Butyric Acid Fermentation in Xylose and Glucose by *Clostridium tyrobutyricum*

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The objective of this study was to understand the effect of different concentrations of xylose and glucose on butyric acid production by *Clostridium tyrobutyricum*. *C. tyrobutyricum* was cultured in a medium containing xylose, glucose, or mixtures of xylose and glucose as the main carbon source. The butyric acid concentration increased from 3.5 to 16.3 g/L in the xylose media, and from 2.6 to 27.0 g/L in the glucose media when the initial sugar concentration increased from 5 to 75 g/L. The yield from xylose to butyric acid started to decrease as the sugar concentration was above 35 g/L, while for glucose media higher glucose concentration resulted in higher yield. At low sugar concentrations (5 g/L or 15 g/L), xylose was more efficient than glucose for butyric acid generation, but at high concentrations (55 or 75 g/L), glucose was more efficient. In mixtures containing both sugars, glucose was the preferred sugar for bacteria growth and xylose was rapidly consumed only after the glucose was exhausted. The xylose to glucose ratio affected bacterial growth and butyric acid production. High xylose to glucose ratios (4:1 or 3:2) showed better butyric acid production than low ratios (1:1, 2:3, or 1:4) when the total initial sugar content in the media was kept at 30 g/L.

Keywords: Clostridium tyrobutyricum; Butyric acid; Glucose; Xylose; Lignocellulosic biomass; Fermentation

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INTRODUCTION

Butyric acid is a short-chain fatty acid (C_3H_7COOH). In nature, the ester forms of butyric acid exist in animal fat and some plant oils (Liu *et al.* 2013). Butyric acid has many applications. It can serve as an intermediate to produce potential biofuels, such as butanol, ethyl butyrate, and butyl butyrate (Wu *et al.* 2010; Al-Shorgani *et al.* 2012; Dwidar *et al.* 2012; Li *et al.* 2013). As a carbon source, butyric acid can be converted to butanol, which has been shown to reduce the toxicity of alcohol in fermentation broth, and to enhance the theoretical yield of butanol by 67% (Du *et al.* 2012; Dwidar *et al.* 2012). Second, in the pharmaceutical industry, butyric acid is used to treat hemoglobinopathies, cancer, and gastrointestinal diseases (Dwidar *et al.* 2012). Various derivatives of butyric acid are produced as vasoconstrictor drugs and antioxidants. Other potential prodrugs that are produced from butyric acid are still under investigation (Du *et al.* 2012). Third, one major application of butyric acid in the chemical industry is plastics production, especially cellulose acetate butyrate plastics. These plastics have better flexibility and greater resistance to light and cold than traditional plastics (Cao *et al.*

2011; Dwidar *et al.* 2012). In addition, plasticizers, surfactants, and textile auxiliaries also use butyric acid as an important ingredient (Liu *et al.* 2013). Last but not least, in the form of esters and salts, butyric acid can be utilized as fragrance and flavoring agents in foods, beverages, and cosmetics (Dwidar *et al.* 2012; Liu *et al.* 2013).

Generally, there are three methods of producing butyric acid. At the industrial scale, butyric acid is obtained mostly from petroleum feedstocks by a petrochemical method (Jones and Woods 1986; Liu *et al.* 2013). This chemical process is related to the oxidation of butyraldehyde. Butyraldehyde comes from propylene, which is derived from crude oil by oxo synthesis (Cascone 2008). This method is preferred due to its relatively low production cost and the availability of the crude material (Dwidar *et al.* 2012). The second method is extracting butyric acid from butter directly. The concentration of butyric acid in butter ranges from 2% to 4%, but the extraction process is so complex and expensive that this method is not a promising alternative (Zigová and Šturdík 2000). The third method is a biological method via microbial fermentation (Jones and Woods 1986; Liu *et al.* 2013). Even though the current cost of the biological method is higher than the chemical-synthesis method, the microbial resources are renewable and they are more abundant, compared to limited crude oil (Dwidar *et al.* 2012). In addition, the demand for bio-based organic and natural products makes the biological method more desirable, especially for food additives, cosmetics, and pharmaceuticals (Zhang *et al.* 2009; Dwidar *et al.* 2012; Dwidar *et al.* 2013; Liu *et al.* 2013).

In microbial fermentation, a number of gram-positive, obligate anaerobes, such as *Clostridium sp.* and *Butyrivibrio fibrisolvens,* have the ability to produce butyric acid as the primary metabolite in significant quantities (Liu *et al.* 2013). Among these microorganisms, *Clostridium* strains are preferred in industry because of their higher productivities and greater final-product concentrations (Dwidar *et al.* 2013). The characteristics of these *Clostrium* species are gram-positivity, chemo-organotrophy, strict anaerobiosity, and spore formation (Zigová *et al.* 1999). *Clostridium tyrobutyricum* is one of the most promising strains due to its ability to produce butyric acid with high selectivity, the tolerance of high concentrations of products, and relatively high and stable productions (Zigová and Šturdík 2000; Liu and Yang 2006; Najafpour 2006; Song *et al.* 2010). This strain is able to utilize some common carbon sources, such as glucose, xylose, fructose, and lactate (Dwidar *et al.* 2013). Among these carbon sources, glucose and xylose are most commonly used because they are the main soluble sugars in hydrolysates of lignocellulosic biomass (Zhu and Yang 2004). It is well known that the major components of biomass are cellulose, hemicellulose, and lignin, with small amounts of pectin, protein, extractives, and ash (Jorgensen *et al*. 2007). Different plant species have varying chemical compositions. In general, lignin content is relatively small, comprising 12% to 35% of the biomass. Lignin is composed of heavily cross-linked polysaccharides, which makes it highly stable (Skoog and Hahn-Hagerdal 1988; Rivas *et al*. 2002). Cellulose is the dominant fraction, accounting for 35% to 50% of the dry weight of biomass, while hemicellulose accounts for 20% to 50% (Kumar *et al.* 2008). Cellulose consists of long, crystalline polymers of glucose. After pretreatment and enzymatic hydrolysis of cellulose, glucose can be obtained for biofuel production. Degradation of hemicellulose is the easiest of the three main components of biomass because of its random amorphous structure. For these feedstock-related reasons, this research was focused on using glucose and xylose for butyric acid fermentation by *C. tyrobutyricum.*

Many factors can affect the fermentation process of butyric acid by *C. tyrobutyricum.* In the research of Zhu and Yang (2004), the initial pH was set to five different levels (5.0, 5.3, 5.7, 6.0, and 6.3). It was found that the highest yield of butyrate was at pH 6.3. The production of acetate and lactate increased as the pH was decreased to 6.0, and lactate and acetate were the main products when the pH was below 5.0. In the study by Jiang et al. (2009), a fibrous bed bioreactor with immobilized *C. tyrobutyricum* was used on cane molasses to produce butyric, and pH 6.0 was found optimum. Some researchers focused on the effect of initial sugar concentration on fermentation by *C. tyrobutyricum*. Michel-Savin *et al.* (1990) compared the initial glucose concentrations of 125 g/L and 85 g/L and found that 125 g/L successfully increased the final butyric acid concentration, but led to a longer lag period. Research carried out by Fayolle *et al.* (1990) also demonstrated that the initial glucose concentration of 130 g/L could result in a long lag time. The effect of hydraulic retention time and glucose concentration (30, 40, and 50 g/L) on the yield of butyric acid by *C. tyrobutyricum* was studied (Mitchell *et al*. 2009). When the retention time was fixed at 16.7 h, 50 g/L glucose led to 22.7 g/L butyric acid, which was the highest among the three glucose concentrations studied. Zhu *et al*. (2002) studied the fermentation of a glucose/xylose mixture (1:1) and found that the acid production pattern from xylose did not change in the presence of glucose and no inhibition of xylose uptake was observed.

However, none of the aforementioned research systematically studied the effect of xylose concentration or the mixing ratio of xylose to glucose on the production of butyric acid by *C. tyrobutyricum*. Therefore, the objective of this study was to understand the effect of xylose and glucose concentrations in a single-sugar culture medium, as well as xylose-glucose mixtures, on butyric acid production.

EXPERIMENTAL

Bacteria Strain and Growth Media

C. tyrobutyricum (ATCC 25755) was obtained from American Type Culture Collection (ATCC, Manassas, VA). The synthetic medium (recipe provided by ATCC) with sugar supplements was used for the inoculation and culture experiments. In the single sugar experiments, D-glucose (CAS 50-99-7, Fisher Scientific, Hampton, New Hampshire, USA) or D-xylose (CAS 5328-36-0, Cascade Analytical Reagents & Biochemicals, Corvallis, Oregon, USA) was added to the synthetic medium at varying initial concentrations (5, 15, 35, 55, or 75 g/L).

In the sugar mixture experiments, bacteria were fed on the glucose/xylose mixtures. The total sugar of mixture was set at 30 g/L. The ratios of xylose to glucose used were 1:4, 2:3, 1:1, 3:2, and 4:1. All media contained yeast extract (5 g/L), $(NH_4)_2SO_4$ (3 g/L), K_2HPO_4 (1.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.6 g/L), and $FeSO_4 \cdot 7H_2O$ (0.03 g/L), and were sterilized at 121 °C for 25 min before inoculation.

Fermentation Process

All experiments were carried out in 500-mL Erlenmeyer flasks containing 270 mL of culture medium and 30 mL of bacteria seeds. The flasks were capped with air-lock valves. After inoculation, the bottles were flushed with N_2 gas to ensure anaerobic conditions. The culture was maintained at 37 °C on a reciprocating shaker at 150 rpm with three replicates.

Analytical Methods

All media were adjusted to a pH of 6.4 every day by adding NH_4OH. Cell density was analyzed by measuring the optical density (OD) of a cell suspension at the wavelength of 620 nm (OD_{620}) with a spectrophotometer (model 340, Sequoia-turner Corp, Santa Clara, California, USA). A high-performance liquid chromatography (HPLC) system (Agilent Technologies 1200 Series, Agilent Technologies, Santa Clara, CA) was used to analyze the organic compounds, including xylose, glucose, lactate, butyrate, and acetate in the fermentation broth. The HPLC system consisted of an auto-sampler, an ion-exclusion rezex organic acid column (Phenomenex, Torrance, California, USA) in a column oven at 45 °C, a diode array detector, and an evaporative light scattering detector. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min.

Statistical analysis was performed on the butyric acid production (peak concentration and yield), obtained from various initial conditions, to determine the level of significance. Multiple one-way analyses of variance (ANOVA) were conducted to evaluate the effect of the initial sugar concentration on butyric acid production by *C. tyrobutyricum* (ATCC 25755). The concentration of the initial glucose or xylose was used as the independent variable, while butyric acid peak concentration was used as the dependent variable. All results were expressed as mean ± standard deviation (SD) ($n \leq 3$). Tukey's adjustment was applied to the general linear model for the determination level of significance ($P < 0.05$) among various treatments. The LS-means with the same letter were not significantly different. SAS Version 9.1.3 (SAS Institute Inc., Cary, NC) was used for all statistical analysis.

RESULTS AND DISCUSSION

Fermentation in Single Sugars

Cell growth

In the xylose-only media, bacteria had approximately a 3-day lag period, but the bacteria in 75 g/L xylose were inhibited more seriously at the beginning than in all the other initial concentrations (Fig. 1, left). When the initial sugar concentration was too high, the excess carbon source was often the reason for osmotic dehydration, which affects the formation of acids, membrane transport, and cell lysis (Edwards 1970). The bacteria in xylose media had a longer lag phase compared with those in the glucose media.

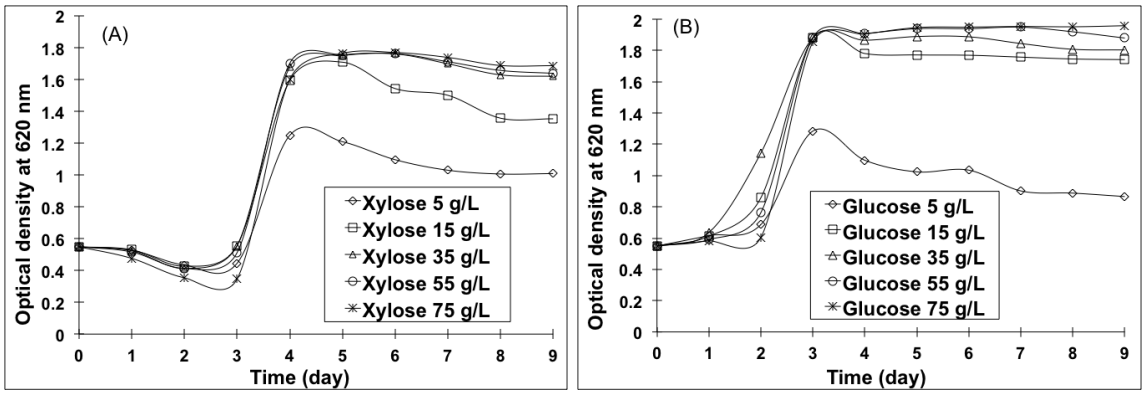

Fig. 1. Growth of *C. tyrobutyricum* in (A) xylose media and (B) glucose media

One possible reason for this lag was that the seed was cultured in the glucose medium, so the bacteria may have needed a longer time to adapt to the xylose environment. Another reason could be that the glucose was more easily metabolized than xylose by the *C. tyrobutyricum*. The maximum OD significantly increased as the initial xylose concentration increased from 5 to 15 g/L. When xylose concentration further increased, cell growth did not change significantly.

In the glucose media, the bacteria exhibited a shorter lag period (approximately 2 days), which had little to do with the initial glucose concentration (Fig. 1, right). The maximum OD increased as the initial glucose concentration increased from 5 to 15 g/L because higher glucose concentrations provided more energy for the bacteria to grow in the logarithmic phase. When the glucose concentrations further increased, the peak biomass concentration was more or less the same, meaning too much glucose was not necessary. Substrate inhibition on cell growth was observed at 75 g/L of sugar for both glucose and xylose, but such inhibition was only at the beginning of the culture.

Butyric acid production

The major acid product of *C. tyrobutyricum* was butyric acid. Lactic acid and acetic acid were detected, but because their concentrations were very low (<1 g/L), they were not shown in the results. Butyric acid production increased as the initial sugar concentration increased in both xylose and glucose media. Peak butyric acid concentration increased from 2.59 to 27.02 g/L in the glucose media, and from 3.53 to 16.29 g/L in the xylose media as the sugar concentration was increased from 5 to 75 g/L (Fig. 2). Comparing Fig. 2A to 2B, it can be found that there were some sudden butyric acid increases for xylose of 35 g/L or higher after day 6, while the butyric acid increases for glucose media were gradual to the end. This may be due to the different metabolic pathways by which *C. tyrobutyricum* utilizes glucose or xylose. It has been known that extra energy is needed to transport xylose across the cell membrane (Jiang *et al.* 2010). The transport of xylose is energized by a high-energy phosphate compound, which is implied to be ATP. It was reported that one mol of ATP is required for the transport of one mol of xylose into the cells of *C. butyricum* (Heyndrickx *et al.* 1991). Glucose is transported into cells by means of a phosphoenolpyruvate-dependent phosphotransferase system without requiring extra energy (Jiang *et al.* 2010). Because of this difference, ATP accumulation may be needed before rapid butyric acid synthesis was possible especially at high xylose concentrations as shown in Fig. 2A.

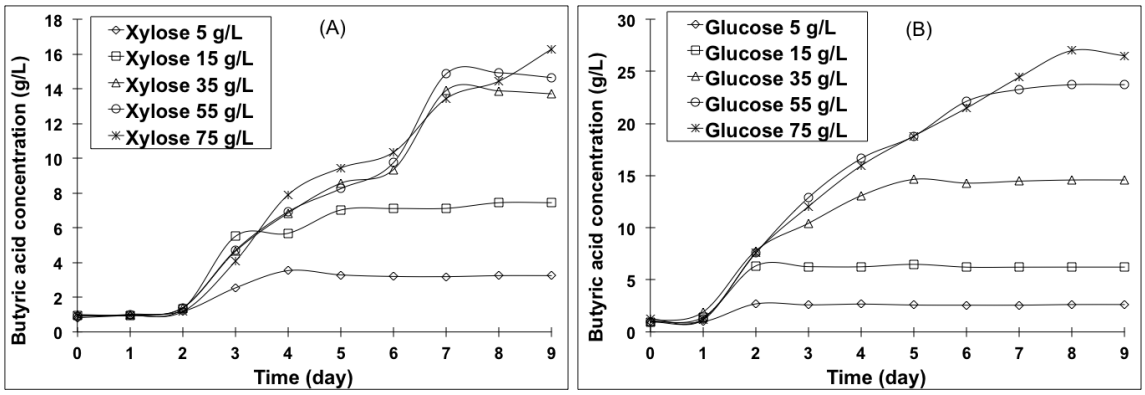

Fig. 2. Butyric acid concentration in (A) xylose media and (B) glucose media

In nine days, all the glucose was utilized, but not all of the xylose was consumed (Fig. 3). High xylose concentrations (>35 g/L) could not be completely used within nine days. Because the bacteria in xylose grew at a lower rate and the amount of butyric acid did not increase near the end, the experiments were stopped at the end of day 9 before the xylose was completely used.

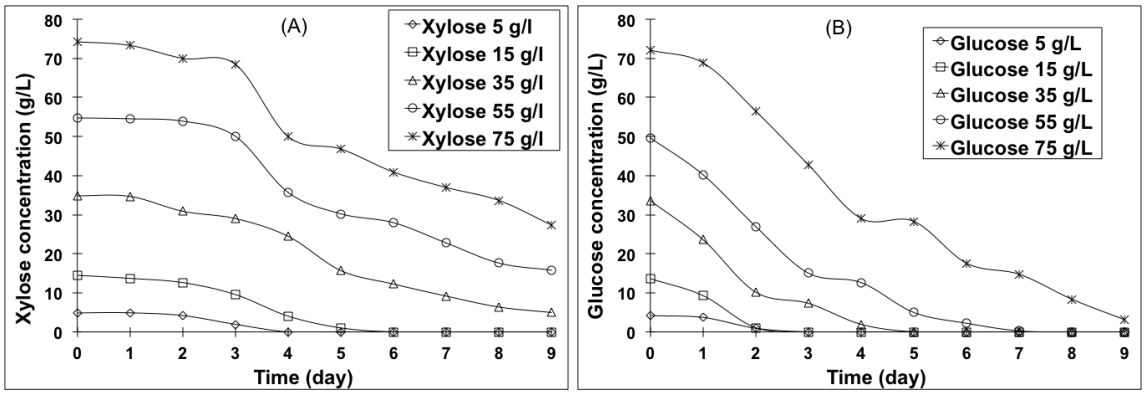

Fig. 3. Sugar consumption in (A) xylose media and (B) glucose media

Table 1 summarizes the conversion efficiencies from sugars to butyric acid. It can be seen that the yield from xylose to butyric acid started to decrease as the sugar concentration was above 35 g/L, while for glucose media higher glucose concentration resulted in higher yield. This suggested that *C. tyrobutyricum* preferred high concentrations of glucose but low xylose concentrations. At low sugar concentrations (5 and 15 g/L), the butyric acid concentration and yield from the xylose media were higher than that from glucose media, whereas the opposite was true at higher sugar concentrations (55 and 75 g/L).

Table 1. Final Concentration and Yield of Butyric Acid in Single-Sugar Media

	In the glucose media			In the xylose media		
Initial sugar concentration (g/L)	Glucose at the peak BA (g/L)	Peak BA concentration[#] (g/L)	Peak BA yield (g/g)	Xylose at the peak BA (g/L)	Peak BA concentration[#](g/L)	Peak BA yield (g/g)
5	0	1.57 ± 0.13 (e)	0.31 ± 0.05 (a)	0	2.25 ± 0.21 (e)	0.45 ± 0.04 (a)
15	0	5.25 ± 0.24 (d)	0.35 ± 0.09 (b)	1.02 ± 0.01	6.09 ± 0.32 (d)	0.44 ± 0.02 (a)
35	0	13.56 ± 0.46 (c)	0.42 ± 0.02 (b)	4.96 ± 0.02	12.78 ± 0.39 (c)	0.43 ± 0.01 (a)
55	0	22.70 ± 0.74 (b)	0.41 ± 0.02 (b)	17.61 ± 0.06	13.99 ± 0.48 (b)	0.37 ± 0.01 (b)
75	8.35 ± 0.03	26.00 ± 0.68 (a)	0.39 ± 0.01 (b)	27.30 ± 0.11	15.37 ± 0.62 (a)	0.32 ± 0.01 (c)

[#]Butyric acid concentration was adjusted by deducting the initial butyric acid coming from the seed inoculum

Fermentation in Xylose and Glucose Mixtures

Cell growth

Bacteria were cultured in five different media with varying xylose to glucose ratios, but the total initial sugar concentration in every medium was kept at 30 g/L. The 30 g/L total sugar concentration was chosen based on the observation that only approximately 30 g/L xylose was consumed during the fermentation process (Table 1). The media with more glucose clearly exhibited more cell growth in the first few days; however, as time progressed, the cells in the media with more xylose caught up and reached similar or even greater peak OD. One exception was for xylose to glucose ratio of 2:3, in which cell growth was the highest among all cases by day 3, but thereafter cell growth could not continue due to the exhaustion of sugars after day 3.

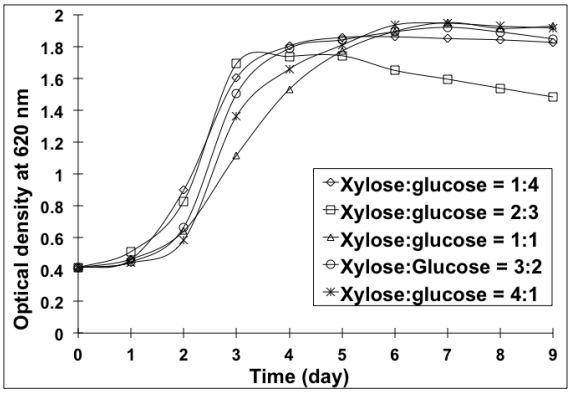

Fig. 4. Growth of *C. tyrobutyricum* in xylose and glucose mixtures

From Fig. 5, it can be seen that the glucose was consumed at a faster rate than the xylose in the mixtures. It was also apparent that xylose consumption was rapid only after the glucose was exhausted. This was different from what was found out by Zhu *et al*. (2002) who reported that in the fed-batch fermentation of xylose and glucose mixtures (1:1 ratio), the bacteria metabolized glucose and xylose simultaneously without preference for either. From Fig. 5, it is clear that in the 1:1 ratio case, glucose was completely used in about 5 days, but the xylose was used very slowly in the first 5 days, and then the consumption of xylose became rapid after day 5 when the glucose had been used up. Other xylose to glucose ratios also showed similar phenomenon of glucose consumption first, followed by rapid consumption of xylose.

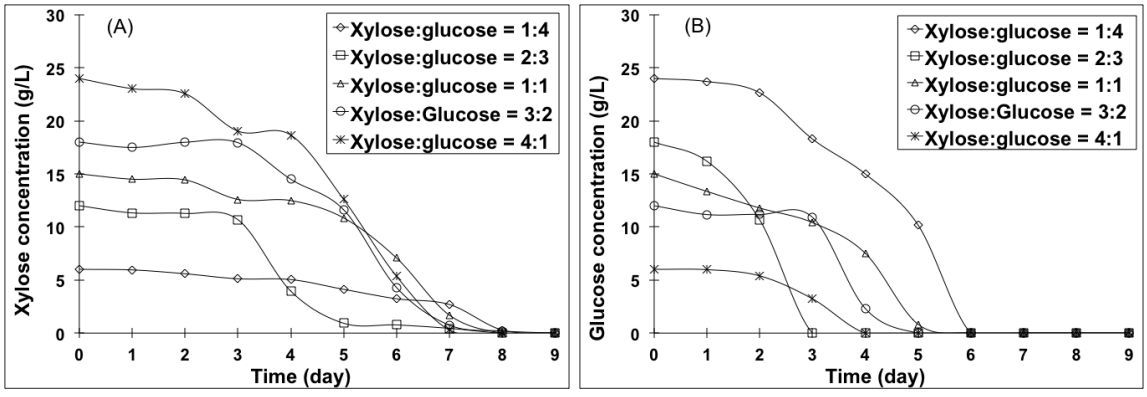

Fig. 5. (A) Xylose and (B) glucose consumptions in xylose and glucose mixtures

It is interesting to note that the xylose to glucose ratio of 2:3 showed the fastest sugar consumption among all cases. The glucose was exhausted in 3 days, and the xylose was used up in 5 days. Both consumptions were the fastest among all cases, indicating that the xylose to glucose ratio significantly influenced sugar consumption. This was consistent with cell growth (Fig. 4), in which cells grew the fastest at a xylose to glucose ratio of 2:3 in the first 3 days. However, glucose was completed exhausted at day 3, and thus, cell growth could not keep up after day 3.

Butyric acid production in xylose and glucose mixtures

Butyric acid concentration in the five different media was in the range of 14.24 ± 0.48 g/L to 15.23 ± 0.55 g/L. Statistically, the two media with more xylose (xylose:glucose = 4:1 or 3:2) had a higher butyric acid concentration than in the other three media, suggesting that xylose was effective in butyric acid production. Using linear interpolation between 15 and 35 g/L sugar concentration data (Table 1), it can be calculated that butyric acid concentration was estimated to be 12.5 g/L in 30 g/L glucose media, and 12.2 g/L in 30 g/L xylose media. Mixtures of glucose and xylose all had better results than the single sugar media, having butyric acid concentrations of 30 g/L. This suggested that mixing the two sugars could promote bacteria growth and butyric acid generation. It was speculated that a small amount of glucose in the media provided energy for the butyric acid synthesis that was needed during xylose metabolism.

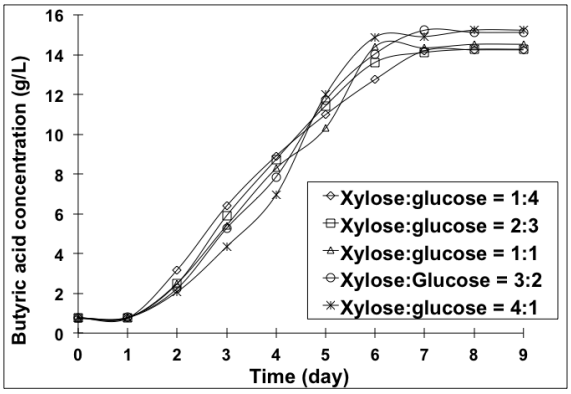

Fig. 6. Butyric acid concentration in xylose and glucose mixtures

CONCLUSIONS

1. The final butyric acid concentration increased with increasing initial sugar concentrations in both xylose- and glucose-only media. In terms of higher sugar to butyric acid yield, *C. tyrobutyricum* preferred higher concentrations of glucose but lower concentrations of xylose in the media.
2. At low sugar concentrations (5 or 15 g/L), xylose was more efficient than glucose for butyric acid production, but at higher concentrations (55 or 75 g/L) glucose was more efficient.
3. In mixtures containing both sugars, glucose was the preferred sugar for bacterial growth, and it was utilized before the xylose was rapidly consumed. The xylose to glucose ratio affected bacterial growth and butyric acid production.

4. High xylose to glucose ratios (4:1 or 3:2) had a better butyric acid production than low ratios (1:1, 2:3, or 1:4) when the total initial sugar content in the media was kept at 30 g/L.

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