High-rate continuous n -butanol production by Clostridium acetobutylicum from glucose and butyric acid in a single-pass fibrous bed bioreactor

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Abstract

Biobutanol produced in acetone-butanol-ethanol fermentation at batch mode cannot compete with chemically derived butanol because of the low reactor productivity. Continuous fermentation can dramatically enhance productivity and lower capital and operating costs but are rarely used in industrial fermentation because of increased risks in culture degeneration, cell washout, and contamination. In this study, cells of the asporogenous *Clostridium acetobutylicum* ATCC55025 were immobilized in a single-pass fibrous-bed bioreactor (FBB) for continuous production of butanol from glucose and butyrate at various dilution rates. Butyric acid in the feed medium helped maintaining cells in the solventogenic phase for stable continuous butanol production. At the dilution rate of 1.88 h^{-1}, butanol was produced at 9.55 g/L with a yield of 0.24 g/g and productivity of 16.8 g/L[?]h, which was the highest ever achieved for biobutanol fermentation and an 80-fold improvement over the conventional ABE fermentation. The extremely high productivity was attributed to the high density of viable cells (˜100 g/L at >70% viability) immobilized in the fibrous matrix, which also enabled the cells to better tolerate butanol and butyric acid. The FBB was stable for continuous operation for an extended period of over one month.

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butanol production. At the dilution rate of 1.88 h^{-1}, butanol was produced at 9.55 g/L with a yield of 0.24 g/g and productivity of 16.8 g/L[?]h, which was the highest ever achieved for biobutanol fermentation and an 80-fold improvement over the conventional ABE fermentation. The extremely high productivity was attributed to the high density of viable cells (˜100 g/L at >70% viability) immobilized in the fibrous matrix, which also enabled the cells to better tolerate butanol and butyric acid. The FBB was stable for continuous operation for an extended period of over one month.

Keywords : Acetone-butanol-ethanol fermentation; continuous fermentation; *Clostridium acetobutylicum* ; fibrous bed bioreactor

1. Introduction

Biobutanol with a 30% higher energy content and lower water miscibility, volatility, flammability, and corrosiveness than ethanol is an attractive drop-in biofuel that can fit with the existing fuel infrastructure and be used in car engines without modification (Zhao et al., 2013). *n* -Butanol can also be dehydrated to 1-butene and further converted to longer-chain aviation fuels. As one of the oldest industrial fermentations (Jones and Woods, 1986; Moon et al., 2016; Soni et al. 1987), acetone-butanol-ethanol (ABE) fermentation by solventogenic clostridia including *Clostridium acetobutylicum* and*Clostridium beijerinckii* has been extensively studied in various process modes, including batch, fed-batch, and continuous (Ezeji et al., 2004; Huang et al., 2004; Jiang et al., 2014; Lu et al., 2012; Qureshi et al., 2008). However, commercial application of ABE fermentation for*n* -butanol and acetone production is hindered by the high production cost (Kumar et al., 2013; Lu et al., 2013; Li et al., 2019). Even after extensive efforts to develop engineered strains and novel process strategies, batch ABE fermentation suffers from low productivity and is unable to compete with solvents produced through petrochemical routes (Cheng et al., 2019; Lee SY et al., 2008; Wang et al., 2014; Xue et al., 2017; Zhao et al., 2013).

A typical batch ABE fermentation usually completes in ˜72 h with a productivity of ˜0.2 g/L[?]h and a final butanol concentration of ˜12 g/L and yield of ˜0.2 g/g glucose (Xu et al., 2015). Compared to batch fermentation, continuous fermentation offers several advantages including higher productivity and little downtime. For ABE fermentation in a bioreactor with continuous supply of nutrients at a high dilution rate, solvent productivity was improved by over 10-fold to >2 g/L[?]h; however, the final solvent titer decreased to ˜5 g/L, which would drastically increase downstream processing costs (Pierrot et al., 1986; Qureshi and Maddox, 1991). In order to enhance butanol productivity, various techniques, including cell immobilization and *in situ* butanol recovery, have been incorporated into ABE fermentation to increase cell density, alleviate butanol toxicity, and increase overall productivity (Cai et al., 2016; Lu et al., 2012; Nguyen et al., 2018; Xue et al., 2016a; 2016b). Cell immobilization on solid support materials, such as brick, bonechar, chitosan, and corn stalk, increased cell density in fermentation and improved ABE productivity to as high as ˜10 g/L[?]h (Frick and Schugerl, 1986; Qureshi et al., 1988; Zhang et al. 2009). Notably, cell immobilization in a highly porous fibrous matrix not only greatly increased cell density in the bioreactor but also facilitated the adaptation of cells to better tolerate environmental stress with over 50% improvements in product titer, yield, and productivity in fermentations for organic acids (Suwannakham and Yang, 2005; Wei et al., 2013; Yang et al., 1994; Zhu et al., 2002; Zhu and Yang, 2003) as well as butanol production (Huang et al., 1998; Huang et al., 2019; Jiang et al., 2014; Li et al., 2019).

However, ABE fermentation is difficult to operate and control in a continuous fermentation process (Al-Shorgani et al., 2019) because of its complex life cycle involving acidogenesis, solventogenesis, and sporulation that are highly regulated by multiple gene regulators involving various kinases, transcription factors, and interlocking signal transduction pathways (Al-Hinai et al., 2015; Steiner et al., 2011; Yang et al., 2018). Acid crash (due to failed transition from acidogenesis to solventogenesis), sporulation (induced by environmental stresses such as butanol toxicity), and strain degeneration (lost solvent production due to cells losing the mega-plasmid carrying the*sol* operon) are among the major causes for low productivity and short production duration associated with *C. acetobutylicum* (Long and Jones, 1984; Lütke-Eversloh and Bahl, 2011). Under stress, clostridia sporulate and halt their metabolism (Diallo et al., 2021), limiting their ability to produce butanol at desirable titers, rates, and yields and longevity for continuous operation.

In this study, the asporogenous *C. acetobutylicum* ATCC55025 derived from the Weizmann strain ATCC4259 was used for continuous*n* -butanol production from glucose and butyrate in a single-pass fibrous bed bioreactor (FBB). *C. acetobutylicum* ATCC 55025 is deficient in forming endospores and has a high butyrate uptake rate and butanol productivity (Xu et al., 2017). With butyric acid in the feed medium and cells immobilized in the FBB, continuous ABE fermentation can be stably maintained in the solventogenic phase at a high rate with >16.5 g/L[?]h butanol productivity, which was the highest productivity ever reported for a biobutanol fermentation process. The effects of feed butyrate concentration and dilution rate on the single-pass continuous bioreactor were studied and the results are reported in this paper.

2. Materials and Methods

2.1 Cultures and Media

C. acetobutylicum ATCC55025 was cultured in modified P2 medium containing (per liter, at pH 5.5): 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 1 g yeast extract, 0.5 g tryptone, 0.2 g $MgSO_4$[?]$7H_2O$, 0.01 g $MnSO_4$[?]H_2O, 0.01 g NaCl, and 0.01 g $FeSO_4$[?]$7H_2O$, 0.1 mg *p* -aminobenzoic acid, 0.1 mg thiamine, 1 μg biotin, and 50 g glucose as the carbon source, unless otherwise noted (Xu et al., 2015). Concentrated mineral ($MgSO_4$[?]$7H_2O$, $MnSO_4$[?]H_2O, NaCl, $FeSO_4$[?]$7H_2O$) and vitamin (*p* -aminobenzoic acid, thiamine, biotin) solutions were prepared separately, filter-sterilized with 0.2 μm pore size filter and stored in a refrigerator until use. The medium without glucose was sterilized by autoclaving at 121 ^{o}C for 30 minutes. The main carbon source, glucose, was sterilized separately in a concentrated solution and added aseptically to the medium to a final concentration of ˜50 g/L or as specified. Butyric acid at various amounts (up to 10 g/L) was also added to the medium to study its effects on cell growth and fermentation kinetics. The medium was adjusted to pH 5.5 with NH_4OH and purged with sterile N_2 for ˜30 min to reach anaerobiosis before use.

2.2 Batch fermentation

Batch fermentation in serum bottles and bioreactors was conducted to evaluate the effect of butyric acid on the growth of *C. acetobutylicum* ATCC55025. Each bottle containing 100 ml of P2 medium with 20 g/L glucose and various amounts of butyric acid at an initial concentration of 0, 2, 5, or 10 g/L was inoculated with 3 mL of 24-h active culture and incubated at 37 °C without shaking. Liquid samples were withdrawn from the bottles periodically to monitor changes in the cell density by measuring the optical density at 600 nm (OD_{600}). Batch fermentation was also conducted in a 500-mL stirred-tank bioreactor containing 250 mL P2 medium with ˜50 g/L glucose and 4 g/L butyric acid as the carbon sources. The reactor pH was controlled at ˜5.0 with ammonium hydroxide (20% w/v). Liquid samples were withdrawn periodically for analyses of cell (optical density, OD_{600}), glucose, butyric and acetic acids, and solvents (acetone, butanol, and ethanol).

2.3 Continuous Fermentation

Continuous fermentation was conducted with cells immobilized in a fibrous bed bioreactor, which was made of a water-jacketed glass column. The glass column with a working volume of 500 mL was packed with a spirally wound cotton towel laminated with a stainless-steel wire mesh as spacers between adjacent fibrous matrices (**Figure 1**). The FBB was autoclaved at 121 °C for 30 min, left overnight at room temperature to allow spore germination, and autoclaved again for one hour. The FBB was purged with N_2 from the bottom of the bioreactor for ˜1 h to remove air and then aseptically connected to a 10-L feed medium bottle containing sterile P2 medium. The bioreactor was then partially filled with the medium while continued being sparged with N_2 for 10 min and inoculated with 60˜100 mL of an overnight culture grown in a serum bottle. The FBB was controlled at 35 °C and held static for 12˜24 h to allow cells to grow and attach/adsorb onto the fibrous bed. When significant gas production (bubbles) was observed and the fermentation broth had reached ˜1.0 OD_{600}, continuous fermentation was started by pumping the feed medium containing 50 g/L glucose and 4 g/L butyric acid into the bottom of the FBB at a constant dilution rate of 0.06 h^{-1}. The continuous fermentation study was operated for over 28 days or until reaching a pseudo-steady state. The continuous fermentation was then studied in the same FBB fed with P2 medium containing 60 g/L glucose and 6.8 g/L butyric acid at 0.12 h^{-1} dilution rate. A smaller FBB with a 125-mL working volume was also

set up for studying the continuous fermentation at a higher dilution rate of 1.88 h^{-1}. Fermentation broth samples were taken periodically from the effluent stream throughout the continuous fermentation studies and analyzed for $OD_{600}$and concentrations of glucose, butyric and acetic acids, and solvents (acetone, butanol, and ethanol). At the end of the continuous fermentation studies, the fibrous matrix with immobilized cells was removed from the FBB and examined for cell density, viability, and morphology.

2.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to study cells immobilized on the fibrous matrix. Small pieces of fibrous matrices with cells cut from various locations in the FBB were examined at 20 kV with FEI Quanta 200 SEM. Samples were fixed in 2.5% glutaraldehyde solution at 4 °C overnight, dehydrated with ethanol progressively from 20% to 100% at 20% increment for 30 min at each step, dried with hexamethyldisilazane (HMDS), and spotter-coated with gold particles under argon gas. The samples were scanned, photographed, and analyzed visually for cell morphology.

2.5 Cell Viability Assay

The modified method of Glenner (Glenner, 1977) was used. Briefly, cells were collected from 1 mL culture broth by centrifugation (16,000 rpm for 10 min) and resuspended in 1 mL 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) solution (1 g/L) by vigorously vortexing. After incubation in dark at room temperature for 30 min, cells were collected by centrifugation and mixed with 1 mL methanol to extract the pink pigment. The color intensity in the supernatant was measured at 485 nm using a spectrophotometer (UV-16-1, Shimazu, Columbia, MD). The viability of tested cells was determined using the exponential-phase cells as the control with 100% viability and boiled (dead) cells as the negative control with 0% viability.

2.6 Analytical Methods

The optical density at 600 nm (OD_{600}) was measured in a 1.5-mL cuvette (1 cm light path length) with a spectrophotometer (UV-16-1, Shimazu). One unit of OD_{600} was equivalent to 0.534 g/L cell dry weight. Glucose in the fermentation broth samples was determined with a YSI 2300 glucose analyzer (Yellow Spring, OH). All metabolites including acetone, butanol, ethanol, acetic acid, and butyric acid in the fermentation broth samples were analyzed, after removing cells by centrifugation or microfiltration using 0.2-μm syringe filters and acidified with phosphoric acid (1% v/v), with a gas chromatograph (GC-2014, Shimadzu) following the method described elsewhere (Xu et al., 2015).

3. Results and Discussion

3.1 Batch Fermentation Kinetics

Butyric acid is a strong growth inhibitor and plays a critical role in regulating cell metabolism in ABE fermentation, which switches from acidogenesis to solventogenesis when the butyric acid concentration reaches a critical level in the culture medium (Bahl et al., 1982). Previous studies have shown that adding a small amount of butyric acid in the culture medium could induce early solventogenesis and increase butanol production (Huang et al., 2004; Lee SM et al., 2008). The effect of butyric acid on cell growth was first investigated in serum bottles and the results are shown in **Figure 2** . As expected, cell growth was strongly inhibited by butyric acid and no growth was observed at 10 g/L of butyric acid. Then, batch fermentation kinetics with medium initially containing 4 g/L butyric acid in a stirred-tank bioreactor with pH controlled at ˜5.0 was investigated. After a 12-h lag phase, cells grew to reach the maximum OD of ˜2.0 at 34 h and then decreased gradually, whereas both butyric acid consumption and solvent production started when cell growth ceased at 34 h (**Figure 3**). About 13.1 g/L butanol, 5.6 g/L acetone, and 0.9 g/L ethanol were produced from ˜50 g/L glucose and ˜2 g/L butyric acid consumed in the fermentation ended at ˜311 h, which was much longer than the typical ˜72 h for batch ABE fermentation with glucose but without butyric acid addition in the medium (**Figure S1**). The butanol yield of ˜0.26 g/g glucose obtained in the fermentation with 4 g/L butyric acid was significantly higher than that obtained in typical ABE fermentation (˜0.20 g/g glucose), whereas the productivity was much lower at ˜0.05 g/L[?]h (vs. ˜0.2 g/L[?]h) due to the strong inhibition by butyric acid resulting in a much lower cell density of <2.0 OD (vs. Max. OD of ˜14 reached

in batch fermentation without butyric acid added in the culture medium). The results indicated that the addition of butyric acid could maintain stable butanol production in ABE fermentation, but the optimal butyric acid concentration must be explored in order to minimize its inhibition effect on cell growth and reactor productivity.

3.2 Continuous Fermentation

Continuous ABE fermentation with cells immobilized in a FBB was studied at different dilution rates and butyric acid concentrations in the feed medium. **Figure 4** shows the time-course data on OD, and glucose, butyrate, acetate, and ABE concentrations in the effluent from the single-pass FBB fed with the medium containing 50 g/L glucose and 4 g/L butyric acid at the dilution rate of 0.06 h^{-1}. Cell OD and butanol production increased rapidly and reached ˜3.0 and ˜5 g/L, respectively, at 181 h but then decreased. The bioreactor was drained and refilled aseptically with a fresh medium at 290 h, which restored and increased butanol production to ˜8.4 g/L at 490 h. The continuous fermentation maintained a pseudo-steady state for the next 160 h, with butanol production fluctuated between 8.4 and 9.4 g/L. During this period, about 25% of the butyric acid (1 g/L) in the feed medium was consumed and the average butanol yield and productivity were ˜0.24 g/g and ˜0.52 g/L[?]h, respectively. There was continuous cell bleeding from the FBB throughout the continuous fermentation, which remained at ˜OD 1.0 during the pseudo-steady state. To investigate if more butyrate could be consumed by cells, the feed butyrate concentration was raised to 8 g/L at 700 h. Soon after increasing the feed butyric acid concentration to 8 g/L, butanol concentration in the outlet stream started to drop rapidly to less than 1 g/L and both glucose and butyric acid concentrations approached the feed concentrations at 890 h (see **Figure S2**), indicating cells were completely inhibited by butyric acid at 8 g/L.

The continuous fermentation was then studied at a higher dilution rate of 0.12 h^{-1}, with the feed medium containing 60 g/L glucose and 6.8 g/L butyric acid. As shown in **Figure 5** , butanol production increased to ˜10 g/L while glucose concentration decreased to ˜20 g/L and butyric acid decreased to ˜2.6 g/L when the fermentation reached a pseudo-steady state at ˜480 h. Afterward, the continuous fermentation produced butanol at the average yield of ˜0.24 g/g and productivity of ˜1.2 g/L[?]h, which was 2.3-fold of that obtained at the lower dilution rate of 0.06 h^{-1}. It should be noted that butanol production dropped from 9.1 g/L at 340 h to 7.3 g/L at 390 h, probably because cells were strongly inhibited by butanol at >8 g/L. However, the reactor was able to recover by itself and continue to increase butanol production to reach ˜10.5 g/L at the pseudo-steady state. The ability of the self-recovery from butanol stress could be attributed to the adaptation of cells in the FBB, which showed the resilience of the immobilized cell bioreactor. The effluent cell OD reached the maximum value of ˜2.6 at 170 h and then decreased to ˜0.8 during the pseudo-steady state. To evaluate the reproducibility of the FBB performance, the reactor was drained and refilled with fresh media at ˜610 h. The effluent butanol concentration reached ˜10.5 g/L in 200 h and stayed in a pseudo-steady state for the next 200 h with average butanol production of 10.8 g/L (see **Figure S3**), similar to that obtained in the previous pseudo-state state.

In general, the reactor productivity would increase with increasing the dilution rate in the continuous fermentation. To explore the optimal dilution rate for maximum productivity, the continuous fermentation was further studied in a smaller FBB with a 150-mL working volume at a high dilution rate of 1.88 h^{-1}. The feed medium contained 60 g/L glucose and 5 g/L butyric acid. Soon after the feeding started, the outlet butanol concentration and cell OD rapidly increased and reached 11.6 g/L and 6.47, respectively, while glucose dropped to 2.5 g/L in ˜140 h (**Figure 6**). Then butanol concentration dropped to less than 8 g/L with glucose back up to >20 g/L in the next 20 h. The sudden drop in butanol production could be attributed to butanol toxicity as well as glucose starvation. An excessive amount or a high concentration of sugar is essential for both onset and maintenance of solventogenesis in clostridial fermentation (Jones and Woods, 1986). When the glucose was back up to ˜20 g/L, butanol production was restored to ˜9.5 g/L and then fluctuated between 8.5 and 10.5 g/L for the next 200 h in the pseudo-steady state. The fluctuation in butanol production could be attributed to the response of cells to butanol stress. At the high dilution rate, butanol production in the FBB reached ˜9.5 g/L at a high productivity of ˜16.8 g/L[?]h and with an average

yield of 0.24 g/g glucose. About 3 g/L or 60% of the butyric acid in the feed medium was co-metabolized with glucose in the fermentation. The effluent cell OD reached the maximum value of 6.47 at ˜140 h and then decreased to ˜3.0 by ˜200 h and stayed at that level during the pseudo-steady state period (200 – 380 h).

Table 1 summarizes and compares the results from batch and continuous fermentations at different dilution rates and feed butyric acid concentrations. In general, butanol yields were ˜0.24 g/g in the continuous fermentation at all dilution rates studied while the reactor productivity increased proportionally with the dilution rate from 0.52 g/L[?]h at 0.06 $h^{-1}$to 16.8 g/L[?]h at 1.88 h^{-1}, which was much higher than the maximum specific growth rate (0.2˜0.45 h^{-1}) observed for *C. acetobutylicum* . Cells immobilization allowed the continuous fermentation to operate at a dilution rate much higher than the specific growth rate without cell washout. Cells had a higher activity and faster growth at the higher dilution rate, as evidenced by the higher max. OD of 6.47 at 1.88 h^{-1} (vs. 3.0 at 0.06 h^{-1}) and steady-state OD in the effluent (3.0 at 1.88 h^{-1} vs. 1.0 at 0.06 h^{-1}). The continuous immobilized-cell bioreactor is thus advantageous to operate at a high dilution rate although the final butanol titer and yield were slightly lower than those in batch fermentation.

3.3 Effects of Butyric Acid

In a typical ABE fermentation, acetic and butyric acids produced in acidogenesis were reassimilated via the CoA transferase, which transfers the CoA from acetoacetyl-CoA to acetate and butyrate to form acetyl-CoA and butyryl-CoA, respectively, with acetone, ethanol, and butanol as the final products (Long and Jones, 1984; Zhao et al., 2013). Previous studies have shown that overexpressing *ctf* AB encoding the CoA transferase could increase butanol production and the robustness of solventogenic clostridia (Lu et al., 2017; Yu et al., 2015) and butyric acid could upregulate *ctf* AB (Bahl et al., 1982; Lee SM et al., 2008). In this study, butyric acid was thus supplemented in the feed medium to promote solventogenesis in clostridial fermentation, resulting in ˜20% higher butanol yield (0. 24 g/g vs. ˜0.20 g/g) compared to without butyric acid addition. The increased butanol yield could also be attributed to reduced cell growth and acetone and ethanol production, which resulted in a much higher ratio of butanol present in the total solvents (˜68% vs. ˜60% w/w in typical ABE fermentation with C. *acetobutylicum* (Xu et al., 2015). Similar results were also reported for *C. beijerinckii* (Lee SM et al., 2008). The addition of butyric acid as a carbon source and precursor for butanol biosynthesis inhibited the conversion of butyryl-CoA to butyric acid and thus increased carbon flow toward butanol production. However, butyric acid at >5 g/L strongly inhibited cell growth and reduced cell viability and productivity in free cell fermentation (see Figs. 2 and 3). Nevertheless, cells immobilized in the FBB were resilient to butyric acid toxicity and could tolerate butyric acid at ˜5 g/L, although butyric acid at 8 g/L completely halted cell metabolism in the FBB (Fig. S2).

3.4 Cell Density, Viability, and Morphology in FBB

Cells in the fibrous matrices were collected from the FBB at the end of the continuous fermentation study and analyzed for their density, viability, and morphology. In general, more cells were observed at the lower parts closer to the entrance of the reactor with higher glucose and lower butanol concentrations since cell metabolism would be faster with higher concentrations of nutrients and lower concentrations of inhibiting end products. Biofilms with dense and thick layers of elongated rod-shaped cells covering the fibers in the fibrous matrices were observed throughout the fibrous bed, as can be seen in the scanning electron micrographs (**Figure 7**). The total cell density in the FBB was approx. 100 ± 15 g cell dry weight/L reactor with >70% cell viability. No obvious sporulation nor autolysis was observed even for cells from the top part of the bioreactor, suggesting that cells in the FBB remained healthy and were highly active throughout the entire process over a period of more than 400˜600 h. It should be noted that there were continuous cell bleedings (˜1.0 OD at 0.06 $h^{-1}$and 0.12 h^{-1} and ˜3.0 OD at 1.88 h^{-1}) from the FBB throughout the continuous fermentation, which allowed for continuous removal of old or stressed cells to be replaced by new and more productive cells (Lewis and Yang, 1992). Furthermore, cells with the elongated morphology in the FBB were adapted to better tolerate toxic metabolites such as butyric acid and butanol. The elongated rod morphology with a higher specific surface area could increase mass transfer and efflux for exchanges of nutrients and metabolites between cells and the environment as previously reported (Suwannakham and

Yang, 2005; Zhu and Yang, 2003). The beneficial effects of cell immobilization in the FBB including enhanced tolerance to toxic metabolic end products and increased product titer, productivity and yield have also been reported for other clostridia such as *C. tyrobutyricum* (Jiang et al., 2011) and *C. formicoaceticum* (Huang et al., 1998) and other bacteria such as propionibacteria (Zhang and Yang, 2009).

3.5 Comparison to Other Studies

Table 2 summarizes and compare the solvent titer, productivity, and yield of ABE fermentation obtained in this study and other notable continuous fermentation studies. In general, continuous fermentation systems can dramatically enhance productivity and lower capital costs by reducing the size of the fermentation system compared to batch fermentation (Vees et al., 2020). Continuous systems can experience little to no downtime between batches and have other processing advantages leading to increased productivity. However, continuous fermentation has rarely been used in industry because of increased risks in culture degeneration, cell washout and contamination during operation for an extended period that may result in catastrophic production lost. Also, the high productivity obtained at a high dilution rate is usually at the expense of incomplete or low substrate conversion. To overcome these problems, continuous fermentation with cell recycling and/or retention via immobilization in the bioreactor has been used to attain a high cell density of ˜100 g DCW/L reactor volume with greatly increased reactor productivity of >2 g/L[?]h (Huang et al., 2004; Jang et al., 2013). Various solid support materials have been applied for cell immobilization via adsorption and entrapment (Badr et al., 2001; Bankar et al., 2012; Chang et al., 2016; Davison and Thompson 1993; Frick and Schugerl, 1986; Gallazzi et al., 2015; Huang et al., 2004; Kong et al., 2015; Qureshi and Maddox, 1988; Qureshi et al., 2000; Survase et al., 2012; Zhang et al. 2009). However, immobilized cell bioreactors such as packed-bed and membrane bioreactors often suffer from lost/declined productivity during prolonged operation due to the accumulation of dead, aging or non-viable cells, which also causes clogging/fouling and limits reactor's operating life to less than a few weeks (Qureshi and Maddox, 1988; Qureshi et al., 2000; Zhang et al., 2009). For continuous fermentation with cell recycling through microfiltration, cell bleeding is necessary to avoid over accumulation of dead and inactive cells and to prolong the reactor life (Tashiro et al., 2005).

In the present study, the ABE productivity of 24.2 g/L[?]h obtained in the single-pass FBB with ATCC55025 at the dilution rate of 1.88 h^{-1} was the highest ever reported to date. In general, a higher productivity can be obtained with a higher dilution rate in a continuous fermentation process. However, a higher dilution rate usually results in lower substrate conversion and butanol production, which will increase production cost (Huang et al., 2019). Furthermore, a high dilution rate usually favors cell growth and acid production in ABE fermentation. To maintain a high productivity while also achieve a high conversion with high butanol yield, continuous fermentation with stirred tank reactors (STR) or recirculating packed bed reactors (PBR) may have to be operated with multiple stages (Badr et al., 2001; Chang et al., 2016; Frick and Schugerl, 1986), which increases the capital cost. In this study, butyric acid was used in the feed medium as a co-substrate with glucose to keep cells in the single-pass FBB in the solventogenesis phase. It has also been reported that adding butyric acid in the feed medium could improve butanol production in a continuous STR (Lee SM et al., 2008).

A PBR with solid support particles like brick (Qureshi et al., 2000) and ceramic beads (Badr et al., 2001) suffered from low void volume, high pressure drop, and clogging and channeling due to the accumulation of cell biomass, which impeded the reactor performance and operating life. In this study, cells were immobilized in the highly porous fibrous matrix spirally wound with gaps between the matrix layers as flow channels to allow for free flow of fermentation broth, suspended solids (cells), and gases (CO_2 and H_2) through the fibrous bed with a low pressure drop without clogging occurring to conventional packed-bed bioreactors (Zhu et al., 2002). Consequently, the FBB could have stable performance throughout the entire operation period of over several months as demonstrated in our previous studies (Lewis and Yang, 1992). Moreover, the FBB with greatly increased cell density also facilitated in-process adaptation or evolutionary engineering of cells to attain higher tolerance to toxic chemicals (e.g., organic acids and butanol) and increase product titer, yield, and productivity as demonstrated in previous studies (Huang et al., 1998; Li et al., 2019;

Suwannakham and Yang, 2005; Wei et al., 2013; Yang et al., 1994; Zhu and Yang, 2003). Since butyric acid and butanol are highly inhibitory to most microorganisms, no contamination was found throughout the continuous fermentation study. The continuous FBB was operated for over 30 days without encountering any performance issues.

The continuous fermentation process can be operated with *in situ*product separation to alleviate butanol toxicity and increase final product titer and reactor productivity (Veza et al., 2021; Yang and Lu, 2013). Gas stripping (Lu et al., 2012; Xue et al., 2016b), adsorption (Xue et al., 2016a), extraction (Bankar et al., 2012; Davison and Thompson 1993), and pervaporation (Cai et al., 2016; Zhu et al., 2018) are the most studied *in situ* butanol separation methods. More recently, vacuum distillation was applied to continuously recover ABE from the fermentation broth in a separate tank, achieving a high final butanol titer of 550 g/L and productivity of 14 g/L[?]h in a continuous ABE fermentation with cell recycling operated at a dilute rate of 0.076 h^{-1} (Nguyen et al., 2018). The process maintained a steady state for ˜170 h. The continuous single-pass FBB can be integrated with gas stripping and pervaporation (or vapor stripping-vapor permeation, VSVP) to further increase productivity and product titer to higher than 600 g/L (Du et al., 2021; Lu et al., 2012).

4. Conclusions

Continuous fermentation with the asporogenous *C. acetobutylicum*ATCC55025 immobilized in a fibrous bed bioreactor was studied for*n* -butanol production from glucose and butyric acid. At a high dilution rate of 1.88 h^{-1}, butanol production at a high productivity of 16.8 g/L[?]h, more than 80 times of that in batch free-cell fermentation, was obtained. Although butyric acid strongly inhibited cell growth, butyric acid added in the feed medium helped maintain stable butanol production and improved butanol yield from glucose by ˜20%. The risk of contamination by commensal bacteria was reduced by butyric acid and butanol, allowing the continuous process to operate for an extended period over 30 days.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version. All other relevant data that support the findings are available from the corresponding author upon request.

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Author Contribution Statement : Chang and Yang contributed to the initial conceptual and experimental design. Chang performed all experiments and data analysis, and wrote the initial draft of the manuscript, which was reviewed with inputs from Xu and Hou. Yang revised and prepared the final version of the manuscript, which was read and approved by all authors.

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Figure Captions

Figure 1 . Experimental setup for the continuous fermentation with clostridial cells immobilized in a fibrous bed bioreactor (FBB). Inset diagram shows the construction of the spirally wound fibrous matrices with stainless steel wire mesh as the mechanical support with gaps between adjacent layers as flow channels for liquid, gas, and solids (cells) to move freely. The highly porous fibrous matrix has a large surface area and void volume (>90% reactor volume) to allow high densities of viable cells (up to 100 g/L cell dry weight) to be immobilized by attachment to fiber surfaces and entrapment within the matrix.

Figure 2 . Effect of butyric acid on cell growth. Cells of*C. acetobutylicum* ATCC 55025 were cultured in P2 medium containing 20 g/L glucose and various amounts of butyric acid (0, 2, 5, or 10 g/L) in serum bottles at 37 °C.

Figure 3 . Kinetics of batch fermentation with free cells cultured in medium containing ˜50 g/L glucose and 4 g/L butyric acid as carbon sources in a stirred-tank bioreactor at 35°C and pH controlled at ˜5.0.

Figure 4 . Kinetics of continuous fermentation with 50 g/L glucose and 4 g/L butyric acid at the dilution rate of 0.06 h^{-1}. (a) Time course data of cell density (OD), glucose, solvents (acetone, butanol, and ethanol), and acids (acetic acid and butyric acid) in the reactor outlet stream; (b) Butanol and ABE yields and butanol productivity.

Figure 5 . Kinetics of continuous fermentation with 60 g/L glucose and 6.8 g/L butyric acid at the dilution rate of 0.12 h^{-1}. (a) Time course data of cell density (OD), glucose, solvents (acetone, butanol, and ethanol), and acids (acetic acid and butyric acid) in the reactor outlet stream; (b) Butanol and ABE yields and butanol productivity.

Figure 6 . Kinetics of continuous fermentation with 60 g/L glucose and 5.0 g/L butyric acid at the dilution rate of 1.88 h^{-1}. (a) Time course data of cell density (OD), glucose, solvents (acetone, butanol, and ethanol), and acids (acetic acid and butyric acid) in the reactor outlet stream; (b) Butanol and ABE yields and butanol productivity.

Figure 7. Scanning electron micrographs of cells immobilized in the fibrous matrix in the fibrous bed bioreactor. Cells adsorbed on the fiber surface formed a thick layer of biofilm. Most of cells had an elongated

rod shape between 3 to 5 μm.

Table 1. Comparison of fermentation kinetics of *C. acetobutylicum* ATCC55025 in batch and continuous fermentations at different dilution rates.

		Batch	**Continuous**	**Continuous**	**Continuous**
			0.06 h^{-1}	0.12 h^{-1}	1.88 h^{-1}
Glucose	Feed Conc. (g/L)	52	50	60	60
	Outlet Conc. (g/L)	1.0 ± 0.2	16.77 ± 1.24	15.87 ± 1.81	13.70 ± 1.62
Butyric Acid	Feed Conc. (g/L)	4.0	4.0	6.8	5.0
	Outlet Conc. (g/L)	2.35 ± 0.11	3.01 ± 0.61	2.59 ± 0.19	1.89 ± 0.28
Acetic Acid	Titer (g/L)	1.83 ± 0.076	3.26 ± 0.23	2.27 ± 0.08	1.85 ± 0.63
	Yield (g/g)	0.032 ± 0.002	0.090 ± 0.016	0.053 ± 0.005	0.042 ± 0.003
	Productivity (g/L[?]h)	0.007 ± 0.001	0.20 ± 0.02	0.27 ± 0.01	3.38 ± 0.10
Acetone	Titer (g/L)	5.58 ± 0.14	3.19 ± 0.24	3.86 ± 0.20	3.38 ± 0.55
	Yield (g/g)	0.11 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
	Productivity (g/L[?]h)	0.02 ± 0.002	0.19 ±0.01	0.46 ± 0.02	7.03 ± 0.36
Butanol	Titer (g/L)	13.08 ± 0.23	8.72 ± 0.51	10.37 ± 0.29	9.55 ± 0.75
	Yield (g/g)	0.25 ± 0.01	0.24 ± 0.02	0.24 ± 0.02	0.24 ± 0.02
	Productivity (g/L[?]h)	0.05 ± 0.01	0.52 ± 0.03	1.24 ± 0.04	16.75 ± 1.68
Ethanol	Titer (g/L)	0.87 ± 0.02	0.62 ± 0.18	0.72 ± 0.12	0.77 ± 0.20
	Yield (g/g)	0.014 ±0.001	0.017 ± 0.006	0.017 ± 0.002	0.02 ± 0.001
	Productivity (g/L[?]h)	0.003 ±0.0002	0.038 ± 0.013	0.083 ± 0.014	1.23 ± 0.07
ABE	Titer (g/L)	19.23 ± 0.38	12.52 ± 0.28	14.98 ± 0.55	13.70 ± 0.32
	Yield (g/g)	0.38 ± 0.02	0.35 ± 0.02	0.35 ± 0.04	0.35 ± 0.04
	Productivity (g/L[?]h)	0.07 ± 0.01	0.72 ± 0.09	1.79 ± 0.19	24.15 ± 2.75

Table 2 . Comparison of continuous ABE fermentation in product titer, yield, and productivity.

Process and Immobilization media	Dilution rate (h^{-1})	Operation duration	Butanol Titer (g/L)	Butanol Yield (g/g)	Butanol Productivity (g/L·h)	ABE Titer (g/L)	ABE Yield (g/g)	ABE Productivity (g/L·h)	Re
STR, free cells	0.02 0.05	200 h 200 h	6.9 9.3	0.24 0.27	0.14 0.46	12.4 16.5	0.43 0.48	0.25 0.82	Al-Sh et 20
STR, free cells STR, corn stover	0.01 0.13 0.44	200 h 100 h 200 h	7.6 10.1 9.5	0.4 0.35 0.33	0.1 1.3 4.2	NA	NA	NA	Ga al.
STR, free cells STR, polyvinyl alcohol	0.04	300 h 140 h	7.1 13.4	0.24 0.44	0.22 0.4	9.4 22.1	0.32 0.73	0.29 0.66	Le al.

Process and Immobilization media	Dilution rate (h^{-1})	Operation duration	Butanol	Butanol	Butanol	ABE	ABE	ABE	Re
STR with cell recycling	0.85	200 h	NA	NA	NA	12.9	NA	11.0	Ta et 200
STR with cell recycling	0.86	40 h	11.9	0.17	10.7	23.5	0.34	21.1	Ja al. 20
STR with cell recycling and *in situ* product separation	0.076	170 h	550	0.35	14.0	NA	NA	NA	Ng et 20
2-stage STR, Ca-alginate	1.0	727 h	NA	NA	NA	3.9	0.21	4.0	Fri an Sc 198
PBR with brick	2.0	450 h	4.5	0.23	9.0	7.6	0.38	15.2	Qu et 200
PBR with sugarcane bagasse	2.0	NA	3.8	0.20	7.5	5.7	0.31	11.3	Ko et 20
PBR with wood pulp fiber	1.5	NA	4.6	0.16	6.9	8.1	0.28	12.1	Su et 20
3-stage PBR with corn stalk	0.04	750 h	12.6	0.24	0.51	19.9	0.38	0.8	Ch et 20
4-stage PBR with ceramic beads	0.13	96	5.5	0.14	0.71	7.3	0.20	1.0	Ba et 200

Process and Immobilization media	Dilution rate (h^{-1})	Operation duration	Butanol	Butanol	Butanol	ABE	ABE	ABE	Re
Recirculating PBR, corn stalk	0.2–1.0	20 days	2.9-5.5	0.18	0.9-2.9	5.5-9	0.32	1.5-5.1	Zh et 20
Recirculating FBB, cotton towel	0.9	600 h	5.1	0.42	4.6	6.8	0.51	6.1	Hu et 20
FBB, cotton towel	0.12 1.88	600 h 300 h	10.4 9.6	0.24	1.24 **16.8**	15.0 13.7	0.35	1.8 **24.2**	**Th wo**
2-stage PBR with extraction, sugarcane bagasse	0.2	30 days	16.9	0.23	1.67	25.3	0.35	2.5	Ba et 20

NA: not available; FBB: fibrous bed bioreactor; PBR: packed bed reactor; STR: stirred-tank reactor

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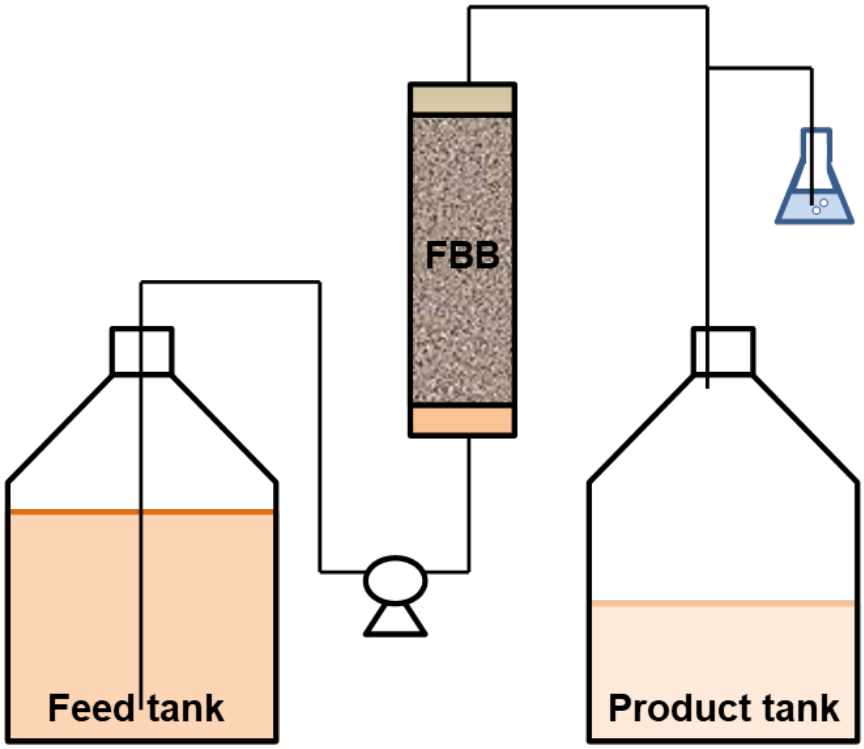

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Figure 2 . Effect of butyric acid on cell growth. Cells of *C. acetobutylicum* ATCC 55025 were cultured in P2 medium containing 20 g/L glucose and various amounts of butyric acid (0, 2, 5, or 10 g/L) in serum bottles at 37 °C.

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Figure 3 . Kinetics of batch fermentation with free cells cultured in medium containing ˜50 g/L glucose and 4 g/L butyric acid as carbon sources in a stirred-tank bioreactor at 35 °C and pH controlled at ˜5.0.

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acid-in-a-single-pass-fibrous-bed-bioreactor

Figure 4 . Kinetics of continuous fermentation with 50 g/L glucose and 4 g/L butyric acid at the dilution rate of 0.06 h^{-1}. (a) Time course data of cell density (OD), glucose, solvents (acetone, butanol, and ethanol), and acids (acetic acid and butyric acid) in the reactor outlet stream; (b) Butanol and ABE yields and butanol productivity.

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Figure 5 . Kinetics of continuous fermentation with 60 g/L glucose and 6.8 g/L butyric acid at the dilution rate of 0.12 h^{-1}. (a) Time course data of cell density (OD), glucose, solvents (acetone, butanol, and ethanol), and acids (acetic acid and butyric acid) in the reactor outlet stream; (b) Butanol and ABE yields and butanol productivity.

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`image7.emf` available at https://authorea.com/users/493818/articles/576120-high-rate-continuous-n-butanol-production-by-clostridium-acetobutylicum-from-glucose-and-butyric-acid-in-a-single-pass-fibrous-bed-bioreactor

Figure 6 . Kinetics of continuous fermentation with 60 g/L glucose and 5.0 g/L butyric acid at the dilution rate of 1.88 h^{-1}. (a) Time course data of cell density (OD), glucose, solvents (acetone, butanol, and ethanol), and acids (acetic acid and butyric acid) in the reactor outlet stream; (b) Butanol and ABE yields and butanol productivity.

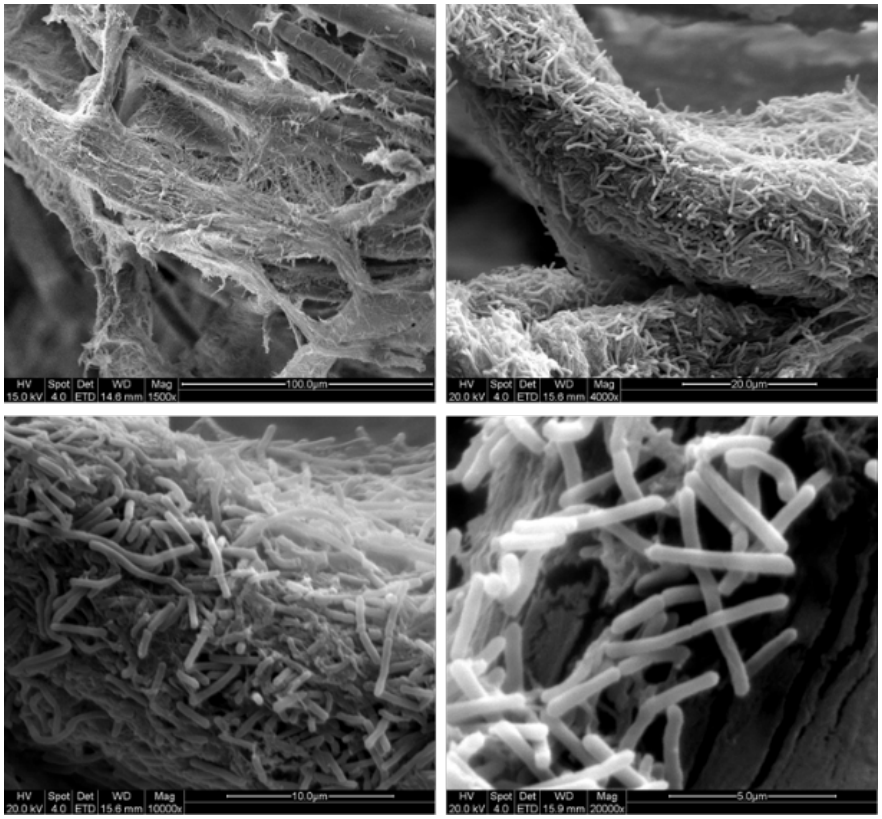

Figure 7. Scanning electron micrographs of cells immobilized in the fibrous matrix in the fibrous bed bioreactor. Cells adsorbed on the fiber surface formed a thick layer of biofilm. Most of cells had an elongated rod shape between 3 to 5 µm.