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BIOTECHNOLOGY and BIOENGINEERING

Prolonged Conversion of *n*-Butyrate to *n*-Butanol with *Clostridium saccharoperbutylacetonicum* in a Two-Stage Continuous Culture with in-situ Product Removal

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Received 11 July 2011; revision received 11 October 2011; accepted 10 November 2011

Published online 17 November 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.24380

ABSTRACT: *n*-Butanol was produced continuously in a two-stage fermentor system with integrated product removal from a co-feed of *n*-butyric acid and glucose. Glucose was always required as a source of ATP and electrons for the conversion of *n*-butyrate to *n*-butanol and for biomass growth; for the latter it also served as a carbon source. The first stage generated metabolically active planktonic cells of *Clostridium saccharoperbutylacetonicum* strain N1-4 that were continuously fed into the second (production) stage; the volumetric ratio of the two fermentors was 1:10. *n*-Butanol was removed continuously from the second stage via gas stripping. Implementing a two-stage process was observed to dramatically dampen metabolic oscillations (i.e., periodical changes of solventogenic activity). Culture degeneration (i.e., an irreversible loss of solventogenic activity) was avoided by periodical heat shocking and re-inoculating stage 1 and by maintaining the concentration of undissociated *n*-butyric acid in stage 2 at 3.4 mM with a pH-auxostat. The system was successfully operated for 42 days during which 93% of the fed *n*-butyrate was converted to *n*-butanol at a production rate of 0.39 g/(L × h). The molar yields $Y_{n\text{-butanol}/n\text{-butyrate}}$ and $Y_{n\text{-butanol/glucose}}$ were 2.0, and 0.718, respectively. For the same run, the molar ratio of *n*-butyrate to glucose consumed was 0.358. The molar yield of carbon in *n*-butanol produced from carbon in *n*-butyrate and glucose consumed ($Y_{n\text{-butanol/carbon}}$) was 0.386. These data illustrate that conversion of *n*-butyrate into *n*-butanol by solventogenic *Clostridium* species is feasible and that this can be performed in a continuous system operating for longer than a month. However, our data also demonstrate that a relatively large amount of glucose is required to supply electrons and ATP for this conversion and for cell growth in a continuous culture.

Biotechnol. Bioeng. 2012;109: 913–921.

KEYWORDS: ABE fermentation; butanol; carboxylate conversion; solventogenic *Clostridium* species; pH auxostat; gas stripping

Introduction

We have recently proposed a novel process for biological conversion of lignocellulosic biomass into the biofuel *n*-butanol (Angenent and Wrenn, 2008). In a first step, the lignocellulose is converted to *n*-butyrate in a mixed-acid fermentation, instead of enzymatically hydrolyzing the cell wall polymers into sugar monomers and fermenting those directly to *n*-butanol (Lee et al., 2008b). In a second step, the *n*-butyrate is subsequently converted to *n*-butanol using a pure culture of *Clostridium*. Rather than generating methane, mixed-acid fermentation with undefined mixed cultures can be maintained by inhibiting the methanogens responsible for converting the intermediate organic acids into methane. Likewise, operating conditions can be set to

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Contract grant sponsor: National Institutes of Food and Agriculture (NIFA)

Contract grant number: 2007-35504-05381

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select for *n*-butyrate production at the expense of other fermentation products (Agler et al., 2011). This process has several advantages over the direct sugar route including: (i) hydrolysis does not require added enzymes; (ii) microbial contamination is not a concern; and (iii) fermentation occurs with hexose and pentose mixtures.

Here, we studied the second step: the conversion of *n*-butyrate into *n*-butanol. Several studies have been published that championed the co-feeding of *n*-butyrate and glucose to increase the performance of the ABE fermentation and the yield of *n*-butanol obtained from sugar fermentation (Table I). The focus of the current study is on the biological conversion of *n*-butyrate into *n*-butanol, instead of on the fermentation of sugars. Adding sugars is necessary but unwanted to the conversion process and our goal is to minimize the use of monomeric sugars for economical and environmental reasons. We continuously converted *n*-butyrate and glucose into *n*-butanol for over a month by establishing a continuous fermentation system for *n*-butyrate conversion by integrating a two-stage fermentation system with gas stripping, while removing the toxic end product *n*-butanol in a more concentrated form. Our conversion rates were high and stripping versus distillation could benefit the overall economics of ABE fermentation (Ezeji et al., 2004).

Nomenclature

Often, *n*-butyrate and *n*-butyric acid are used generally without specifically referring to the state of protonation. Here, we use: "*n*-butyrate" for general use regardless of protonation; "*n*-butyric acid" for the feedstock substrate (>99% purity); "UBA" (undissociated *n*-butyric acid) for only the protonated species; "*n*-butyrate anion" for only the unprotonated species; and "total *n*-butyrate" for the combination of the protonated and unprotonated species. We used the term "metabolic oscillations" to indicate periodical changes of solventogenic activity, while we used "culture degeneration" to describe the irreversible loss of solventogenic activity. We used the term "planktonic cells" to differentiate our study with free floating cells from studies that use a matrix on which bacteria are immobilized and form biofilms.

Background Information

n-Butyrate with a pK_A value of 4.8 is an inhibitor to microbial metabolism and growth (Freese and Sheu, 1973), especially at a pH value below 4.8, where the ratio of UBA/*n*-butyrate anion is high. UBA can freely diffuse across the cell membrane. Once UBA enters the cytoplasm, where the pH is usually one to two units above the extracellular environment (Gottwald and Gottschalk, 1985; Huesemann and Papoutsakis, 1988), protons are released by dissociation. *n*-Butyrate is trapped inside the cell, and the intracellular pH decreases, which can have a negative impact on enzyme activities (Huesemann and Papoutsakis, 1988; Ismaiel et al., 1993). Second, the membrane potential, which is coupled to vital cell processes, is dissipated by the scalar movement of protons into the cytoplasm (Herrero et al., 1985). Therefore, it is mainly the UBA that is toxic to the cells, and it is the UBA concentration in the medium that is relevant to trigger *n*-butanol formation, rather than the concentration of total *n*-butyrate or the concentration of the less hydrophobic acetic acid (Bahl et al., 1982a; Holt et al., 1984; Huesemann and Papoutsakis, 1988, 1990; Maddox et al., 2000; Matta-El-Ammouri et al., 1987; Monot et al., 1984; Tashiro et al., 2004). For a continuous process, the operator must find a suitable UBA concentration for the specific bacterial strain and conditions, that, on one hand, is high enough to trigger solventogenesis, while, on the other hand, is as low as possible to minimize its negative impact on metabolism, cell viability, and reactor performance, and to minimize its loss into the waste.

Materials and Methods

Bacterial Strains

All continuous-culture experiments were conducted with *Clostridium saccharoperbutylacetonicum* type-strain N1-4 (ATCC number 27021). Other strains of solventogenic *Clostridium* species used in this study and their sources are listed in Table SI.

Media

All chemicals were obtained from Sigma–Aldrich, St. Louis, MO (unless specified differently). When pH adjustments were necessary, a 2-M aqueous KOH solution was used. Potato glucose (PG) medium was prepared in the same manner and composition as previously described (Alalayah et al., 2008), except that instead of filtering the medium with cotton cloth after boiling, we let the suspended particles settle in a graduated cylinder at 4°C overnight. Supernatant was aliquoted in 10-mL or 50-mL volumes in anaerobic serum bottles, sparged with nitrogen, sealed with blue butyl-rubber stoppers (Bellco Glas, Vineland, NJ), and autoclaved, resulting in a clear medium. P2 medium (Qureshi and Blaschek, 1999) contained 1 g/L Difco yeast extract (BD, Franklin Lakes, NJ) and 60 g/L glucose. After autoclaving, each 1 vol% of the following filter-sterilized solutions were added: 100 × acetate buffer (50 g/L KH_2PO_4, 50 g/L K_2HPO_4, and 220 g/L NH_4CH_3COO), 100× vitamin solution (0.1 g/L para-amino-benzoic acid, 0.1 g/L Thiamine, and 0.001 g/L Biotin), and 100× mineral solution (20 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L $MnSO_4{\cdot}7H_2O$, 1 g/L·$FeSO_4{\cdot}7H_2O$, and 1 g/L·NaCl). Tryptone-yeast extract-acetate (TYA) medium (Tashiro et al., 2004) contained 60 g/L glucose, 2 g/L yeast extract, and 6 g/L tryptone from casein. After autoclaving, each 1 vol% of

Table I. Comparison of parameters obtained from the literature regarding continuous ABE fermentations as single- or two-stage cultures, with glucose +/− *n*-butyric acid.

Set-up	g/L Substrate glucose: *n*-butyrate	pH	D (1/h)	*n*-Butanol productivity (mg/(L × h))	$Y_{butanol/carbon}$	*n*-Butanol in reactor (g/L)	*Clostridium* strain	Stability of solventogenesis/comments	Reference
Cont. cult; suspended cells	40:0	4.8	0.060	740	0.368	9.0	ATCC824	2 months stable	Fick et al. (1985)
Cont. culture, susp. Cells; gas stripping	1163:0	5.0	0.012	910 (cont.)	0.351	6.8 ± 5.0	BA101	500 h operation with strong oscillations	Ezeji et al. (2005b)
Cont. culture; susp. Cells; +/− *n*-butyrate	3.6:0, 3.6:5.28	4.3	0.133	Glucose concentrations too low for good productivity	0.0337 without *n*-butyrate	8.1	ATCC824	Culture stable, but no duration time reported; *n*-butyrate consumption not reported, 20–80 mM *n*-butyrate at pH4.3 triggered solventogenesis	Bahl et al. (1982a)
Cont. cult; susp. cells + *n*-butyrate	9–36:1.23	4.5–6.3	0.056	ND	ND	ND	LMD 27.6	Culture unstable at all pH values; 14 mM *n*-butyrate did not trigger solventogenesis at pH 5.25.	Joebses and Roels (1983)
Two-stage cont. culture, suspended cells	54:0	4.3 (1), 4.3 (2)	Dl = 0.125, D2 = 0.030	377	0.379	12.6	ATCC 824	1 year stable	Bahl et al. (1982b)
Two-stage cont. culture, suspended cells	60:0	4.5 (1), 5.0 (2)	Dl = 0.080, D2 = 0.040	56	0.344	12.7	ATCC 824	1 month stable run	Godin and Engasser (1990)
Two-stage cont. culture, suspended cells	60:0	6.0 (1), 4.5 (2)	Dl = 0.075, D2 = 0.060	400	0.160	5.93	ATCC 824	1 month stable run	Lai and Traxler (1994)
Two-stage cont. culture, suspended cells	60:0	4.7 (1), 5.1 (2)	Dl = 0.120, D2 = 0.022	27 (total solvents)	0.246	9.1	B592	1,600 h stable	Mutschlechner et al. (2000)
Two-stage cont. culture; immobilized cells; pervaporation	60:0	4.7 (1), 4.7 (2)	Dl = 0.550, D2 = 0.180	1240 (total solvents)	0.241	9.3 (total solvents)	B592	2,500 h stable; 30% concentration buildup did not cause detrimental effects	Gapes et al. (1996)
Cont. culture; susp. or immobil. cells + *n*-butyrate	60:1.58	5.0	0.040	220 (susp.), 400(immob.)	0.388 susp., 0.713 immobil.	7.1 susp., 13.4 immobil.	NCIMB 8052	300 h stable operation; 18 mM *n*-butyrate prevented degeneration	Lee et al. (2008a)
Cont. culture, immobil. cells + *n*-butyrate	54:3.5	4.3	0.900	4600	0.68	5.1	ATCC 824	1,000 h stable; *n*-butyrate was not considered when calculating $Y_{n\text{-butanol/glucose}}$	Huang et al. (2004)
Batch culture, nongrowing cells + *n*-butyrate	20:10	6.5	N.A.	N.D.	0.577	8.6	Nl-4	Highest butyrate: glucose ratio in the literature	Tashiro et al. (2007)
Two-stage cont. culture; suspended cells + *n*-butyrate, gas stripping	60:10.5	5.2	0.025	393	0.388	4.0 (15.7 total)	Nl-4	1,000 h stable	This study

D, dilution rate.
Clostridium species were either freely suspended or immobilized.

the following sterile solutions was added: 300 g/L·NH_4CH_3COO, 50 g/L·KH_2PO_4, 30 g/L·$MgSO_4 \cdot 7H_2O$, and 1 g/L·$FeSO_4 \cdot 7H_2O$.

Culture Conditions

All *Clostridium* strains were grown at 35°C. Spore solutions of *Clostridium* strains were obtained by keeping overnight batch cultures in anaerobic serum bottles on the bench at room temperature (20–25°C) for 14 days. Next, these cultures were kept at 4°C. Precultures were inoculated from spore solution (5 vol%), heat shocked in a water bath at 75°C for 1 min, cooled in ice-water for 1 min, and grown overnight in anaerobic culture tubes with 10 mL of PG medium and nitrogen headspace. Batch cultures were inoculated with 5 vol% of preculture, and grown at an initial pH of 6.0 in 100 mL of either P2 medium or TYA medium, in anaerobic serum bottles (60-mL headspace) connected via sterile needles (BD, 23 gauge) and sterile filters (Pall Corporation, Port Washington, NY; 200-μm pore size) to fermentation airlocks to avoid pressure build-up during gas production. P2 medium was used for growth of *C. beijerinckii* strain P260, for the strain comparison (serum bottles), and for all batch-culture experiments in serum bottles with *n*-butyrate. TYA medium was used for *C. saccharoperbutylacetonicum* strain N1-4, except if otherwise specified. Batch cultures (in serum bottles and fermentors) were usually incubated for 4 days, except when high concentrations of *n*-butyrate were added and slow growth required a longer period of incubation. The fermentor used in our studies was a 2-L Bioflo & Celligen 310 fermentor/bioreactor with a 1-L working volume (New Brunswick Scientific, Edison, NJ). The agitation speed was 50 rpm, and foam development was controlled through a foam sensor by adding 100 × diluted antifoam 204 solution when necessary. The pH was controlled at variable levels by addition of 2-M potassium hydroxide or 2-M hydrochloric acid or in case of pH auxostat operation by addition of >99% *n*-butyric acid.

Continuous cultures were grown in the same fermentor, but connected to a 5-L medium reservoir and a 5-L waste vessel. The dilution rate in the 1-L fermentor was always 0.025/h. For two-stage continuous cultures (Fig. 1), a custom made 250-mL jacketed glass fermentor (stage 1) was introduced to the system. It was placed on a magnetic stirrer at 200 rpm, and connected in between the medium reservoir and the main fermentor (stage 2). The working volume was 100 mL, and therefore the dilution rate was 10 × higher than stage 2. *n*-Butyric acid was initially added with the glucose from the medium reservoir at varying concentrations (see results), but in our final system, *n*-butyric acid was added to stage 2 only through a pH auxostat operation that was triggered at pH > 5.2, thereby maintaining an UBA concentration of ~0.3 g/L. Furthermore, in the final system, the stage 1 fermentor was heat shocked in 5-day intervals at 65°C for 1 h and re-inoculated (5 vol%) from an overnight preculture. Cultures were regularly checked for contamination via visual control using a Labophot phase-contrast microscope (Nikon Inc., Melville, NY) with an oil immersion objective and 1,000× magnification.

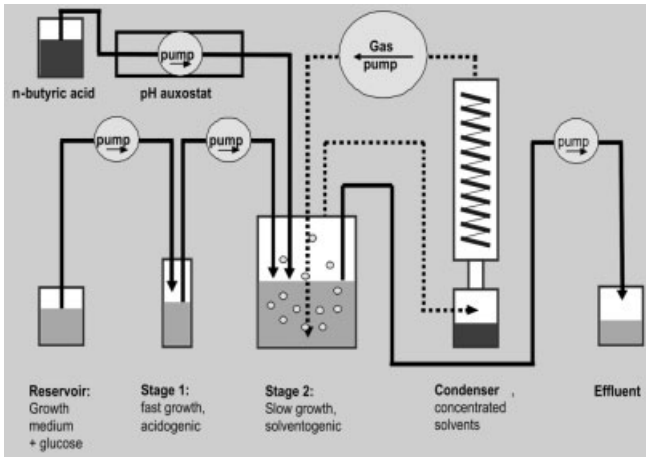

Figure 1. Schematic of two-stage continuous culture with gas stripping. The growth medium contained 60 g/L glucose as a source of carbon (for cell growth), and as a source of ATP and electrons for conversion of *n*-butyrate into *n*-butanol.

Gas Stripping

Gas stripping was performed in all continuous cultures in the fermentor with a 1-L working volume, using a factory-supplied gas sparger similar to that previously described (Ezeji et al., 2005a). The gas-circulating rate was 3 L/min. The rate of *n*-butanol stripping was determined by measuring the *n*-butanol concentration in a model solution containing *n*-butanol (20 g/L), ethanol (1 g/L), acetone (8 g/L), acetic acid (8 g/L), and *n*-butyric acid (2 g/L) at the start for over 140 h in 4–6 h intervals. The *n*-butanol stripping rates were plotted versus the concomitant *n*-butanol concentration, and from the resulting regression curve we obtained a parabolic dependency (r^2 value was 0.9543) of the *n*-butanol stripping rate versus the *n*-butanol concentration with the following equation:

$$B = 0.0022\ C^2 + 0.0236\ C$$

where B is the *n*-butanol removal rate in g/(L × h) and C is the *n*-butanol concentration in g/L. In continuous cultures, the gas flow rate was increased to 6.4 L/min, resulting in a theoretical *n*-butanol removal rate of 0.97 g/(L × h) at an *n*-butanol concentration of 10 g/L.

Analysis of Substrates and Products

The glucose concentration was determined with a HPLC system (Waters Corporation, Milford, MA) with a 600 Controller & Pump, 717 Autosampler, 410 Differential Refractometer, a Supelco Aminex HPX87H column

(Sigma), and 5 mM sulfuric acid as eluant at a flow rate of 0.6 mL/min. A gas chromatography system (HP 5890, Hewlett Packard, Palo Alto, CA), equipped with a 7673 autoinjector and a flame ionization detector was used for the quantification of carboxylates and alcohols. GC columns were purchased from Sigma.

The amount of carbon dioxide formed was calculated from substrate and product concentrations, considering the biochemical pathways involved in CO_2 formation. For each mol of acetone, ethanol, acetate, and *n*-butanol produced, 3, 1, 1, and 2 mol CO_2, respectively, are known to be coproduced (Jones and Woods, 1986). The amount of *n*-butyrate consumed was subtracted from the amount of *n*-butanol produced for this calculation, since no CO_2 is formed during *n*-butyrate to *n*-butanol conversion. It was verified by gas chromatography and high performance liquid chromatography that no significant formation of other possible metabolic end products, such as formate, lactate, isopropanol, acetoin, or butanediol, occurred, which could have changed the stoichiometry of CO_2 production.

Cell mass concentration was measured via optical density at 600 nm, using a Biophotometer (Eppendorf North America, Hauppauge, NY), and determining a correlation-coefficient of 395 mg dry weight/($L \times OD_{600\,nm}$). The concentration of UBA in the medium was calculated from the concentration of total *n*-butyrate and the pH value, using the Hendersson–Hasselbalch equation:

$$pH - pK_A = \log\left(\frac{[A^-]}{[AH]}\right)$$

where pH is the pH value, pK_A has a value of 4.8, which is the negative logarithm of the acid dissociation constant for *n*-butyrate, $[A^-]$ is the concentration of *n*-butyrate anions and [AH] is the concentration of UBA.

Results and Discussion

Organism and Growth Conditions

In a series of experiments (some are described in the Supplementary Materials of this publication), the optimum conditions for the biological conversion of *n*-butyrate into *n*-butanol were determined. This included a comparison of different solventogenic *Clostridium* strains, the selection of growth medium, *n*-butyrate concentration, and pH. As a result of the initial batch culture studies, we used *Clostridium saccharoperbutylacetonicum*, TYA medium, a pH of 5.2, and an *n*-butyrate concentration of 1.0 g/L (3.4 mM UBA) for optimizing the continuous culture.

Preventing Metabolic Oscillations and Culture Degeneration in Continuous Culture

Adding *n*-butyrate and glucose simultaneously to batch cultures (fermentors) of strain N1-4 in TYA medium had resulted in molar ratios of total *n*-butyrate and glucose consumption of up to 1.5 during the highest specific rates of *n*-butanol production. Similar ratios had previously been reported for fed batch cultures with strain N1-4 (Tashiro et al., 2004). We started feeding glucose and *n*-butyric acid at this ratio to a single-stage continuous fermentation while utilizing gas stripping to maintain an average *n*-butanol concentration at ~4.0 g/L, which is below a concentration that significantly inhibits *n*-butanol production by strain N1-4. Therefore, the removal rates from gas stripping were sufficient to prevent product inhibition of *n*-butanol production. However, in single-stage continuous cultures, feeding *n*-butyrate and glucose at a molar ratio of 1.5 from a medium reservoir (i.e., 44 g/L *n*-butyric acid and 60 g/L glucose) resulted in the inhibiting accumulation of total *n*-butyrate (i.e., acid crash) after short operating periods (1–2 days; data not shown). Such acid crashes have been previously reported for continuous cultures operating at low dilution rates (Clarke et al., 1988; Gapes et al., 1996; Jones and Woods, 1986). Subsequent lowering of the molar ratio of total *n*-butyrate and glucose to 0.5 in the reservoir of the single-stage fermentor did not circumvent this problem. Instead, we were able to overcome these short-term acid crashes in our one-stage set-up by feeding *n*-butyric acid separately via a pH auxostat (automatic feeding until a pH set point of 5.2 is reached) while supplying only glucose (60 g/L) from the growth-medium reservoir, similarly as reported previously (Tashiro et al., 2004). However, these operating conditions still resulted in dramatic oscillations of high and low *n*-butanol productivity with a 4–6 day period of oscillation (Fig. 2A).

We dampened these oscillations considerably by operating the fermentation in two stages (Fig. 1), as suggested by others (Afschar et al., 1985; Bahl et al., 1982b; Gapes et al., 1996; Godin and Engasser, 1990; Lai and Traxler, 1994; Mutschlechner et al., 2000). The first stage served as a cell propagator and its volume was set at 1/10th that of the second production stage. The first stage fermentor was fed with glucose at a short hydraulic retention time to support a metabolically active culture as a continuous inoculum for stage 2, while the rate of *n*-butyric acid fed into stage 2 was controlled using a pH-auxostat operating mode. These modifications resulted in a more stable *n*-butanol concentration during 7 days of the operating period (Fig. 2B). However, the system still underwent culture degeneration (lost the ability to produce solvents) after 12 days (Fig. 2B) accompanied by the accumulation of acids (not shown here), as had been observed previously in another report with glucose-fed *C. saccharoperbutylacetonicum* (Kosaka et al., 2007).

Prior studies have observed that also feeding *n*-butyric acid to the cell propagator prevented culture degeneration (Assobhei et al., 1998; Kashket and Cao, 1995; Lee et al., 2008a). We, therefore, restarted the continuous two-stage system and added *n*-butyric acid at concentrations varying between 1 and 3 g/L directly to the medium reservoir for stage 1 in addition to the pH-auxostat controlled feed of

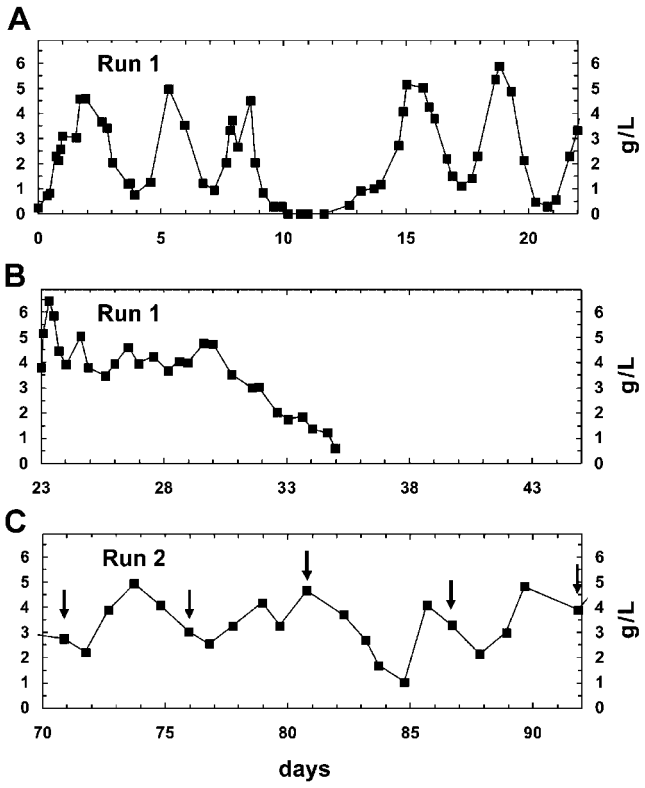

Figure 2. Effects of operating conditions on metabolic oscillations and culture degeneration. Comparison of *n*-butanol concentration (squares) over time at three different operating conditions in 1-L continuous culture fermentors with gas stripping and *n*-butyrate feeding by pH auxostat. **A**: Single stage continuous culture with data from day 1–22 of the operating period (Run 1) displaying pronounced oscillations; (**B**) On day 22 of run 1, the fermentor of the single stage fermentation from panel (A) became stage two by introducing a first fermentation stage, data of stage 2 are shown for day 23–35 of the operating period of Run 1; and (**C**) butanol concentrations from stage 2 of a separate two-stage continuous culture (Run 2, day 70–92 of the operating period) with additional 5-day-interval-heatshocking/re-inoculation of stage 1 are shown. For complete Run 2 see Figs. 3, S2, S3, S4.

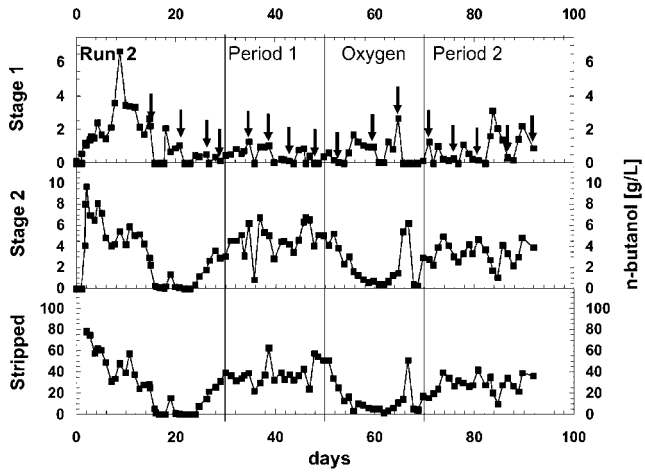

Figure 3. *n* -Butanol concentrations in optimized two-stage system with gas-stripping and periodical heat shocking and re-inoculation during an operating period of 92 days of Run 2. *n*-Butanol concentrations over time in stage 1, stage 2, and in the stripped solvent are shown. Period 1 and 2 indicate the time periods during which the system operated stable and for which the performance parameters were calculated. Oxygen indicates the period where oxygen leaked into the system. Arrows indicate heat shocking and re-inoculation events.

n-butyric acid into stage 2 (Fig. 3). Measurements of metabolic products throughout the entire operating period of the run showed that acidogenesis and solventogenesis occurred in both stages 1 and 2 (Figs. S2 and S3). However, culture degeneration set in during the first 18 days of the operating period (Fig. 3). Next, in addition to feeding both *n*-butyrate and glucose to stage 1, we heat shocked and re-inoculated stage 1 from spore solutions every 5 days starting on day 18 of the operating period (after confirming that stage 2 did not crash during the 1-day period without continuous inoculation from stage 1). Recovery of *n*-butanol production occurred after removal of *n*-butyric acid from the feed reservoir to stage 1 on day 20 of the operating period (Fig. 3). Indeed, including periodic re-inoculation of stage 1 maintained a high *n*-butanol production rate without culture degeneration during days 30–50 and 70–92 of the operating period. During the interim period with arrested *n*-butanol production, oxygen infiltration into stage 2 from days 55–64 of the operating period had resulted in oxygen inhibition (Jones and Woods, 1986). After fixing this problem on day 64, it took only a couple of days to restore *n*-butanol production (Fig. 3).

Numerous ABE fermentation studies exist, which feature continuous cultures with either freely suspended or immobilized cells—these cultures were run successfully for prolonged periods of time; in one case for up to 1 year (Table I). These studies differed from our study in several aspects, the major one being that either no *n*-butyric acid was co-fed with glucose, or the molar ratio of *n*-butyrate and glucose was an order of magnitude lower compared to the present study. The authors of these studies had used *C. acetobutylicum*, *C. beijerinckii*, or *C. saccharoperbutylacetonicum* strains. Generally, two-stage cultures with low dilution rates in the second stage, phosphate limitation, relatively low pH values, or comparably low amounts of *n*-butyric acid were used to optimize *n*-butanol yields and productivity, and to avoid culture degeneration and acid crashes. We applied all these strategies; but in our study, a prolonged stable *n*-butyrate to *n*-butanol conversion with *C. saccharoperbutylacetonicum* was only achieved when the first stage was heat shocked and re-inoculated in regular time intervals. With this strategy, *n*-butanol will be produced indefinitely or until other obstacles, such as phage contamination, occur.

Comparing *n*-butanol concentrations over the operating period for the two-stage, pH-auxostat fermentor without (Fig. 2B) and with periodic re-inoculation (Fig. 2C), shows that preventing culture degeneration by re-inoculation did increase the oscillatory behavior of *n*-butanol production in

stage 2 again, but not to the extent of the single-stage, pH-auxostat fermentor (Fig. 2A). We anticipate that this oscillatory behavior can be dampened by periodically switching between two newly heat shocked and re-inoculated stage 1 fermentors so that stage 2 is always fed with metabolically active cells; however, this was not tested. The added costs associated with operating duplicate stage 1 fermentors is somewhat diminished by its smaller volume (one stage 1 fermentor is 1/10th in volume compared to a stage 2 fermentor).

Performance Parameters of the Optimized 2-stage Continuous Culture

The average performance parameters (Table II) were calculated over the 42 days during which the system operated without disturbance (day 30–50 and 70–92; Fig. 3). The average *n*-butanol production rate and concentration in stage 2 was 0.39 g/(L × h) and 4.0 g/L, respectively. The gas stripper was successful in capturing 85% of *n*-butanol. The alcohol was stripped and recovered at a rate of 0.33 g/(L × h) at an *n*-butanol concentration of 32.8 g/L, corresponding to a concentration factor of 8.2. Further optimization of the continuous stripping set-up is necessary because 15% of the *n*-butanol was not removed prior to exiting the second stage.

Table II. Performance parameters of the continuous two-stage fermentation with integrated product removal calculated for the operating period during which the reactor was stable (i.e., from day 30 to 50 and from day 70 to 92).

	mg/(L × h)	% of total
Rates		
n-Butyrate feeding	251	100
n-Butyrate loss	18	7
n-Butyrate conversion	233	93
n-Butanol production	393	100
Glucose conversion	1390	93
Cell mass production (dry weight)	57	100
Glucose consumption catabolic	1333	89
n-Butanol production	393	100
Acetone production	198	100
Ethanol production	17	100
Acetate production	10	100
Molar ratios (catabolic)		
n-Butyrate: glucose	0.358	
n-Butanol: glucose	0.718	
n-Butanol: *n*-butyrate	2.007	
Acetone: glucose	0.462	
Ethanol: glucose	0.049	
Acetate: glucose	0.023	
Carbon recovery (% C)	88	
Redox balance (O/R)	0.94	
	Stage 2	Stripped
% Product recovery		
Acetone	22	78
n-Butanol	15	85
Ethanol	19	81

Glucose was consumed at an average rate of 1.39 g/(L × h), amounting to 93% of the total supplied glucose. Based on the cell dry weight, we estimate that 4% of the consumed glucose went into anabolism to produce cell material, while the remaining 96% (1.33 g/(L × h)) were directed into energy metabolism (fermentation). The *n*-butyric acid was fed at an average rate of 0.25 g/(L × h) and 93% was converted to *n*-butanol. The remaining 7% was lost either into the waste reserve (0.016 g/(L × h) or gas stripped (0.002 g/(L × h)). The molar yield of *n*-butanol produced to *n*-butyrate consumed ($Y_{n\text{-butanol}/n\text{-butyrate}}$) in our system was 2.0, which means that approximately 50% of the *n*-butanol production originated through the more conventional ABE fermentation (i.e., acidogenesis and solventogenesis) from glucose; the molar yield of *n*-butanol produced to glucose consumed ($Y_{n\text{-butanol/glucose}}$) was 0.718. To summarize, the average experimental molar stoichiometry of the fermentation was:

$$0.36\ n\text{-butyrate} + 1.00\ \text{glucose} \rightarrow 0.46\ \text{acetone} + 0.72\ n\text{-butanol} + 0.05\ \text{ethanol} + 0.02\ \text{acetate} + 2.18\ CO_2$$

Based upon this equation, we calculated that 88% of the carbon atoms from *n*-butyrate and glucose were recovered in the fermentation products (including CO_2) with an oxidation/reduction (O/R) balance of 0.94 (Buckel, 1999), indicating that ~12% of the total carbon is unaccounted for, and that the missing product is an oxidized one. The concentration of other potential fermentation products, such as lactate, formate, isopropanol, diacetyl, acetoin, and butanediol never exceeded 0.02 g/L, which amounts to <0.2% of the missing carbon. Similar observations had been made by others (Gottschal and Morris, 1981), but they did not have an explanation. A more thorough day-by-day analysis of our data revealed that the overall imbalanced fermentation had occurred due to the "oxygen accident", even before we had documented the problem and *n*-butanol concentrations had been affected. An average carbon recovery of 99 (±16) percent was calculated when omitting the days with low carbon recovery (around the oxygen accident) from the calculation (Fig. S5).

From the molar ratio of *n*-butyrate and glucose consumed (0.358) it can be calculated that only 18% of the consumed glucose served as a source of electrons and ATP for the conversion of *n*-butyrate into *n*-butanol. This value would be 100% in a system optimized for minimum glucose consumption. A maximum theoretical molar *n*-butyrate and glucose consumption ratio of 2.0 would be achieved when all electrons derived from glucose oxidation (4 mol of NAD(P)H) would be transferred to butyryl-CoA, according to:

$$2\ n\text{-butyrate} + 1\ \text{glucose} \rightarrow 2\ n\text{-butanol} + 1\ \text{acetone} + 3\ CO_2$$

The overall ATP yield permitting cell growth would be 1 ATP/glucose or 0.5 ATP/*n*-butyrate, since 2 mol of ATP are produced during glycolytic oxidation of 1 mol of glucose to 2 acetyl-CoA. Of the 2 mol of external *n*-butyrate, 1 mol would be converted into butyryl-CoA using the CoA-transferase reaction of the acetone-forming pathway starting with acetacetyl-CoA (Wiesenborn et al., 1989), while the other mol of *n*-butyrate can be converted to butyryl-CoA by investing one mol ATP through the butyratekinase/phosphotransbutyrylase (*buk*/*ptb*) pathway. The latter pathway is up-regulated during solventogenesis in *C. acetobutylicum* (Alsaker and Papoutsakis, 2005), and this was also suggested to be the case for our strain N1-4, based on a kinetic modeling approach (Shinto et al., 2007). The molar ratio of consumption of *n*-butyrate and glucose has previously been reported to be at least 1.6 in fed-batch cultures of strain N1-4 (Tashiro et al., 2004), and 1.4 in nongrowing cells (Tashiro et al., 2007), indicating that the *buk*/*ptb* pathway is employed to a substantial extent by this organism. From another recent study that used batch cultures in an inexpensive phosphate free mineral medium, a molar *n*-butyrate and glucose consumption ratio of up to 2.79 can be calculated (Al-Shorgani et al., 2011). However, this ratio was not obtained due to conversion of *n*-butyrate into *n*-butanol, since the molar yield $Y_{n\text{-butanol}/n\text{-butyrate}}$ was only 0.49. The authors did not give any explanation regarding the fate of *n*-butyrate that was consumed but not reduced to *n*-butanol. The authors claimed that *C. saccharoperbutylacetonicum* N1-4 was able to convert small amounts of *n*-butyrate into *n*-butanol without the presence of glucose, but they did not suggest what the source of electrons and activation energy necessary for the conversion was, nor did they present evidence that there was no glucose carryover from their 10% inoculum.

While we only achieved a molar ratio of 0.358 for *n*-butyrate and glucose consumption in our continuous system (Table II), this is still the highest value published for continuous cultures. Even with its lower ratio, the optimized continuous system is preferred to the batch system because the former is known to provide higher *n*-butanol production rates at comparable yields (Li et al., 2011; Ni and Sun, 2009), the downtime between fermentation runs is avoided, and there are operating advantages when the continuous fermentation can be maintained stable for prolonged periods of time (Gapes, 2000). In addition, our ultimate goal is to utilize molecules other than sugars as a source of reducing equivalents and energy for the conversion of *n*-butyrate into *n*-butanol to avoid the competition of fuel production with food production. Therefore, we did not attempt to further optimize the molar ratio of *n*-butyrate and glucose consumption.

Our experimental molar yield of carbon in *n*-butanol produced to carbon in *n*-butyrate and glucose consumed ($Y_{n\text{-butanol/carbon}}$) was 0.386 and slightly higher than reported in literature for continuous cultures with planktonic cells (single- and two-stage fermentors) and glucose as the only carbon source (Table I; yields of 0.241–0.379). We achieved a similar result compared to a carbon yield of 0.388 reported in the literature by adding *n*-butyrate to growing cells in continuous cultures (Lee et al., 2008a). Theoretically, 1 mol of glucose (without *n*-butyrate) can be converted into 1 mol *n*-butanol and 2 mol CO_2 with a resulting $Y_{n\text{-butanol/carbon}}$ of 0.667 if we assume no anabolism. At the other extreme theoretical situation where 2 mol *n*-butyrate and 1 mol glucose would be co-fermented into 2 mol *n*-butanol and 1 mol acetone, the $Y_{n\text{-butanol/carbon}}$ would be 0.571. Therefore, maximizing the *n*-butyrate conversion with glucose should not necessarily result in a higher overall $Y_{n\text{-butanol/carbon}}$. We conclude that the observed slight increase in $Y_{n\text{-butanol/carbon}}$ must have been caused by stimulation of the conventional ABE fermentation of glucose by *n*-butyrate, and not because the added *n*-butyrate was converted into *n*-butanol. Thus, it is beneficial to only add relatively small amounts of *n*-butyrate to trigger solventogenesis when the goal is to maximize $Y_{n\text{-butanol/carbon}}$. We, on the other hand, aimed for a maximum molar ratio of *n*-butyrate and glucose consumption to reduce the utilization of sugars, which stands in direct contrast to maximizing $Y_{n\text{-butanol/carbon}}$.

Conclusions

Here, we describe the operation of a two-stage continuous fermentation system that is optimized for conversion of *n*-butyrate into *n*-butanol with co-fermentation of glucose by slow growing planktonic cells of *C. saccharoperbutylacetonicum* strain N1-4 and with integrated product removal. The establishment of a stable conversion process is a stepping stone for our ongoing research, investigating alternative sources of carbon, energy, and reducing power for the conversion of *n*-butyrate into *n*-butanol, which are derived from lignocellulose.

This project was supported by the USDA through the National Institute of Food and Agriculture (NIFA), grant number 2007-35504-05381. We also want to thank Professor Larry Walker (Cornell University) for his support and the permission to conduct a large part of our research within the Biofuel Research Laboratory.

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