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The Use of High Pressure CO_2-Facilitated pH Swings to Enhance in situ Product Recovery of Butyric Acid in a Two-Phase Partitioning Bioreactor

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ABSTRACT: Through the use of high partial pressures of CO_2 (pCO_2) to facilitate temporary pH reductions in two-phase partitioning bioreactors (TPPBs), improved pH dependent partitioning of butyric acid was observed which achieved in situ product recovery (ISPR), alleviating end-product inhibition (EPI) during the production of butyric acid by *Clostridium tyrobutyricum* (ATCC 25755). Through high pressure pCO_2 studies, media buffering effects were shown to be substantially overcome at 60 bar pCO_2, resulting in effective extraction of the organic acid by the absorptive polymer Pebax® 2533, yielding a distribution coefficient (D) of 2.4 ± 0.1 after 1 h of contact at this pressure. Importantly, it was also found that *C. tyrobutyricum* cultures were able to withstand 60 bar pCO_2 for 1 h with no decrease in growth ability when returned to atmospheric pressure in batch reactors after several extraction cycles. A fed-batch reactor with cyclic high pCO_2 polymer extraction recovered 92 g of butyric acid to produce a total of 213 g compared to 121 g generated in a control reactor. This recovery reduced EPI in the TPPB, resulting in both higher productivity (0.65 vs. 0.33 $g\,L^{-1}\,h^{-1}$) and yield (0.54 vs. 0.40). Fortuitously, it was also found that repeated high pCO_2-facilitated polymer extractions of butyric acid during batch growth of *C. tyrobutyricum* lessened the need for pH control, and reduced base requirements by approximately 50%. Thus, high pCO_2-mediated absorptive polymer extraction presents a novel method for improving process performance in butyric acid fermentation, and this technique could be applied to the bioproduction of other organic acids as well.

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KEYWORDS: *Clostridium tyrobutyricum*; butyric acid; two-phase partitioning bioreactor (TPPB); in situ product recovery (ISPR); carbon dioxide; high pressure

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Introduction

The biological production of organic acids as commodity chemical feedstocks represents a sustainable alternative to the use of petrochemicals, and industrial bioproduction is on the increase, with interest in a range of organic acids (Sauer et al., 2008; Weusthuis et al., 2011). To be competitive with petrochemically derived organic acids, cost reduction is a critical consideration (Kurzrock and Weuster-Botz, 2010), and both increased biological performance and improved acid recovery would generate significant benefits, whether through increased productivity, or reduced separation costs. Solid–liquid two-phase partitioning bioreactors (TPPBs) (Daugulis et al., 2011) can be used to achieve both these goals simultaneously, by absorbing an end-product into an inexpensive, easy-to-handle absorptive polymer phase, which is typically added directly to the bioreactor, achieving in situ product recovery (ISPR) (Craig and Daugulis, 2013; Dafoe and Daugulis, 2011; Daugulis, 2004; Khan and Daugulis, 2010; Morrish and Daugulis, 2008; Peterson and Daugulis, 2014; Prpich and Daugulis, 2004). Aside from the ease of recovery afforded by physical separation of solid polymers from fermentation broth, the reduction of end-product concentrations reduces end-product inhibition (EPI) during a fermentation, which in turn can improve productivities, yields and titers.

In the case of organic acids, however, absorptive removal by polymers is particularly challenging. Partitioning will occur only with protonated species of a given acid (Kertes and King, 1986), and thus partitioning improves significantly at lower pH values, which can be problematic for fermentations, as they often require near-neutral pH values during operation. To overcome these mutually exclusive pH values, previous work (Peterson and Daugulis, 2014) focused on the use of CO_2 sparging during butyric acid fermentations with *Clostridium tyrobutyricum* to lower pH values temporarily while recycling reactor contents through a column packed with an absorptive polymer, after which the pH could be readily returned to near neutral values. Unlike reactive extraction

techniques, which employ toxic extractants to recover organic acids, extraction through CO_2-mediated pH swings does not result in cell toxicity (Peterson and Daugulis, 2014) and these swings are easily and quickly reversible (Hepburn and Daugulis, 2012). While previous work has shown that CO_2 at atmospheric pressure makes a significant improvement in polymer extraction over CO_2 free controls, relatively modest extraction was achieved (Peterson and Daugulis, 2014), resulting in no substantial improvement in process performance. This was because while atmospheric CO_2 can quickly achieve low pH values in water, medium components and butyric acid both increased buffering, which in turn prevented the desired pH drop and thus limited subsequent extraction. However, it may be possible that the increased solubility of CO_2 afforded by increased pressures (Dodds et al., 1956) may be able to overcome such buffering from fermentation broth.

High pCO_2-faciliated extraction presents a promising method for improving extraction of organic acids by lowering pH substantially more than is possible under atmospheric conditions due to increases in CO_2 solubility. The relationship between pH and CO_2 partial pressures is well documented (Meyssami et al., 1992), and elevated pressures have been shown to lower pH values to as low as 3.2 in water. However, medium components have been shown to restrict pH swings under high pCO_2 similar to tests under atmospheric conditions, as buffering effects from phosphate (Bortoluzzi et al., 2011) and complex medium additives (Garcia-Gonzalez et al., 2010) have been observed, and minimization of such components has shown increased pH swings (Peterson and Daugulis, 2014). Additionally, the presence of an organic acid has also been shown to restrict pH drops under high pCO_2 (Meyssami et al., 1992). Therefore, if high pCO_2 extractions are to be applied to fermentations, it is important to determine to what extent high pressure can improve pH depression in the presence of medium components, while also determining the effectiveness of such pressures to facilitate butyric acid extraction in a given medium under increasing acid concentrations.

Importantly, additional concerns need to be addressed if ISPR of butyric acid is to be achieved through high pCO_2. Specifically, as the goal of ISPR is to improve process performance, it is essential that any extraction techniques applied do not impart adverse effects on microbial growth or activity. Numerous studies have documented the use of high pressure and supercritical CO_2 for inactivation of various microorganisms (Bertoloni et al., 2006; Debs-Louka et al., 1999; Spilimbergo et al., 2002, 2009), highlighting the importance of such considerations. However, it has been shown that tolerance to high pCO_2 varies between organisms (Jones and Greenfield, 1982), and also that microbial inactivation requires exposure for defined periods of time to be effective (Debs-Louka et al., 1999). Thus, for a given microorganism it may be possible to pressurize fermentation broth for short periods without inflicting serious harm on cells. This is supported by the work of L'italien et al. (1989), who demonstrated that *S. cerevisiae* was capable of repeatedly tolerating 70 bar pCO_2 for 1 h, with an immediate return to ethanol production upon depressurization to atmospheric conditions, suggesting online cyclical pressurization with elevated pCO_2 is feasible in some cases. Overall, if high pCO_2 is to be used as an agent for achieving ISPR, it is paramount that extractive conditions be identified that do no harm to cell growth or process performance.

The objective of this study was to investigate the use of elevated partial pressures of CO_2 during fermentation to overcome buffering and permit improved ISPR of butyric acid. This was achieved through study of the effect of medium components on pH swings, extraction afforded by increasing pressures in medium with or without butyric acid, and cell tolerance to such pressures, followed by integration of high pressure extraction into batch and fed-batch reactors for ISPR demonstration. The techniques outlined herein represent a novel, non-toxic extractive approach to improving fermentation of butyric acid, and may be applied to the production of other organic acids.

Materials and Methods

Organism, Medium, and Materials

C. tyrobutyricum (ATCC 25755) was initially grown on medium described elsewhere (Wu and Yang, 2003) and cryopreserved in 15% glycerol at −75°C until needed. All further tests utilizing medium employed a formula comprised of yeast extract, $5\,g\,L^{-1}$; $(NH_4)_2SO_4$, $3\,g\,L^{-1}$; $MgSO_4{\cdot}7H2O$, $0.6\,g\,L^{-1}$; K_2PO_4, $0.3\,g\,L^{-1}$; $FeSO_4{\cdot}7H2O$, $0.03\,g\,L^{-1}$. Pebax® 2533, an absorptive polymer, was kindly donated by Arkema, Inc. (Colombes, France). All chemicals used in this study were purchased from Fisher Scientific Company, Ltd (Ottawa, Canada).

Polymer Selection

Pebax® 2533 (Arkema, Inc.), a polyether block amide copolymer, was selected as an absorbent phase on the basis of both its chemical affinity for butyric acid as well as its handling properties, as previous work (Peterson and Daugulis, 2014) identified several polyether copolymers demonstrating good affinity for butyric acid. Pebax® 2533, which recovers butyric acid through absorption rather than adsorption, yields a partitioning coefficient of 4.1 for butyric acid (Peterson and Daugulis, 2014). It must be noted that distribution coefficients (D), which are widely discussed in this work, represent dynamic pH-dependent partitioning at a given pH, unlike partitioning coefficients which are constant under acidified conditions.

pH Shifting Under High Pressure and Atmospheric Conditions

To determine what effect high pCO_2 can have on pH reduction, changes to pH were studied in the presence of dibasic phosphate and yeast extract, as previous work had

demonstrated the buffering effects of these medium components (Hepburn and Daugulis, 2012; Peterson and Daugulis, 2014). Direct pH determination at high pCO_2 was achieved by observing the spectra of samples loaded with Bromophenol Blue (10 mg L^{-1}) in a 10 mL stainless steel high-pressure view cell (Parr Instrument Company, Moline, IL) with sapphire cell windows equipped for use in a UV–Vis spectrometer (Toews et al., 1995). A maximum pressure of 60 bar pCO_2 was selected, as this was determined to be the upper limit of CO_2 solubility before reaching critical conditions under moderate temperatures (i.e., 25–37°C) (Dodds et al., 1956), thus providing a maximum theoretical potential for pH reduction. Ten milliliters samples were pressurized for 1 h, with stirring provided by a magnetic stirplate. Concentrations of 0.3 g L^{-1} potassium dibasic phosphate and 5 g L^{-1} yeast extract, which reflect medium composition levels used for fermentations, were tested in RO water to determine achievable pH reductions. Atmospheric CO_2 sparging tests were also performed to provide comparison to high pressure experiments in 5 L Bioflo III reactors with a 2 L working volume (New Brunswick Scientific, Edison, NJ) at 1 VVM CO_2, 200 rpm, and 25°C without pH control to provide similar conditions to that in the pressure vessel for comparison.

High pCO_2-Mediated Extraction of Butyric Acid

To determine the impact of increased pCO_2 and butyric acid extraction from medium, 100 g of Pebax® 2533 was added to a 1 L Parr pressure vessel equipped with an internally threaded sampling tube (Parr Instrument Company) along with 500 mL of medium as described above, which also contained 5 g L^{-1} butyric acid. Three-molar KOH was used to adjust pH to 6.0, as this value reflects optimal pH values maintained in butyric acid fermentation by *C. tyrobutyricum*. The vessel was then sealed and pressurized to 15, 30, 45, and 60 bar pCO_2 and mixed at 500 rpm for 1 h, after which the vessel was drained under pressure, and the final aqueous concentration of butyric acid was determined to ascertain achieved extraction. A similar experiment was performed to determine the extent to which increasing butyric acid concentrations reduced extraction through elevated buffering strength. In this case, extraction at 60 bar pCO_2 at butyric acid concentrations of 5, 10, 15, and 20 g L^{-1} in medium pH-adjusted to 6.0 was tested as described above. All tests were performed in triplicate and compared to untested medium samples as controls.

Culture Conditions

Unless otherwise stated, tests involving *C. tyrobutyricum* were performed with 100 g L^{-1} glucose in 5 L BioFlo III reactors with a 2 L working volume under anaerobic conditions at 37°C, 200 rpm agitation and 0.25 VVM N_2 sparging, while pH was controlled to 6.0 by addition of 3 M KOH and H_2SO_4. All reactors were inoculated with cells grown on 10 g L^{-1} glucose over 18 h first in 150 mL anaerobic serum bottles, then in sealed anaerobic 500 mL shake flasks with closable vents and a spargeline, after which the inoculum was added anaerobically to reactors (10% v/v). For all reactors, when the butyric acid concentrations were determined to remain constant for at least 12 h the earliest time point at this concentration was considered to be the fermentation endpoint.

Cell Tolerance to High pCO_2

To determine tolerance of *C. tyrobutyricum* to 60 bar pCO_2 exposure, a batch reactor was prepared as described above. Once the cells had achieved stationary phase, 750 mL of fermentation broth was anaerobically transferred to an autoclaved 1 L Parr vessel, and pressurized to 60 bar pCO_2. One hundred milliliters aliquots were drawn off under pressure after 1, 2, and 3 h intervals into aseptic anaerobic flasks. These aliquots were then used as inocula (10% v/v) for 24 h serum bottle growth studies using 10 g L^{-1} glucose in medium, and the difference in growth between treatments was used to ascertain how long cells could withstand exposure without affecting cell viability.

To more definitively test the effect that a pressurized extraction regime could exert on the cells, a batch reactor was prepared as described above. Once acid production was observed to be well under way, as indicated by consumption of 250 mL of 3 M KOH, 500 mL of fermentation broth was transferred to a sterilized pressure vessel in the absence of a polymer phase and exposed to 60 bar pCO_2 for 1 h with mixing at 500 rpm. After 1 h the contents of the high pressure vessel were returned to the fermenter and after a rest period of 3 h, the process was repeated, up to a total of four times after which the fermentation proceeded without further disruption. To determine what effect the cyclical pressurization regime exerted on process performance, a batch reactor was ran under identical conditions without pressurization to act as a control.

Batch Mode High pCO_2 Online Extraction of Butyric Acid

To demonstrate butyric acid extraction with an absorptive polymer through use of high pCO_2 cycling during fermentation, a batch reactor coupled to a cyclical high pressure extraction procedure was performed. Once acid production was observed to be well under way, as indicated by consumption of 250 mL of 3 M KOH, 500 mL of fermentation broth was transferred to a sterilized pressure vessel loaded with 300 g Pebax® 2533, and pressurized to 60 bar pCO_2 and mixed at 500 rpm for 1 h, after which the liquid contents were returned to the bioreactor. After this extraction, a rest period of 3 h was allowed, and the extractive procedure was repeated for a total of four extraction cycles. Extractions were commenced once substantial acid production was observed, as indicated by the consumption of 250 mL 3 M KOH for pH control. Within the time frame in which extraction were performed, pH control was turned off, and the pH of the system was monitored. Polymers masses employed in extractions were desorbed sequentially three times for 12 h in 0.25 M KOH under shaking (30% polymer

fraction [w/w]), and butyric acid concentrations at the end of each desorption step were recorded.

Fed Batch pCO_2 Online Extraction of Butyric Acid and Polymer Regeneration

To demonstrate that high pCO_2 extraction can be repetitively performed over the span of extended fermentations, a fed-batch reactor was employed. The reactor was operated in batch mode until glucose values were observed to be less than 10 g L^{-1}, at which point a 500 mL bolus consisting of glucose and medium components was added to yield a working volume of 2.5 L at original glucose and medium concentrations. Cyclical extraction events as described above were initiated once 250 mL of 3 M KOH had been consumed, and three 1-h extractions separated by 3-h resting periods were performed, for a combined extraction window of 9 h. After the initial three extractions were completed, the fermentation proceeded uninterrupted for 15 h, excluding sampling. At this point (i.e., 24 h from commencement of extraction), the same regime of three extractions was repeated, with identical resting times. This 24 h extraction procedure was performed twice more for a total of four 24-h cycles, and represents twelve 1-h extractions over the span of the fermentation. After each extraction, the polymer mass was removed from the pressure vessel, and soaked aseptically in 0.25 N KOH (30% polymer fraction [w/w]) on a shaker for 12 h, after which the alkali desorption solution was sampled and quantified to determine extracted butyric acid. Following this, the polymer mass was rinsed with sterile RO water twice (30% polymer fraction [w/w]) and dried aseptically in a laminar flow hood, after which it was reused for a second round of extractions. To provide a reference for comparison of process performance, a fed-batch reactor was performed under identical conditions without a high pCO_2 extraction regime to act as a control.

Analytical Methods

Aqueous samples were analyzed for glucose, butyric and acetic acid using HPLC (Varian Prostar, Mississauga, ON) with a Varian Hi-Plex H column (300 mm × 7.7 mm) at 60°C with a 10 mM H_2SO_4 mobile phase at 0.7 mL min^{-1}, with either a refractive index detector (PS 356, Varian Prostar) for glucose quantification, or a UV–Vis detector (PS325, Varian Prostar) at 220 nm for quantification of butyric and acetic acid. Cell concentration was measured using optical density at 600 nm, and translated to cell dry weight via a predetermined calibration curve.

Results and Discussion

High pCO_2 Extraction and the Buffering Effect of Medium Components

If higher pCO_2 levels are to be employed for increased polymer extraction, to ensure that pH values are less or equal to the pK_a of butyric acid (4.8) it is important to first characterize how low the pH can be reduced at elevated pressures, compared to reductions achieved through atmospheric gas sparging. As yeast extract and phosphate were earlier identified to be the primary sources of buffering in the medium used here, concentrations of these two components reflecting medium composition were tested at 60 bar. As can be seen in Figure 1, the pH values achieved for both yeast extract (pH 3.8) and phosphate (pH 3.2) under 60 bar pCO_2 are significantly lower than respective values of 5.2 and 4.7, demonstrating that elevated pCO_2 improves the extent of pH swings, which indicates that the use of high partial pressures of CO_2 could improve pH-dependent extraction. This pH dependency is determined by pK_a, and only pH values near or below this value will demonstrate appreciable uptake by an absorptive polymer. Thus, as the pK_a of butyric acid is 4.8, significant partitioning could be expected in the presence of 5 g L^{-1} yeast extract and 0.3 g L^{-1} phosphate.

To quantify the relationship between pressure and polymer absorption, tests were performed at increasing pCO_2 values as shown in Figure 2A, which plots distribution coefficients achieved after 1 h against increasing pressure conditions, and it is apparent that a direct relationship exists between pCO_2 and uptake, with results rising to a maximum value at 60 bar pCO_2 ($D = 2.4 \pm 0.1$). Thus, as 60 bar pCO_2 provided the highest possible polymer extraction without risk of reaching undesirable supercritical conditions, this level of pCO_2 was selected for online butyric acid extraction.

However, as has been previously noted (Peterson and Daugulis, 2014), butyric acid also contributes buffering strength to fermentation medium, potentially limiting the extent to which pH can be reduced by CO_2 sparging. Thus, as a fermentation proceeds and butyric acid accumulates, a decrease in distribution coefficients would be anticipated. Figure 2B demonstrates distribution coefficients achieved at

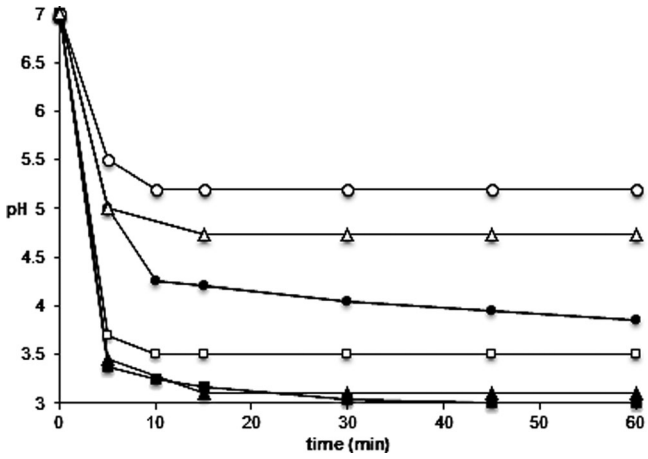

Figure 1. pH values achieved with pCO_2 in water (squares), 5 g L^{-1} yeast extract (circles) or 0.3 g L^{-1} phosphate (triangles) at through pressurization to 60 bar pCO_2 (closed symbols) or through sparging at atmospheric pressures (open symbols).

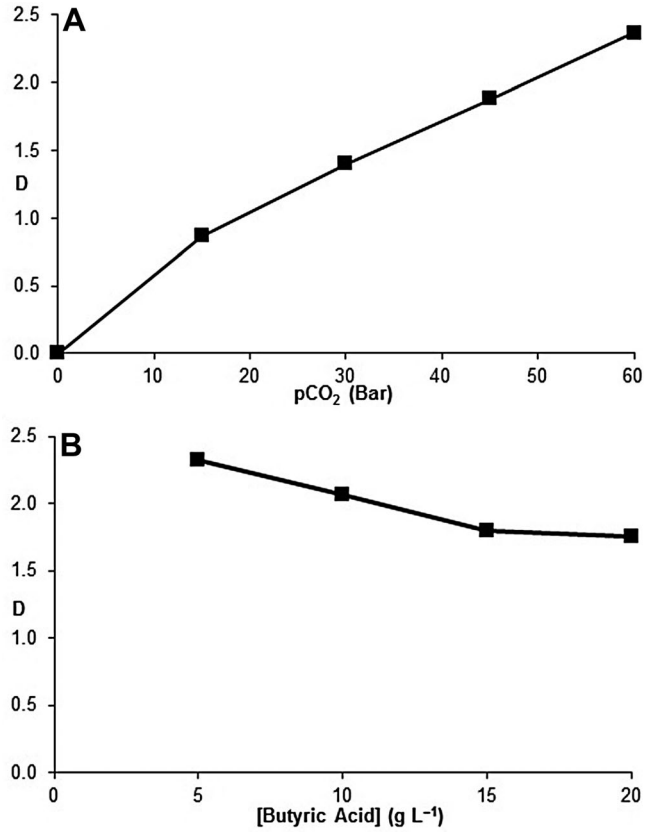

Figure 2. The effect of (**A**) pCO_2 on distribution coefficients for 5 g L^{-1} butyric acid (**B**) butyric acid concentration on distribution coefficients for butyric acid at 60 bar pCO_2. All tests were performed with Pebax® 2533 as the absorptive polymer phase.

60 bar pCO_2 as a function of butyric acid concentration, and it can be seen that D decreases with increasing acid concentration, and this effect was most pronounced with 20 g L^{-1} butyric acid, yielding a distribution coefficient of 1.8 ± 0.03. However, such reductions do not critically limit butyric acid removal, as D represents the ratio of butyric acid between polymer and aqueous phases, and thus at higher acid concentrations, although a reduced ratio of uptake is achieved, the higher amounts of butyric acid provides a higher concentration driving force for equilibrium-based partitioning. For example, in the above-described experiments, while 60 bar pCO_2 in the presence of 5 g L^{-1} butyric acid removes 0.8 g acid ($D = 2.4$), in the presence of 20 g L^{-1} butyric acid 2.6 g L^{-1} ($D = 1.8$) is absorbed. Thus, while proportionally less acid is recovered at increasing concentrations, the overall amount of butyric acid absorbed by the polymer is increased, demonstrating that the buffering effect of the acid is outweighed by the increased driving force provided by elevated concentrations. Therefore, high pCO_2 polymer extractions can be performed over a seemingly wide range of butyric acid concentrations, and will not lose effectiveness as butyric concentration rise toward the end of fermentations.

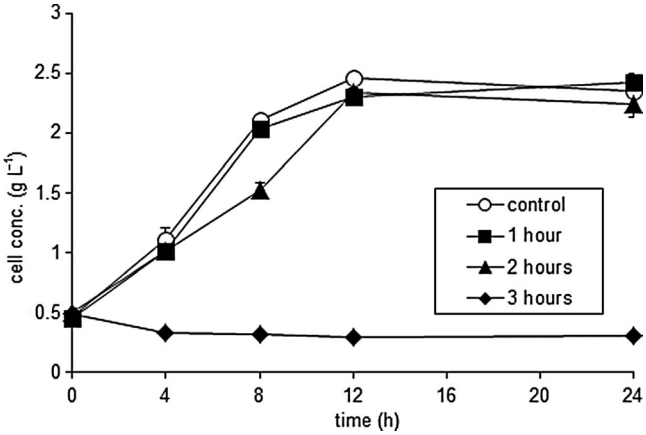

Figure 3. Cell tolerance to 60 bar pCO_2, as determined by subsequent growth after 1 h (squares), 2 h (triangles), or 3 h (diamonds) exposures, compared to a non-pressurized control (open circles).

Cell Tolerance to High pCO_2

While it has been demonstrated that elevated pCO_2 results in increased distribution coefficients, the impact of increased pressure on cell viability and performance are of critical importance, if such an extraction process is to be applied to achieve ISPR in fermentations. Figure 3 shows OD growth curves for serum bottles inoculated with cells exposed to 60 bar pCO_2 for 1, 2, or 3 h and it can be seen that no difference was observed between a 1 h exposure and a non-exposed control, while a 2 h exposure showed a slight decrease in subsequent cell growth. A 3 h exposure however failed to show signs of growth after 24 h, indicating that *C. tyrobutyricum* cannot withstand 60 bar pCO_2 indefinitely. These results suggest that elevated pressure intervals of 1–2 h could be performed during a fermentation without deleterious effect. Figure 4B shows a batch reactor which was subjected to repeated 1 h pressurization cycles in the absence of polymer alongside a control run (Fig. 4A) operated at atmospheric pressure, to investigate what effect such a cycling regime would exert on an actual fermentation. As can be seen in Table I, which summarizes the performance shown in Figure 4A and B, no differences were observed between the batch control and the cyclically pressurized batch run in terms of butyric acid titer (68–72 g L^{-1} butyric acid), yield (0.35–0.37 g butyric acid produced/g glucose consumed) or productivity (0.50 g L^{-1} h^{-1}). However, the selectivity for butyric acid in the cyclically pressurized reactor was somewhat lower at 0.74 (g butyric acid/g total acid) compared to 0.84 in the control as a result of increased acetic acid by-product formation. This result is not unanticipated, as studies have shown selectivity is decreased at pH values lower than 6 (Zhu and Yang, 2004). However, if the extraction of butyric acid confers marked advantages, it is possible that the benefits of such a process could outweigh this disadvantage. Regardless, these results confirm that high

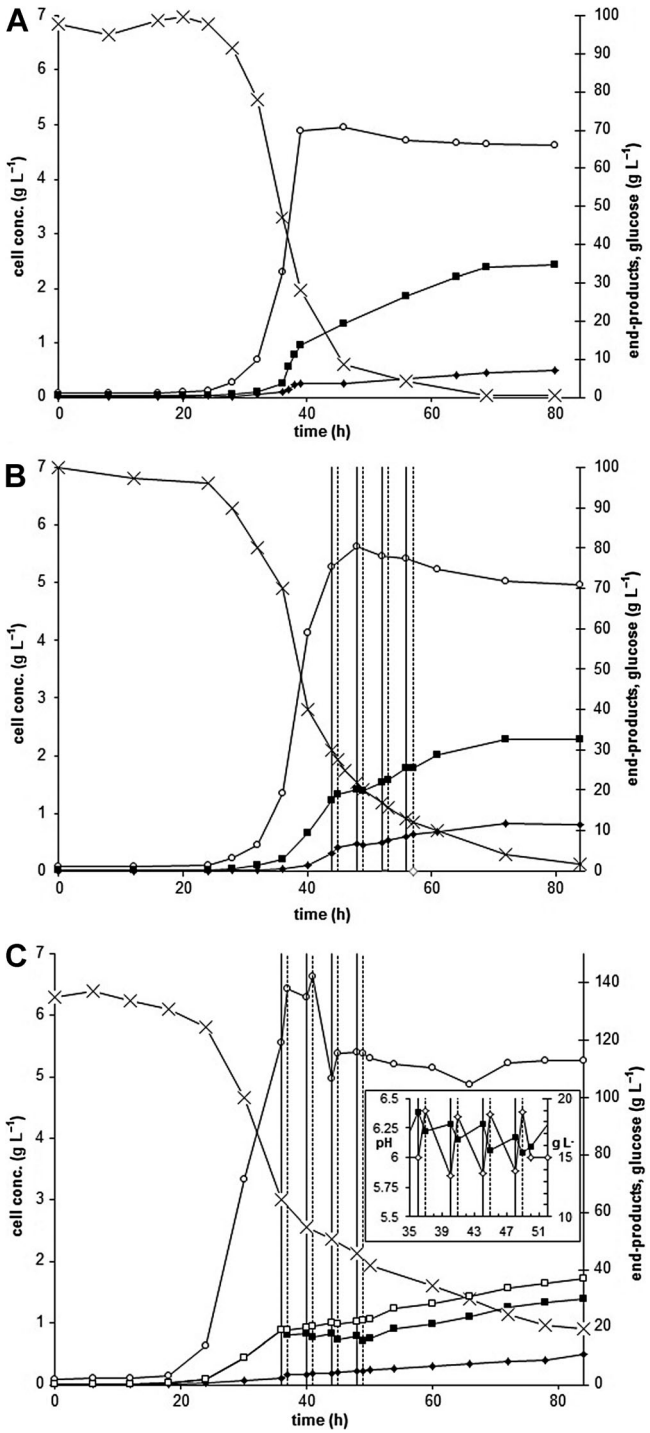

Figure 4. Batch fermentation of butyric acid from *C. tyrobutyricum* under (**A**) conventional culture techniques, (**B**) cyclical pressurization at 60 bar pCO_2, or (**C**) cyclical pressurization and use of an absorptive polymer to achieve ISPR. Open circles represent OD, solid squares represent aqueous butyric acid, open squares represent butyric acid including extracted acid, solid diamonds represent acetic acid, crosses represent glucose, and open diamonds represent pH (inset). Dashed vertical lines indicate CO_2 sparging initiation and solid vertical lines represent CO_2 sparging termination.

pCO_2 extractions as outlined herein do not adversely affect cell viability, or process performance.

Batch Reactor Extraction and pH Effects

A batch reactor coupled with a polymer extraction regime facilitated by high pCO_2 pH swings was performed as shown in Figure 4C, and it is apparent that butyric acid concentrations in the reactor were decreased by extraction events at 36, 40, 44, and 48 h (see inset Fig. 4C). These decreases in butyric acid concentration coincide with the amount of butyric acid recovered from the polymer phases (Table II), which on average removed 3.5 ± 0.1 g butyric acid, for a total of 14.1 g butyric acid recovered, which combined with a final aqueous concentration of 30 g L^{-1} in 2 L yields 74 g of butyric acid. It is interesting to note that during polymer desorptions approximately 90% of acid recovery was achieved in the first of three sequential 12 h alkali soakings (Table II), suggesting that a single stage desorption of the acid may be sufficient for polymer regeneration and reuse within the time frame of a fermentation. Thus, absorptive polymer extraction mediated through high pCO_2-mediate pH swings was clearly achieved during the batch fermentation shown in Figure 4C. However, total butyric acid produced was similar in both the control and the polymer-free pressurized runs, which produced 68.4 and 72.4 g butyric acid, and all three runs had yields of 0.35–0.37 g butyric acid/g glucose consumed. Also, a decrease in selectivity (0.78) was observed similar to polymer-free pressurized runs, but this decrease was diminished, likely as a result of butyric acid removal, which may affect end-product ratios.

An additional interesting observation arising from this extractive fermentation was the effect that butyric acid extraction exerted on reactor pH values. Specifically, between the first and last extraction cycles shown in Figure 4C the pH control was shut off and the pH was maintained exclusively by butyric acid removal. As can be seen in the inset of Figure 4C, reactor pH values initially dropped to 5.8 due to ongoing acid production in the bioreactor during extraction, but increased to 6.3 after extracted broth was returned to the reactor as a result of decreased butyric acid concentration through polymer absorption. This phenomenon of pH control through acid recovery has been reported previously for both the production of butyric (Wu and Yang, 2003) and lactic acid (Ataei and Vasheghani-Farahani, 2008) through use of reactive extraction and ion-exchange resin techniques respectively, but to date this effect has not been demonstrated using absorptive polymers in TPPBs. The major benefit arising from acid extraction is apparent by examination of the total based added during the fermentations, as seen in Table I, which shows that the extractive batch run utilized 0.44 g KOH per g butyric acid produced, compared to 0.86 g KOH per g butyric acid in the control, which translates into an almost 50% reduction of base required. Furthermore, the intentional omission of pH control during the extractive batch run (Fig. 4C) did not seem to affect process performance when

Table I. Parameters from batch and fed-batch fermentations of butyric acid by *C. tyrobutyricum* compared to treatments with 60 bar pCO_2 pressure cycling and absorptive polymer extractions.

	Batch			Fed-batch	
Parameter	Control	Pressure only polymer-free	Polymer ISPR	Control	Polymer ISPR
Aqueous butyric acid (g)	68.4	72.4	60	128	121
Butyric acid extracted (g)	—	—	14	—	92
Total butyric acid (g)	68.4	72.4	74	128	213
Acetic acid (g)	12.8	26	21	26	56
$Y_{P/S}$ (g butyric/g gluc.)	0.35	0.37	0.35	0.40	0.54
Productivity (g/h)	0.50	0.50	0.44	0.33	0.65
Selectivity (g butyric/g tot. acid)	0.84	0.74	0.78	0.83	0.79
g base/g butyric acid	0.86	0.79	0.44	0.67	0.62

compared to the pressurized polymer-free batch run (Fig. 4B), which also employed conventional pH control. This reduction would also decrease ion accumulation and the associated osmotic stress, and thus could potentially improve performance if optimized, especially over longer fermentations. Regardless, while overall this extractive regime achieved online extraction and lead to the simultaneous elimination of pH control, the lack of improvement in reactor performance indicates that such removal did not confer the benefits usually associated with ISPR. This is likely due to insufficient removal of product coupled with late stage extraction, as the alleviation of EPI achieved through this removal does not leave sufficient time for cells to benefit from reduced end-product toxicity. However, it is clear that extraction was achieved through use of high pressure pCO_2 and absorptive polymers, and more frequent extractive regimes utilizing higher polymer fractions, along with culture techniques employing higher substrate loadings could be applied to potentially increase butyric acid removal and improve reactor performance.

Fed-Batch Reactor Extraction

Extractive fed-batch techniques were subsequently employed to ensure that sufficient substrate and nutrients were available for extended fermentation after extraction, and as can be seen in Figure 5, a fed-batch reactor coupled with high pressure pCO_2 polymer extraction was performed alongside a fed-batch control reactor. This extraction regime was similar to that employed for the above mentioned extractive batch reactor, except that 12 extractions were performed, compared to four extractions performed in batch. These 12 extractions were distributed into four groups over the span of the fermentation, which was replenished with substrate and nutrients after 68 h, and the results are displayed in Figure 5B. Also similar to the extractive batch reactors, extraction cycles demonstrated a clear reduction in aqueous butyric acid concentrations, as can be seen in Figure 4B at 36–44, 60–68, 84–92, and 108–116 h. Further confirming the successful extraction of butyric acid, post-extraction polymer desorptions yielded recovered butyric acid and distribution coefficients as reported in Table III. As anticipated, due to the increased concentration driving force arising from higher accumulated levels of butyric acid, the butyric acid absorbed by the polymers increased during the course of the fermentation, with the averages of each group of three extractions yielding recovered butyric acid values of 5.9 ± 0.8, 6.8 ± 0.4, 8.0 ± 1.0, 10.1 ± 1.0 g. Thus, even though it has been demonstrated that higher butyric acid concentrations increase medium buffering and reduce the distribution coefficient for a given extraction, the elevated concentrations translate into a higher driving force, with more acid being extracted overall despite diminished distribution ratios. It is also interesting to note a single step alkali desorption was successfully employed for regeneration of polymer beads in a rapid manner, permitting their reuse for further extractions while demonstrating no obvious negative effects on extraction performance.

As seen in Table I, a total of 92 g butyric acid was extracted during the course of the fed-batch fermentation, which

Table II. Butyric acid recovery from three sequential 12 h polymer desorptions in 0.25 M KOH after high pCO_2 polymer extractions during batch fermentation of butyric acid.

		Desorption #1		Desorption #2		Desorption #3		
Extraction #	t (h)	Butyric (g)	% rec.	Butyric (g)	% rec.	Butyric (g)	% rec.	Total butyric acid (g)
1	36	3.2	88.9	0.3	8.3	0.1	2.8	3.6
2	40	3.2	88.9	0.3	8.3	0.1	2.8	3.6
3	44	3.2	88.8	0.3	8.4	0.1	2.8	3.6
4	48	3.0	88.2	0.3	8.9	0.1	3.0	3.4

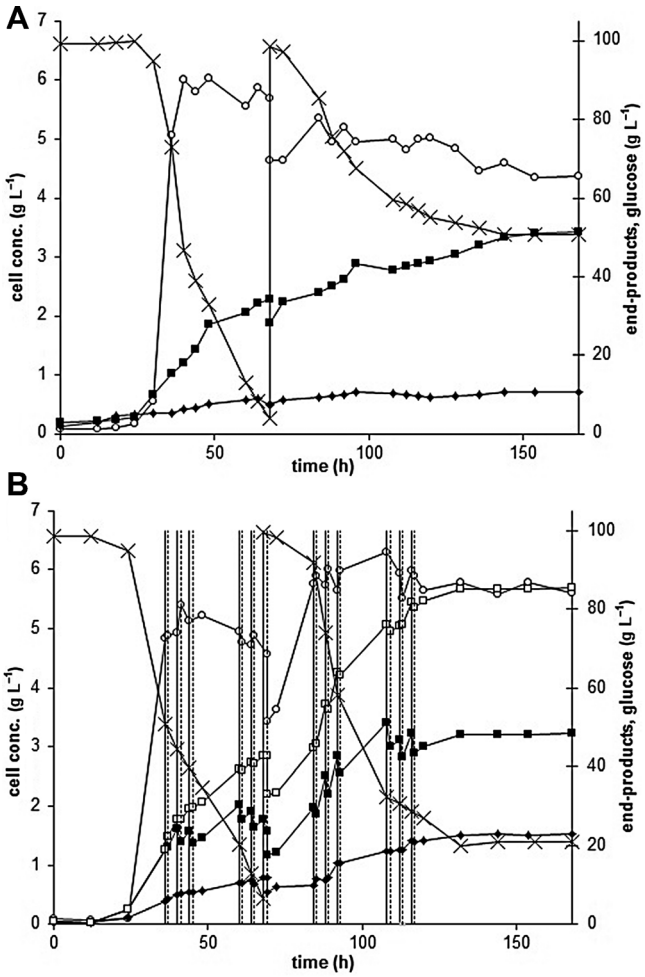

Figure 5. Fed-batch fermentation of butyric acid from *C. tyrobutyricum* under (**A**) conventional culture techniques or (**B**) cyclical pressurization and use of an absorptive polymer to achieve ISPR. Open circles represent OD, solid squares represent aqueous butyric acid, open squares represent butyric acid including extracted acid, solid diamonds represent acetic acid, and crosses represent glucose. Dashed vertical lines indicate CO_2 sparging initiation and solid vertical lines represent CO_2 sparging termination.

Table III. Total butyric acid recovered and estimated distribution coefficients (*D*) from single 12 h desorptions in 0.25 M KOH after high pCO_2 polymer extraction intervals during fed-batch fermentations.

Extraction	Time of extraction (h)	Acid recovered (g)	*D*
1	36	4.9	1.8
2	40	6.4	1.9
3	44	6.3	1.9
4	60	7.2	1.5
5	64	7.0	1.6
6	68	6.4	1.5
7	84	7.0	1.5
8	88	8.0	1.2
9	92	9.0	1.2
10	108	10.7	1.2
11	112	10.6	1.4
12	116	8.9	1.0

represented 43% of total acid produced, compared to 19% of total acid produced by the extractive batch run, clearly demonstrating the advantage of an increased number of extractions and higher acid concentrations afforded through fed-batch operation. Overall, the extractive fed batch system had superior performance to the control fed batch reactor, whether in total acid produced (212 vs. 128 g), yield (0.54 vs. 0.40 g butyric acid/g glucose consumed), or productivity (0.65 vs. 0.33 $g L^{-1} h^{-1}$). Although, as noted, the extractive batch run in this study was operated without pH control, the fed-batch run was performed using traditional pH control, to clearly demonstrate that any benefits are a result of EPI rather than reduced osmotic stress, and thus further improvements to this extractive fed-batch technique could be made through utilization of extraction to control pH. The approach used here represents a semi-continuous removal system for butyric acid, and could potentially extend fermentation times not only through batch feeding coupled with a regular extraction regime, but also through the reduction or elimination of pH control and subsequent ion accumulation, while also potentially reducing water use. The use of high pCO_2-mediated absorptive polymer extractions has been demonstrated to be an effective ISPR method that confers significant benefits on fermentation performance through alleviation of end-product inhibition.

Conclusion

Elevated pCO_2 significantly increases the reversible pH drop of fermentation medium with subsequent improvements in pH-dependent partitioning of butyric acid, and a direct relationship exists between pCO_2 and distribution coefficients up to 60 bar pCO_2. In contrast to atmospheric pressure conditions, it has also been shown that medium components and typical end-products do not prevent CO_2-mediated extraction, and effective removal can be performed. Furthermore, it has been demonstrated that cultures of *C. tyrobutyricum* can tolerate repeated 1 h cyclical exposures to 60 bar pCO_2. Multiple extractions and high substrate loadings achieved through fed-batch operation have been shown to significantly increase overall production, yields and titers of butyric acid generated by *C. tyrobutyricum*, and these benefits are clearly a result of alleviation of EPI. Through utilization of online extraction to control pH, it may also be possible to further increase productivity through reduced ion accumulation and osmotic stress over longer fermentations, and optimization studies of extraction-mediated pH control would be merited. Finally, for the first time a TPPB utilizing synchronous polymer regeneration and reutilization has been employed, and this work also points to the feasibility of the TPPB platform for producing other hydrophilic bioproducts such as organic acids.

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