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Butanol Production from Cane Molasses by *Clostridium saccharobutylicum* DSM 13864: Batch and Semicontinuous Fermentation

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Abstract *Clostridium acetobutylicum* strains used in most Chinese ABE (acetone–butanol–ethanol) plants favorably ferment starchy materials like corn, cassava, etc., rather than sugar materials. This is one major problem of ABE industry in China and significantly limits the exploitation of cheap waste sugar materials. In this work, cane molasses were utilized as substrate in ABE production by *Clostridium saccharobutylicum* DSM 13864. Under optimum conditions, total solvent of 19.80 g/L (13.40 g/L butanol) was reached after 72 h of fermentation in an Erlenmeyer flask. In a 5-L bioreactor, total solvent of 17.88 g/L was attained after 36 h of fermentation, and the productivity and yield were 0.50 g/L/h and 0.33 g ABE/g sugar consumption, respectively. To further enhance the productivity, a two-stage semicontinuous fermentation process was steadily operated for over 8 days (205 h, 26 cycles) with average productivity (stage II) of 1.05 g/L/h and cell concentration (stage I) of 7.43 OD_{660}, respectively. The average batch fermentation time (stage I and II) was reduced to 21−25 h with average solvent of 15.27 g/L. This study provides valuable process data for the development of industrial ABE fermentation process using cane molasses as substrate.

Keywords Acetone–butanol–ethanol · Fermentation · Cane molasses · *Clostridium saccharobutylicum* · Semicontinuous

Introduction

Butanol can be produced by *Clostridium*-based fermentations, and is regarded as one of the next-generation biofuels. As a biofuel, it has remarkable advantages over bioethanol [1–3].

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Butanol is less corrosive and could be distributed through gasoline pipeline system; it is less hygroscopic and tolerates water contamination better; it is less evaporative and explosive owing to its lower vapor pressure; it has 30% higher energy density than ethanol and is closer to gasoline; and importantly, it can be mixed in higher ratios with gasoline in existing cars without retrofitting the engine.

Global crude oil price has risen dramatically over the past decades, which opens new opportunities as well as challenges for the biofuel industry. Many nations and organizations have recently joined in new biofuel activities, and a lot of research works have been carried out to produce biofuel [such as butanol from acetone–butanol–ethanol (ABE) fermentation] worldwide [4, 5]. Challenging the biofuel development, the cost of substrate is the most important economic factor in ABE production [6–8]. In China, as well as in many developing countries, grain and certain lands are banned for biofuel production use due to the "food versus fuel" debate.

In order to lower the cost, various cheap substrates such as molasses [9, 10], sago starch [11], wheat bran [12], barley straw hydrolysate [13], palm oil waste [14], and lignocellulosic materials [15] have been studied in the ABE fermentation. Cane molasses, a major by-product of sugar industry, consist of approximately 50–55% reducing sugars (sucrose, fructose, and glucose), suspended colloids, heavy metals, vitamins, nitrogenous compounds, etc. [16–18]. The recent price of cane molasses is around 800−1,000 yuan RMB per ton, which is cheaper than corn (around 2,100 −2,300 yuan RMB per ton) in China (based on statistical data as of February 2012). Due to its high carbohydrate contents and relatively low price, cane molasses are excellent substrates for fermentation. Also, the use of cane molasses in ABE production is economically advantageous: relatively cheap, convenient to operate as a liquid, and no additional hydrolysis step is required [19, 20]. Cane molasses have been reported for the fermentative production of a number of important chemicals, including butyric acid [21], gluconic acid [22], and citric acid [23].

A few groups reported the ABE fermentation of molasses. Syed et al. [19] utilized 6% cane molasses for ABE fermentation by *Clostridium acetobutylicum* PCSIR-5 and PCSIR-10, and total solvent of 15.2 and 19.2 g/L was attained, respectively. Recently, 18.3 g/L of butanol was produced by a mutant strain *C. acetobutylicum* MEMS-7 using 6% blackstrap molasses [24]. Shaheen et al. [25] studied the ABE fermentation using 6% cane molasses by four species of solvent-producing *Clostridia*, the maximum total solvent of 18.9 g/L was produced by *Clostridium beijerinckii* NCP P260. When 80 g/L of soy molasses together with 25.3 of g/L glucose was used, Qureshi et al. [26] acquired total ABE solvent up to 22.8 g/L by a mutant strain *C. beijerinckii* BA101.

Recently, Qureshi et al. studied the ABE fermentation, using *C. beijerinckii* P260, from various agricultural waste hydrolysates (such as wheat straw hydrolysate, corn stover hydrolysate, and switchgrass hydrolysate), and 21.37−26.64 g/L of total solvent and 0.55 g/L/h of productivity were reached [27, 28]. The annual production of cane molasses is around 3 million tons in China, which is mostly available in Southern China (such as Guangxi province). As far as we know, no Chinese solvent plant is currently using cane molasses as substrate or as a supplement in ABE fermentation, especially in a continuous process. It is therefore necessary to investigate the ABE fermentation from cane molasses as well as its continuous process. In this work, cane molasses were used as substrate for ABE fermentation by *Clostridium saccharobutylicum* DSM 13864, a saccharolytic solvent-producing strain. Semicontinuous fermentation was investigated in this study for improved solvent productivity as it is more economical and less time-consuming compared with batch fermentation [29, 30].

Materials and Methods

Microorganisms and Growth Conditions

The *C. saccharobutylicum* DSM 13864 strain used in this study was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures). The strain was grown anaerobically in DSMZ medium 104b.pyx containing trypticase peptone 5.0 g/L, meat peptone (pepsin-digested) 5.0 g/L, yeast extract 10.0 g/L, glucose 5.0 g/L, resazurin 1.0 mg/L, salt solution (DSMZ medium 104) 40.0 mL/L, and cysteine HCl×H_2O 0.5 g/L at 35 °C for 12–18 h in a vacuum desiccator. The spores were obtained after cultivating anaerobically at 35 °C for 2 weeks. The spore suspension was maintained in 30% glycerol at −80 °C.

Reinforced *Clostridium* medium was used as seed media, containing: yeast extract 3 g/L, beef extract 10 g/L, peptone 10 g/L, soluble starch 1 g/L, glucose 5 g/L, cysteine HCl×H_2O 0.5 g/L, sodium chloride 3 g/L, sodium acetate 3 g/L, and resazurin 3 mg/L. The medium was adjusted to pH 6.5 with 5 M NaOH solution, and then it was sterilized at 115 °C for 20 min. Cells were transferred into a fermentation medium after 12–18 h at 35 °C in a vacuum desiccator.

Pretreatment of Cane Molasses

Cane molasses used in this study were purchased from Jiangmen Sugarcane Chemical Plant Co., Ltd (Guangdong), and it contains sucrose 30% (*w*/*w*), glucose 3.2% (*w*/*w*), fructose 10.8% (*w*/*w*), other carbohydrates 2.5% (*w*/*w*), ash 9.6% (*w*/*w*), metal ions 8.9% (*w*/*w*), salt 4.6% (*w*/*w*), crude protein 4.3% (*w*/*w*), crude fat 0.06% (*w*/*w*), etc. Pretreatment of cane molasses is necessary to eliminate some of the inhibitory components, such as colloidal material, ash, and other suspended materials, which could affect the growth of microorganisms [16, 31]. Three liters of tap water was added to 7 L cane molasses, and the mixture was then acidified to pH 3.5 with 5 M H_2SO_4. Following water bath at 60 °C for 2 h, the mixture was centrifuged at 10,800×*g* for 15 min. The supernatant was diluted before using as fermentation substrate.

Fermentation

The fermentation medium and conditions were optimized to achieve a high level of total solvents and butanol using cane molasses as fermentation substrate. The initial fermentation medium contains 6% (*w*/*w*) cane molasses, and the pH was adjusted to 6.5 with saturated NaOH before autoclaving for 20 min at 121 °C. Fermentation parameters including sugar content of cane molasses, nitrogen source, pH, temperature, buffering agent, trace element, and inoculum size were optimized. The experiments were carried out in a 250-mL Erlenmeyer flask with 150 mL working volume for 72 h.

Batch culture was carried out in a 5-L stirred bioreactor with a 2.5-L fermentation medium under optimized fermentation conditions except that 10 g/L instead of 20 g/L of corn steep powder was used to prevent too much foam during the fermentation process. The pH of medium was carefully adjusted with freshly prepared saturated NaOH to avoid the introduction of HCl since sodium chloride was reported to be a strong inhibitor to cell growth in ABE fermentation [26]. To ensure an anaerobic environment, external CO_2 gas was aerated into a bioreactor through microbial filter before inoculation. To measure the rate of gas production, an inverted cylinder filled with saturated $NaHCO_3$ solution was placed in a container with same $NaHCO_3$ solution, and the end of gas outlet was extended to the bottom of the cylinder.

Semicontinuous fermentation system can usually be divided into two stages: a feasible culture is supplied in stage I, and a high concentration of product is acquired in stage II [31]. In this study, cyclic operation of semicontinuous fermentation was carried out in four reactors, including one stage I reactor and three stage II reactors. Two supplementary tanks were used for adding fresh medium into stage I and stage II reactors. The number of stage II reactors was decided by dividing stage II fermentation time (t_2) by stage I fermentation time (t_1). Stage I fermentation was conducted with 200 mL medium in a 250-mL reactor, while stage II fermentation was carried out in a 500-mL reactor with 300 mL working volume. The size of two supplementary tanks is 4 L. The stage I supplement medium (SSM I) consists of 2% cane molasses, 2 g/L $(NH_4)_2SO_4$, 10 g/L corn steep powder, 3.2 g/L $CaCO_3$, 0.5 g/L K_2HPO_4, and 0.01 g/L $MnSO_4{\cdot}H_2O$. The stage II supplement medium (SSM II) consists of 8% cane molasses, 2 g/L $(NH_4)_2SO_4$, 10 g/L corn steep powder, 3.2 g/L $CaCO_3$, 0.5 g/L K_2HPO_4, and 0.01 g/L $MnSO_4{\cdot}H_2O$. The schematic drawing of reactors and procedures for two-stage semicontinuous ABE fermentation is shown in Fig. 1.

Analytical Methods

The concentration of cell was measured by the optical density (OD) at 660 nm using a spectrophotometer. Before measuring, the cell suspension was diluted five to ten times with 2% hydrochloric acid to eliminate insoluble medium ingredients.

Sugars (sucrose, fructose, and glucose) were determined using a Waters 600 high-performance liquid chromatography (Waters, USA) equipped with a calcium cation exchange column SugarPak1 (6.5 mmid×300 mm; Waters, USA) and 2410 refractive index detector. The mobile phase was ultra pure water at a flow rate of 0.4 ml/min and 85 °C.

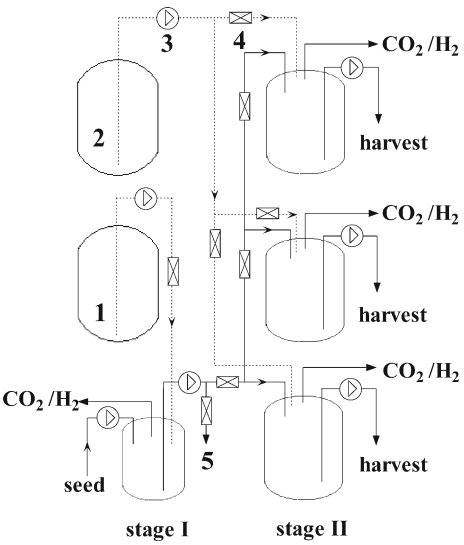

Fig. 1 The schematic flow chart of reactors and procedures for two-stage semicontinuous ABE fermentation. *1* When the cell concentration of stage I reached the threshold value, 150 mL seed solution from stage I (250-mL reactor) is transferred into stage II reactor (500-mL reactor); *2* Fresh SSM I (150 mL) is added to the stage I, while 150 mL fresh SSM II is added to the stage II; *3* Then, new cultivations are started; *4* When the solvent concentration of stage II reached the threshold value, the fermentation broth of stage II is harvested; *5* When the cell concentration of stage I reached the threshold value again, the procedure is repeated (1-SSM I; 2-SSM II; 3-peristaltic pump; 4-pinchcock; 5-sampling port)

The concentration of acetone, butanol, ethanol, acetate, and butyrate was assayed by a Varian 3900 gas chromatography (Varian, USA) equipped with PEG-20M column (30 m×0.32 mm×0.4 μm) and a flame ionization detector. The column temperature program was as follows: holding 70 °C for 0.5 min, and then rising to 170 °C at 20 °C/min to 190 °C at 10 °C/min, finally holding 1 min at 190 °C. The flow rate of column was 1 ml/min. Isobutanol was used as an internal standard for quantification.

The ABE yield was calculated as follows: Yield(grams ABE/grams sugar) = grams ABE/grams(sucrose + fructose + glucose)

Results

Optimization of Fermentation Medium and Conditions

To investigate the feasibility of molasses as economic substrates, the utilization of various sugars (sucrose, fructose, and glucose) presented in cane molasses was studied in ABE fermentation by *C. saccharobutylicum* (Table 1). The results indicate that the strain could ferment glucose, fructose, or sucrose to produce 7.82−9.15 g/L of butanol. When a mixture of three sugars in the ratio of pretreated molasses (262.28 g/L of sucrose, 76.18 g/L of fructose, and 56.31 g/L of glucose) was used, the strain could produce 13.15 g/L of total solvent (including butanol 8.79 g/L, acetone 3.88 g/L, and ethanol 0.48 g/L) at 48 h, indicating that cane molasses could be used for butanol production.

The appropriate sugar concentration of cane molasses for ABE fermentation was investigated within the range of 2% to 12% (percent, *w*/*w*; Fig. 2a). The total solvent production rose when the sugar concentration was increased from 2% to 6%, and the highest total solvent of 13.01 g/L was obtained at sugar concentration of 6%. A dramatic decrease was however observed as the sugar concentration was further increased from 6% to 12%, which was coincident with the report by Shaheen et al. [25].

The effect of various carbonates including K_2CO_3, $CaCO_3$, $MgCO_3$, $KHCO_3$, and $NaHCO_3$, acting as pH regulator for ABE fermentation using cane molasses, was investigated. It was remarkable that $CaCO_3$ significantly promoted solvent accumulation in which the total solvent was doubled, while other buffering agents barely had an effect (Fig. 2b).

Additionally, initial fermentation pH and temperature were optimized. Over the range of 30 °C to 40 °C, the solvent production increased steadily from 30 °C to 37 °C; further increasing to 38 °C however resulted to a sharp fall in total solvent from 18.90 to 11.78 g/L, and less than 5 g/L solvent was produced at 40 °C (Fig. 2c). *C. saccharobutylicum* showed good solvent production between initial pH 6.0 and 6.8, and the highest solvent level was reached at pH 6.0 (see Electronic Supplementary Material (ESM), Fig. A1).

Moreover, various inorganic and organic nitrogen sources were tested. Although $(NH_4)_2HPO_4$ (total solvent 16.22 g/L) gave a bit higher solvent level, $(NH_4)_2SO_4$ (total solvent, 15.69 g/L) was chosen as the inorganic nitrogen source since it is much cheaper (see ESM, Fig. A1). As for organic nitrogen source, corn steep powder (CSP) was the best one (see ESM, Fig. A1). Therefore, the combination of $(NH_4)_2SO_4$ and CSP was used in the fermentation medium. Based on the result of an orthogonal test, it was noted that CSP has a stronger influence towards the ABE produced compared with $(NH_4)_2SO_4$ (data not shown). The content of CSP was thus optimized (Fig. 2d). The result shows that the solvent production increased with CSP content from 5 to 20 g/L, and the maximal total solvent of 19.39 g/L (butanol 13.03 g/L, acetone 4.99 g/L, and ethanol 1.37 g/L) was achieved at CSP content of 20 g/L.

Table 1 ABE fermentation of various sugars by *C. saccharobutylicum* DSM 13864 in TYA medium

Sugars (g/L)	Acetone (g/L)	Ethanol (g/L)	Butanol (g/L)	Acetate (g/L)	Butyrate (g/L)	Total solvent (g/L)	Residual sugars (g/L)	Sugar utilization[a] (%)	Cell concentration (OD_{660})
Glucose	3.55±0.06	0.43±0.04	7.82±0.16	3.14±0.04	0.90±0.06	11.80±0.26	0.00±0.00	100±0.00	3.80±0.03
Fructose	3.40±0.18	0.45±0.04	8.46±0.11	3.13±0.04	0.73±0.04	12.06±0.00	0.00±0.00	100±0.00	3.85±0.03
Sucrose	4.22±0.08	0.52±0.01	9.15±0.01	3.12±0.01	0.68±0.01	13.89±0.11	0.27±0.00	99.33±0.11	3.3±0.10
Sugar mixture[b]	3.88±0.25	0.48±0.06	8.79±0.28	3.25±0.08	0.72±0.06	13.15±0.03	0.00±0.00	100±0.00	4.18±0.15

TYA medium: total sugar 40 g/L, beef extract 2 g/L, yeast extract 2 g/L, tryptone 6 g/L, ammonium acetate 3 g/L, KH_2PO_4 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, and $FeSO_4{\cdot}7H_2O$ 0.01 g/L; fermentation time, 48 h

[a] Sugar utilization(percentage) = (total sugar consumption/total sugar) × 100%

[b] Sugar mixture: sucrose 28 g/L, glucose 4 g/L, and fructose 8 g/L (total sugar, 40 g/L)

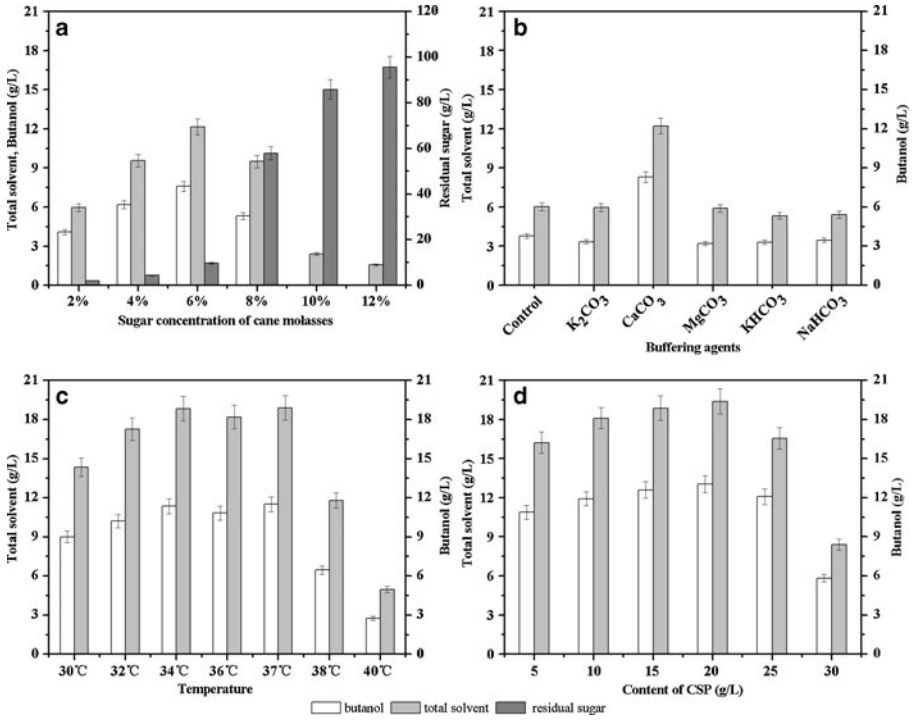

Fig. 2 The effect of sugar concentration of cane molasses (**a**), buffering agent (**b**), temperature (**c**), and content of CSP (**d**) on ABE fermentation by *C. saccharobutylicum* DSM 13864. The fermentation medium and conditions were as follows: **a** 2−12% cane molasses, 3.2 g/L $CaCO_3$, pH 6.5, 35 °C; **b** 6% cane molasses, 3.2 g/L buffering agent, pH 6.5, 35 °C; **c** 6% cane molasses, 3.2 g/L $CaCO_3$, 2 g/L $(NH_4)_2SO_4$, 10 g/L CSP, 0.5 g/L K_2HPO_4, 0.01 g/L $MnSO_4{\cdot}H_2O$, pH 6.0, 30−40 °C; **d** 6% cane molasses, 3.2 g/L $CaCO_3$, 2 g/L $(NH_4)_2SO_4$, 5−30 g/L CSP, 0.5 g/L K_2HPO_4, 0.01 g/L $MnSO_4{\cdot}H_2O$, pH 6.0, 37 °C

The effect of trace element (Fe^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, and Ba^{2+}) was also studied. It was apparent that Mn^{2+} and Ni^{2+} were beneficial for the solvent accumulation (see ESM, Fig. A1). Since Mn^{2+} is much cheaper than Ni^{2+}, it was selected as a supplementary component in the fermentation medium to boost the solvent production. Mn^{2+} is one of the commonly used trace elements in ABE fermentation [32, 33].

The optimum fermentation medium and conditions were determined as follows: 6% cane molasses, 2 g/L $(NH_4)_2SO_4$, 20 g/L corn steep powder, 3.2 g/L $CaCO_3$, 0.5 g/L K_2HPO_4, and 0.01 g/L $MnSO_4{\cdot}H_2O$, inoculum size 8% (*v*/*v*), pH 6.0, and 37 °C. The total solvent reached 19.80 g/L after 72 h of fermentation in a 250-mL Erlenmeyer flask, including 13.40 g/L butanol, 5.01 g/L acetone, and 1.39 g/L ethanol.

Batch Fermentation

The ABE fermentation by *C. saccharobutylicum* using cane molasses in a 5-L stirred bioreactor is illustrated in Fig. 3. The fermentation process could be divided into two phases, the acidogenic phase and solventogenic phase. During the acidogenic phase (the first 13 h), acetate and butyrate were produced quickly, causing a decrease in pH value from 5.52 to 4.99. Then, the solventogenic phase started with conversion of organic acid (acetate and

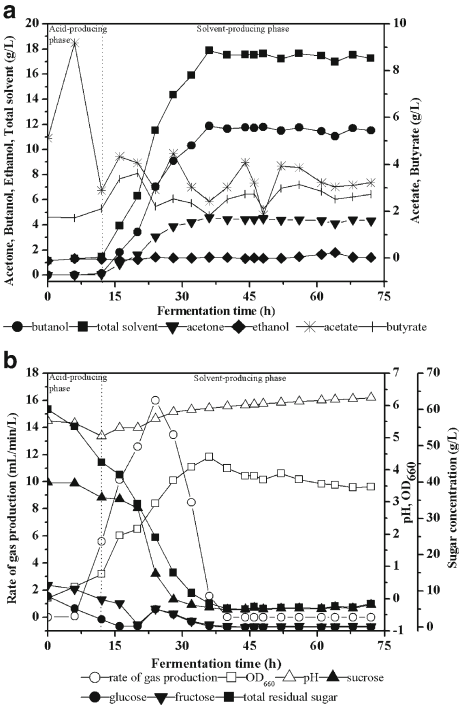

Fig. 3 Time course of ABE fermentation by *C. saccharobutylicum* DSM 13864 in a 5-L stirred bioreactor. **a** Butanol, total solvent, acetone, ethanol, acetate, and butyrate; **b** Rate of gas production, OD_{660}, pH, and sugar concentration. The fermentation medium and conditions were as follows: 6% cane molasses, 3.2 g/L $CaCO_3$, 2 g/L $(NH_4)_2SO_4$, 0.5 g/L K_2HPO_4, 10 g/L CSP, 0.01 g/L $MnSO_4 \cdot H_2O$, pH 6.0, 37 °C, inoculum size 8% (*v*/*v*)

butyrate) into solvent (acetone, butanol, and ethanol). At the end of the fermentation, the pH of fermentation broth increased to 6.24.

The batch fermentation was carried out for 72 h, and the ABE accumulation reached its highest level at 36 h. A total solvent of 17.88 g/L was obtained at 36 h, including acetone 4.60 g/L, butanol 11.86 g/L, ethanol 1.42 g/L, and the productivity and yield were 0.50 g/L/h and 0.33 g ABE/g sugar consumption, respectively. Total organic acid (acetate and butyrate) was up to 10.90 g/L (acetate 9.18 g/L and butyrate 1.72 g/L) at 6 h, and decreased to 5.93 g/L (acetate 3.21 g/L and butyrate 2.72 g/L) at the end of fermentation (at 72 h; Fig. 3a).

Sugar consumption during the batch fermentation was also determined (Fig. 3b). At 60.01 g/L of initial sugar (sucrose 39.87 g/L, glucose 8.56 g/L, and fructose 11.58 g/L), glucose and fructose were almost depleted at 16 h (0.24 g/L) and 20 h (0.74 g/L), respectively. During 20−28 h, sucrose was rapidly decomposed into glucose and fructose for further consumption, suggesting a lagged metabolism of sucrose than that of glucose and fructose. During the first 36 h of batch fermentation, 89.04% of total sugar was consumed, and the residual sugar was 6.58 g/L.

The gas production of *C. saccharobutylicum* started off quickly after 5 h of fermentation, peaked at 24 h, and stopped at 36 h, while a rapid increase in cell concentration was also noticed during 5−36 h, suggesting that the cell growth and gas production were possibly coupled (Fig. 3b). The cell concentration was up to a maximum (4.4 OD_{660}) at 36 h when the

highest total solvent was reached, and then reduced to 3.5 OD_{660} mainly due to the toxicity of butanol.

Semicontinuous Fermentation

To attain a desirable ABE productivity and yield in stage II, high cell concentration and activity should be ensured at the end of stage I. Prior to two-stage semicontinuous fermentation, the appropriate sugar concentration of SSM I (stage I supplement medium) and SSM II (stage II supplement medium) was therefore investigated by evaluating the cell growth with different sugar concentrations of cane molasses (Table 2). The result indicates that lower level of sugar concentration is beneficial for the cell growth of *C. saccharobutylicum*. Sugar concentration of 2% was thus used in SSM I. For SSM II, cane molasses with 8% sugar was used, and the actual sugar concentration in stage II fermentor was determined to be 41.45 g/L, representing a mixture of 150 mL of fresh SSM II and 150 mL of broth from stage I (as shown in Fig. 1).

In order to determine the suitable fermentation time in stage I, parameters including cell concentration, gas production, residual sugar, and solvent production were investigated during 9 h of cultivation (Fig. 4). Since a slowing down in cell growth was observed at 8 h, corresponding to an increasing solvent concentration (butanol 3.55 g/L and total solvent 6.92 g/L) and a low sugar level (0.70 g/L residual sugar), a fermentation time of 7 h was thus used in stage I to obtain appropriate cell concentration. In the first cycle of stage I, 16 mL of seed solution (8% inoculum size) and 200 mL of fresh SSM I were transferred into stage I reactor, the medium was then incubated at 37 °C for a prolonged 12 h to achieve the desired cell density (7.75 OD_{660}), and 7 h of incubation was sufficient for the following cycles (Fig. 5). The cell concentration in stage I was able to maintain steadily between 7.54±0.07 and 8.18±0.16 OD_{660} during the 26 cycles until it reduced to 4.51 OD_{660} in the last cycle (the 28th cycle). During the 26 cycles, the average total solvent was up to 4.00±0.19 g/L (butanol 2.61±0.12 g/L), and the average residual sugar was 2.89±0.14 g/L at the end of fermentation.

For stage II, the fermentation finished in 14−18 h during 26 cycles. The fermentation process was operated steadily for 205 h (26 cycles), and the average total solvent of 15.27±1.2 g/L was attained. At 14 h, the average productivity and yield were 1.05±0.09 g/L/h and 0.37±0.03 g ABE/g sugar consumption. It is noted that the total solvent was up to 18.50 and 19.11 g/L in the 15th and 17th cycle, respectively (Fig. 5).

Discussion

In this study, cane molasses could be utilized for ABE production by *C. saccharobutylicum* DSM 13864, a saccharolytic strain. In the optimization of fermentation medium, lower solvent production was observed at higher sugar concentration (>6%; Fig. 2a), likely due to

Table 2 Effect of sugar concentration on cell growth of *C. saccharobutylicum* DSM 13864

Sugar concentration	2%	4%	6%	8%	10%
OD_{660} at 6 h	6.81±0.11	4.96±0.24	3.76±0.27	2.61±0.23	2.49±0.14
OD_{660} at 12 h	11.32±0.34	9.13±0.15	8.55±0.34	3.92±0.11	2.61±0.08

The fermentation medium and conditions are the same as Fig. 3 except 2–10% cane molasses was used

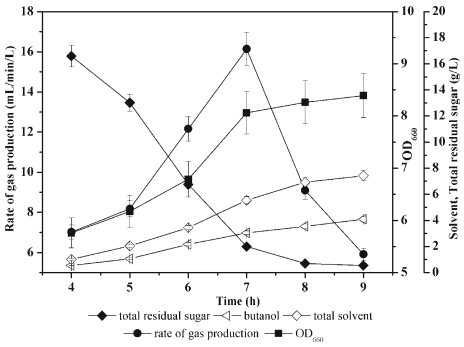

Fig. 4 Time course of stage I fermentation by *C. saccharobutylicum* DSM 13864. The fermentation medium and conditions were the same as in Fig. 3 except the concentration of cane molasses was 2%

the existence of excessive salt and other inhibitors such as sodium chloride, coloring substances, and heavy metals in cane molasses [26, 34]. Thus, cane molasses sugar concentration of less than 6% was used in ABE fermentation as reported by Shaheen et al. [25]. A combination of $(NH_4)_2SO_4$ and CSP was used as the nitrogen source in this study. Abou-Zeid et al. also reported that $(NH_4)_2SO_4$ was utilized very well as inorganic nitrogen source in ABE fermentation with blackstrap molasses [35]. CSP, a natural organic nitrogen source, is widely used as a low-cost nutrient for microbial fermentation including ABE production [6, 36]. A higher content of CSP (>20 g/L) resulted in decreased solvent production (Fig. 2d). The reason might be due to the presence of growth inhibitors (lactic acid, heavy metals, minerals, and their salts) in CSP as reported by Qureshi et al. [26]. Besides, it was noticed that excessive foam was generated during the fermentation process with high content of CSP. As a pH regulator, calcium carbonate plays an important role in improving ABE production [37]. In ABE production from cane molasses by various *Clostridium* strains, the amount of $CaCO_3$ required to neutralize the excess acidity differs significantly, 2−4 g/L is appropriate for *C. saccharobutylicum* (this study), while 10−30 g/L is necessary for *C. beijerinckii* DSM 6422 (unpublished data), keeping pH within the range of 4.99−6.20 (*C. saccharobutylicum*) (this study), and 5.20−6.00 (*C. beijerinckii*) (unpublished data). Under the optimum fermentation medium and conditions, total solvent of 19.80 g/L was attained in a 250-mL Erlenmeyer flask.

During the batch fermentation by *C. saccharobutylicum* in a 5-L stirred bioreactor, an earlier decrease in acetate was observed in the late acidogenic phase (Fig. 3a). This phenomenon could be due to the complex metabolic pathway involved in the conversion

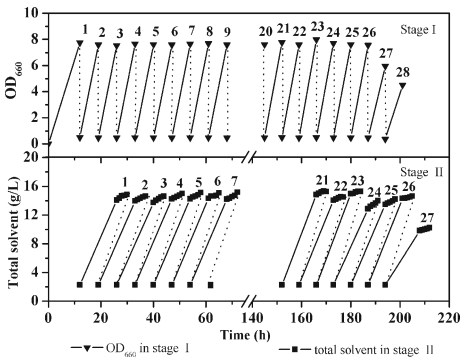

Fig. 5 Time course of two-stage semicontinuous ABE fermentation by *C. saccharobutylicum* DSM 13864. SSM I: 2% cane molasses, SSM II: 8% cane molasses. The fermentation medium was the same as in Fig. 3 except the concentration of cane molasses

of acetate into acetone, in which several intermediates such as acetyl-CoA, acetoacetyl-CoA, and acetoacetate are produced. During the batch fermentation, it was interesting to find that gas production was closely related to the accumulation of organic acids and total solvent, and the solvent accumulation ceased at 36 h as gas production stopped (Fig. 3). It was also noticed that the glucose was completely used at 40 h, while a small amount of sucrose and fructose remained to the end of the fermentation (Fig. 3b). The fast consumption of glucose is as expected since glucose has been the preferred sugar for most fermenting microorganisms [38]. Interestingly, a slight increase in sucrose was observed during 36−72 h of the fermentation, which presumably came from the resolving clumps of substrate particles which released encaged sucrose.

Continuous and semicontinuous fermentations have been reported as important fermentation processes. Chaiklahan et al. [39] reported the cultivation of *Spirulina platensis* using pig wastewater in a semicontinuous process for more than 17 days. Bauer et al. [40] and Sanada et al. [41] utilized semicontinuous culture system to achieve higher product titer. Afschar et al. reported the ABE production from molasses using an economic two-stage continuous fermentation with cell recycling, resulting to 8.1−8.9 g/L of butanol, 12.8−13 g/L of total solvent, and 3.0−3.6 g/L/h of productivity with a yield of 0.3 g ABE/g carbohydrate [42]. In this study, a two-stage semicontinuous fermentation process without cell recycling equipment was established, and a higher average solvent of 15.27 g/L was attained. Compared with that of the batch fermentation (0.50 g/L/h at 36 h), the average productivity of semicontinuous fermentation (1.05 g/L/h at 14 h) was enhanced for more than 100%. The average fermentation time (stage I, 7 h and stage II, 14−18 h) in semicontinuous fermentation was reduced to 21−25 h, which was greatly shortened compared with that of batch fermentation (36 h). The semicontinuous fermentation process could be steadily operated for over 8 days (205 h, 26 cycles; Fig. 5). Moreover, the sugar utilization was calculated to be up to 99.22%. Sucrose and glucose were exhausted, while a little fructose was left at the end of fermentation, and the average residual sugar was only 0.32 g/L. The results demonstrate that, compared with batch fermentation, a better sugar utilization, shorter fermentation time, and improved productivity could be achieved in semicontinuous fermentation by *C. saccharobutylicum* DSM 13864 from cane molasses.

Furthermore, our preliminary experiments indicate that it is not efficient to perform the continuous ABE process in a two-fermentor system due to the high residual sugar (data not shown). Continuous ABE fermentation in a four 3-L fermentor system is therefore being conducted in our laboratory to develop the commercial ABE process from substrates such as cane molasses and agricultural waste hydrolysate.

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