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Mathematical Differentiation to Reveal the Fermentative Characteristics of *Lactobacillus helveticus* ATCC 15009

[1]Chiung-Chi Peng, [2,3]Ming-Hua Yang, [4]Chao-Ming Chuang, [2,3]Chiu-Lan Hsieh, [2]Hui-Er Wang, [3]Chun-Hung Chiu, [3]Charng-Cherng Chyau, [5]Kuan-Chou Chen and [3,6]Robert Y. Peng
[1]Department of Nursing, Cardinal Tien College of Healthcare and Management 112, Min-Zu Rd., Sin-Dian City, Taipei County, Taiwan 23143
[2]Department of Food and Nutrition, [3]Research Institute of Biotechnology, [4]Department of Nursing, Hungkuang University No. 34, Chung-Chie Road, Shalu County, Taichung Hsien, Taiwan 43302
[5]Department of Urology, Taipei Medical University Hospital, [6]Research Institute of Medical Sciences, Taipei Medical University 252, Wu-Xin Street, Xin-Yi District, Taipei 116

Abstract: Fermentative characteristics of *Lactobacillus helveticus* ATCC15009 were investigated by varying size of initial inocula, the pH values of fermentation, respectively by a pH-controlled and a pH-uncontrolled processes at 38°C. The first and the second differentiation of lactic acid production (P vs. t) and growth (X vs. t) data were taken. Their maximum peaks and reflection points were solved, whence the relationship of the model was derived. Alternatively, Henderson-Hasselbalch equation was utilized to criticize the dependencies of the feed back inhibition on the total lactate cncentration and pH. Results revealed that in lactose medium lactic acid production rate (dP/dt) of *L. helveticus* B1075 obeys the growth (βdX/dt) associated model, dP/dt = βdX/dt. Furthermore, the non-competitive end product inhibition is entirely dependent on the total lactate concentration.

Key words: *Lactobacillus helveticus* ATCC15009, lactic fermentation model, noncompetitive feed back inhibition

INTRODUCTION

The lactic acid production has received much attention due to its numerous uses in food and biochemical industries (Dikroy, 1985) and increasing numbers in biomedical applications have been reported (Lipinsky and Sinclair, 1986; Naude, 1989).

Lactic acid fermentation is an end-product-inhibited reaction. Kinetics of lactic acid fermentation by *Lactobacillus delbruckii* on glucose-yeast extract medium in batch cultures have been reported by Luedeking (Luedeking, 1956; Luedeking and Piret, 2000). They stated that the formation rate of lactic acid product was simultaneously related to both the biomass growth rate and the total biomass. However, most of the available mathematical models for fermentation processes are unstructured, i.e., the biomass composition is assumed constant during all operating conditions. These models are unable to simulate experiments carried out under conditions widely different (Nielsen *et al*., 1991) and consequently are normally restricted to one reactor type and to one model of operation (Esener *et al*., 1982).

Kinetic analysis of lactic acid fermentation by batch wise experiments had been reported (Edwards and Wilke, 1968; Pirt, 1987; Roy *et al*., 1987). Hanson and Tsao (1972) reported the kinetic analysis and simulation of continuous fermentation using results obtained from batch experiments. In addition to search for the inherent relationship between the product formation and the bacterial cell growth rates, this article also examined the relevant lactate species that might exhibit the non-competitive end product inhibition.

MATERIALS AND METHODS

Microorganism and inocula: The strain *Lactobacillus helveticus* ATCC15009 purchased from Food Industry Research and Development Institute (Singui, Taiwan) was used. The fermentation process was performed according to Roy *et al.* (1987). The strain was maintained in an SM medium. Transfer of cultures was periodically conducted every 2 weeks. Initially, the stabs were incubated at 38±0.1°C for 24 h in an anaerobic chamber and then stored at 4°C. The stab cultures were transferred

Corresponding Author: Kuan-Chou Chen, Department of Urology, Taipei Medical University Hospital, Taipei Medical University 252, Wu-Xin Street, Xin-Yi District, Taipei 116

to the seed culture LS medium which contained in per litre: Lactose (ACS standard grade) 50 g, yeast extract (Difco) 30 g, $MgSO_4$ 0.6 g, $FeSO_4$ 0.03 g, $MnSO_4$ 0.03 g, Na-acetate 1.0 g, K_2HPO_4 0.5 g and KH_2PO_4.0.5 g. Values of pH were adjusted to 6.4-6.5 with 2 MNaOH before cultivation. The seed stab culture transfer was repeated 4-5 times in order to let the strain adapted to the fermentation environment. An amount of inoculum was added into the fermentor to bring up the initial inocula concentration with cell counts of 3.00×10^8 mL^{-1}, 1.20×10^9 mL^{-1} and 2.40×10^9 mL^{-1}, respectively.

Preparation of SM medium: The sterile SM medium was prepared by dissolving 12% (w/w) skim milk powder (low fat grade) in distilled water. Ten milliliter portions of which were transferred to test tubes. The test tubes were stoppered with cotton plugs and covered with aluminum foils. The medium was sterilized in an autoclave at 121°C for 20 min.

Bioreactor: LS medium was used as described by Roy *et al.* (1987) with slight modification. After inoculation, pH values were readjusted to 6.3-6.4. Fermentations were started for both the pH-controlled and the pH-uncontrolled runs. Batch fermentation of lactic acid was conducted in a 5 L bioreactor (Firstek Scientific, Model F-5-D, Taiwan) with a three-liter working volume. For the pH-controlled experiments, pH values were kept constant at 6.0±0.02 by automatic adjustment with NaOH (2 M). A laboratory pH meter was connected to a titrator (pH controller, DO controller and AF controller, Firstek Scientific, Model FC-1000-D, Taiwan). The temperatures were held at 38±0.1°C for both the pH-controlled and the pH-uncontrolled runs. The impeller-speed was maintained at 100 rpm. Aliquots of sample (1 mL) were withdrawn hourly for analysis of lactic acid and biomass.

Analytical methods

Measurement of biomass: The cell dry weight (in g L^{-1}) was determined from the optical density at 620 nm using a spectrophotometer (HACH spectrophotometer DR 2000, HACH CO., U.S.A.). A calibration curve of the dry weight vs. the optical density (Ohara *et al.*, 1992) was used to calculate the cell dry mass.

The cell counts were measured with a hemacytometer according to the Thoma's method (Liu and Wang, 1983). The final biomass data in g L^{-1} and the cell counts in #/mL^{-1} were re-correlated to the spectrophototurbidimetric readings (Data unpublished).

Assay of lactic acid: Since, *Lactobacillus helveticus* ATCC15009 is a type of homofermentative *Lactobacillus*, it exclusively produced lactic acid as the only product, which had been previously confirmed by the HPLC method (data not shown) before this investigation. Therefore, an acid-base titration method was adequately to track the lactic acid product. Thus NaOH solution (2.5 mol L^{-1}) was used as the titrant. For each titration, one milliliter of phenolphthalein solution (10%) was used as the indicator.

Calculation: Each milliliter of NaOH (2.5 mol L^{-1}) is equivalent to 0.2252 g of lactic acid produced.

Mathematical treatment of the results: The experimental data including the cell population (X) and product formation (P) vs. time of cultivation were plotted in X-t and P-t curves. The maximum onset for each set of data was further searched by taking the first derivatives, dX/dt, or dP/dt. In order to search for the peak onset points, the second derivatives (d^2X/dt^2 and d^2P/dt^2, respectively) were further operated to check the exact onset times of both the cell growth and the product formation. To obtain clearer determinations, smoothing was applied by the polynomial regressions.

In addition, Henderson-Hasselbalch Equation (Eq. 1) (Harper *et al.*, 1977) adopting the value pKa = 3.88 for lactic acid was used to calculate the dissociated and the undissociated species of lactate at feedback inhibition point.

$$pH = pKa+\log [A^-]/[HA] \quad (1)$$

Table 1: Condition at the onset of inhibition and at infinitely replased time (*)

Initial inocula (cell mL^{-1})	pH	Lactic acid formed [a] (g L^{-1})	(mol L^{-1})	Cultivation time [a] (h)	Cell count (cells mL^{-1})	Max production rate [a] g L^{-1} h^{-1}	Final lactic acid concentration (g L^{-1})
pH-controlled							
3.00×10^8	6.0	8.000	0.0888	22.00	1.25×10^{10}	2.48	20.67
1.20×10^9	6.0	8.200	0.0910	16.30	1.80×10^{10}	3.02	22.50
2.40×10^9	6.0	8.333	0.0925	5.83	1.29×10^{10}	3.40	22.84
pH-uncontrolled							
3.00×10^8	4.0	8.318	0.0923	120.00	2.25×10^{10}	1.50	19.33
1.20×10^9	3.7	8.333	0.0925	66.00	1.50×10^{10}	2.17	18.67
2.40×10^9	4.0	8.318	0.0923	19.00	1.63×10^{10}	3.20	18.00

(*) Operation condition: Temperature, 38±0.1°C; agitation speed, 10 rpm, (a) Items measured at the onset of feedback inhibition, (b) Quantities measured at final relapsed time

RESULTS

Cell counts at inhibition point are not correlated with initial inocula: The time course variations of cell growth and lactic acid production were, respectively differentiated for the pH-controlled (Fig. 1-3) and the pH-uncontrolled experiments (Fig. 4-6). Results indicated that for the pH-controlled runs, the cell concentrations at the onset of feedback inhibition were 1.25×10^{10}mL^{-1}, 1.80×10^{10}mL^{-1} and 1.29×10^{10}mL^{-1}, respectively (Fig. 1-3;

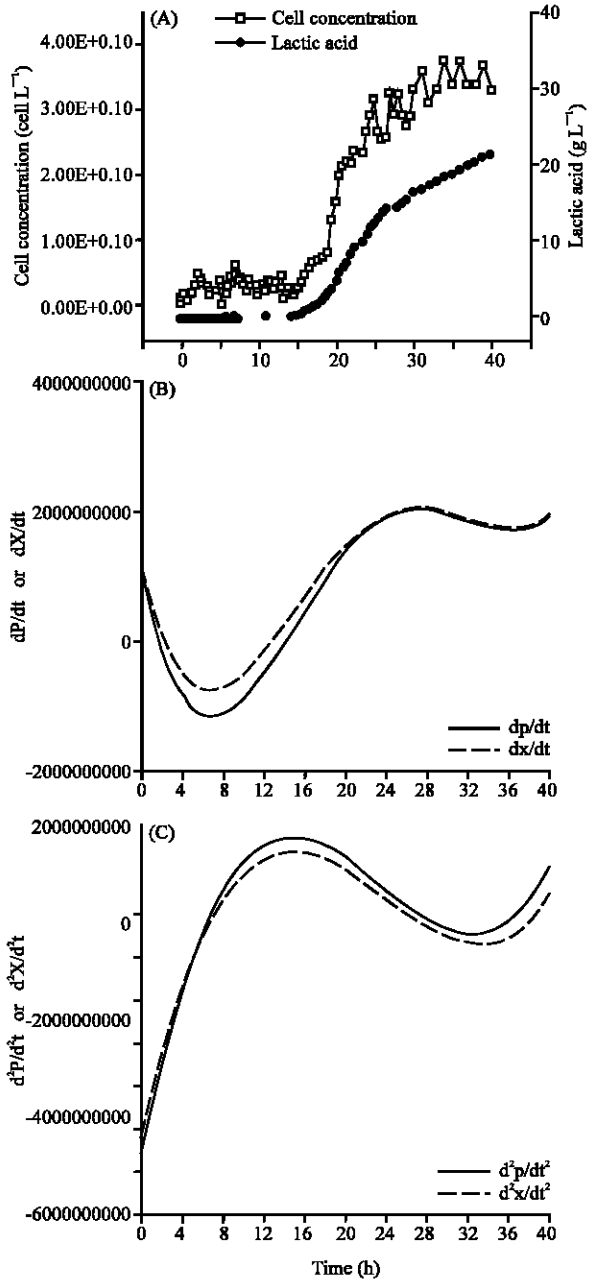

Fig. 1: Time variations of (a) cell concentration (blank square) and lactic acid production (solid circle), (b) first derivatives, dP/dt (black line) and dX/dt (gray line), weighted with a factor β, β = 3.33 × 10^{-10} g cell^{-1} and (c) second derivatives, d^2P/dt^2 (black line) and d^2X/dt^2 (gray line)

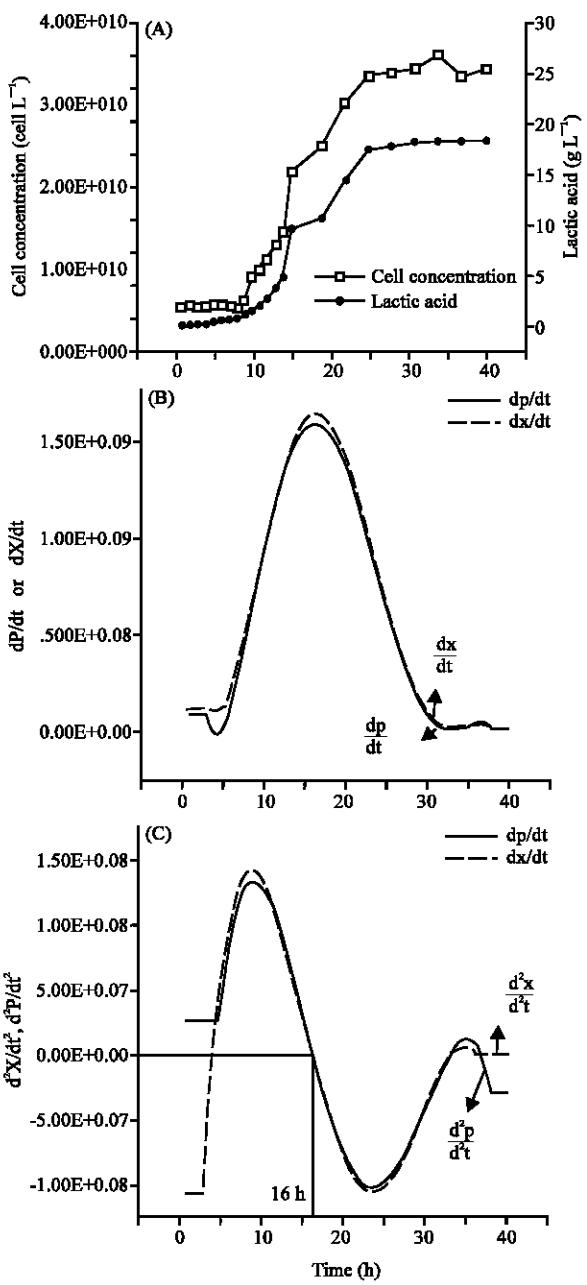

Fig. 2: Time variations of (a) cell concentration (blank square) and lactic acid production (solid circle), (b) first derivatives, dP/dt (solid line) and dX/dt (dotted line), weighted with a factor β, β = 8.33 × 10^{-10} g cell^{-1} and (c) second derivatives, d^2P/dt^2 (solid line) and d^2X/dt^2 (dotted line)

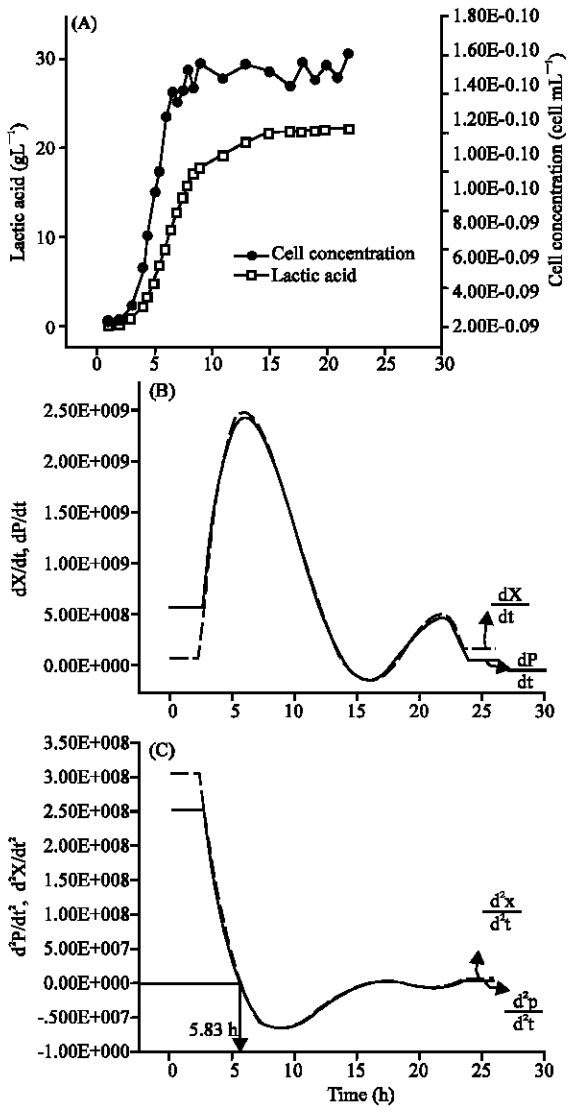

Fig. 3: Time variations of (a) cell concentration (solid circle) and lactic acid production (blank square), (b) first derivative, dP/dt (solid line) and dX/dt (dotted line), weighted with a factor β, β = 7.69 × 10^{-10} g $cell^{-1}$ and (c) second derivative, d P/dt (solid line) and d^2X/dt^2 (dotted line)

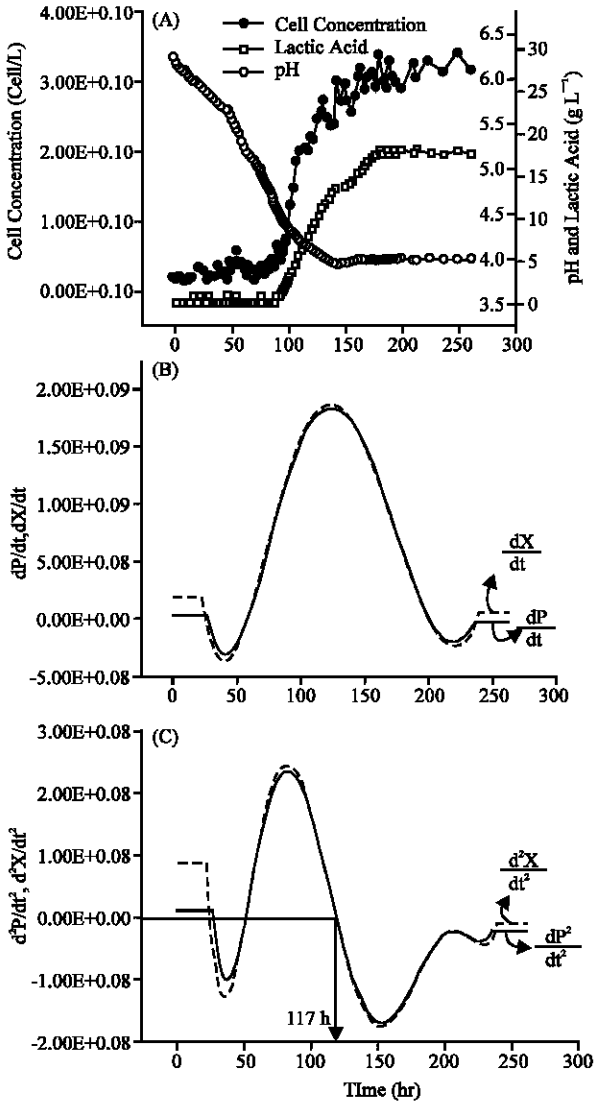

Fig. 4: Time variations of (a) cell concentration (solid circle), lactic acid production (blank square) and pH (blank circle) (b) first derivative, dP/dt (solid line) and dX/dt (dotted line), weighted with a factor β, β = 6.66×10^{-10} g $cell^{-1}$ and (c) second derivative, d^2P/dt^2 (solid line) and d^2X/dt^2 (dotted line)

Table 1) and those for the pH-uncontrolled runs, the cell counts reached 2.25×10^{10}mL^{-1}, 1.50×10^{10}mL^{-1} and 1.63×10^{10}mL^{-1}, respectively (Fig. 4-6; Table 1), contrasting with the inocula of 3.00×10^{8} mL^{-1}, 1.20×10^{9} mL^{-1} and 2.4×10^{9} mL^{-1}, respectively (Table 1).

The less initial inocula, the longer time required to reach feedback inhibition: However, data revealed that the less initial inocula, the longer fermentation times were needed to initiate feedback inhibition. In this regard, the pH-controlled process required 5.83-22 h, whereas much longer (19-120 h) were required by the pH-uncontrolled runs (Table 1).

Lactic acid production is more decreased in ph-uncontrolled fermentations: Although, the cell counts in the initial inocula and at the onset of feed back inhibition were comparable for both categories, yet a slightly lower

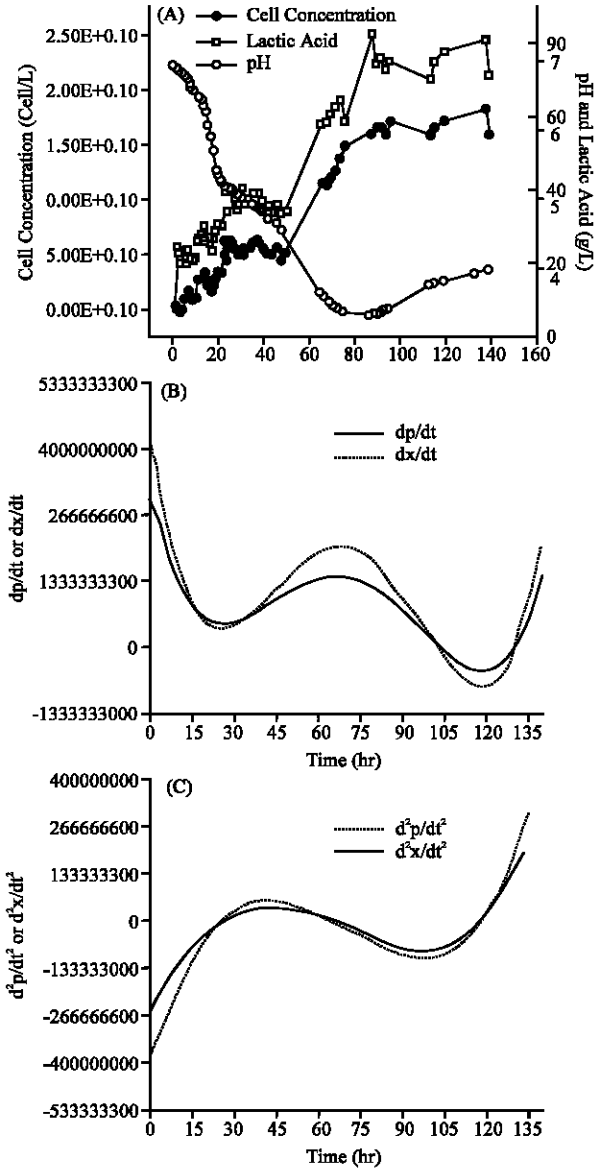

Fig. 5: Time variations of (a) cell concentration (blank square), lactic acid production (solid circle) and pH (solid diamond) (b) first derivative, dP/dt (black line) and dX/dt (gray line), weighted with a factor β, $\beta = 8.33\times10^{-10}$ g cell^{-1} and (c) second derivative, d^2P/dt^2 (black line) and d^2X/dt^2 (gray line)

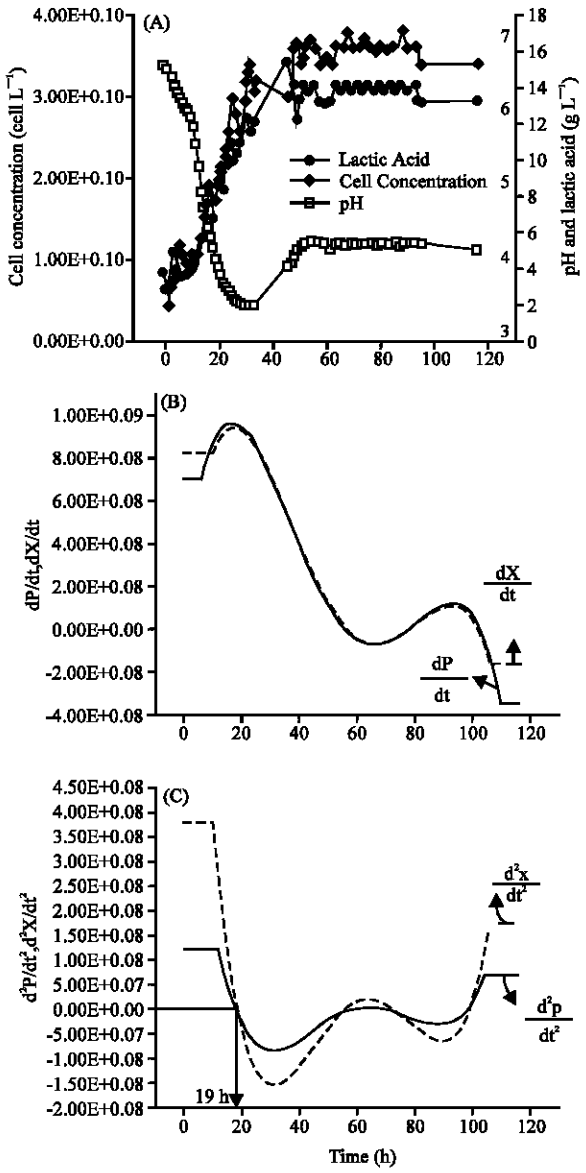

Fig. 6: Time variations of (a) cell concentration (solid circle), lactic acid production (solid diamond) and pH (blank square) (b) first derivative, dP/dt (solid line) and dX/dt (dotted line), weighted with a factor β, $\beta = 7.69\times10^{-10}$ g cell^{-1} and (c) second derivative, d^2P/dt^2 (solid line) and d^2X/dt^2 (dotted line)

maximum lactic acid production rates and less final lactic acid productions with an average decrease of 15.14% at the infinitely relapsed time were found with the pH-uncontrolled fermentations (Table 1).

Feedback inhibition is associated only with the concentration of total lactate species: As reported pKa = 3.86 at 37°C for lactic acid (Yeh *et al*., 1991), which is consistent with the measured value pKa = 3.88 at 38°C (unpublished data in our lab). Based on this, ratio of A^- to HA were computed with Henderson-Hasselbalch Equation (Eq. 1) (Naude, 1989) by neglecting the correction from the ionic strength effect (Laidler and Meiser, 1995). Ratio of A^- to HA were seen to be constant in pH-controlled, nonetheless, varying very much in pH-uncontrolled runs. Results in Table 2 show that the

Table 2: Concentration of various lactate species at the onset of inhibition under pH- controlled and pH-uncontrolled conditions*

	Controlled run			Uncontrolled run		
Experimental items	1	2	3	1	2	3
pH	6.0	6.0	6.0	4.0	3.7	4.0
Total lactate (A^-+HA)						
(g L^{-1})	8.000	8.200	8.333	8.318	8.333	8.318
(mol L^{-1})	0.0888	0.0910	0.0925	0.0923	0.0925	0.0923
Dissociated lactate (A^-)						
(g L^{-1})	7.9401	8.1383	8.271	4.7387	3.3153	4.7387
(mol L^{-1})	0.0881	0.0903	0.0918	0.0526	0.0368	0.0526
Undissociated lactic acid (HA)						
(g L^{-1})	0.060	0.0617	0.0627	3.5792	5.0180	3.5792
(mol L^{-1})	6.686×10^{-4}	6.853×10^{-4}	6.965×10^{-4}	0.0397	0.0557	0.0397
(A^-/HA) ratio	131.82	131.82	131.82	1.318	0.661	1.318

*Operation temperature, 38±0.1°C; agitation speed, 100 rpm, Data were calculated with Henderson-Hasselbalch Equation: pH = pKa+log [A^-]/[HA] adopting the value pKa = 3.88 for lactic acid

noncompetitive inhibition was pH-independent, which was apparently associated with only the total lactate (A^-+HA) concentration rather than the undissociated species (HA) as having been often cited in literature (Yeh *et al*., 1991; Bajpai and Iannotti, 1988; Harrero, 1993).

DISCUSSION

Cell counts at inhibition point are correlated only with the lactic acid produced: Obviously, the cell counts at inhibition point are more likely correlated with the lactic acid produced. As evidenced by the fact that less initial inocula required a longer time for fermentation to attain the final lactic acid concentration, by which feed back inhibition apparently occurred at the same concentration of lactic acid (around 8.00-8.333 g L^{-1}), despite of pH-controlled or uncontrolled (Table 1). Further evidencing that the noncompetitive inhibition by lactic acid was pH-independent for *L. helveticus*. To repeat, such feed back inhibition is in reality associated with only the total lactate (A^-+HA) concentration (Table 2) rather than the undissociated species (HA) as having been often cited in literature (Yeh *et al*., 1991; Bajpai and Iannotti, 1988; Harrero, 1993).

Only slight or not at all lactic acid consumption may occur at the late inhibition stage: Moreover, it seemed that some lactic acid had been consumed in the late stage of fermentation (Fig. 4-6) as seen from the re-elevation of pH values at the late stage of fermentation, which was excluded by the fact that the comparable final cell populations were found in both the pH-controlled and the pH-uncontrolled runs (Table 1).

Lactic acid production by *L. helveticus* is only growth rate associated: The first derivative curves of the lactic acid production rate dP/dt whose peaks were actually reflecting the onset of inhibition points coincided consistently with those of the cell growth rate curves dX/dt (Fig. 1-6), implying that the Luedeking's model (Eq. 2) is inapplicable in this respect, consequently the pertinent model is speculated to be merely Eq. 3.

$$dP/dt = \alpha X+\beta dX/dt \quad (2)$$

$$dP/dt = \beta dX/dt \quad (3)$$

The controversial point may be reflected from the time scale of the onset points for both the maximum cell growth rates (dX/dt) and the production formation rates (dP/dt), however smoothing by a polynomial regression on the X-t and P-t curves showed deviations were only within a fraction of an hour (Fig. 1-6). Obviously, results seem to be rather contradictory to Roy *et al.* (1987) who reported that in a lactose synthetic medium, the contribution of both mechanisms was approximately equal at the end of fermentation, however the nature of the culture medium had a significant effect on the product synthesis mechanisms.

In glucose synthetic medium, production of lactic acid is associated with the non-growth associated mechanism, whereas in whey-yeast extract permeate medium, the non-growth-associated mechanism contributes the major part (Luedeking, 1956). In addition, temperature and pH also could influence the relative contributions in whey-yeast-extract permeate medium (Luedeking, 1956). Under optimal temperature and pH, the contribution by non-growth-associated product formation was found to be more significant with all temperatures, while insignificant with all pH values tested (Roy *et al*., 1987).

Results (Fig. 1-6) reveal that in lactic fermentation by *L. helveticus* ATCC15009, the growth-associated type was the only mechanism at a temperature of 38±0.1°C., being independent on the pH values used. Altogether strongly supports our model (Eq. 3) of *L. helveticus* ATCC15009 fermentation with lactose.

Recently, distinctive difference was found in the production potential of cells (cell-density-related parameter in Luedeking-Piret equation) when lactic acid concentration reached over 55 g L^{-1}, which led to a more precise estimation of bioreactor performance (Kwon *et al*., 2001).

To summarize, the lactic production by *L. helveticus* ATCC15009 is confirmed to obey a growth-associated model, i.e., $dP/dt = \beta dX/dt$. Moreover, the total concentration of all lactate species present in the fermentation medium is pertinently responsible for the non-competitive end product inhibition mechanism.

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