Kinetics of butyric acid fermentation of glucose and xylose by 
Clostridium tyrobutyricum wild type and mutant

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Received 9 August 2005; received in revised form 27 September 2005; accepted 3 October 2005

Abstract
The kinetics of butyric acid fermentation by Clostridium tyrobutyricum at pH 6.0 and 37 °C were studied with the wild type ATCC 25755 and its mutant PPTA-Em, which was obtained from integrational mutagenesis to inactivate the chromosomal pta gene, encoding phosphotransacetylase (PTA). The potential of using this mutant to improve butyric acid production from glucose and xylose was evaluated in both free and immobilized cell fermentations. Compared to the wild type, in free cell fermentations PPTA-Em produced 15% more butyrate (0.38 g/g versus 0.33 g/g) from both glucose and xylose, at much higher concentrations (37.2 g/L versus 22.9 g/L from glucose and 33.5 g/L versus 19.4 g/L from xylose). The increased butyrate production in the mutant can be attributed to the reduced acetate production as well as reduced specific growth rate. The acetate yield in the mutant was reduced by 13.5% (0.058 g/g versus 0.067 g/g) and 32% (0.045 g/g versus 0.066 g/g) from glucose and xylose, respectively. The mutant’s specific growth rate was reduced by 36% (0.137 h⁻¹ versus 0.214 h⁻¹) on glucose and 26% (0.086 h⁻¹ versus 0.116 h⁻¹) on xylose. A fibrous-bed bioreactor (FBB) was used to immobilize PPTA-Em mutant cells and further improve butyric acid production. The final butyric acid concentrations in fed-batch fermentations reached 49.9 g/L from glucose and 51.6 g/L from xylose, with the butyrate yield increased to 0.44 g/g glucose and 0.45 g/g xylose. As evidenced by the greatly increased butyrate/acetate ratio in the final product profile, it is concluded that the mutant’s metabolic pathway has been shifted to favor butyrate production due to the knockout of pta gene even though acetate production remains at a significant level. The observed metabolic shift is corroborated by the changed protein expression patterns as seen in two-dimensional protein electrophoresis and SDS-PAGE.

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Keywords: Clostridium tyrobutyricum; Butyric acid fermentation; Fibrous-bed bioreactor

1. Introduction

Clostridium tyrobutyricum, a gram-positive, rod-shaped, spore-forming, and obligate anaerobic bacterium, can produce butyric acid, acetic acid, hydrogen and carbon dioxide from various carbohydrates including glucose and xylose [1]. Butyric acid is a short-chain fatty acid naturally generated by anaerobic fermentation of dietary substrates in intestines. It is currently produced by petrochemical routes and has many applications; in the chemical industry, it is mainly used to synthesize butyryl polymers; in the food industry, it is used to enhance butter-like notes in food flavors; and in the pharmaceutical industry, it can be used to treat colorectal cancer and hemoglobinopathies [2,3]. Also, the esters of butyrate are used as additives for increasing fruit fragrance and as aromatic compounds in perfumes.

There has been increasing interest in the production of butyric acid from biomass using C. tyrobutyricum and C. butyricum [4–7]. However, the conventional butyric acid fermentation process is not yet economically competitive because of its low reactor productivity, low final product concentration, and low product yield. Recently, C. tyrobutyricum cells immobilized in a fibrous-bed bioreactor (FBB) were successfully used for butyrate fermentation with increased reactor productivity and final product concentration [7]. A novel extractive fermentation process has also been developed, using Alamine 336 in oleyl alcohol as the extractant contained in a hollow-fiber membrane extractor for selective removal of butyric acid from the fermentation broth, that can further enhance product concentration and purity while lowering the recovery and purification costs [1,8,9].

Creating metabolically engineered mutants of clostridial strains is another approach for improving fermentation. Recently, integralional mutagenesis has been successfully used to create gene knockout mutants [10,11]. In this technique, a non-replicative integralional plasmid containing a fragment of...
the target gene and a selection marker is constructed and then inserted into the parental chromosome by homologous recombination to knockout the target gene, resulting in mutants with altered metabolic pathways and fermentation characteristics. Using a non-replicative plasmid containing a partial pta gene, encoding PTA (phosphotransacetylase), Green et al. obtained mutants of solventogenic *C. acetobutylicum* ATCC 824 with much reduced PTA and AK (acetate kinase) activities and acetate production [10]. Similarly, by using the integrational plasmid pPTA-Em, a pta-deleted *C. tyrobutyricum* mutant (PPTA-Em) with decreased PTA activity and increased butyrate production was obtained [11].

The main objective of this work was to evaluate the potential of using the metabolically engineered *C. tyrobutyricum* mutant PPTA-Em for butyric acid production from glucose and xylose. In this study, free cell and immobilized cell FBB fermentations were carried out at 37 °C and pH 6.0 with PPTA-Em and the wild type for comparison purpose. The effects of integrational mutagenesis, sugar source, and cell adaptation in the FBB on butyric acid fermentation kinetics were studied and are discussed in this paper. Finally, SDS-PAGE and two-dimensional protein electrophoresis were used to study and characterize protein expression changes as affected by the mutation in PPTA-Em and different sugar sources used in the fermentations.

2. Materials and methods

2.1. Cultures and medium

*C. tyrobutyricum* ATCC 25755 was maintained on reinforced clostridial medium (RCM; Difco) plates in an anaerobic chamber (95% N2, 5% H2). Working cultures were grown at 37 °C in a previously described synthetic medium (CGM) with glucose as the substrate [12]. The metabolically engineered mutant, PPTA-Em, was obtained by transforming *C. tyrobutyricum* competent cells with non-replicative plasmids containing erythromycin (Em) resistant gene and partial pta fragment obtained from PCR amplification [11]. The pta knockout mutant, created through homologous recombination of the plasmids with the chromosome, was selected and stored on the RCM plates containing 40 µg/mL Em.

2.2. Fermentation kinetic studies

Fed-batch fermentations of *C. tyrobutyricum* were performed in a 5-L stirred-tank fermentor (Marubishi MD-300) containing 2 L of the medium with either glucose or xylose as the substrate. Erythromycin was not used in the fermentation study as the mutant PPTA-Em was genetically stable without the selection marker. Anaerobiosis was reached by initially sparging the medium with nitrogen. The medium pH was adjusted to ~6.0 with 6N HCl before inoculation with ~100 mL of cell suspension prepared in a serum bottle. Experiments were carried out at 37 °C, 150 rpm for agitation, and pH 6.0 ± 0.1 controlled by adding NH4OH. The fed-batch mode was operated by pulse feeding concentrated substrate solution when the sugar level in the fermentation broth was close to zero. The feeding was continued until the fermentation ceased to produce butyrate due to product inhibition. Samples were taken at regular intervals from the fermentation broth for the analyses of cell, substrate and acid products.

2.3. Fermentation in fibrous-bed bioreactor

The fibrous-bed bioreactor (FBB) was made of a glass column packed with spiral wound cotton towel and had a working volume of ~480 mL. Detailed description of the reactor construction has been given before [12]. The reactor was connected to the 5-L stirred-tank fermentor containing the medium through a recirculation loop (~1 m long, tubing i.d.: 3.1 mm; Microflex Norprene 06402-16, Cole Parmer, Chicago, IL) and operated under well-mixed conditions with pH and temperature controls. The FBB was first operated at a repeated batch mode to increase the cell density in the fibrous-bed to a stable, high level (>50 g/L). To adapt the culture to a higher butyrate concentration, the reactor was then operated at fed-batch mode by pulse-feeding a concentrated substrate solution whenever the sugar level in the fermentation broth was close to zero. At the end of each fed-batch experiment, the medium in the fermentor was completely replaced with 2 L of fresh medium to start new fed-batch fermentation.

2.4. Analytical methods

Cell density was analyzed by measuring the optical density of the cell suspension at a wavelength of 600 nm (OD600) with a spectrophotometer (Spectorea-turner, Model 340). One unit of OD600 corresponded to 0.68 g/L of cell dry weight for cells grown in the glucose medium. A high-performance liquid chromatography (HPLC) was used to analyze the organic compounds, including glucose, xylose, lactate, acetate, and butyrate, in the fermentation broth. The HPLC system consisted of an automatic injector (Shimazu SIL-10A), a pump (Shimadzu LC-10A), organic acid analysis column (Bio-Rad HPRX-874), a column oven at 45 °C (Shimadzu RID-10A), and a refractive index detector (Shimadzu RID-10A). The eluent was 0.01N H2SO4, at a flow rate of 0.6 mL/min. Hydrogen and carbon dioxide production during the fermentation was monitored using an on-line respirometer (Micro-oxymsystem, Columbus Instrument, Columbus, OH). The fermentor was connected to a tightly sealed bottle (volume: 5400 mL) for the collection of the gas products, which was flushed with nitrogen periodically. The change of gas composition inside the bottle was monitored and used to estimate the gas
production, which was also measured by collecting the produced gas in an inverted 4-L plastic graduate cylinder in a water trough.

2.5. Preparation of cell extract and protein electrophoresis

Cells cultivated in 100 mL of CGM at 37 °C were allowed to grow to the exponential phase (OD600 = 1.5), and then harvested and washed. The cell pellets suspended in 10 mL of 25 mM Tris–HCl buffer (pH 7.4) were sonicated, and the protein extract was collected by centrifugation.

Protein samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared from the cell extract after sonication and centrifugation. The cell extract (10 mL) was concentrated using four volumes of acetone (40 mL) to precipitate protein at 20 °C overnight, and re-dissolved in 2 mL of 25 mM Tris–HCl buffer (pH 7.4), following the standard protocol (Bio-Rad, Hercules, CA). Protein samples, 24 µg per well, were loaded into 12.5% SDS-PAGE gel, and run at 100 V per 2.5 h with PROTEAN II xi Cell (Bio-Rad) and stained following the instruction of the manufacturer.

For two-dimensional protein electrophoresis (2DE), cell extract was concentrated by acetone and then dissolved in the rehydration buffer (8 M urea, 4% CHAPS, 10 mM DTT, 0.2% (w/v) Bio-Lytes 3/10) for sample preparation. The first dimension was performed on an 11 cm IPG strip with a nonlinear immobilized pH 3–10 gradient (Amersham, Piscataway, NJ). The IPG strip was rehydrated in the rehydration buffer with 20 µg proteins at 50 V for 12 h using PROTEAN IEF Cell (Bio-Rad). After rehydration, the protein was focused on IPG strip by preset method, at 250 V for 15 min to remove excess salts, then ramped linearly from 250 to 5000 V for 2 h, and finally maintained at 8000 V for 10–15 min and in equilibrate buffer II (6 M urea, 2% SDS, 0.375 M Tris–HCl, pH 8.8, 20% glycerol and 135 mM iodoacetamide) for 10–15 min. The equilibrated strip was applied to a polyacrylamide/PDA SDS gel to run the second dimension electrophoresis at 100 V for 120–180 min with Mini-PROTEAN 3 Cell (Bio-Rad). The protein spots were developed using silver staining kit (Amersham). The two-dimensional protein electrophoresis maps were analyzed by using Phoretix 2D software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK).

3. Results and discussion

3.1. Fermentation kinetics

Typical fed-batch fermentation kinetics with C. tyrobutyricum wild type and PPTA-Em mutant grown on glucose and xylose are shown in Figs. 1 and 2, respectively. In general, there was a short lag phase with little gas (H2 and CO2) and acid (acetic and butyric acids) production, which then increased during the exponential phase. For the wild type, acid production either stopped or slowed down dramatically when cells entered
the stationary phase, although gas production and glucose (or xylose) consumption continued for a substantial period. For the PPTA-Em mutant, acetic acid production also stopped in the stationary phase; however, butyric acid production continued to reach a much higher level. Consequently, the mutant produced much more butyric acid and reached a higher final butyric acid concentration when the fed-batch fermentations were finally stopped. The fermentations were stopped when the sugar substrate was no longer consumed by the cells due to inhibition by butyric acid [10].

It is clear that PPTA-Em is more tolerant to butyric acid inhibition, a result from the genetic manipulation of the pta gene in the mutant [11]. The specific growth rates and product yields from glucose and xylose in these fermentations were estimated and are listed in Tables 1 and 2, respectively. The specific growth rate was determined from the slope of the semi-logarithmic plot of OD versus time using the time-course data in the exponential phase. The product yield was determined from the linear plot of product concentrations versus substrate concentrations at various fermentation times during the batch fermentation. The effects of the mutation on C. tyrobutyricum and the butyric acid fermentation are further discussed in the following sections.

### 3.2. Effect on cell growth

The mutant’s specific growth rate was reduced by 36% (0.137 h\(^{-1}\) versus 0.214 h\(^{-1}\)) on glucose (Table 1) and 26% (0.086 h\(^{-1}\) versus 0.116 h\(^{-1}\)) on xylose (Table 2), respectively. However, the cell biomass yields for the mutant appeared to be higher than those for the wild type, although the difference was within the error range. The lower specific growth rate for the mutant can be attributed to the metabolic burden on cells caused by possibly less energy (ATP) generation in the sugar metabolism due to pta knockout. This is mainly because that pyruvate oxidation to acetate can generate more ATP than its oxidation to butyrate. The lower specific growth rate and biomass yield with xylose as the substrate is because that xylose fermentation gives lower energy efficiency as compared with glucose fermentation. The different specific growth rate and biomass yield from the mutant suggest that the carbon and energy fluxes have been redistributed in the metabolic pathways of the mutant, which also led to the significant changes in acids production.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>PPTA-Em</th>
<th>Immobilized cell</th>
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<tbody>
<tr>
<td></td>
<td>Free cell</td>
<td>Free cell</td>
<td>Immobilized cell</td>
</tr>
<tr>
<td><strong>Cell growth</strong></td>
<td></td>
<td></td>
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<tr>
<td>Specific growth rate (h(^{-1}))</td>
<td>0.116 ± 0.009</td>
<td>0.086 ± 0.020</td>
<td>0.048 ± 0.006</td>
</tr>
<tr>
<td>Biomass yield (g/g)</td>
<td>0.095 ± 0.003</td>
<td>0.109 ± 0.013</td>
<td>0.069 ± 0.003</td>
</tr>
<tr>
<td><strong>Acid production</strong></td>
<td></td>
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</tr>
<tr>
<td>Butyric acid concentration (g/L)</td>
<td>19.42 ± 1.195</td>
<td>33.49 ± 2.89</td>
<td>51.55 ± 3.49</td>
</tr>
<tr>
<td>Butyric acid yield (g/g)</td>
<td>0.33 ± 0.02</td>
<td>0.38 ± 0.02</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>Acetic acid concentration (g/L)</td>
<td>3.31 ± 0.01</td>
<td>3.89 ± 0.26</td>
<td>6.42 ± 1.60</td>
</tr>
<tr>
<td>Acetic acid yield (g/g)</td>
<td>0.066 ± 0.006</td>
<td>0.045 ± 0.001</td>
<td>0.045 ± 0.016</td>
</tr>
<tr>
<td>Butyrate/acetate ratio (g/g)</td>
<td>5.87</td>
<td>8.61</td>
<td>8.02</td>
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<tr>
<td><strong>Gas production</strong></td>
<td></td>
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<tr>
<td>H(_2) yield (g/g)</td>
<td>0.017 ± 0.001</td>
<td>0.017 ± 0.001</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>CO(_2) yield (g/g)</td>
<td>0.365 ± 0.001</td>
<td>0.373 ± 0.031</td>
<td>0.348 ± 0.004</td>
</tr>
<tr>
<td>H(_2)/CO(_2) ratio (mol/mol)</td>
<td>1.07 ± 0.02</td>
<td>1.05 ± 0.02</td>
<td>0.96 ± 0.01</td>
</tr>
</tbody>
</table>

Note: average ± standard deviation were calculated from two (wild type) or three (mutant) batch fermentations.

![SDS-PAGE of cellular proteins from C. tyrobutyricum.](image-url)
3.3. Effects on butyric and acetic acids production

Compared to the wild type, the PPTA-Em mutant not only can tolerate and produce a much higher concentration of butyric acid, it also gives higher butyric acid yields from glucose and xylose. As can be seen in Tables 1 and 2, PPTA-Em produced 15% more butyrate (0.38 g/g versus 0.33 g/g) from both glucose and xylose. The increased butyrate production in the mutant can be attributed to the reduced acetate production as well as reduced specific growth rate. The acetate yield in the mutant was reduced by 13.5% (0.058 g/g versus 0.067 g/g) from glucose and 32% (0.045 g/g versus 0.066 g/g) from xylose, respectively. Although the mutant still produced a significant amount of acetate, the inactivation of pta gene reduced acetic acid and increased butyric acid production because more substrates were directed toward the butyric acid formation pathway, as evidenced by the increased butyrate/acetate (B/A) ratio (from ~5.5 to ~8.9 g/g) in the fermentations. This metabolic shift was the direct result of pta knockout mutation.

The final butyric acid concentrations produced from glucose and xylose by PPTA-Em increased by 62% (from 22.9 to 37.2 g/L) and 72% (from 19.4 to 33.5 g/L), respectively. Our previous study has shown that the acetic acid-forming enzymes (PTA and AK) are more sensitive to butyric acid inhibition than butyric acid-forming enzymes (phosphotransbutyrylase and butyrate kinase) [13]. The mutant’s reduced sensitivity to butyric acid inhibition thus can be attributed to the reduced carbon flux through the PTA–AK pathway. However, the final acetic acid concentration produced in the fermentation by

![Two-dimensional protein electrophoresis for wild type (A) and mutant (B) grown on glucose.](image)

Fig. 4. Two-dimensional protein electrophoresis for wild type (A) and mutant (B) grown on glucose.
PPTA-Em was not much affected even though the acetic acid yield from sugar was reduced significantly. This is because that there may be other enzymes or pathways present in C. tyrobutyricum that can also produce acetate from pyruvate or acetyl CoA [11].

3.4. Effect on gas production

As discussed before, both hydrogen and carbon dioxide were produced throughout the fermentation. The pta knockout mutation did not appear to have any significant effect on gas production by the PPTA-Em mutant. The average hydrogen and carbon dioxide yields were found to be \(~0.017\) and \(~0.37\) g/g, respectively, for both the mutant and the wild type using either glucose or xylose as the substrate.

3.5. SDS-PAGE and 2-DE analyses of protein expression

The effects of pta knockout and fermentation conditions on protein expression were first studied with SDS-PAGE. As can be seen in Fig. 3, there are notable differences in the SDS-PAGE protein profiles obtained from the wild type and the mutant. For example, the level for the proteins with molecular weight ($M_w$) of \(~32\) kDa was significantly lowered for PPTA-Em mutant grown on glucose. Interestingly, this group of \(~32\) kDa proteins in the mutant was much higher when xylose was used as the growth substrate. This substrate effect, however, was not observed with the wild type. It is clear that a single gene knockout can induce rather complicated responses in protein expression by the cells due to gene and metabolic regulatory networks.

![Fig. 5. Two-dimensional protein electrophoresis for wild type (A) and mutant (B) grown on xylose.](image)
The effects on protein expression were further studied with two-dimensional protein electrophoresis (2DE). There were ~200 protein spots on the 2DE maps obtained for both the wild type and mutant PPTA-Em grown on glucose (Fig. 4). A smaller number of protein spots were obtained for growth on xylose (Fig. 5). These 2DE protein maps were analyzed with the Phoretix 2D AdvancedTM software, which compared and identified proteins with changed expression levels after normalizing the different intensities of the protein spots on these 2DE gels. As expected, the number of protein spots and their intensities (expression levels) on the 2DE gels were different between the wild type and the mutant. For example, in the region circled on the gels (PI: 5.5–7.5, \( M_W: \sim 32 \text{ kDa} \)) there are two more protein spots for the wild type than for the mutant. These missing protein spots are very likely to include PTA and AK, but cannot be identified in this work due to lack of proteomic information for \( C. tyrobutyricum \). Table 3 shows the results of 2DE analysis of proteins in this region. The expression levels of these protein spots with different PI’s and \( M_W \)’s are compared based on the normalized protein expression volume. With the wild type grown on glucose as the reference, above 100% indicates up regulation while below 100% indicates down regulation of the protein expression in the mutant or growth on xylose. For growth on glucose, two proteins (spot #89, PI: 6.3, \( M_W: 31.1 \); spot #93, PI: 6.77, \( M_W: 33.6 \)) found in the wild type were missing and at least one protein (spot #95, PI: 6.90 and \( M_W: 33.55 \)) highly expressed in the wild type (N volume = 0.802) was dramatically down-regulated in the mutant. Similarly, for growth on xylose, the protein spot #93 (PI: 6.77, \( M_W: 33.6 \)) found in the wild type was also missing in the mutant. It is noted that protein expression is also significantly affected by the carbon source used in the fermentation. However, the \( pta \) knockout has similar effects on protein expression and metabolic pathway shift, regardless of the carbon source used in the fermentation.

### 3.6. Effects of carbon source

\( C. tyrobutyricum \) is one of a few bacteria that can use xylose to produce butyric acid. As observed in this work, both glucose and xylose can be efficiently used in butyric acid fermentation and there is no obvious preference for either glucose or xylose as the carbon source, although cell growth and biomass yield from xylose were significantly lower than those from glucose. The lower growth rate from xylose should not be a concern for immobilized cell fermentation using the FBB, which is discussed below.
3.7. Immobilized cell fermentation in FBB

Immobilized cell bioreactors have the potential in improving a fermentation process by increasing cell density, reactor productivity and final product concentration. We have previously developed a fibrous-bed bioreactor (FBB) for immobilized cell fermentations to produce several organic acids from biomass with significantly improved productivity, yield, and final product concentration [12–14]. The FBB was thus applied to the PPTA-Em mutant in this work to further improve butyric acid production from glucose and xylose. As shown in Fig. 6, the FBB fermentations produced much more butyric acid from glucose and xylose than those obtained in free cell fermentations. As cells adapted in the FBB became more tolerant to butyrate inhibition, they were able to produce butyric acid at a final concentration of ~50 g/L with a butyrate yield of ~0.45 g/g sugar (see Tables 1 and 2). The improvements over the free cell fermentations were over 16.5% in butyrate yield and 34–54% in the final butyrate concentration. The improved butyrate yield can be attributed to the reduced cell growth in the immobilized cell fermentation, whereas the improved butyrate tolerance is the result of adaptation and possibly physiology changes in the FBB environment [13,15]. SDS-PAGE protein expression profiles (see Fig. 3) for cells from the FBB showed similar patterns to those in the free cell fermentations, however. Further study and characterization of cells adapted in the FBB will be necessary in order to fully understand why the FBB fermentation allowed the cells to tolerate and produce more butyric acid.

4. Conclusions

The potential of using the C. tyrobutyricum mutant obtained from integrational mutagenesis that selectively inactivated the chromosomal pta gene, to produce butyrate from glucose and xylose was studied. Compared with the wild type, butyric acid production from glucose and xylose by this mutant is improved with higher butyrate yields, final product concentrations, and product purity (B/A ratio). The butyric acid fermentation was further improved by immobilizing the mutant in the fibrous-bed bioreactor to facilitate cell adaptation to attain even higher product yields and concentrations. Since both glucose and xylose, which are rich in corn fiber hydrolysate [16], can be used equally well to produce butyric acid, it is feasible to produce butyric acid from mixed sugars derived from abundant plant biomass such as corn fiber. As demonstrated in this work, the butyric acid fermentation can be more efficiently carried out in the FBB using the metabolically engineered mutant of C. tyrobutyricum, allowing economical production of bio-based butyric acid.

Acknowledgements

This work was supported in part by research grants from the Department of Energy (DE-FG02-00ER86106), the U.S. Department of Agriculture (CSREES 99-35504-7800), and the Consortium for Plant Biotechnology Research Inc. (CPBR; R-82947901).

Reference