gradient was formed in the gel beads and it was caused by the accumulation of the inhibitory product (free lactic acid) and not by substrate starvation. From this, they proposed the following expression to describe growth, which involves in addition to the substrate both forms of the lactic acid, the dissociated and the undissociated form:

$$\mu = \frac{\mu_{max} s}{K_S + s}\left(1 - \frac{L^{-1}}{L_m^{-1}}\right)\exp\left(1 - \frac{[HL]}{[HL_m]}\right) \qquad \text{(I-30)}$$

where L^{-1} and *[HL]* were the concentration of lactate and that of undissociated lactic acid respectively (g/l), L_m^{-1} and $[HL_m]$ are constants. According to these authors, the results calculated from this model matched experimental data.

Kumar Dutta et al., (1996) proposed to describe lactic acid fermentation on glucose using *L. delbrueckii* strain NCIM 2365 a growth kinetic model based on the Monod, (1942) equation:

$$\frac{dx}{dt} = \left[\mu_{max} \left(1 - \frac{C_P}{C_P^*} \right)^n \right] \left[\frac{sx}{K_S + s} \right] \quad \text{(I-31)}$$

where C_P* was the maximum concentration of inhibitory product in a batch process, K_S was the Monod constant and n the toxic power.

Monteagudo and co-workers, (1997) used the model of Monod (1942), (Eq.I-3). However they considerd that the growth is limited by the carbon substrate and the accumulated lactic acid. From this, they proposed the following equation to describe their experimental results:

$$\frac{dx}{dt} = \mu x \left(1 - \frac{p}{p_{max}} \right) \quad \text{(I-32)}$$

where *p and* p_{max} were the concentrations of the product at time t and at the end of the fermentation, respectively. Calculated data (Eq.I-32) were found to accurately describe the relationship between the biomass concentration and the lactic acid production, according to these authors.

Peeva and Peev, (1997) proposed a new method for *pH* stabilization of the lactoacidic fermentation using *L. casei-NBIMCC*-1013 based on the following model:

$$\frac{dx}{dt} = \mu_{max} \left(1 - k_i p^{\alpha} \right) - k_d x \quad \text{(I-33)}$$

with:

$$\alpha = 6{,}13 p_{max} - 0{,}056$$

where k_d was the coefficient of inhibition by the death cells and p_{max} was the theoretical lactic acid concentration (mol l^{-1}),

To describe growth to *Lactococcus lactis ssp lactis ATTC* 19435, Akerberg and co-workers, (1998) added to the Monod relation (Eq.I-3) terms for substrate and product inhibitions, as well as terms to account for the influence of pH and temperature:

$$r_x = \frac{\mu_{max}.s_g.x}{s_g + K_s + \frac{\left(s_g^*\right)^2}{K_i}}\left(1 - K_p.p^*\right)^n \qquad \text{(I-34)}$$

with:

$$\mu_{max} = \frac{\mu_m}{1 + \left(k_{\mu 1}/[H^+]\right) + \left(k_{p2}[H^+]\right)} \qquad \text{(I-35)}$$

$$K_p = \frac{K_{pm}}{1 + \left(k_{p1}/[H^+]\right) + \left(k_{p2}/[H^+]\right)} \qquad \text{(I-36)}$$

where K_s was the saturation constant, K_i the substrate inhibition parameter, Kp the parameter representing the pH dependence of product inhibition, n the parameter used to describe product inhibition, μ_m, K_μ, Kp_m and k_p are kinetic parameters which describe the effect of the pH on μ_{max} and K*p*.

By using the method of neuronal networks as a method of resolution, Gonzalo et al. (1998) proposed an implicit law to describe their results, which involved the temperature *T*, the product *P* and the *pH*:

$$\frac{dx}{dt} = \mu(p, T, pH)x \qquad \text{(I-37)}$$

Berry et al., (1999) used a modified unstructured model proposed initially by Yabannavar and Wang, (1991) to describe the cell growth during batch fermentation of *Lactobacillus rhamnosus.* The cell growth kinetic can be described by equation (I-27):

$$r_x = \frac{dx}{dt} = \mu x - K_d x \qquad \text{(I-27)}$$

where the specific growth rate is inhibited by both the dissociated and the undissociated lactic acid (Eq.I-38):

$$\mu = \left[\frac{(\mu_{max} s)}{(K_S + s)}\right]\left[1 - \frac{L^{-1}}{K_{\mu L}}\right]\exp\left(-LH/K_{\mu LH}\right) \quad \text{(I-38)}$$

where K_d, x, μ_{max}, k_s, $K_{\mu L}$, $K_{\mu LH}$, L^{-1}, LH denote the cell death constant, the cell dry weight, the maximum cell specific growth rate, the substrate saturation constant, the dissociated and undissociated lactic acid inhibition constants, the dissociated and undissociated lactic acid concentrations respectively. This model described experimental results accurately.

Diaz et al., (1999) developed an on-line estimator based on the biomass concentration for detecting and quantifying the growth phases encountered in batch cultures. *Escherichia coli* TP2339:1291 was used in this study. The model that best simulated the microbial growth over all the culture was:

$$\frac{dx}{dt} = 0{,}70.\left(1 - \frac{x}{45{,}65}\right).x \quad \text{(I-39)}$$

According to the authors, the simulation of the microbial growth was highly accurate.

Fu and Mathews, (1999) examined the effect of pH and carbon substrate on both growth and lactic acid production for *Lactobacillus plantarum* growing on lactose in batch culture. Examination of growth curves shows on one hand, that values of pH close to 4 inhibit growth, whereas it is not the case for pH values between 5 and 6. These authors reported that this effect is probably due to the strong concentration of both dissociated and undissociated form of the lactic acid. On the other hand, by studying the effect of the carbon substrate, Fu and Mathews, (1999) found that the phase of latency (lag phase) is very short for a concentration of 20 g/l, whereas it is prolongated for 60 g/l (5h). The stationary phase and the decline phase remain unchanged. In conclusion, these authors proposed to describe their experimental curves the Monod model with μ_m and K_S functions of the pH:

$$\mu x = \frac{dx}{dt} = \frac{\mu_{max}(pH) s x}{K_S(pH) + s} \quad \text{(I-40)}$$

where $\mu_{max} = 0.523\exp\left(-0.16(pH-5.0)^2\right) - \frac{0.265}{0.614(pH-4.0)}$ (I-41)

and

$$K_S = 0.605\exp\left(0.85(pH-5.0)^2\right) - \frac{106.4}{0.65+(pH-4.0)}$$

According to these authors, this model described accurately the experimental results.

In their studies for lactic acid production on glucose using *Lactobacillus delbrueckii*, Moldes et al., (1999) modified the original equation proposed by Mercier et al., (1992) for biomass growth by replacing the term $\left(1-\frac{x}{x_m}\right)$ by an inhibition term $\left(1-\frac{p}{p_m}\right)$ since inhibition is caused by the product:

$$\frac{dx}{dt} = \mu_m\left(1-\frac{p}{P_m}\right).x \quad \text{(I-42)}$$

where p and p_m were product concentrations at time t and at the end of the batch respectively. According to these authors, this model showed a remarkable ability to describe growth kinetic.

Conception et al. (2000) studied the effect of different substrates (lactose, glucose and galactose) on lactic acid production using *Lactobacillus bulgaricus* in batch fermentation. They showed that during the first hours of fermentation glucose and galactose accumulated in the medium, since the rate of hydrolysis of lactose to glucose and galactose was faster than the assimilation rate of these substrates. They observed a short fermentation times with a very short lag phase. To describe their experimental results, these authors used an unstructured Monod, (1942) type model:

$$\mu = \frac{\mu_{max} s}{K_S + s}\left(\frac{K_{PI}}{p+K_{PI}}\right) \quad \text{(I-43)}$$

$$\mu = \frac{\mu_{max} s}{K_S + s}\left(1 - \frac{p}{K_{PI}}\right) \quad \text{(I-44)}$$

$$\mu = \mu_{max}\left(1 - \frac{p}{K_{PI}}\right) \quad \text{(I-45)}$$

where K_{PI} was product inhibition constant.

According to these authors, on the one hand the first two equations are non-competitive inhibition laws, and the inhibition by the lactic acid affects only the specific growth rate. On the other hand, the third equation, which is only a simplified equation 2, leads to a better fit compared to both other equations.

Leroy and De Vuyst, (2001) noticed that the specific growth rate of *Lactobacillus sakei strain* CTC494 reaches its maximum value at the end of the phase of latency, before decreasing with the increase of the biomass x; consequently, they proposed the following equation:

$$\mu = \mu_{max} . \gamma_i \quad \text{(I-46)}$$

where:

$$\gamma_i = \gamma_{[s]} . \gamma_{[HL]} . \gamma_{[CNS]} \quad \text{(I-47)}$$

The inhibitory function $\gamma_{[s]}$ is given by the equation of Monod:

$$\gamma_{[s]} = \frac{s}{s + K_s} \quad \text{(I-48)}$$

The inhibition action of lactic acid production $\gamma_{[HL]}$ is due to the toxic effect of the lactic acid molecules produced:

$$\gamma_{[HL]} = \left(1 - \frac{[HL]}{[HL]_{max}}\right)^n \quad \text{(I-49)}$$

n, is a constant

$\gamma_{[CNS]}$ is the remaining self-inhibition coefficient which was ascribed to limited availability of nutrients.

According to these authors, this simple model has considerable advantages compared to commonly used descriptive models such as the logistic growth equation. It offers a better fit and a more realistic description of the growth data by taking into account both growth inhibition due to lactic acid production and changes in growth rates due to nutrient depletion

According to the results obtained by Amrane and Prigent, (1996, 1998), the effect of the *pH* and the undissociated lactic acid concentration was examined during growth of *L. helveticus* on whey permeate. Amrane and Couriol, (2002) noted that the specific growth rate decreased with the increase of the undissociated lactic acid concentration, and consequently proposed the following logistics equation:

$$\mu = \mu_{max}\, e^{-\frac{[HL]}{[HL]_C}} - \mu_0 \qquad \text{(I-50)}$$

where μ_0 and $[HL]_C$ are constants.

It was shown that the model proposed by these authors matched well growth kinetics in various culture conditions.

As expected, maximum biomass concentration increase with increasing nitrogen supplementation, (Belhocine, 1987, Amrane and Prigent, 1997, Leh and Charles, 1989), from this, Schepers et al., (2002) tried to put into equation the nitrogen limitation recorded during growth of *L. helveticus*. They proposed a model which contains four variables, carbon and nitrogen substrate concentrations, the lactic acid concentration and the pH

$$: \mu = \left[\frac{(\mu_{max} s)}{(K_S + s)}\right] \qquad \text{(I-3)}$$

$$\mu(pH) = \mu e^{-\left(\frac{(|pH_{opt} - pH|)^n}{\sigma^2}\right)} \qquad \text{(I-51)}$$

This last equation is known under the name of the Gauss equation:

$$\mu(L^-) = \mu\left(\frac{1}{1+e^{K_p(L^- - K_i)}}\right) \quad \text{(I-52)}$$

$$\mu(HL) = \mu.e^{-k_i.HL} \quad \text{(I-53)}$$

where σ , n are parameters in the Gaussian equation, pH_{opt} is an optimal pH.

The concentration of the both dissociated [L^-] and undissociated [HL] forms of lactic acid were calculated using the Henderson – Hasselbach equation.

$$[HL] = \frac{P}{1+10^{(pH-pKa)}} \quad \text{(I-54)}$$

$$[L^-] = \frac{P}{1+10^{(pKa-pH)}} \quad \text{(I-55)}$$

where pKa=-log (K_a) and p= [HL] + [L^-]

From the combination of equations (I-52) and (I-53) and after rearrangement of equations (I-54) and (I-55) one obtains the following relation:

$$\mu(p,pH) = \mu\left(\frac{e^{-K_{i,[HL]}\frac{p}{1+10^{(pH-pKa)}}}}{1+e^{K_{p,L^-}\left(\frac{p}{1+10^{(pKa-pH)}} - K_{i,L^-}\right)}}\right) \quad \text{(I-56)}$$

Equations (I-51) and (I-52) can be introduced in the above equation (I-56) to deduce the growth rate:

$$\frac{dx}{dt} = (\mu_{max} + \beta.pH_C.WP_C)\frac{s}{s+K_S}.\frac{z}{z+K_z}\left(\frac{e^{-K_{i,[HL]}\frac{s}{1+10^{(pH-pKa)}}}}{1+e^{K_{p,L^-}\left(\frac{s}{1+10^{(pKa-pH)}} - K_{i,L^-}\right)}}\right).e\left(\frac{(|pH_{opt}-pH|)^n}{\sigma^2}\right)x$$

(I-57)

In this relation *WP* was the concentration of whey permeate, *s* and *z* were the carbon and nitrogen substrate concentrations respectively, K_s and K_z the Monod parameters for both carbon and nitrogen substrates, $Ki_{,L}$, $Ki_{,,HL}$ are lactate and undissociated lactic acid inhibition parameters respectively..

There are no direct methods to follow the concentration of "usable" nitrogen; Schepers et al., (2002) calculated the initial concentration of nitrogen substrate by the following relation:

$z(0) = 2.889 + 1.219YE_C + 0.384YE_C.pH_C - 0.274WP_C.YE_C - 0.221WP_C.pH_C$

Then they determined $Y_{x/z}$ using the Gauss equation:

$$Y_{x/z}(pH) = e^{-\left(\frac{(5.51-pH)^{2.18}}{1.86^2}\right)} \qquad \text{(I-58)}$$

where YE_C and were Coded yeast extract concentration (YE_C = *(YE -10))/5.5)*, WP_C whey permeate concentration (WP_C = *(WP -60))/30) and pHc* Coded pH ((*pHc = (pH -5.5))/0.8).* This model describes well their experimental results in various culture conditions.

Some authors use a complex model to validate their experimental results. Biazar et al.,(2003) tried to solve the equation relating to the growth kinetics of *L. helveticus*, and previously proposed by Tango et al.,(1999) using the Adomian decomposition method (1983, 1986):

$$\frac{dx}{dt} = \left[\mu_m \frac{s}{K_S + s} e^{-(S/K_i)^{n_1}} e^{-(P/K_{ip})^{n_2}} - K_d\right] x \qquad \text{(I-59)}$$

where K_i was the substrate concentration at which the substrate inhibition factor was: $e^{-(S/K_i)^{n_1}} = 0.368$; and K_{ip} was the lactic acid inhibition concentration at which the product inhibition factor was: $e^{-(P/K_{ip})^{n_2}} = 0.368$.

This method appears very useful compared to the other numerical methods, but requires the knowledge of all model parameters.

Boonmee et al., (2003) showed in their studies on batch cultures of *L. lactis NZ133* that growth kinetics was predominantly influenced by lactose limitation and lactate product inhibition. They consequently proposed to describe experimental data the following equation:

$$\frac{dx}{dt} = \mu_{max}\left(\frac{s}{K_{SX} + s}\right)\left(1 + \frac{p - p_{iX}}{p_{mX} - p_{iX}}\right)\left(\frac{K_{iX}}{K_{iX} + s}\right) x \qquad \text{(I-60)}$$

where K_s and K_i were the lactose limitation constant and the lactate inhibition constant respectively; p_m and p_i were the maximum inhibitory lactate concentration and the threshold level of lactate before an inhibitory effect, respectively. When compared to the experimental data, the model provided good prediction for growth.

Jyoti et al., (2003) developed an optimal model to describe the kinetics of growth and product formation by *L. rhamnosus* on multiple substrates and proposed the following expression:

$$\mu_i = \frac{\mu_{max,i} \frac{e_i}{e_{max,i}} s_i}{K_i + s_i} \qquad \text{(I-61)}$$

where *i*= 1, 2 and $\mu_{max,i}$ was the maximum specific growth rate on the substrate s_i (glucose or product lactate), $\frac{e_i}{e_{max,i}}$ was the specific relative growth enzyme levels inside the cell and K_i was the substrate saturation constant. The net specific growth rate μ on a medium containing two substrates in terms of individual growth rates can be defined as:

$$\mu = \alpha_1 \mu_1 + \alpha_2 \mu_2 \qquad \text{(I-62)}$$

where μ_i was the specific growth on the substrate *i*, α_1 and α_2 were the control coefficients corresponding to the genetic and metabolic regulations inside the cell, respectively. According to these authors, the kinetic model developed can be used for the design and operation of batch and continuous reactors, such as fed-batch and chemostat reactors.

Baati et al., (2004) developed a model which takes into account the inhibition of the growth under sub-optimal temperature conditions of culture which was accompanied by an increase of the maintenance energy. The growth rate must take into account the inhibition by the lactic acid and the variation of the energy of maintenance as a function of the temperature:

$$\mu = 0 \quad \text{if} \quad \Theta \quad \rangle \quad \Theta_m \qquad \text{(I-63)}$$

$$\mu = \alpha v_P - m \quad \text{if} \quad \Theta \quad \langle \quad \Theta_m \tag{I-64}$$

where Θ_m was a maximum temperature beyond which there was no more growth. It was assumed that the average maintenance varied hyperbolically with temperature until a certain limit (Θ_m). According to these authors, this maximum temperature of growth was experimentally identified and was fixed in the model with the aim of decreasing the complexity of the parametric identification. This value was set at Θ_m = 45°C.

$$m = \delta \frac{\Theta_m - \Theta}{\beta + (\Theta_m - \Theta)} \tag{I-65}$$

By taking account the residual substrate concentration at low temperatures we used a model of the Monod's type:

$$m = b \frac{s}{K_a + s} \tag{I-66}$$

Thus:

$$m = b \frac{\Theta_m - \Theta}{c + (\Theta_m - \Theta)} \frac{s}{K_a + s} \tag{I-67}$$

From this, the final law governing the cell multiplication if the temperature was lower than the maximum temperature of growth Θ_m was:

$$\mu = a v_P \_ b \frac{\Theta_m - \Theta}{c + (\Theta_m - \Theta)} \frac{S}{K_\alpha + S} \tag{I-68}$$

The terms α (biomass on lactate yield), β (constant of affinity) and δ (maximal maintenance) were constants. K_α was the substrate catabolic constant of affinity of the non-proliferating cells.

According to Bâati et al., (2004), the model described satisfactorily the kinetic behaviour for discontinuous cultures carried out at various growth temperatures. The evolution of the growth rate must take into account the inhibition by the produced lactic acid and the variation of the maintenance energy according to the temperature.

In the model developed by Amrane, (2005), growth kinetics was divided into five phases: the lag phase, the exponential growth, the deceleration growth phase, the stationary state and the decline phase. Each phase can be mathematically described by simple model kinetics.

Ben Youssef et al.,(2005) studied the effects of nutritional medium limitation on the growth in batch culture of *L. casei ssp. rhamnosus* which it is resistant to high lactic acid concentrations and has multiple nutritional requirements. In their study, numerous models were tested to fit experimental results:

$$\mu = \bar{\mu}_{max}\left(\frac{\overline{K}_p^{gc}}{\overline{K}_p^{gc} + p}\right)\left(\frac{s}{K_s^{gc} + s}\right)\left(1 - \frac{p}{p_c^{gc}}\right) \qquad \text{(I-69)}$$

where $\overline{K}_p^{gc}$ the lactic acid inhibition constant, K_s^{gc} the affinity constant o the growing cells for glucose and p_c^{gc} the critical lactic acid concentration. Similarly to Fredereco et al. (1994), Ben Youssef et al., (2005) included in their model an additive parameter which represents the phase of decline of the cells:

$$\frac{dx}{dt} = \mu x - K_d x \qquad \text{(I-70)}$$

Where k_d was the decline constant.

The biomass concentration decreases at the end of culture, confirming that it is necessary to integrate a cell death constant in the process model. The above model described accurately growth kinetics until the end of fermentation under various culture conditions.

Altiok et al., (2006) studied the effect of various initial whey lactose concentrations on growth kinetics of *L. casei*. They took into account in their modelling both growth (x) and production (p) inhibition. While an inhibition by the carbon substrate was not observed, the Monod model previously modified by Levenspiel, (1980):

$$\frac{dx}{dt} = \mu\left(1 - \frac{p}{p_m}\right)^h \quad \text{(I-71)}$$

$$\frac{dx}{dt} = \mu\left(1 - \frac{x}{x_m}\right)^f \left(1 - \frac{p}{p_m}\right)^h \quad \text{(I-72)}$$

where x_m and p_m were the inhibitory biomass and lactic acid concentrations respectively, f and h were the toxic power for both biomass and lactic acid inhibition. These authors show in their study that the inhibitory effects on both biomass and product increased for increasing toxic powers h and f. The above equation matched their results obtained for *L. casei NRRL B-441*.

The Haldane equation (Eq. I-73) where K_i is the substrate inhibition constant and exponential substrate inhibition model (Aiba et al, (1969)) (Eq. I-74) were used. However according to Altiok et al., (2006), the model with a substrate inhibition term did not result good predictions of experimental data:

$$\mu = \frac{\mu_{max} s}{K_S + s + s^2/K_i} \quad \text{(I-73)}$$

$$\mu = \frac{\mu_{max} s}{K_S + s} \exp\left(-s/K_i\right) \quad \text{(I-74)}$$

According to Lan and al., (2006), growth kinetics can be described by a modified logistic model which take into account the inhibitory effect of the product through an exponent *n* (Messen et al., 2003):

$$\mu = K\left(1 - \frac{x}{x_{max}}\right)^n .x \quad \text{(I-75)}$$

where x is biomass concentration, K an empirical equation constant depending on the maximum specific growth rate, x_{max} the maximum biomass concentration achievable under the specified conditions. The modified Messen et al., (2003) model was able to describe the experimental results obtained by Land et al. (2006).

Nandasana and Kumar, (2008) modified the model developed by Boonmee et al., (2003) by adding a cell death coefficient (K_d) to the growth kinetic of *Enterococcus faecalis* RKY1 growing on molasse:

$$\mu = \frac{\mu_{max}.s.K_{ix}}{(K_{sx}+s)(K_{ix}+s)} e^{-P/Kpx} \quad \text{(I-76)}$$

where Kp is a product inhibition constant

$$\frac{dx}{dt} = (\mu - K_d).x \quad \text{(I-27)}$$

This model describes satisfactorily growth kinetics, according to these authors.

During batch cultures of *L. casei subsp. rhamnosus* on date juice for lactic acid production, Nancib, (2007) examined the effect of both glucose (s_1) and fructose (s_2) carbon substrates concentrations. The model developed accounts for the effects of substrates limitation and cell death on the cell growth, specific growth rate μ is usually expressed as a function of the limiting substrate concentration by a Monod model (Eq. I-3):

$$\frac{dx}{dt} = \mu x \quad \text{(I-1)}$$

$$\frac{dx_{ns_1}}{dt} = \frac{dx_{s_1}}{dt} - k_{Ds_1} x_{s_1} \quad \text{(I-77)}$$

$$\frac{dx_{ns_2}}{dt} = \frac{dx_{s_2}}{dt} - k_{Ds_2} x_{s_2} \quad \text{(I-78)}$$

$$\frac{dx_n}{dt} = \left[\mu_{maxs_1} \frac{s_1}{K_{s_1}+s_1} - K_{Ds_1}\right] x_{s_1} + \left[\mu_{maxs_2} \frac{s_2}{K_{s_2}+s_2} - K_{Ds_2}\right] x_{s_2} \quad \text{(I-79)}$$

According to this author, this model describes the growth kinetics only at the beginning of the fermentation (15 h). After this period, the model does not describe satisfactorily the experimental results.

V´azquez and Murado, (2008a and b) developed a mathematical model to evaluate various peptidic sources for biomass, lactic acid and bacteriocin production by two lactic acid

bacteria, *Pediococcus acidilactici* NRRL B-5627 and *Lactococcus lactis subsp.lactis*. The following growth model was considered:

$$x = \frac{x_{max}}{1 + \exp\left[2 + \frac{4\upsilon_{mX}}{x_{max}}(\lambda_X - t)\right]} \qquad \text{(I-80)}$$

where υ_{mX} and λ_X were maximum growth rate (h^{-1}) and growth lag phase (h), respectively. This model is that of Verlhust whose parameters are modified mathematically in order to give direct biological significance of its parameters. The model predictions were found to match well with the experimental data of *Pediococcus acidilactici* NRRL B-5627 and *Lactococcus lactis subsp.lactis* growth on sugar.

I-2.a.2. Product kinetics:

The model of Luedeking and Piret, (1959a and b) is the most widely used for the kinetics of production. Indeed these authors showed, according to their experimental results, that lactic acid production is partially associated with growth, and then proposed the following relation:

$$q_P = \frac{dp}{xdt} = A\mu + B \qquad \text{(I-81)}$$

In this relation q_p was the specific productivity rate, A was a coefficient for the growth-associated production and B was a coefficient for the non-growth-associated production.

Amrane, (1991) showed that the Luedeking and Piret relation, (1959a and b) accounted well for the experiment results, except at pH = 5.4, lactic acid production was completely linked to growth ($q_P = A\mu$).

Rogers et al., (1978) put forward two models each containing a substrate dependent term. In these expressions A and B are constants. Based on their experiments with *S. cremoris*

fermenting lactose they concluded that the substrate dependent models (Eq. I-80, I-83) fitted their data better than that of the simple Luedeking and Piret, (1959) (Eq. I-81 see below).

$$\frac{dp}{dt} = A\frac{dx}{dt} + B\left(\frac{s}{K_S^{'} + s}\right)x \qquad \text{(I-82)}$$

$$\frac{dp}{dt} = A\frac{dx}{dt} + Bxs \qquad \text{(I-83)}$$

During exponential growth phase, namely for μ constant and equal to μ_{max}, Cogan, (1978) solved the system formed by the equations (I-1) and (I-81) and obtained the following kinetic of production:

$$p = x_0 \frac{A.\mu_{max} + B}{\mu_{max}}.(e^{\mu_{max} t} - 1) \qquad \text{(I-84)}$$

According to Amrane, (1991), this relation describes only a part of the production, during the exponential growth phase, but can be useful to determine μ_{max}.

Similarly to Cogan, (1978) but in the general case, Tayeb and co-workers, (1984) solved the system involving equations (I-1), (I-16) and (I-81), they obtained this relationship:

$$\frac{d^2p}{dt^2} + \frac{A\mu_{max}K_P}{(K_P + p)(A)\mu_{max}K_P + BK_P + Bp}\left(\frac{dp}{dt}\right)^2 - \frac{\mu_{max}K_P}{p + K_P}.\frac{dp}{dt} = 0 \qquad \text{(I-85)}$$

This equation had an approached solution in three zones: when $p << K_p$, i.e. at the beginning of culture, the specific growth rate μ was constant and equal to μ_m, one led to the reduced equation (58).

When p was different from K_p, μ was always constant but equal to $\mu_{max} / 2$. Inhibited constant K_p was equal to the lactic acid concentration for which the slope of the curve is equal to $\mu_{max} / 2$.

Finally when $p >> K_p$, μ was given by the following expression: $\mu = \mu_{max}. K_p / p$, and equation (57) can be simplified:

$$\frac{d^2p}{dt^2} + \frac{\alpha\mu_m K_P}{\alpha\mu_m K_P + \beta p}\left(\frac{dp}{dt}\right)^2 - \mu_m K_P \frac{dp}{dt} = 0 \qquad \text{(I-86)}$$

The authors propose to use a nonlinear regression for the calculation of the parameters *A* and *B*. However the examination of the term in $(dp/dt)^2$ of the equation (I-86) shows that this equation cannot give A and B separately, but only their ratio.

According to Jorgenson and Nikolajsen, (1987) the models proposed by Rogers et al. (1978) do not explain metabolic pathways; nevertheless the models can be very useful during actual fermentation because the models mirror some general behaviour like the expression for rate suggested by Monod, consequently they proposed the following expression with a negative term including substrate concentration to describe their experiments:

$$\frac{dp}{dt} = A\frac{dx}{dt} + B.x - c.x.s \qquad \text{(I-87)}$$

Experimental data obtained by Jorgenson and Nikolajsen, (1987) were accurately fitted by this equation.

Roy and co-workers, (1987) also considered a partial association between growth and production (Eq.I-81) to describe production kinetics. They determined the part of lactic acid synthesized by both mechanisms, growth-associated (p_A) (Eq.I-88) and non-growth-associated (p_B) (Eq.I-89):

$$p_A = A\frac{dx}{dt} = Ax_0\left(\frac{e^{\mu_{max}t}}{1 - \frac{x_0}{x_{max}}(1 - e^{\mu max t})} - 1\right) \qquad \text{(I-88)}$$

$$p_B = Bx = B\frac{x_{max}}{\mu_{max}}\ln\left(1 - \frac{x_0}{x_{max}}(1 - e^{\mu max t})\right) \qquad \text{(I-89)}$$

According to Amrane, (1991), experimental data obtained by Roy and co-workers, (1987) were not satisfactorily described by the model.

Leh and Charles, (1989) also used a law of production which involved carbon substrate consumption:

$$\frac{dp}{dt} = -Y_{P/S,G}\left(\frac{ds}{dt}\right)_G - Y_{P/S,M}\left(\frac{ds}{dt}\right)_M \quad \text{(I-90)}$$

Where $Y_{P/S,G}$ and $Y_{P/S,M}$ were the product on carbon substrate yield related to growth (G) and maintenance (M), respectively. Determination of both yields cannot be experimentally checked, since only their sum can be experimentally calculated.

Amrane, (1991) and Amrane and Prigent, (1994a and b) noted during their experiments that the experimental points obtained at the beginning of the production are well described by a Luedeking and Piret relation, $q_p = A\,\mu + B$, namely for significant values of the specific growth rate. Contrarily, almost half of the lactic acid is produced during the deceleration growth phase and the stationary state, whereas the specific growth rate tended to a zero value. This part of production is not satisfactorily described by the Luedeking and Piret relation, which cannot account for the decrease of the specific production rate for nil value of the specific growth rate. Consequently, these authors modified the model of Luedeking and Piret, (1959) by introducing an additive term:

$$q_p = \frac{dp}{x.dt} = A\mu + B\left[1 - \exp(-F.\mu)\right] \quad \text{(I-91)}$$

Where *A* and *B* are both coefficients for growth-associated and non-growth-associated production respectively, *F* an additional term (dimensionless). This relation allowed to describe the part of lactic acid described during stationary state.

Similarly for growth, Fredereco et al, (1994) developed for production, the following equations:

$$\frac{d[La_{t,s}]}{dt} = Y_{La/x}\left(\mu[x_v] + y_s[x_v]\right) \quad \text{(I-92)}$$

$$\frac{d[Ma_t]}{dt} = -y_{Ma}[x_v] \quad \text{(I-93)}$$

$$\frac{d[La_{t,Ma}]}{dt} = -\frac{d[Ma_t]}{dt} \quad \text{(I-94)}$$

$$\frac{d[La_t]}{dt} = \frac{d[La_{t,s}]}{dt} + \frac{d[La_{t,Ma}]}{dt} \qquad \text{(I-95)}$$

This model, where $[La_{t,s}]$ $[Ma_t]$ $[La_{t,Ma}]$ $[La_t]$ were total lactic acid concentration from hexose fermentation, total malic acid concentration, total lactic acid concentration from molate utilization and total lactic acid concentration, represents the data obtained by Fredereco et al, (1994) in batch culture, very well.

Concerning the production of lactic acid, Wang et al., (1995) also considered the Luedeking and Piret relation (Eq.I-81) which proved to be valid and applicable in many fermentation processes (Keller and Gerhardt, (1975); Roy et al., (1987); Bibal et al., (1989)). The agreement between the calculated and the experimental data was reasonably good, according to theses authors. On the other hand, values of 8.77 and 0,33 (1/h) for growth- and non-growth-associated coefficients were found, lets to say that production was almost completely linked to growth.

Dutta et al., (1996) proposed the following modified Luedeking-Piret equation for product formation:

$$\frac{dp}{dt} = \left[A\mu_{max}\left(1 - \frac{C_P}{C_P^*}\right)^n + B \right]\left[\frac{sx}{K_S + s}\right] \qquad \text{(I-96)}$$

According to these authors, this modified model would be helpful in both batch and continuous lactic acid fermentation of glucose.

During the lactose fermentation with the *strain Lactobacillus casei* lactic acid production is mainly non growth associated according to Peeva and Peev, (1997). Hence they proposed the following relationship to describe their experimental data:

$$\frac{dp}{dt} = \beta x\left(1 - k_i P^{\alpha}\right) \qquad \text{(I-97)}$$

Where β was the biomass productivity coefficient [mol/ g.s]

The exponent α strongly depends on the lactose initial concentration and for its calculation, the following relationship was derived:

$A = 6.13\ p_{max} - 0.056$

where p_{max} is the theoretical lactic acid concentration which should be obtained after a complete fermentation of the substrate.

In their experimental work dealing with the conversion of Beet Molasses to lactic acid by the homofermentative organism *Lactobacillus delbruckii C.E.C.T 286* Owing to the inhibitory effect of lactic acid, Monteagudo et al., (1997) modified the Luedeking-Piret, (1959) model by the addition of a term indicating the dependence of the rate of lactic acid production on its concentration:

$$\frac{dp}{dt} = \left(A\frac{dx}{dt} + Bx \right)\left(1 - \frac{p}{p'_{max}} \right) \qquad \text{(I-98)}$$

Where the production rate tended towards a nil value when the maximum lactic acid concentration p_{max} was achieved. According to these authors, the developed model described accurately their experimental results. Indeed, on one hand they concluded that the accumulation of lactic acid inhibits the development of *L. delbruckii* C.E.C.T 286 on Beet Molasses, and on the other hand bacteria were able to produce lactic acid even after growth ceased (the corresponding growth- and non-growth-associated parameters were A=0.235 and B=0.087 1/h).

Akerberg et al., (1998) also considered the equation of Luedeking and Piret, (1959). According to their analyses, these authors noted that the production of acid lactic was growth-associated. Indeed, the values of the growth- and non-growth-associated parameters were 13.2 and 0.064 h^{-1}, respectively.

Gonzalo Acuna et al., (1998) used the neurone network method to describe their experimental results:

$$\frac{dp}{dt} = \nu(P, T, pH)x \qquad \text{(I-99)}$$

In this equation, biomass and lactic acid concentrations were chosen as the state variables of the network while pH and temperature corresponded to the control variables. According to theses authors, this model offered more stable responses, due to an implicit corrective action arising from the training methodology and the associated method for biomass estimation.

Similarly to growth, Moldes et al., (1999) proposed for lactic acid production the following relation:

$$\frac{dp}{dt} = P_0^{'}.\left(1 - \frac{p}{p_m}\right).p \qquad \text{(I-100)}$$

where p_0 was the initial product concentration.

The experimental data were closely interpreted by the model, according to these authors.

The Luedking-Priet, (1959a) was also slightly modified by Berry et al, (1999):

$$r_p = \frac{dp}{dt} = Ax\mu + xs(K_S + s) \qquad \text{(I-101)}$$

Values of 0,389 and 0,0025 were found for the parameters A and B, indicating that lactic acid production by *L. rhamnosus* was predominantly growth-associated. A yield of 1.67 moles lactic acid per mole glucose consumed was found.

Fu and Mathews, (1999) showed that lactic acid fermentation of *L. plantarum* was a homolactic process mainly growth-associated. Consequently, they proposed the following equation:

$$p = Y_{P/S}(pH)(s_0 - s) + p_0 \qquad \text{(I-102)}$$

where

$Y_{P/S} = 1.036\exp\left(-0.092(pH - 6.0)^2\right)$ was the product yielding coefficients, p_0, and s_0 were the initial values of product and substrate, respectively.

This model proved to describe accurately the experimental results.

Amrane, (2001) used two medium supplementations, a rich medium and a poor medium, differing by the quantity of available nitrogen. As expected, the rich medium resulted in a higher maximum biomass concentration. Cessation of growth can be related to the available “usable” nitrogen, while cessation of production always occurred when lactose became exhausted from the medium, since resting cells are unable to use carbon from death cells (Amrane and Prigent, 1997). A corrective term was therefore added to the Luedeking-Piret expression taking into account the substrate limitation:

$$\frac{dp}{dt} = A\frac{dx}{dt} + Bx\left(1 - \frac{x_{res}}{s}\right) \qquad \text{(I-103)}$$

where s and s_{res} were the lactose concentrations at time t and at the end of the batch, respectively. Experimental data obtained by Amrane, (2001) were accurately fitted by this equation.

Burgos-Rubio et al., (2000) found a good agreement by using the Luedeking-Piret model to fit their experimental results. These authors reported that the conversion into lactic acid is more significant by using glucose ($Y_{P/S}$ = 0.9) than galactose and affirmed that the fermentative process is growth-associated (A = 9).

Leroy and De Vuyst, (2001) proposed this simple model, to describe their experimental data using *Lactobacillus Sakei* strain CTC494:

$$\frac{dp}{dt} = -Y_{p/s}\frac{ds}{dt} \qquad \text{(I-104)}$$

Experimental data obtained by Leroy and De Vuyst, (2001) were accurately fitted by this model.

Amrane and Couriol, (2002) proposed the following relationship to describe culture without ph control, based on the variations of the specific growth rate with the undissociated lactic acid concentration:

$$[HL] = -[HL]_C . \ln\left[\frac{\mu_{max} - c}{\mu_{max} - c + ce^{d.t}} + \frac{\mu_0}{\mu_{max}}\right] \quad \text{(I-105)}$$

where c, d were constants, μ_0 and $[HL]_C$ are constants coefficients in the inhibition relation respectively.

This equation account well for the experimental values recorded in batch culture without pH control. It should be observed that the equation of Handerson–Hasselbatch was used for the calculation of the undissociated lactic acid concentration [HL].

Schepers et al., (2002b) considered the Luedeking and Piret relation but with variable growth- and non-growth-associated coefficients vary with the various operating conditions:

$$\frac{dp}{dt} = a(pH, s, z, p)\frac{dx}{dt} + b(pH, s, z, p)x \quad \text{(I-106)}$$

In agreement with previous work (Schepers and co-workers, 2002a), the effect of the growth-associated parameter was not significant; hence this parameter was maintained constant in the model:

$$\frac{dp}{dt} = A\frac{dx}{dt} + (b + B.pH_C)\frac{s}{s + K_S}.\left(\frac{e^{-K_{i,[HL]}\frac{p}{1+10^{(pH-pKa)}}}}{1 + e^{K_{P,La-}\left(\frac{p}{1+10^{(pKa-pH)}} - K_{i,[L^-]}\right)}}\right).x \quad \text{(I-107)}$$

This equation describes well the results of Schepers et al., (2002b) under various culture conditions, but appears somewhat complex.

The rate at which the product is accumulated in the bioreactor was expressed by the following relationship according to Biazar et al, (2003)**:**

$$\frac{dp}{dt} = \left[\left(\mu_m A\frac{s}{K_S + s}e^{-(s/K_i)^{n_1}}e^{-(p/K_{ip})^{n_2}} - K_d\right) + B\right]x \quad \text{(I-108)}$$

where K_s and K_d were the initial substrate concentrations at half the maximum specific cell growth rate and specific cell death, respectively. Experimental data were well fitted by this model.

Boonmee et al. (2003) proposed for lactic acid production the following equation:

$$\frac{dp}{dt} = A.\frac{dx}{dt} + q_{p,max} B.\left(\frac{s}{K_{SP} + s}\right)\left(1 + \frac{p - p_{iP}}{p_{mP} - p_{iP}}\right)\left(\frac{K_{iX}}{K_{iP} + s}\right)x \qquad \text{(I-109)}$$

where K_s and K_i were the lactose limitation constant and the lactate inhibition constant respectively; qp_{max} is the maximum specific lactate production rate; p_m and p_i were maximum and threshold lactate concentrations respectively.

These authors showed that the growth-associated production was relatively low (0.932), while the non-growth-associated production was higher (3.02).

According to Bâati et al, (2004) the evolution of the specific lactate production rate *(q_P)* as a function of the lactate concentration can be described using an exponential type function:

$$q_P = q_{Pmax} e^{-K_P P} \qquad \text{(I-110)}$$

The maximum specific lactic acid production rate was given by the following expression:

$$q_{Pmax} = \left(K_b \Theta - K_C\right)^2 \qquad \text{(I-111)}$$

According to their results, at low temperatures growth ceased before the exhaustion of glucose from the culture medium. The effect of a substrate limitation was expressed by the following equation:

$$q_P = q_{Pmax} \frac{s}{K_a + s} \qquad \text{(I-112)}$$

By taking into account both effects (Eqs.I-111 and I-112), the specific lactic acid production rate can be written in the following form:

$$q_P = q_{Pmax} \frac{s}{K_a + s} e^{-K_P p} \qquad \text{(I-113)}$$

In this equation q_{Pmax} was the maximum specific lactic acid production rate, the terms K_b and K_c were two constants, K_S was a substrate anabolic constant of affinity of the proliferating cells and K_P was a product inhibition constant.

According also to these authors, this model allows to describe correctly the observed lactic acid production in discontinuous cultures carried out at various growth temperatures.

Similarly to growth, production kinetic can be deduced for each phase (Amrane, 2005). According to this author, this model was applied to the case of low supplementation of culture medium, namely in the case of nitrogen limitation. The model was also successfully applied to the more simple case of high supplementation of culture medium, namely in absence of nitrogen limitation.

During the experiments, partial coupling between growth and production and substrate limitation on uncoupled production were observed. Consequently the specific production rate was modelled as follows Ben Youssef et al., (2005):

$$q_P = A\mu + B\left(\frac{s}{\overline{K}_S^{gc} + s}\right) \qquad \text{(I-114)}$$

where A and B were the coefficients for growth- and non-growth-associated production, and $\overline{K}_S^{gc}$ was the affinity constant of the resting cells for glucose. The model matched experimental data under various culture conditions (Ben Youssef and al. 2005).

Altiok et al., (2006) showed that only the growth-associated coefficient of the Luedeking-Piret relation displays a great variability depending on the initial carbon substrate concentration. According to a previous study (Amrane and prigent, 1997), Altiok et al., (2006) calculated this coefficient using the following equation:

$$p - p_0 = A(x - x_0) \qquad \text{(I-115)}$$

However these authors noted that the model developed by Amrane, (2001) was not appropriate to describe their experimental results. However, this conclusion seems obvious, owing to the absence of carbon limitation.

Nandasana and Kumar, (2007) observed a significant effect of lactic acid inhibition, while the effects of substrate limitation and substrate inhibition were found to be relatively weak. They consequently propose a modified Boonmee et al., (2003) equation (Eq.I-109):

$$\frac{dp}{dt} = A\frac{dx}{dt} + q_{p,max}\left(\frac{s.K_{ip}}{(K_{SP}+s)(K_{ip}+s)}\right)e^{-P/Kpp}.x \qquad \text{(I-116)}$$

The model was found to provide good predictions of experimental lactic acid production data

For lactic acid production from both glucose and fructose substrates from date juice by *L. casei subsp. Rhamnosus* Nancib, (2007) noted that production was growth associated, and hence proposed a modified Luedcking-Piret, (1959) equation:

$$\frac{dp}{dt} = A\frac{dx}{dt} \qquad \text{(I-117)}$$

$$\frac{dp_{s_1}}{dt} = A_{S_1}\frac{dx_{S_1}}{dt} \qquad \text{(I-118)}$$

$$\frac{dp_{S_2}}{dt} = A_{S_2}\frac{dx_{S_2}}{dt} \qquad \text{(I-119)}$$

After rearrangement, the following equation was obtained:

$$\frac{dp}{dt} = \left[x_{S_1}\left[A_{S_1}\left(\mu_{\max S_1}\frac{s_1}{K_{s_1}+s_1}\right) - K_{DS_1}\right] + x_F\left[A_{S_2}\left(\mu_{\max S_2}\frac{s_2}{K_{s_2}+s_2} - K_{Ds_2}\right)\right]\right] \qquad \text{(I-120)}$$

where s_1 and s_2 were substrates concentrations for glucose and fructose respectively.
The model gave a good fit of production kinetics until only about 15 h of culture, according to these authors.

In the case of the production of lactic acid, Vazquez and Murado, (2008a) proposed the following equation, with the same calculation applied to the biomass:

$$p = \frac{p_{max}}{1+\exp\left[2+\frac{4\upsilon_{mp}}{p_{max}}(\lambda_p - t)\right]} \qquad \text{(I-121)}$$

where p was the product concentration, υ_{mp} and λ_p were maximum production rate (h^{-1}) and product lag phase (h), respectively.

Vazquez and Murado, (2008b) also proposed another model witch based on the Luedicking-Piret model as follow:

$$p = \frac{A.x_{max}}{1+\left(\frac{x_{max}}{x_0}-1\right)\exp\left(-\frac{4v_{mx}.t}{x_{max}}\right)} - Ax_0 + \frac{B.x_{max}^{\ 2}}{4.v_{mx}}.Ln\left[\frac{x_0.\left(e^{\frac{(4v_{mx}t)}{x_{max}}}-1\right)+x_{max}}{x_{max}}\right] \qquad \text{(I-122)}$$

where υ_{mX} and λ_X were maximum growth rate (h^{-1}) and growth lag phase (h), respectively. Theses two models predictions were found to match well with the experimental data of *Pediococcus acidilactici* NRRL B-5627 and *Lactococcus lactis subsp.lactis* growth on sugar, according to theses authors

I-2-b. Continuous fermentation

Continuous Fermentation is characterized by continuous fresh substrate supply and the removal of an equivalent volume of broth to maintain constant the volume of medium in the bioreactor; consequently, after an initial phase of transition all parameters remained constants. At steady state, the following assumptions can be made: (a) the reactor is completely mixed; the composition of the effluent is identical to that in the reactor ($s=s_e$, $x=x_e$), (b) there are no microbial cells entering the system ($x_i=0$), (c) the microbial cell concentration in the reactor does not vary with time; the quantity of microbial cells in the system is equal to that removed from the system for a given time increment ($dx/dt=0$) and (d) the substrate concentration in the reactor does not change with time ($ds/dt=0$). Based on these assumptions, the microbial mass balance, substrate mass balance and product mass balance can be computed.

A microbial mass balance for one stage continuous fermentation can be described as follows:

Accumulation = Inlet + Production – Outlet – Consumption

It was drawn for each species

- For biomass:

$Vdx = Fx_0dx + V\mu xdt - Fxdt$

By dividing the two parts of the equation by Vdt, and by replacing F/V by D, it comes:

$$\frac{dx}{dt} = (\mu - D).x \qquad \text{(I-123)}$$

- For product:

If q_p is the specific productivity of the cells, the mass balance for the product is:

$Vdp = Fp_0dt + Vq_pxdt - Fpdt.$

While dividing by *Vdt* and replacing *F/V* by *D*, it comes:

$$\frac{dp}{dt} = q_P x - D.p \qquad \text{(I-124)}$$

with no product in the feeding ($p_0 = 0$).

- For carbon substrate:

Carbon substrate consumption can be expressed as follows:

$$Vds = Fs_0dt - V\frac{q_P x}{Y_{P/S}}dt - Fsdt$$

Dividing by Vdt, one obtains:

$$\frac{ds}{dt} = D(s_0 - s) - \frac{q_P x}{Y_{P/S}} \qquad \text{(I-125)}$$

- For Nitrogen substrate:

Nitrogen is only used for biomass formation. The consumption of nitrogen can be then expressed as follows:

$$Vdz = Fz_0dt - V\frac{\mu x}{Y_{X/Z}}dt - Fzdt$$

z and z_0 are the nitrogen concentration at a given time and its initial value; and $Y_{x/z}$ is the biomass on nitrogen substrate yield. The preceding relation becomes after divided by Vdt:

$$\frac{dz}{dt} = D(z_0 - z) - \frac{\mu x}{Y_{X/Z}} \qquad \text{(I-126)}$$

The above system cannot always be analytically solved. In the case of a substrate limitation (Monod behaviour (1942)), the resolution of the above system leads at stationary state to:

$$\frac{ds}{dt} = 0 \Rightarrow \bar{s} = \frac{K_S.D}{\mu_m - D} \qquad \text{(I-127)}$$

Combination of equations (I-125) at steady state and (I-127) leads to:

$$\bar{x} = D(s_0 - \frac{K_S.D}{\mu_m - D}).\frac{Y_{P/S}}{q_P} \qquad \text{(I-128)}$$

Volumetric productivity is given by the following expression, after substitution equation (I-128) into equation (I-129):

$$D\,\bar{p} = q_P\,\bar{x} \qquad \text{(I-129)}$$

$$D.\bar{p} = D.Y_{P/S}(s_0 - \frac{K_S.D}{\mu_m - D}) \qquad \text{(I-130)}$$

The first derivative of this expression is equal to zero for $D=D_{opt}$

$$D_{opt} = \mu_m(1 - \sqrt{\frac{K_S}{K_S + s_0}}) \qquad \text{(I-131)}$$

Since K_S (about a few tens of mg/l for sugars) is negligible in front of s_0 (about 48 g/l) for whey, D_{opt} is practically equal to μ_m. Dilution rates higher than D_{opt} leads to bioreactor washout (sudden decrease of the biomass). Working at lower dilution rates is therefore necessary.

According to Amrane, (1991), this model is not applicable to lactic fermentation, since it does not take into account the product inhibition. Luedeking-Piret, (1959b) resolved in the

case of a law inhibition proportional given by the equation (I-8) the system established by the equations balance:

By substituting the relationship (I-8) in Equation (I-123) at steady state one can be obtained for product:

$$\bar{p} = (\mu_m - D)/h \qquad \text{(I-132)}$$

If we consider the above equation (I-132) by combining the preceding equation with the partially growth associates production law the (I-81) and the equations (I-123) at steady state and (I-129), it possible to obtain x at steady state:

$$\bar{x} = D\frac{\mu_m - D}{h(\alpha(+ \beta)} \qquad \text{(I-133)}$$

Similarly we can obtain the substrate concentration at steady state as follows:

$$\bar{s} = s_0 - \frac{\mu_m - D}{h.Y_{P/S}} \qquad \text{(I-134)}$$

Maximum volumetric productivity $D.\bar{p}$ is obtained for $D_{opt} = \mu_m/2$. From this point the biomass starts to decrease but not sharply. As in the case of a substrate limitation, the biomass becomes nill for a dilution rate D equal to μ_m. This model accounts for the experimental results of Luedeking – Piret, (1959b) rather well, and was also considered by several authors, since they also observed a linear relation between $\bar{p}$ and *D* (Major et Bull, 1985; Mehaia et Cheryan, 1986).

Herbert (1962) modelled continuous two stages fermentation, with different volume. If the first or the second reactors or both reactors are fed with medium containing the limiting substrate *s*, but with different substrate concentrations *s* in the sterile feed of each stage.

Microbial mass balance for the first stage can be described as follows:

$$\frac{dx_1}{dt} = \mu_1.x_1 - D_1x_1 \qquad \text{(I-123)}$$

For substrate concentration:

$$\frac{ds_1}{dt} = D_1 s_{01} - D_1 s_1 - \frac{\mu_1 x_1}{Y_{X/S}} \quad \text{(I-135)}$$

At steady state conditions ($\frac{dx}{dt} = 0 ; \frac{ds}{dt} = 0$) and after rearrangement the following expression was obtained:

$$\bar{s}_1 = K_S \frac{D_1}{\mu_m - D_1} \quad \text{(I-127)}$$

For the second stage, the accumulation was given by the following expression:

$$\frac{dx_2}{dt} = D_{12} x_1 - D_2 x_2 + \mu_2 x_2 \quad \text{(I-136)}$$

In this expression, the dilutions rates D_1, D_{12} and D_2 where equal to F_1/V_1, F_1/V_2 and F_2/V_2, respectively. $D_{12}x_1$ represented the biomass arrived from the first reactor.

For substrate concentrations:

$$\frac{ds_2}{dt} = D_{12} s_1 + D_0 s_{02} - D_2 s_2 - \frac{\mu_2 x_2}{Y_{X/S}} \quad \text{(I-137)}$$

where D_{02} equal to F_{02}/V_2

After elimination the specific growth rate between these equations, biomass in the steady state becomes ($\frac{dx_2}{dt} = 0 \ ; \frac{ds_2}{dt} = 0$):

$$\bar{x}_2 = Y_{X/S}\left(\frac{D_{12}\,\bar{s}_1}{D_2} + \frac{D_{02} s_{02}}{D_2} - \bar{s}_2\right) + \frac{D_{12}\,\bar{x}_1}{D_2} \quad \text{(I-138)}$$

After rearrangement one obtained, for $\bar{x}$:

$$\bar{x}_2 = \frac{Y_{X/S}}{\mu_m s_2}\left(K_S + s_2\right)\left(D_{12} s_1 + D_{02} s_{02} - s_2\right) \quad \text{(I-139)}$$

An implicit expression for the substrate concentration $\bar{s}_2$ versus D_2 at steady state can be deduced by rearrangement of the above equations:

$$(\mu_m - D_2)\bar{s_2^2} - (\frac{\mu_m D_{12} s_{01}}{D_2} + \frac{(\mu_m - D_2) D_{02} s_{02}}{D_2} - K_S D_{12} \frac{D_1}{\mu_m - D_1} + K_S D_2)\bar{s_2} + K_S D_{02} s_{02} + K_S D_{12} \bar{s_1} = 0 \quad \text{(I-140)}$$

The same mathematical treatment can be used if the process involved more than two stages.

The author supposed that the yield $Y_{X/S}$ was constant throughout culture. However, lactic acid production and hence substrate consumption continued after growth cessation. Moreover, this model assumed a substrate limitation (Monod law), which is not necessarily the case in lactic acid fermentation.

Keller and Gerhardt, (1975) modelled several stages continuous fermentation, and considered a substrate limitation and a proportional inhibition by the formed product, as well as a partial association between growth and production:

$$\mu = \mu_m \frac{s}{s + K_S}.(1 - p/p_m) \quad \text{(I-141)}$$

This model, where p_m is maximum product concentration, based on the Monod (1942) equation, According to Amrane, (1991) this give a good agreement with a few experimental data presented.

Rogers et al., (1978) applied their kinetic model developed for batch culture of *S. cremoris* growing under lactose limitation to the continuous culture

$$k_1 \left(\frac{s}{K_S + s} \right) \left(\frac{K_P}{K_P + p} \right).x - D.x = 0 \quad \text{(I-142)}$$

$$D.L_0 - \frac{1}{Y_P} \frac{dp}{dt} - D.L = 0 \quad \text{(I-143)}$$

$$k_4 \frac{dx}{dt} + k_5 \left(\frac{s}{K_S' + s} \right).x - D.p = 0 \quad \text{(I-144)}$$

YE and al., (1996) studied the performance improvement of lactic acid fermentation by multistage extractive fermentation using *Lactobacillus delbrueckii* IAM1928 in continuous

mode. Mass balances for biomass, lactic acid and substrate concentrations are described as follows, respectively:

$$\frac{dx}{dt} = \mu x \tag{I-1}$$

$$\frac{dp}{dt} = Q_p + \frac{F_e}{V} p_a + \frac{F_{if}}{V} p - \frac{F_{ef}}{V} p \tag{I-145}$$

$$\frac{ds}{dt} = -\frac{1}{Y_{X/S}} \mu x - \frac{1}{Y_{P/S}} Q_P \tag{I-146}$$

The flow rate of the aqueous phase can be computed by subtracting F_{if} from F_{ef} as:

$F_e = F_{ef} - F_{if}$

where F_e, F_{ef}, F_{if} were flow rate of aqueous phase, removal rate of filtrate, recycle rate of cell concentrated broth, ml. min^{-1} respectively.

qp is the production rate of lactic acid and can be expressed, according to the Luedeking – Piret relation (1959a) as follows:

$$q_P = (A + B\mu)x \tag{I-147}$$

The specific cell growth rate, μ, was assumed to be only a function of the lactic acid concentration, since the substrate concentration was usually high in homofermentative production of lactic acid. Therefore, μ may be given by the following equation:

$$\mu = \mu_m \exp(ap) \tag{I-148}$$

where a is a model parameter (g/l).

Pinelli and al., (1997) proposed for L-and D-Lactic acid productions using *Lactobacillus casei* DMS 20011 and *Lactobacillus coryniformis* DMS in continuous fermentation a kinetic model basing on the following mass balance:

$$\frac{dx}{dt} = (\mu - D)x \tag{I-123}$$

They analysed their experimental result using two following non competitive product inhibition models:

$$\mu = \frac{\mu_{max} s}{\left(K_S + s\right)\left(1 + {}^{p}/_{K_i}\right).} \quad \text{(I-149)}$$

$$\mu = \frac{\mu_{max} s}{\left(K_S + s\right).} \exp\left(-{}^{p}/_{K_i}\right) \quad \text{(I-150)}$$

where K_i and K_S were product inhibition and saturation constants g l^{-1} respectively.

Pinelli and al., (1997) concluded that the first non-competitive product inhibition model proved quite attractive for the description of L(+) lactic acid fermentation, while the second one was more relevant to describe the D(-) Lactic acid fermentation.

To describe continuous Production of Lactic Acid by *Lactobacillus rhamnosus* in a two-stage membrane cell-recycle bioreactor, Kown et al., (2001) developed a kinetic model based on mass balance. The growth, production and substrate consumption rates were:

$$\frac{dx}{dt} = (1-\theta)Dx_{in} + r_x - BDx \quad \text{(I-151)}$$

$$\frac{dp}{dt} = (1-\theta)Dp_{in} + r_x - Dp \quad \text{(I-152)}$$

$$\frac{ds}{dt} = (1-\theta)Ds_{in} + r_x - Ds \quad \text{(I-153)}$$

where the parameter θ was calculated by incorporating a titration constant γ which depends on the composition and concentration of the base solution, assuming that the pH drop in the reactor was only affected by the lactic acid formation:

$$\theta F = \gamma . r_p . V \quad \text{(I-154)}$$

Or

$$\theta = \frac{\gamma . r_p}{D}$$

And γ could be deduced from experimental data.

For cell growth and by taking into account the product inhibition on cell growth, the Levenspiel's model with Monod equation was used (Levenspiel, (1980)):

$$\mu = \frac{r_X}{x} = \mu_m \frac{s}{K_S + s}\left(1 - \frac{p}{p_m}\right)^c \qquad \text{(I-155)}$$

where K_S was the saturation constant in Monod equation (g/l), c was the toxic-power constant in the Levenspiel's product-inhibition model.

And for lactic acid formation, the Luedeking-Piret equation was considered in this work. According to these authors, the model was found to be applicable to most of the existing data with MCRB (performance of membrane cell recycle bioreactor) and was in good agreement with Levenspiel's product-inhibition model and the Luedeking-Piret equation appeared to be effective to describe production kinetics.

According to Ajbar and Fakeeha, (2002), the continuous bioreactor was described by the following unsteady-state mass balance equations for the limiting substrate s, the biomass x and the product P:

$$\frac{dx}{dt} = (\mu - D).x \qquad \text{(I-123)}$$

$$\frac{ds}{dt} = D(s_f - s) - a\mu x \qquad \text{(I-156)}$$

$$\frac{dp}{dt} = b\mu x - D.p \qquad \text{(I-157)}$$

where s_f was the substrate feed concentration, D the dilution rate, $\mu(s, p)$ the specific cell growth rate, and a, b are constant stochiometric coefficients.

The model (Eqs. I-123, I-156 and I-157) became dimensionless by introducing the following variables:

$$\bar{s} = \frac{s}{s_{ref}};\ \bar{x} = \frac{ax}{s_{ref}};\ \bar{p} = \frac{p}{p_{ref}};\ \alpha = \frac{bs_{ref}}{ap_{ref}};\ \bar{D} = \frac{D}{\mu_{ref}};\ \bar{t} = t\mu_{ref};\ \bar{\mu} = \frac{\mu}{\mu_{ref}}$$

where s_{ref}, p_{ref}, and μ_{ref} were reference values for s, p, and μ respectively. The model in its dimensionless form was given by:

$$\frac{d\bar{x}}{dt} = \bar{\mu}.\bar{x} - \bar{D}.\bar{x} \quad \text{(I-158)}$$

$$\frac{d\bar{s}}{dt} = \bar{D}\left(\bar{s}_f - \bar{s}\right) - \bar{\mu}\bar{x} \quad \text{(I-159)}$$

$$\frac{dp}{d\bar{t}} = \alpha\bar{\mu}.\bar{x} - \bar{D}.\bar{p} \quad \text{(I-160)}$$

The steady-state values of *x* and *p* were related to *s* by the following simple relations:

$$\bar{x} = \left(\bar{s}_f - \bar{s}\right) \quad \text{(I-161)}$$

$$\bar{p} = \alpha.\left(\bar{s}_f - \bar{s}\right) \quad \text{(I-162)}$$

Three models are tested in this study; the first model was based on the well-known Haldane substrate inhibition kinetics with the addition of an inhibitory effect of the product. The growth rate μ(s, p) was assumed to have the following form:

$$\mu(s,p) = \mu_1(s)\mu_2(p)$$

$$\mu_1(s) = \frac{\mu_{max} s}{K_S + s + s^2/K_i} \quad \text{(I-163)}$$

$$\mu_2(p) = \left(1 - \frac{p}{p_{max}}\right)^n \quad \text{(I-164)}$$

The second example pertained to the following form:

$$\mu_2(p) = \frac{K_P}{K_P + p} \quad \text{(I-165)}$$

The third and last example consisted in the following form of μ_2 (p):

$$\mu_2(p) = e^{-\lambda P} \quad \text{(I-166)}$$

where K_i, Kp, Ks were substrate inhibition constant in cell growth rate, kinetic parameter respectively and p_m was the saturation constant in cell growth rate.

Boonmee et al. (2003) applied their kinetic model developed for batch fermentation to continuous cultures. The model equations were derived from the batch model equations. Resolution of the system for steady state conditions for biomass, substrate and product concentrations led to:

$$\frac{dx}{dt} = \mu_{max}\left(\frac{s}{K_{SX}+s}\right)\left(1+\frac{p-P_{iX}}{P_{mX}-P_{iX}}\right)\left(\frac{K_{iX}}{K_{iX}+s}\right)x - Dx \qquad \text{(I-167)}$$

$$\frac{dp}{dt} = \alpha\frac{dx}{dt} + q_{p,max}\left(\frac{s}{K_{SP}+s}\right)\left(1+\frac{p-P_{iP}}{P_{mP}-P_{iP}}\right)\left(\frac{K_{iX}}{K_{iP}+s}\right)x - Dp \qquad \text{(I-168)}$$

$$\frac{ds}{dt} = D(s_0 - s) + q_{S,max}\left(\frac{s}{K_{SS}+s}\right)\left(1+\frac{p-P_{iS}}{P_{mS}-P_{iS}}\right)\left(\frac{K_{iX}}{K_{iP}+s}\right)x \qquad \text{(I-169)}$$

According to theses authors, the simulation results provided a very good agreement between the model and the experimental data.

Richter and Nottelmann, (2004) proposed to describe their experimental data using Lactobacillus *paracasei* ATB160111 in a membrane reactor system, an empirical steady state model:

$$p = a + b\Big/\left(1+\exp\left(-\left(s_N - c\right)/d\right)\right) = a + SIGC_N(b,c,d) \qquad \text{(I-170)}$$

where *p* was lactic acid conentration, SIG abbreviation for sigmoid s_N was the nutrients supply and a, b, c, d were constants.

Lin and Wang, (2007) considered a multistage integrated continuous fermentation process for producing lactic acid. Each stage consists of a mixing tank, a bioreactor, a cell recycle unit, and an extractor. A generalized mathematical model was formulated to express the integrated process. Lin and Wang, (2007). have compared the overall productivity and conversion of the integrated process with those of two simplified processes.

I-3. Structured Models:

I-3.a. Growth Kinetics

During their studies for lactic acid fermentation using Lactococcus lactis subsp, lactis biovar. Diacetylactis in batch culture Cachon and Diviès, (1993) proposed a kinetic structured model based on the inhibitory effect of lactic acid on the cellular activity and this inhibition was described as non-competitive (Rogers et al., 1978; Ohara et al., (1992)). Thus, the specific growth rate (μ) and the specific lactate production rate (π) can be respectively described as follow:

$$\mu(p) = \mu_{max} . \frac{K_{P\mu}}{K_{P\mu} + p} \qquad \text{(I-171)}$$

$$\pi(p) = \pi_{m} . \frac{K_{P\pi}}{K_{P\pi} + p} \qquad \text{(I-172)}$$

where p was the lactic acid concentration, μ_{max} the maximum specific growth rate, π_m the maximum lactate production rate, and $K_{p\mu}$ and $K_{p\pi}$ were the inhibition constants for growth and lactate production, respectively.

The increase in the total population can be then expressed as:

$$\frac{dx}{dt} = \mu_{m} \frac{s}{K_S + s} . \frac{K_{P\mu}}{K_{P\mu} + p} . x_g \qquad \text{(I-173)}$$

$x = x_g + x_{ng}$

where $x_{g,,}$ x_{ng} are biomass in state of growth and in one of non-growth.

Concerning the structured models, Gadgil and Venkatesh, (1997) proposed to describe growth of *Lactobacillus bulgaricus* in batch fermentation a modified Monod (1942) equation:

$$\frac{dx}{dt} = \frac{\mu_{max} exs}{K_S + s} \qquad \text{(I-174)}$$

where e is the specific level of the enzyme, s and x are the concentration of the substrate (s) and the biomass (x), respectively. μ_{max} the maximum specific growth rate (h^{-1}) and K_s represents saturation constant of the limiting substrate.

$$\frac{dx}{dt} = \left(\frac{\mu_{max} \left(e/e_m\right)}{1 + \left[L^{-1}/K_I\right]} \right) \frac{sx}{K_S + s} \qquad \text{(I-175)}$$

$$\frac{d\left(e/e_m\right)}{dt} = \left(\frac{\mu_{max}}{1 + \left[L^{-1}/K_I\right]} + \beta \right) \frac{sx}{K_S + s} - B\frac{e}{e_m} - \frac{e}{x}\frac{dx}{dt} \qquad \text{(I-176)}$$

According to these authors, the model is able to predict biomass growth profile, at various values of pH.

I-3.b. Product kinetics:

Similarly to growth, the specific lactate production rate (π) can be describing, as follows (Cachon and Diviès, (1993)):

$$\pi(P) = \pi_m . \frac{K_{P\pi}}{K_{P\pi} + p} \qquad \text{(I-177)}$$

Production rate can thus be expressed as:

$$\frac{dp}{dt} = \pi . x_{g+ng} = \pi_m \frac{s}{K_S + s} . \frac{K_{P\pi}}{K_{P\pi} + p} . x_{g+ng} \qquad \text{(I-178)}$$

The model using non-competitive inhibition equations was satisfactory for lactic acid production, according to theses authors.

Gadgil and Venkatesh, (1997) proposed a model which took into account both effects of the pH and the lactate ion on the activity of the β-galactosidase. The amount of the synthesized enzyme was involved in the production rate. The developed model simulated the effect of both the pH and the lactic acid concentration on the expression and the degradation of the enzyme.

II. MATERIALS AND METHODS

II-1. 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 - 16°C. As required, these cultures were thawed and reactivated by two transfers in 10 % ($w\ v^{-1}$) skim milk (42°C, 24h).

II-2 Medias and Cultures Conditions

1. Cultures with and without pH control (Chapter III § 1 and 2)

Whey permeate powder (SIAB, Chateaubourg, France) was used as a carbon source; the powder was reconstituted at 57g l^{-1}, corresponding to a lactose concentration of 48g l^{-1}. Before use, permeate was clarified by a heat / calcium process (Fauquant et al. 1985). It was supplemented with 3g l^{-1} $CaCl_2$, $2H_2O$, and the pH was settled at 7.3. The solution was pumped through two heat exchangers at 80 and 16°C respectively (mean residence time: 20 seconds). The solution was left to decant overnight at 4°C, and the supernatant was then supplemented with a range of yeast extract concentrations, 5, 10, 20g l^{-1}, or the following *RM* supplementation (g l^{-1}): yeast extract (*YE*), 20; trypsic and pancreatic casein peptones, 5 each (all from Biokar, Pantin, France), Tween 80, 1 (Merk, Darmstadt, Germany).

Cultures were carried out in a 2 L reactor (Set 2M, SGI, Toulouse, France, magnetically stirred (300 rpm) at 42°C. pH was controlled at 5.9 by automatic addition of 10 mol L^{-1} NaOH.

Seed culture was carried out in a 0.25 L laboratory-designed glass fermentor equipped with a sterilizable combination glass electrode (Ingold, Paris, France), cotton plug filter, magnetic stirrer, infra-red lamp temperature control (set at 42°C), and an aseptic transfer line

In addition, both fermentors were equipped with an aseptic recirculation loop (Watson-Marlow 501 U peristaltic pump; Volumax, Montlouis, France) incorporating a laboratory-made turbidimeter.

Bacteria were precultivated 9 h without pH control on a sterile *RM* medium. Then 1.6L of pasteurised culture medium was inoculated with 0.2 L seed culture, and the reaction was left to proceed at 42°C at the required pH or without pH control.

Total biomass was deduced on-line from turbidimetric measurements after dry weight calibration; the observed standard deviation was ± 0.2 g L^{-1}. The amount of 10 mol L^{-1} of NaOH used for pH control corresponded to the quantity of lactate anion produced at a given pH. The concentration of the total (*p*) and undissociated (HL) lactic acid was then calculated using the Henderson-Hasselbach equation (pK_A = 3.8), the lactate concentration (L^-) and the corresponding pH value:

$$[HL] = \frac{p}{1+10^{pH-pK_A}} \qquad \text{(II-1)}$$

and

$$p = [HL] + [L^-] \qquad \text{(II-2)}$$

The observed standard deviation was ± 1 g L^{-1} for lactic acid concentrations.

2. Cultures at different controlled pH (Chapter III (*§*1 and 3)

Whey permeate powder (Armor-Protéines, St Brice, France) was used as a carbon source; the powder was reconstituted as previously described (*§III*-1).

Two sets of cultures were carried out at various pH control on *RM* medium (Amrane and Prigent 1999b) 1.6g L^{-1} of the culture medium were supplemented with aliquots of 1 mol L^{-1} of lactic acid in order to achieve initial concentrations p_i of 0, 2 and 5 g/l corresponding to pH values of 5.90, 4.63 and 4.04 respectively. During each run, the pH was maintained at its initial value by automatic addition of 10 mol L^{-1} of NaOH. The same procedure was applied

to culture media for which the initial pH was adjusted to the same values with hydrochloric acid instead of lactic acid.

A set of cultures were also carried out without pH control on whey supplemented with 5 or 10 g L^{-1} YE or the *RM* supplementation.

Whey based media contained 48g L^{-1} lactose, reconstituted from 67 g L^{-1} sweet cheese whey powder (EVEN Ltd). For the preparation of culture medium and just before, whey proteins were hydrolysed by means of 0.8g L^{-1} *Bacillus subtilis* endroprotease B500 (Gist-Brocades, Séclin, France) at 50°C and pH= 7.20 for 7 h (Leh and Charles, 1989). The hydrolysis progress was followed by continuous monitoring of the rate of 1 mol L^{-1} NaOH addition for pH control (Jacobsen et al., 1957; Adler-Nissen, 1984). No supplementation was added to the culture medium.

Seed culture medium (*RM*) was prepared as previously described (*§III*-1).

3. Continuous cultures (Chapter III § 4)

A schematic description of the system is given in Figure II-1.

For the first stage, a 250 mL glass reactor (§II-2.1) was used.

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 inoculated in the first-stage 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 h (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 h, 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 changed by varying the sole feed flow rate of sterile culture medium F_0 at constant culture volume V_c = 800 mL.

II-3.Analytical methods

1. Bacterial concentration

Bacterial concentration is determined by measurement of the dry weight, in the following way:

A known volume of broth is centrifuged at 300 rpm, during the required time for biomass separation, namely 20 mn.

- After supernatant removal, biomass is washed in distillated water and centrifuged again.
- After supernatant removal, the washed biomass is collected in crystallizers, dried (105°C for 16 h) and weighed.

The dry cellular weight is then deduced from the initial volume of centrifuged broth (Amrane, 1991)

2. Lactate concentration

Lactic acid concentrations in the supernatant were determined spectrophotometrically by the Fe^{3+} lactate complex method according to the method of Ling, (1951), modified by

Ayroulet - Martin and Fournaud (1979), which added to the original protocol a method of protein defecation by $NaOH\text{-}ZnSO_4$, in the presence of $BaCl_2$ (Amrane, 1991).

3. Sugar concentration

Lactose (as total sugars) concentrations in the supernatant was determined spectrophotometrically by the phenol-sulphuric acid method (Herbert et al., 1971), (Montgommery1961) this method is more precise than the Anthrone one (Herbert and al.,1971a).

4. Nitrogen concentration

The primary amino groups are determined spectrophotometrically by the method of Satake and al., (1959) with the trinitrobenzene-sulphonic acid (TNBS)(Amrane, 1991).

III.4 Numerical methods

For instantaneous rates, the derivative is taken by averaging the slopes of two adjacent data points as follows:

$$\left(\frac{\mathrm{d}\,y}{\mathrm{d}\,t}\right)_i = \frac{1}{2}\left(\frac{y_{i+1}-y_i}{t_{i+1}-t_i}+\frac{y_i-y_{i-1}}{t_i-t_{i-1}}\right) \qquad \text{(II-3)}$$

Where y corresponded to the cellular (x) , the undissociated lactic acid [HL] concentration or the lactic acid (p) concentration.

From an experimental array [t_i, $x_{i,\,exp}$], i = 1....N, and an initial parameters vector $\mathbf{P_0}$ = [x_0, μ_{max}, x_{max}]$_0$ for growth (Eq.III-2, Chapter III) the initial value for the target function χ^2 (no weighted sum of deviation squares) was calculated as follows:

$$\chi^2 = \sum_{i=1}^{N}\frac{[\mathbf{D_0}(i)]^2}{x_{i,cal}} \qquad \text{(II-4)}$$

The ith term of the initial deviations vector $\mathbf{D_0}$ was $\mathbf{D_0}$ (i) = $x_{i\ exp}$ - $x_{i,\,cal}$, where $x_{i,\,cal}$ was calculated by introducing t_i and $\mathbf{P_0}$ in equation ((Eq.III-2, Chapter III)) for growth.

Then a 'better' vector **P**1 (in the least squares sense) was drawn by a Levenberg–Marquardt algorithm (1977):

$$P_1 = P_0 + \Delta P_0 = P_0 + \left[J'.J + \lambda.I\right]^{-1}.\lambda.J'.D_0 \qquad \text{(II-5)}$$

Where *I* was the identity matrix, *J* the Jacobian matrix (partial derivatives of *xi* with respect to parameters), J' its transposed form, and λ an arbitrary scalar. In order to avoid any exponential divergence, an initial value λ=5000 for example was chosen; λ was decremented while the target function χ^2 decreased, until a relative change less than a predetermined tolerance was noticed for χ^2 between two successive iterations.

Since no analytical solution was found for Eqs.III-7, III-12 Chapter III, the identification of parameters vector [A,B] was carried out by means of a Newton–Gauss algorithm ; in equation (II-5) λ was made equal to zero, while *J* had to be calculated term by term through numerical integration (Runge-Kutta method) of the variational equation:

$$\frac{d}{dt}\left(\frac{\partial p}{\partial \mathbf{P}}\right) = \frac{\partial}{\partial \mathbf{P}}\left(\frac{dp}{dt}\right) \qquad \text{(II-6)}$$

The following definition has been used for the determination of SD^2 the sum of the residual squares:

$$SD^2 = \sum_{i=1}^{N}[y_{i\,\exp} - y_{ical}]^2 \qquad \text{(II-7)}$$

With y_i a growth or production parameter.

For Chapter III *§III*- 3, the same method was used in the first and second part except D_0 that was replaced by $D_0(i) = \left(\frac{dx}{dt}\right)_{i,exp} - \left(\frac{dx}{dt}\right)_{i,calc}$

and

$$\chi^2 = \sum_{i=1}^{N}\frac{[\mathbf{D_0}(i)]^2}{\left(\frac{dx}{dt}\right)_{i,cal}} \qquad \text{(II-8)}$$

Equations (III-11, Chapter III) for production and (III-17, Chapter III) for growth, were solved numerically with an iterative algorithm; the program flow chart is given in Figure II.1.

The target function χ_P^2 was calculated as follows:

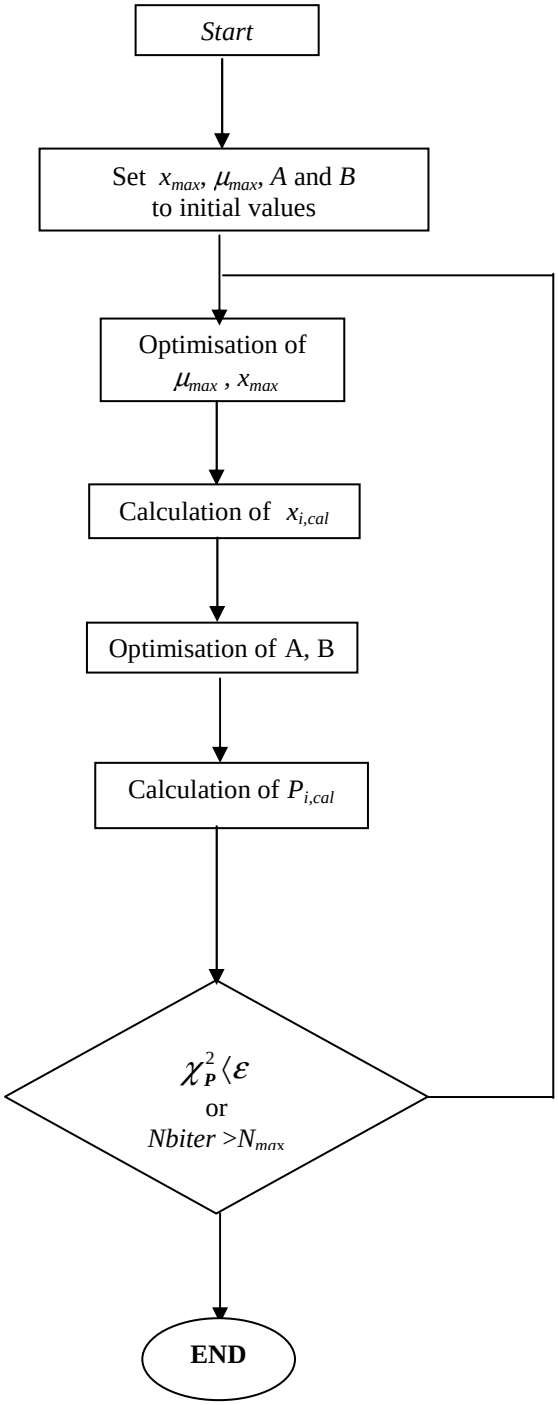
$$\chi_P^2 = \sum_{i=1}^{N} \frac{\left(\mathbf{P}_{i,calc} - \mathbf{P}_{i,\exp}\right)^2}{\mathbf{P}_{i,calc}} \qquad \text{(II-9)}$$

For Chapter III section 4, 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*:

$$RSD = \sqrt{\frac{\sum_{i=1}^{N}\left[Y_{i,\exp} - Y_{i,calc}\right]^2}{n-q}} \qquad \text{(II-10)}$$

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



FigureII.1. Program flow chart of the iterative algorithm used for the numerical resolution ('*GM2*' model) of growth, equation (III-17, chapter III), and production, equation (III-11, chapter III).

III. MODELLING

Unstructured models have proven to accurately describe lactic acid fermentation in a wide range of experimental conditions and media. In this aim, specific growth rate was previously described by means of a logistic function of time (Amrane, 2001):

$$\mu = \mu_{max} \frac{1}{1 + \frac{c * e^{d*t}}{\mu_{max} - c}} \quad \text{(III-1)}$$

Integration of equation (VI-1) gave the following growth time-course:

$$x = x_0 * \exp\{\mu_{max} * t - \frac{\mu_{max}}{d}[\ln \frac{\mu_{max} - c + c * e^{d*t}}{\mu_{max}}]\} \quad \text{(III-2)}$$

Growth time-course was accurately fitted by means of the above model; however it is not completely satisfactory from a cognitive point of view. Indeed, all growth parameters have not an obvious biological meaning (Amrane and Prigent, 1994a). Consequently, in the present work, the Verlhust model which proved to describe satisfactory growth kinetic (Moraine and Rogovin, 1996; Norton et al., 1994, Pandey et al., 2000) was preferred to the above model:

$$\frac{dx}{dt} = \mu_{max} * \left(1 - \frac{x}{x_{max}}\right) * x \quad \text{(III-3)}$$

Integration of equation (VI-3) gave:

$$x = x_0 * x_{max} * \frac{e^{\mu_{max} * t}}{x_{max} - x_0 + x_0 * e^{\mu_{max} * t}} \quad \text{(III-4)}$$

where x_0 and x_{max} are the initial and maximal values of the biomass concentration and μ_{max} is the maximal specific growth rate.

During lactic acid fermentation, accumulated lactic acid decreases the pH value. The acidic pH inhibits fermentation (Luedeking, and Piret, 1959a, Gonçalves et al., 1997, Fu and Mathews 1999, Amrane and Prigent, 1999a). To overcome this inhibition, the pH is

maintained during culture at its optimal value for lactic acid production (5.9) (Hanson and Tsao, 1972; Venkatesh et al.,1993), at which the final free lactic acid concentration (approximately 0.3 g l^{-1}) is below the inhibitory threshold (Gätje and Gottschalk, 1991). In absence of inhibition, cessation of growth is due to nutritional limitations, deficiency in peptide sources (Turner and Thomas, 1975; Mozzi et al., 1994) or in growth factors (Major and Bull 1985, Aeschlimann and von Stockar, 1989). In order to account for cessation of production, observed in the beginning of the decline phase (Amrane and Prigent, 1997), due to carbon limitation, the Luedeking-Piret model was previously modified (Amrane, 2001, Amrane and Prigent, 1999b). The modified Luedeking-Piret model is not convenient for culture when lactose became limiting and for seed culture with inhibitory effects. Consequently this model will be improved for culture (the corresponding SLM model is developed in section III-2) and seed culture (the corresponding IM model is developed in section III-1). To avoid the use of two different models for production rate, depending on the culture conditions, the above models can be merged, leading to a general model (GM1 model, developed in section III-3) taking into account both effects, a nutritional limitation effect and an inhibitory effect. However in some cases, an inhibitory effect can be observed during growth, which however ceased when carbon became limiting. Such cultures were described by means of a 'new generalized model' *(*GM2 model, developed in section III-3*)*. The development and the validation of the above models were also carried out for two stage continuous cultures (section III-4); the first stage acting as a continuous seed culture (no pH control), inoculating continuously the second bioreactor, the production reactor (pH control at 5.9), which was continuously fed with sterile culture medium.

To help the reader, all the developed models with the corresponding equations for growth and production are summarized in appendix.

III-1. INHIBITION MODEL FOR SEED CULTURE (IM model)

III-1.1. INTRODUCTION

Since the positive effects of precultivating without pH control was shown (Amrane and. Prigent, 1996, Amrane and Prigent, 1998a), the previously developed models are not convenient for seed culture. Indeed, they do not take into account the inhibition observed in absence of pH control. Several models involving lactic acid inhibition can be found in the available literature, which consider non-competitive product inhibition (Kumar Dutta, 1996, Ohara et al., 1992), or other types of inhibition (Pinelli et al., 1997, Åkerberg et al., 1998, Biazar 2003), by the total lactic acid produced.

However, it is now recognised that the main inhibitory species is the undissociated form of the lactic acid: inhibition by weak organic acids is related to the solubility of the non-dissociated form within the cytoplasm membrane and the insolubility of the ionised acid form (Gätje and Gottschalk, 1991; McDonald et al., 1990). The result is an acidification of the cytoplasm and the collapse of the motive force, causing an inhibition of nutrient transport (Kashket 1987, Bender and Marquis, 1987). Amongt the available bibliography, only few models involve the undissociated form of the lactic acid, assuming a non-competitive inhibition (Yeh, 1991), or an exponential decay to describe the linking of specific (Vereecken, and Van Impe, 2002) growth rate (Venkatesh et al., 1993) with the undissociated lactic acid concentration.

In this part, the Luedeking-Piret model (1959a) was modified by introducing an additional term to account for the undissociated lactic acid inhibition.

The relationship between pH, also involved in growth inhibition (Amrane and Prigent, 1999a), and both the dissociated [HL] and the undissociated [L^-] forms of lactic acid was described by the Henderson-Hasselbach equation:

$$[HL] = \frac{[L^-]}{10^{pH-pK_A}} \qquad \text{(III-5)}$$

The total lactic acid concentration corresponded to the sum of both forms of the organic acid:

$$p = [HL] + [L^-] \quad \text{(III-6)}$$

III-1.2. RESULTS AND DISCUSSION

For all the tested supplementations, the Verlhust model (Eq.III-4) matched experimental growth data (Fig. III-1), leading to sum of the residual squares in the range 0.01-0.03 (Table III-1). As expected, maximum biomass concentration increased for increasing nitrogen supplementation of culture medium (Table III-1). However, owing to the inhibitory effects of pH and undissociated lactic acid concentration, maximum biomass concentrations were in all cases low, if compared to the values recorded at controlled pH (Amrane and Prigent, 1998b).

In the beginning of culture, owing to the low undissociated lactic acid concentrations, pH was the main inhibitory factor and was shown to highly affect growth rates; maximum specific growth rate μ was reduced by half for pH control of 4.6 instead of the optimal pH (5.9) during *L. helveticus* growth on the same culture medium (Amrane and Prigent, 1999a). Contrarily, medium supplementation has a more limited effect on μ, which decreased by only 28 % for increasing *YE* concentration from 5 to 20g l^{-1} (Amrane and Prigent, 1998b). From this, in the beginning of culture, pH was the main factor affecting growth, accounting for the similar maximum specific growth rates recorded for all experiments (Table III-1).

Table III-1. Parameters extracted from the model for growth and lactic acid production data of batch cultures of L. *helveticus* carried out without pH control on whey permeate supplemented with 5, 10, 20 g l^{-1} YE and the RM supplementation

		Nitrogen supplementation			
		Yeast extract YE (g l^{-1})			RM
		5	10	20	
x_0	(g l^{-1})	0.04	0.02	0.04	0.02
x_{max}	(g l^{-1})	0.87	1.05	1.45	1.54
μ_{max}	(h^{-1})	0.63	0.66	0.64	0.68
SD^2		0.013	0.012	0.034	0.024
A	(-)	2.68	2.29	2.52	3.89
B	(h^{-1})	0.422	0.648	0.527	0.295
$p_{calc, f}$	(g l^{-1})	4.44	6.10	7.56	7.98
$p_{ass, f}$	(g l^{-1})	2.22	2.34	3.52	5.88
$p_{non\text{-}ass, f}$	(g l^{-1})	2.22	3.76	4.03	2.10
SD^2		0.69	0.30	0.67	0.60

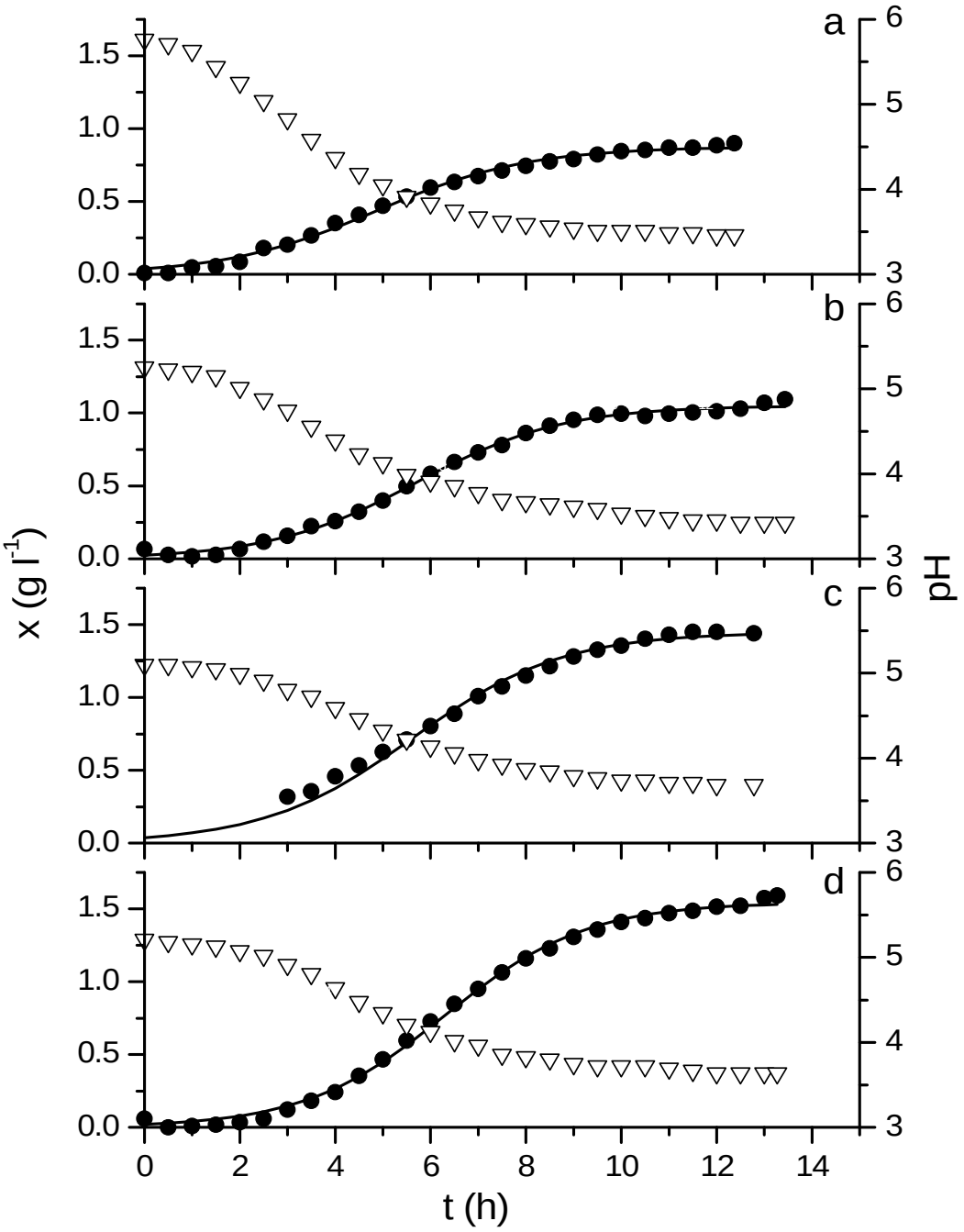


Figure III-1. Experimental growth (•) and pH (∇) data recorded during L. *helveticus* growth on whey permeate supplemented with 5 (a), 10 (b) and 20 (c) g l^{-1} yeast extract and the RM supplementation (d); growth model, Eq. III-4 (continuous line).

It can be noticed that, similarly to maximum biomass concentrations, final lactic acid productions increased with the nitrogen supplementation of culture medium, leading to

increasing final amounts of undissociated lactic acid, owing to the similar final culture pH (Table III- 2).

To describe lactic acid production data, the Luedeking-Piret model (1959a) was modified by introducing an additional term to account for the undissociated lactic acid inhibition:

$$\frac{dp}{dt} = A * \frac{dx}{dt} + B * x * \left(1 - \frac{[HL]}{[HL]_{inh}}\right) \quad \text{(III-7)}$$

Where A and B were the coefficients for growth- and non-growth-associated production, respectively; $[HL]_{inh}$ was the inhibitory undissociated lactic acid concentration, 8.5g l^{-1} (Amrane and Prigent, 1999a). The pH was also involved in the model through the Henderson-Hasselbach equation (Eq. III-5).

For all the tested supplementations, the above model (Eq.III-7) matched experimental production data (Fig. III-2), leading to sum of the residual squares SD^2 in the range 0.3-0.7 (Table III-1).

Table III-2. Parameters extracted from experimental growth and lactic acid production data of batch cultures of *L. helveticus* carried out without pH control on whey permeate supplemented with 5, 10, 20 g l^{-1} YE and the RM supplementation

		Nitrogen supplementation			
		Yeast extract YE (g l^{-1})			RM
		5	10	20	
x_{max}	(g l^{-1})	0.90	1.09	1.44	1.59
$p_{exp,\,f}$	(g l^{-1})	4.29	5.91	7.23	7.92
$[HL]_{exp,\,f}$	(g l^{-1})	2.96	4.16	4.09	4.69
pH_f		3.46	3.42	3.68	3.64

The highest value of the coefficient *A* for growth-associated production and the lowest value for the coefficient *B* for non-growth-associated production were recorded for the more important nitrogen supplementation of culture medium, the *RM* supplementation (Table III-1).

It was expected, since growth and production linking increased with the nitrogen supplementation of culture media (Amrane and Prigent, 1998a and b).

Since the early work of Luedeking and Piret, (1959a), the most common way to characterize the partial association of a production with growth was to draw the specific rate of production ($q_p = \frac{dp}{x * dt}$) versus the specific rate of growth ($\mu = \frac{dx}{x * dt}$).

In this aim and in a first step, by considering the optimized parameter values for growth and production (Table III-1), calculated specific growth and production rates, deduced from equations III-3 and III-7, were compared to the corresponding experimental values, which corresponded to the ratio of the instantaneous rates (Eq.II-3 Material and methods) on the experimental biomass concentration data. The corresponding graph is reported in Figs.III-3a and b for 5 g l^{-1} YE and the RM supplementation, chosen as examples. A fairly good agreement between the calculated and the experimental values was observed.

The Luedeking and Piret plot (Fig.III-3c) confirmed the increase of the growth-associated mechanism for increasing nitrogen supplementation of culture medium. Indeed, lowering culture medium supplementation led to a clear decrease of the q_p values and hence a decrease of the slope, which corresponded to the growth-associated parameter *A*; while the effect on the ordinate intercept, which corresponded to the non-growth-associated parameter *B,* was not obvious. The model validated this behaviour, since the calculated values matched experimental data (Fig.III-3c). The experimental values recorded at the beginning of cultures, namely at low biomass concentrations and then high specific growth rates, can be regarded as of little significance, due to the high error in the differentiation of experimental data (Levenspiel 1962). However, it was previously shown that the best criterion to characterize growth and production linking was their determination, instead of the direct comparison of coefficients *A* and *B* which could lead to contradictory results (Amrane and Prigent 1997).

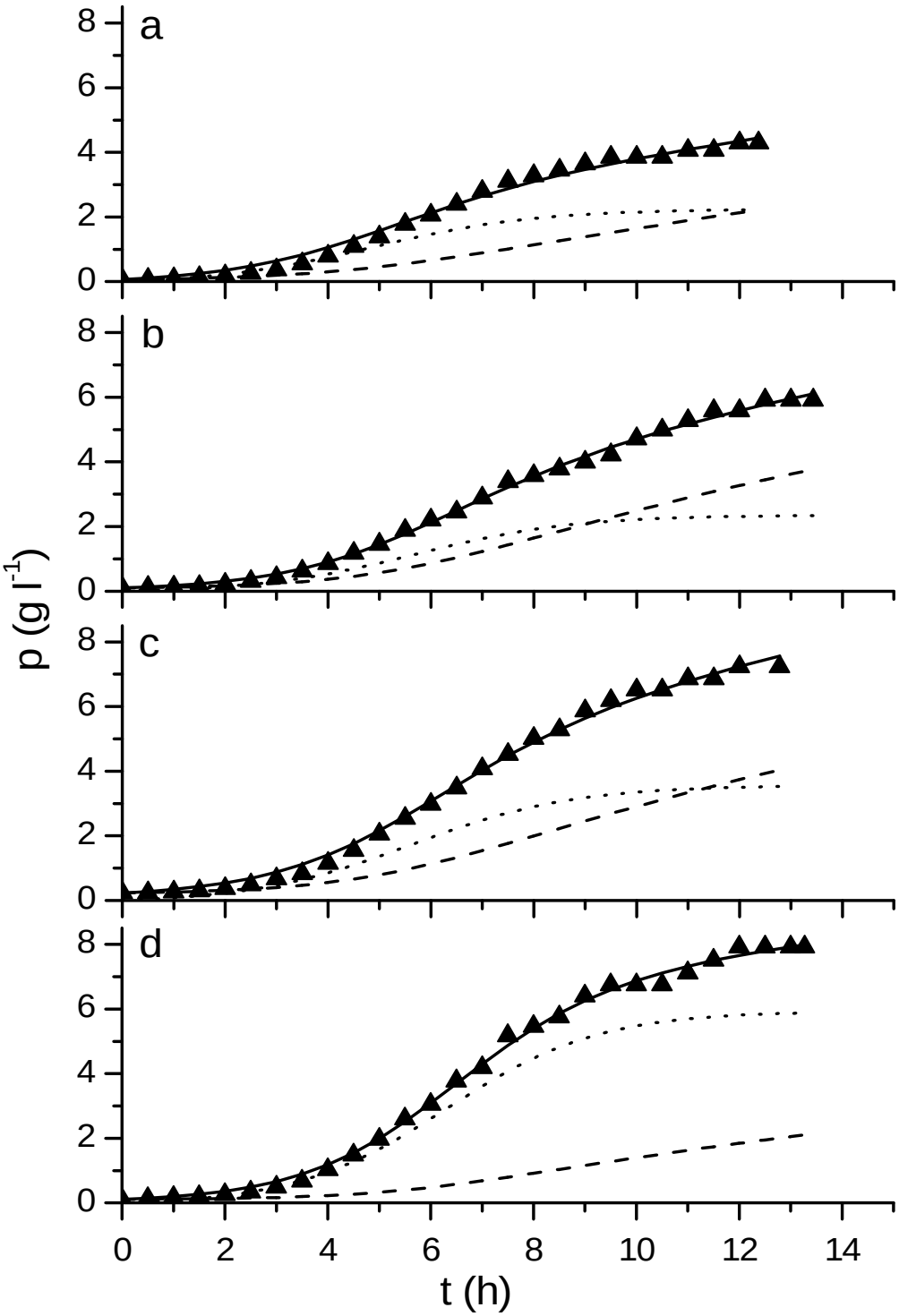


Figure III-2. Experimental total lactic acid production (▲) data recorded during L. *helveticus* growth on whey permeate supplemented with 5 (a), 10 (b) and 20 (c) g l^{-1} yeast extract and the RM supplementation (d). Calculated production (numerical integration of Eq.III-7) time-courses (continuous line), as well as the calculated growth-associated (numerical integration of Eq.III-8) (dot line) and non-growth-associated (numerical integration of Eq.III-9) (dash line) parts of the total lactic acid production

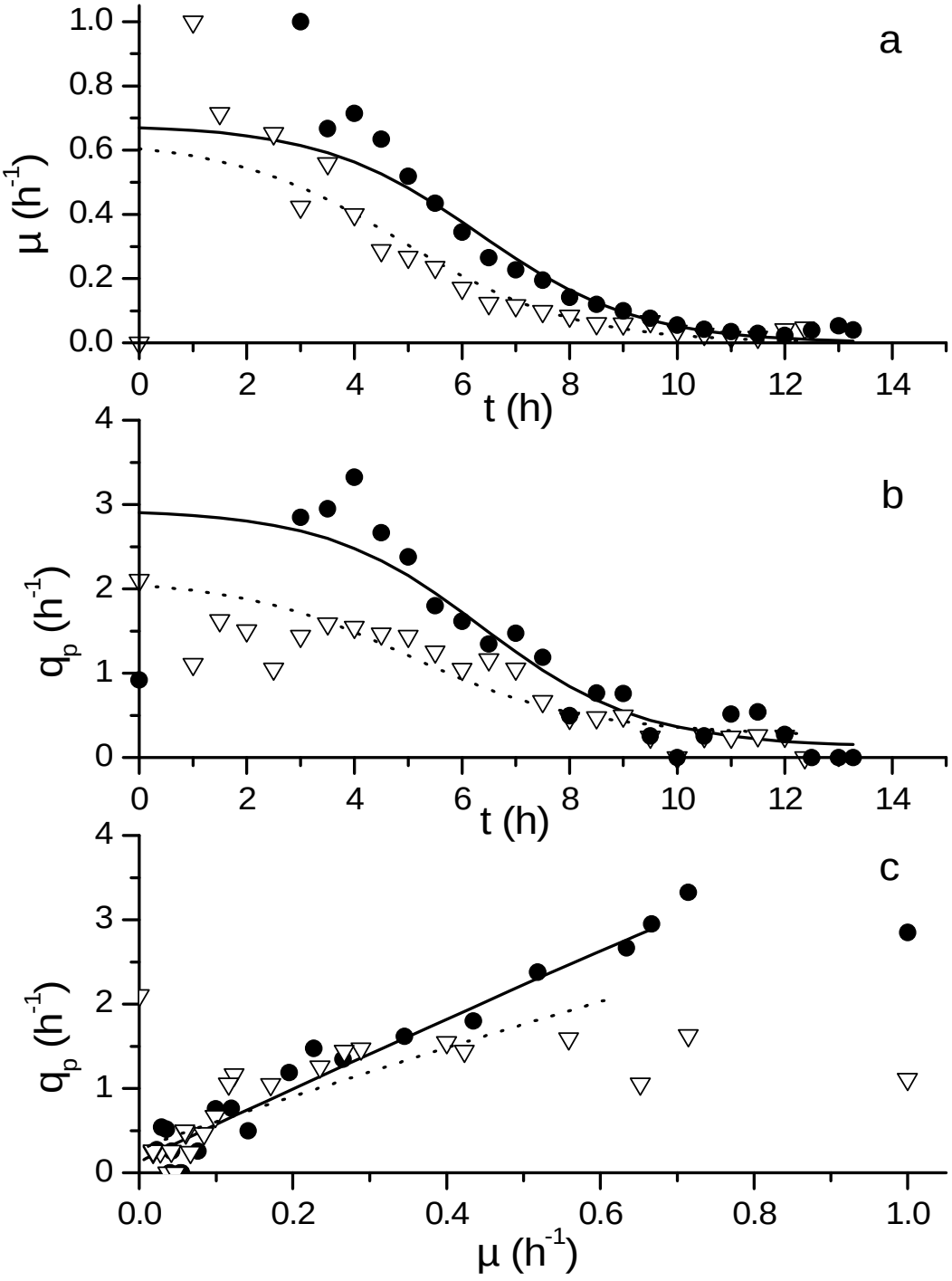


Figure III-3. Specific growth μ (a) and production q_p (b) rate time-courses, as well as the Luedeking-Piret plot, q_p vs. μ (c), recorded during *L. helveticus* growth on whey permeate supplemented with 5 g l^{-1} yeast extract, experimental data (∇) and calculated values (dot line) and the RM supplementation, experimental data (•) and calculated values (continuous line).

The growth-associated part of the production corresponded to the integration of:

$$\frac{dp}{dt} = A * \frac{dx}{dt} \qquad \text{(III-8)}$$

and the non-growth-associated part corresponded to the integration of:

$$\frac{dp}{dt} = B * x * \left(1 - \frac{[HL]}{[HL]_{inh}}\right) \qquad \text{(III-9)}$$

Both parts are displayed in Fig.III-2. As observed, for low supplementation of culture media, viz. 5 and 10g l^{-1} YE, similar growth-associated productions were recorded all culture long; while the associated part of production increased for high nitrogen supplementation of culture media. It was especially the case when whey permeate was supplemented with RM, leading to the main part of lactic acid produced by a growth-associated mechanism, nearly 74 %, in agreement with the behaviour recorded in case of pH control (Amrane and Prigent, 1999b). Conversely, during growth on whey permeate supplemented with RM, the amount of lactic acid produced by a non-growth-associated mechanism remained all culture long lower than those recorded for lower nitrogen supplementation of culture medium (Fig.III-2), and the final amount of non-growth-associated production was 2.1g l^{-1} (Table III-1).

III-1.3. CONCLUSION

In the model developed in this first part, all the parameters have a clear biological meaning. The Verlhust model was considered to describe growth kinetics (Eq.III-3), which can easily integrated to give growth time-courses (Eq.III-4). Since, seed cultures were considered in this work, namely experiments carried out without pH control, the Luedeking-Piret model, (1959) was modified by introducing an additional term to account for the undissociated lactic acid inhibition (Eq.III-7). The model was found to match both experimental growth and production data, and was validated in various culture conditions, namely for a large range of nitrogen supplementation of whey permeate.

III 2: SUBSTRATE LIMITATION MODEL AND GENERALIZED MODEL FOR SEED CULTURE AND CULTURE (SLM and GM1 models)

III-2. 1. INTRODUCTION

An inhibitory effect of the undissociated lactic acid is now undoubtedly proven (Vick Roy et al.1983, Gätje and Gottschalk, 1991), which affect in close relation, with the pH effect (Amrane and Prigent, 1999a), growth and hence lactic acid production during cultures without pH control. To overcome this inhibition, pH is maintained at its optimal value, 5.9 (Hanson and Tsao, 1972; Venkatesh et al., 1993), leading to nitrogen and carbon limitations causing cessation of growth and lactic acid production, respectively (Amrane and Prigent, 1997).

To describe lactic acid production data, the Luedeking-Piret model (1959a) was modified by introducing an additional term to account for the undissociated lactic acid (and pH) inhibition in case of cultures without pH control (IM model : Eq.III-7) (see § III-1.). While during cultures at pH controlled at 5.9, a corrective term was introduced to account for cessation of production due to carbon substrate limitation (Eq. III-10) (Amrane, 2001):

$$\frac{dp}{dt} = A * \frac{dx}{dt} + B * x * \left(1 - \frac{s_{res}}{s}\right) \qquad \text{(III-10)}$$

Where *A* and *B* were the coefficients for growth- and non-growth-associated production, respectively. *s* and s_{res} were the lactose concentration at time *t* and the end of the batch, respectively (Amrane, 2001).

In case of cultures carried out at controlled pH, lactic acid production ceased when lactose became limiting, leading to final lactose concentrations in the range 1-3g L^{-1} (Amrane, 2001). Obviously, it was not the case for cultures carried out without pH control, leading to high residual lactose concentrations, and hence aberrant amount of growth- and non-growth-associated productions as it will be shown below (Table III-5). Moreover, without pH control, the residual lactose concentration varied with the culture conditions (see § III-1.). From all

these, equation III-10 was modified by introducing the constant s_{lim}, in place of the residual lactose concentration s_{res}; s_{lim} corresponded to the limiting lactose concentration, 3g L^{-1}, deduced from several runs on whey supplemented with various yeast extract concentrations (Amrane and Prigent, 1998b, Amrane and Prigent, 1999b):

$$\frac{dp}{dt} = A * \frac{dx}{dt} + B * x * \left(1 - \frac{s_{lim}}{s}\right) \qquad \text{(III-11)}$$

The following abbreviation will be given to this model and considered throughout the text: SLM model.

To avoid the use of two expressions for production rate (Eqs.III-7 and III-11), depending on the culture conditions, the above expressions can be merged, leading to an unique expression taking into account both effects, a nutritional limitation effect (Eq.III-11) and an inhibitory effect (Eq. III-7):

$$\frac{dp}{dt} = A * \frac{dx}{dt} + B * x * \left(1 - \frac{s_{lim}}{s}\right) * \left(1 - \frac{[HL]}{[HL]_{inh}}\right) \qquad \text{(III-12)}$$

Validation of this generalized model (Eq.III-12, abbreviation: GM1 model) and its comparison to the above lactic acid production models (Eqs.III-7 IM model and III-11 SLM model) was the aim of this section (§ III-2). Cultures on whey supplemented with various nitrogen supplementations, at pH 5.9 to consider the case of carbon substrate limitation (Amrane and Prigent, 1997; Amrane and Prigent, 1998b), and without pH control to consider the case of an inhibition by the pH and the undissociated lactic acid (Amrane and Prigent, 1999a) were considered to compare the three models.

To describe growth and in agreement with part one, the Verlhust model which proved to describe satisfactory growth kinetics (Eq. III-3) was considered.

III-2. 2. Results and Discussion

Parameters extracted from the growth model (Eq.III-4) are displayed in Table III-3. It could be observed that growth model accounted well for experimental data recorded at pH controlled at 5.9 (Fig.III-4a) and without pH control (Fig.III-5a) until nearly the end of stationary state, leading to sum of the residual squares in the ranges 0.06-0.13 and 0.01-0.03 respectively (TableIII-3). As expected, maximum biomass concentration increased for increasing nitrogen supplementation of culture medium (TableIII-3). However, due to the inhibitory effect of pH and undissociated lactic acid concentration, maximum biomass concentrations were low in all cases, if compared to the values recorded at pH controlled at the optimal value for growth, 5.9 (Table III-3).

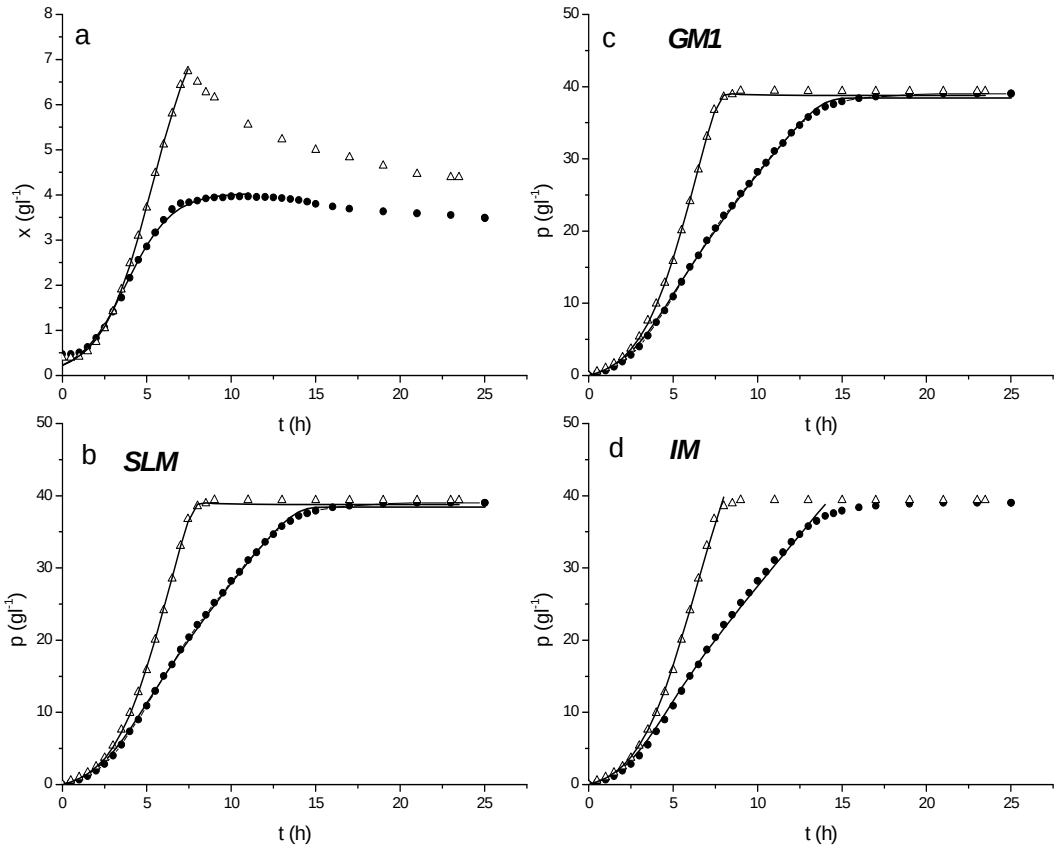


Figure III- 4. Growth (a) and lactic acid production (b, c, d) kinetics during batch cultures of *L. helveticus* growing at pH controlled at 5.9 on whey supplemented with 10 g L^{-1} yeast extract (Δ) and the RM supplementation (•); calculated data (—) by means of the generalized model GM1, Eq.III-12 (b), the substrate limitation model SLM, Eq.III-11 (c) and the inhibition model IM, Eq.III-7 (d).

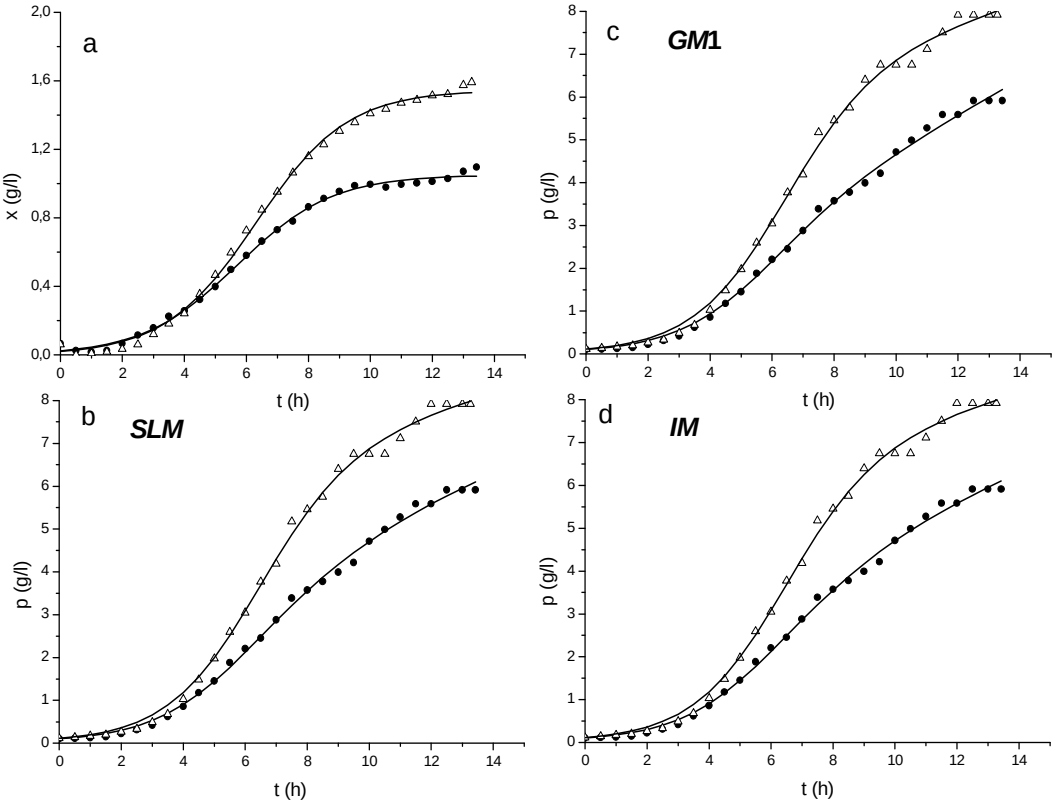


Figure III-5. Growth (a) and lactic acid production (b, c, d) kinetics during batch cultures of *L. helveticus* growing without pH control on whey supplemented with 10 g L^{-1} yeast extract (Δ) and the RM supplementation (•); calculated data (—) by means of the generalized model GM1, Eq.III-12(b), the substrate limitation model SLM, Eq.III11(c) and the inhibition model IM, Eq.III-7 (d).

In the beginning of culture, pH was close to the optimal value 5.9, in the case of cultures without pH control, leading to similar maximum specific growth rates recorded for all experiments carried out at pH controlled at 5.9 or without pH control (Table III-3), since μ_{max} were recorded in the beginning of growth. From this, the limited effect of medium supplementation on μ is confirmed, in agreement with the low decrease (28 %) of μ_{max} recorded for increasing *YE* concentration from 5 to 20g l^{-1} (Amrane and Prigent, 1998b). In addition, in the beginning of culture, owing to the low undissociated lactic acid concentrations, pH was the main inhibitory factor and was shown to highly affect growth rates, maximum specific growth rate μ was reduced by half for pH control of 4.6 instead of

the optimal pH (5.9) during *L. helveticus* growth on the same culture medium (Amrane and Prigent, 1999a).

Table III -3. Parameters given by the growth model (Eq.III-4) for batch cultures of *L. helveticus* carried out at pH controlled at 5.9 and without pH control on supplemented whey

Cultures		Without pH control				At pH controlled at 5.9	
		YE (g l^{-1})			RM	YE (g l^{-1})	RM
		5	10	20		10	
x_0	(g l^{-1})	0.04	0.02	0.04	0.02	0.23	0.22
x_{max}	(g l^{-1})	0.87	1.05	1.45	1.54	4.04	8.41
μ_{max}	(h^{-1})	0.63	0.66	0.64	0.68	0.74	0.68
SD^2		0.01	0.01	0.03	0.02	0.13	0.06

III-2.2.*a. Cultures at pH controlled at 5.9*

Fig.III-4b shows that as expected the 'substrate limitation model' (SLM), involving a corrective term for carbon substrate limitation (Eq.III-11), as was the case when pH was controlled at 5.9 (Amrane, 2001), matched lactic acid production data recorded during cultures at pH 5.9, in agreement with previous results (Amrane, 2001). The 'generalized model' (GM) (Eq.III-12), which involved both terms, a term for pH and undissociated lactic acid inhibitions and a term for carbon substrate limitation also shows a good agreement with experimental data (Fig.III-4c). From this, the parameters for growth- and non-growth-associated production given by the GM ((Eq.III-12)) and the SLM ((Eq.III-11)) models were similar (TableIII-4). During cultures at controlled pH (5.9), final free lactic acid concentration (approximately 0.3g l^{-1}) is below the inhibitory threshold (Gätje and Gottschalk, 1991), namely almost negligible compared to the inhibitory undissociated lactic acid concentration, 8.5g l^{-1} (Amrane and Prigent, 1999a). Consequently, the inhibition term $\left(1-\frac{[HL]}{[HL]_{inh}}\right)$ had no effect, since it remained throughout culture close to unit.

The growth-associated part of the production corresponded to the integration of:

$$\frac{dp}{dt} = A * \frac{dx}{dt} \qquad \text{(III-8)}$$

and the non-growth-associated part corresponded to the integration of:

$$\frac{dp}{dt} = B * x * \left(1 - \frac{s_{lim}}{s}\right) \qquad \text{(III-13)}$$

for the 'substrate limitation model' (Eq.III-11) and integration of:

$$\frac{dp}{dt} = B * x * \left(1 - \frac{s_{lim}}{s}\right) * \left(1 - \frac{[HL]}{[HL]_{inh}}\right) \qquad \text{(III-14)}$$

for the 'generalised model GM1'.

Both models led to similar parts of growth- and non-growth-associated productions (Table III -4). As expected and in agreement with previous results (Amrane and Prigent, 1998b), the growth-associated part of production was higher for the largest nitrogen supplementation of culture medium (RM). This has to be related to the higher maximum biomass concentration recorded in absence of nitrogen or growth factors limitation, as was the case during culture on RM medium.

Fig.III-4d shows that the 'inhibition model' (IM) matched experimental production data during growth. However, it did not account for cessation of production when carbon substrate became limiting, leading to high sum of the residual squares if compared to those given by the GM1 and the SLM models (Table III-4), owing to the negligible effect of the inhibition term $\left(1 - \frac{[HL]}{[HL]_{inh}}\right)$ (Eq.III-7), which remained close to unit. Thus, at pH controlled at 5.9, Equation 1 can be assimilated to the Luedeking and Piret (1959a) equation; and parameters A and B given by the IM model differed from those given by SLM and GM1 models.

Table III-4. Parameters extracted from the production models (Eqs.III-7, 11 and 12) for lactic acid production data of batch cultures of *L. helveticus* carried out at pH controlled at 5.9 on whey permeate supplemented with 10 g l^{-1} yeast extract and the RM supplementation.

Media		GM (Eq.III-12)		SLM (Eq.III-11)		IM (Eq.III-7)	
		10g l^{-1}YE	RM	10g l^{-1}YE	RM	10g l^{-1}YE	RM
A	(-)	2.32	2.34	2.36	2.29	2.62	3.27
B	(h^{-1})	0.83	1.20	0.81	1.18	0.72	0.72
$p_{calc, f}$	(g l^{-1})	38.4	38.8	38.4	38.8	-	-
$p_{ass, f}$	(g l^{-1})	8.8	19.2	9.0	18.8	-	-
$p_{non\text{-}ass, f}$	(g l^{-1})	29.6	19.6	29.4	20.0	-	-
SD^2		1.04	1.61	1.05	1.57	6.98	4.28

III-2.2.*b. Cultures without pH control*

Table III-5 illustrates the high impact of the residual lactose concentration on the production parameters A and B, and hence the growth-and non-growth-associated parts of lactic acid production. Indeed, during cultures without pH control, inhibition account for cessation of growth and lactic acid production, leading to high residual lactose concentration, namely approximately 39g L^{-1} for cultures carried out without pH control on whey supplemented with 10g L^{-1} yeast extract. However, high values of s_{res} led to low values of the substrate limitation term $\left(1-\frac{s_{res}}{s}\right)$; hence the parameter B increased, while the parameter A decreased. Aberrant values were consequently obtained for growth- and non-growth-associated parts of production (TableIII-5). From this, the modification of the 'Substrate limitation model' by introducing the limiting lactose concentration s_{lim} (Eq. III-11), in place of the residual concentration s_{res} (Eq.III-10) was clearly validated.

Table III-5.Effect of the residual lactose concentration s_{res} on the growth- and non-growth-associated parts of production obtained in case of a substrate limitation term, $\frac{dp}{dt} = A * \frac{dx}{dt} + B * x * \left(1 - \frac{s_{res}}{s}\right)$ (Eq.III-10), and both substrate limitation and inhibition terms, namely the following expression $\left(\frac{dp}{dt} = A * \frac{dx}{dt} + B * x * \left(1 - \frac{s_{res}}{s}\right) * \left(1 - \frac{[HL]}{[HL]_{inh}}\right)\right)$ (Eq.III-15), during batch cultures of *L. helveticus* carried out without pH control on whey permeate supplemented with 10 g L^{-1} yeast extract.

		Equation III-10		Equation III-15	
s_{res}	(g l^{-1})	3	39	3	39
A	(-)	2.87	0.32	2.28	-2.92
B	(h^{-1})	0.42	12.59	0.70	25.56
$p_{calc, f}$	(g l^{-1})	6.17	5.73	6.10	5.71
$p_{ass, f}$	(g l^{-1})	2.93	0.33	2.32	-2.99
$p_{non-ass, f}$	(g l^{-1})	3.24	5.40	3.78	8.70

Fig.III-5d shows that as expected (§ III-1) 'inhibition model' (Eq.III-7) matched lactic acid production data recorded during cultures without pH control, as shown as an examples for cultures carried out on whey supplemented with 10g L^{-1} yeast extract and the RM supplementation. It was also the case for the 'generalized model' and also the 'substrate limitation model'. Parameters for growth- and non-growth-associated production given by the 'inhibition model' and the 'generalized model' were similar, while those given by the 'substrate limitation model' differed, leading to slightly higher sum of the residual squares SD^2, if compared to those given by the GM1 and the IM models (TableIII-6). Consequently, IM and GM1 models gave similar parts of growth- and non-growth-associated production; while SLM model overestimated the growth-associated part of production, until values of growth-associated lactic acid produced 26 and 21 % higher than the growth-associated production given by the IM and GM1 models, during *L. helveticus* cultures on whey supplemented with 10 and 20g L^{-1} yeast extract (Table III-6). Indeed, during cultures without

pH control, low amount of lactic acid were produced, leading to high residual lactose concentrations; the substrate limitation term $\left(1-\frac{s_{lim}}{s}\right)$ remained then always close to unit.

To counterbalance this effect, the parameter B given by the SLM model was always lower than the values given by the IM and GM1 models, leading to an underestimation of the non-growth-associated production (Table III-6). The SLM model appears therefore inappropriate to describe culture without pH control. It is illustrated at the examination of figureIII-6, showing that throughout culture, the model largely overestimated growth-associated production, shown as an example for the worst case, namely growth on whey supplemented with 10g L^{-1} yeast extract.

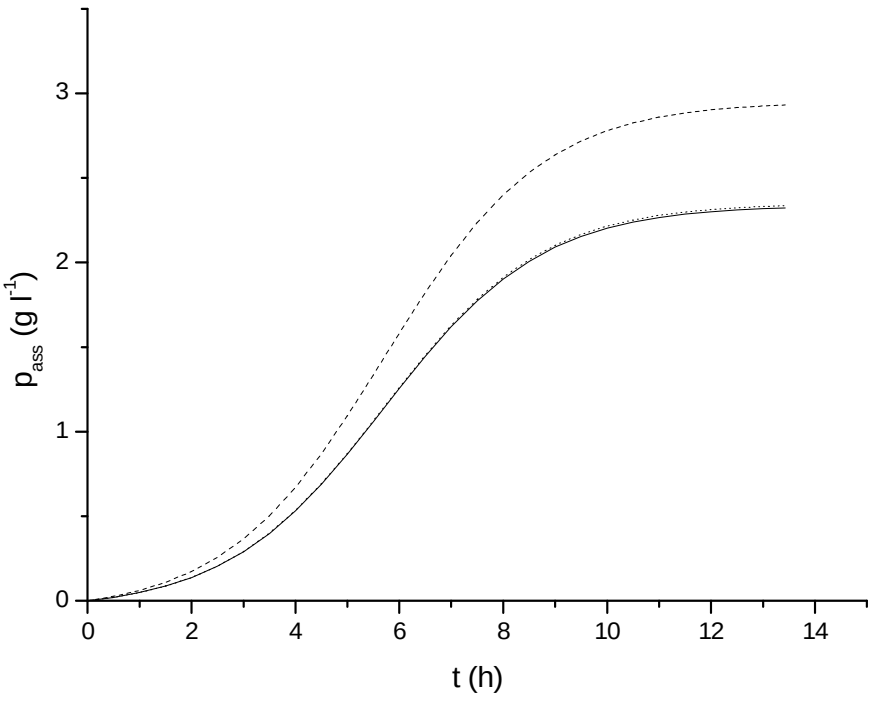


Figure III-6. Growth-associated production determined by the 'generalised model' (dot line), the 'inhibition model' (dash line) and the 'substrate limitation model' (continuous line) during batch cultures of *L. helveticus* growing without pH control on whey supplemented with 10 g L^{-1} yeast extract

Table III-6.Parameters derived from the models for lactic acid production (Eqs.III-7, 11 and 12) during batch cultures of *L. helveticus* carried out without pH control on whey permeate supplemented with 5, 10, 20g l^{-1} YE and the RM supplementation.

		Generalized model (GM1)				Substrate limitation model (SLM)				Inhibition model (IM)			
		Yeast extract (g l^{-1})			RM	Yeast extract (g l^{-1})			RM	Yeast extract (g l^{-1})			RM
		5	10	20		5	10	20		5	10	20	
A	(-)	2.67	2.28	2.51	3.88	3.00	2.87	3.05	4.17	2.68	2.29	2.52	3.89
B	(h^{-1})	0.46	0.70	0.57	0.32	0.31	0.42	0.33	0.16	0.422	0.648	0.53	0.30
$p_{calc, f}$	(g l^{-1})	4.44	6.10	7.55	7.98	4.44	6.17	7.58	8.00	4.44	6.10	7.56	7.98
$p_{ass, f}$	(g l^{-1})	2.22	2.32	3.50	5.87	2.49	2.93	4.24	6.30	2.22	2.34	3.52	5.88
$p_{non\text{-}ass, f}$	(g l^{-1})	2.22	3.78	4.05	2.11	1.95	3.24	3.34	1.70	2.22	3.76	4.03	2.10
SD^2		0.69	0.30	0.67	0.60	0.76	0.35	0.89	0.59	0.69	0.25	0.67	0.61

III-2.3. Conclusion

The above results show that the generalized model gave a satisfactory description of experimental data in various culture conditions, since it was validated during cultures at pH controlled and in absence of pH control, as well as for different nitrogen supplementation of culture media.

During cultures at pH controlled, nutritional limitations caused cessation of growth and lactic acid production; then the ‘inhibition model’ was obviously inappropriate as experimentally confirmed, since it did not account for cessation of production.

In absence of pH control, growth and hence lactic acid production were inhibited by pH and the undissociated lactic acid. The ‘generalised model’ and the ‘inhibition model’ gave similar calculated data; while the parameters A and B for growth- and non-growth-associated production given by the ‘substrate limitation model’ differed, leading to an overestimation of the growth-associated production.

III-3 GROWTH MODEL IMPROVEMENT (GM2 model) AND COMPARISON OF THE GENERALIZED MODELS (GM1 and GM2).

III-3.1. INTRODUCTION

The ‘generalized model’ (GM1, §III-2) gave a satisfactory description of experimental data in various culture conditions, since it was validated in case of nitrogen limitation, namely during cultures at pH controlled at 5.9 for various nitrogen supplementation of culture medium, and in case of an inhibition, namely during cultures in absence of pH control (see §III-2). However, in some cases, especially during cultures carried out at acidic pH control, an inhibitory effect can be observed during growth, which however ceased when carbon became limiting (or when the undissociated lactic acid concentration reached its inhibitory threshold

value at highly acidic pH control) (Amrane and. Prigent, 1999a). Description of such cultures by means of the 'new generalized model' (GM2) was examined in this part.

Moreover, in the growth model (Verlhust model – Eq.III-3), possible limitation or inhibition of growth was only indirectly taken into account. Growth model can be therefore improved by introducing an inhibition term, as previously proposed (Altiok et al., 2006):

$$\frac{dx}{dt} = \mu_{max} * \left(1 - \frac{x}{x_{max}}\right)^{f} * \left(1 - \frac{p}{p_{max}}\right)^{h} * x \qquad \text{(III-16)}$$

Where *f* and *h* were parameters related to the "toxic power" for biomass and product inhibition.

However, since the main inhibitory species is the undissociated form of the lactic acid (Gätje and Gottschalk 1991, Kashket 1987), the term added to the growth model in this work was related to the undissociated lactic acid inhibition $\left(1 - \frac{[HL]}{[HL]_{inh}}\right)$, instead of the total lactic acid concentration [27], leading to the following modified Verlhust model (Eq.III-3):

$$\frac{dx}{dt} = \mu_{max} * \left(1 - \frac{x}{x_{max}}\right) * \left(1 - \frac{[HL]}{[HL]_{inh}}\right) * x \qquad \text{(III-17)}$$

This expression allowed a dissociation of the inhibitory and the nutritional effects; the term $\left(1 - \frac{x}{x_{max}}\right)$ accounted in "a global way" for an increasing lack of nutrients, namely for a nitrogen limitation (Amrane , 2001; Amrane and Prigent, 1998b; Diaz et al., 1999).

Equation III-11 involving a carbon substrate limitation to account for cessation of production was considered for production.

In addition, lactic acid and lactose concentrations were assumed to be linearly correlated (Amrane, 2001):

$$Y_{P/S} = \frac{p - p_0}{s_0 - s} = \text{constant} \qquad \text{(III-18)}$$

Validation of the second 'generalized model' (GM2), namely equation (Eq.III-17) for growth and equation (Eq.III-11) for production, and its comparison to the 'generalized model' (GM1), namely equations (Eq.III-3) and (Eq.III-12) for growth and production respectively, was also examined in this section.

III-3.2. Results and Discussion

Figures III-8 and III-9 show for both models a strong correlation between experimental and calculated data. Indeed, the group of data points are homogeneously distributed around the first bisectrix, with almost all the data points included in the range ± 5 % around the bisectrix. The model matched both experimental growth (Fig.III-8) and production (Fig.III-9) data in a wide range of culture conditions. Indeed, it was validated in absence of inhibitory effect, namely during growth on various culture media at the optimal pH, 5.9 (Fig.III-8a – b and Fig.III-9a – b); and in case of an inhibitory effect by the pH and the undissociated lactic acid accumulation, namely during growth on various culture media in absence of pH control (Fig.III-8c – d and Fig.III-9c – d), or during growth on RM medium at various acidic pH control, initially adjusted by lactic acid addition (Fig.III-8e – f and Fig.III-9e – f).

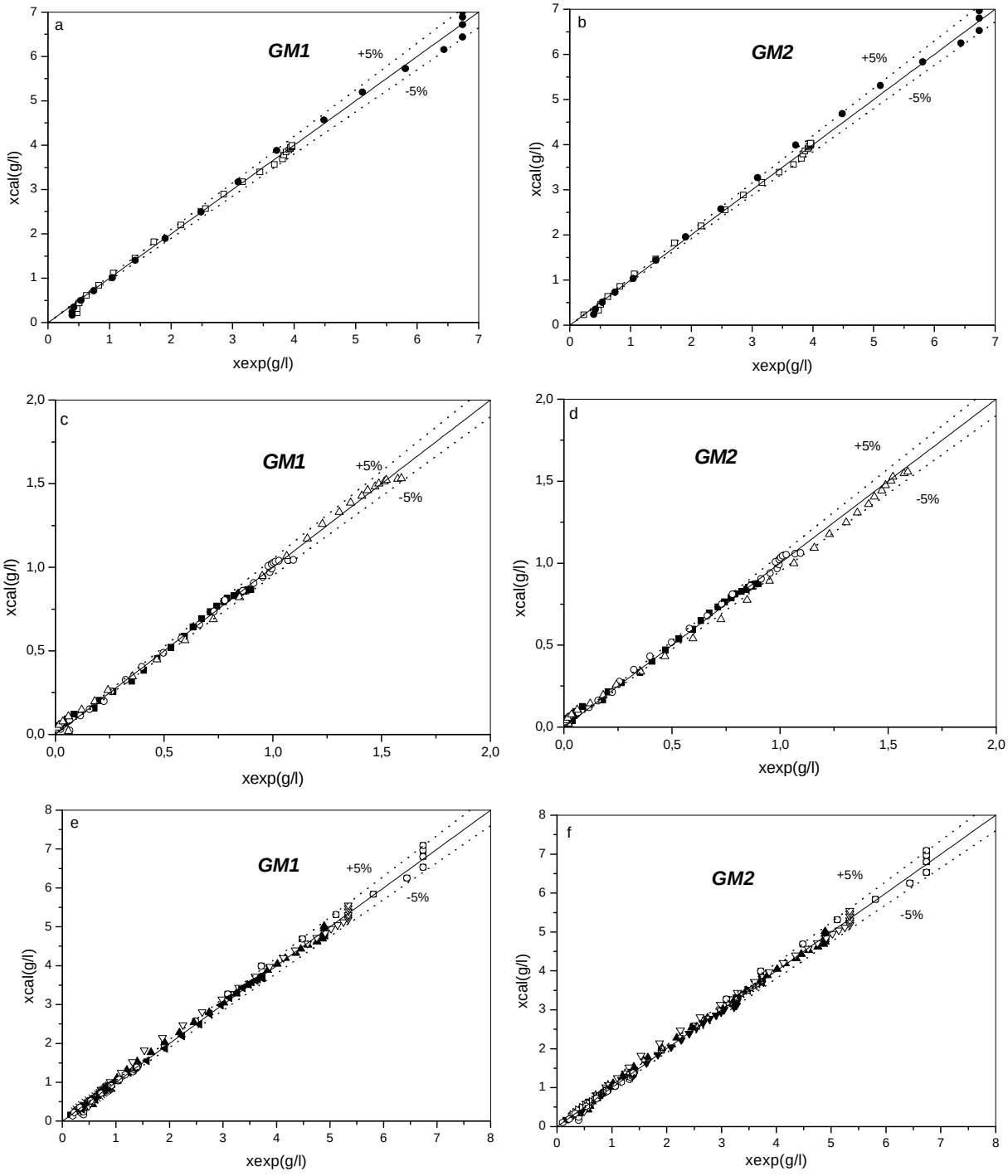


Figure III-7. Parity plot of predicted growth, equation (III-3) (Figs.III-7a, c and e) 'GM1' model, and numerical integration of equation (III-17) (Figs.III-7b, d and f) 'GM2' model, versus experimental growth time-course data during batch cultures of L. *helveticus* at pH controlled at 5.9 on whey supplemented with 10 g L^{-1} yeast extract (●) and the RM supplementation (□) (Figs. III-7a and b), without pH control on whey supplemented with 5 (■) and 10 (○) (g L^{-1}) yeast extract and the RM supplementation (Δ) (Figs. III-7c and d), and at pH controlled at 5.9 (■,○), 4.63 (▲,●), 4.34 (▼, Δ) and 4.04 (►,□) on whey supplemented with RM (Figs.III-7e and f).

Except in one case, namely production model for L. helveticus growing at strong acidic pH control (4.04), low residual standard deviation (RSD) were recorded. Indeed, the RSD values given by the first model ‘GM1’were always below 0.16 and 0.78 for growth and production models respectively (TableIII-7), and those given by the second model ‘GM2’ were below 0.18 and 0.83 for growth and production models (TableIII-8). Moreover, for a given experiment, the RSD given by both models were of the same order of magnitude. Contrarily, in case of strong acidic pH control (4.04), the generalized model ‘GM2’ gave a reasonable residual standard deviation (1.54), owing to the high number of data points since the corresponding culture lasted 80 h, while the RSD value increased drastically to 11.67in case of the ‘GM1’ model.

Some remarks can be drawn at the comparison of the parameters given by both models (Tables III-7 and 8). In absence of any inhibitory effect, namely during cultures carried out at pH 5.9, nearly similar parameters values were given by both models. Contrarily, in case of an inhibition, namely during cultures in absence of pH control, the ‘GM2’ model gave higher values for the growth-associated parameter A and lower values for the non-growth-associated parameter B, if compared to the values given by the ‘GM1’ model, showing a clear effect of the term related to the undissociated lactic acid inhibition $\left(1-\frac{[HL]}{[HL]_{inh}}\right)$.

Table III-7. Calculated growth (Eq.III-3) and production (Eq.III-12) parameters given by the generalized models 'GM1' for batch cultures of L. *helveticus* carried out on supplemented whey permeate at pH controlled at 5.9, without pH control and at various acidic pH control.

							Acidic pH control					
		pH controlled at 5.9		Without pH control			Lactic acid initially added			HCl initially added		
		YE($g\ L^{-1}$)	RM	YE ($g\ L^{-1}$)		RM						
		10		5	10		5.9	4.63	4.04	5.9	4.63	4.04
x_0	($g\ L^{-1}$)	0.22	0.16	0.04	0.02	0.02	0.16	0.37	0.23	0.16	0.3	0.14
μ_{max}	(h^{-1})	0.77	0.78	0.63	0.66	0.68	0.78	0.41	0.13	0.78	0.5	0.29
x_{max}	($g\ L^{-1}$)	3.98	7.28	0.87	1.05	1.54	7.28	5.10	0.89	7.28	5.47	1.39
RSD		0.067	0.16	0.023	0.022	0.03	0.16	0.004	0.0002	0.16	0.015	0.0005
A	(-)	2.36	2.30	2.67	2.28	3.88	2.30	-0.025	-6	2.30	0.69	88.83
B	(h^{-1})	0.81	1.22	0.46	0.7	0.32	1.21	1.94	1.46	1.21	1.83	-17.15
$p_{calc,\ f}$	($g\ L^{-1}$)	40.1	40.2	4.44	6.10	7.98	40.2	40.1	22.5	40.2	40.1	40.2
RSD		0.76	0.57	0.18	0.11	0.15	0.57	0.61	0.78	0.57	0.50	11.7

During cultures at acidic pH control, an effect of the above inhibitory term was observed, since the 'GM2' model (Table III-8) led to slightly higher growth parameter values (μ_{max} and x_{max}), if compared to the 'GM1' model (TableIII-7). As expected, maximum specific growth rate μ_{max} and maximum biomass concentration x_{max} decreased for decreasing pH control, owing to an increasing inhibitory effect. For both acidic pH, 4.63 and 4.04, and irrespective of the model used, higher μ_{max} and x_{max} were recorded when the pH was initially adjusted using HCl addition instead of lactic acid addition, in agreement with previous results (Amrane and. Prigent, 1999a), confirming the inhibitory effect of the undissociated lactic acid. It can also be noted that during culture at pH 4.04 initially adjusted by lactic acid addition, 8.9g L^{-1} of undissociated lactic acid was produced, which have to be added to the 1.8 g L^{-1} initially added (corresponding to 5g L^{-1} of total lactic acid). This total HL concentration has to be related to the values of 8.5 and 13.4g L^{-1} leading to a full inhibition of growth (leading to a stationary phase) and acid production respectively (Amrane and Prigent, 1999a). The inhibitory effect led to cessation of lactic acid production before the carbon source was exhausted (Fig.III-9b).

The generalized model 'GM2' led to increasing parameters A for growth-associated production (and decreasing parameters B for non-growth associated production), for increasing inhibitory effect, namely decreasing pH values (TableIII-8), in agreement with previous results (Amrane and. Prigent, 1999a). It should however be noted that lower parameter A values were given by the model (TableIII-8), if compared to the experimental values corresponding to the slope of the linear part of the product on biomass yield (Amrane and Prigent, 1999a), except at low pH control (4.04) for which the calculated values corresponded to the experimental ones. High experimental values for the parameter A led to an overestimation of the growth-associated production (Amrane and. Prigent, 1999a), as shown during culture at pH 5.9 (see §III-2).

Table III-8.Calculated growth (Eq.III-17) and production (Eq.III-11) parameters given by the generalized models 'GM2' for batch cultures of *L. helveticus* carried out on supplemented whey permeate at pH controlled at 5.9, without pH control and at various acidic pH control.

							Acidic pH control					
		pH controlled at 5.9		Without pH control			Lactic acid initially added			HCl initially added		
		YE(g L^{-1})	RM	YE (g L^{-1})		RM						
		10		5	10		5.9	4.63	4.04	5.9	4.63	4.04
x_0	(g L^{-1})	0.23	0.16	0.038	0.024	0.02	0.16	0.35	0.25	0.16	0.3	0.1
μ_{max}	(h^{-1})	0.75	0.8	0.65	0.68	0.67	0.8	0.45	0.15	0.8	0.55	0.37
x_{max}	(g L^{-1})	4.03	7.35	0.90	1.09	1.69	7.35	5.59	0.95	7.36	5.85	1.4
RSD		0.062	0.17	0.020	0.023	0.044	0.17	0.005	0.004	0.18	0.027	9.6 E-05
A	(-)	2.41	1.94	3.29	3.02	4.95	1.93	2.69	13.14	1.93	2.54	13.33
B	(h^{-1})	0.79	1.32	0.27	0.39	0.05	1.32	0.93	0.29	1.32	0.93	0.33
$p_{calc, f}$	(g l^{-1})	40.2	40.2	4.42	6.19	7.76	40.2	40.2	25.8	40.2	40.2	40.2
RSD		0.76	0.51	0.15	0.11	0.19	0.51	0.83	0.77	0.51	0.76	1.54

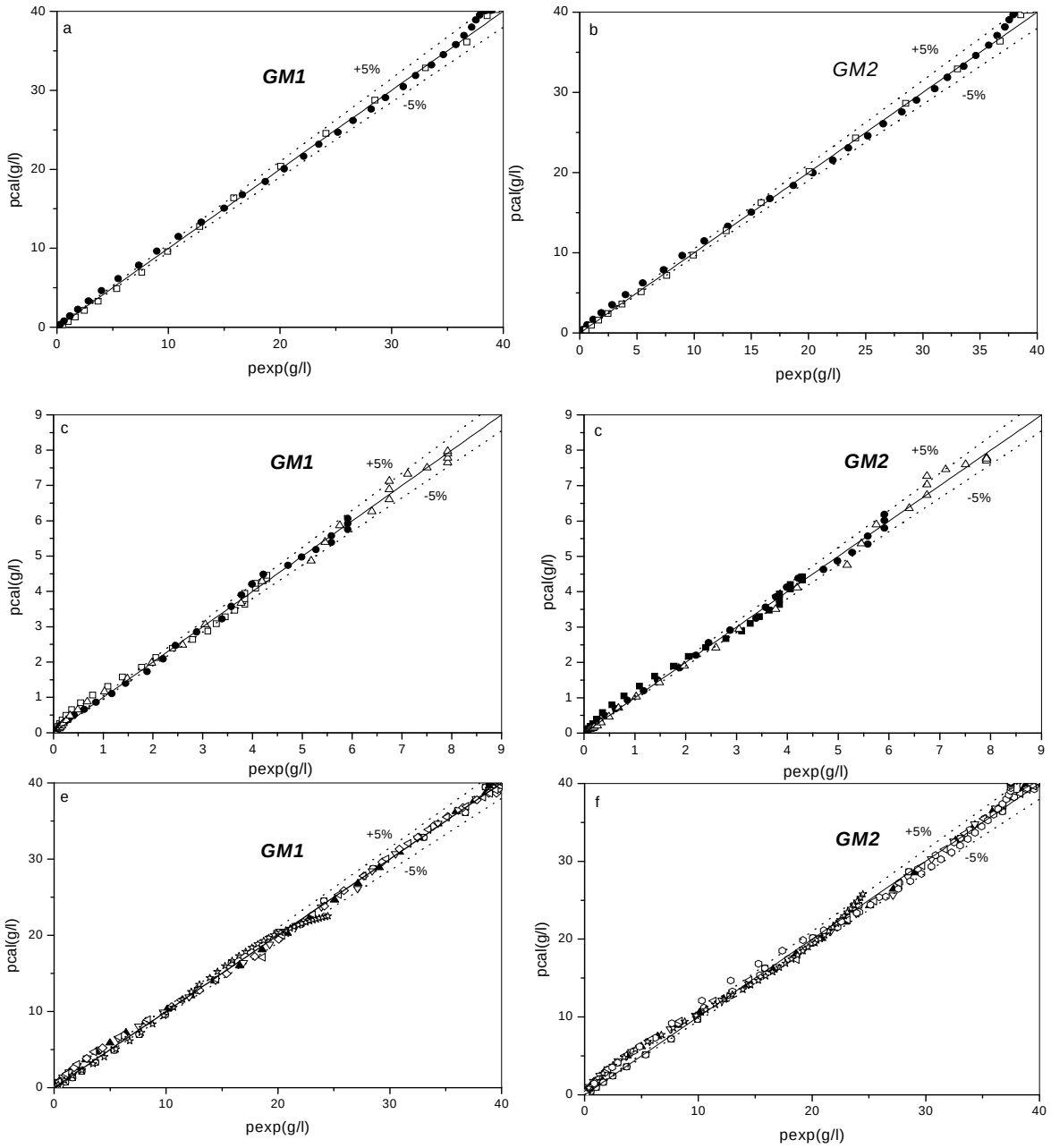


Figure III-8. Parity plot of predicted lactic acid production by numerical integration of equation (III-12) (Figs.III-8a, c and e) 'GM1' model and equation (III-11) (Figs.III-8b, d and f) 'GM2' model versus experimental production time-course data during batch cultures of L. *helveticus* at pH controlled at 5.9 on whey supplemented with 10 g L^{-1} yeast extract (●) and the RM supplementation (□) (Figs.III-8a and b), without pH control on whey supplemented with 5 (□) and 10 (●) (g L^{-1}) yeast extract and the RM supplementation (Δ) (Figs.III-8c and d), and at pH controlled at 5.9 (■,○), 4.63 (▲,●), 4.34 (◊,*) and 4.04 (Δ,□) on whey supplemented with RM (Figs.III-8e and f).

Examination of growth- and non-growth-associated parameters A and B given by the generalized model 'GM1' shows some negative values, as well as an aberrant value for a pH control of 4.04, initially adjusted with HCl (TableIII-8). The term related to the undissociated

lactic acid inhibition $\left(1-\frac{[HL]}{[HL]_{inh}}\right)$ was therefore more appropriate in the growth rate relation (Eq.III-17), instead of the production rate relation (Eq.III-12), confirming the above assumption (see Introduction). Indeed, 'GM2' model involved two terms in the growth relation (Eq.III-17), the Verlhust term accounting in "a global way" for an increasing lack of nutrients, namely for a nitrogen limitation (Amrane, 2001; Amrane and Prigent, 1998b, Diaz et al. 1999), and an inhibitory term; while carbon substrate limitation was considered for cessation of production (Eq.III-13). Obviously, the addition of the inhibitory term in the production rate ('GM1' model) was not appropriate in case of both an inhibitory and a nutritional effect, as was the case at acidic pH control. Consequently, only the fitting obtained by means of the generalized model 'GM2' was displayed in Figure III-9. As observed, the model matched growth (Fig.III-9a) and lactic acid production (Fig.III-9b) experimental data. It should however be noted that the decline phase, when occurring, was not described by the model. Indeed, when growth ceased concomitantly to cessation of lactic acid production, namely when carbon source became exhausted, growth history displayed a sharp peak, corresponding to a sudden shift from growth to autolysis, in agreement with previous results (Amrane, 2001; Amrane and Prigent, 1997). It was the case when pH was controlled at 5.9 and 4.63 (Fig.III-9a). When cells were cultivated at pH 4.04, a long stationary phase was recorded, since only 40 h of growth was needed to achieve the growth inhibitory concentration of undissociated lactic acid, namely 8.5g L^{-1} (Fig.III-9a). At this acidic pH control, cessation of production was recorded before carbon source exhaustion, owing to the final HL concentration achieved, 13.4g L^{-1} (Fig.III-9b), which inhibited lactic acid production (Amrane and. Prigent, 1999a). This inhibitory HL concentration was not achieved when pH was controlled at 5.9 and 4.63, leading to cessation of lactic acid production, due to carbon source exhaustion (Fig.III-9b).

The Luedeking and Piret plot, q_p vs. μ, is a valuable tool to analyse the linking between growth and lactic acid production (Luedeking and Piret, 1959a, Amrane and Prigent, 1997), As observed (Fig.4c), the model 'GM2' matched experimental data. For an increasing inhibitory effect, namely decreasing pH control, decreasing q_p and μ values were recorded; this behaviour was satisfactory described by the model (Fig.III-9c). The experimental values recorded at the beginning of cultures, namely at low biomass concentrations and then high specific growth rates, can be regarded as of little significance, due to the high error in the differentiation of experimental data (Levenspiel, 1962).

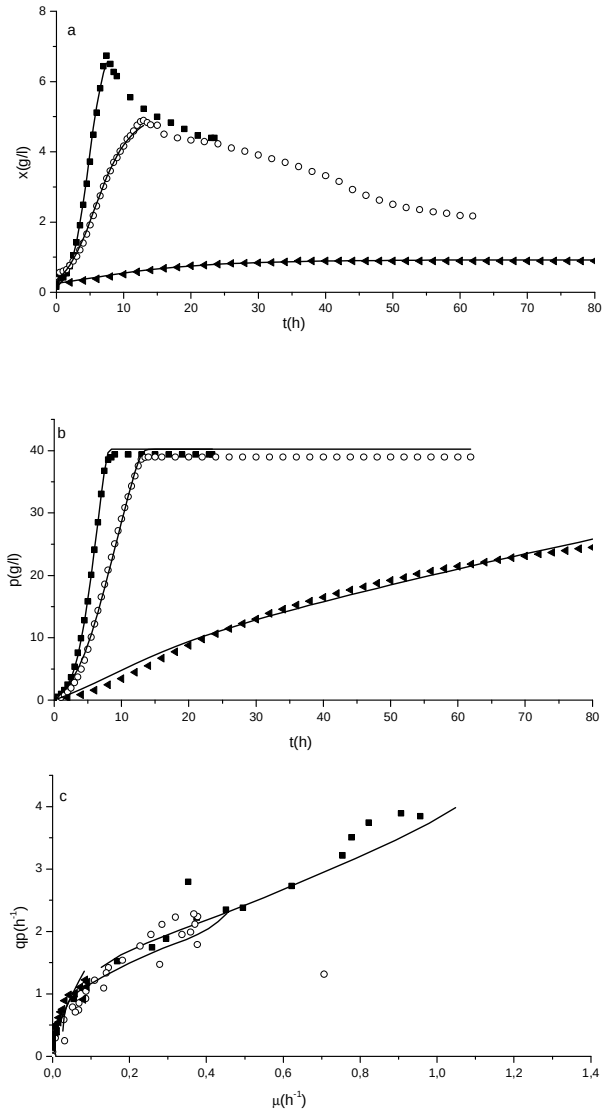


Figure III-9. Growth (Fig.III-9a), lactic acid production (Fig.III-9b) time-courses and specific production rate versus specific growth rate (Fig.III-9c) during batch cultures of *L. helveticus* growing at various controlled pH (■) 5,9; (○) 4,63; (◀) 4,04 initially adjusted by addition of lactic acid; calculated data (continuous lines).

III-3.3.Conclusion

Two generalized models were compared in this section. In the first generalized model ‘GM1’, both inhibitory and nutritional effects were taken into account in the production rate expression; while the inhibitory effect was introduced in the growth rate expression in the second model ‘GM2’. Both matched experimental growth and lactic acid production data in various culture conditions and media, namely in case of growth inhibition (cultures without pH control or at acidic pH control) or nutritional limitations. Discrepancies was only observed between experimental and calculated data using the generalized model ‘GM1’ at low pH control (4.04), namely in case of a high inhibitory effect. The better adequation of the ‘GM2’ model was confirmed at the examination of growth- and non-growth-associated parameters, A and B. Indeed, some aberrant A and B values were given by the ‘GM1’ model at acidic pH control, namely in case of an inhibitory effect on growth, which however ceased due to carbon source exhaustion.

III-4: MODELLING OF A TWO STAGE CONTINUOUS CULTURE

III-4.1. INTRODUCTION

Batch fermentation remains the most commonly used approach in industrial lactic acid production. However, volumetric productivities are low due to end-product inhibition (Amrane and Prigent, 1996, Kwon et al., 2001), and it is now well established that lactic acid production is strictly dependent on cell growth. Continuous bioreactors can be therefore a useful alternative (Amrane and Prigent, 1996). However, volumetric productivities reported for continuous one stage bioreactors remain very low. The efficiency of continuous two-stage bioreactors was previously successfully demonstrated (Amrane and Prigent, 1996, Schepers et al., 2006). In addition to high volumetric productivities, this process present the useful and usual advantages of continuous systems, namely only one sterilisation is needed at the beginning of the culture, process control is very simple and possible problem of inoculum reproducibility is avoided, owing to the availability of continuous seed culture (Amrane and Prigent, 1996). Conversion rates of lactose into lactic acid (above 90 %) for a weak residence time (8 h) are similar to those recorded in batch culture under comparable conditions, namely a final lactic acid concentration of 44g L^{-1}, corresponding to nearly 90 % conversion rate after 8 h of culture. Contrarily, a long residence time is needed to achieve an interesting conversion rate during one stage continuous culture.

The objective of this section was the development and the validation of the above models in the case of a two stage continuous culture (Amrane and Prigent, 1996); the first stage acting as a continuous seed culture (no pH control), inoculated continuously the second bioreactor, the production reactor (pH control at 5.9), which was continuously fed with sterile culture medium.

III-4.2. Overall Mass Balance

A schematic diagram of the system is given in Figure III-10.

The following assumptions were considered:

(i) The fermentation process was carried out in continuous stirred-tank reactors (CSTR),

(ii) There was no micro-organisms in the feeds of each stage ($x_0 = 0$),

(iii) At steady state conditions, there was no variation of growth and product concentrations with time ($\frac{dx}{dt} = 0 ; \frac{dp}{dt} = 0$).

The mass balance can be expressed as follows:

Accumulation = Inlet + Production – Outlet – Consumption (III-19)

It was drawn for each stage.

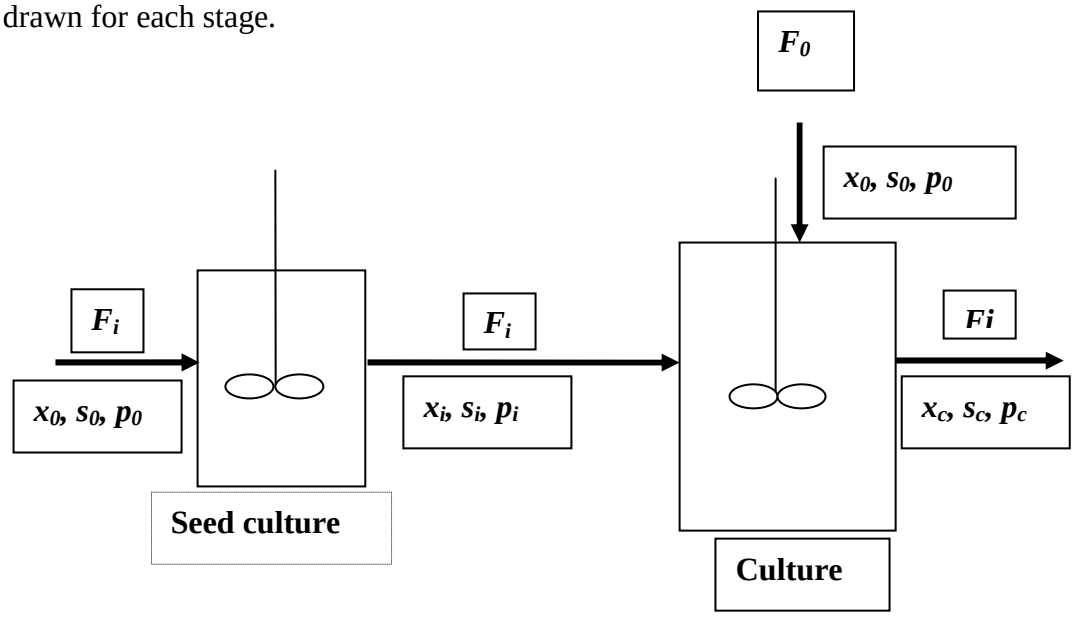


Figure III-10.Diagram of the two-stage continuous process.

III-4.2.*1. First stage (Seed culture – no pH control)*

a. For growth

$$\frac{dx_i}{dt} = (\mu_i - D_i)x_i \quad \text{(III-20)}$$

where μ_i and $D_i = \frac{F_i}{V_i}$ were the specific growth rate (h^{-1}) and the dilution rate in the first stage (h^{-1}).

Under steady-state conditions $\left(\frac{dx}{dt} = 0\right)$, Eq. (III-20) became:

$$D_i = \mu_i \qquad \text{(III-21)}$$

b. For production

$$\frac{dp_i}{dt} = D_i p_o - q_{p_i} x_i - D_i p_i \qquad \text{(III-22)}$$

By considering a negligible lactic acid concentration in the feed of the continuous seed culture, at steady-state (Eq.III-22) can be written as follows:

$$q_{p_i} \bar{x}_i = D_i \bar{p}_i \qquad \text{(III-23)}$$

III-4.2.*2. Second stage (Culture – pH controlled at 5.9)*

a. For growth

$$\frac{dx_c}{dt} = D_i x_i \frac{V_i}{V_c} + \left(\mu_c - D_c\right) x_c \qquad \text{(III-24)}$$

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

Under steady-state conditions $\left(\frac{dx_C}{dt} = 0\right)$, Eq (III-24) became:

$$\frac{V_i}{V_c} D_i \bar{x}_i + \left(\mu_c - D_c\right) \bar{x}_c = 0 \qquad \text{(III-25)}$$

b. For production

$$\frac{dp_c}{dt} = \frac{V_i}{V_c} D_i \bar{p}_i - D_c \bar{p}_c + q_{p_c} \bar{x}_c \qquad \text{(III-26)}$$

Under steady-state conditions $\frac{dp_c}{dt} = 0$, and Eq.(III-26) can therefore be written as follows :

$$\frac{V_i}{V_c} D_i \bar{p}_i - D_c \bar{p}_c + q_{p_c} \bar{x}_c = 0 \qquad \text{(III-27)}$$

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 conditions in the seed culture and the culture reactors, respectively.

The above equation (Eq. III-27) can be rearranged as follows:

$$\bar{p}_c = \left[\frac{V_i}{V_c} D_i \bar{p}_i + q_{p_c} \bar{x}_c \right] \frac{1}{D_c} \qquad \text{(III-28)}$$

III-4.3. Model development

The Verlhust model which proved to describe satisfactory growth kinetics (Moraine and Rogovin, 1996; Pandey et al., 2000) was considered for all the models developed above:

$$\mu = \mu_{max} \left(1 - \frac{x}{x_{max}} \right) \qquad \text{(III-3)}$$

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

III-4.3..1. Growth model

a. First stage (Seed culture – no pH control)

Under steady-state conditions ($D_i = \mu_i$, Eq. III-21), Eq. (III - 3) became:

$$\bar{x}_i = x_{max_i} \left(1 - \frac{D_i}{\mu_{max_i}} \right) \qquad \text{(III-29)}$$

b. Second stage (Culture – pH controlled at 5.9)

the introduction of the Verlhust model (Eq. III - 3) in the mass balance for biomass in the second stage (III-24) led to the following implicit equation of $\bar{x}_c$:

$$\frac{V_i}{V_c}\bar{x}_i D_i + \left[\frac{\mu_{max_c}}{x_{max_c}}\left(x_{max_c} - \bar{x}_c\right) - D_c\right]\bar{x}_c = 0 \qquad \text{(III-30)}$$

Eq.(III-30) indicated that the biomass concentration at steady-state $\bar{x}_c$ was a function of the biomass concentration at steady-state in the first stage $\bar{x}_i$, and the dilution rates in the first (D_i) and in the second (D_c) stages.

III-4.3.2. Production models

III-4.3.2.A. Luedeking-Piret model (*LP* model)

To describe the production kinetics, the Luedeking-Piret model was considered:

$$q_p = A\mu + B \qquad \text{(III-31)}$$

Where A and B were the coefficients for growth- and non-growth-associated productions.

III-4.3.2.A.1. *First stage (Seed culture – no pH control)*

By introducing the equation for mass balance of the product at steady-state (Eq. III-22) into the Luedeking-Piret model (Eq. III-31) and by considering the biomass concentration at steady-state in the first stage (Eq. III-20), it came:

$$\bar{p}_i = \frac{x_{max_i}}{D_i}\left[1 - \frac{D_i}{\mu_{max_i}}\right]\left(A_i D_i + B_i\right) \qquad \text{(III-32)}$$

III-4.3.2.A.2. ***Second stage (Culture – pH controlled at 5.9)***

By introducing the Verlhust expression (Eq. III-3) into the Luedeking-Piret relation (Eq. III-31), the specific production rate in the second stage q_{p_c} can be expressed as a function of the biomass concentration at steady-state in the second stage $\bar{x}_c$:

$$q_{p_c} = A_c \frac{\mu_{max_c}}{x_{max_c}} (x_{max_c} - \bar{x}_c) + B_c \qquad \text{(III-33)}$$

From the mass balance for the product in the second stage (Eq. III-28), the lactic acid concentration at steady-state in the second stage $\bar{p}_c$ can then be expressed as a function of the dilution rates in the first D_i and the second D_c stages, the lactic acid concentration at steady-state in the first stage $\bar{p}_i$ and the biomass concentration at steady-state in the second stage $\bar{x}_c$:

$$\bar{p}_c = \left[\frac{V_i}{V_c} D_i \bar{p}_i + \left(A_c \frac{\mu_{max_c}}{x_{max_c}} (x_{max_c} - \bar{x}_c) + B_c \right) \bar{x}_c \right] \frac{1}{D_c} \qquad \text{(III-34)}$$

III-4.3.2.B. Modified Luedeking-Piret model

III-4.3.2.B.1 ***First stage (Seed culture – no pH control)*** **(*IM* model)**

The first stage of the system acts without pH control, owing to the positive effects of pre-cultivating without pH control (Amrane and Prigent, 1996, Amrane and Prigent, 1998a). Since the main inhibitory species is the undissociated form of the lactic acid (Kashket, 1987; Gätje and Gottschalk, 1991), the Luedeking-Piret model was previously modified to account for the undissociated lactic acid inhibition (see *§III-1*):

$$q_p = A * \mu + B * \left(1 - \frac{[HL]}{[HL]_{inh}} \right) \qquad \text{(III-7)}$$

Where $[HL]$ and $[HL]_{inh}$ were the undissociated lactic acid concentration and its inhibitory concentration, namely 8.5g L^{-1} (Amrane and Prigent, 1999a). The concentration of the undissociated form of lactic acid [HL] was given by the Henderson-Hasselbach equation:

$$[HL] = \frac{p}{1+10^{pH-pK_A}} \qquad \text{(III-5)}$$

An implicit expression for the lactic acid concentration at steady-state can be deduced by introducing the mass balance of the product in the first stage (Eq. III-23) into the above modified Luedeking-Piret model (Eq. III - 7) and by considering the biomass concentration at steady-state in the first stage (Eq. III - 29):

$$\bar{p}_i = \frac{x_{max_i}}{D_i}\left(1-\frac{D_i}{\mu_{max\,i}}\right)\left[A_i D_i + B_i\left(1-\frac{\bar{p}_i}{[1+10\text{^}(pH_i - pK_A)][HL]_{inh}}\right)\right] \qquad \text{(III-35)}$$

III-4.3.2.B.2.*Second stage (Culture – pH controlled at 5.9)* (*SLM* model)

To overcome the inhibitory effects of pH (Gonçalves et al., 1997, Fu and Mathews 1999) and undissociated lactic acid species (Gätje and Gottschalk, 1991), the pH is maintained during culture, namely in the second stage, at its optimal value for lactic acid production (5.9) (Hanson and Tsao, 1972, Venkatesh et al., 1993), leading to cessation of production when carbon became limiting. Indeed, the lactic acid bacteria are unable to use the carbon content arising from the autolysed cells (Amrane and Prigent, 1997). In order to take this limitation into account, the Luedeking-Piret expression was previously modified (Amrane, 2001):

$$q_p = A\mu + B\left(1-\frac{s_{res}}{s}\right) \qquad \text{(III-10)}$$

Recently, the above expression was improved by introducing the limiting lactose concentration s_{lim}, in place of the residual lactose concentration s_{res} (see § III-2):

$$q_p = A\mu + B\left(1 - \frac{s_{lim}}{s}\right) \qquad \text{(III-11)}$$

s_{lim} corresponded to the limiting lactose concentration, 3g L^{-1}, deduced from several runs on whey supplemented with various yeast extract concentrations (Amrane and Prigent, 1999b, Amrane and Prigent, 1998b).

There was a linear relationship between the carbon substrate concentration s and the lactic acid production p, if we consider a constant product on substrate yield $Y_{P/S}$:

$$Y_{P/S} = \frac{p - p_0}{s_0 - s} \qquad \text{(III-18)}$$

By introducing the Verlhust expression (Eq.III-3) into the above modified Luedeking-Piret relation (Eq. (III-11) and by considering a constant product on substrate yield $Y_{P/S}$ (Eq. (III-18), 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 $\bar{p}_c$:

$$q_{p_c} = A_c \frac{\mu_{max_c}}{x_{max_c}}(x_{max_c} - \bar{x}_c) + B_c\left(1 - \frac{s_{lim} Y_{P/S}}{s_0 Y_{P/S} - \bar{p}_c + p_0}\right) \qquad \text{(III-36)}$$

Introduction of the specific production rate (Eq. III-36) into the mass balance for the product in the second stage (Eq. III-28) led to the lactic acid concentration at steady-state in the second stage $\bar{p}_c$:

$$\bar{p}_c = \left[\frac{V_i}{V_c} D_i \bar{p}_i + \left(A_c \frac{\mu_{max_c}}{x_{max_c}}(x_{max_c} - \bar{x}_c) + B_c\left(1 - \frac{s_{lim} Y_{P/S}}{s_0 Y_{P/S} - \bar{p}_c + p_0}\right)\right)\bar{x}_c\right]\frac{1}{D_c} \qquad \text{(III-37)}$$

This implicit expression of $\bar{p}_c$ (Eq. III-37) involved the lactic acid concentration in the first stage $\bar{p}_i$, the biomass concentration in the second stage $\bar{x}_c$ and the dilution rates in the first D_i and the second D_c stages.

III-4.3.2.C. generalized model (*GM1* model)

To avoid the use of two expressions to describe the production rate, depending on the culture conditions, the above Luedeking-Piret expressions (Eqs.III-7 and III-11) were merged, leading to an unique expression taking into account both effects, an inhibitory effect (Eq.III-7) and a nutritional limitation effect (Eq. III-11):

$$q_p = A * \mu + B * \left(1 - \frac{s_{lim}}{s}\right) * \left(1 - \frac{[HL]}{[HL]_{inh}}\right) \qquad \text{(III-12)}$$

III-4.3.2.C.1. ***First stage (Seed culture – no pH control)***

Similarly to the inhibition model (Eq. III-35), an implicit expression for the lactic acid concentration at steady-state can be easily derived from the above relation (Eq. III-12) by considering a constant product on substrate yield $Y_{P/S}$:

$$\bar{p}_i = \frac{x_{max_i}}{D_i}\left(1 - \frac{D_i}{\mu_{max\,i}}\right)\left[A_i D_i + B_i\left(1 - \frac{Y_{P/S}.s_{Lim}}{s_0 Y_{P/S} - \bar{p}_i + p_0}\right)\left(1 - \frac{\bar{p}_i}{[1+10\text{^}(pH_i - pKA)][HL]_{inh}}\right)\right] \qquad \text{(III-38)}$$

It can be noted that the product concentration at steady-state in the first stage was only function of the dilution rate D_i.

III-4.3.2.C.2. ***second stage (Culture – pH controlled at 5.9)***

Similarly to the substrate limitation model Eq.(III-36), 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 $\bar{p}_c$, if the Henderson-Hasselbach equation (Eq. III - 5) was considered for the undissociated lactic acid concentration:

$$q_{p_c} = A_c \frac{\mu_{max_c}}{x_{max_c}}(x_{max_c} - \bar{x}_c) + B_c\left(1 - \frac{s_{lim} Y_{P/S}}{s_0 Y_{P/S} - \bar{p}_c + p_0}\right)\left(1 - \frac{\bar{p}_c}{[1+10\hat{}(pH_c - pK_A)][HL]_{inh}}\right) \quad \text{(III-39)}$$

The introduction of the above specific production rate (Eq. III - 39) into the mass balance for the product in the second stage (Eq. III-28) led to the lactic acid concentration at steady state in the second stage $\bar{p}_c$:

$$\bar{p}_c = \left[\frac{V_i}{V_c} D_i \bar{p}_i + \left(A_c \frac{\mu_{max_c}}{x_{max_c}}(x_{max_c} - \bar{x}_c) + B_c\left(1 - \frac{s_{lim} Y_{P/S}}{s_0 Y_{P/S} - \bar{p}_c + p_0}\right)\right)\left(1 - \frac{\bar{p}_c}{[1+10\hat{}(pH_c - pK_A)][HL]_{inh}}\right)\bar{x}_c\right]\frac{1}{D_c} \quad \text{(III-40)}$$

As for the other models, the expression of $\bar{p}_c$ (Eq. III-40) involved $\bar{p}_i$, $\bar{x}_c$, D_i and D_c.

III-4.4. Results and Discussion

III-4.4.1. First Stage (Seed culture)

The calculated value (Eq. III-29) for the biomass concentration at steady-state $\bar{x}_i$ in the first stage $\bar{x}_i$ (continuous seed culture) was 1.4g L^{-1}, *i.e.* rather close to the experimental value 2.0g L^{-1} – (Amrane and Prigent, 1996). The considered values for the maximum biomass concentration x_{max_i} and the maximum specific growth rate μ_{max_i} involved in Eq.III-29 were 1.54 g L^{-1} and 0.68 h^{-1} respectively, taken from previous batch cultures of *L. helveticus* carried out without pH control on the similar medium (see section 1). The dilution rate in the first stage D_i was 0.083 h^{-1} (Amrane and Prigent, 1996).

The calculated values for the lactic acid concentration at stationary state $\overline{p}_i$ in the first stage are given in Table III-9. Three models were considered for the production kinetics, the Luedeking-Piret (LP) (Eq. III-31), the 'Inhibition model' (IM), i.e. the Luedeking-Piret model modified to account for the undissociated lactic acid inhibition (Eq.III-7) and the 'Generalized model1' (GM1) which take into account both an inhibition and a substrate limitation (Eq.III-12). The lactic acid concentration at steady state $\overline{p}_i$ was deduced from production kinetics by considering the above biomass concentration at steady-state $\overline{x}_i$. In the relation for the lactic acid concentration at steady-state given by the Luedeking-Piret model (Eq. III-32), the growth- (A_i) and non-growth-associated (B_i) parameters were deduced from the fitting of production time-courses recorded during batch culture of L. helveticus carried out without pH control on the similar medium (Amrane and Prigent, 1999a)and are collected in TableIII-9. Similarly, the 'Inhibition model' (Eq.III-7) and the 'Generalized model' (Eq.III-12) led to an implicit relation for the lactic acid concentration at steady-state, Eqs.(III-35) and (III-38) respectively; the previously calculated values for parameters A_i and B_i deduced from batch cultures of L. helveticus carried out without pH control on the same medium were considered (see section 1) and are given in Table III-9. The experimental pH in the seed culture reactor was also taken into account in a first approach, 3.9 (Amrane and Prigent, 1996). In the 'Generalized model1', s_{lim} corresponded to the limiting lactose concentration, 3g L^{-1}, deduced from several runs on supplemented whey (Amrane and Prigent, 1999b, Amrane and Prigent, 1998b); the corresponding product on substrate yield $Y_{P/S}$ (0.9) was also addressed. We shall keep in mind that during cultures without pH control, a low amount of lactic acid was produced, leading to high residual lactose concentrations; then the substrate limitation term $\left(1-\frac{s_{lim}}{s}\right)$, namely the term $\left(1-\frac{Y_{P/S}.s_{Lim}}{s_0 Y_{P/S}-\overline{p}_i+p_0}\right)$ in Eq. III-38, remained always close to

unit. Therefore, 'inhibition term' $\left(1-\frac{\bar{p}_i}{[1+10\text{^}(pH_i - pKA)][HL]_{inh}}\right)$ in Eq.III-38 appeared the most significant. It should also be noted that the lactic acid concentration in whey was neglected ($p_0 = 0$).

As observed inTableIII-9, similar $\bar{p}_i$ values were given by the three considered models. Indeed, the undissociated lactic acid concentration was 4.1g L^{-1} (pH_i = 3.9), namely lower than the inhibitory undissociated lactic acid concentration (8.5g L^{-1}(Amrane and Prigent, 1999a), leading to a low inhibitory effect. The average calculated value was 8.0g L^{-1} with a standard error of 0.2g L^{-1}. This value was rather close to the experimental one, 9.2g L^{-1} (Amrane and Prigent, 1996).

Reliable prediction was therefore recorded for both the biomass and the lactic acid concentrations at steady-state in the seed culture stage.

Table III-9.Lactic acid concentration at steady-state $\bar{p}_i$ in the seed culture stage obtained by considering the growth- (A_i) and non-growth-associated (B_i) parameters deduced from the fitting of batch cultures of L. helveticus carried out on the same medium.

		Model		
		LP	IM	GM1
		(Eq.III-32)	(Eq.III-35)	(Eq.III-38)
A_i		4.24	3.89	3.88
B_i	(h)	0.14	0.30	0.32
$\bar{p}_{i,calc}$	(g L^{-1})	8.0	7.8	8.1

III-4.4.2. Second Stage (Culture)

Figure 2 shows the experimental and the calculated (Eq. III-30) biomass concentration data at steady-state in the second stage (RSD = 0.5). As observed, the model did not account for the important decrease of the biomass concentration at high dilution rate, namely close to

wash out (Amrane and Prigent, 1996), and could be subsequently improved. Complex growth models have been avoided in this work to avoid too complex production models.

Table III-10.Growth- (A_c) and non-growth-associated (B_c) parameters deduced from the fitting of batch cultures of *L. helveticus* carried out on the same medium and used for the determination of the lactic acid concentrations at steady-state $\bar{p}_c$ in the culture stage.

		Model		
		LP	SLM	GM1
		(Eq.III-34)	(Eq.III-37)	(Eq.III-40)
A_c		5.16	2.36	2.32
B_c	(h)	0.3	0.81	0.83

From the production kinetics, namely the 'Ludeking-Piret model' (Eq. III-31), the 'Substrate limitation model' (SLM) (Eq. III-7) and the 'Generalized model' (GM1) (Eq. III-12), the lactic acid concentration at steady-state $\bar{p}_c$ in the second stage (production reactor) was expressed as a function of the dilution rates in both stages, D_i and D_c, the lactic acid concentration in the seed culture stage $\bar{p}_i$ and the biomass concentration in the culture stage $\bar{x}_c$, namely Eqs. III-34, 37 and 40 for LP, SLM and GM1 models respectively.

In the models for lactic acid concentration at steady-state (Eqs.III-34,37and 40), the maximum biomass concentration and the maximum specific growth rate in the second stage x_{max_c} and μ_{max_c} were deduced from the fitting (Verlhust model – Eq.III-11) of batch cultures of L. helveticus carried out at pH controlled at 5.9 on the same medium. Similarly to the first stage, a value of 3g L^{-1} for s_{lim} and the corresponding product on substrate yield $Y_{P/S}$ (0.9) were considered. Moreover, it should be noted that whey contained 48g L^{-1} (s_0) lactose and a negligible amount of lactic acid (p_0).

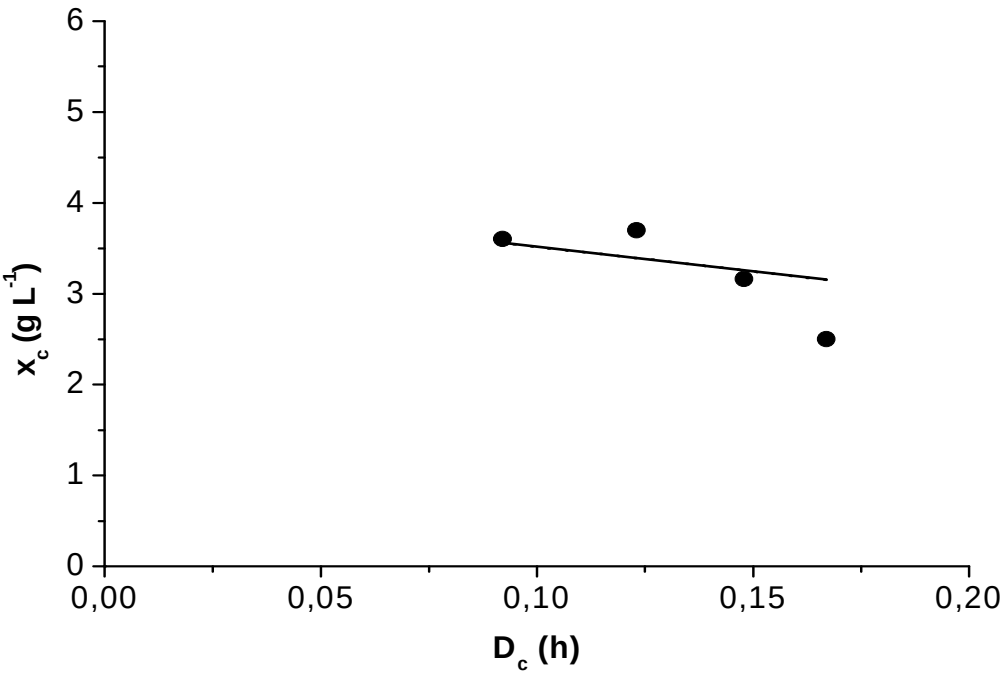


Figure III-11.Experimental (symbol) and calculated (continuous line) (Eq. III-30) biomass concentrations at steady-state in the second stage of the system.

In Figure III-12a, the parameters for the growth and the non-growth associated, A_c and B_c, were taken from the fitting (Eqs.III-34, 37 and 40) of batch culture data of L. helveticus growing at a pH 5.9 on the same medium. The corresponding values are collected in Table III-10. As observed and similarly to the behaviour recorded for the seed culture stage, all the tested models underestimated the lactic acid concentrations at steady-state. The 'Luedeking-Piret model' appeared to fail in the description of the experimental data by the largest amount; while the 'Substrate limitation model' and the 'Generalized model' led to some similar and fairly reliable predicted values, the residual standard deviations decreased to 8.1 and 8.8 when compared to the value given by the LP model (RSD = 24.7). Indeed, at controlled pH (5.9), the undissociated lactic acid concentration (approximately 0.3g L^{-1}) is below the inhibitory threshold (Gätje and Gottschalk, 1991), namely almost negligible compared to the inhibitory undissociated lactic acid concentration, 8.5g L^{-1} (Amrane and Prigent, 1999a). Consequently,

the inhibition term $\left(1-\frac{[HL]}{[HL]_{inh}}\right)$, namely the term $\left(1-\frac{\bar{p}_i}{[1+10\text{^}(pH_i-pKA)][HL]_{inh}}\right)$ in Eq.40, had no effect, since it was close to unit; and the main term was therefore the substrate limitation term $\left(1-\frac{Y_{P/S}.s_{Lim}}{s_0 Y_{P/S}-\bar{p}_i+p_0}\right)$ (Eqs.III-34 and 40).

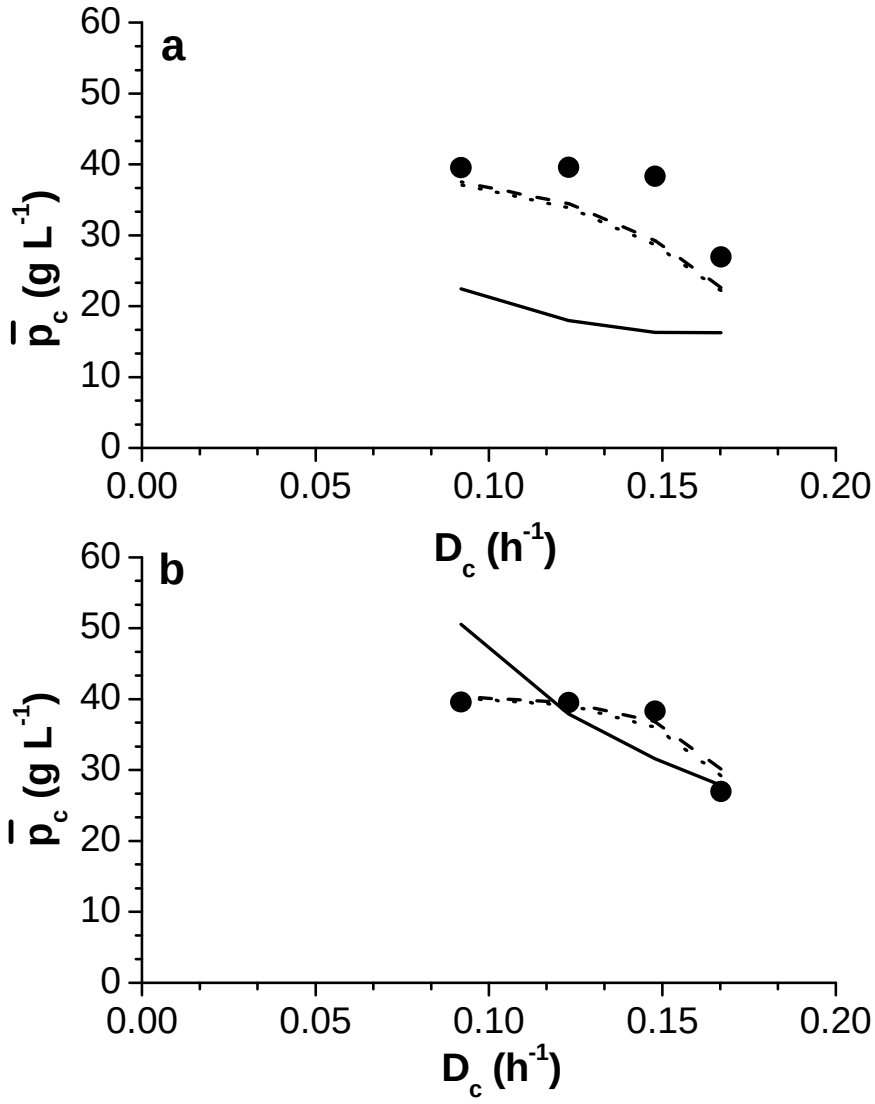


Figure III-12. Experimental (symbol) and calculated (continuous lines) lactic acid concentrations at steady-state in the second stage of the system by considering the optimized growth (A_c) and non-growth associated (B_c) production parameters (b) or the parameter values deduced from the fitting of batch cultures of *L. helveticus* carried out on the same medium (a). Lactic acid concentrations were calculated by considering the Luedeking and Piret model ((Eq. III-34 – solid line), the substrate limitation model (Eq. III-25 – short dot line) and the generalized model (Eq. III-40 – dash dot line).

The predictive potential of the modified Luedeking and Piret models (IM, SLM and GM1) were confirmed, since fairly reliable predicted concentrations were recorded for the biomass and the lactic acid concentrations at steady-state in both stages, seed culture and culture. However for a comprehensive model validation, some reliable predicted data should be confirmed for continuous two-stage cultures on various media; the corresponding work requires subsequent experiments.

The calculated data displayed in Figure III-12b were the result of an optimization of the parameters A_c and B_c, which were 3.32 and 1.10 for the three models. As observed and confirming the above results, the 'Luedeking-Piret model' failed in describing experimental data; while both other models (SLM and GM1)led to similar calculated data (as discussed above), which was close to experimental data (RSD = 2.3-2.6). It can be observed, that both optimized parameter values were higher than the parameter values taken from the fitting of batch culture data.

The 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 productivity displayed in Figure III-13 was calculated using the optimized parameters A_c and B_c; per the previous observations, it matched the experimental values for the SLM and GM1 models (RSD = 0.4), since the calculated $\overline{p}_c$ values matched the experimental values (Fig.III-13).

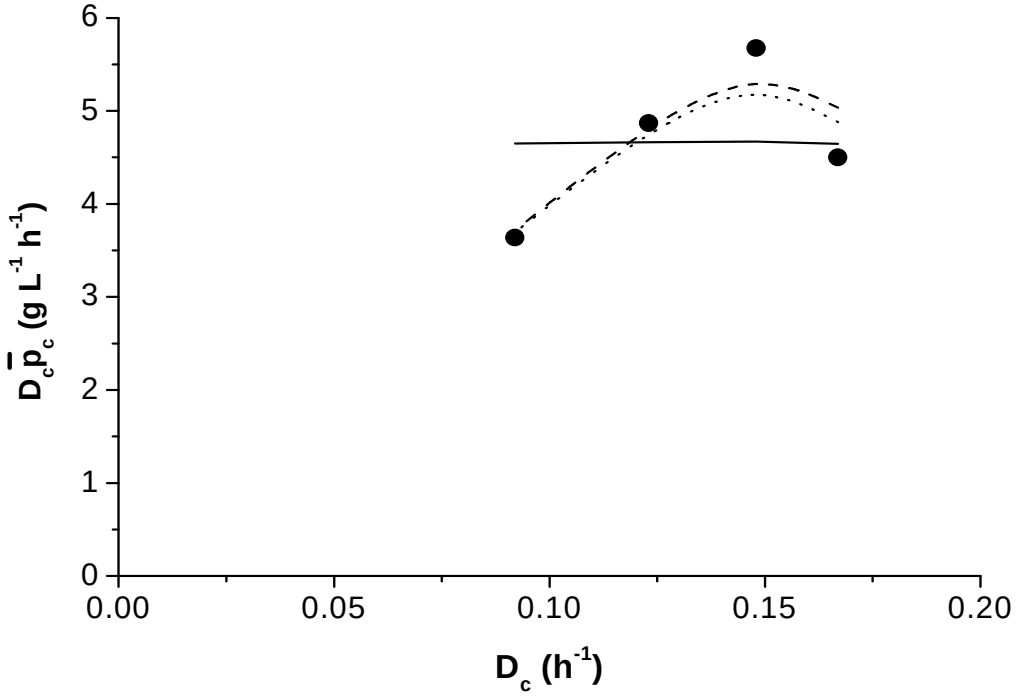


Figure III-13. Experimental (symbol) and calculated (continuous lines) volumetric productivity $D_c \overline{p}_c$ at steady-state in the second stage of the system by considering the optimized growth- (A_c) and non-growth-associated (B_c) production parameters. The Luedeking and Piret (Eq.III-34 – short dot line), the substrate limitation (Eq.III-37–dash line) and the generalized (Eq.III-40– dash dot line) models were respectively considered for calculations.

The parity plot (FigureIII-14) confirmed that the 'Substrate limitation model' and the 'Generalized model were appropriate, owing to the strong correlation between experimental and calculated data. Indeed, the group of data points was homogeneously distributed around the first bisectrix; the correlation coefficient was 0.97 for the growth data and was at least 0.99 for the production data and the volumetric productivity data.

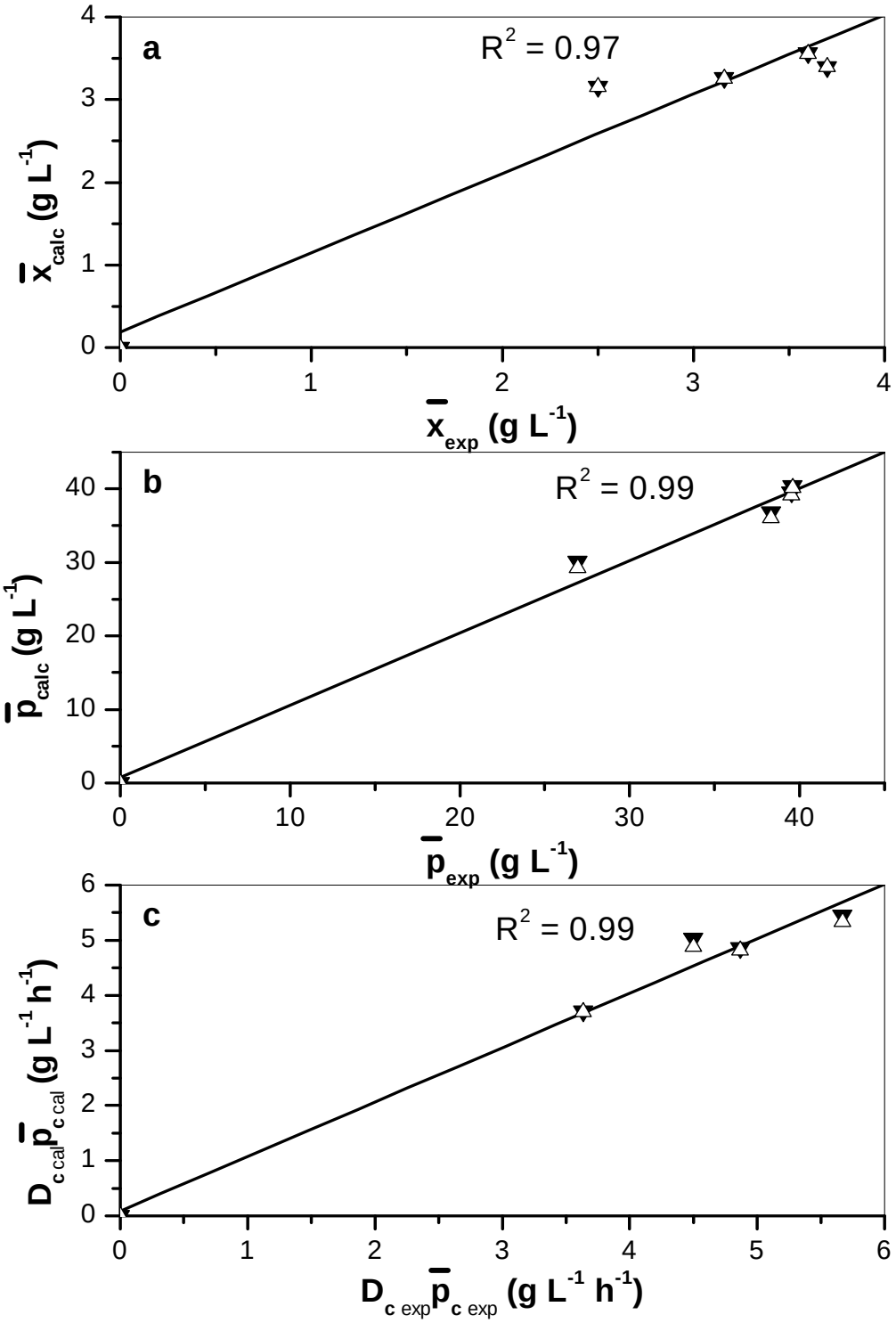


Figure III-14. Parity plot of the predicted biomass concentrations (a), the lactic acid concentrations (b) and the volumetric productivities $D_c \bar{p}_c$ (c) at steady-state in the second stage by considering the substrate limitation (B) and the generalized (Δ) models versus experimental time-course data.

III-4.5.Conclusion

Experimental data were accurately described in the case of a two stage continuous culture, by the above models; the first stage acting as a continuous seed culture (no pH control), inoculated continuously the second bioreactor, the production reactor (pH control at 5.9), which was continuously fed with sterile culture medium.

As observed for growth, the model did not account for the important decrease of the biomass concentration at high dilution rate and could be therefore subsequently improved. The corresponding work is in progress.

As expected, the ‘Luedeking-Piret model’ failed in describing experimental data; while both other models, SLM and GM1, led to similar calculated data.

Conclusion

A study on modelling lactic acid fermentation in batch and continuous cultures was carried out. The most important conclusions are:

- The model developed concerning seed culture, namely experiments carried out without pH control (inhibition model), shows that all the parameters have a clear biological meaning. The Verlhust model was considered to describe growth kinetics (Eq.III-3), which can easily be integrated to give growth time-courses (Eq.III-4). The Luedeking-Piret model was modified by introducing an additional term to account for the undissociated lactic acid inhibition (Eq.III-7). The model was found to match both experimental growth and production data and was validated in various cultures conditions, namely for a large range of nitrogen supplementation of whey permeate.

- During cultures at pH controlled at 5.9, nutritional limitations caused cessation of growth and lactic acid production; the 'inhibition model' was therefore obviously inappropriate as experimentally confirmed, since it did not account for cessation of production. From this, to describe lactic acid production data, a corrective term was introduced into the Luedeking-Piret model (Eq. III-10) to account for cessation of production owing to carbon substrate limitation. Moreover, this last model has been improved by introducing the limiting lactose concentration s_{lim}, in place of the residual lactose concentration s_{res} (Eq. III-11). This model (substrate limitation model) was successfully tested for a large range of nitrogen supplementation; the model matched whole production kinetics recorded during cultures at pH controlled, namely in case of nutritional limitations.

To avoid the use of two expressions (inhibition model and substrate limitation model) for production rate depending on the culture conditions, the above expressions were merged, leading to a unique expression that takes into account both effects, a nutritional limitation effect and an inhibitory effect (Eq.III-12). Results obtained show that the generalized model

gave a satisfactory description of experimental data in various culture conditions, since it was validated during cultures at pH control and in absence of pH control, as well as for different nitrogen supplementation of culture media. However, in some cases, especially during cultures carried out at acidic pH control, an inhibitory effect can be observed during growth, which, however, ceased when carbon became limiting (or when the undissociated lactic acid concentration reached its inhibitory threshold value at highly acidic pH control).In this case the above model (Eq.III-12) appeared inappropriate. From this, another general model was considered. This model was based on the modified Verlhust expression (Eq.III-17). In this model, the inhibitory term related to the undissociated lactic acid inhibition was added in the growth relation instead of the production model. Both generalized model matched experimental growth and lactic acid production data in various culture conditions and media, namely in case of growth inhibition (cultures without pH control or at acidic pH control) or nutritional limitations. Discrepancies were only observed between experimental and calculated data using the first generalized model (Eq.III-3 and Eq.III-12) at low pH control (4.04), namely in case of a high inhibitory effect. The better adequation of the second generalized model (Eq.III-17 and Eq.III-11) was confirmed at the examination of growth- and non-growth-associated parameters, A and B. Indeed, some aberrant A and B values were given by the first generalized model at acidic pH control, namely in case of an inhibitory effect on growth, which however ceased due to carbon source exhaustion.

- Finally, the above models were developed in the case of a two stage continuous culture: the first stage acting as a continuous seed culture (no pH control), inoculating continuously the second bioreactor, the production reactor (pH control at 5.9), which was continuously fed with sterile culture medium. The models matched experimental data accurately but for growth, the model did not account for the important decrease of the biomass concentration at high dilution rate and could be therefore subsequently improved. On

the other hand and as expected, the ‘Luedeking-Piret model’ failed in describing experimental data; while both other models, the substrate limitation model and the first generalized model led to similar and satisfactory calculated data.

In the near future, it would be interesting to improve the growth model in the case of two stage continuous cultures using a neural networks method to describe lactic acid fermentation. To generalize the models developed in this work to other types of fermentation, such as ethyl fermentation, alcohol fermentation and others may be also subsequently considered. The generalization of the models developed for one substrate in this work to multi-substrate fermentation would be also helpful.

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Appendix

APPENDIX

	Growth Model : Verlhust model	
	$\mu = \mu_{\max} * \left(1 - \frac{x}{x_{\max}}\right)$	

Production Model	**Ludeking-Piret (*LP*) (1959)**	**Substrate limitation Model (*SLM*)**	**Inhibition Model (*IM*)**
	$qp = A * \mu + B$	$qp = A * \mu + B * \left(1 - \frac{s_{\lim}}{s}\right)$	$qp = A * \mu + B * \left(1 - \frac{[HL]}{[HL]_{inh}}\right)$

Models	**General Model 1 (*GM1*)**	**General Model 1 (*GM2*)**
Growth	$\mu = \mu_{\max} * \left(1 - \frac{x}{x_{\max}}\right)$	$\mu = \mu_{\max} * \left(1 - \frac{x}{x_{\max}}\right) * \left(1 - \frac{[HL]}{[HL]_{inh}}\right)$
Production	$qp = A * \mu + B * \left(1 - \frac{s_{\lim}}{s}\right) * \left(1 - \frac{[HL]}{[HL]_{inh}}\right)$	$qp = A * \mu + B * \left(1 - \frac{s_{\lim}}{s}\right)$

Abstract

During lactic acid fermentation in batch and continuous culture using Lactobacillus *helveticus,* seed culture is usually carried out without pH control, while culture is carried out at pH controlled at the optimal value to overcome inhibitory effects. In this study, novel mathematical models are set up to describe lactic acid production in batch and continuous fermentation. The Luedeking-Piret expression was therefore previously modified by introducing additional terms involving the undissociated form of the lactic acid, the main inhibitory species, in case of batch cultures without pH control. To describe growth, the Verlhust model which proved to describe satisfactory growth kinetics was considered. The model was found to match both experimental growth and production data. Another model was also developed involving the residual lactose concentration to account for carbon substrate limitation, responsible for cessation of production during batch cultures of *Lactobacillus helveticus* at controlled pH. This model matched experimental data accurately. Two generalized models were then deduced from the above expressions. The results obtained show that the generalized models gave a satisfactory description of experimental data in various culture conditions, since they were validated during cultures at pH control and in absence of pH control, as well as for different nitrogen supplementation of culture media. Both models, as well as the Luedeking-Piret model, were developed to describe successfully continuous two-stage culture of *L. helveticus*

Keywords: lactic acid, fermentation, modeling

Résumé

Au cours de la fermentation lactique en discontinu ou en continu, la préculture se fait généralement à pH libre tandis que la culture se fait à pH régulé, et ce pour éviter une inhibition par l'acide formé. Dans ce travail, de nouveaux modèles mathématiques ont été

développés pour décrire la croissance et la production d'acide lactique. Le modèle de Luedeking-Piret a été modifié en introduisant, d'une part, l'effet inhibiteur de l'acide lactique non dissocié dans le cas de la préculture. Pour la croissance, le modèle de Verlhust a été utilisé dans ce travail. Le modèle développé décrit correctement les résultats expérimentaux jusqu'à la fin de la fermentation. D'autre part, afin de rendre compte d'une limitation nutritionnelle, ce qui est le cas lors de l'étape de culture (à pH régulé) un second modèle a été développé en introduisant la concentration résiduelle en substrat carboné. Pour éviter l'utilisation des deux modèles précités, deux modèles généralisés ont été développés qui tiennent compte à la fois de l'effet inhibiteur de l'acide lactique non dissocié et des limitations nutritionnelles. Ces modèles décrivent bien les résultats expérimentaux à pH libre et à pH régulé. Ces modèles ont été appliqués avec succès au cas d'un bioréacteur biétagé en continu.

Mots Clés: acide lactique, fermentation, modélisation

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. Luedeking-Piret $\left(1-\frac{[HL]}{[HL]_{inh}}\right)$ □□□□□□□ □□□□□□□ □□□□□□□ □ □□ □□□□□□□ □□□□□□ □□ □□□□ □□□□□□□□ □□□

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Mots Clés: acide lactique, Fermentation, Modélisation

خلاصه

تتم عملية تحضير حمض اللبنيك بطريقة متقطعة و متواصلة عبر مرحلتين. في المرحلة الأولى عادة ما تنفذ دون مراقبة أل pH بينما المرحلة الثانية يثبت فيها أل pH وذلك لتفادي تأثيره على عملية الإنتاج.
في هذا الإطار تم إيجاد نماذج رياضيه جديدة لوصف عملية إنتاج حمض اللبني ك [وذلك بتعديل نموذج Luedeking-Piret. إلى النموذج الأول تم إضافة تركيز حمض اللبنيك الغير متفكك $\left(1-\frac{[HL]}{[HL]_{inh}}\right)$ الذي له تأثير مباشر على عملية الإنتاج عندما يكون أل pH دون مراقبة النموذج الجديد أعطى نتائج جيدة مقارنة بالنتائج التجريبية. بينما النموذج الثاني أضيفت إلى معادلة Luedeking-Piret عبارة $\left(1-\frac{S_{\lim}}{S}\right)$ و هذا لشرح عملية الإنتاج عند نهاية التخمير و ذالك لما يؤل تركيز اللاكتوز إلى الصفر عندما يكون أل pH ثابت لتفادي استعمال هذان النموذجان استطعنا إيجاد نموذج معمم يحتوي على خصائص النموذجين السالفين الذكر. النتائج التي أعطاها النموذج الجديد جاءت مطابقة تماما لنتائج النماذج السابقة. كل هذه النماذج طبقت على مفاعل بيولوجي يشتغل بطريقة متواصلة و أعطت نتائج جد ايجابية مقارنة بنتائج التجربة.

الكلمات الدالة: حامض لبنى، التخمير، النمذجه