

Impact of Lactic Acid and Hydrogen Ion on the Simultaneous Fermentation of Glucose and Xylose by the Carbon Catabolite Derepressed *Lactobacillus brevis* ATCC 14869

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Lactobacillus brevis ATCC 14869 exhibited a carbon catabolite derepressed phenotype that has ability to consume fermentable sugars simultaneously with glucose. To evaluate this unusual phenotype under harsh conditions during fermentation, the effects of lactic acid and hydrogen ion concentrations on *L. brevis* ATCC 14869 were examined. Kinetic equations describing the relationship between specific cell growth rate and lactic acid or hydrogen ion concentration were deduced empirically. The change of substrate utilization and product formation according to lactic acid and hydrogen ion concentration in the media were quantitatively described. Although the simultaneous utilization has been observed regardless of hydrogen ion or lactic acid concentration, the preference of substrates and the formation of two-carbon products were changed significantly. In particular, acetic acid present in the medium as sodium acetate was consumed by *L. brevis* ATCC 14869 under extreme pH of both acid and alkaline conditions.

Keywords: Hydrogen ion, lactic acid, simultaneous sugar consumption, *Lactobacillus brevis* ATCC 14869, carbon catabolite repression

Introduction

Lactic acid (LA) has been used in fermented foods, beverages, and dairy products [6]. Recently, lactic acid has been reported as a platform chemical and precursor for biodegradable plastics and green solvents [1, 2, 10, 15]. More than 80% of lactic acid is currently produced by fermentation of glucose from starch biomass. Although lignocellulosic biomass has been considered as an alternative renewable substrate for producing lactic acid [24], it has polyphenolic inhibitors and mixture of carbohydrates, including glucose, xylose, and arabinose. This substrate heterogeneity has made it difficult to be used for industrial utilization.

Carbon catabolite repression (CCR) is a common microbial response termed as metabolism of carbohydrate from a carbon mixture source. [32]. In this process, bacteria use only one preferred carbon as source for faster growth, whereas utilization of other sources of carbon remains neglected due to certain regulatory processes. It has been reported that *Lactobacillus brevis* ATCC 14869 does not have apparent CCR and is capable of metabolizing every fermentable sugar in the medium simultaneously without any inhibition [16]. *L. brevis* is an obligatory heterofermentative lactobacilli. It has a broad range for utilization of sugars, including many pentose sugars. *L. brevis* is considered as a major spoilage bacterium for fermented foods and beverages. Because it has tolerance for highly acidic conditions [23,

31], it is considered as favorable for industrial production of lactic acid from biomass.

A high concentration of lactic acid is required for its industrial production. Therefore, it is necessary to examine the effect of lactic acid concentration on growth of host strain fermentation as well as lactic acid production. Various models and equations have been proposed to explain the influence of lactic acid and hydrogen ion concentration on microbial cell growth, including *Lactobacillus* spp. [4, 8, 9, 12, 13, 20, 21, 25–27, 29, 30, 33]. However, most studies have focused on utilization of single carbon sources for production of lactic acid. There is not much work published for production of lactic acid under the influence of mixed substrates. It has been reported previously in our published study that *L. brevis* ATCC 14869 is capable of metabolizing pentose and hexose sugars at the same time [18, 19] and the discussed the importance of simultaneous utilization of mixed sugars in lignocellulosic fermentation [17]. The present study demonstrates the impact of lactic acid and hydrogen ion concentration on *L. brevis* ATCC 14869 growth. In particular, their influence on the simultaneous sugar consumption and product formation were also examined.

Materials and Methods

Bacterial Strains and Growth Conditions

L. brevis ATCC 14869 used for this study was obtained from the American Type Culture Collection (ATCC). Modified MRS medium (pH-6.0) was used for the experiments, consisting of (g/l) bactopectone, 15 g; yeast extract, 5 g; ammonium citrate, 2 g; sodium citrate, 5 g; and dipotassium phosphate, 2 g. Concentrated carbon source (10×) was prepared and added into the inoculum.

Fermentation

L. brevis ATCC 14869 was cultivated in MRS medium for 12 h at 37°C. After that, it was used for inoculation. Glucose and xylose were prepared separately and then added into the modified MRS medium making 20 g/l each with 5% (v/v) of inoculation. Fermentation was carried out at 37°C and pH 6.0 was controlled by 10 N NaOH. A 2 ml sample was taken at selected time intervals and used for analysis. For the effect of hydrogen concentration, the initial pH was controlled at prescribed values by addition of 10 N HCl. For the impact of lactic acid concentration, 0, 20, 40, 60, and 80 g/l of lactic acid were added initially.

HPLC Analysis

Substrate and end-product concentrations were analyzed using an HPLC system (Shimadzu, Japan) with a BioRad HPX-87H column (BioRad, USA). Fermentation broth (1 ml) was centrifuged at 10,000 ×g for 10 min and the supernatant was transferred into

new microcentrifuge tubes prior to analysis. For HPLC separation of the supernatant, the BioRad HPX-87H column was heated and maintained at 65°C and an RI detector was used for identification of substrate and product. H₂SO₄ 0.01 N was used as the mobile phase with a flow rate of 0.6 ml/min.

To determine cell density, the cell pellet was resuspended in the same volume of deionized water and the OD₆₀₀ was measured by using a Beckman DU 7400 spectrophotometer (Beckman, CA, USA).

Calculation of Specific Values

Specific cell growth rates were calculated by the linear regression between the natural log of cell OD and time during the exponential phase ($r^2 > 0.95$). The specific substrate utilization and product formation rates were calculated during the exponential phase within the specified experimental conditions (described in the Results section). The following equations were used in this calculation:

$$q_s = \frac{1}{X} \frac{ds}{dt} = \frac{\Delta S}{X \cdot \Delta t} = \frac{1}{n} \sum_{i=1}^n \frac{(S_i - S_{i+1})}{(t_{i+1} - t_i) \{ (X_i + X_{i+1}) / 2 \}} \quad (1)$$

$$q_p = \frac{1}{X} \frac{dp}{dt} = \frac{\Delta P}{X \cdot \Delta t} = \frac{1}{n} \sum_{i=1}^n \frac{(P_{i+1} - P_i)}{(t_{i+1} - t_i) \{ (X_i + X_{i+1}) / 2 \}} \quad (2)$$

n = number of segments

where X, S, and P stands for the cell mass (OD), concentration of substrates, and concentration of products, respectively. The experiments were done in duplicates.

Results

Simultaneous Fermentation of Glucose and Xylose by *L. brevis* ATCC 14869

Mixed sugar fermentation of glucose and xylose was carried out to determine atypical characteristics of *L. brevis* ATCC 14869. Glucose and xylose were used simultaneously by *L. brevis* ATCC 14869 for 24 h of fermentation. Both 10 g/l of glucose and xylose were completely consumed with similar consumption rates (Fig. 1). This pattern of simultaneous consumption of glucose and xylose was continuously observed throughout the experiments even after using different hydrogen or lactic acid concentrations.

Effect of Lactic Acid Concentration on Cell Growth

The impact of lactic acid on *L. brevis* ATCC 14869 was determined after carrying out fermentations with initial lactic acid concentrations of 0, 20, 40, 60, and 80 g/l. Cell growth was monitored during the time required for de novo synthesis of 20 g/l of lactic acid. The initial lactic acid

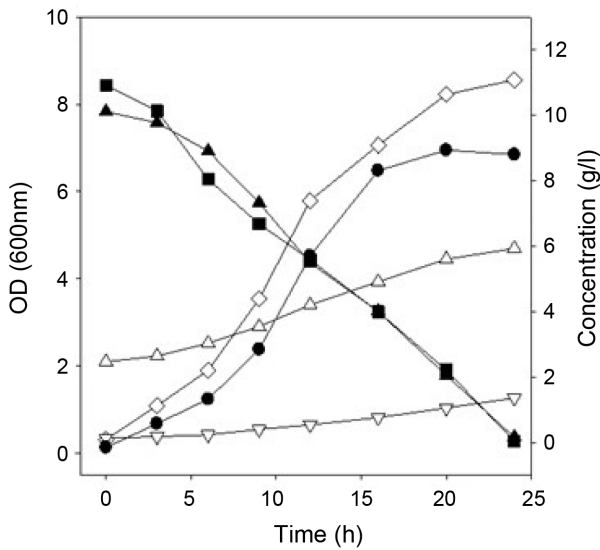


Fig. 1. Simultaneous fermentation of glucose and xylose by the carbon catabolite depressed *Lactobacillus brevis* ATCC 14869. Symbols: (●) cell OD, (■) glucose, (▲) xylose, (◇) lactic acid, (△) acetic acid, (▽) ethanol.

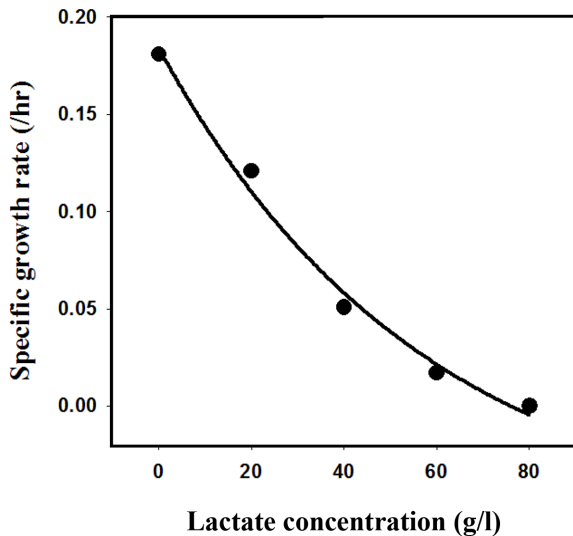


Fig. 2. The effect of lactic acid concentration on the specific cell growth rates of *Lactobacillus brevis* ATCC 14869. The solid line is the simulation of Eq. (2) in text.

concentration influenced the specific cell growth rate and lag time (Fig. 2A). As the initial lactic acid concentration increased, the specific cell growth rate decreased exponentially from the maximum cell growth rate of 0.184/h. The lag time for cell growth was increased exponentially, extending up to 18 and 50 h after using the initial concentration of lactic

acid as 40 and 60 g/l, respectively. There was no cell growth, substrate utilization, or lactic acid production in the fermentation containing an initial concentration of lactic acid of 80 g/l.

The parameters of the relationship between the initial lactic acid concentration and specific cell growth rate were deduced by nonlinear regression ($r^2 = 0.991$).

$$\frac{d\mu}{d[L]} = a \cdot \exp(-k_{[L]} \cdot [L])$$

$$[L] = 0 \text{ then } \mu = \mu_{\max} \quad (3)$$

The following equation was derived from Eq. (3):

$$\mu = \mu_{\max} + \frac{a}{k_{[L]}} \{1 - \exp(-k_{[L]} \cdot [L])\} \quad (4)$$

$$\mu_{\max} = 0.184 \text{ (/h)}$$

where $k_{[L]} = 1.74 \times 10^{-2} \text{ (1/g)}$

$$a = -4.38 \times 10^{-3} \text{ (g l}^{-1} \text{ h}^{-1}\text{)}$$

The rate of specific cell growth responded to the change of lactic acid concentration exponentially as described in Eq. (4). The solid line shown in Fig. 2B represents the simulated results of the equation.

Effect of Initial Lactic Acid on Substrate Utilization and Product Formation

The specific substrate consumption and product formation rates were calculated during production of an additional 20 g/l of lactic acid from amount varied from the starting concentration of lactic acid. It was found that specific glucose and xylose consumption rates decreased during cell growth with increasing initial lactic acid concentration (Fig. 3A). There was no consumption of glucose and xylose at the initial lactic acid concentration of 60 g/l. However, a small amount of lactic acid (21 mM) was produced during cell growth after a long lag phase. Thus, the rate of specific cell growth was found to be directly affected by lactic acid concentration, whereas specific production rates of lactic acid and acetic acid were not influenced significantly and maintained similar values regardless of the initial lactic acid concentration. However, ethanol production has been found to decrease continuously regarding to initial concentration (Fig. 3B). There was no consumption of carbohydrates at the initial lactic acid concentration of 60 g/l. Thus, the specific lactic acid production rate was similar to those from different initial lactic acid concentrations.

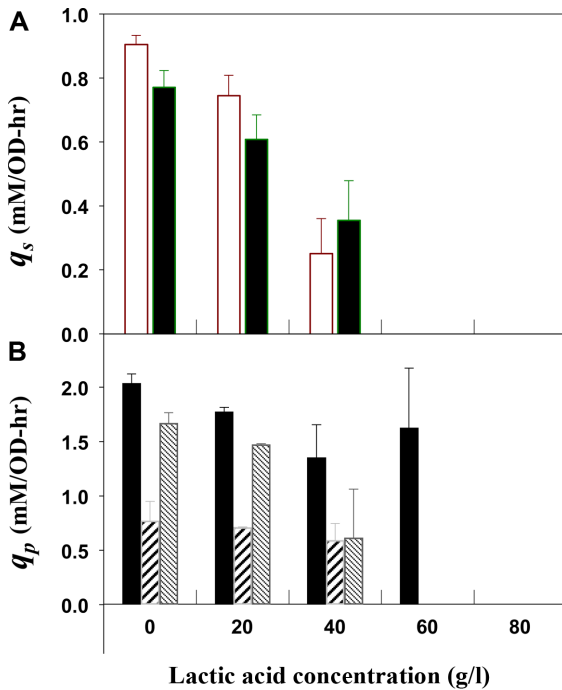


Fig. 3. Effect of lactic acid concentration on the specific (A) substrate consumption and (B) product formation rates of simultaneous glucose and xylose fermentation by *Lactobacillus brevis* ATCC 14869.

Symbols: (A) (□) the specific glucose consumption rates (mM glucose/OD-hr), (■) the specific xylose consumption rates (mM xylose/OD-hr), (B) (■) the specific lactic acid production rate (mM lactate/OD-hr), (▨) the specific acetic acid production rate (mM acetate/OD-hr), (▩) the specific ethanol production rate (mM ethanol/OD-hr).

Effect of H⁺ Concentration on Cell Growth

The effect of hydrogen ion concentration on growth of *L. brevis* ATCC 14869 was investigated. Fermentation kinetic values were obtained during production of 10 g/l of lactic acid in order to minimize the interference by lactic acid.

As shown in Fig. 4A, maximum cell growth rate was observed at pH 6 and 7. *L. brevis* ATCC 14869 showed strong resistance to acidic conditions but dramatically lost viability under alkaline conditions. At pH 8, the specific cell growth rate reduced to 50% of the maximum growth rate obtained at pH 7. *L. brevis* ATCC 14869 could not grow at pH 9. However, *L. brevis* ATCC 14869 maintained metabolic functions in acidic conditions down to pH 3. However, the optical density of the cell was reduced during fermentation at pH 2 and below. The relationship between specific cell growth rate and hydrogen ion concentration in alkaline and acidic pH ranges are shown in Figs. 4B and 4C, respectively. The simulated equations and parameters obtained by non-

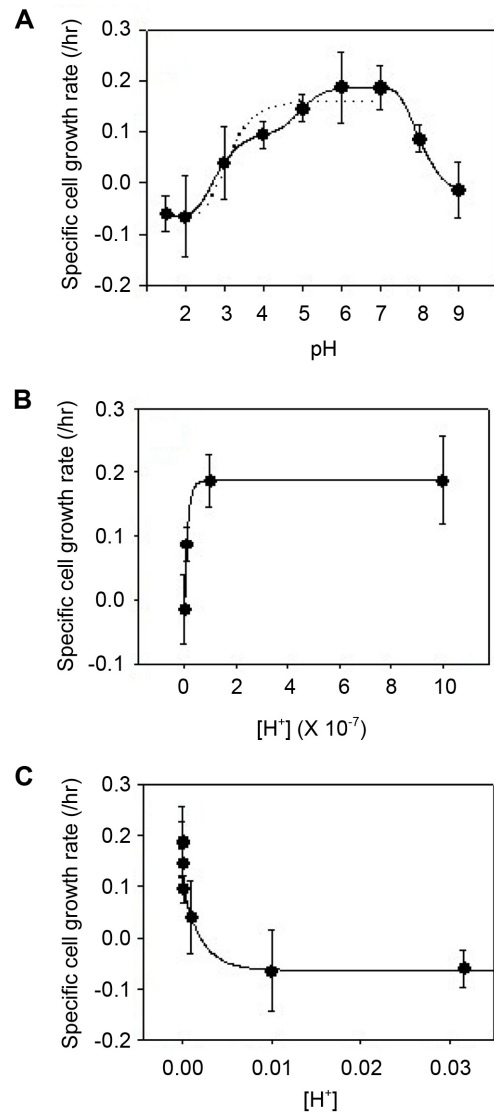


Fig. 4. Relationship between pH and specific cell growth rates. (A) Effect of hydrogen ion concentration on specific cell growth rates; (B) Alkaline region (from 10⁻⁹ to 10⁻⁶); and (C) Acidic region (from 10⁻⁶ to 10⁻¹⁵). Solid lines in each figure represent the simulation of equations described in the text.

linear regression are as follows:

$$\begin{aligned}
 &6 < \text{pH} < 9 \\
 &\mu = y_0 + a_2 \cdot \{1 - \exp(-b_2 \cdot [\text{H}^+])\} \\
 &y_0 = -0.0304 \\
 &a_2 = 0.217 \\
 &b_2 = 7.74 \times 10^7 \\
 &r^2 = 0.999
 \end{aligned}
 \tag{5}$$

$$1.5 < \text{pH} < 7$$

$$\begin{aligned} \mu &= y_0 + a_3 \cdot \exp(-b_3 \cdot [\text{H}^+]) + c \cdot \exp(-d \cdot [\text{H}^+]) \\ y_0 &= -0.0637 \\ a_3 &= 0.0869 \\ b_3 &= 6.65 \times 10^4 \\ c &= 0.167 \\ d &= 483 \\ r^2 &= 0.995 \end{aligned} \quad (6)$$

The hydrogen ion concentration changed the specific cell growth rate exponentially both in alkaline and acidic conditions.

The effect of $[\text{H}^+]$ on specific cell growth rates was exponentially increased toward optimum pH at 6.0 with inhibition constant ($k_{[\text{H}^+] \text{ high}}$) of $-b_2 = -7.74 \times 10^7$. In the low pH range, *L. brevis* ATCC 14869 showed strong acidic tolerance and cumulative exponential decay by $[\text{H}^+]$ with two inhibition constants, $k_{[\text{H}^+] \text{ low}} = -b_3 = -6.65 \times 10^4$ and $k_{2[\text{H}^+] \text{ low}} = -d = -4.83 \times 10^2$. Fig. 4A describes the specific growth rate as a function of pH. It clearly shows that the derived equations and measured data have significant relationship. The dotted line in the acidic region in Fig. 4A represents the simulation using single exponential decay Eq. (6), similar to high pH.

$$\begin{aligned} \mu &= y_0 + a_4 \cdot \exp(-b_4 \cdot [\text{H}^+]) \\ y_0 &= -0.0624 \\ a_4 &= 0.2138 \\ b_4 &= 8.26 \times 10^2 \\ r^2 &= 0.947 \end{aligned} \quad (7)$$

The single exponential decay as shown in Fig. 4A has high r^2 values. Thus, a double exponential decay curve was more appropriate to describe the relationship between hydrogen ions $[\text{H}^+]$ and the specific growth rate.

Effect of H^+ Concentration on Substrate Utilization and Product Formation

The substrate consumption and end-product formation profile of glucose and xylose fermentation by *L. brevis* ATCC 14869 beyond the pH range are shown in Figs. 5A and 5B respectively. In spite of decreasing in optical density of cells, the carbohydrates were found to be consumed at both extreme alkaline and acidic pH (pH <2.0 and >9.0).

A small amount of glucose (~5 mM) was used at pH 3.0 and below. Glucose consumption increased with increasing pH and decreased rapidly after pH 7.0. Glucose was not

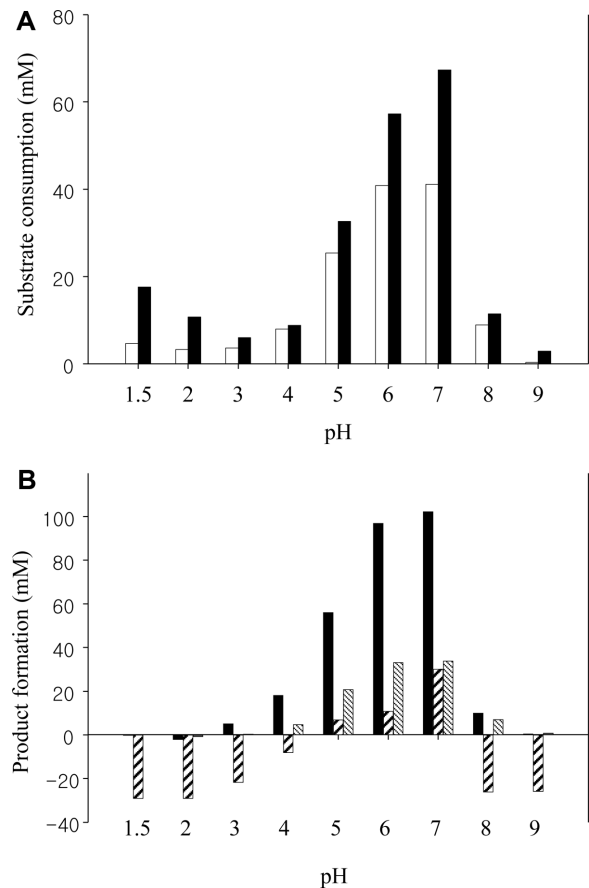


Fig. 5. Effect of pH on (A) substrate consumption and (B) product formation of simultaneous glucose and xylose fermentation by *Lactobacillus brevis* ATCC 14869.

Negative concentration of acetic acid in product formation represents the decrease of initial acetic acid concentration. It is one of the medium components present as sodium acetate (5 g/l). Symbols: (A) (□) glucose mM, (■) xylose mM, (B) (■) lactic acid mM, (▨) acetic acid mM, (▩) ethanol mM.

utilized at pH 9.0. Xylose was always preferentially utilized regardless of media pH. The minimum xylose consumption was observed at pH 3.0 (6.0 mM). Interestingly, xylose consumption increased at pH 2.0 (11 mM) and pH 1.5 (18 mM). The xylose consumption pattern was found to resemble that with glucose at pH above 3.0. However, there was no utilization of xylose at pH 9.0 (4 mM).

End-product formation, particularly acetic acid, showed interesting results (Fig. 5B). Lactic acid production was observed between pH 3.0 and 8.0. Ethanol was produced from pH 4.0 to 8.0 and was the dominant two-carbon end-product. Acetic acid was produced only at pH range of 5.0 to 7.0. Acetic acid in the media decreased in both extreme acidic and alkaline conditions. MRS medium initially

contains 5 g/l of sodium acetate and 2.4 g/l of acetic acid. It was found that 30 mM of acetic acid was consumed by *L. brevis* ATCC 14869 at pH 1.5, 2.0, 8.0, and 9.0, whereas consumption of acetic acid was reduced as pH moved towards the optimum pH 7.0.

Discussion

Glucose and xylose are the prominent carbon sources obtained from lignocellulosic biomass. Glucose derived from cellulose is only ~50% of dry cell mass. Therefore, consumption of heterologous sugar including xylose is crucial to meet the economical aspect of lignocellulosic biomass utilization. Carbon catabolite repression hinders the utilization of xylose in media having glucose. However, it shows sequential consumption of glucose over xylose. *L. brevis* ATCC 14869 naturally exhibited an atypical but important aspect of sugar consumption. It has been shown the simultaneous utilization of mixed sugars that virtually allowed to use the mixture of xylose and glucose as a single carbon source in the media. Both glucose and xylose were readily used by *L. brevis* ATCC 14869, however, the production of NAPH was different. It might be the impact on the redox balance of the cell resulting in the perturbation of substrate consumption and product formation. Concentrations of hydrogen ion and lactic acid are one of the key factors that impact the redox balance of the cell in any given media.

Depending on the pH, lactic acid could result in three different products that could impact cell growth. Hydrogen ion ($[H^+]$), an anionic lactic acid ion ($[LA^-]$), and an undissociated form of lactic acid ($[LAH]$). Most studies have focused on the undissociated lactic acid as a primary inhibitor [33]. However, it has been shown in Fig. 6 that only 0.8% of total lactic acid is $[LAH]$ at pH 6.0. It is commonly considered as the optimum pH for most microbial cell growth, whereas environmental pH is typically controlled at pH 6.0 or 7.0 in industrial fermentation conditions. Furthermore, cytosolic pH of bacteria is maintained around pH 6.0 (pH homeostasis). The impact of lactic acid concentration on these processes should be interpreted as the impact of the dissociated form of lactic acid ($[LA^-]$).

Cell growth rate of *L. brevis* ATCC 14869 decreased exponentially in proportion to lactic acid concentration. The mechanism of bacterial growth inhibition by organic acids is not yet clear. However, the inhibitory effect of high concentration of lactic acid in the media on cell growth and additional lactic acid production appears to be related to the difficulties in excretion and subsequent accumulation

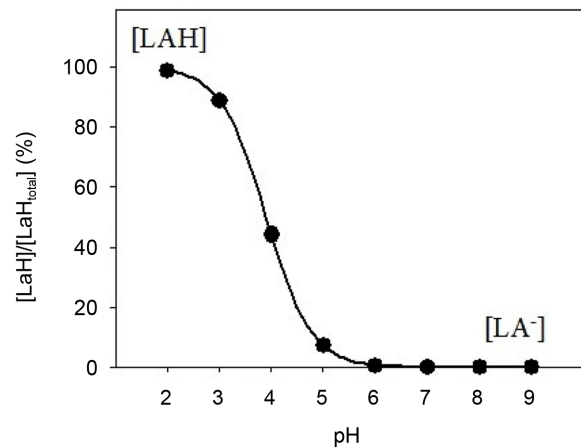


Fig. 6. Ratio of non-dissociated lactic acid to total lactic acid at different pH.

of cytosolic lactic acid. The lactic acid excretion system of LAB has been characterized as carrier-mediated that is facilitated by diffusion with proton ions [5, 11]. Therefore, when a high concentration of lactic acid exists outside the cell, the cytosolic lactic acid concentration has to be increased to fulfill the Gibb's free energy for translocation. Consequently, the accumulation of cytosolic lactic acid might decrease the central carbon metabolic flux by sequential feedback inhibition or increase the Gibb's free energy barrier of the overall metabolic reaction.

The accumulation of lactic acid in the cytosol because of a high extracellular lactic acid concentration could affect cell growth by altering the intracellular hydrogen ion concentration. However, if pH is above 6.0 intracellularly, then lactic acid is produced as a dissociated form (lactate anion and hydrogen ion). The hydrogen ion, one of the fermentation end-products, is eliminated from the cytosol through efflux with the lactic acid anion [11, 14]. Thus, inhibition of lactic acid excretion can lead to internal accumulation of protons, although the internal pH of the cell is tightly regulated by various mechanisms [14, 22, 28]. It increases cytosolic hydrogen ion concentration and presents an additional burden on the cell and decreases overall sugar metabolic rates.

The growth rate of *L. brevis* ATCC 14869 decreased sharply at alkaline pH. *L. brevis* ATCC 14869 reduced more than 80% of cell growth rate at pH 8.0 and no growth was observed at pH 9.0. A probable cause for susceptibility to alkaline conditions could be the loss of the proton motive force (PMF). In particular, sugar transport systems of *L. brevis* ATCC 14869 have been shown to be driven by the PMF [3, 7, 34, 35]. Thus, the loss of PMF might cause the

sharp decrease in sugar uptake and thereby slows cell growth.

Substrate utilization and product formation profiles at different pH were also an interesting aspect. Despite the negative values of the specific cell growth rates under extreme acidic conditions, metabolic activity of *L. brevis* ATCC 14869 was observed. It has been found that 18 and 11mM of xylose were consumed with 3 mM of glucose at pH 1.5 and 2.0, respectively. In contrast, sugars were not used by *L. brevis* ATCC 14869 at high pH, which might support the hypothesis of alkaline susceptibility resulting from the loss of PMF. Lactic acid production was observed with the cell growth at pH 3.0. At extreme pH, acetic acid is used for cellular materials rather than energy generation because it can be readily converted into two-carbon molecular intermediates, such as acetyl-CoA or acetyl phosphate.

It has been found that consumption of glucose relative to xylose increased with decrease in pH from 7.0 to 4.0. A possible driving force that changed this input flux ratio might be the NADH requirement of the cell. In addition to controlling the cellular redox by the distribution of acetyl phosphate flux (*i.e.*, consumption of NADH), *L. brevis* ATCC 14869 is able to control the production of NADH by changing specific utilization of pentose or glucose mixtures.

Acknowledgments

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