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Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*

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Abstract

The effect of pH (between 5.0 and 6.3) on butyric acid fermentation of xylose by *Clostridium tyrobutyricum* was studied. At pH 6.3, the fermentation gave a high butyrate production of 57.9 g l^{-1} with a yield of 0.38–0.59 g g^{-1} xylose and a reactor productivity up to 3.19 g l^{-1} h^{-1}. However, at low pHs (<5.7), the fermentation produced more acetate and lactate as the main products, with only a small amount of butyric acid. The metabolic shift from butyrate formation to lactate and acetate formation in the fermentation was found to be associated with changes in the activities of several key enzymes. The activities of phosphotransbutyrylase (PTB), which is the key enzyme controlling butyrate formation, and NAD-independent lactate dehydrogenase (iLDH), which catalyzes the conversion of lactate to pyruvate, were higher in cells producing mainly butyrate at pH 6.3. In contrast, cells at pH 5.0 had higher activities of phosphotransacetylase (PTA), which is the key enzyme controlling acetate formation, and lactate dehydrogenase (LDH), which catalyzes the conversion of pyruvate to lactate. Also, PTA was very sensitive to the inhibition by butyric acid. Difference in the specific metabolic rate of xylose at different pHs suggests that the balance in NADH is a key in controlling the metabolic pathway used by the cells in the fermentation.

Keywords: *Clostridium tyrobutyricum*; Xylose; Butyric acid; pH; Metabolic pathway

1. Introduction

Because of its wide applications in food and pharmaceutical industries (Pouillart, 1998; Sharpell, 1985; Zigová et al., 1999), butyric acid production by fermentation from natural resources has become an increasingly attractive alternative to currently used petroleum-based chemical synthesis. It has been demonstrated that butyrate can be produced as the major fermentation product by *Clostridium tyrobutyricum* grown on the acid hydrolysate of corn fiber containing mainly glucose and xylose as the carbon sources, with acetate as a byproduct (Zhu et al., 2002). Corn fiber is an abundant byproduct from corn wet milling, and it can be readily hydrolyzed by inorganic acid to yield fermentable sugars, mainly glucose and pentoses. The butyric acid fermentation was found to be best operated at pH ∼6, but a lower pH is desirable for producing free acids that are easier to be separated from the fermentation broth (Wu and Yang, 2003). Clostridium bacteria are able to grow in a pH range of 4.5–7.0 (Zigová and Sturdik, 2000); however, little is

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known about the effect of pH on butyrate production from xylose.

It is well known that the pH is an important factor affecting most organic acid fermentations, including lactic acid (Silva and Yang, 1995), propionic acid (Hsu and Yang, 1991; Stinson and Naftulin, 1991), acetic acid (Goodwin and Zeikus, 1987; Tang et al., 1989), butyric acid (Vandak et al., 1997), and succinic acid fermentations (Van der Werf et al., 1997; Weimer, 1993). In general, the medium pH not only affects cell growth and fermentation rate, but also changes final product yield and purity. Changing the medium pH also may induce a metabolic shift. As was discovered in this study, butyric acid fermentation of xylose by *C. tyrobutyricum* changed from a predominant butyrate production at pH 6 to predominant lactate and acetate production at pH 5. This metabolic shift in *C. tyrobutyricum* has never been reported in the past, and thus, is the focus of this study since little is known about the factors controlling the intermediary metabolism of butyrate fermentation. The main objective of this work was to understand the physiological behaviors of *C. tyrobutyricum* under different pHs at the biochemistry level.

In the metabolic pathway of *C. tyrobutyricum* (Fig. 1), acetyl-CoA produced from pyruvate is a branch-point intermediate located at the node dividing two analogous pathways leading to the formation of acetate and butyrate. In these pathways, acetyl-CoA and butyryl-CoA are first converted to acetyl phosphate and butyryl phosphate by phosphotransacetylase (PTA) and phosphotransbutyrylase (PTB), respectively. These acyl phosphates are then converted to acetate and butyrate by acetate kinase (AK) and butyrate kinase (BK), respectively. The activities of these four enzymes, thus, play critical roles in affecting the production of acetic and butryric acids in *C. tyrobutyricum*. Also, there are two key enzymes involved in lactate production—lactate dehydrogenase (LDH), which is the enzyme catalyzing the formation of lactate from pyruvate with the regeneration of NAD^+ and NAD-independent LDH (iLDH), which is responsible for the production of pyruvate from lactate. The activities and expression levels of these enzymes as affected by the fermentation pH would determine the acid formation pattern in *C. tyrobutyricum*.

In this study, fed-batch fermentations using immobilized *C. tyrobutyricum* in a fibrous-bed bioreactor

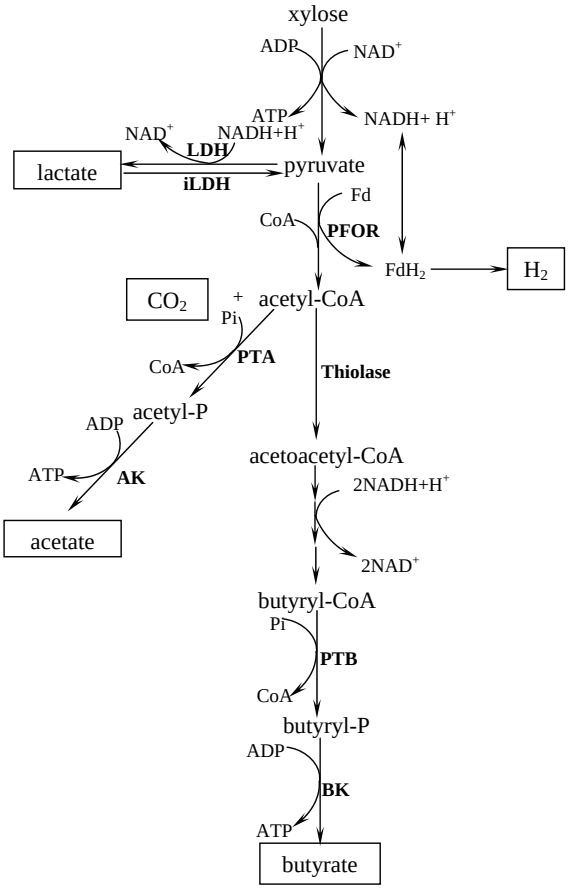

Fig. 1. The metabolic pathway for xylose fermentation by *Clostridium tyrobutyricum*. The enzymes involved as indicated by their abbreviations are: phosphotransacetylase (PTA); acetate kinase (AK); phosphotransbutyrylase (PTB); butyrate kinase (BK); acetyl-CoA acetyltransferase (or thiolase); pyruvate-ferredoxin oxidoreductase (PFOR); lactate dehydrogenase (LDH); NADH-independent lactate dehydrogenase (iLDH).

(FBB) were carried out to study the effects of pH, in the range of 5.0–6.3, on product yield, butyrate selectivity, and concentration. The FBB has been developed for several organic acid fermentations (Huang et al., 2002), including butyric acid fermentation (Zhu and Yang, 2003), and was thus, chosen in this study. The extracts from the immobilized cells from the FBB were analyzed for the pH dependence and butyrate tolerance of several key enzymes specifically involved in the production of acetate, butyrate, and lactate. By collecting the cells from different physiological states (pH 5.3 and 6.3), we were able to compare in the

extracts from the two cell types the expression levels of those key enzymes. The results can be used to explain the metabolic shift and are discussed in this paper.

2. Materials and methods

2.1. Culture and media

Clostridium tyrobutyricum ATCC 25755 was cultured in a synthetic medium described previously (Huang et al., 1998) with xylose as the substrate. The stock culture was maintained in serum bottles under anaerobic conditions at 4 °C. All media were sterilized by autoclaving at 121 °C, 15 psig, for 30 min.

2.2. Fermentation

The fermentation system consisted of a 0.5-l fibrous-bed bioreactor connected to a 5-l stirred-tank fermentor (Marubishi MD-300). The FBB was made of a glass column packed with spiral wound cotton towel and had a working volume of ∼480 ml. The FBB was connected to the stirred-tank fermentor through a recirculation loop and operated under well-mixed condition with pH and temperature controls. Anaerobiosis was reached by sparging the fermentor medium with N_2. Detailed description of the reactor construction has been given elsewhere (Zhu et al., 2002). To start up the reactor, 100 ml of cell suspension prepared in serum bottles were inoculated into the fermentor and then allowed to grow for 3 days at 37 °C, agitated at 150 rpm, and pH controlled at 6.0 by adding NH_4OH. Cell immobilization was then carried out by circulating the medium in the fermentor through the fibrous bed at a pumping rate of ∼25 ml min^{-1}. After about 36–48 h of continuous circulation, most of the cells were immobilized and no change in cell density in the medium could be identified. The spent medium in the fermentor was then replaced with fresh medium, and the recirculation rate increased to ∼100 ml min^{-1} for new batch fermentation. The batch fermentation process was repeated several times to increase the cell density in the fibrous bed before the fermentation kinetics at five different pH values (6.3, 6.0, 5.7, 5.3, and 5.0) were studied under the fed-batch mode. Unless otherwise noted, all fed-batch fermentations were started with 2 l of fresh medium, followed with pulse feeding concentrated xylose solution whenever the xylose concentration in the fermentation broth was close to zero until the fermentation ceased to produce butyrate due to product inhibition. Samples were taken at regular intervals for the analysis of cell, substrate and product concentrations.

At the end of the fermentation study, cells immobilized in the FBB were washed off from the fibrous matrix, collected and stored at 4 °C. These cells, which had already been adapted in the FBB, were maintained as suspended cultures in serum bottles with glucose as the carbon source. They were used for further fermentation studies and assays of intracellular acid-forming enzyme activities.

2.3. Preparation of cell extracts and acid-forming enzyme assays

Cells were grown in a synthetic glucose medium (50 ml) at 37 °C to the exponential phase (OD_{620} = ∼ 1.5). Cells were harvested, washed and suspended in 5 ml of 25 mM Tris–HCl (pH 7.4). The cell suspension was sonicated, and cell debris was removed by centrifugation. The protein content of extracts was determined by the method of Bradford with bovine serum albumin as the standard (Bio-Rad protein assay).

The activities of acetate kinase and butyrate kinase were measured in the direction of acyl phosphate formation with potassium acetate and sodium butyrate as the enzyme substrates, respectively, at 30 °C in 200 mM Tris–HCl buffer (pH 7.4), which also contained 10 mMATP, 10 mM $MgCl_2$, and 6% (w/v) hydroxylamine hydrochloride (neutralized with KOH). The reaction was initiated by adding cell extract and stopped after 15 min by adding 10% (w/v) ice-cold trichloroacetic acid. Color was then developed by adding 2.5% (w/v) $FeCl_3$ in 2.0 N HCl, and the absorbance at 540 nm was measured (Cary et al., 1988). One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of hydroxamic acid per min. Phosphotransacetylase and phosphotransbutyrylase were assayed with 0.2 mM acetyl-CoA and butyryl-CoA as the enzyme substrates, respectively, in 0.1 M potassium phosphate buffer (pH 7.4) following the method of Andersch et al. (1983). The enzyme activity was monitored by following the liberation of coenzyme A at 405 nm, and one unit of enzyme is

defined as the amount of enzyme converting 1 μmol of acetyl-CoA or butyryl-CoA per min. The specific activities of AK, BK, PTA, PTB are defined as the units of enzyme activity per milligram of total protein. Details about these enzyme assays can be found elsewhere (Zhu and Yang, 2003).

Lactate dehydrogenase was assayed spectrophotometrically using a modified method of Diez-Gonzalez et al. (1995) by recording the rate of oxidation of NADH at 37 °C with pyruvate as the substrate (ε of 6.22 $mM^{-1}\,cm^{-1}$ at 340 nm) in SpectraMax 250 (Molecular devices). The assay mixture contained (per 200 μl): 50 mM sodium succinate (pH 5.4), 10 mM sodium pyruvate, 3 mM fructose-1,6-diphosphate (FDP), 0.1 mg ml^{-1} NADH, and protein crude extract (10 μg). One unit of LDH is defined as the amount of enzyme that oxidizes 1 μmol of NADH per min. NAD-independent lactate dehydrogenase activity was measured by the method of Diez-Gonzalez et al. (1995). The assay mixture contained (per 200 μl): 0.1 mM potassium phosphate (pH 7.4), 12 μg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 24 μg phenazine methosulfate (PMS), 30 mM DL-lactate, 20–300 μg of protein extract. The rate of increase in absorbance (570 nm) for reduced MTT was determined. An ε of 17 $mM^{-1}\,cm^{-1}$ was used. One unit of iLDH is defined as the amount of enzyme that reduces 1 nmol of MTT per min. The specific enzyme activity is reported as per mg of protein (U mg^{-1}).

For the pH dependence study of enzymes, sodium phosphate/citrate buffer with a pH from 5.0 to 7.0 was used for enzyme assays instead of the buffer mentioned above. To study the effects of butyrate on enzyme activities, sodium butyrate was added to the assay mixtures at a concentration ranging from 0 to 200 mM. Enzyme activities were measured and expressed as the relative specific activity of the reaction without butyrate addition.

2.4. Analytical methods

Cell density was analyzed by measuring the optical density of the cell suspension at a wavelength of 620 nm (OD_{620}) with a spectrophotometer (Sequoia-turner, model 340). One unit of OD_{620} corresponded to 0.797 g l^{-1} cell dry weight for cells grown in the xylose medium. Gas (H_2 and CO_2) production in the fermentation was monitored using an on-line respirometer system equipped with both H_2 and CO_2 sensors (Micro-oxymax, Columbus Instrument). A high performance liquid chromatography (HPLC) system was used to analyze the organic compounds, including xylose, lactate, butyrate, and acetate in the fermentation broth. The HPLC system consisted of an automatic injector (Shimadzu SIL-10Ai), a pump (Shimadzu LC-10Ai), an organic acid analysis column (Bio-Rad HPX-87H), a column oven at 45 °C (Shimadzu CTO-10A), and a refractive index detector (Shimadzu RID-10A). The eluent was 0.01 N H_2SO_4 at a flow rate of 0.6 ml min^{-1}.

3. Results and discussion

3.1. Fermentation kinetics

The kinetics of xylose fermentations at various pH values between 5.0 and 6.3 are shown in Figs. 2 and 3. As can be seen in these figures, the products from the fermentation were strongly affected by the pH. In general, butyrate production increased and acetate decreased with increasing the pH. A maximum butyrate concentration of 57.9 g l^{-1} was produced at pH 6.3, with only 8 g l^{-1} of acetate and negligible amount (<1 g l^{-1}) of lactate as the acid byproducts (Fig. 2). In contrast, acetate and lactate production increased dramatically as the pH decreased to below 6.0 (Fig. 3). When pH dropped to 5.0, lactate and acetate became the major products and only a small amount of butyrate was produced in the fermentation, a clear metabolic shift from butyrate forming to acetate and lactate forming pathways (see Fig. 1). It appeared that at pH 6 and higher, acetate production was inhibited by butyric acid and reduced to almost a halt when the concentration of butyric acid reached ∼15 g l^{-1} (Fig. 2). In contrast, at pH 5.7 and lower, acetate production continued with butyrate production. When the pH was further reduced to 5.0, lactate, instead of butyrate, became the major fermentation product (Fig. 3). Some gases (H_2 and CO_2) were also produced as byproducts in these fermentations.

Table 1 summarizes the kinetic data for fed-batch fermentations at various pH values. It is clear that a higher pH favored butyrate production and gave higher butyrate yield, productivity, and final concentration.

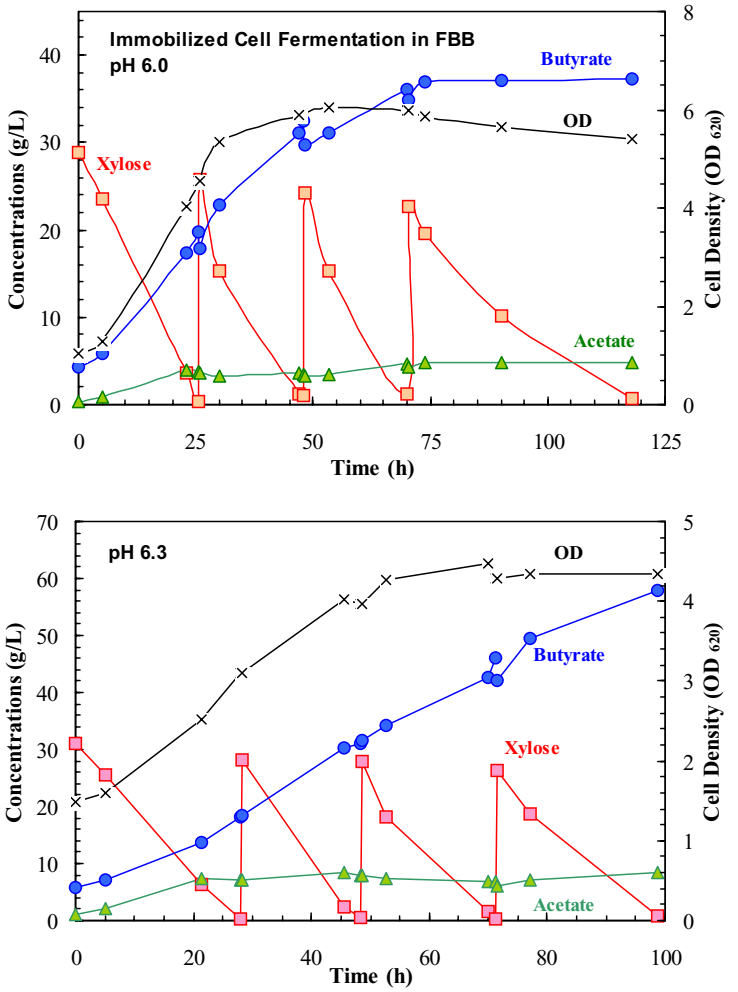

Fig. 2. Kinetics of fed-batch fermentations of xylose by *C. tyrobutyricum* immobilized in the fibrous-bed bioreactor at pH 6.3 and 6.0.

The selectivity for butyrate was 0.87 at pH 6.0, but decreased to less than 0.1 at pH 5.0. It is noted that the product yields and reactor productivities obtained at each pH varied significantly, with lower yields and productivities at higher butyrate concentrations due to butyrate inhibition. At the optimal pH of 6.3 for butyrate production, the overall reactor productivity was $2.63\,g\,l^{-1}\,h^{-1}$ based on the fibrous bed reactor volume of 0.4 l at the final butyrate concentration of $57.9\,g\,l^{-1}$, and the overall butyrate yield from xylose was $\sim 0.47\,g\,g^{-1}$. However, the total amount of acids, including butyrate, acetate, and lactate, produced from xylose fermented was higher at lower pHs and approached $1\,g\,g^{-1}$ at pH 5, indicating that more xylose was consumed for gas generation and/or cell growth at higher pHs. The optimal pH for cell growth on xylose was ~5.7, where the cell concentration in the medium reached a maximum OD_{620} of 9.1. It is noted that there was a high density of cells immobilized in the fibrous matrix and only a small portion of the total cell population was present as suspended cells during the fermentation. However, changes in the suspended cell concentration were the results of new cell growth activities in the reactor system, and thus, can be used as an indicator of cell growth rate. Based on the OD data for the suspended cells in the medium,

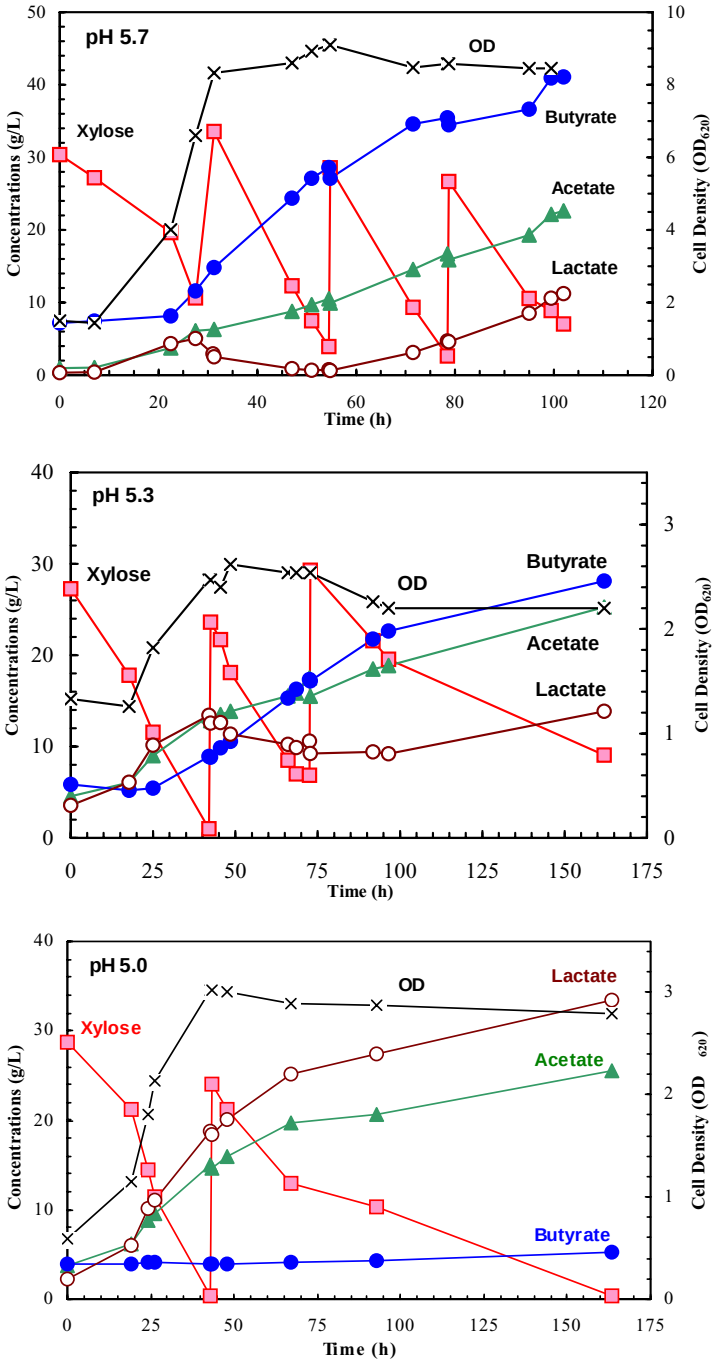

Fig. 3. Kinetics of fed-batch fermentations of xylose by *C. tyrobutyricum* immobilized in the fibrous-bed bioreactor at pH 5.7, 5.3, and 5.0.

Table 1
Effects of pH on butyrate selectivity and total acid production of *C. tyrobutyricum* in fed-batch fermentations

	pH				
	5.0	5.3	5.7	6.0	6.3
Specific growth rate (h^{-1})	0.040 ± 0.006	0.026 ± 0.008	0.073 ± 0.004	0.057 ± 0.002	0.027 ± 0.002
Product yield ($g\,g^{-1}$)					
Butyrate	0.004–0.06	0.23–0.54	0.31–0.47	0.28–0.54	0.38–0.59
Acetate	0.40–0.46	0.17–0.49	0.14–0.32	0.06–0.11	0.07
Lactate	0.58–0.64	0.24–0.39	0.15–0.32	0.002	0.005
Total acid yield ($g\,g^{-1}$)	1.00	0.84	0.67	0.48	0.54
Final concentration ($g\,l^{-1}$)					
Butyrate	5.3	28.1	41.1	37.3	57.9
Acetate	25.5	25.3	22.6	4.9	8.4
Lactate	33.5	13.9	11.2	0.5	1.0
Biomass (OD_{620})	3.0	2.6	9.1	6.0	4.5
Butyrate selectivity ($g\,g^{-1}$)[a]	0.08	0.42	0.55	0.87	0.86
Reactor productivity ($g\,l^{-1}\,h^{-1}$)	0.02–0.06	1.15–1.42	1.42–2.96	1.45–3.30	2.20–3.19

[a] The weight ratio of butyrate produced to total acids produced.

the maximum specific growth rate, μ, for cells grown on xylose was found to be $\sim$0.073 h^{-1} at pH 5.7.

Although lactate production was considerably small at optimal butyrate production when pH is above 6.0, this bacterium is able to switch to lactate production and high lactate accumulation occurs at pH < 6.0. This result is similar to those found in *C. acetobutylicum* fermentation in low-iron glucose medium (Bahl et al., 1986) or under sulfate limitation (Bahl and Gottschalk, 1984), *C. butyricum* fermentation in an atmosphere of carbon monoxide (Kubowitz, 1934), and *C. pasteurianum* under iron limitation (Dabrock et al., 1992).

3.2. Effects of pH on expression levels of acid-forming enzymes

After the fed-batch fermentation study, cells in the fibrous bed bioreactor were grown as suspended cultures at pH 6.3 and 5.3, respectively. These cells were then harvested at the end of the exponential phase. Cell extracts were prepared after ultrasonication, and the intracellular acid-forming enzyme activities were determined. Table 2 shows the effects of pH on the expression levels of various key acid-forming enzymes in the fermentation pathways. As indicated by the specific enzyme activity, acetate formation is controlled by the enzyme PTA, which had a higher expression level at pH 5.3. On the other hand, butyrate formation is controlled by the enzyme PTB, which has a higher expression level at pH 6.3. Consequently, butyrate production was higher at the higher pH and acetate was higher at the lower pH. Also, at the lower pH 5.3, the activity of LDH was 15-fold higher than that of iLDH, thus, favoring lactate production. In contrast, at pH 6.3, iLDH was 80-fold higher than LDH, and thus, little or no lactate was produced since iLDH catalyzes lactate utilization. It can be concluded that the metabolic shift from butyrate formation to lactate and acetate formation in the fermentation is associated with decreases in the activities of PTB and iLDH and

Table 2
Specific activity of enzymes involved in acetate, butyrate, and lactate formation

Enzyme	Specific activity ($U\,mg^{-1}$ protein)	
	pH 6.3 culture	pH 5.3 culture
PTA	0.691 ± 0.011	1.148 ± 0.027
AK	15.3 ± 0.4	3.4 ± 0.1
PTB	0.0138 ± 0.0004	0.0048 ± 0.0001
BK	0.15 ± 0.01	0.17 ± 0.01
LDH	0.59 ± 0.002	3.47 ± 0.02
iLDH	47.7 ± 3.8	0.24 ± 0.03

increases in PTA and LDH. It is noted that AK was higher at pH 6.3 than at 5.3, and BK was not significantly affected by the pH. However, changes in AK and BK activities would not significantly affect acetate and butyrate production, respectively, since their activities were much higher than those of PTA and PTB.

3.3. Effects of pH on acid-forming enzyme activities

Cells from the FBB were grown in serum bottles at an initial pH of ~6.0. The cells were harvested and their intracellular proteins were extracted and assayed for AK, BK, PTA, PTB, LDH, and iLDH activities at various pH values from 5.0 to 7.0. As shown in Fig. 4, AK and BK in the direction of acyl phosphate were not significantly affected by the pH between 5.0 and 7.0. However, in the acyl-phosphate-forming direction, the activity of PTA increased while PTB decreased with increasing the pH. A similar pH effect on PTA from *C. tyrobutyricum* CNRZ510 has also been reported (Touraille and Bergere, 1974). From the pH dependency of these acid-forming enzymes, it seems that acetate production would be inhibited and butyrate production would be enhanced at lower pHs, which is contradictory to the fermentation results (Figs. 2 and 3). Both LDH and iLDH had lower enzyme activities with decreasing the pH (Fig 4c). However, the activity of LDH was reduced by only 20% when pH dropped to 5.0, while only 13% of iLDH activity remained at pH 5.0. Consequently, a higher pH was favorable for pyruvate formation from lactate and lower pH helped lactate production, which is consistent with our fermentation results.

The observed pH effects on AK, BK, and PTB are somewhat unexpected. For the enzyme AK from *Escherichia coli*, AK activities in both directions of the reaction increased rapidly with pH in the range of 5.5–7.4, and had a sharp optimum at pH 7.4 (Rose et al., 1954). For BK isolated from *C. acetobutylicum* ATCC824, BK activity decreased sharply as pH dropped from 8.0 to 5.5 (Hartmanis, 1987). For PTB from *C. acetobutylicum* ATCC 824, PTB activity peaked between pH 7.5 and 8.0 and dropped precipitously when pH decreased from 7.5 to 6.0 (Wiesenborn et al., 1989). Although PTA was virtually inactive at pH 5.0, and PTA activity increased and PTB decreased with increasing pH, the medium pH would have much smaller effects on these enzymes since the intracellular pH was usually kept within a small range and was significantly higher than the medium pH when it was acidic. A constant ΔpH of 0.9–1.1 was observed in acidogenic *C. acetobutylicum* (Terracciano and Kashket, 1986). It is, thus, clear that the observed pH effects on acetate, lactate, and butyrate production were mainly attributed to the effects on the expression levels, instead of enzyme activities, of various acid-forming enzymes.

3.4. Inhibition effects of butyrate on enzyme activities

Butyric acid is an inhibitor to cell growth. Its uncoupling effect of oxidative phosphorylation interferes with the establishment and maintenance of a functional pH-gradient across the membrane for the transport of metabolites. Its anesthetic effect on the membrane also leads to membrane expansion and interface effects (Soni et al., 1987; Herrero et al., 1985). Butyrate may also affect the activities of various acid-forming enzymes, and thus, the metabolic pathway. The effects of butyrate on the activities of PTA, AK, PTB, LDH, and iLDH were studied at pH 7.4 and the results are shown in Fig. 5. BK was not studied because butyrate was the substrate used in the enzyme assay. As can be seen in Fig. 5a, AK, PTA, and PTB all were strongly inhibited by butyrate but with PTA being the most sensitive one, losing almost all of its activity at 50 mM butyrate. This may explain why the fermentations at pH 6.0 and 6.3 ceased to produce acetate when the butyrate concentration reached ~15 g l^{-1} (Fig. 2). The activities of AK and PTB were reduced by ~45 and ~68%, respectively, at 200 mM butyrate. For LDH and iLDH (Fig. 5b), they were also inhibited by butyrate but not as strongly. The activity of LDH decreased by ~20% at 20 mM; however, further increasing the butyrate concentration did not seem to affect LDH activity. The activity of iLDH was reduced to ~70% at 200 mM butyrate. It is noted that the inhibition of butyrate on PTA, AK, and PTB followed the non-competitive inhibition kinetics represented by the following equation:

$$\frac{1}{v} = \frac{1}{v_{max}} + \frac{1}{v_{max} K_i} I$$

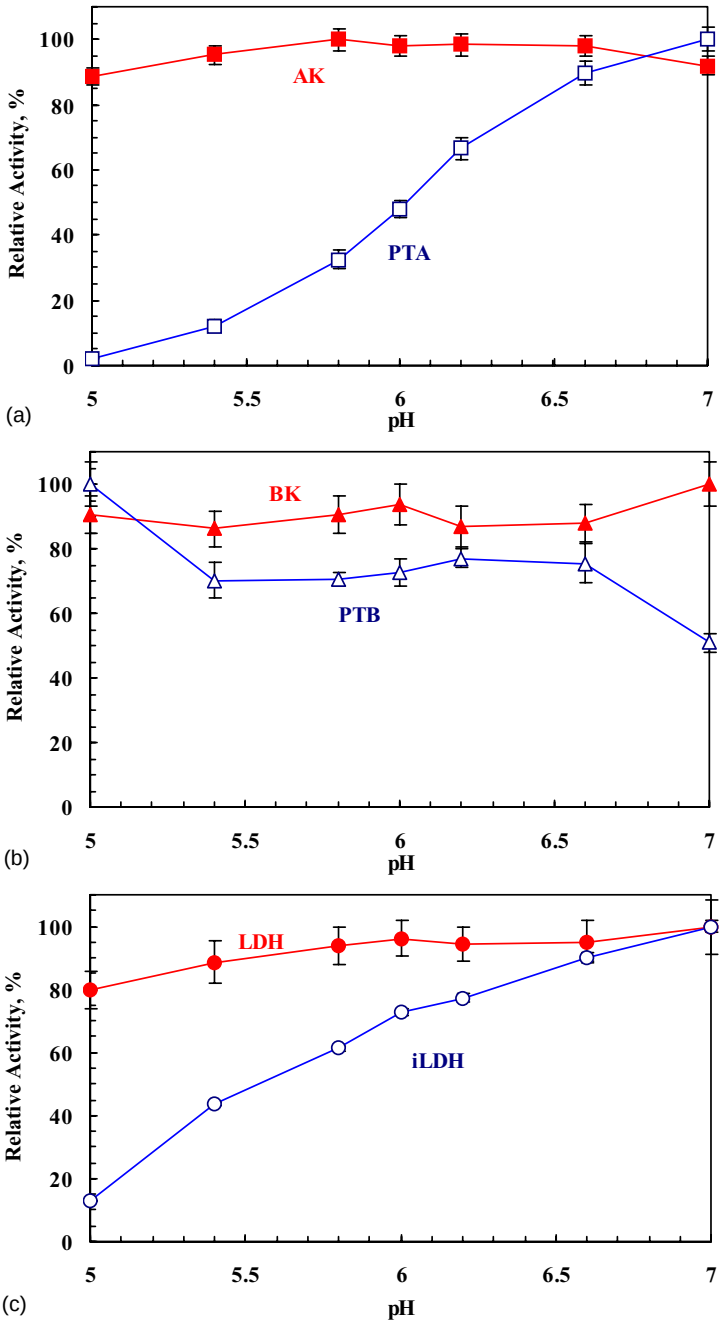

Fig. 4. Effects of pH on enzyme activities. (a) AK and PTA; (b) BK and PTB; (c) LDH and iLDH.

where ν is the specific activity of enzyme (U mg^{-1}), ν_{max} the maximum activity, K_i the inhibition rate constant (mM), and I is the butyrate concentration. The values of ν_{max} and K_i for each enzyme, as determined from the linear plot of $1/\nu$ versus I, are listed in Table 3. A higher K_i value indicates that the enzyme is less sensitive to butyrate inhibition.

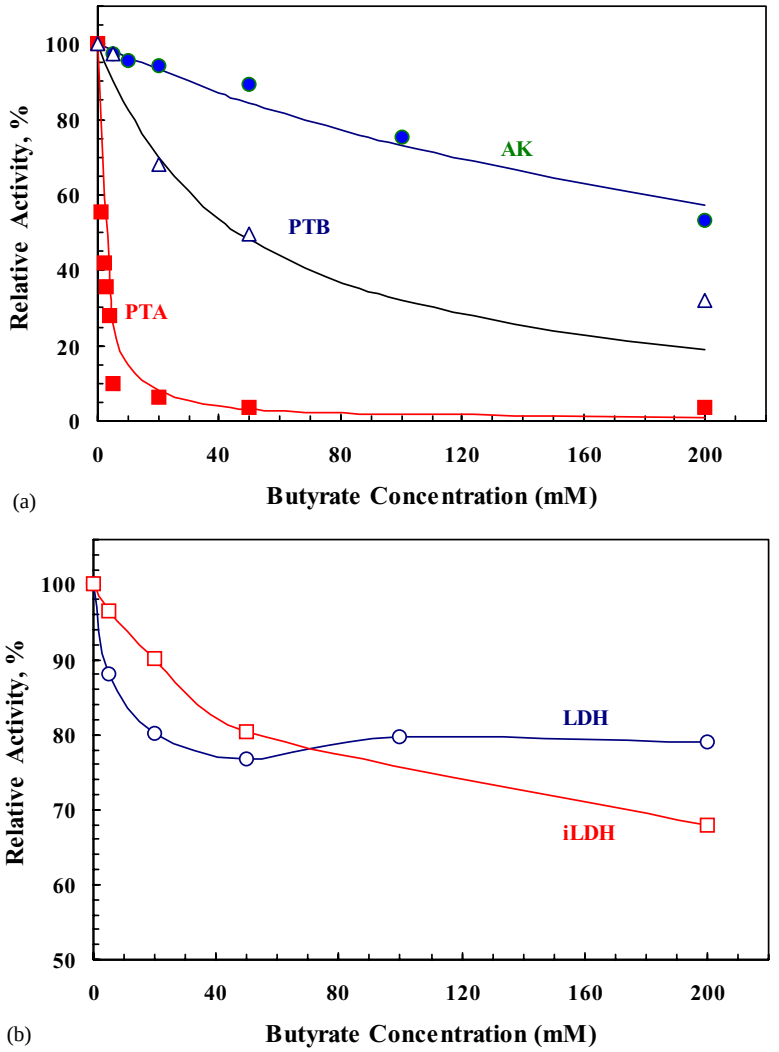

Fig. 5. Effects of butyric acid on enzyme activities. (a) PTA, AK, and PTB; (b) LDH and iLDH. For PTA, AK, and PTB, butyric acid inhibition followed non-competitive models as indicated by the simulated curves.

3.5. *Effect of pH on metabolic pathway*

It has been reported that changing the medium pH may induce a metabolic shift; such as from homolactic to heterolactic fermentation for certain lactobacilli (Borch et al., 1991; Torino et al., 2001) and lactococci (Hofvendahl et al., 1999) and from acidogenesis to solventogenesis in ABE fermentation

Table 3
Non-competitive butyrate inhibition kinetics of enzymes

Parameters	PTA	AK	PTB	iLDH
ν_{max} (U mg^{-1})	0.301 ± 0.025	6.34 ± 0.17	0.0037 ± 0.0002	0.00196 ± 0.00001
K_i (mM)	1.73 ± 0.14	270 ± 41	46.6 ± 2.1	210.3 ± 1.2

by *C. acetobutylicum* (Girbal et al., 1995; Rogers and Gottschalk, 1993). Different patterns of organic acid production were also observed in *C. sporogenes* fermentation at different pHs, and butyric acid production was favored at pH 5.5 (Montville et al., 1985). *Clostridium acetobutylicum* is able to direct its metabolic pathway from acid to solvent production associated with some physiological changes when pH decreased to a critical point. It was, thus, not surprising to observe that butyric acid fermentation of xylose by *C. tyrobutyricum* changed from a predominant butyrate production at pH 6 to predominant lactate and acetate production at pH 5, although this metabolic shift in *C. tyrobutyricum* has never been reported in the past.

As found in this study, lactate can be consumed by *C. tyrobutyricum* at pH 5.7 and higher, which is consistent with the reports that *C. tyrobutyricum* used lactic acid as a substrate during cheese making (Huchet et al., 1997; Thylin et al., 1995). However, production of lactate as a major fermentation product by *C. tyrobutyricum* has not been reported before. LDH catalyzes the formation of lactate from pyruvate with the regeneration of NAD^+, but it does not normally function as lactate-oxidizing enzyme (Garvie, 1980). Studies on LDH from *C. acetobutylicum* DSM 1731 showed that the enzyme was unidirectional, catalyzing only the reduction of pyruvate and was activated by the presence of fructose-1,6-diphosphate with calcium or magnesium ions as positive effectors (Freier and Gottschalk, 1987). Since the activity of LDH in *C. tyrobutyricum* was not significantly affected by the pH, the NAD-independent LDH, which is responsible for the conversion of lactate to pyruvate as previously found in *C. acetobutylicum* P262 (Diez-Gonzalez et al., 1997), must be present in *C. tyrobutyricum* and played the critical role in regulating lactate production or consumption by the cells.

To further understand how the pH might have caused the shift in the fermentation pathway, two fed-batch free-cell fermentations were carried out at pH 5.3 and 6.3 using the adapted culture from the FBB (Figs. 6 and 7). The concentration data obtained were used to calculate the total consumption or production of the metabolites, which were then divided by the time elapsed and the averaged OD_{620} from the initial and the final data points to estimate the specific metabolic rates or metabolic fluxes for xylose consumption, and lactate, acetate, butyrate, CO_2, and H_2 production during these fermentations. As can be seen in Table 4, the fermentation at pH 6.3 had twice higher butyrate production rate than that at pH 5.3, although its xylose consumption rate was only two thirds of that at pH 5.3. The increased butyrate formation rate at pH 6.3 was coupled with a greatly reduced acetate formation rate, which was less than 10% of that at pH 5.3. Furthermore, the lactate production rate was also high at pH 5.3, but was negligible at pH 6.3. Clearly, a significant amount of substrate carbon was directed toward lactate formation at the branch point of pyruvate in the pathway (see Fig. 1). Also, there was a higher CO_2 production rate at pH 6.3, although similar H_2 production rate was observed for both pHs studied. However, it should be noted that a significant amount of CO_2 produced in the fermentation was dissolved in the fermentation broth, which could not be easily accounted for. Also, there might be significant re-utilization of these gases by the microorganism. It is known that many acetogenic clostridia can utilize H_2 and CO_2 for acetate production under certain conditions (Rogers and Gottschalk, 1993). It is possible that *C. tyrobutyricum* also has a similar pathway to utilize the gaseous metabolites, although no such

Table 4
Specific metabolic rates at pH 5.3 and 6.3

Specific metabolic rate ($mM\,h^{-1}\,OD^{-1}$)	pH 5.3	pH 6.3
3Xylose → 5pyruvate		
Xylose consumption	2.53	1.85
Pyruvate production[a]	4.22	3.08
Pyruvate → lactate		
Pyruvate consumption[a]	1.06	0
Lactate production	1.06	0
Pyruvate → AcCoA + H_2 + CO_2		
CO_2 production	~0.85	~1.36
H_2 production	~1.39	~1.43
AcCoA production[a]	~1.39	~1.43
Pyruvate → AcCoA → acetate		
Pyruvate or AcCoA consumption[a]	1.57	0.12
Acetate production	1.57	0.12
2Pyruvate → 2AcCoA → butyrate		
Pyruvate or AcCoA consumption[a]	1.36	3.06
Butyrate production	0.68	1.53
Total pyruvate consumption	3.99	3.18

[a] Rate shown is from simple mass balance based on the stoichiometric equation given for the reaction.

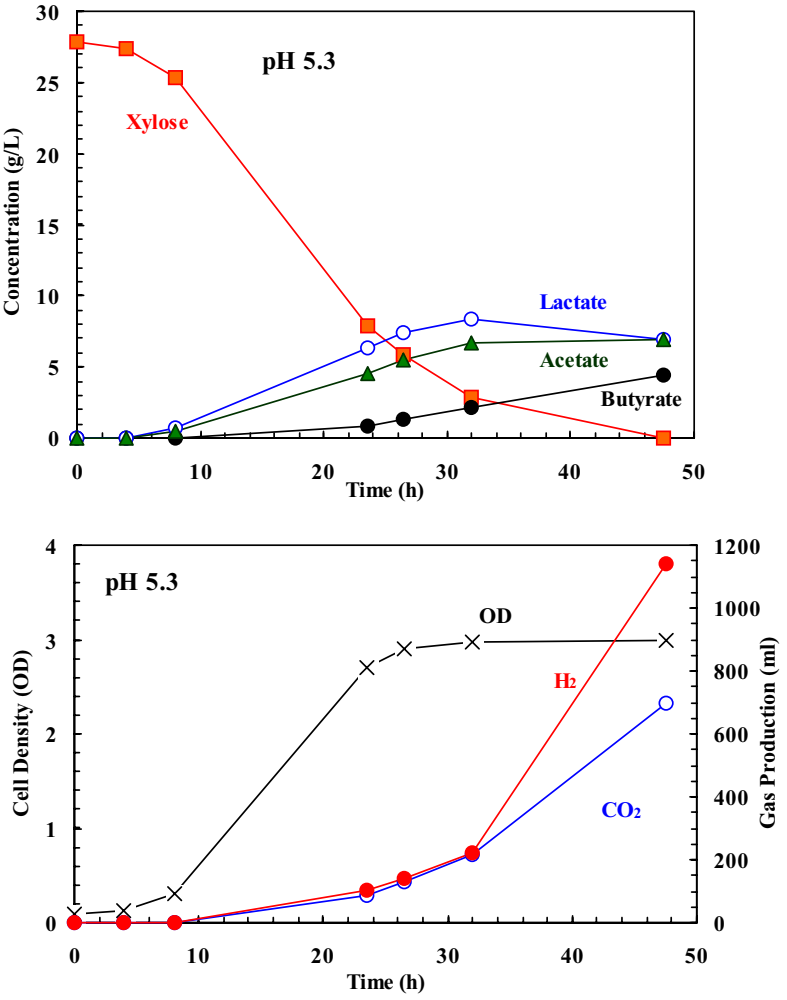

Fig. 6. Kinetics of xylose fermentation by free cells of *C. tyrobutyricum* at pH 5.3.

finding has been reported. The specific rates for pyruvate production, which can be estimated from xylose consumption, and pyruvate consumption, as estimated from the formation of various acid products, are also shown in Table 4. A total carbon recovery of ~100%, based on the ratio of total pyruvate consumption to its production from xylose, was obtained at pH 6.3, while only 95% of carbon recovery was obtained at pH 5.3, indicating that more carbon was used for cellular maintenance at pH 5.3, which is consistent with the observed lower biomass production at the lower pH.

The metabolic rate data clearly show a dramatic metabolic shift from lactate and acetate production to butyrate production with pH increase from 5.3 to 6.3. A similar metabolic flux analysis of *C. thermosuccinogenes* fermentation also showed that the flux of carbon towards lactate formation decreased significantly with increasing the pH from 6.50 to 7.25 (Sridhar and Eiteman, 2001). The metabolic pathway shift can be attributed to the control of balancing the redox potential or NADH and NAD^+. It has been reported that regulation of pyruvate metabolism in *Lactococcus lactis* is dependent on the imbalance between catabolism and anabolism (Garrigues et al., 2001; Mercade et al., 2000). A high NADH/NAD^+ ratio resulted from the imbalance between glycolysis and biomass synthesis can inhibit glyceraldehydes-3-phosphate dehydrogenase (GAPDH) greatly and decrease the

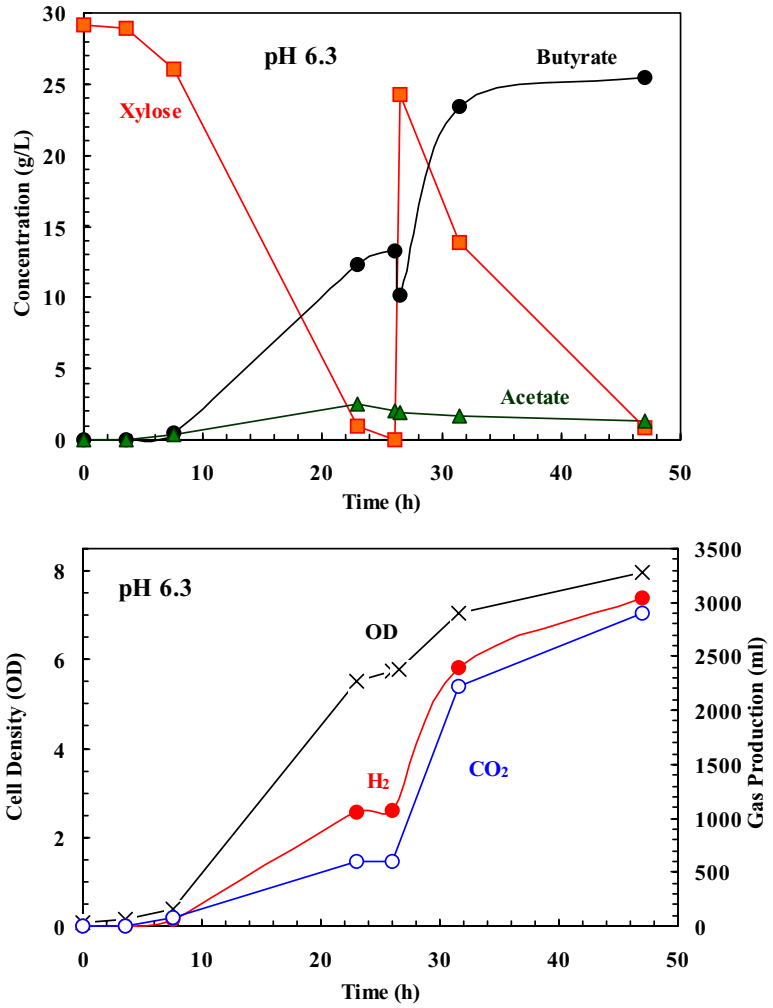

Fig. 7. Kinetics of xylose fermentation by free cells of *C. tyrobutyricum* at pH 6.3.

glycolysis flux. Meanwhile, LDH can be activated strongly by the high coenzyme ratio, and thus, lactic acid production is induced. With the higher xylose metabolism and lower PTB activity at pH 5.3, there probably were initial accumulations of NADH and pyruvate in *C. tyrobutyricum* cells, and the higher NADH/NAD^+ ratio activated the LDH (Garrigues et al., 2001) and led to lactate production from pyruvate. On the other hand, the lower xylose metabolic rate and higher PTB activity at pH 6.3 would result in a lower NADH/NAD^+ ratio, which would not activate LDH. Furthermore, the activity of LDH could be inhibited by NAD^+ (Fitzgerald et al., 1992). Therefore, there was no lactate production and butyric acid was the main acid product in the fermentation at pH 6.3. It is, thus, clear that the accumulation of NADH (NADH/NAD^+ ratio), the LDH activity, and the activities of the enzymes catalyzing the production of butyryl-CoA involving NADH oxidation, all control the switch of the carbon flow from lactate to butyrate production. This metabolic shift is similar to the NADH induced shift from acid production to solvent production in *C. acetobutylicum* (Woods and Santangelo, 1993) and the shift from homolactic acid to mixed-acid fermentation in *L. lactis* (Garrigues et al., 1997) and *Enterococcus faecalis* (Snoep et al., 1990).

For the fermentation at pH 6.3, the rate-limiting step probably resides in the pathway converting acetyl-CoA to butyryl-CoA and then to butyrate. The limiting step could be at the reaction from acetyl-CoA to acetoacetyl-CoA catalyzed by thiolase or from butyryl-CoA to butyryl phosphate catalyzed by phosphotransbutyrylase. The much lower PTB activity, as compared to butyrate kinase (see Table 2), suggests that the conversion of butyryl-CoA to butyryl phosphate is likely the rate-limiting step. However, further study of the activities of key enzymes (e.g. thiolase) and intermediates (such as NADH, acetyl-CoA, and butyryl-CoA) in the metabolic pathway is necessary in order to fully elucidate the control mechanism.

4. Conclusion

The pH in the range of 5.0–6.3 had profound effects on butyrate fermentation of xylose by *C. tyrobutyricum*. The high pH of 6.3 allowed for good butyrate production, achieving a high butyrate concentration of 58 g l^{-1} and a butyrate yield of 0.38–0.59 g g^{-1} xylose fermented. Butyrate production was lower at lower pHs, with acetate and lactate as the main acid products at pH 5.0. The metabolic shift from butyrate formation at pH 6.3 to lactate and acetate formation at pH 5.0 is associated with decreased activities of PTB and iLDH and increased activities of PTA and LDH. PTB and PTA are responsible for the production of butyrate and acetate, respectively. LDH is responsible for lactate formation from pyruvate, while iLDH catalyzes the reverse reaction. This work not only demonstrates but also explains why and how the metabolic pathways shift at different fermentation pHs. The results also provide a better understanding of the complex metabolic network of the butyric acid fermentation.

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