

Article

## Continuous Fermentation of *Clostridium tyrobutyricum* with Partial Cell Recycle as a Long-Term Strategy for Butyric Acid Production

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**Abstract:** In making alternative fuels from biomass feedstocks, the production of butyric acid is a key intermediate in the two-step production of butanol. The fermentation of glucose via *Clostridium tyrobutyricum* to butyric acid produces undesirable byproducts, including lactic acid and acetic acid, which significantly affect the butyric acid yield and productivity. This paper focuses on the production of butyric acid using *Clostridium tyrobutyricum* in a partial cell recycle mode to improve fermenter yield and productivity. Experiments with fermentation in batch, continuous culture and continuous culture with partial cell recycle by ultrafiltration were conducted. The results show that a continuous fermentation can be sustained for more than 120 days, which is the first reported long-term production of butyric acid in a continuous operation. Further, the results also show that partial cell recycle via membrane ultrafiltration has a great influence on the selectivity and productivity of butyric acid, with an increase in selectivity from  $\approx 9\%$  to 95% butyric acid with productivities as high as 1.13 g/Lh. Continuous fermentation with low dilution rate and high cell recycle ratio has been found to be desirable for optimum productivity and selectivity toward butyric acid and a comprehensive model explaining this phenomenon is given.

**Keywords:** butyric acid; continuous fermentation; ultrafiltration; *Clostridium tyrobutyricum*

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## 1. Introduction

### 1.1. Background

Butyric acid is an organic acid produced via fermentation that is used in perfumes, as a food additive, and as an intermediate in alternative fuels [1]. When making alternative fuels, butyric acid production is the first step in a two-step fermentation route to produce butanol [2]. There is a renewed interest in butanol production from biomass and agricultural waste for sustainability, energy independence and reduction of dependence on foreign oil. Although the liquid fuels of primary focus from biomass to date have been bioethanol and biodiesel, the DOE Roadmap has identified butanol as a second generation biofuel [3]. Butanol is a potential gasoline substitute or additive which can favorably compete with or complement bioethanol manufacture. However, several technical and economic challenges remain in commercial butanol production, including many of the challenges addressed by Ramey [2].

The single-step process, one method of which is referred to as ABE [4], forms a product stream containing acetone, butanol and ethanol. The ABE process yields 0.23 g butanol/g sugar [5]. Ramey's description of a two-step fermentation process in which sugar is converted to butyric acid in step one, and butyric acid to butanol in step two, raises the theoretical yield of butanol by 67% [2,6]. However, there has been concern that in order to upgrade butyric acid to butanol, additional energy in the form of glucose is needed, and this adversely affects the selectivity of the two-step fermentation process [7,8]. Thus, a second two-step option is fermentation production of butyric acid, followed by catalytic hydrogenation to produce butanol. The catalytic hydrogenation process has been experimentally verified to produce yields of 0.34 g butanol/g sugar [9]. Even given these conceptual breakthroughs, there remains a challenge to continuous production of butyric acid: the development of a process with high productivity and selectivity that maximizes inhibition of acetic acid and lactic acid byproducts. The purpose of this paper is to examine the continuous long term production of butyric acid via *Clostridium tyrobutyricum* with partial cell recycling as a strategy for this process.

*C. tyrobutyricum* is an anaerobic bacterium which produces lactic acid, butyric acid and acetic acid as its main fermentation products from sugars, while also producing hydrogen and carbon dioxide as gaseous byproducts. Comparable yields of butyric acid from glucose were obtained using either *C. tyrobutyricum* or *C. thermobutyricum* [10,11]. Liu *et al.* [12,13] developed *C. tyrobutyricum* mutants which gave higher butyrate yields (>0.4 g/g) and concentrations (43 g/L). *C. tyrobutyricum* has become the organism of choice because it converts both five- and six-carbon sugars to butyric, acetic and lactic acids. Elevated pH (6.3) is favorable for the production of butyric acid, and lower pH (<5.7) is more favorable for the production of acetic and lactic acids [11,14]. Higher total acid yields are attained at reduced pH, but higher butyrate selectivities and concentrations are attained at increased pH. To date, most of the research [1,11–13,15–18] conducted with *C. tyrobutyricum* was done at relatively low pH because of recovery concerns, as solvent extraction recovers free acid only, although

organic acid separation techniques such as electrodeionization [19,20] are being developed to separate organic acids at high pH. Thus, butyric acid can be produced in high yield by employing elevated pH.

Productivity in a fermentation is important because the higher the productivity, the smaller the fermenter volume that is needed for the desired product. There are two generally accepted methods for increasing productivity beyond the maximum allowed by batch growth kinetics: cell immobilization and cell recycle. Cell immobilization is a technique for retaining cells inside the reactor through attachment to a surface [21], entrapment within porous matrices [22], or containment behind a barrier or self-aggregation [23]. Cell recycle is a technique for separating the cells from the product stream by centrifugation, filtration or settling in a conical tank, followed by returning the cells back to the reactor [24]. An assessment of cell recycle technologies shows that centrifugation to remove cells can be cost-prohibitive and simple settling with or without the addition of flocculating agents requires large tanks due to the similarity in densities between cells and the fermentation broth. *C. tyrobutyricum* fermentation systems coupled with ultrafiltration or microfiltration have had limited investigation [16,17]. A complete cell recycle was used in Michel-Savin's work, recycling 100% of the cell mass back to the fermenter while removing all of the products. A higher cell density was achieved, and as a consequence the productivity was considerably higher with more butyric acid formed [16,17]. However, total cell recycle is not preferred in long-term operation because of the accumulation of dead cells, which can easily block the membrane and prevent long-term production cycles; and oscillation in metabolic activities, which had been previously explained as aging of cell mass [25].

Table 1 shows the major studies that have been conducted for increasing cell mass and thus increasing productivity including batch [1], immobilized cells [11], extractive fermentation [11], fed-batch fermentation with total cell recycle [17], and fermentation with partial cell recycle (this study).

**Table 1.** Comparison of fermentation results from free cells, immobilized cells, extractive fermentation, and this study.

Parameters	Free cells (pH 6.0)	Immobilized cells (pH 6.0)	Immobilized cells (pH 5.5)	Extractive fermentation (pH 5.5)	Complete cell recycling (pH 5.8)	Partial cell recycling (pH 6.8)
OD <sub>max</sub>	5.8	11.5	8.2	8.1	35	15.1
Butyrate concentration (g/L)	16.3	43.4	20.4	301	33.0	7.58
Butyrate yield (g/g)	0.34	0.42	0.38	0.45	0.41	0.45
Acetate yield (g/g)	0.120	0.095	0.115	0.111	0.012	0.02
Butyrate productivity (g/Lh)	0.193	6.77	5.11	7.37	5.3	1.13
Product selectivity	0.74	0.81	0.77	0.80	0.97	0.95
Operation days	2	2	2	14	24	120
Reference	[1]	[11]	[11]	[11]	[17]	This study

A single result from this study is presented in the introduction to show the need for research in partial cell recycle fermentation processes. When compared to batch studies, partial cell recycle gave a higher butyrate yield (0.45 g/g compared to 0.34 g/g), higher butyrate productivity (1.13 g/Lh

compared to 0.193 g/Lh), and increased butyric product selectivity (0.95 to 0.74). Further, the operation time for this study was 120 days (stopped only to switch fermenter to another product) as compared to 24 days for full recycle (stopped because of membrane fouling). However, even though the yield for partial cell recycle was as high as extractive fermentation and better than immobilized cells, the overall productivity was lower than immobilized schemes (1.13 g/Lh as compared to 5–7 g/Lh). Following is an attempt to develop the first comprehensive model for partial cell recycling of *C. tyrobutyricum* so that the high selectivity and long term growth can be kept while maximizing the overall productivity. The challenge in this fermentation is to convert all of the sugars to butyric acid, rather than a combination of organic acids and solvents, at as high of a productivity as possible.

### 1.2. Theory

*C. tyrobutyricum* has the capacity for producing several acid species including routes to lactate, acetate, and butyrate. All of these liquid products as well as the gaseous byproducts of CO<sub>2</sub> and H<sub>2</sub> have been shown to be possible end products for *C. tyrobutyrium* under various culture conditions [14,17,18]. The pathway in Whu and Zhang [14], for instance, shows that pyruvate may be converted directly to lactate via lactate dehydrogenase, or alternately undergo oxidoreduction to make acetate or butyrate. The pathway also shows that the cell must make CO<sub>2</sub> and H<sub>2</sub> in producing either butyrate or acetate.

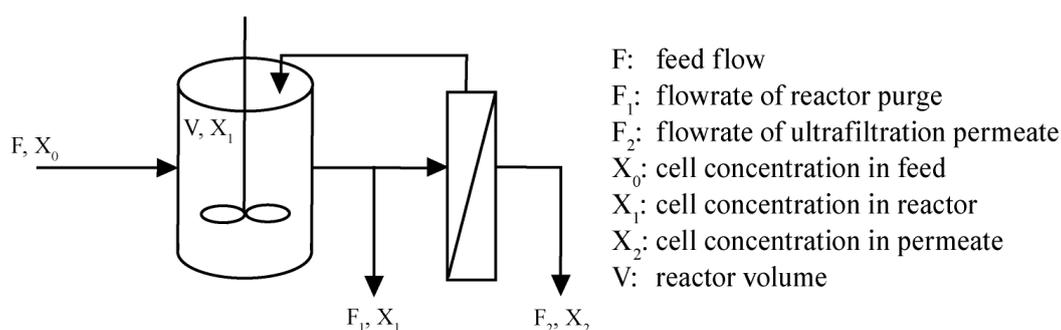
To better understand batch and continuous fermentation systems, a mass balance accounting for the specific growth rate,  $\mu$ , and cell retention time,  $\sigma$ , was derived. For the batch fermentation, the specific growth rate can be approximated as  $\frac{\Delta X}{\Delta t} / X$  and the productivity of butyric acid can be approximated as  $\frac{\Delta C_b}{\Delta t}$ . For the continuous fermentation, as shown as Figure 1, a material balance for cell mass yields:

$$F * X_0 + V * \mu * X_1 - F_1 * X_1 - F_2 * X_2 = V * \frac{dX_1}{dt} \quad (1)$$

$$F = F_1 + F_2 \quad (2)$$

$$F_2 = R * F \quad (3)$$

**Figure 1.** Schematic diagram for mass balance of continuous fermentation with cell recycle by *C. tyrobutyricum*.



In this model,  $F$  is the feed flow rate,  $F_1$  is the flowrate of the purge, and  $F_2$  is the flowrate of the permeate from the UF unit while the parameter  $X$  indicates the cell mass present in the same positions. When the continuous fermentation reaches steady state  $\frac{dX_1}{dt} = 0$ :

$$\mu = (1 - R) * \frac{F}{V} = (1 - R) * D \quad (4)$$

The cell retention time for continuous fermentation is defined as:

$$\sigma = \frac{1}{D} = 1/\mu \quad (5)$$

and  $\sigma$  for continuous fermentation with cell recycle is defined as:

$$\sigma = \frac{V}{F_1} = \frac{V}{(1 - R) * F} = 1/\mu \quad (6)$$

These equations give the ability to compare the growth rate of batch and continuous systems. This model is not media-independent: the media used as described below affects both the cell growth rate and the quantities of butyric acid produced, and differing media would produce different effects. Finally, for the purposes of this paper, selectivity is defined as the weight percentage (g/g) of butyric acid produced as compared to the total organic acids produced.

## 2. Results and Discussion

### 2.1. Batch Fermentation Kinetics with *Clostridium Tyrobutyricum*

In order to determine growth parameters for the continuous fermentation model with partial cell recycle, a batch study was performed. Batch fermentation data are shown in Table 2. The batch fermentation was operated for about 18 hours in a cycle. In the lag phase, little acetic and butyric acids were produced. However, in the exponential phase, the production of both acetic acid and butyric acid increased. The butyric acid selectivity in the last four hours of the fermentation (late exponential or early stationary phase) was 81%, compared to the overall butyric acid selectivity of 68%. Reported batch system results showed a butyric acid selectivity of 70%–89% [16,17], which depended mainly on the glucose concentration. A higher glucose concentration generally resulted in higher butyric acid selectivity. Analyzing the two runs in Table 2 shows the highest instantaneous butyric acid selectivity (all instantaneous values calculated by taking the difference between indicated time point and the one before) at or near the end of the runs. This indicates butyric acid selectivity is higher when the cell growth rate slows and thus a high glucose concentration would extend this time in the stationary phase and thus increase the overall butyric acid selectivity of the fermentation.

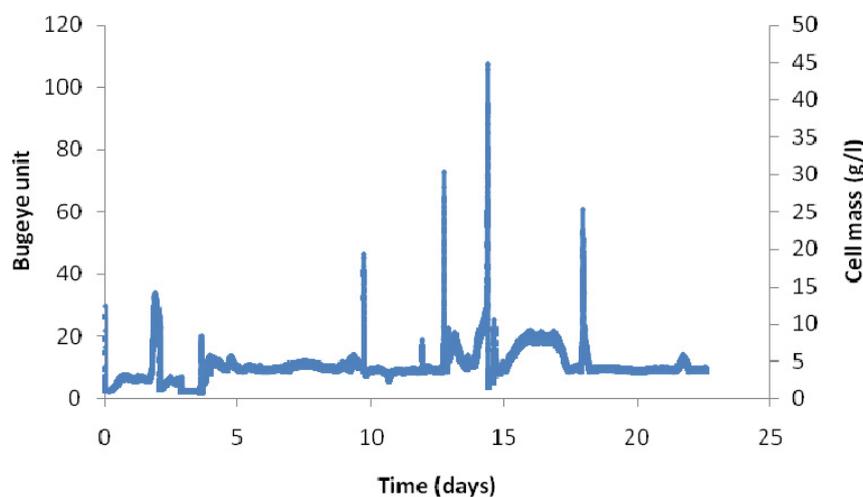
**Table 2.** *C. tyrobutyricum* batch fermentation kinetics at pH 6.8.

Run	Time (h)	Butyric acid (g/L)	Acetic acid (g/L)	Glucose (g/L)	Cell mass (g/L)	$\Delta x/\Delta t$ (g/Lh)	Instantaneous productivity of butyric acid (g/Lh)	Instantaneous productivity of acetic acid (g/Lh)	Instantaneous selectivity of butyric acid
Run #1	0	1.29	0.52	17	0.8	-	-	-	-
	1	1.48	0.63	16.5	0.9	0.1	0.271	0.157	0.63
	4.7	2.22	1.16	14.2	1.42	0.135	0.301	0.193	0.609
	8.3	3.31	1.68	11.1	2.0	0.167	0.433	0.206	0.677
	12.9	5.23	2.36	6.2	2.68	0.152	0.596	0.211	0.739
	16.6	7.13	2.72	1.7	2.77	0.081	0.734	0.139	0.841
	18.0	7.95	2.75	0.03	3.1	0.071	0.806	0.031	0.963
Run #2	0	0.4	0.21	12	0.6	-	-	-	-
	10	1.71	0.97	7.4	1	0.09	0.131	0.076	0.633
	23.3	4.38	2.41	3.06	2.4	0.241	0.20	0.108	0.649
	24.4	4.65	2.455	2.4	2.65	0.636	0.245	0.041	0.857
	26.7	4.85	2.462	1.86	2.75	0.1	0.087	0.003	0.967
	27.5	5.13	2.5	1.26	2.77	0.0625	0.35	0.048	0.879
	28.5	5.77	2.71	0.96	2.75	-0.04	0.64	0.21	0.751

## 2.2. Long-Term Fermentation Performance with *C. Tyrobutyricum*

Some of the principal reasons for using partial cell recycle are to facilitate long-term fermentations and to increase cell concentration inside reactors, which is a problem in anaerobic systems. In order to prove that partial cell recycle increases cell concentrations over the long term, we performed a 120 day fermentation run. Figure 2 shows real-time cell mass monitoring during continuous fermentation for the first 23 days of a 120 day fermentation run.

**Figure 2.** Real-time cell mass monitoring with Bugeye biomass monitor for continuous fermentation with *C. tyrobutyricum* at pH of 6.8.



These Bugeye™ readings, calibrated with periodic cell mass readings, were taken non-continuously over the entire run with variations in data due to (1) changes in conditions; (2) removal of the Bugeye™

unit for visual observation of the reactor and use of the unit in other experiments; and (3) changing the nitrogen tank. This long time period monitoring of the cell mass with the Bugeye™, however, shows that the Bugeye™ can be used as an accurate way to track cell mass. *C. tyrobutyricum* is a rod-shaped gram-positive bacteria, and microscopic inspection showed that the appearance of the bacteria was not altered with fermentation time. With 120 days of fermentation as compared to a more traditional 30 hour run, the fermenter performed about 100 times longer than a typical batch *C. tyrobutyricum* fermentation. This, in an industrial process, could allow for much higher productivity due to lack of downtime, with smaller overall reactor volumes. It should be noted that the membrane was taken offline and cleaned once a week without ever having to take the fermenter offline.

### 2.3. Effect of Dilution Rate on Continuous Fermentation Performance with No Cell Recycle

A series of studies with continuous fermentation were performed to examine the effects of dilution rate on fermentation performance. The fermentation generally took approximately three residence times to adjust to new conditions, and no transient data are incorporated in any of the yield or selectivity measurements. Continuous fermentation data with and without partial cell recycle are summarized in Table 3.

**Table 3.** Comparison of continuous fermentation with *C. tyrobutyricum*.

Dilution rate (h <sup>-1</sup> )	R	wt% butyric	wt% acetic	wt% lactic	Cell mass (g/L)	Yield of	Productivity of	Y <sub>PS</sub>
						butyric (g/g sugar)	butyric (g/Lh)	
0.077	0	33.3	9.1	57.6	4.34	0.22	0.28	0.66
0.077	95	95.8	3.2	0	15.1	0.446	0.567	0.47
0.15	0	19.3	14.7	66	4	0.143	0.36	0.74
0.15	80	88.9	7.2	3.8	14.3	0.435	1.06	0.49
0.15	95	94.3	4	1.7	14.8	0.442	1.11	0.47
0.21	0	35.3	40.4	24.2	4.01	0.221	0.79	0.63
0.21	40	23.4	17.3	59.3	5.3	0.184	0.65	0.79
0.21	80	70.9	16.3	12.7	14.1	0.332	1.17	0.47
0.21	95	53	14.6	32.4	10.8	0.318	1.12	0.6
0.38	0	30.2	48.8	21	2.4	0.074	0.48	0.25
0.38	26	9.5	14.4	76.1	4.2	0.066	0.43	0.69
0.38	43	5.8	11.1	83.2	4.5	0.051	0.33	0.88
0.38	80	37.2	10.3	52.5	13.9	0.149	0.96	0.40
0.38	95	42.5	12.8	44.7	15.5	0.159	1.26	0.37

Conditions were picked such that the fermenter went from a low cell growth rate to cell wash-out conditions. As shown, the selectivity of butyric acid in a continuous fermentation without cell recycle (R = 0) varied from 19% to 35%. Lactic acid was also present as a product. Although many times lactic acid is not seen as a product in *C. tyrobutyricum* fermentations, it has been shown to be present at levels >25% under certain media and pH conditions [14]. The highest ratios of butyrate to acetate are at the lowest dilution rates. These values are quite low compared to the batch results reported by Michel-Savin *et al.* [16,17] of 70%–89%, or the fed-batch results reported by Wu and Yang [11] of

74% to 81%. They are also much lower than our own batch results of 68%. Cell wash-out occurred at  $0.38 \text{ h}^{-1}$  dilution rate and is as evident by the Yp/s column where at this condition this value dropped to 0.25. Since butyric acid is the desired product, it is also noted the highest yield and concentration occurred at conditions of  $0.15 \text{ h}^{-1}$  dilution rate and 95% cell recycle.

#### 2.4. Effects of Recycle Rate on Continuous Fermentation

Improvements in productivity were made by employing continuous culture with partial cell recycle. Since batch results showed higher selectivity in the late exponential or early stationary phase than the lag phase, it was theorized that increasing cell mass in a continuous system at low dilution rates might also increase the selectivity of butyric acid. In order to increase the selectivity of butyric acid, partial cell recycle was used to increase the cell mass. Continuous fermentation with partial cell recycle was operated at dilution rates of 0.077, 0.15, 0.21 and  $0.38 \text{ h}^{-1}$ . As shown in Table 3, at a dilution rate of  $0.21 \text{ h}^{-1}$ , the cell mass increased when  $R$ , the amount of effluent leaving the reactor recycled back to the reactor, increased but never exceeded 14.1 g/L. This is a higher cell mass than in any of the immobilized results [11] shown earlier in Table 1. At extremely high cell mass ( $>14.3 \text{ g/L}$ ) and low dilution rate ( $<0.15 \text{ h}^{-1}$ ), the yield of butyric acid was the highest of the entire study at  $>94.3\%$ . However, at high cell masses of  $\approx 14 \text{ g/L}$  for dilution rates of 0.21 and  $0.38 \text{ h}^{-1}$ , the selectivity was 70.9% and 37.2%, respectively. It should be noted that as butyric acid selectivity increases, acetic acid becomes more prevalent than lactic acid as the primary byproduct. The cell mass for the continuous system without cell recycle was almost constant for different dilution rates at about 4.0 g/L. When the system was operated continuously with a high rate of cell recycle, the cell mass was significantly higher. A maximum cell mass at a dilution rate of  $0.21 \text{ h}^{-1}$  of 14.1 g/L was achieved when the system was operated at an  $R$  of 80%. But when  $R$  was increased to 95%, the cell mass decreased to 10.8 g/L instead of increasing. A possible reason for this phenomenon is cell aging [16,17]. It should be noted that in a short-term study when the media concentration was halved and the glucose was kept constant, no difference in productivity was seen, showing that the media limitations are not the reason for the observed shift in products from acetic and lactic acids to butyric acid.

#### 2.5. Correlation of Productivity with Cell Growth Rate

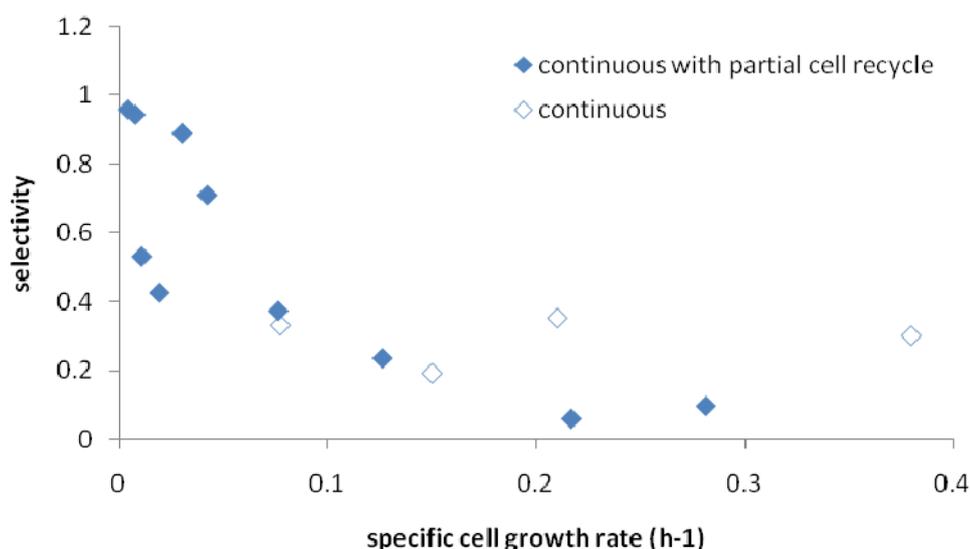
The specific growth rate of cells, or increase in cell mass over time, represents a shift in selectivity at different growth rates, which has a significant impact on this fermentation process. Rapid cell growth has a higher energy demand and preferentially produces acetic acid. At low growth rates, the production of butyric acid is favored over acetic acid. For continuous fermentation, the productivity of butyric acid is higher when  $\mu$  is lower. When  $\mu$  approaches zero, an oscillation in productivity occurs, as has been reported by Michel-Savin *et al.* [16,17], and Ferras *et al.* [26]. The oscillation in productivity is predicted to either be from product accumulation, lack of nutrients, or cell aging.

The selectivity and productivity of butyric acid varied significantly with fermentation modes, dilution rates, and extent of cell recycle for continuous fermentation. Thus, it was attempted to use fermentation results to correlate productivity with cell growth rate. The butyrate selectivity vs. the growth rate in continuous culture with and without cell recycle was calculated using Equations (4–6) and compared to the data derived from batch culture. Since the specific growth rate  $\mu = D \cdot (1 - R)$  and

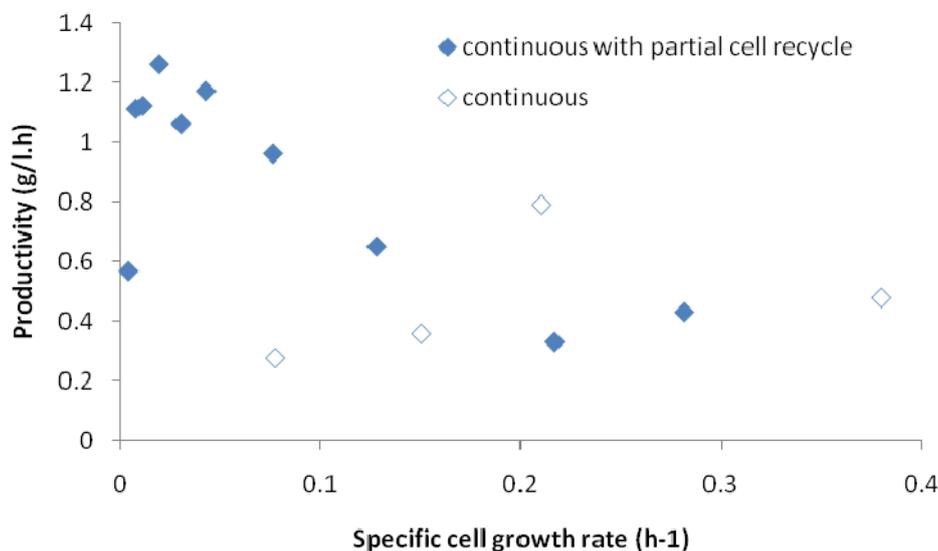
$D$  is known,  $\mu$  can be calculated; these results are shown in Figure 3. It is important to note that although all of the continuous data with and without partial cell recycle fit on the same trend line in this figure, the inclusion of continuous culture without cell recycle on this line would require a feed at extremely low flow rates, artificially skewing the selectivity high and productivity low.

If the maximum productivity of butyric acid can be assumed to be 1.2 g/Lh when  $\mu$  is  $0.02 \text{ h}^{-1}$ , an operating condition can be evaluated to achieve a high selectivity of butyric acid by applying Equation (4) and the definitions of dilution rate and productivity. Since butyric acid is produced at low growth rates, a stoichiometric ratio of glucose to butyric acid would predict that one mole of glucose gives one mole butyric acid, two moles carbon dioxide, and two moles hydrogen. Converting to g butyric acid/g sugar gives a value of 0.489. We assume that this theoretical assumption is correct since it is above but close to the maximum productivity of 0.45 [11], 0.41 [17], and 0.45 (this study) shown in literature. Thus, the concentration of butyric acid is:  $17 \text{ g/L (the glucose concentration)} \times 0.489 \times 0.95$  (the assumed maximum productivity for these calculations) = 7.9 g/L. The dilution rate for this maximum productivity can then be calculated as  $1.2 \text{ g/Lh} / 7.9 \text{ g/L} = 0.15 \text{ h}^{-1}$ . Because maximum productivity is achieved at  $\mu = 0.02 \text{ h}^{-1}$  as shown in Figure 4,  $R$  can be calculated directly from Equation (4), and is 87%. These results suggest that a continuous fermentation with low dilution rate and a relatively high ratio of cell recycle of about 85% can achieve high butyric acid selectivity and productivity. To test this assumption, a continuous fermentation at a dilution rate of  $0.15 \text{ h}^{-1}$  and  $R$  of 85% was performed, with the result of a butyric acid selectivity of 89%. This is very close to the calculated results, and shows that the model accurately describes the system.

**Figure 3.** Influence of specific cell growth rate to selectivity of butyric acid in continuous fermentation by *C. tyrobutyricum* at pH of 6.8.



**Figure 4.** Influence of specific cell growth rate to productivity of butyric acid in continuous fermentation by *C. tyrobutyricum* at pH of 6.8.



### 3. Experimental Section

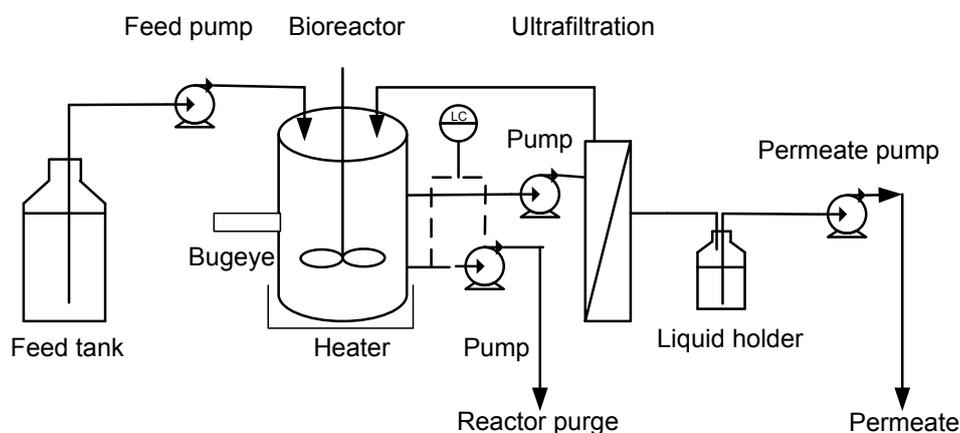
#### 3.1. Cultures and Medium

Stock culture of *C. tyrobutyricum* (ATCC 25755) was kept in bottles under anaerobic conditions at 4 °C. For pre-culture, 10 mL stock cultures were used to inoculate 100 mL of PYG (Peptone, Yeast extract, Glucose) medium in an incubator at 37 °C for about 48 h. A medium with 6.5 g/L peptone, 3.5 g/L yeast extract and 17 g/L D-(+)-glucose (all from Aldrich-Sigma, St. Louis, MO, USA) was used in batch and continuous culture studies, both with and without cell recycle. All media were autoclaved at 121 °C for 30 minutes before use.

#### 3.2. Fermentation Modes

Batch and continuous fermentations were carried out in a 1 L reactor (Applikon, Foster City, CA, USA). A schematic of the entire system is given in Figure 5. The fermentation was controlled at a temperature of 37 °C, and a pH of 6.8. The stirring rate was controlled at 500 rpm, which was high enough to keep the cells suspended without leading to significant foaming. Batch fermentation started with 700 mL of medium inoculated with 100 mL pre-culture. Nitrogen was sparged into the culture at a flowrate of 20 mL/min to maintain the anaerobic state of the fermenter. When the concentration of glucose decreased to approximately 0.5 g/L, the fermentation was switched from batch to continuous mode. For continuous fermentation, the flowrate of the feed medium was controlled by a peristaltic pump (Masterflex, Cole-Palmer, Vernon Hills, IL, USA). The total volume of 700 mL was maintained by a liquid level control, which was part of the fermentation system. Two 4 L bottles were used as the feed bottles.

**Figure 5.** Process diagram of continuous *C. tyrobutyricum* fermentation/membrane separation system.



An ultrafiltration membrane cartridge (Koch Membrane, Boston, MA, USA) was used for cell recycle. This cartridge was made of polysulfone, under the brand of Romicon, with a 100,000 MWCO. A peristaltic pump was used at the feed side of the cartridge. Another peristaltic pump was used to remove permeate, which contains the product solution. A pinch valve at the feed side was used to adjust the pressure difference between the feed and permeate side. The permeate flowrate was adjusted by the valve and permeate pump controller. The membrane was cleaned by cycling 8 L of a solution of 10 g/L sodium hydroxide and 10 g/L sodium hypochlorite through the membrane for 30 minutes sequentially, about once a week. When the membrane was being cleaned, the system was switched to continuous mode without cell recycle. After the cleaning procedure, the membrane was rinsed in pH 5 water to adjust the pH in the membrane to less than 7. The membrane cartridge, along with all the tubing and connectors, was replaced when the pressure at the feed side was greater than 5 psig.

For all fermentations, the feed glucose concentration was 17 g/L. The dilution rate was varied from  $0.077 \text{ h}^{-1}$  to  $0.38 \text{ h}^{-1}$  to investigate the influence of dilution rate on selectivity and productivity of butyric acid. A steady state of constant cell mass, acid and glucose concentrations was obtained after a minimum of three residence times. In all cases except for wash-out conditions, the glucose exiting the fermenter was  $<1 \text{ g/L}$ , indicating that glucose was the limiting nutrient. For continuous runs with cell recycle, culture was run through the feed side of the ultrafiltration membrane for approximately two hours without any permeate flowrate (closed permeate side) before continuous fermentation with cell recycle was initiated. Partial cell recycle was applied to the fermentation by varying the permeate flowrate, as shown as Figure 5. The parameter  $R$  was used to evaluate the extent of cell recycle and was defined as the flowrate of permeate over the feed flowrate; for example, when the permeate flowrate is equal to the feed flowrate,  $R$  is 100%.  $R$  was varied from 0% to 95% at dilution rates of  $0.38 \text{ h}^{-1}$  to  $0.077 \text{ h}^{-1}$  to investigate the influence of  $R$  on the selectivity and yield of butyric acid. The system ran for approximately four months without major interruptions or shut-downs. Cells were inspected via microscope for any change in culture morphology. The membrane held a good permeate flux during operation. The feed flowrate to the membrane was 60 mL/min and the average pressure at feed side was 1.6 psig when fouling was not serious. The pressure at permeate side was adjusted, depending on the desired permeate flowrate.

### 3.3. Analytical Methods

High-performance liquid chromatography (HPLC) was used to analyze limiting substrate products, including glucose, lactic acid, acetic acid and butyric acid. The HPLC system consisted of a Waters 717 autosample injector, a Waters 1525 binary HPLC pump, an IC-Pak™ ion-exclusion column and a Waters 2414 refractive index detector (Waters, Milford, MA, USA). The solvent was 0.0005 mol/L sulfuric acid at a flowrate of 1.0 mL/min and a fast HPLC analytical method originally developed for ethanol fermentation analysis was used [27]. Cell density was monitored by a Bugeye™ 200 noninvasive biomass monitor (Buglab, Foster City, CA, USA), a biomass detector with the ability to record cell growth in real time using software, which was calibrated against a dry cell mass curve.

## 4. Conclusions

This paper demonstrates the first continuous fermentation with partial cell recycle of glucose to butyric acid via *C. tyrobutyricum* by performing a 120 day continuous run. A comprehensive model to predict selectivity and productivity is derived and tested showing very good agreement between the prediction and the actual results. However, although a very good productivity of 1.13 g/Lh at 94.3% selectivity is achieved, high productivities comparable to those achieved using cell immobilization or selective recycling were not reached. Therefore, we have shown that partial cell recycle should be performed in conjunction with selective separation, allowing for adaptation of the model to differing media for varying effects on selectivity and productivity. The use of this model proves that long-term fermentation runs are a viable option for butyric acid and ultimate butanol production; with extremely long run times available, the process could be operated industrially for extremely long time periods.

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## References

1. Zigova, J.; Sturdik, E.; Vandak, D.; Schlosser, S. Butyric acid production by *Clostridium tyrobutyricum* with integrated extraction and pertraction. *Process. Biochem.* **1999**, *34*, 835–843.
2. Ramey, D.E. Continuous Two Stage, Dual Path Anaerobic Fermentation of Butanol and Other Organic Solvents Using Two Different Strains of Bacteria. U.S. Patent 5,753,474, 19 May 1998.
3. Binder, T.; Canavera, D.; Cavalieri, R.; Chen, S.; Conway, R.; Downing, M.; Drumm, L.; Eidman, V.; Foster, H.; Green, K. Vision for bioenergy and biobased products in the United States: Bioeconomy for a sustainable future. In *Proceedings of the Biomass Research and Development Initiative Vision Workshop*, Sacramento, CA, USA, 2–3 March 2006.
4. Zhang, C.; Yang, H.; Yang, F.; Ma, Y. Current progress on butyric acid production by fermentation. *Curr. Microbiol.* **2009**, *59*, 656–663.
5. Qureshi, N.; Ezeji, T.C.; Ebener, J.; Dien, B.S.; Cotta, M.A.; Blaschek, H.P. Butanol production by *Clostridium beijerinckii*. Part I: Use of acid and enzyme hydrolyzed corn fiber. *Biosource Technol.* **2008**, *99*, 5915–5922.

6. Ramey, D.E. *Production of Butyric Acid and Butanol*; DE-F-G02–00ER86106; U.S. Department of Energy: Morgantown, WV, USA, 2006.
7. Potts, T.M.; Du, J.; Paul, M.; May, P.; Beitle, R.; Hestekin, J. The production of butanol from Jamaica Bay macroalgae. *Environ. Prog. Sustain. Energy* **2012**, *31*, 29–36.
8. Tashiro, Y.; Takeda, K.; Kobayashi, G.; Sonomoto, K.; Ishizaki, A.; Yoshino, S. High butanol production by *Clostridium saccharofermentans* N1-4 in fed-batch culture with pH-stat continuous butyric acid and glucose feeding method. *J. Biosci. Bioeng.* **2004**, *98*, 263–268.
9. Potts, T.M.; Ackerson, M.; Hestekin, J.A. The production of fuel grade butanol from algal carbohydrates utilizing a hybrid biological and chemical process. Presented at AIChE Annual Meeting, Minneapolis, MN, USA, 16–21 October 2011.
10. Weigel, J.; Seung-Uk, K.U.K.; Kohring, G.W. *Clostridium thermobutyricum*: A moderate thermophile isolated from a cellulolytic culture that produces butyrate as the major product. *Int. J. Syst. Bacteriol.* **1989**, *39*, 199–204.
11. Wu, Z.; Yang, S.T. Extractive fermentation for butyric acid production from glucose by *Clostridium tyrobutyricum*. *Biotechnol. Bioeng.* **2003**, *82*, 93–102.
12. Liu, X.; Yang, S.T. Kinetics of butyric acid fermentation of glucose and xylose by *Clostridium tyrobutyricum* wild type and mutant. *Process Biochem.* **2006**, *41*, 801–808.
13. Liu, X.; Zhu, Y.; Yang, S.T. Butyric acid and hydrogen production by *Clostridium tyrobutyricum* ATCC 25755 and mutants. *Enzyme Microb. Technol.* **2006**, *38*, 521–528.
14. Zhu, Y.; Yang, S.T. Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. *J. Biotechnol.* **2004**, *110*, 143–157.
15. Van An del, J.G.; Zoutberg, G.R.; Crabbendam, P.M.; Breure, A.M. Glucose fermentation by *Clostridium butyricum* grown under a self generated gas atmosphere in chemostat culture. *Appl. Microbiol. Biotechnol.* **1985**, *23*, 21–26.
16. Michel-Savin, D.; Marchal, R.; Vandecasteele, J.P. Butyrate production in continuous culture of *Clostridium tyrobutyricum*: Effect of end-product inhibition. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 127–131.
17. Michel-Savin, D.; Marchal, R.; Vandecasteele, J.P. Control of the selectivity of butyric acid production and improvement of fermentation performance with *Clostridium tyrobutyricum*. *Appl. Microbiol. Biotechnol.* **1990**, *32*, 387–392.
18. Zhu, Y. Enhanced Butyric Acid Fermentation by *Clostridium Tyrobutyricum* Immobilized in a Fibrous-Bed Reactor. Ph.D. Dissertation, Ohio State University, Columbus, OH, USA, 2003.
19. Arora, M.B.; Hestekin, J.A.; Snyder, S.W.; St. Martin, E.J.; Lin, Y.J.; Donnelly, M.I.; Millard, C.S. The separative bioreactor: A continuous separation process for the simultaneous production and direct capture of organic acids. *Sep. Sci. Technol.* **2007**, *42*, 2519–2538.
20. Du, J.; Lorenz, N.; Beitle, R.R.; Hestekin, J.A. Application of wafer-enhanced electrodeionization in continuous fermentation process to produce butyric acid with *Clostridium tyrobutyricum*. *Sep. Sci. Technol.* **2012**, *47*, 43–51.
21. Hu, W.; Dodge, T.C. Cultivation of mammalian cells in bioreactors. *Biotechnol. Prog.* **1985**, *1*, 4–10.
22. Cheetham, P.S.J.; Blunt, K.W.; Bucke, C. Physical studies on cell immobilization using calcium alginate gels. *Biotechnol. Bioeng.* **1979**, *21*, 2155–2168.

23. Karel, S.F.; Libicki, S.B.; Robertson, C.R. The immobilization of whole cells: Engineering principles. *Chem. Eng. Sci.* **1985**, *40*, 1321–1354.
24. Shuler, M.L.; Kargi, F. *Bioprocess Engineering: Basic Concepts*, 2nd ed.; Prentice Hall: Upper Saddle River, NY, USA, 2002.
25. Mason, C.A.; Hamer, G.; Bryers, J.D. The death and lysis of microorganisms in environmental processes. *FEMS Microbiol. Lett.* **1986**, *39*, 373–401.
26. Ferras, E.; Minier, M.; Goma, G. Acetonobutylic fermentation: Improvement of performances by coupling continuous fermentation and ultrafiltration. *Biotechnol. Bioeng.* **1986**, *28*, 523–533.
27. Young, J.; Lee, P.J.; di Gioia, A.D. Fast HPLC Analysis for Fermentation Ethanol Process. *Waters* **2007**, 720001896EN. Available online: <http://www.waters.com/waters/library.htm?cid=511436&lid=1512671> (accessed on 26 July 2012).

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