

Acetone–Butanol–Ethanol Production With High Productivity Using *Clostridium acetobutylicum* BKM19

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ABSTRACT: Conventional acetone–butanol–ethanol (ABE) fermentation is severely limited by low solvent titer and productivities. Thus, this study aims at developing an improved *Clostridium acetobutylicum* strain possessing enhanced ABE production capability followed by process optimization for high ABE productivity. Random mutagenesis of *C. acetobutylicum* PJC4BK was performed by screening cells on fluoroacetate plates to isolate a mutant strain, BKM19, which exhibited the total solvent production capability 30.5% higher than the parent strain. The BKM19 produced 32.5 g L⁻¹ of ABE (17.6 g L⁻¹ butanol, 10.5 g L⁻¹ ethanol, and 4.4 g L⁻¹ acetone) from 85.2 g L⁻¹ glucose in batch fermentation. A high cell density continuous ABE fermentation of the BKM19 in membrane cell-recycle bioreactor was studied and optimized for improved solvent volumetric productivity. Different dilution rates were examined to find the optimal condition giving highest butanol and ABE productivities. The maximum butanol and ABE productivities of 9.6 and 20.0 g L⁻¹ h⁻¹, respectively, could be achieved at the dilution rate of 0.85 h⁻¹. Further cell recycling experiments were carried out with controlled cell-bleeding at two different bleeding rates. The maximum solvent productivities were obtained when the fermenter was operated at a dilution rate of 0.86 h⁻¹ with the bleeding

rate of 0.04 h⁻¹. Under the optimal operational condition, butanol and ABE could be produced with the volumetric productivities of 10.7 and 21.1 g L⁻¹ h⁻¹, and the yields of 0.17 and 0.34 g g⁻¹, respectively. The obtained butanol and ABE volumetric productivities are the highest reported productivities obtained from all known-processes.

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Introduction

Our increasing concerns on the limited fossil resources and environmental problems including climate change have resulted in worldwide interests in renewable bio-based chemicals, such as acetone–butanol–ethanol (ABE), which are important solvents used in industries including polymer, paint, lacquer, and coating (Lee et al., 2008; Ranganathan and Maranas, 2010; Schmidt and Weuster-Botz, 2012). ABE are naturally produced by a genus *Clostridium* (Crown et al., 2011), but the biological production is not sufficient to compete with the petro-based production yet (Green, 2011). In order to develop a sustainable and economically viable ABE fermentation process, the process should combine the properties of high product titer along with high productivity. Contrary to this, ABE production by conventional fermentation process is limited by low product titer and productivity (Green, 2011).

The major factors affecting ABE production by fermentation are low production titer caused by low solvent tolerance of clostridia and low solvent productivity. To overcome the problem of low solvent titer, strain

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improvement by traditional random mutagenesis as well as knowledge based rational metabolic engineering have been applied to develop improved strains (Annous and Blaschek, 1991; Jang et al., 2012a,b; Tomas et al., 2003). Similarly, various bioprocess strategies such as batch, fed batch and continuous fermentation and integrated fermentation process with in situ product recovery techniques have been applied to improve the product titer and productivity (Ezeji et al., 2004, 2005; Lee et al., 2008; Roffler et al., 1988; Soni et al., 1987). However, the obtained ABE productivities in all these systems were not high enough, one of the reasons for which being the cell concentrations.

Continuous fermentation is considered as one of the suggested strategies for improving the solvent productivity of ABE fermentation (Soni et al., 1987). However, traditional continuous fermentation has the inherent limitation of cell wash-out when operated at high dilution rates. This problem can be addressed by applying high cell density continuous fermentation. Two different reactor systems are reported to carry out continuous ABE fermentation with high cell density: biofilm based bioreactor (Lienhardt et al., 2002; Qureshi et al., 2005) and membrane cell-recycle bioreactor (Tashiro et al., 2005). Fermentation of *Clostridium beijerinckii* BA101 in the biofilm based bioreactor resulted in a maximum solvent productivity of $16.2 \text{ g L}^{-1} \text{ h}^{-1}$ (Lienhardt et al., 2002). High cell density cultivation of *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) in the membrane cell-recycle bioreactor showed a maximum ABE productivity of $11.0 \text{ g L}^{-1} \text{ h}^{-1}$ (Tashiro et al., 2005).

In this study, a hyper-ABE producing *Clostridium acetobutylicum* BKM19 was developed by random mutagenesis, followed by determination of the ABE production performances in batch fermentation. Additionally, it was also aimed to find the optimal condition for improved ABE volumetric productivity of the BKM19 strain during continuous fermentation with membrane cell recycling. Different dilution rates with and without cell bleeding were examined to find the optimal condition. The maximum butanol and solvent productivities of 10.7 and $21.1 \text{ g L}^{-1} \text{ h}^{-1}$, respectively, could be achieved when the fermenter was operated at dilution rate of 0.86 h^{-1} with the bleeding rate of 0.04 h^{-1} . The obtained butanol and ABE volumetric productivities in this study are the highest reported to date.

Material and Methods

Bacterial Strain, Chemical Reagents, and Culture Condition

C. acetobutylicum strain PJC4BK (Green et al., 1996) was provided by Professor Eleftherios T. Papoutsakis, University of Delaware (Newark, DE) and Professor George Bennett, Rice University (Huston, TX). All the chemicals were of reagent grade, and unless otherwise stated, all chemicals used in this study were purchased from Difco (Detroit,

MD), Sigma–Aldrich (St. Louis, MO), or Junsei Chemicals (Tokyo, Japan). Except for the bioreactor studies, all the cultures were carried out in an anaerobic chamber (Forma Scientific, Marietta, OH), which contained 96% (v/v) nitrogen and 4% (v/v) hydrogen at 37°C . Stock cultures were stored at -80°C as 15% (v/v) glycerol stocks of cells, which were grown to an OD_{600} of 0.8–1.0. Clostridial Growth Medium (CGM) was used as a basal medium for the cultivation of *C. acetobutylicum*. The CGM contained $0.75 \text{ g L}^{-1} \text{ K}_2\text{HPO}_4$, $0.75 \text{ g L}^{-1} \text{ KH}_2\text{PO}_4$, $0.7 \text{ g L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, $0.017 \text{ g L}^{-1} \text{ MnSO}_4 \cdot 5\text{H}_2\text{O}$, $0.01 \text{ g L}^{-1} \text{ FeSO}_4 \cdot 7\text{H}_2\text{O}$, $2 \text{ g L}^{-1} (\text{NH}_4)_2\text{SO}_4$, $1 \text{ g L}^{-1} \text{ NaCl}$, 2 g L^{-1} asparagine, 0.004 g L^{-1} *p*-aminobenzoic acid, 5 g L^{-1} yeast extract. In all experiments, the initial pH of the medium was adjusted to 6.5 with 1 M NaOH and heat sterilized at 121°C for 15 min. Glucose was autoclaved separately and mixed in an anaerobic chamber. *p*-Aminobenzoic acid was also added separately as a filter sterilized solution. Static plate cultures were grown on $2 \times$ YTG agar medium which contained 16 g L^{-1} bacto tryptone, 10 g L^{-1} yeast extract, $4 \text{ g L}^{-1} \text{ NaCl}$, 5 g L^{-1} glucose and 1.5% (w/v) agar. Inoculums were prepared in CGM.

Mutation and Screening

Mutagenesis of the *C. acetobutylicum* PJC4BK strain was carried out by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) following the previously reported method (Annous and Blaschek, 1991). For this purpose, cells were initially grown to an OD_{600} of 1.0 in $2 \times$ YTG medium. Then, cells were harvested by centrifugation at $7,000 \text{g}$ for 15 min at 4°C and resuspended in fresh $2 \times$ YTG medium to an OD_{600} of 5.0. The resuspended cells were aliquoted into three different parts, and were treated with 25, 50, and $100 \mu\text{g mL}^{-1}$ NTG, respectively, at 37°C for 5 min in an anaerobic chamber (Forma Scientific). After the NTG-treatment was over, all three aliquots of NTG-treated cells were mixed together and washed twice with fresh $2 \times$ YTG medium. Finally, cells were resuspended in 5-volume of fresh $2 \times$ YTG medium and incubated anaerobically at 37°C for 12 h. Cells were subjected to selection pressure by plating them onto $2 \times$ YTG medium containing 2 mg L^{-1} fluoroacetate. And the plates were incubated anaerobically at 37°C until mutant colonies appear. All the colonies appearing on selection plate were screened for their solvent producing capabilities. For this purpose, single colonies were picked up from the selection plates and inoculated into the individual wells of 24-well microtiter plate containing 2 mL of CGM. The PJC4BK strain was also inoculated into one of the wells in the microtiter plate as a control. Microtiter plates were incubated in the anaerobic chamber at 37°C for 48 h, and culture broths were taken out to analyze the production of metabolites. Finally, mutants showing improved ABE production along with reduced production of acetate were selected for further studies.

Batch Fermentation

Batch fermentations were carried out at 32°C in a 5 L jar fermenter (LiFlus GX; Biotron, Kyunggi-Do, Korea) containing 1.8 L of CGM supplemented with 80 g L⁻¹ glucose. Seed cultures were anaerobically prepared in the same medium. A single colony was inoculated into a test tube containing 10 mL medium, grown over night, and was transferred to the 500 mL flask containing 200 mL medium. When the cell density reached OD₆₀₀ of 2.0 in the flask, cells were inoculated into the bioreactor. The culture pH was automatically controlled above 5.0 by addition of 28% (v/v) ammonia solution during the acidogenic phase, and was not controlled when it became higher than the set value during the solventogenic phase. Small amount of antifoam (Antifoam 204; Sigma-Aldrich) was added into the bioreactor to control foaming during the late acidogenic phase. Oxygen-free nitrogen gas, obtained by passing it through an oxygen trap (Agilent, Waldbronn, Germany), was sparged throughout the fermentation at a flow rate of 0.5 L min⁻¹. The agitation speed was controlled at 200 rpm.

High Cell Density Continuous Cultivation With Cell Recycling

Continuous fermentation in membrane cell recycle bioreactor was carried out in a glass fermenter (1 L Bioflow 110; New Brunswick Scientific Co., Edison, NJ) with a working volume of 400 mL. The initial medium used was GCM supplemented with 80 g L⁻¹ glucose. A hollow fiber ultrafiltration module (4,800 cm² surface area; Spectrum Laboratories Inc., Rancho Dominguez, CA) was connected to the bioreactor for cell recycling. The experimental set up for the continuous fermentation with total cell recycling using membrane cell bioreactor is represented in Figure 1. A diaphragm pump (Model P-07090-40; Cole Parmer

Instrument Co., Vernon Hills, IL) was used for continuous recirculation of fermentation broth through the hollow fiber module. Cells were recycled into the fermenter while the cell-free permeate was collected out. The inflow of the feeding medium was balanced with the permeate outflow. Oxygen-free nitrogen was continuously sparged through the medium to maintain the strictly anaerobic condition. During the acidogenic phase of fermentation, the minimum value of pH was maintained at 5.0 by automatically adding ammonia solution. Fermentations were carried out 100 rpm and 32°C. The fermenter was initially inoculated with 10% (v/v) inoculum (40 mL) and the experiment was operated in batch mode until the residual glucose concentration in the medium approached 20 g L⁻¹. The cultivation was then switched to continuous mode with total cell recycling. Several dilution rates were examined to find the optimal condition. Since cells become old during the long-term operation, the culture OD₆₀₀ was adjusted to ca. 150 before changing to a new dilution rate; in this way, solvent production performance at different dilution rates could be better compared. The steady-state operation at each dilution rate was performed for 27–30 h, which correspond to ca. 9–10 generations.

High Cell Density Continuous Cultivation With Cell Recycling and Bleeding

The experimental set up for high cell density cultivation with cell recycling and bleeding was similar to that described above, except that one additional peristaltic pump was used for controlled cell bleeding (Fig. 1). All the experiments were carried out at the dilution rate of 0.9 h⁻¹. Cells were removed from the fermenter by bleeding at two different rates to find the optimal condition. The inflow of the feeding medium was balanced by the combined outflow of permeate

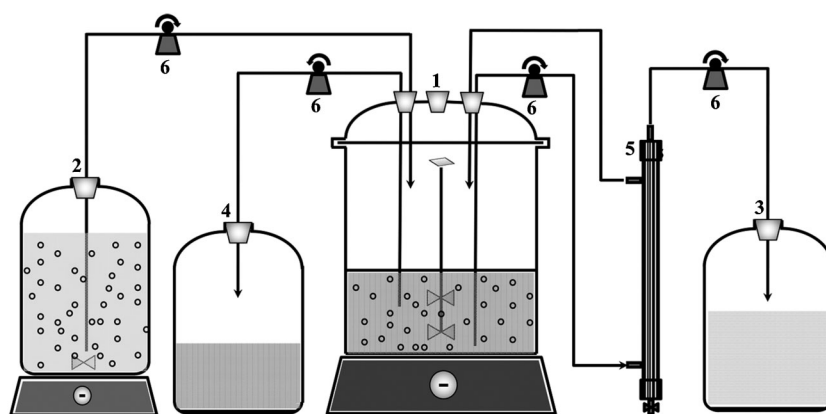


Figure 1. Schematic of the membrane cell-recycle bioreactor used for high cell density continuous fermentation. Symbols are: (1) jar fermenter used for continuous cultivation of cells; (2) feed tank; (3) reservoir for permeate collection; (4) reservoir for the collection of culture broth during cell bleeding; (5) hollow fiber ultra-filtration module; (6) peristaltic pump.

and culture broth as a bleed. The dilution rates were calculated as per the following equation:

$$D_1 = \frac{F_1}{V}$$

$$D_2 = \frac{F_2}{V}$$

$$D_t = D_1 + D_2$$

where D_1 is the dilution rate 1 (h^{-1}), F_1 is the outflow rate of the permeate from the hollow fiber ultrafiltration module (L h^{-1}), V is the working volume (L), D_2 is dilution rate 2 (h^{-1}), F_2 is the outflow rate (L h^{-1}) of the broth by bleeding and D_t is the total dilution rate (sum of dilution rates 1 and 2) of the system. Solvent volumetric productivity was calculated by using the formula:

$$\text{Volumetric productivity} = P \times D_t$$

where, P is the solvent (ABE or butanol) concentration (g L^{-1}) and D_t is the total dilution rate (h^{-1}).

Analytical Methods

For the analysis of residual sugar and metabolites produced, samples were centrifuged at 7,000g for 10 min. Cell pellet was discarded while the supernatant was used for analysis. Metabolite concentrations were measured by gas chromatography (Agilent 6890N; Agilent Technologies, Santa Clara, CA) as described previously (Lee et al., 2009). Cell growth was monitored by measuring the OD_{600} using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

Results

Isolation of ABE Hyper-Producing *C. acetobutylicum* Strain BKM19 by Mutagenesis

The *C. acetobutylicum* strain PJC4BK is a butyrate kinase inactivated mutant showing enhanced solvent production capability (Green et al., 1996). The PJC4BK strain was subject to NTG mutagenesis. Mutants were screened on a selection medium containing 2 mg L^{-1} fluoroacetate. More than 100 fluoroacetate resistant colonies were obtained during the first round of screening. The obtained mutants were subsequently screened for their solvent producing capabilities in 24-well microtiter plates. About 12.5% of the obtained mutants showed increased solvent (ABE) production, especially ethanol. Among the mutants, an ABE hyper-producing BKM19 strain was isolated. The obtained BKM19 strain was able to produce 19.0 g L^{-1} of ABE (9.3 g L^{-1} butanol, 9.2 g L^{-1} ethanol, and 0.5 g L^{-1} acetone) together with 2.1 g L^{-1} acetate and no butyrate from 80 g L^{-1} glucose in the microtiter culture. The performance of the BKM19 strain is much better than that of the parental PJC4BK strain, which produced 13.9 g L^{-1} ABE (7.6 g L^{-1} butanol,

4.1 g L^{-1} ethanol, and 2.2 g L^{-1} acetone) with 4.7 g L^{-1} acetate and 0.2 g L^{-1} butyrate under the same culture condition.

Comparative Batch Fermentation of PJC4BK and BKM19 Strains

Batch fermentations were carried out to evaluate the differences in ABE producing capabilities of the PJC4BK and BKM19 strains under the same controlled experimental condition. The BKM19 strain produced 32.5 g L^{-1} of ABE (17.6 g L^{-1} butanol, 10.5 g L^{-1} ethanol, and 4.4 g L^{-1} acetone) from 85.2 g L^{-1} glucose (Fig. 2), while the PJC4BK strain produced 24.9 g L^{-1} of ABE (15.9 g L^{-1} butanol, 6.1 g L^{-1} ethanol, and 2.9 g L^{-1} acetone) from 73.7 g L^{-1} of glucose (Fig. 3). In comparison, the wild-type *C. acetobutylicum* ATCC 824 strain produced 10.9 g L^{-1} of butanol and 17.1 g L^{-1} of ABE from 67.2 g L^{-1} of glucose (Lee et al., 2009). The mutant BKM19 strain produced 30.5% and 90.5% more ABE than the PJC4BK and ATCC 824 strains, respectively. The yields of butanol and ABE obtained were 0.16 and 0.25 g g^{-1} for ATCC 824, 0.22 and 0.34 g g^{-1} for PJC4BK, and 0.21 and 0.38 g g^{-1} for BKM19, respectively, suggesting that the BKM19 strain is a superior solvent producer.

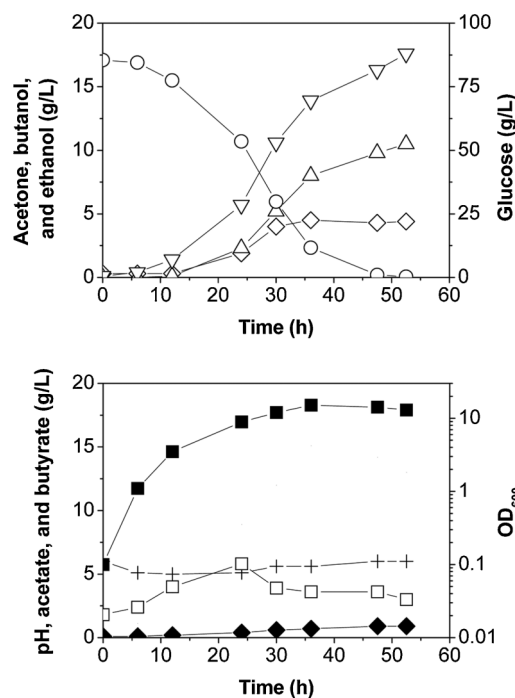


Figure 2. Time profiles of batch fermentation of the *C. acetobutylicum* BKM19 strain. Symbols are: ■, OD_{600} ; ○, glucose; ▽, butanol; Δ, ethanol; ◇, acetone; □, acetate; ♦, butyrate; +, pH.

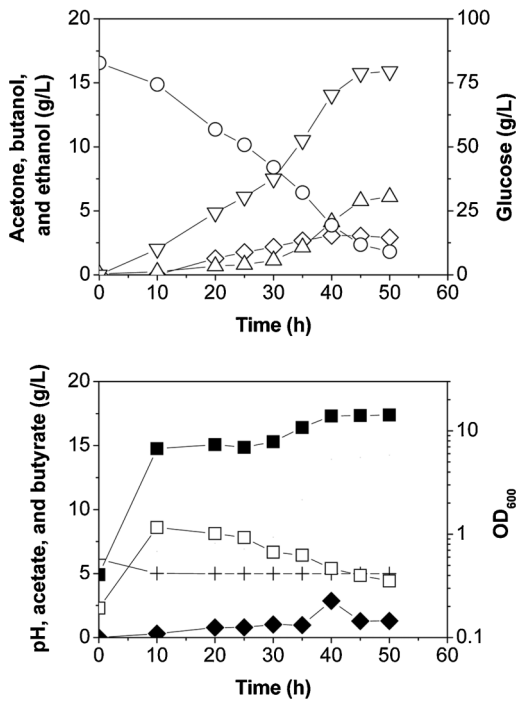


Figure 3. Time profiles of batch fermentation of the *C. acetobutylicum* PJC4BK strain. Symbols are: ■, OD₆₀₀; ○, glucose; ▽, butanol; △, ethanol; ◇, acetone; □, acetate; ◆, butyrate; +, pH.

High Cell Density Continuous ABE Fermentation of BKM19 Using Membrane Cell-Recycle Bioreactor

In order to increase the butanol volumetric productivity, high cell density continuous fermentation of the *C. acetobutylicum* BKM19 strain was performed. Several different dilution rates were examined to find an optimal condition. During the conventional continuous ABE fermentation, the dilution rate could not be increased beyond 0.2–0.3 h⁻¹ due to the sluggish growth of *Clostridium* sp. (Tashiro et al., 2005). Thus, increasing the dilution rate beyond these values normally results in cell wash out. This is why high cell density continuous

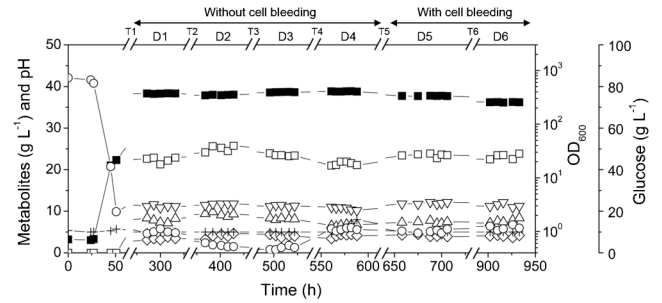


Figure 4. Time profiles of high cell density continuous fermentation of the *C. acetobutylicum* BKM19 strain using an external membrane cell recycling system. Several different dilution rates were first examined to find the best dilution rate, which was followed by the cultivation with cell bleeding at two different bleed rates. D_1 , D_2 , D_3 , and D_4 represents the steady states obtained at the dilution rates of 0.7, 0.6, 0.85, and 0.9 h⁻¹, respectively. D_5 and D_6 represent the steady states obtained at the dilution rate of 0.9 h⁻¹ with cell bleeding at the bleeding rates of 0.04 and 0.08 h⁻¹, respectively. T_1 , T_2 , T_3 , T_4 , T_5 , and T_6 represent the transition states before reaching the steady state for corresponding dilution rate. Symbols are: ■, OD₆₀₀; ○, glucose; □, acetone–butanol–ethanol (ABE); ▽, butanol; △, ethanol; ◇, acetone; +, pH.

fermentation offers the possibility of applying much higher dilution rates.

First, continuous fermentation was started at the dilution rate of 0.7 h⁻¹, which was chosen based on the previous report (Tashiro et al., 2005) as well as our own preliminary studies. At this dilution rate, a cell density (OD₆₀₀) of 370.4 could be achieved. The steady state butanol and ABE concentrations achieved were 11.2 and 22.4 g L⁻¹, respectively (Fig. 4), resulting in the butanol and ABE productivities of 7.9 and 15.7 g L⁻¹ h⁻¹, respectively (Table I). Next, the dilution rate was decreased to 0.6 h⁻¹. At this dilution rate, a cell density (OD₆₀₀) reached 349.6 (Fig. 4). The steady state butanol and ABE concentrations were 11.7 and 25.0 g L⁻¹, respectively, resulting in the butanol and ABE productivities of 7.0 and 15.0 g L⁻¹ h⁻¹, respectively (Table I).

Since the solvent volumetric productivity was decreased by lowering the dilution rate, we examined the effect of increasing the dilution rate on the fermentation

Table I. Effect of dilution rates on butanol and ABE production during high cell density continuous fermentation of the BKM19 strain using membrane cell-recycle bioreactor.

D_t (h ⁻¹)	OD (600 nm)	Glucose consumption (g L ⁻¹)	Concentration (g L ⁻¹)		Yield on glucose (g g ⁻¹)		Volumetric productivity (g L ⁻¹ h ⁻¹)	
			Butanol	ABE	Butanol	ABE	Butanol	ABE
0.60	349.6 ± 4.5	76.4 ± 0.8	11.7 ± 0.1	25.0 ± 0.7	0.15 ± 0.0	0.33 ± 0.0	7.0 ± 0.1	15.0 ± 0.4
0.70	370.4 ± 3.4	69.9 ± 0.8	11.2 ± 0.3	22.4 ± 0.7	0.16 ± 0.0	0.32 ± 0.0	7.9 ± 0.2	15.7 ± 0.5
0.85	394.3 ± 2.9	77.7 ± 0.8	11.3 ± 0.2	23.5 ± 0.3	0.15 ± 0.0	0.30 ± 0.0	9.6 ± 0.2	20.0 ± 0.2
0.90	406.5 ± 2.6	68.6 ± 0.6	10.7 ± 0.3	21.4 ± 0.4	0.16 ± 0.0	0.31 ± 0.0	9.6 ± 0.3	19.3 ± 0.4

± Represents standard deviation.

performance; two higher dilution rates of 0.85 and 0.9 h⁻¹ were examined. Increasing the dilution rate to 0.85 h⁻¹ resulted in dramatic increase of both butanol and ABE volumetric productivities to 9.6 and 20.0 g L⁻¹ h⁻¹, respectively, while their steady state concentrations decreased slightly (Table I). The cell concentration also increased to the OD₆₀₀ of 394.3 (Fig. 4). When the dilution rate was further increased to 0.9 h⁻¹, however, the butanol and ABE volumetric productivities were similar while the cell concentration was slightly higher (the OD₆₀₀ of 406.5; Table I). These results indicate that the solvent volumetric productivities as well as cell concentration are highly influenced by the dilution rate applied, and the high cell density continuous fermentation of the solvent hyper-producing *C. acetobutylicum* BKM19 allows highly efficient production of solvents. In particular, the butanol and ABE volumetric productivities obtained at the dilution rate of 0.85 h⁻¹ (or 0.90 h⁻¹) are the highest reported productivities among the all known processes reported to date. Also, the yields of butanol and ABE were 0.15–0.16 and 0.30–0.33 g g⁻¹, respectively, during the high cell density continuous cultivation, suggesting that relatively high yield solvent production is possible in a continuous fermentation mode.

High Cell Density Continuous ABE Fermentation of BKM19 With Cell Bleeding

During the above experiments carried out at different dilution rates, it was found that cell concentration increased with the increase of dilution rate. However, the high cell concentration made it rather difficult to perform the experiment for a longer period due to intensive bubbling, occasional broth overflow through the condenser and the blockage of hollow fiber membrane column. To solve these problems and to maintain a desired cell concentration during the continuous cell recycle fermentation, cell bleeding was applied (Lee and Chang, 1987). Since the dilution rate of 0.85–0.9 h⁻¹ showed good fermentation performance, the high cell density continuous fermentation with cell bleeding was performed at the dilution rate (D_1) of 0.9 h⁻¹ with two different bleeding rates ($D_2 = 0.04$ and 0.08 h⁻¹).

Initially, the bleeding rate (D_2) was set at 0.04 h⁻¹ while keeping the D_1 at 0.86 h⁻¹ (thus, D_t of 0.9 h⁻¹). Cell bleeding resulted in the decrease of cell concentration to the OD₆₀₀ of

335.0 as compared to the OD₆₀₀ of 406.5 achieved during the continuous fermentation without bleeding (Fig. 4). However, the maximum obtained butanol and ABE productivities were increased to a value of 10.7 and 21.1 g L⁻¹ h⁻¹, respectively (Table II). Next, the bleeding rate (D_2) was increased to 0.08 h⁻¹ while keeping the D_1 at 0.82 h⁻¹ (thus, D_t of 0.9 h⁻¹). Increase of bleeding rate resulted in the further decrease of cell concentration to the OD₆₀₀ of 255.5 (Fig. 4). However, the solvent productivity was not significantly affected by this further increase of bleeding rate; the butanol and ABE productivities achieved were 10.3 and 20.8 g L⁻¹ h⁻¹, respectively (Table II). The yields of butanol and ABE were 0.17 and 0.34 g g⁻¹, respectively, which are slightly higher than those obtained with the high cell density continuous cultivation without cell bleeding. These results suggested that cell bleeding allows further increase of butanol and ABE productivities and yields.

Operational Stability of *C. acetobutylicum* BKM19 During the High Cell Density Continuous Fermentation

It has been well known that ABE fermentation suffers from the problems of strain degeneration and instability. Thus, the performance and operational stability of *C. acetobutylicum* BKM19 during the long-term continuous ABE fermentation were examined. Loss or decrease of solvent production capability was considered as the representation of strain degeneration. Figure 4 shows the fermentation profile of the long-term continuous fermentation of the *C. acetobutylicum* BKM19 strain. High cell density continuous fermentation could be successfully run for 935 h (about 39 days) without strain degeneration. After 935 h, some strain degeneration was seen as exhibited by the decrease of butanol and ABE productivities (data not shown).

Discussion

In order to make the clostridial ABE fermentation process competitive again, it is necessary to develop a cost efficient process employing a superior performing strain. Thus, this study aimed at developing a solvent hyper-producing mutant strain of *C. acetobutylicum* and a continuous fermentation process to increase the productivity. First, random chemical mutagenesis of an engineered *C. acetobutylicum* PJC4BK

Table II. Effect of cell bleeding rates on butanol and ABE production during high cell density continuous fermentation of the BKM19 strain using membrane cell-recycle bioreactor.

D_1 (h ⁻¹)	D_2 (h ⁻¹)	D_t (h ⁻¹)	OD ₆₀₀	Glucose consumption (g L ⁻¹)	Concentration (g L ⁻¹)		Yield on glucose (g g ⁻¹)		Volumetric productivity (g L ⁻¹ h ⁻¹)	
					Butanol	ABE	Butanol	ABE	Butanol	ABE
0.82	0.08	0.90	255.5 ± 2.1	67.9 ± 1.1	11.4 ± 0.5	23.2 ± 0.6	0.17 ± 0.0	0.34 ± 0.0	10.3 ± 0.4	20.8 ± 0.6
0.86	0.04	0.90	335.0 ± 2.2	69.7 ± 0.8	11.9 ± 0.2	23.5 ± 0.4	0.17 ± 0.0	0.34 ± 0.0	10.7 ± 0.2	21.1 ± 0.3

± Represents standard deviation.

Table III. A comparison of ABE productivities achieved so far by using cell recycling.

Strategy	Microorganism used	D_1 (h^{-1})	D_2 (h^{-1})	ABE concentration (g L^{-1})	Volumetric ABE productivity ($\text{g L}^{-1} \text{h}^{-1}$)	Refs.
Cell recycling	<i>C. acetobutylicum</i> BKM19	0.85	—	23.5 ± 0.3	20.0 ± 0.2	This study
Cell recycling with bleeding	<i>C. acetobutylicum</i> BKM19	0.86	0.04	23.5 ± 0.4	21.1 ± 0.3	This study
Cell recycling	<i>C. saccharoperbutylactonicum</i> N1-4	0.85	—	12.9	11.0	Tashiro et al. (2005)
Cell recycling with bleeding	<i>C. saccharoperbutylactonicum</i> N1-4	0.76	0.09	11.5	9.77	Tashiro et al. (2005)
Cell recycling with bleeding	<i>C. acetobutylicum</i> P262	0.39	0.02	9.9	4.06	Ennis and Maddox (1985)
Cell recycling	<i>C. acetobutylicum</i>	0.33	—	9.3–20.5	4.34	Ferras et al. (1986)
Cell recycling with bleeding	<i>C. acetobutylicum</i> ATCC 824	0.435	0.065	13.0	6.50	Pierrot et al. (1986)
Cell recycling	<i>C. acetobutylicum</i>	0.40	—	10.3	4.10	Schlote and Gottschalk (1986)
Cell recycling	<i>C. acetobutylicum</i>	0.64	—	8.44	5.40	Afschar et al. (1985)

± Represents standard deviation.

strain, which already shows improved solvent formation capability, was carried out. Random mutagenesis has recently been used to develop superior solvent producing strains (Connor et al., 2010; Malaviya et al., 2012). Screening was performed on a selection medium containing 2 mg L^{-1} fluoroacetate, as it has previously been used to isolate variants that lost acetate production capability (Rothstein, 1986). Once inside the cell, fluoroacetate is converted to fluoroacetyl-CoA in a reversible reaction catalyzed by phosphotransacetylase (Pta) and acetate kinase (Ack) in some microbes, such as *Aspergillus niger* (Sealy-Lewis and Fairhurst, 1998), *Azotobacter vinelandii* (McKenney and Melton, 1986), and *Clostridium thermosaccharolyticum* (Rothstein, 1986). In the next step, fluoroacetate (or fluoroacetyl-CoA) condenses with oxaloacetate to form fluorocitrate, which causes cell death by irreversibly binding to aconitase. Thus, it was expected that fluoroacetate resistant variants of *C. acetobutylicum* would have mutations in genes encoding CoA-transferase to reduce the reaction leading to the formation of fluoroacetyl-CoA. Another possibility for the cells to survive under the fluoroacetate condition is the increased carbon flow to solvent production from acetyl-CoA to reduce the formation of fluorocitrate. Thus, mutants selected on fluoroacetate-containing medium would have improved capabilities for solvent production from acetyl-CoA. Among the mutants, a hyper ABE producing strain BKM19 could be isolated; it was able to produce 36.7% more ABE compared with the parental PJC4BK strain with a high yield. However, sequencing of the *ctfAB* genes encoding CoA-transferase of the BKM19 strain revealed that there was no mutation in the *ctfAB* genes. Thus, the tolerance to fluoroacetate seems to be due to the mutation(s) in other genes or non-coding DNA, which can be identified by whole genome sequencing.

The cell concentrations obtainable during the typical ABE fermentation by *Clostridium* sp. remains very low, which consequently results in rather low solvent volumetric productivity (Tashiro et al., 2005). To address this problem, we performed for the first time high cell density continuous

fermentation of *C. acetobutylicum* using a cell recycling bioreactor. It could be demonstrated that the BKM19 strain could be used for the long-term continuous ABE fermentation with high solvent productivity. At the optimal dilution and bleed rates, the high cell density continuous fermentation resulted in the butanol and ABE productivities of 10.7 and $21.1 \text{ g L}^{-1} \text{h}^{-1}$, respectively. Comparison of solvent productivities by using cell recycling bioreactors reported to date has been presented in Table III. The maximum ABE volumetric productivity obtained in this study is 1.9-fold higher than the previously highest one ($11.0 \text{ g L}^{-1} \text{h}^{-1}$) obtained by cell recycling fermentation of *C. saccharoperbutylacetonicum* N1-4 (Tashiro et al., 2005). Furthermore, the productivity of $21.1 \text{ g L}^{-1} \text{h}^{-1}$ obtained in this study is 1.3-fold higher than the highest productivity reported to date ($16.2 \text{ g L}^{-1} \text{h}^{-1}$) achieved by biofilm reactor fermentation of *C. beijerinckii* BA101 (Lienhardt et al., 2002).

One of the frequently reported problems of continuous ABE fermentation is strain degeneration (Ferras et al., 1986). The *C. acetobutylicum* BKM19 strain developed in this study showed stable solvent productivity without strain degeneration during the high cell density continuous fermentation for more than a month (935 h; about 39 days).

In conclusion, we have isolated the *C. acetobutylicum* BKM19 strain by random mutagenesis and performed high cell density continuous fermentation by optimizing the dilution and bleeding rates. The long-term stable fermentation with the highest butanol and ABE productivities suggests that the *C. acetobutylicum* BKM19 strain will allow cost effective production of solvents. As demonstrated recently (Jang et al., 2012b), further improvement in the yield and productivity of solvents can be achieved by integrating the in situ product recovery system such as gas stripping.

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