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Effects of three main sugars in cane molasses on the production of butyric acid with *Clostridium tyrobutyricum*

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Abstract−The effects of three main sugars in cane molasses were investigated systematically to prepare a cost-effective medium for butyric acid bioproduction. Additionally, 30 g/L corn steep liquor was screened out as the suitable nitrogen source. In the batch fermentation of free cells, when 60 g/L glucose was the only carbon source, 21.28 g/L butyric acid was achieved after 30 h cultivation. Similar product concentration, productivity and yield were obtained when 60 g/L fructose was applied. The utilization of sucrose would bring about lower productivity (0.29 g/L·h) and product concentration (18.15 g/L), but the yield of butyric acid/sucrose (0.34 g/g) is almost the same as that from glucose or fructose (0.35 g/g). Finally, the sugar mixture (15 g/L glucose, 20 g/L fructose and 35 g/L sucrose) was employed to produce butyric acid in a fibrous-bed bioreactor (FBB), and 40.11 g/L butyric acid was produced with one simple fed-batch strategy.

Key words: Butyric Acid, *Clostridium tyrobutyricum*, Cane Molasses, Fibrous-bed Bioreactor

INTRODUCTION

Butyric acid and its derivatives have been applied widely in foodstuff, chemical and pharmaceutical industries. Esters of butyric acid are used as additives to increase fruit fragrance and butter-like note in the food industry [1], as well as aromatic compounds for perfume production [2]. In the chemical industry, butyric acid is a fundamental chemical and also the building block of butyryl polymers. Recently, increasing interest has been paid to producing butanol at higher rate using butyric acid as the substrate. Moreover, butyric acid, as one of the short-chain fatty acids generated by anaerobic fermentation of dietary substrates, is known to have therapeutic effects on colorectal cancer and hemoglobinopathies [3].

Currently, butyric acid is mainly produced by oxidation of butyraldehyde, which is obtained from the oxosynthesis of propylene. With the rocketing price, petroleum is no longer cheap and abundant. Thus, the fermentation production of butyric acid is becoming an attractive alternative to replace the petroleum-based chemical synthesis. Moreover, the public concerns on the environmental pollution caused by the petrochemical industry, and the preferences of consumers for the bio-based natural ingredients in foods, cosmetics and pharmaceuticals, are also stimulating the bioproduction of butyric acid. Butyric acid can be produced by a variety of genera, including *Clostridium*, *Butyrivibrio*, *Butyribacterium*, *Sarcina*, *Eubacterium*, *Fusobacterium* and *Megasphera* [4]. Among these strains, *Clostridium* species are the best-producing ones, yielding a high and relatively stable productivity of butyric acid under anaerobic conditions. Especially, *Clostridium tyrobutyricum* has been frequently employed to produce butyric acid from various carbohydrates [5-7]. Recently, a fibrous-bed bioreactor (FBB) with immobilized cells has been successfully developed for the fermentation production of acetic acid, propionic acid and butyric acid with greatly increased reactor productivity, final product concentration and product yield [8]. These good performances could be attributed to the regenerative ability and high mass transfer capability of the reactor. Compared to conventional immobilized cell bioreactors, the FBB has more advantages, including efficient and continuous operation without repeated inoculations, the elimination of cell lag phase, the good long-term stability, and easier downstream processing [9].

Normally, the cost of fermentation could be significantly reduced when a cheap and suitable carbon source is applied. Plant biomass, such as low-value agricultural commodities and food processing byproducts, represents a useful and valuable resource for microbial fermentations. Cane molasses is a byproduct from the sugar industry that consists of water, approximately 45-50% (w/w) total sugars (sucrose, glucose and fructose), suspended colloids, heavy metals, vitamins and nitrogenous compounds, etc. It has been used as a raw material for the fermentation production of highly valuable organic acids including succinic acid, lactic acid, citric acid, and so on [10-12]. In our previous work, the utilization of glucose and xylose has been evaluated for the production of butyric acid in the FBB bioreactor [9], and the primary results for the bioproduction of butyric acid in our laboratory came by using cane molasses as the medium components [13]. To formulate one optimal fermentation medium for the cost-effective butyric acid production, further work was carried out to investigate the effects of three main sugars (glucose, fructose, sucrose) in cane molasses and seven nitrogen sources. Finally, high and cost-effective production of butyric acid was achieved in FBB with the cane molasses-simulated medium.

MATERIALS AND METHODS

1. Microorganism and Media

C. tyrobutyricum ATCC 25755 was donated by Prof. Shang-Tian Yang (Dept. of Chemical and Biomolecular Engineering, the Ohio

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Table 1. Comparison of fermentation results of *C. tyrobutyricum* using various nitrogen sources

Nitrogen source	Urea	KNO_3	CSL	Fish meal	Yeast extract	Peptone	Beef extract	CGM
Butyric acid (g/L)	6.09	6.32	9.44	6.69	8.13	8.88	10.91	10.96

The initial glucose concentration was 30 g/L

State University, Columbus, OH). The stock culture was kept in serum bottles in a synthetic medium under anaerobic conditions at 4 °C [14]. Unless otherwise noted, the cultivation medium (*Clostridium* growth medium, CGM) contained (per liter of distilled water): 30 g glucose, 5 g yeast extract, 5 g peptone, 3 g $(NH_4)_2SO_4$, 1.5 g K_2HPO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.03 g $FeSO_4 \cdot 7H_2O$. All media were sterilized by autoclaving at 121 °C, 15 psig, for 20 min.

2. Production of Butyric Acid in an FBB System

The batch fermentations with suspended cells in a 5-*l* stirred-tank fermentor were carried out to investigate the effects of different carbon and nitrogen sources on butyric acid production. The fed-batch fermentations with an FBB system were employed to examine the effects of sugar mixture and cane molasses-based medium. The FBB was made of a glass column packed with spiral-wound cotton towel in a working volume of 480 mL. The entire FBB system consisted of a 5 L fermentor connecting to a 0.5 L fibrous-bed bioreactor through a recirculation loop. A detailed description of the reactor construction has been given elsewhere [15]. The system was operated at 37 °C, and pH was controlled at 6.0 by an on-line sensing and dosing system.

3. Analytical Methods

Cell density was analyzed by measuring the optical density of the cell suspension at 600 nm (OD_{600}) with a spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience). One unit of OD_{600} corresponded to 0.68 g/L cell dry weight for cells growing in the glucose medium [16]. A high performance liquid chromatography system was used to analyze the carbohydrate compounds, including glucose, fructose, and sucrose in the fermentation broth. The HPLC system consisted of an automatic injector (Agilent 1100, G1313A), a pump (Agilent 1100, G1311A), a Zorbax carbohydrate analysis column (250 mm×4.6 mm, 5 μm; Agilent, USA), a column oven at 30 °C (Agilent 1100, G1316A), and a refractive index detector (Agilent 1100, G1362A). The eluent phase was ethyl nitrile solution (ethyl nitrile/water=75/25 v/v), and the flow rate was controlled at 1.4 mL/min. Quantitative analysis of butyric acid and acetic acid was performed by gas chromatography (Agilent 6820 GE, Agilent Technologies) under the following conditions: glass column packed with HP-innovax (30 m×0.320 mm), N_2 as the carrier gas, temperatures of the flame ionization detector and injection port at 250 °C, and column temperature at 250 °C. The concentrations of butyric and acetic acid were determined according to a standard calibration curve [17].

RESULTS AND DISCUSSION

1. Effect of Different Nitrogen Sources on Butyric Acid Production

In many previous studies, *Clostridium* growth medium (CGM) was always used as one preferable nitrogen source to support the cell growth and butyric acid production. However, CGM is too expensive to produce butyric acid as the bulk chemical. To optimize the nitrogen, the effects of different inorganic and organic nitrogen sources were compared with the free cells of *C. tyrobutyricum* in the shaken flask, including urea (5 g/L), KNO_3 (5 g/L), corn steep liquor corn (CSL) (15 g/L), fish meal (10 g/L), yeast extract (10 g/L), peptone (10 g/L) and beef extract (10 g/L). As shown in Table 1, the inorganic nitrogen sources, e.g., urea and KNO_3, could not bring about high concentration of butyric acid. Among a variety of organic nitrogen sources, three nitrogen sources (corn steep liquor, peptone and beef extract) could generate more than 80% of butyric acid concentration which was achieved with CGM. As the cheapest one, corn steep liquor was employed as a desirable nitrogen source for the substitution of CGM in the following experiments.

The effects of different CSL concentration on cell growth and butyric acid production were further investigated. As shown in Fig. 1(a), the butyrate concentration, yield and productivity were improved with the increasing of CSL concentration from 2.5 g/L to 10 g/L, but decreased over 10 g/L. In Fig. 1(b), the similar profiles of cell growth were observed. The lag time of 2.5 g/L and 15 g/L CSL was longer than those of 5 g/L and 10 g/L CSL, which sug-

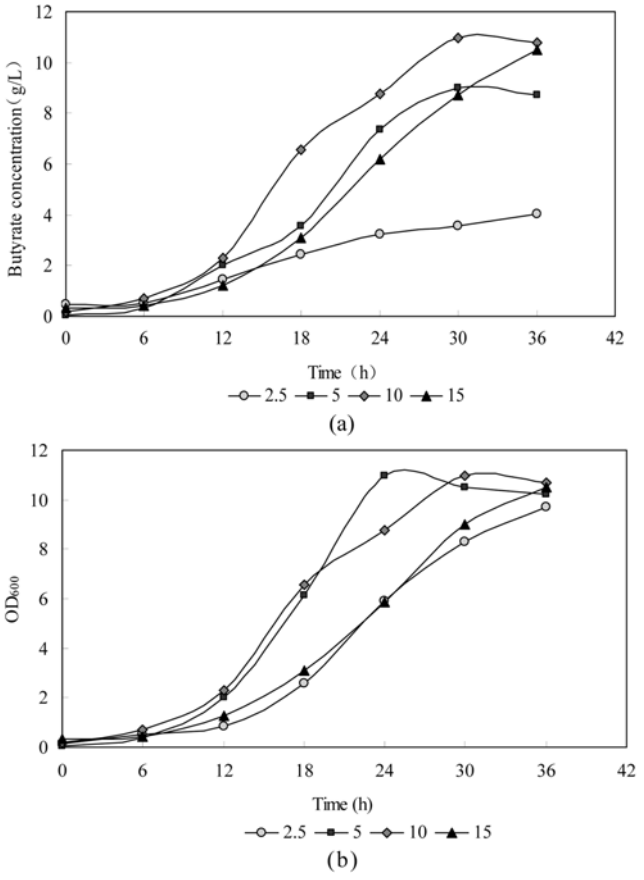

Fig. 1. Effect of initial corn steep liquor concentrations (2.5, 5, 10 and 15 g/L) on butyric acid production (a) and cell growth (b).

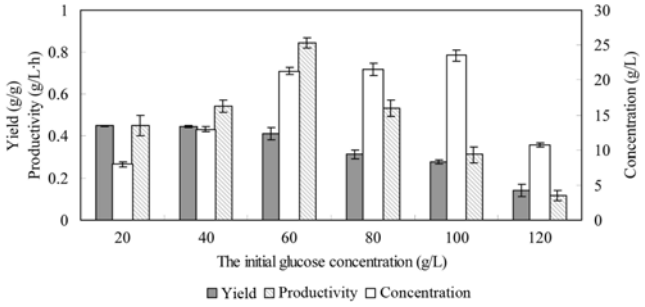

Fig. 2. Effect of initial glucose concentrations on the production of butyric acid.

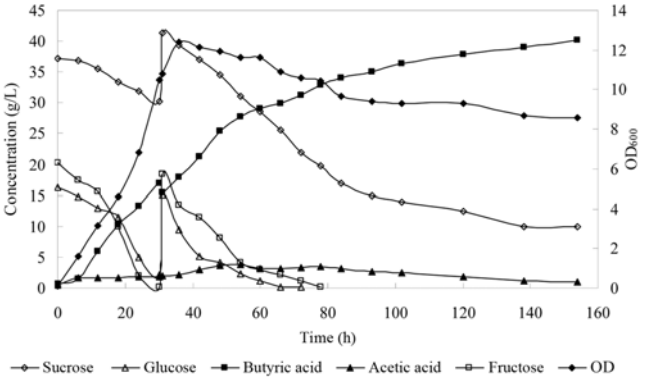

Fig. 3. Time courses of cell growth and production of butyric acid in fed-batch mode by *C.tyrobutyricum* immobilized in the FBB.

gested an optimal concentration of nitrogen source for the growth of *C. tyrobutyricum*. Thus, 10 g/L CSL was adopted for butyric acid production in the succeeding experiments.

2. Effect of Initial Concentration of Glucose on Butyric Acid Production

Although the effect of glucose on butyric acid production has been investigated before, it is still necessary to be examined again in the CSL-based medium for compatibility check. Different concentrations of glucose were tested for butyric acid production with the free cells of *C. tyrobutyricum* in the 5-liter bench-top fermentor. As shown in Fig. 2, the production of butyric acid increased significantly with the increase of initial concentrations from 20 g/L to 60 g/L. The maximum butyric acid concentration (21.28±0.51 g/L) and productivity (0.845 g/L·h) were obtained with the initial glucose concentration of 60 g/L, and the yield of 0.41 g/g was quite close to the theoretical maximum value of 0.49 g/g [8]. Due to the substrate inhibition, further concentration increase of glucose over 60 g/L would decrease the yield of butyric acid.

3. Effects of Fructose and Sucrose on the Production of Butyric Acid

It was well known that three existing sugars in the cane molasses were glucose, fructose, and sucrose. To evaluate the feasibility of utilizing cane molasses for the production of butyric acid, the effects of fructose and sucrose on the production of butyric acid were further examined, respectively. As shown in Table 2, three different sugars (glucose, fructose and sucrose) could be efficiently utilized to produce butyric acid. The highest butyric acid concentration (21.28 g/L) was obtained with 60 g/L glucose in the medium. Compared to glucose, fructose was also a very good carbon source to generate similar product concentration, productivity and yield of butyric acid. However, when sucrose was used as the sole carbon source, the productivity (0.29 g/L·h) and product concentration (18.15 g/L) of butyric acid decreased significantly. But the yield of butyric acid/sucrose (0.34 g/g) was almost the same as that with from glucose or fructose (0.35 g/g). These results suggest that all three main sugars in cane molasses could be utilized efficiently by *C. tyrobutyricum* to produce butyric acid, only sucrose was utilized at a relatively lower rate.

4. Efficient Production of Butyric Acid with the Simulated Sugar Mixture in the FBB System

The approximated ratio of three sugars in molasses was about 10 g/L glucose, 15 g/L fructose and 35 g/L sucrose. However, the glucose and fructose could be utilized much faster that sucrose. To improve the efficiency of the fermentation, one simulated sugar mixture was prepared as the carbon source which consisted of 15 g/L glucose, 20 g/L fructose and 35 g/L sucrose. As shown in Fig. 3, the components of this simulated complex carbon source were utilized simultaneously in the FBB bioreactor, and the consumption rates of glucose and fructose are much faster than that of sucrose. When the concentrations of glucose and fructose were below 0.5 g/L, a concentrated sugar mixture (glucose : fructose : sucrose=15 : 20 : 10) was supplemented to ensure the efficient production of butyric acid in the broth. After 120 h fermentation, 40.11 g/L butyric acid was achieved, which is about one-fold improvement over that from the batch fermentation using glucose as the sole carbon source. This high production of butyric acid from the simulated sugar mixture suggested that the application of low-cost cane molasses as the raw substrate was promising for cost-effective production of butyric acid.

Although some primary results were reported in our previous work [9,13], some expensive nitrogen components, such as yeast extract or peptone, were employed in the reported formulated medium for butyric acid production. Further efforts were carried out in the present work to provide one low-cost fermentation medium by investigating the effects of different sugars in cane molasses and

Table 2. Fermentation results of *C. tyrobutyricum* using various carbon sources

Sugar[a]	Sugar residue (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Butyric acid productivity (g/L·h)	Yield of butyric acid (g/g)
Glucose	0.45±0.12	7.06±0.47	21.28±0.51	0.85±0.01	0.35±0.01
Fructose	0.96±0.33	7.29±0.30	20.26±0.24	0.70±0.01	0.34±0.00
Sucrose	4.48±0.50	7.69±0.48	18.85±0.35	0.29±0.01	0.34±0.01

[a]The initial total sugar concentration was 60 g/L

the supplementary nitrogen sources. The present work would be helpful in the development of cost-effective bioproduction of butyric acid in the future.

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REFERENCES

1. D. W. Armstrong and H. Yamazaki, *Trends Biotechnol.*, **4**, 264 (1986).
2. F. H. J. Sharpel, *Comprehensive biotechnology*, Oxford, UK: Pergamon Press, 965 (1985).
3. E. A. Willims, J. M. Coxhead and J. C. Mathers, *Proc. Nutr. Soc.*, **62**, 107 (2003).
4. J. Zigova and E. Sturdik, *J. Ind. Microbiol. Biotechnol.*, **24**, 153 (2000).
5. D. Michel-Savin, R. Marchal and J. P. Vandecasteele, *Appl. Microbiol. Biotechnol.*, **32**, 387 (1990).
6. X. G. Liu, Y. Zhu and S. T. Yang, *Enzyme Microb. Technol.*, **38**, 521 (2006).
7. Z. T. Wu and S. T. Yang, *Biotechnol. Bioeng.*, **82**, 93 (2003).
8. Y. Zhu, Z. T. Wu and S. T. Yang, *Process Biochem.*, **38**, 657 (2002).
9. L. Jiang, J. F. Wang, S. Z. Liang, X. N. Wang, P. L. Cen and Z. N. Xu, *Appl. Biochem. Biotechnol.*, **160**(2), 350 (2010).
10. Y. P. Liu, P. Zheng, Z. H. Sun, Y. Ni, J. J. Dong and L. L. Zhu, *Bioresour. Technol.*, **99**, 1736 (2008).
11. C. Kotzamanidis, T. Roukas and G. Skaracis, *World J. Microbiol. Biotechnol.*, **18**, 441 (2002).
12. J. L. Wang, X. H. Wen and D. Zhou, *Bioresour. Technol.*, **75**, 231 (2000).
13. L. Jiang, J. F. Wang, S. Z. Liang, X. N. Wang, P. L. Cen and Z. N. Xu, *Bioresour. Technol.*, **100**, 3403 (2009).
14. Y. Huang and S. T. Yang, *Biotechnol. Bioeng.*, **60**, 499 (1998).
15. E. M. Silva and S. T. Yang, *J. Biotechnol.*, **41**, 59 (1995).
16. X. G. Liu and S. T. Yang, *Process Biochem.*, **41**, 801(2006).
17. G. He, Q. Kong, Q. Chen and H. Ruan, *J. Zhejiang Univ. Sci.*, **11**, 1076 (2005).