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Performance of batch, fed-batch, and continuous A–B–E fermentation with pH-control

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ABSTRACT

Batch, fed-batch, and continuous A–B–E fermentations were conducted and compared with pH controlled at 4.5, the optimal range for solvent production. While the batch mode provides the highest solvent yield, the continuous mode was preferred in terms of butanol yield and productivity. The highest butanol yield and productivity found in the continuous fermentation at dilution rate of 0.1 h^{-1} were 0.21 g-butanol/g-glucose and 0.81 g/L/h, respectively. In the continuous and fed-batch fermentation, the time needed for passing acidogenesis to solventogenesis was an intrinsic hindrance to higher butanol productivity. Therefore, a low dilution rate is suggested for the continuous A–B–E fermentation, while the fed-batch mode is not suggested for solvent production. While 3:6:1 ratio of acetone, butanol, and ethanol is commonly observed from A–B–E batch fermentation by *Clostridium acetobutylicum* when the pH is uncontrolled, up to 94% of the produced solvent was butanol in the chemostat with pH controlled at 4.5.

1. Introduction

Industrial-scale A–B–E fermentation (acetone–butanol–ethanol) was in practice worldwide from the early 20th century until the 1970s, before being supplanted by petroleum-based chemicals. The revival of A–B–E fermentation is currently being stimulated by the consideration of butanol for biofuel. Butanol has recently been proposed as a gasoline additive, or even as a complete gasoline replacement (Lee et al., 2008a). Butanol is superior to ethanol because it has higher energy content, lower volatility and less corrosiveness (Lee et al., 2008a).

Clostridium acetobutylicum and *Clostridium beijerinckii* are two bacterial strains often used for A–B–E fermentation. A–B–E fermentation is typically characterized as a biphasic growth pattern in terms of metabolite production. The first stage is called acidogenesis. During acidogenesis, the bacterial culture mainly produces acetic and butyric acids. This occurs during exponential growth of the bacterial culture. The second stage is called solventogenesis. This begins late in the exponential growth phase and continues into the stationary phase of the bacterial culture, where acetone, butanol, and ethanol are the main products. During solventogenesis, the acids produced from the first stage of acidogenesis are re-assimilated and converted to solvents (Jones and Woods, 1986). Although the phase transition from acidogenesis to solventogenesis is a complex metabolic process, several important factors have been identified. The pH-acid effect, where the pH is below 5.5, and the threshold concentration of undissociated butyric acid of 1.5 g/L are two necessary factors for triggering the onset of solventogenesis in *C. acetobutylicum* (Monot et al., 1984; Hüsemann and Papoutsakis, 1988).

In addition to being one factor required for triggering the onset of solventogenesis, the effect of pH on butanol production during solventogenesis has also been studied. While acidic pH is important for triggering the onset of solventogenesis, one report indicated that pH of 4.3 is the optimal pH for butanol production using *C. acetobutylicum* (Bahl et al., 1982a). Since the technique of pH-control is easily managed, A–B–E fermentation with pH-control becomes a practical technique for optimizing fermentative butanol production (Bahl et al., 1982b; Soni et al., 1987; Geng and Park, 1993; Huang et al., 2004; Napoli et al., 2010; Salleh et al., 2008). The other advantage of running A–B–E fermentation at low pH is to inhibit the nitrogen availability to bacterial culture since nitrogen limitation is arguably important for triggering and maintaining solventogenesis (Monot and Engasser, 1983; Roos et al., 1985). This is especially important when the complex culture media is used. The technique of pH-control also prevents the common problem of acid-crash, in which A–B–E fermentation culture

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fails to switch from acidogenesis to solventogenesis due to the production of excess acids (Maddox et al., 2000).

Batch fermentation is the most often used operation mode in industries such as pharmaceutical or brewing due to its high efficiency and good control (Ghose and Tyagi, 1979; Cardona and Sánchez, 2007). Fed-batch fermentation is another option and is usually considered when substrate inhibition or catabolite repression might occur (Modak et al., 1986; Luli and Strohl, 1990). Typically, fed-batch fermentation is started with a low substrate concentration. When the fermentation culture consumes the substrate, more substrate is then added to maintain the fermentation process while not exceeding the detrimental substrate level. Also, the dilution effect during the addition of the substrate solution may also solve the problem of catabolite toxicity, such as the acetate problem in *E. coli* fermentation (Luli and Strohl, 1990). Substrate inhibition is not a major concern in A–B–E fermentation when glucose is used as carbon source; however, the dilution effect may relieve the problem of butanol toxicity and thus improve the solvent productivity. Continuous operation is a third option and has several advantages over batch and fed-batch operations, including minimizing equipment downtime and time loss due to the lag phase of the microbial culture.

Performance of A–B–E fermentation is a function of clostridial strain, media composition, fermentation techniques, and fermentation conditions (Monot et al., 1984; Bahl et al., 1982a,b; Geng and Park, 1993; Napoli et al., 2010; Stephens et al., 1985; Barbeau et al., 1988; Woolley and Morris, 1990; Gottschal and Morris, 1982; Mutschlechner et al., 2000; Qureshi and Blaschek, 1999; Lee et al., 2008b). In this study, pH-controlled A–B–E fermentation at acidic pH 4.5 for solvent production was conducted in batch, fed-batch, and continuous modes. Comparisons of the three modes were made in terms of the fermentation performance. To the authors' knowledge, this is the first time this comparison has been conducted for A–B–E fermentation.

2. Methods

2.1. Bacterial strains and culture medium

C. acetobutylicum ATCC® 824™ was used for fermentative solvent production. Each experiment was started with the spore culture of *C. acetobutylicum*. To germinate the spores of *C. acetobutylicum*, spore cultures were transferred to fresh Difco™ reinforced clostridial medium (19 g/L) diluted to a concentration of 10%. The freshly transferred culture was heat shocked for 1 min at 80 °C to induce spore germination, followed by incubation at 37 °C for 20–24 h. Two percent inoculum was transferred to a serum bottle containing clostridia reactor media (CRM). CRM is composed of the following solutions (per liter): solution I [60 g glucose]; solution II [5 g yeast extract]; solution III [0.75 g KH_2PO_4, 0.75 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 1 g NaCl]; solution IV [0.2 g $MgSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$]; solution V [0.5 g L-cysteine·HCl]. Solution I, II and III were sterilized by autoclave and solution IV and V were sterilized by 0.22 μm syringe filtration (Mermelstein et al., 1994). Initial pH was adjusted to around 6.

2.2. Batch A–B–E fermentation

A–B–E fermentation was carried out using a BioFlo 3000 Bioreactor (New Brunswick Scientific). Throughout the batch, fed-batch, and continuous A–B–E fermentation, the pH was maintained at 4.5 with 2 N NaOH and 1 N HCl. The temperature was kept at 37 °C. Ultra high purity nitrogen was allowed to flow through the headspace of the fermentor. The initial nitrogen flow rate was 250 mL/min and decreased to 120 mL/min when the OD_{540} reached 0.6 (Mermelstein et al., 1994). Batch fermentation was started by transferring 2% inoculum to the fermentor containing CRM.

2.3. Fed-batch A–B–E fermentation with in situ butanol pervaporation

Fed-batch fermentation with *in situ* butanol pervaporation was carried out as follows: Starting with an initial volume of 330 mL, A–B–E fermentation was allowed to run in batch mode for 21 h. Fresh medium was then fed into the fermentor at a volumetric feed rate of 66 mL/h. The medium contained exactly amount of nutrient as CRM, except the glucose concentration was 85 g/L. The feed of medium was stopped after 12 h and the fermentation continued in batch mode.

In situ butanol pervaporation was carried out as follows: when fed-batch fermentation started, the fermentation culture solution was re-circulated over the pervaporation membrane, enclosed in a membrane holder, at a rate of 150 mL/min. At the same time, a vacuum pump was turned on to generate a vacuum condition (total pressure was less than 1 mm Hg) on the downstream side of the pervaporation membrane to start *in situ* butanol pervaporation. The composite pervaporation membrane used was polydimethylsiloxane (PDMS, Sylgard® 184) supported with the dual layers of a porous polyethylene sheet and a perforated brass metal sheet. The membrane fabrication and the details of the PDMS/dual support composite membrane are described elsewhere (Li et al., 2010). The PDMS/dual support composite membrane was pre-sterilized by circulating 30% of ethanol solution through for 12 h followed by a wash with 500 mL of sterilized DI water before the pervaporation experiment started. The solution–diffusion model, a Fick's first law-based equation, was used to describe the pervaporation process (Wijmans and Baker, 1995):

$$J_i = K_{i,ov} C_i$$

where J_i is the flux of the permeate i with the units of g/h/m^2. $K_{i,ov}$ is the overall mass transfer coefficient of permeate i with the units of mm/h. C_i is the concentration of permeate i in the fermentor with the units of g/dm^3.

2.4. Continuous A–B–E fermentation

Continuous fermentation was carried out as follows: A–B–E fermentation was allowed to run in batch mode for 21 h, until a significant amount of butanol was produced. Fresh medium was then fed into the fermentor. The medium contained exactly the same amount of nutrient as CRM, except the glucose concentration. The glucose concentration of the medium and the feed rate used are indicated in the Section 3. The volume of fermentation culture was kept constant by setting a purge stream with the same volumetric flow rate as the feed stream. Dilution rate (D, h^{-1}) is defined as the ratio of the volumetric feed rate of medium (F, mL/h) to the volume of fermentation culture solution (V, mL). Note that since there was no cell retention technique applied during the continuous fermentation, D is the reciprocal of both the fluid residence time and the cell residence time in the fermentor.

2.5. Analytical methods

Cell density was measured at 540 nm using a BioMate™ 3 spectrophotometer (Thermo Spectronic, USA). Glucose concentration was measured using YSI 2700 Biochemistry Analyzer. Acetone, ethanol, butanol, acetic acid, and butyric acid in the fermentation culture solutions were analyzed by gas chromatography (GC) using the DB-FFAP capillary column and a FID detector. Temperatures of injector and detector were maintained at 250 °C. Samples were first filtered through 0.2 μm syringe filter with an injection volume of 1 μL. GC oven temperature was initially held at 40 °C for 2 min.

The temperature was raised with a gradient of 5 °C/min until 45 °C and then the gradient was changed to 20 °C/min and held for 3 min at 225 °C. Pervaporation permeates were also analyzed by GC.

3. Results and discussion

3.1. Batch A–B–E fermentation

Batch A–B–E fermentation with pH-control at 4.5 was conducted for comparison to fed-batch and continuous fermentation behavior. Fig. 1 illustrates the solvent production profiles and more extensive data on the batch behavior will be published elsewhere. The solvent and butanol yields of batch A–B–E fermentation were 0.32 and 0.23 (g/g-glucose), respectively. The solvent and butanol productivities of batch A–B–E fermentation were 0.52 and 0.37 g/L/h, respectively.

The effect of pH on the solvent production in batch A–B–E fermentation with *C. acetobutylicum* ATCC® 824™ was reported previously (Monot et al., 1984). When pH was controlled at 4.5, the solvent and butanol yields were 0.35 and 0.24 (g/g-glucose), respectively, which are nearly identical to the values reported above in the current work. Without pH control, the solvent yields and productivities are significantly lower (Jones and Woods, 1986; Keis et al., 2001).

3.2. Fed-batch A–B–E fermentation with in situ butanol pervaporation

In this study, fed-batch A–B–E fermentation with pH-control at 4.5 was conducted. Fermentation culture was first operated in batch mode. By 21 h the fermentation culture had transitioned from acidogenesis to solventogenesis. The fresh media, containing 85 g/L of glucose, was then fed at the rate of 66 mL/h into the fermentor for 12 h. The changes of the fermentation culture volume and the dilution rate during the fed-batch mode are shown in Fig. 2A. During the fed-batch mode, from 21 to 33 h, the biomass and the acid concentrations first decreased slightly but then increased rapidly as shown in Fig. 2B and C. This indicated a vigorous growth of the fermentation culture and the dominance of acidogenic culture, even though the fermentation culture had already progressed into solventogenesis, see Fig. 2D for the profile of solvent concentrations. During the fed-batch mode, the butanol concentration first decreased and then remained at 4.8 g/L until the feed was stopped. The butanol concentration of 4.8 g/L was significantly less than the threshold concentration of butanol toxicity at 12–15 g/L (Jones and Woods, 1986). The butanol concentration was kept low by the continuing dilution during fed-batch operation, and the low butanol concentration probably contributed to the rapid rise in biomass concentration as well as the rapid rise in acid concentrations. It should be noted here that *in situ* pervaporative butanol separation also contributed to the maintenance of low butanol concentration. However, due to the small surface area of the pervaporation membrane, the mass of butanol removed was limited, as discussed below.

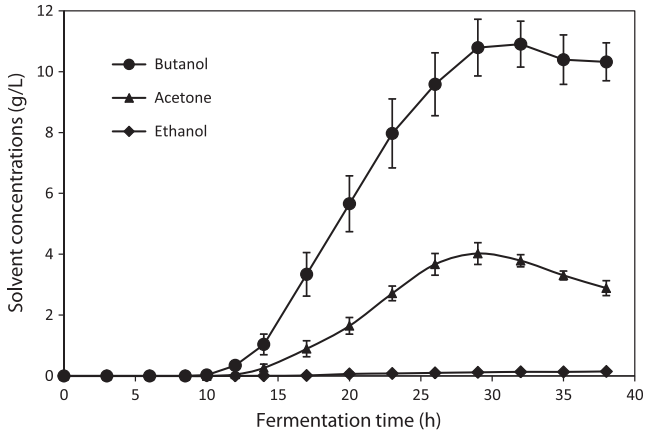

Fig. 1. Solvent profiles of batch A–B–E fermentation controlled at pH 4.5.

The butanol inhibition effect was observed when the feed was stopped and the fermentation process reverted back to batch mode. Fig. 2B and D illustrate that when the butanol concentration rose after 33 h, the biomass concentration as well as the acid concentrations went down. Furthermore, it can be seen from Fig. 2B that the glucose consumption rate decreased dramatically at 43 h when the butanol concentration reached the threshold concentration of 12 g/L. The gradual decrease in butanol concentration after 43 h was due to butanol removal by pervaporation.

There were subtle changes in the concentrations of solvents and acetic acid in the time frame of 30–33 h, where the solvent concentrations increased and the concentration of acetic acid decreased. This indicates a gradual transition from acidogenesis to solventogenesis and can be attributed to the decrease in dilution rate during the fed-batch mode. The transition is also in agreement with the results presented below in Figs. 4 and 5A, i.e. when continuous A–B–E fermentation is operated at low dilution rates, the fermentation culture will be dominated by the solventogenic culture.

The performance of fed-batch A–B–E fermentation was evaluated based on the data in the first 43 h. The overall volumetric glucose consumption rate was 1.59 g/L/h, where the total fermented glucose was 75.8 g. The overall volumetric glucose consumption rate for batch A–B–E fermentation was 1.62 g/L/h, where the total fermented glucose was 63.3 g. Batch and fed-batch modes had similar performance on the overall volumetric glucose consumption rate. However, in terms of the total amount of glucose utilization, the fed-batch mode showed an advantage over the batch mode. This can be attributed to the same reason for the increase in biomass and acid concentrations during the fed-batch mode, i.e. the butanol toxicity problem was relieved by the dilution effect during the fed-batch mode. In fact, fed-batch mode is expected to have better performance in terms of glucose utilization when a better feeding strategy is adopted, such as the exponential feeding strategy (Modak et al., 1986). In terms of solvent yield and productivity, however, the batch mode showed better performance than the fed-batch mode, see Fig. 6A and B below.

Compared to batch A–B–E fermentation, a low solvent productivity is generally observed using the fed-batch mode even though a butanol removal unit was integrated with the fermentation to reduce the butanol inhibition effect (Qureshi et al., 2001). Several possible reasons for low solvent productivity proposed by Qureshi et al. were the presence of inactive or dead cells, accumulation of inhibitor macromolecules, and deficiency of nutrients (Qureshi et al., 2001). Ezeji et al. has reported that fed-batch fermentation with gas stripping provided better performance than the control batch fermentation, where the solvent yield and solvent productivity for fed-batch fermentation were 0.47 g-solvent/g-glucose/L and 1.16 g/L/h, respectively, and the solvent yield and productivity for batch fermentation were 0.39 g-solvent/g-glucose/L and 0.29 g/L/h, respectively (Ezeji et al., 2004). The contradictory results can be attributed to the different *Clostridia* strains used. In this study, as shown in Fig. 6A and B below, the batch mode was shown to be better than the fed-batch mode, which is in agreement with Qureshi, et al. In addition to those reasons proposed by Qureshi et al. for the poor performance of the fed-batch fermentation, we proposed that the time needed for passing acidogenesis was another factor responsible for the low solvent productivity, see Fig. 2 for the dominance of the acidogenic culture during the addition of fresh feed solution. This intrinsic hindrance for solvent production was also found to be a rate-limiting step in continuous

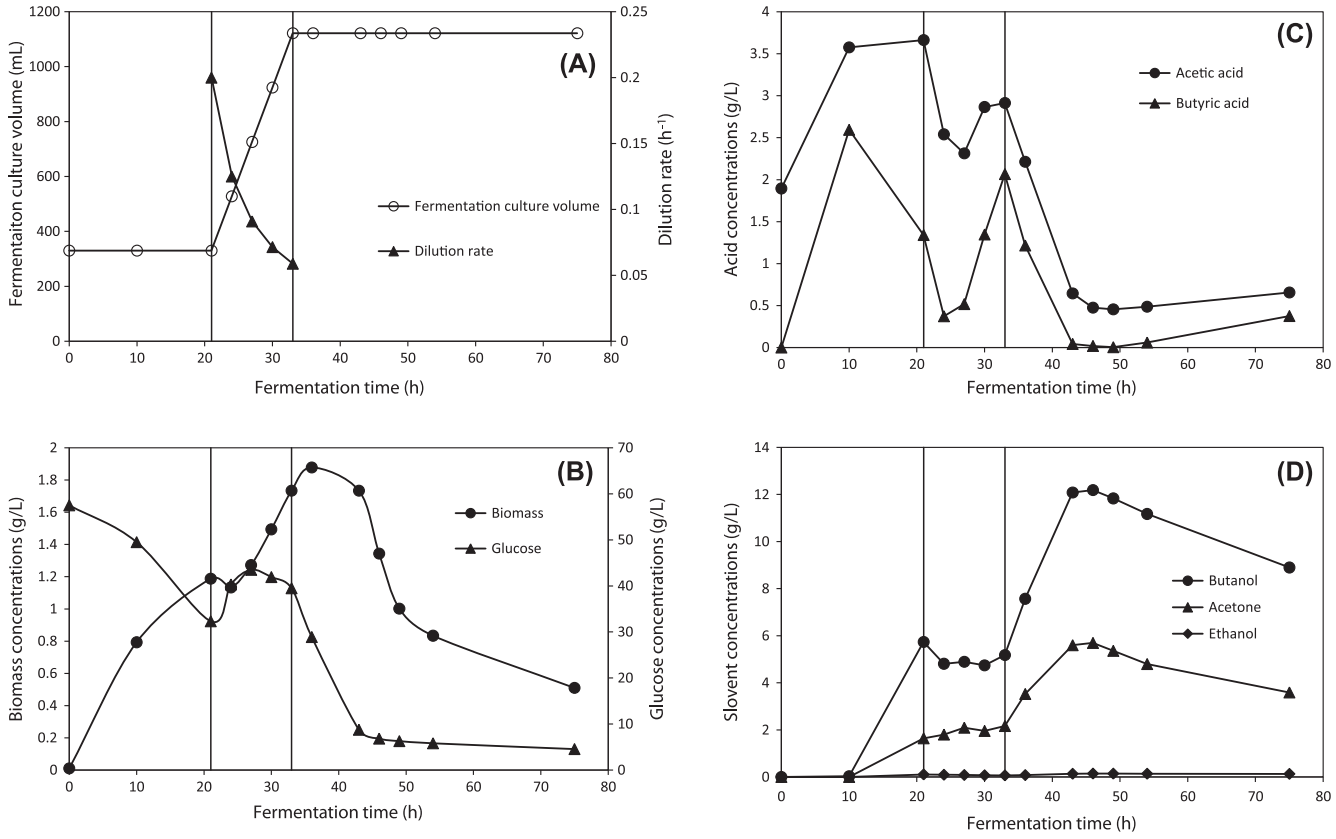

Fig. 2. Performance of fed-batch A–B–E fermentation. Profiles of (A) fermentation volume and dilution rate; (B) biomass and glucose concentrations; (C) acid concentrations; (D) solvent concentrations. Two vertical lines in each figure indicate the start and end of the fed-batch mode.

A–B–E fermentation, even though the pH of the fermentation culture was controlled at 4.5, which is favored for the solvent production, as discussed in Section 3.4. Although the fed-batch mode is not suggested for solvent production, fed-batch mode may still be considered for producing hydrogen, which is the major metabolite generated from the culture at the acidogenic phase (Chin et al., 2003).

As noted above, a pervaporation system was operated during the fed-batch experiment to remove butanol from the fermentation broth. Details on the polydimethyl siloxane/porous polyethylene dual support composite membrane and its use during A–B–E batch fermentation have been discussed in previous studies (Li et al., 2010). The pervaporation system was operated to simply demonstrate *in situ* butanol removal during fed-batch operation, and had only a minor effect on the experiment due to the very small membrane area employed. About 11% of total produced butanol was removed in the first 43 h, which is the time frame used to evaluate the performance of fed-batch fermentation.

3.3. Profiles of continuous A–B–E fermentation

Continuous A–B–E fermentation with pH control at 4.5 was conducted at several dilution rates. Each of four dilution rates were used for a period of time leading to the identification of four steady-state conditions that were used to characterize the performance of the continuous A–B–E fermentation.

The overall fermentation profiles are presented in Fig. 3A–C. The fermentation culture was operated in batch mode for the first 26 h. After transitioning from acidogenesis to solventogenesis, the fermentation operation was switched to continuous mode. The fermentation culture did not reach steady state in the first 190 h due to control difficulties, including a power outage and minor temperature fluctuations. However, after 190 h, the fermentation culture reached 4 distinct steady states in correspondence to the 4 dilution rates investigated. To clearly illustrate one of the steady-state conditions for the fermentation culture, Fig. 4 shows a small portion of the metabolite profiles from Fig. 3 on an expanded time scale for a dilution rate of 0.13 h^{-1}. All four of the steady state concentrations of biomass, consumed glucose, and major metabolites at the 4 different dilution rates are listed in Table 1. In addition, a second run was completed where power and control difficulties did not occur, and the steady state results at the two dilution rates investigated are also listed in Table 1.

The metabolite profiles in run 1 changed in correspondence to the dilution rate until 336 h. Solvent degeneration was observed after 336 h, as indicated by the third vertical solid line in Fig. 3A–C. When solvent degeneration occurred, the butanol concentration dropped dramatically from 8–9 g/L to 2–3 g/L, while the dilution rate was maintained at 0.10 h^{-1} in the time frame of 286–353 h. Along with the decrease in the butanol concentration was an increase in the acid concentrations. This indicated that the fermentation culture was still alive, but the activity of the acid re-assimilation to produce solvents decreased. Also observed was a decrease in the biomass concentration. Consequently, the fermentation culture consumed less glucose. The existence of a true steady-state condition for A–B–E fermentation has long been challenged due to the issue of solvent degeneration, in which the solvent producing ability of the bacteria was irreversibly lost after 2–3 weeks of continuous operation using a single-stage fermentor (Stephens et al., 1985; Barbeau et al., 1988; Woolley and Morris, 1990; Chiao and Sun, 2007).

The fermentation culture reached another steady-state condition during the solvent degeneration, in which the fermentation culture still steadily produced low concentrations of butanol at the dilution rate of 0.13 h^{-1}. Nevertheless, the butanol and the biomass concentrations cannot build up in the fermentor after switch-

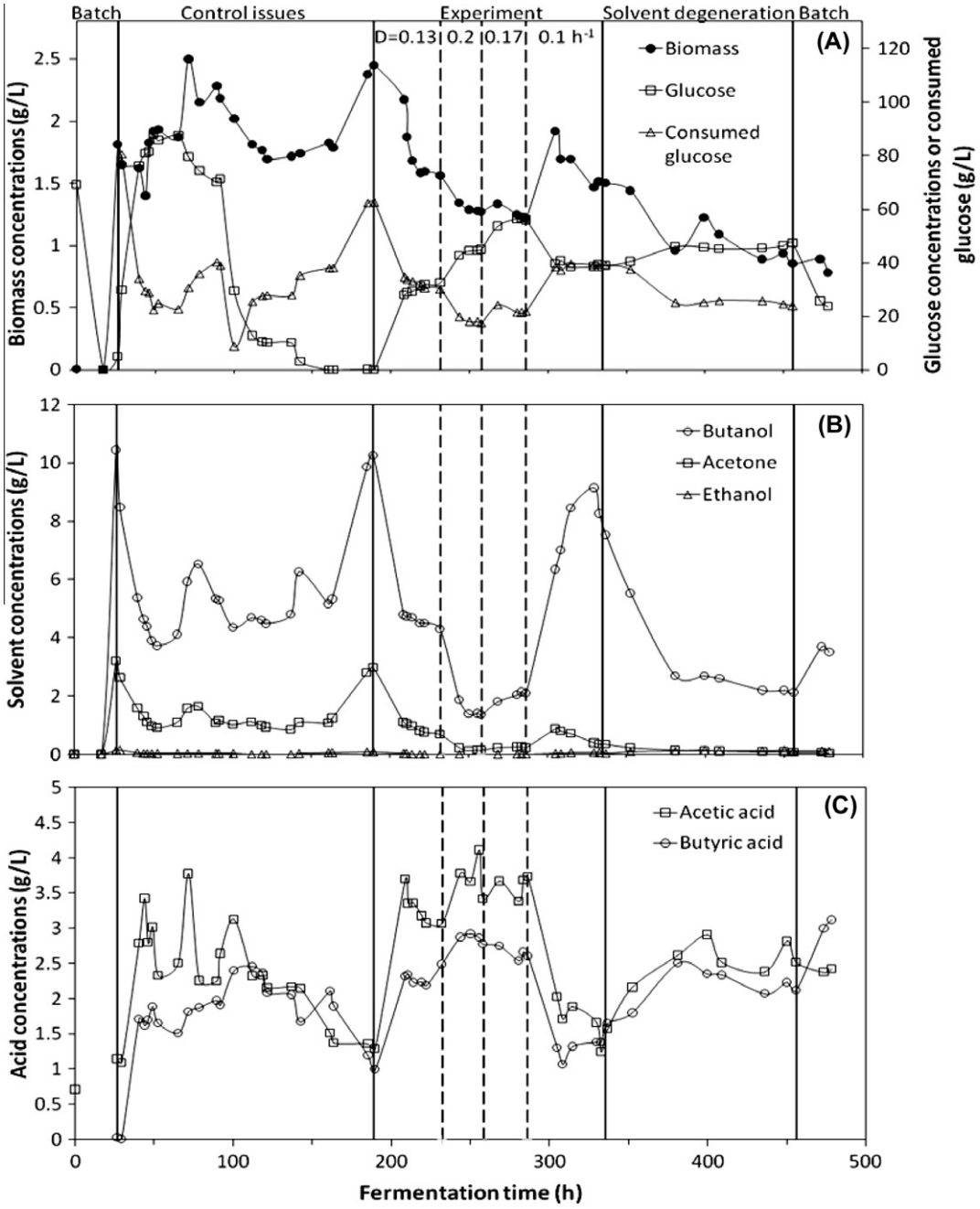

Fig. 3. Profiles for continuous fermentation of (A) Biomass, glucose concentration, and consumed glucose; (B) solvent concentrations; (C) acid concentrations. Consumed glucose (g/L) in (A) was calculated by subtracting glucose concentration in the fermentor from the glucose concentration in the feed solution. Four vertical solid lines in each figure represent five major experimental events as labeled above (A). Note that in the time frame of 26–456 h, the A–B–E fermentation was operated in the continuous mode. In the experimental period of 189–456 h, three vertical dotted lines were further added to indicate the dilution rates used.

ing from continuous to batch modes, see the vertical solid line at 456 h in Fig. 3A–C. Therefore, it is concluded that the solvent-degenerative fermentation culture loses not only the ability to produce solvents but also loses the normal growth profile.

3.4. Performance of continuous fermentation for solvent production

The performance of continuous fermentation for solvent production was evaluated based on the data of run 1 in Table 1. The volumetric glucose consumption rates and the metabolite productivities (g/L/h) are presented in Fig. 5A. There were two ranges of the volumetric glucose consumption rates, which can be correlated with the solvent/acid ratio. When the solvent/acid ratio was less than 1, the acid concentrations dominated in the fermentation culture and the volumetric glucose consumption rates were at a lower value of 3.57 g/L/h. When the solvent/acid ratio was greater than 1, the solvent concentrations dominated in the fermentation culture and the volumetric glucose consumption rates were at higher values of 3.80 and 3.95 g/L/h. Although the data are insufficient to statistically prove a decline in glucose consumption at low solvent/acid ratios, the small decrease observed is consistent with previous reports (Soni et al., 1987).

Fig. 5A indicates that the relative production of solvents and short fatty acids depends on the dilution rate. At a lower dilution rate, the fermentation culture mainly produced solvents while at

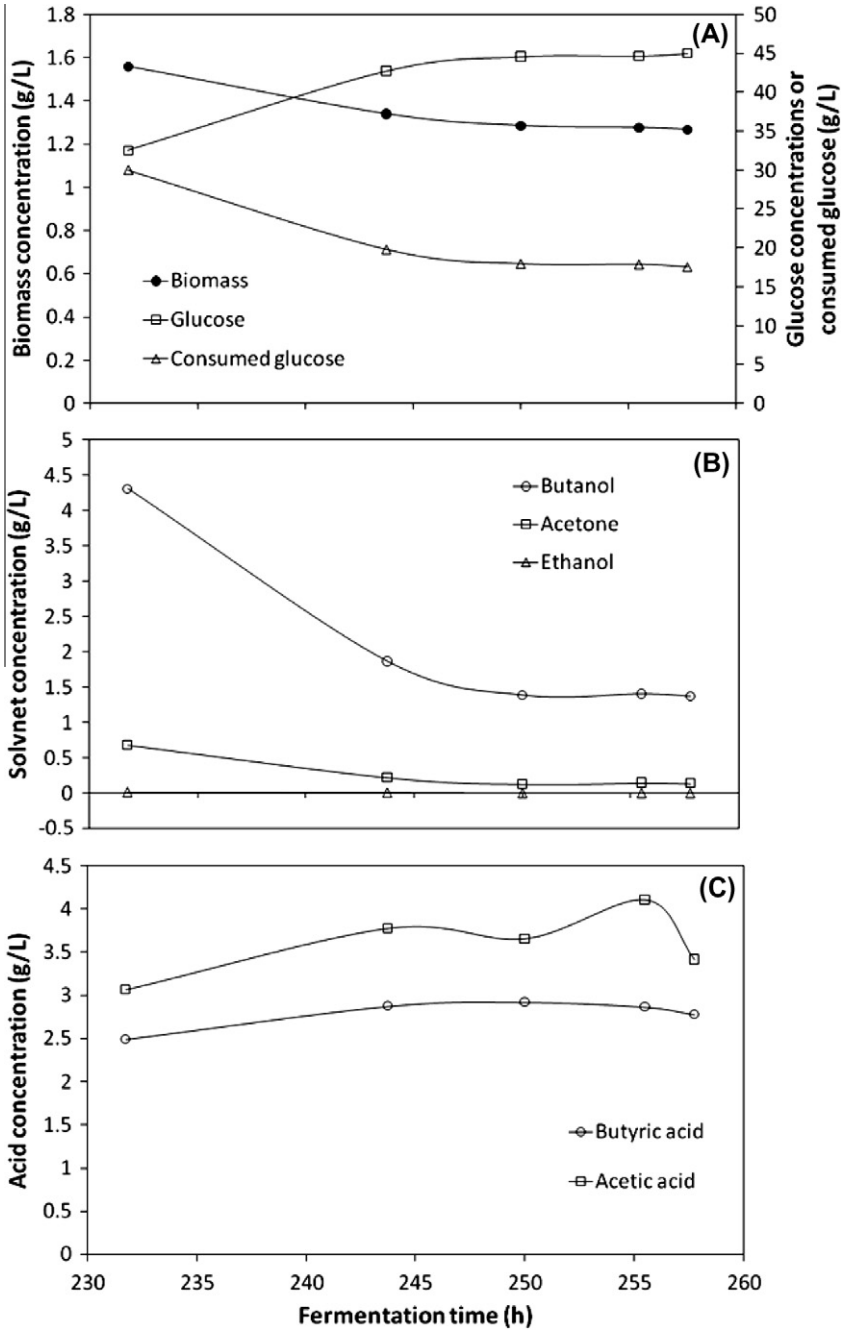

Fig. 4. Expanded fermentation profiles of figure in the time frame of 189–232 h, where the dilution rate was 0.13 h^{-1}. (A) Biomass, glucose concentration, and consumed glucose; (B) solvent concentrations; (C) acid concentrations. The last three data points for the concentrations of the biomass, consumed glucose, and major metabolites were averaged to represent the steady-state conditions of continuous A–B–E fermentation for each quantity.

a higher dilution rate, the fermentation culture mainly produced acids. A–B–E fermentation is known by its biphasic growth pattern: acidogenesis followed by solventogenesis (Lee et al., 2008a; Jones and Woods, 1986; Mitchell, 1997). Therefore, at a higher dilution rate, i.e. a shorter residence time, the fermentation culture was dominated by cells in the acidogenic phase, where acetic and butyric acids were the main metabolites. At a lower dilution rate, i.e. a longer residence time, the fermentation culture had enough time to fully enter solventogenesis phase and hence increased solvent productivity was observed. The decreased acid productivities at the lower dilution rates were due to re-assimilation, where acetic acid and butyric acid were re-assimilated by the fermentation culture for the synthesis of acetone and 1-butanol, respectively (Jones and Woods, 1986). Fig. 5A shows that the dilution rate of 0.20 h^{-1}, a residence time of 5 h, was sufficient for the clostridial fermentation culture to enter the solventogenesis phase where the pH was at 4.5. The highest total solvent productivity of 0.86 g/L/h was found at the dilution rate of 0.1 h^{-1}, where the butanol productivity was 0.81 g/L/h. This indicates that 94% of solvent produced was butanol (Table 1). In fact, butanol was observed to be the majority among the total solvents at all chosen dilution rates. The 3:6:1 solvent ratio of acetone, butanol, and ethanol is usually observed at the end of batch A–B–E fermentation using *C. acetobutylicum* (Jones and Woods, 1986; Beesch, 1953). However, when the pH of batch A–B–E fermentation was controlled at 4.5, the shift of metabolic activity caused the fermentation culture to produce less ethanol (Monot et al., 1984). In this study, the fermentation culture in pH-controlled continuous mode produced not

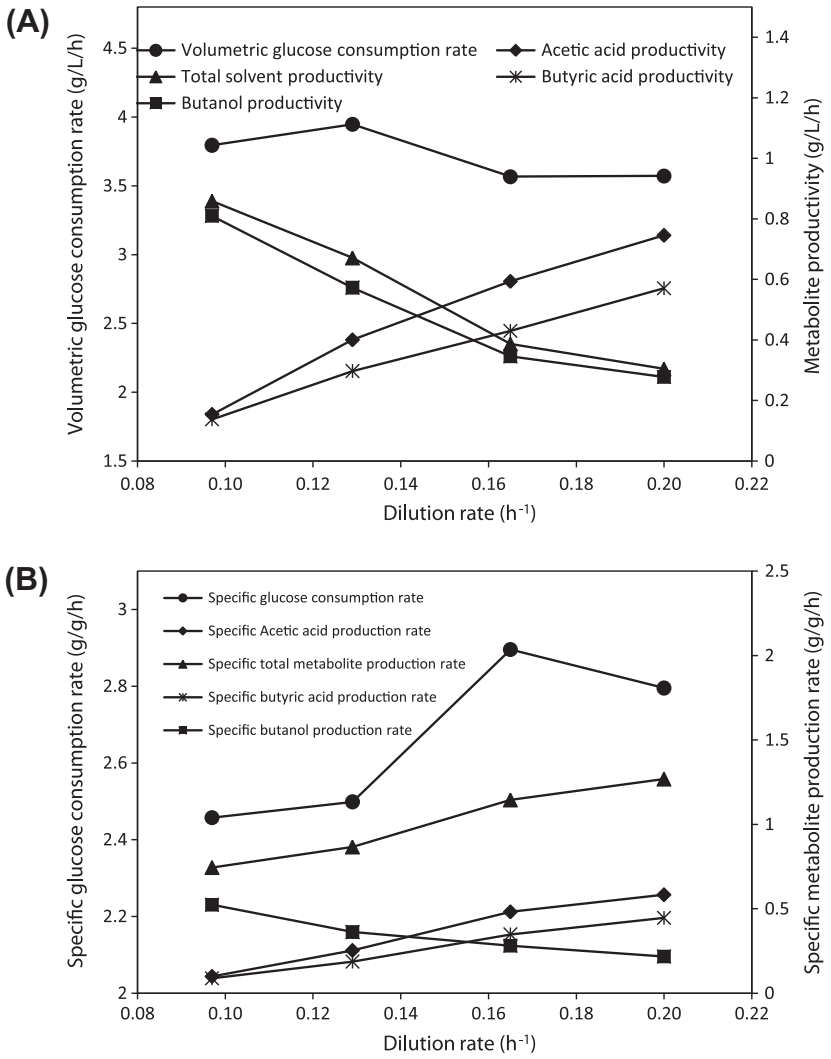

Fig. 5. (A) Volumetric glucose consumption rates and metabolite productivities of continuous A–B–E fermentation at pH 4.5; (B) specific glucose consumption, metabolite production, and total metabolite production rates of continuous A–B–E fermentation at pH 4.5. Total solvent production rate are not shown in (B) since butanol accounted for 94% of the solvents produced.

only less ethanol but also less acetone, where the amount of acetone produced was one order of magnitude less than butanol produced. Consequently, the total solvent productivities and the butanol productivities were close to each other at the dilution rates investigated.

The solvent distribution of A–B–E fermentation depends on the electron flow or redox balance (Rao and Mutharasan, 1987; Girbal et al., 1995). For example, alcohol formation has been shown to be NADH-dependent, during which NADH is oxidized to NAD^+. In contrast, the oxidation of NADH is not coupled with acetone formation (Rao and Mutharasan, 1987; Girbal et al., 1995). Under the "NADH pressure" or "redox imbalance" created by the acidic fermentation environment of pH 4.5, the increase in alcohol formation rather than acetone formation can relieve those stresses. Furthermore, 94% butanol produced in the total solvent indicates that the formation of butanol rather than ethanol is preferred during the "NADH pressure" or "redox imbalance". This selectivity for butanol is due to the four moles of NADH needed for creating 1 mol of butanol, while only two moles of NADH are needed for creating 1 mol of ethanol (Rao and Mutharasan, 1987; Gheshlaghi et al., 2009).

The specific glucose consumption rates and specific metabolite production rates (g-metabolite/g-biomass/h) are presented in Fig. 5B. Fig. 5B indicates that while the specific metabolite production rates had the same trend as the metabolite productivities shown in Fig. 5A, the specific glucose consumption rate had the opposite trend to the volumetric glucose consumption rate. The specific glucose consumption rates, however, were observed at lower values of 2.4–2.5 g-glucose/g-biomass/h when the solvent/acid ratio was less than 1. The specific glucose consumption rate was observed to increase to higher values of 2.8–2.9 g glucose/g biomass/h when the solvent/acid ratio was greater than 1. The high specific glucose consumption rates at the low solvent/acid ratios can be attributed to the high demand for glucose during acidogenesis to synthesize carbon dioxide (Jones and Woods, 1986; Roos et al., 1985; Mitchell, 1997). The fermentation culture produced the most total metabolites at the highest dilution rate of 0.20 h^{-1} and the least total metabolites at the lowest dilution rate of 0.10 h^{-1}. This further verifies the two regimes of bacterial metabolic behavior corresponding to solventogenesis and acidogenesis observed in batch fermentation. In addition to the high demand of glucose during acidogenesis, the data presented in Fig. 5 regarding the glucose consumption could also be an indication that the activity of the fermentation culture decreased as the residence time increased. The decreased cell activity can be attributed to the combination effects of butanol inhibition as well as the increased activity of endospore formation triggered by the activity

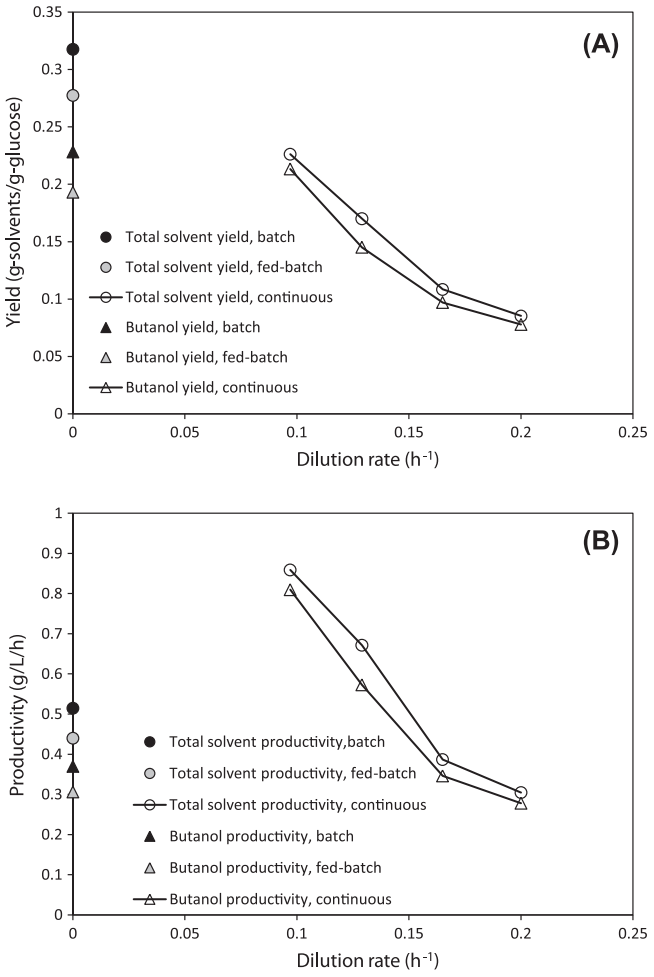

Fig. 6. Comparisons of the performance of batch, fed-batch, and continuous A–B–E fermentation in terms of (A) yield and (B) productivity.

of solventogenesis (Schuster et al., 1998; Dürre and Hollergschwandner, 2004).

Comparisons of the solvent yields (g-solvent/g-glucose) and solvent productivities are illustrated in Fig. 6A and B. Fig. 6A indicates that when all three fermentation processes were conducted with pH-control at 4.5, batch fermentation was the most efficient operation mode in terms of the total solvent production, where the total solvent yield was 0.32. However, in terms of the butanol yield, batch fermentation showed no advantage over continuous fermentation ($D = 0.1\ h^{-1}$), where the butanol yield was 0.21 g/g, see Fig. 6A and Table 1. As to the solvent and butanol productivities, continuous fermentation ($D = 0.1\ h^{-1}$) was the preferential operation mode, where the highest solvent and butanol productivities were found to be 0.86 and 0.81 g/L/h, see Fig. 6B and Table 1.

To verify the trends observed in continuous A–B–E fermentation at pH 4.5, a second continuous fermentation run at dilution rates of 0.13 and 0.17 h^{-1} was conducted. The data listed in Table 1 indicate that the second run had better performance than the first run in terms of both butanol yield and productivity. This may be attributed to the culture history, where there were no power outages and control difficulties in run 2 as occurred in run 1. Therefore, the culture of run 2 may have been healthier than in run 1, which is supported by the higher biomass concentrations at the same dilution rates. The other explanation to the quantitative difference between the two runs could also simply be experimental noise, since *C. acetobutylicum* is notorious for its culture instability. Although the data of run 2 are not quantitatively equal to the data of run 1 at similar dilution rates, the data of run 2 show the same trends observed in the first run for all measured variables. Therefore, data presented in Fig. 6 can be treated as a conservative boundary for the chemostat at pH 4.5.

The selectivity for butanol is listed in Table 1 as the butanol solvent fraction (g-butanol/g-solvent) and the butanol total fraction (g-butanol/g-total metabolite). Clearly, the butanol solvent fraction is several percentage points lower in run 2 than in run 1, primarily because of the higher acetone production in run 2. Nevertheless, the selectivity for butanol production is still much higher than the 60% commonly found in uncontrolled pH fermentation, and follows the same trend as in run 1. Furthermore, acid production in run 2 is significantly lower than in run 1, so the butanol fraction of total metabolites listed in the "Butanol Total Fraction" column is higher in run 2 than in run 1 by a large margin, and yet the trends seen in the two runs are the same as the dilution rate, *D*, is changed.

Bahl et al. used a two-stage phosphate limited continuous process to run A–B–E fermentation using *C. acetobutylicum* DSM 1731 (Bahl et al., 1982b). While the pH was maintained at 4.3 for the two fermentors, the first fermentor was operated at a high dilution rate of 0.125 h^{-1} at 37 °C to achieve a vigorous cell growth and a low butanol concentration in the fermentor. The second fermentor was operated at a low dilution rate of 0.04 h^{-1} at 33 °C. In this manner, a butanol yield of 0.23 g/g and a butanol productivity of 0.38 g/l/h were achieved (Bahl et al., 1982b). It can be concluded here that the single-stage continuous process used in this study provided higher butanol productivity than the two-stage continuous process did. However, the two-stage continuous process had greater long-term culture stability than the single-stage process. It was claimed by Bahl et al. that by using two-stage phosphate-limited chemostat process, A–B–E fermentation can run for over a year without losing the cell activity (Bahl et al., 1982b).

In this study, performance of batch, fed-batch, continuous A–B–E fermentation are compared and it is concluded from

Table 1
Steady-state concentrations of the biomass, consumed glucose, and major metabolites at different dilution rates.

Dilution rate (h^{-1})	Biomass (g/L)	Consumed glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric acid (g/L)	Acetic acid (g/L)	Butanol yield (g/g)	Butanol productivity (g/L/h)	Butanol solvent fraction (g/g-total solvent)	Butanol total fraction (g/g-total metabolite)
RUN 1											
0.20	1.28	17.86	1.39	0.14	0.00	2.85	3.73	0.08	0.28	0.91	0.17
0.17	1.23	21.62	2.10	0.25	0.00	2.61	3.60	0.10	0.35	0.89	0.25
0.13	1.58	30.60	4.44	0.75	0.02	2.31	3.11	0.15	0.57	0.85	0.42
0.10	1.54	39.13	8.35	0.45	0.05	1.44	1.59	0.21	0.81	0.94	0.70
RUN 2											
0.17	1.41	27.37	3.21	0.68	0.01	2.06	3.50	0.12	0.53	0.82	0.34
0.12	1.97	38.69	7.08	1.79	0.04	1.31	2.33	0.18	0.81	0.79	0.56

Fig. 6A and B that the continuous mode is preferred in terms of both the butanol yield and productivity. Performance of A–B–E fermentation is a function of clostridial strain, media composition, fermentation techniques, and fermentation conditions (Monot et al., 1984; Bahl et al., 1982a,b; Geng and Park, 1993; Napoli et al., 2010; Stephens et al., 1985; Barbeau et al., 1988; Woolley and Morris, 1990; Gottschal and Morris, 1982; Mutschlechner et al., 2000; Qureshi and Blaschek, 1999; Lee et al., 2008b). Due to the lack of thorough understanding of the effect of those variables on fermentation performance, it is difficult to compare data from different investigations. Nevertheless, Monot and Engasser reported also that continuous operation was preferred for A–B–E fermentation in terms of productivity (Monot and Engasser, 1983). When pH was controlled at 5.0, the solvent productivities of batch and continuous (D = 0.04–0.08 h^{-1}) A–B–E fermentation were 0.23 and 0.31 g/L/h, respectively (Monot and Engasser, 1983). In this study, at pH 4.5, the solvent productivity in continuous fermentation was 0.86 g/L/h, as indicated in Fig. 6B, which is higher than the batch productivity of 0.51 g/L/h. Thus, reducing pH from 5 to 4.5 gives higher solvent productivity in both batch and continuous fermentation, but the improvement in the continuous fermentation is much greater than in the batch fermentation.

Continuous operation has several advantages over batch and fed-batch operations, including minimizing downtime and the lag phase on the microbial culture. Nevertheless, in major fermentation industries, the batch mode is still widely adopted due to its high efficiency and good control (Ghose and Tyagi, 1979; Cardona and Sánchez, 2007). In this study, the highest solvent productivity of continuous A–B–E fermentation occurred at the lowest dilution rate, see Fig. 6B. This indicates the importance of the residence time for butanol productivity, where a high residence time was needed to allow fermentation culture to fully enter solventogenesis from acidogenesis. While even better results may be obtained at lower dilution rate, higher butanol concentration in the fermentor will lead to toxicity (Jones and Woods, 1986), limiting any further improvement that can be attained at lower dilution rates. As discussed by Shuler and Kargi, a mathematical analysis to quantify the advantages of one method over another is possible when a detailed kinetics model for A–B–E fermentation is available (Shuler and Kargi, 2001).

4. Conclusions

In this study, different fermentation modes were compared, all controlled at the acidic pH of 4.5 for solvent production. While the batch mode provides the highest solvent yield, the continuous mode was preferred for butanol yield and productivity. In the continuous and fed-batch fermentations, the time needed for passing acidogenesis to solventogenesis was an intrinsic hindrance to butanol productivity. Therefore, a low dilution rate is suggested for the continuous A–B–E fermentation, while the fed-batch mode is not suggested for solvent production. While 3:6:1 ratio of acetone, butanol, and ethanol is commonly observed from A–B–E fermentation by *C. acetobutylicum* when the pH is uncontrolled, the percentage of butanol among produced solvent was greatly improved by controlling pH at 4.5 in a chemostat.

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References

Bahl, H., Andersch, W., Braun, K., Gottschalk, G., 1982a. Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture. Appl. Microbiol. Biotechnol. 14, 17–20.

Bahl, H., Andersch, W., Gottschalk, G., 1982b. Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Appl. Microbiol. Biotechnol. 15, 201–205.

Barbeau, J.Y., Marchal, R., Vandecasteele, J.P., 1988. Conditions promoting stability of solventogenesis or culture degeneration in continuous fermentations of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 29, 447–455.

Beesch, S.C., 1953. Acetone–butanol fermentation of starches. Appl. Environ. Microbiol. 1, 85–95.

Cardona, C.A., Sánchez, Ó.J., 2007. Fuel ethanol production: process design trends and integration opportunities. Bioresour. Technol. 98, 2415–2457.

Chiao, J., Sun, Z., 2007. History of the acetone–butanol–ethanol fermentation industry in China: development of continuous production technology. J. Mol. Microbiol. Biotechnol. 13, 12–14.

Chin, H., Chen, Z., Chou, C.P., 2003. Fedbatch operation using *Clostridium acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production. Biotechnol. Prog. 19, 383–388.

Dürre, P., Hollergschwandner, C., 2004. Initiation of endospore formation in *Clostridium acetobutylicum*. Anaerobe 10, 69–74.

Ezeji, T.C., Qureshi, N., Blaschek, H.P., 2004. Acetone–butanol–ethanol (ABE) production from concentrated substrate: reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. Appl. Microbiol. Biotechnol. 63, 653–658.

Geng, Q., Park, C., 1993. Controlled-ph batch butanol–acetone fermentation by low acid producing *Clostridium acetobutylicum* B18. Biotechnol. Lett. 15, 421–426.

Gheshlaghi, R., Scharer, J.M., Moo-Young, M., Chou, C.P., 2009. Metabolic pathways of clostridia for producing butanol. Biotechnol. Adv. 27, 764–781.

Ghose, T.K., Tyagi, R.D., 1979. Rapid ethanol fermentation of cellulose hydrolysate I. Batch versus continuous systems. Biotechnol. Bioeng. 21, 1387–1400.

Girbal, L., Vasconcelos, I., Saint-Amans, S., Soucaille, P., 1995. How neutral red modified carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH. FEMS Microbiol. Rev. 16, 151–162.

Gottschal, J.C., Morris, J.G., 1982. Continuous production of acetone and butanol by *Clostridium acetobutylicum* growing in turbidostat culture. Biotechnol. Lett. 4, 477–482.

Huang, W., Ramey, D., Yang, S., 2004. Continuous production of butanol by *Clostridium acetobutylicum* immobilized in a fibrous bed bioreactor. Appl. Biochem. Biotechnol. 115, 887–898.

Hüsemann, M.H.W., Papoutsakis, E.T., 1988. Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic acid and proton concentrations. Biotechnol. Bioeng. 32, 843–852.

Jones, D.T., Woods, D.R., 1986. Acetone–butanol fermentation revisited. Microbiol. Mol. Biol. Rev. 50, 484–524.

Keis, S., Shaheen, R., Jones, D., 2001. Emended descriptions of *Clostridium acetobutylicum* and *Clostridium beijerinckii*, and descriptions of *Clostridium saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov.. Int. J. Syst. Evol. Microbiol. 51, 2095–2103.

Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J., Jung, K.S., 2008a. Fermentative butanol production by clostridia. Biotechnol. Bioeng. 101, 209–228.

Lee, S.M., Cho, M.O., Park, C.H., Chung, Y., Kim, J.H., Sang, B., et al., 2008b. Continuous butanol production using suspended and immobilized *Clostridium beijerinckii* NCIMB 8052 with supplementary butyrate. Energy Fuels 22, 3459–3464.

Li, S.-Y., Srivastava, R., Parnas, R.S., 2010. Separation of 1-butanol by pervaporation using a novel tri-layer PDMS composite membrane. J. Membr. Sci. 363, 287–294.

Luli, G.W., Strohl, W.R., 1990. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl. Environ. Microbiol. 56, 1004–1011.

Maddox, I.S., Steiner, E., Hirsch, S., Wessner, S., Gutierrez, N.A., Gapes, J.R., et al., 2000. The cause of "Acid Crash" and "Acidogenic Fermentations" during the batch acetone–butanol–ethanol (ABE) fermentation process. J. Mol. Microbiol. Biotechnol. 2, 95–100.

Mermelstein, L.D., Welker, N.E., Petersen, D.J., Bennett, G.N., Papoutsakis, E.T., 1994. Genetic and metabolic engineering of *Clostridium acetobutylicum* ATCC 824. Ann. NY Acad. Sci. 721, 54–68.

Mitchell, W.J., 1997. Physiology of Carbohydrate to solvent conversion by clostridia. In: Advances in Microbial Physiology. Academic Press, pp. 31–130.

Modak, J.M., Lim, H.C., Tayeb, Y.J., 1986. General characteristics of optimal feed rate profiles for various fed-batch fermentation processes. Biotechnol. Bioeng. 28, 1396–1407.

Monot, F., Engasser, J.M., 1983. Production of acetone and butanol by batch and continuous culture of *Clostridium acetobutylicum* under nitrogen limitation. Biotechnol. Lett. 5, 213–218.

Monot, F., Engasser, J., Petitdemange, H., 1984. Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 19, 422–426.

Mutschlechner, O., Swoboda, H., Gapes, J.R., 2000. Continuous two-stage ABE-fermentation using *Clostridium beijerinckii* NRRL B592 operating with a growth rate in the first stage vessel close to its maximal value. J. Mol. Microbiol. Biotechnol. 2, 101–105.

Napoli, F., Olivieri, G., Russo, M., Marzocchella, A., Salatino, P., 2010. Butanol production by *Clostridium acetobutylicum* in a continuous packed bed reactor. J. Ind. Microbiol. Biotechnol. 37, 603–608.

Qureshi, N., Meagher, M.M., Huang, J., Hutkins, R.W., 2001. Acetone butanol ethanol (ABE) recovery by pervaporation using silicalite–silicone composite membrane from fed-batch reactor of *Clostridium acetobutylicum*. J. Membr. Sci. 187, 93–102.

Qureshi, N., Blaschek, H.P., 1999. Production of acetone–butanol–ethanol (ABE) by a hyper-producing mutant strain of *Clostridium beijerinckii* BA101 and recovery by pervaporation. Biotechnol. Prog. 15, 594–602.

Rao, G., Mutharasan, R., 1987. Altered electron flow in continuous cultures of *Clostridium acetobutylicum* induced by Viologen dyes. Appl. Environ. Microbiol. 53, 1232–1235.

Roos, J.W., McLaughlin, J.K., Papoutsakis, E.T., 1985. The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of *Clostridium acetobutylicum*. Biotechnol. Bioeng. 27, 681–694.

Salleh, M., Tsuey, L., Ariff, A., 2008. The profile of enzymes relevant to solvent production during direct fermentation of sago starch by *Clostridium saccharobutylicum* P262 utilizing different pH control strategies. Biotechnol. Bioproc. Eng. 13, 33–39.

Schuster, K.C., van, d.H., Gutierrez, N.A., Maddox, I.S., 1998. Development of markers for product formation and cell cycle in batch cultivation of *Clostridium acetobutylicum* ATCC 824. Appl. Microbiol. Biotechnol. 49, 669–676.

Shuler, M.L., Kargi, F., 2001. In: Bioprocess Engineering: Basic Concepts. Prentice Hall, pp. 245–247.

Soni, B.K., Soucaille, P., Goma, G., 1987. Continuous acetone–butanol fermentation: a global approach for the improvement in the solvent productivity in synthetic medium. Appl. Microbiol. Biotechnol. 25, 317–321.

Stephens, G.M., Holt, R., Gottschal, J., Morris, J., 1985. Studies on the stability of solvent production by *Clostridium acetobutylicum* in continuous culture. J. Appl. Microbiol. 58, 597–605.

Wijmans, J.G., Baker, R.W., 1995. The solution–diffusion model: a review. J. Membr. Sci. 107, 1–21.

Woolley, R., Morris, J., 1990. Stability of solvent production by *Clostridium acetobutylicum* in continuous culture: strain differences. J. Appl. Microbiol. 69, 718–728.