Metabolic Engineering of *Clostridium tyrobutyricum* for Production of Biofuels and Bio-based Chemicals

Dissertation

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ABSTRACT

The depletion of petroleum resource is aggravated by the increasing demand for fossil fuels. Moreover, the burning process of fossil fuels causes global warming and pollution. These concerns promote the intensive investigation on the development of economical processes to produce fuels and chemicals based on renewable resources. In this respect, plant biomass is the current sustainable source for the production of bio-based chemicals and biofuels.

*C. tyrobutyricum* is a rod-shape, gram-positive bacterium that produces butyrate, acetate, and hydrogen from various saccharides, including glucose and xylose, under strict anaerobic conditions. Phosphotransbutyrylase (PTB) is a key enzyme in the butyric acid synthesis pathway. However, its role in affecting metabolic flux distribution and production of various metabolites is not well understood. In this work, a mutant with inactivated *ptb* gene, encoding phosphotransbutyrylase, was created by integrational mutagenesis through homologous recombination. Compared to the wild-type, the activities of phosphotransbutyrylase (PTB) and butyrate kinase (BK) in the mutant decreased 76% and 42%, respectively; meanwhile, phosphotransacetylase (PTA) and
acetate kinase (AK) increased 7% and 29%, respectively. In addition, the mutant displayed a higher tolerance to butyric acid.

The fermentation conditions were optimized for the ptb mutant fermentation. The pH value was set at 6 and the initial substrate concentration was 50 g/l by using one-factor-a-time screening. The impact of ptb-disruption on the fermentation profile was studied using glucose and xylose as substrate. Compared to the wild type, the b/a ratio decreased by 34.7% for glucose and 28.1% for xylose, this could be attributed to the inactivation of ptb. The productivity of hydrogen increased 38% for glucose and 46% for xylose, which could be partially attributed to a higher specific growth rate (33% increase for glucose and 40% increase for xylose) in addition to the lower b/a ratio. The butyric acid fermentation and hydrogen production were further improved by immobilizing the cells in the fibrous-bed bioreactor to facilitate cell adaptation to attain a higher final product concentration.

The genome of C. tyrobutyricum was sequenced by using the 454 technology. Several genes encoding enzymes involved in the solvent formation pathway were annotated. Interestingly, the only missing gene for a complete butanol production route is for butyrylaldehyde dehydrogenase. Moreover, the mutants of C. tyrobutyricum showed higher tolerance to butanol than the butanol producing C. acetobutylicum. Plasmids carrying aldehyde/alcohol dehydrogenase gene aad or adhE2 from C. acetobutylicum
were constructed and introduced into *C. tyrobutyricum*. The engineered mutants showed significant butanol production in batch fermentation with glucose as the substrate. This work demonstrated that it is feasible to use *C. tyrobutyricum* to produce butanol.
Dedicated to my parents and boyfriend Bingxu Song
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PUBLICATIONS

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FIELDS OF STUDY

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CHAPTER 1

INTRODUCTION

*Clostridium tyrobutyricum* is a member of *clostridia* family and was discovered as cause for the late-blowing defect in cheese. It produces butyric acid, acetic acid, and hydrogen from diverse carbon sources including glucose and xylose (Klijn *et al.* 1995). Butyric acid is a 4-carbon carboxylic acid and has many applications in food, perfume and pharmaceutical industries (Dziedzak 1986; Pouillart 1998; Williams *et al.* 2003). The production of butyric acid from sustainable biomass has become a promising alternative to the current petroleum chemical route due to public concerns about environmental pollution and the depletion of petroleum resources. Compared with other bacteria in butyric acid fermentation, *C. tyrobutyricum* has many advantages, such as high product purity and yield (Wu and Yang 2003). Furthermore, hydrogen as the gas by-product is a clean, efficient and sustainable energy source with the highest energy content per unit weight (143 GJ/ton) among all known fuels (Boyles 1984). In addition, hydrogen does not contribute to any environmental problems since its oxidation product is H₂O only.

The suggested metabolic pathway in *C. tyrobutyricum* is shown in Figure 1.1. In this pathway, the substrate, hexose or pentose, is oxidized to acetyl-CoA, with the releasing of CO₂ and hydrogen. Then, acetyl-CoA can be either converted to acetyl
phosphate resulting in the excretion of acetate, or condensed and reduced to form butyryl-CoA resulting in the excretion of butyrate. Phosphotransacetylase (PTA) and acetate kinase (AK) catalyze the formation of acetate and generate ATP for cell growth (Green et al. 1996). Mutants with disrupted pta and ack have been developed, and both of them showed increased butyrate and decreased acetate production (Zhu et al. 2004; Liu et al. 2006; Liu and Yang 2006).

The butyrate formation pathway comprising of two consecutive enzymatic reactions catalyzed by phosphotransbutyrylase (PTB) and butyrate kinase (BK), encoded by ptb and buk, respectively (Walter et al. 1993), plays a critical role in butyric acid biosynthesis in C. acetobutylicum (Green et al. 1996; Green and Bennet 1997).

\[
\begin{align*}
(1) \text{butyryl-CoA} + \text{Pi} & \rightarrow \text{Butyryl-P} + \text{CoA} \\
(2) \text{butyryl-P} + \text{ADP} & \rightarrow \text{Butyrate} + \text{ATP}
\end{align*}
\]

This pathway is important in the energy metabolism of the organism, as ATP is produced during the conversion of butyryl-CoA to butyrate (Valentine and Wolfe 1960). Both enzymes have been purified and characterized from C. acetobutylicum strain ATCC 824. PTB is an octamer of identical 31-kDa subunits consisting of 302 residues and BK was reported to be a dimer of identical 30-kDa subunits consisting of 356 residues. ptb and buk are located in the same operon with ptb proceeding buk 44 bp in C. acetobutylicum (Hartimanis and Gatenbeck 1984; Wisenborn et al. 1989). buk has been interrupted in C. acetobutylicum and the mutant showed a decreased butyrate production and increased acetate production (Desai et al. 1999). The butyrate formation consumes
NADH, in which the reduction equivalence could be transferred to ferredoxin by NADH-ferredoxin oxidoreductase, then the reduced ferredoxin could be oxidized and the hydrogen is released under the catalysis of hydrogenase.

The main problem associated with conventional carboxylic acid fermentation is the final product inhibition, which causes low product concentration, yield and productivity, resulting in a high cost in product recovery (Michel-Savin et al. 1990a; Michel-Savin et al. 1990b; Michel-Savin et al. 1990c). This problem can be partially addressed by using the fibrous-bed bioreactor in which cells are immobilized in a fibrous matrix to achieve a high cell density (Yang 1996). The fibrous-bed bioreactor has been widely applied in the production of organic acids, including propionic acid, lactic acid and acetic acid, to increase cell density, reactor productivity, titer and yield (Yang et al. 1994; Silva and Yang 1995; Huang et al. 1998). The fibrous-bed bioreactor has also been used to adapt C. tyrobutyricum to increase its butyrate tolerance and to enhance the final product concentration in the fermenter (Zhu et al. 2004; Liu et al. 2006).

Genome sequencing discovered that C. tyrobutyricum has butanol dehydrogenase and it could produce butanol if the butyryl aldehyde dehydrogenase is functional in the cell. Moreover, pta and ack mutant have shown higher butanol tolerance (16 g/l) than wild type strain (12 g/l), which is similar to C. acetobutylicum. These two facts make it rational to construct C. tyrobutyricum mutants as novel butanol producing bacteria with higher butanol tolerance.

1.1 Objectives
The overall goal of this research was to explore the potential of *Clostridium tyrobutyricum* for production of biofuels and bio-based chemicals. First of all, a *ptb*-disrupted mutant was constructed and characterized in order to evaluate the effect of *ptb* knock-out on the fermentation. Secondly, the fermentation kinetics was compared between the wild type and *ptb* mutant. In addition, several fermentation modes were investigated. The fibrous bed bioreactor was also used to increase the butyric acid tolerance. The third objective of this study was to establish butanol production in *C. tyrobutyricum* by heterologous gene expression. The 454 genome sequencing study suggested that *C. tyrobutyricum* can be a good butanol producing bacterium if butyryl CoA can be reduced to butyrylaldehyde in the cell. Therefore, efforts were put on the expression of aldehyde dehydrogenase in *C. tyrobutyricum*. Figure 1.2 provides an overview of the research objectives, approaches, and the scope of this study, which is briefly described below.

**Task 1: Construction and characterization of *ptb*-disrupted mutant by homologous recombination**

Gene disruption has been widely used in the study of protein function and gene expression regulation. An antibiotic resistant gene (*Em*) was inserted into the target gene fragment (*ptb*), resulting in the target gene interruption. However, the genome of *C. tyrobutyricum* has not been completely sequenced and its *ptb* gene sequence was not available. In this case, the amino acid sequence in the highly conserved region of PTB
and the *Clostridium* preferred codon were used to design degenerate primers to achieve the aim. After the mutant was proved by PCR identification, the related acid forming enzyme activities were evaluated. The butyrate tolerance and the fermentation profile were also compared between the wild type and the *ptb*-disrupted mutant. The results are reported in Chapter 3.

**Task 2: Fermentation kinetics and process development for butyric acid and hydrogen production in *ptb* mutant.**

The effects of *ptb*-disrupted on cell growth as well as butyric acid, acetic acid, and hydrogen production from glucose and xylose in free-cell batch fermentations were studied and the results are reported in chapter 4. In addition, medium composition was simplified and different low cost substrates were evaluated. The fermentation conditions were optimized for the *ptb* mutant fermentation. The fibrous-bed bioreactor was used to adapt cells for increased tolerance to butyric acid and to attain high final butyric acid concentration in the fermenter. The potential of using *ptb*-disrupted mutant for butyrate and hydrogen production are also discussed.

**Task 3: Establishment of butanol production in *Clostridium tyrobutyricum*.**

The genome of *C. tyrobutyricum* has been sequenced by using 454 technology. The genes encoding the key enzymes in the suggested metabolic pathway were located in
the database and the homology was compared with other *Clostridium* species. Expression plasmid pCAAD and pSOS-adhE2 containing *aad* and *adhE2*, respectively, were constructed and transformed into *C. tyrobutyricum*. Fermentations were carried out in serum bottles to verify butanol production in the mutants. Free cell fermentation in a stirred-tank fermenter was also performed to evaluate the kinetics of the mutant, and the results are reported in Chapter 5.
1.2 References


Figure 1.1 The metabolic pathway for the biosynthesis of butyrate, acetate, and hydrogen in *Clostridium tyrobutyricum*. (*pfor*: pyruvate: ferredoxin oxidoreductase; *nfor*: NADH:ferredoxin oxidoreductase; *hyd*: hydrogenase; *pta*: phosphotransacetylase; *ack*: acetate kinase; *hbd*: 3-hydroxybutyryl CoA dehydrogenase; *ptb*: phosphotransbutyrylase; *buk*: butyrate kinase)
Objective

Improve bio-based chemicals and biofuels production by metabolically engineered *C. tyrobutyricum*

- Metabolic engineering & strain improvement
  - PCR amplification of *pib*
  - Construct the plasmid
  - Develop metabolic mutants: *pib* mutants
  - Characterize the mutant
  - Investigate the knock-out impact

- Fermentation kinetics & process development
  - Medium optimization
  - Feedstock selection
  - Condition optimization
  - Batch fermentation
  - Fed-batch fermentation
  - Immobilized cell fermentation

- Metabolic engineering for butanol production
  - Genome sequencing
  - Metabolic pathway reconstruction
  - Identify target gene
  - Expressive plasmid construction
  - Transformation
  - Fermentation

**Figure 1.2** Research objectives and scope of this study
CHAPTER 2
LITERATURE REVIEW

2.1 Bio-based chemicals production

2.1.1 Biomass

The depletion of petroleum resource is aggravated by the increasing demand for fossil fuels. The gasoline price per gallon increased to $4.10 in 2008 from $2.56 in 2005 and $3.23 in 2006 (News). Although the gasoline price has decreased in 2009, the fluctuating and unstable oil price brings concerns on both economies and politics. Moreover, the burning process of fossil fuel produces pollutants, causing global warming and acid rain (Yat et al. 2008). These concerns promote intensive efforts on the development of economical processes for producing fuels and chemicals from renewable resources (Huber et al. 2006). In this respect, plant biomass is the most sustainable feedstock for production of organic chemicals and biofuels (Klass 1998; Kumar et al. 2009).

The United States consumed ~25% of global energy and was responsible for ~25% of global CO$_2$ emissions with only 4.5% of the world’s population (Yat et al. 2008). It has been estimated that the U.S. could produce $1.3 \times 10^9$ metric tons of dry biomass per year (Perlack et al. 2005). This amount of biomass has the energy content of $3.8\times10^9$ boe (barrels of oil energy equivalent) which will account for around 52% of energy demand
while the U.S. consumes $7.3 \times 10^9$ barrels of oil (Book CIA)[7]. The worldwide raw biomass energy potential in 2050 is estimated to be between $25 \times 10^9$ to $76 \times 10^9$ boe (Haug 2004). These data indicate the great potential application of biomass in the energy industry and the chemical industry.

Plant biomass is one of the most abundant renewable resources and constantly being formed by the interaction of CO$_2$ in the air, water, and sunlight by plant during the process of photosynthesis (Kumar et al. 2009). It consists of lignin and polysaccharides, including starch, cellulose and hemicellulose. Lignocellulosic materials including agricultural residues (corn stover, wheat straw, cane bagasse,), forest products (hardwood and softwood), and dedicated crops (switchgrass, salix) are renewable sources of energy. Lignin in lignocelluloses forms a protective barrier preventing the cellulose and hemicellulose accessible to fungi and bacteria for hydrolysis. In order to convert the biomass to fermentable sugars, plant biomass needs to be pretreated to remove the lignin and make the cellulose in the plant fibers exposed for hydrolysis. Pretreatment techniques include ammonia fiber explosion, chemical treatment, biological treatment, and steam explosion (Hsu et al. 1980; Broder et al. 1995). Then, acids or enzymes can be used to break down the cellulose or hemicellulose into its monomer as fermentable substrate. Some microorganisms even can utilize the hemicellulose or cellulose directly. For example, Clostridium thermocellum has the ability to convert cellulose to ethanol without hydrolysis. Three major hydrolysis processes are typically used to produce a variety of sugars suitable for ethanol production: dilute acid, concentrated acid and enzymatic hydrolysis (Broder et al. 1995). In a word, the polysaccharide in plant biomass could be
hydrolyzed to glucose, xylose and other fermentable sugar, then the hydrolytes are used as feedstock for biological fermentation to produce biofuels or bio-based chemicals (Mosier et al. 2005).

2.1.2 Bio-based chemicals

2.1.2.1 Hydrogen

Hydrogen is an efficient and clean energy source for the future. It has the highest energy content per unit weight as high as 143 GJ/ton among all known fuels (Boyles 1984). Not chemically bound to carbon and other elements, the only oxidation product of hydrogen is H2O. Therefore, it doesn’t contribute to the greenhouse effect and pollution. Moreover, hydrogen can be easily converted to electricity by fuel cell to overcome the problems caused by inconvenient transportation and storage.

Currently, 95% of hydrogen is produced through water gas shift reaction, steam reforming of natural gas, and electrolysis of water (Elam et al. 2003). However, all of these methods either use fossil fuel as materials, or are highly energy intensive. The alternative method to those chemical routes is biological production, which includes direct or indirect biophotolysis, fermentation, photosynthetic production and in vitro enzymatic conversion of biomass (Woodward et al. 1996). Among these biological routes, fermentation is one of the most promising technologies contributed to the following advantages: (1) it takes renewable biomass as substrate; (2) it produces valuable metabolites as byproducts; (3) the bioreactor design is simple for the dark fermentation (Nath and Das 2004). Many microorganisms, such as Clostridium,
Enterobacter, Rhodopseudomonas, etc., have the ability to produce hydrogen during anaerobic fermentation process via reactions catalyzed by iron- or nickel-containing hydrogenases (Kataoka et al. 1997; Kumar et al. 2001; Zhu et al. 2004; Liu et al. 2006; Liu and Yang 2006). These hydrogenases catalyze the reversible reaction between molecular hydrogen and its component two protons and two electrons.

\[ 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2 \]

Generally molecular hydrogen is formed during the transfer of reducing equivalents produced during pyruvate oxidation using pyruvate-ferredoxin oxidoreductase (PFOR) coupling with hydrogenase (Gray and Gest 1975; Cammack et al. 2001; Hallenbeck and Benemann 2002). The reactions are illustrated by the following equations:

Pyruvate + CoA + ferredoxin (ox) → Acetyl-CoA + CO₂ + ferredoxin (red)

Ferredoxin (red) → Ferredoxin (ox) + H₂

The theoretical molar yield of hydrogen is two per mol glucose if the reducing equivalent from pyruvate is the only reducing source of the hydrogen. However, Clostridium also contains NADH: ferredoxin oxidoreductase (NFPR) which can produce additional hydrogen using the reducing equivalent stored in NADH during the glycolysis (Jungermann et al. 1971; Hallenbeck 2005).

NADH + ferredoxin (ox) → NAD⁺ + ferredoxin (red)

Ferredoxin (red) → Ferredoxin (ox) + H₂

The oxidation of NADH by ferredoxin (ox) is energetically unfavorable under standard condition and \( \Delta G \) could be negative only at low partial pressures of hydrogen.
This challenge could be addressed by coupling with the ATP hydrolysis which is highly energetically favorable.

The key issue for the fermentative hydrogen production is the low yield. The maximum hydrogen yield from Clostridium is 4 if the acetate is the only end-product. However, if butyrate, which consumes 2 mol of NADH during its formation, is the end-product, only 2 mol of hydrogen can be achieved (Thauer et al. 1977). It has been estimated the overall cost of hydrogen production using fermentable biomass as feedstock is $25/GJ based on some assumptions (Classen et al. 2000). Therefore, the hydrogen is currently more expensive than other alternative fuels. NREL conducted an economic analysis indicating that the a hydrogen price could hit $2.47 kg\(^{-1}\) which is in the range of the hydrogen cost goal with assumptions of that the glucose price is under 0.11 kg\(^{-1}\) and the molar yield of hydrogen is 4 mol per mol glucose (Turner et al. 2008).

Both molecular manipulation and process development have been used to improve hydrogen production. The anaerobic fermentation leads to production of volatile fatty acids and alcohols. The production of alcohol and reducing acid such as lactic acid consumes the reducing equivalent (NADH) and causes low hydrogen production (Levin et al. 2004). In order to enhance the hydrogen yield, the carbon source needs to be directed to the acetate pathway, instead of alcohol, lactic acid and other volatile fatty acid pathways (Hawkes et al. 2002; Levin et al. 2004). The amount of NADH could be increased if the pathway to alcohol or lactic acid was blocked and the hydrogen could be formed during the oxidation of NADH (Das and Veziroglu 2001). Kumar et al. (Kumar et al. 2001) used the proton-suicide technique with NaBr and NaBrO\(_3\) to block the pathways
of organic acid formation and increased hydrogen production to 3.8 mol/mol glucose. A similar enhancement of hydrogen yield was achieved by blocking the biosynthesis of alcoholic and acidic metabolites using both ally alcohol and the proton suicide technique in *E. aerogenes* HU-101 (Mahyudin *et al.* 1997). Gene inactivation has also been employed to improve the hydrogen molar yield from 0.56 to 1.17 in *Enterobacter aerogenes* (Rachman *et al.* 1997). Hydrogenase is another target for hydrogen production. Some hydrogenases interact with NADH in the cytoplasmic side and with proton in the periplasmic side to form hydrogen. The reverse transcription PCR and quantitative real-time PCR methods have been developed to investigate the transcription level of *hydA* during hydrogen fermentation by *C. butyricum* (Tolvanen *et al.* 2008; Wang *et al.* 2008). Wang *et al.* showed linear correlation between the specific hydrogen production rate and the expression level of *hydA* (hydrogenase A) (Wang *et al.* 2008). It is also noticed that the profiles of bacterial growth displayed a similar trend to the *hydA* expression level.

On the other hand, fermentation process conditions were optimized to increase the bio-hydrogen production. The effect of hydraulic retention time on the hydrogen yield was studied (Chu *et al.* 2009). It is concluded that at the optimum HRT range 6–8 h, the hydrogen yield was in the range of 1.4–1.5 mol H$_2$/mol glucose. Cheng *et al.* (Cheng *et al.* 2002a) reported that pH condition of inoculum (sewage-treated plant sludge) enhanced hydrogen production and shortened the lag time. A dual-substrate steady-state model has been developed and the model predicted the relation between the accumulation of the end product and the consumption of glucose and peptone at the dilution rate 0.06-
0.8 per hour (Whang et al. 2006). Other factors such as pH, heat shock, the concentration of substrate, the concentration of Cu$^{2+}$, and the concentration of Fe$^{2+}$ have also been explored (Lee et al. 2001; Cheng et al. 2002b; Lee et al. 2002; Noike et al. 2002; Bai et al. 2004; Lee 2004).

### 2.1.2.2 Butyric acid

Butyric acid is a 4-carbon carboxylic acid and has many applications in food, perfume and pharmaceutical industries. Its acid form could enhance the butter-like flavor in food and the esters of butyric acid serve as aromatic compounds in perfume industries (Dziedzak 1986). It is also used as a raw material for the production of biodegradable polymer β–hydroxylbutyrate. Butyrate has been used as the building blocks to synthesize the plastic material and textile fiber. Moreover, butyrate is considered as an anti-neoplastic drug because of its ability to inhibit the histone deacetylases (HDAC) and the favorable safety profile in humans (Pouillart 1998; Williams et al. 2003). A family of acyloxylalkyl butyrate prodrugs is presently in clinical development (Rephaeli et al. 2000). Butyric acid derivatives have also been developed to produce antithyroid and vasoconstrictor drugs and used in anesthetics (Playne 1985).

Currently, butyric acid is produced by oxidation of butyraldehyde obtained from oxosynthesis of propylene (Pryde 1978; Kroschwitz and Howe-Grant 1997). The production of butyric acid from fermentation of biomass has become an promising alternative to the current petroleum-chemical synthesis. Butyric acid producing bacteria, such as *C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii*, *C. populeti*, *C. barkeri*, and *C.
*thermobutyricum* produce butyric acid as their main product from various substrates and also form acetate as byproduct (Crabbendam *et al.* 1985; Michel-Savin *et al.* 1990a; Michel-Savin *et al.* 1990b; Michel-Savin *et al.* 1990c) (Alam *et al.* 1988; Patel and Agnew 1988; Zigova and Sturdisk 2000). Compared to other bacteria in butyric acid fermentation, *C. tyrobutyricum* has many advantages such as high product purity and yield (Wu and Yang 2003). *C. tyrobutyricum* was discovered as notorious bacteria for its ability to ferment lactate in the cheese to produce gas and high level of butyric acid, which causes the blowing of the cheese. The early researches were focused on how to identify and limit *C. tyrobutyricum* (Klijn *et al.* 1995).

In order to improve the production of butyric acid, a fibrous-bed bioreactor (FBB) was successfully used for butyrate fermentation. The reactor productivity and final product concentration was increased (Zhu and Yang 2003). With the immobilization of cells in the fibrous matrix, high cell density was attained and the reactor productivity, final product concentration, and product yields were improved. The butyrate yield achieved 0.423 g/g or 0.9 mol/mol of glucose consumed in the fermentation (Zhu *et al.* 2002). Molecular methods were also employed to improve strain and direct the carbon source to butyric acid formation. *pta* and *ack* gene encoding PTA and ACK were disrupted by the insertion of erythromycin resistant gene (*erm*) and the mutant shows higher butyric acid production than wild type (Zhu *et al.* 2004; Liu and Yang 2006). Fed batch fermentation was conducted to examine the fermentation kinetics of the mutants. The butyrate final concentration reached 32.5 g/l and 41.6 g/l, for *pta* mutant and *ack* mutant respectively, much higher than the 20.2 g/l for wild type. The fibrous bed
bioreactor was also performed to adapt cells under a high butyric acid concentration to increase the final titer (Zhu et al. 2002; Liu and Yang 2006). Several bio-waste such as corn fiber hydrolysate, corn meal hydrolysate and cane molasses were also used as substrate to produce butyric acid (Huang 2002; Zhu et al. 2002; Jiang et al. 2009). The effect of fermentation pH, the substrate, and the initial concentration of substrate on the fermentation profile has been evaluated (Zhu et al. 2004; Jo et al. 2008). Similar to other fermentation process suffering from product inhibition, C. tyrobutyricum fermentation is strongly inhibited by butyric acid (Michel-Savin et al. 1990a). Researcher developed in situ separation processes to decrease the acid product concentration during the fermentation (Keshav et al. 2009). Micro-filtration, ultra-filtration, and permeate electrodialysis have also been integrated with fermentation of lactic acid (Boyaval et al. 1987). The extractive and pertractive fermentation processes using Hostarex (20% w/w) in oleyl alcohol as the organic phase to produce butyric acid by C. butyricum have been developed. The experimental results showed that the integration of extraction and pertraction with fermentation improved the butyric acid concentration from 7.3 g/l to 10.0 g/l (extractive fermentation) and 20.0 g/l (pertractive fermentation) and that the butyric acid production yield increased from 0.24 g/g to 0.30 g/g (pertractive fermentation) (Zigova et al. 1999). A novel extractive fermentation process by FBB was developed by Wu and Yang (Wu and Yang 2003). 10% (v/v) alamine 336 in oleyl alcohol was used as extractant in a hollow-fiber membrane extractor to selectively remove butyric acid from fermentation broth.
2.1.2.3 Butanol

The acetone-butanol-ethanol (ABE) fermentation was one of the oldest and largest biological fermentation processes in the history, only second to the ethanol fermentation. It was discovered by Louis Pasteur in 1861 (Jones and Woods 1986) and attracted intense attention due to its ability to produce acetone which was highly demanded as the solvent in cordite production during the WWI and the WWII (Killeffer 1927). However, the development of petroleum industry lower the production cost of butanol chemically through oxo process (Lemke 1963) or aldol process (Maiorov et al. 1971), which caused the decline of the ABE fermentation since 1960. Recently, the rising price and the limited resource of the petroleum trigger the renascence of the ABE fermentation since butanol is considered as one of the most promising alternatives to gasoline for several reasons. With longer carbohydrate chain, butanol has lower vapor pressure (0.33 psi) (Minteer 2006) and is much safer and more environmentally friendly. With a higher energy content than ethanol, closer octane number to gasoline and lower affinity to water, butanol can be blended into fuels or directly replace gasoline and act as an alternative fuel in engines (Alasfour 1997).

ABE fermentation is conducted by strictly anaerobic Clostridia. However, due to the complicated origin of the microorganisms and the unsystematic nomenclature based on the differences in the type and the ratio of the solvent produced, the name of different clostridia species led confusion for long time (Jones and Keis 1995; Keis et al. 2001). Keis et al. performed a system analysis based on the 16S rRNA sequence, biotyping and DNA fingerprinting, then categorized the industrial solvent-producing clostridia into 4
species, which are *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum* sp. nov. and *C. saccharobutylicum* sp. nov.. Most industrial solvent-producing clostridia strains are classified as *C. beijerinckii* or *C. acetobutylicum* (Jones and Keis 1995; Keis et al. 1995; Keis et al. 2001).

The ABE fermentation has two stages, acidogenesis and solventogenesis as shown in Figure 2.1. At the exponential stage of fermentation, the Ph value decreases while the bacteria convert saccharide to acetic acid and butyric acid. After the Ph value lower than 4.5-5.0, the fermentation goes to stationary phase and the metabolic pathway will be shifted to solventogenesis. The bacteria will start to produce ethanol, acetone and butanol (Jones and Woods 1986). However, in an environment containing high alcohols like butanol, the bacterial membrane changes and the membrane becomes more fluid and easier to be lysed (Linden and Moreira 1982). Butanol production is thus lowered since the number of solvent producing bacteria decreases. Moreover, ABE fermentation suffers from the low yield, low productivity, and low final concentration due to the end-product inhibition (Huang et al. 2004). Therefore, the ABE fermentation route is still not competitive with the gasoline economically. Extensive metabolic engineering studies have been done and several mutants have been constructed to increase the butanol selectivity or the butanol tolerant.

*C. acetobutylicum* ATCC 824, isolated from the garden soil in Connecticut in 1924 (Weyer and Rettger 1927) and closely related to the historical Weizmann strain (Johnson and Chen 1995; Cornillot and Soucaille 1996), has been used as the genomic model to investigate the gene expression regulation network, although this strain has
never been used in industry. Its genome has been sequenced and annotated (Nolling et al. 2001), containing a 3.94 Mb chromosome and a 192 kb megaplasmid pSol1 where locate genes involved in sporulation and solvent formation, such as \textit{aad}, \textit{ctfA}, \textit{ctfB}, \textit{adc} (Cornillot et al. 1997; Tomas et al. 2003). Several genes involved in the acid production, solvent production and sporulation have been cloned and mutants with inactivated gene or overexpressed gene have been constructed to examine the gene’s function and to explore gene expression regulation by microarray and/or other methods. The mutant without the megaplasmid (M5) displays an asporogenous and cannot produce solvent. This confirmed that the megaplasmid plays an important role in solvent production (Tomas et al. 2003). In the butyrate kinase (\textit{buk}) inactivation mutant, higher level of butyryl phosphate (BuP) is detected, and consistently, solvent is formatted earlier and the concentration is higher than wild type (Desai and Papoutsakis 1999). The results suggested that the intracellular BuP is a putative regulator of solventogenesis in \textit{C. acetobutylicum}, which probably acts as a phosphodonor of transcriptional factor. Transcriptional analysis confirmed that higher BuP level in the buk mutant related to the up-regulation of solvent formation genes (Zhao et al. 2005). In alcohol/aldehyde dehydrogenase (\textit{aad}) overexpression combining antisense RNA downregulation of CoA transferase B (\textit{ctfB}) mutant, \textit{aad} was overexpressed and resulted in the highest reported ethanol production. On the other hand, the butyrate depletion in the fermentation suggested that butyryl CoA maybe the limiting factor on butanol production. The addition of butyric acid increasing the butanol/ethanol ratio confirmed this hypothesis.
Moreover, the enzyme PTB and BK were found able to uptake butyrate without acetone production (Desai et al. 1999).

Besides the gene manipulation, fermentation condition has also been optimized and strains have also been adapted by different methods to increase the solvent production. Cell immobilization to increase butanol production and prolong the fermentation lifetime is also widely studied (Larger et al. 1985; Lienhardt et al. 2002).

Considering the important roles of butyrate, BuP and Butyryl CoA in the solvent production, the butyrate co-feeding with glucose strategy could probably increase butanol yield since both BuP and ButyrylCoA concentrations would be higher during the butyrate/glucose co-feeding. Experiments and stoichiometric modeling indicate that butanol production can be increased by co-feeding sugar with butyric acid (Shinto et al. 2007). A two-step fermentation process has been developed (Ramey 1998) which can increase the butanol production by feeding the C. acetobutylicum with sugar and butyric acid produced from saccharide by C. tyrobutyricum.

The effects of glucose, butyrate, pH and dilution rate on the butanol production in continuous fermentation were also investigated. A high butanol yield of 0.42 g/g glucose was obtained by continuous production in a fibrous-bed bioreactor using butyrate and glucose as cosubstrates (Huang et al. 2004).

2.2 Metabolic engineering and strain development

2.2.1 Clostridium
The genus *Clostridium* is a heterogeneous assemblage of gram-positive, obligatory anaerobic, rod-shaped bacteria forming endospores under extreme conditions (Young and Cole 1993). The heterogeneity of the group is indicated by the remarkable range of d(G+C) contents of their DNAs from 24% to 55% (Johnson and Francis 1975; Cato *et al.* 1986). *Clostridium* attracts intense interest due to its wide application in medical research and fermentation industry. *C. perfringens* is the pathogenic strain causing fatal Necrotic enteritis (pig-bel). *C. tetani* produces tetanospasmin, a biological toxin, and is the causative agent of tetanus. *C. thermocelum, C. stercocellulolyticum* and *C. cellulovorans* have the ability to degrade polysaccharide including cellulose, hemicellulose and starch in biomass. Some *Clostridium* strains produce organic solvents and acids through anaerobic fermentation from sugar, such as extensively investigated *C. acetobutylicum*, which has been well known for the Acetone-Butanol-Ethanol (ABE) fermentation. Until now, the whole genomes have been sequenced for 5 *Clostridium* species, and some other species sequencing projects are undergoing.

*C. tyrobutyricum* is a member of Clostridia family and it was discovered as the causative agent for the late-blowing defect in cheese (Klijn *et al.* 1995). Under anaerobic fermentation condition, it produces butyric acid, acetic acid, and hydrogen. The suggested metabolic pathway was illustrated in Figure 2.2. Compared to other Clostridia bacteria, *C. tyrobutyricum* does not produce any alcohol, simplifying the final product composition. It also has many advantages including simple medium composition, and relatively high product purity and yield (Wu and Yang 2003). Gene inactivation through
homologous recombination has been successfully applied to block the acetate pathway to increase butyrate production (Zhu et al. 2004; Liu and Yang 2006).

2.2.2 Metabolic engineering

Metabolic engineering is defined as the method of optimizing the genetic and regulatory process by recombinant DNA techniques including gene disruption, gene insertion (heterologous expression) and gene overexpression technology. The purpose of the metabolic engineering is to understand the metabolic network and to direct the flux to the desired products. It provided many useful biosynthesis tools for biotechnology applications (Michalodimitrakis and Isalan 2009). Metabolic flux analysis (MFA) has been used to analyze and predict the flux distribution in the metabolic pathway (Varma and Palsson 1994; Stephanopoulos et al. 1998). The stoichiometric models are established by a set of chemical reaction equations using metabolic flux analysis (MFA), where the fluxes are measured by experiment. Linear programming was used as a major tool to develop the MFA. Genome scale models of microbial cells have been developed to predict the impact of a metabolic pathway on cell growth and product biosynthesis (Price et al. 2004). Kayser et al. (Kayser et al. 2005) studied the native metabolism of E. coli under different growth conditions while Ozken (Ozkan et al. 2005) did a similar research for recombinant protein production.

Metabolic engineering has been widely applied to the production of bio-based chemicals or biofuels from renewable biomass. For example, the genes encoding enzymes responsible for biosynthesis of isopropanol (Chen and Hiu 1986; Hanai et al. 2005).
2007) and butanol (Atsumi et al. 2008) in Clostridium were recently expressed and the production of isopropanol or butanol was established in E. coli. The pathway for higher alcohol production from amino acid by S. cerevisiae (Sentheshanmuganathan 1960; Yoshimoto et al. 2002; Schoondermark-Stolk et al. 2006) was expressed in E. coli. Six straight and branched-chain alcohols were produced and 1.28 g/l of isopentanol have been produced by directing the flux to the desired pathway (Kalscheuer et al. 2006; Ladygina et al. 2006; Connor and Liao 2008).

Other method for metabolic engineering is to adjust the redox balance. The availability of NADH or NADPH could be increased or NADH and NADPH could be switched by overexpression of corresponding enzymes (Dos Santos et al. 2004; Sanchez et al. 2006). The expression of heterologous gene gyceraldehydes-3-phosphate dehydrogenase results in a 40% reduction in glycerol production and a 3% increase in ethanol production when the gene was introduced into S. cerevisiae (Bro et al. 2004; Bro et al. 2006). The deletion of NADPH dependent glutamate dehydrogenase led an altered redox metabolism and hence changed expression of other genes encoding NADPH-dependent enzymes. Hydrogen production from glucose was increased by blocking alcohol and some organic acid formation pathways in E. cloacae IIT-BT-08 and hydrogen yield was enhanced by 62% in E. cloacae double mutant than the wild type strain (Kumar et al. 2001).

Instead of one-gene-a-time, combinatorial engineering was developed to express multiple gene (Pfleger et al. 2006) in one mutant. Some other techniques such as ligation-
free assembly (Li and Elledge 2007) and BioBricks (Shetty et al. 2008) could construct pathways from existing DNA fragments or genes.

Introducing genes for uptaking different substrates into cells has also been studied. pUR400 plasmid carrying invertase gene, responsible for breaking down sucrose to glucose and fructose, is transformed into E.coli strain HD701 and successfully utilize sucrose to produce hydrogen (Penfold et al. 2003).

Hydrogenase is another target for hydrogen production. Karube (Karube et al. 1983) cloned and expressed the hydrogenase gene from C. butyricum in E. coli strain HK16 (hyd') in CB medium resulting that the hydrogenase activity increased by 3 folds compared to wild type (Karube et al. 1983). hydA, a [Fe]-hydrogenase gene from E. cloacae IIT-BT-08 was overexpressed E. coli BL-21 which does not produce hydrogen, the hydrogen yield was enhanced (Mishra et al. 2004; Chittibabu et al. 2006) to 3.12, higher than in the wild type IIT-BT-08. Yoshida and colleagues blocked the lactate (ldhA knock-out) and succinate (frdBC knock-out) production pathways, the hydrogen yield was increased by 90% (Yoshida et al. 2006). Multiple knock-out/overexpression E. coli strain has been constructed by the combination of fhlA overexpressed, hycA inactivated, ldhA and frdBC deleted , the effect of those mutation were evaluated step by step (Yoshida et al. 2006; Yoshida et al. 2007).

2.2.3 Key enzymes related to the study

The anaerobic fermentation pathway in C. tyrobutyricum is shown in Figure 2.2. The substrate, hexose or pentose, is firstly catabolized to pyruvate via EMP pathway or
HMP pathway, and then oxidized to acetyl-CoA. The second oxidation process is accompanied by ferredoxin reduction. The resulting FdH$_2$ is then oxidized and the electrons are transferred to proton to produce hydrogen catalyzed by hydrogenase (Hallenbeck and Benemann 2002).

The acetyl-CoA is the branch point intermediate in the metabolic pathway, which can be converted to either acetyl phosphate resulting in the excretion of acetate, or butyryl-CoA, which could be converted to butyryl phosphate resulting in the excretion of butyrate. The following enzymes play critical roles in this anaerobic pathway.

2.2.3.1 PTB and BK

The butyrate formation is comprised of two consecutive enzymatic steps:

(1) butyryl-CoA + Pi $\rightarrow$ Butyryl-P + CoA

(2) butyryl-P + ADP $\rightarrow$ Butyrate + ATP

These two steps are catalyzed by phosphotransbutyrylase (PTB) and butyrate kinase (BK), encoded by $ptb$ and $buk$, respectively (Walter et al. 1993). This pathway is important in the energy metabolism of the organism, as ATP is produced during the conversion of butyryl-CoA to butyrate (Valentine and Wolfe 1960). Both enzymes have been purified and characterized from $C. \text{acetobutylicum}$ strain ATCC 824. PTB is an octomer of identical 31-kDa subunits consisting of 302 residues and BK was reported to be a dimer of identical 30-kDa subunits consisting of 356 residues. $ptb$ and $buk$ are located in the same operon with $ptb$ proceeding $buk$ 44 bp in $C. \text{acetobutylicum}$ (Hartimanis and Gatenbeck 1984; Wisenborn et al. 1989). $buk$ has been interrupted in $C.$
acetobutylicum and mutant shows a decreased butyrate production and increased acetate production.

2.2.3.2 PTA and AK

Similar to the PTB and BK, phosphotransacetylase (PTA) and acetate kinase (AK) catalyze the formation of acetate and generate ATP for cell growth (Boynton et al. 1996). The pta gene has been cloned and characterized in different microorganisms, including C. acetobutylicum (Boynton et al. 1996), E. coli (Matsuyama et al. 1989; Kakuda et al. 1994), M. thermophila (Latimer and Fery 1993), and so on. The fragments of these two genes in C. tyrobutyricum have also been cloned and sequenced (Zhu et al. 2004; Liu et al. 2006). The cloned pta fragment corresponding 244 amino acids and the amino acid sequence is 70% of similarity to the PTA of C. acetobutylicum. The result shows the high degree of similarity in PTA between C. tyrobutyricum and C. acetobutylicum. This fragment was used to construct a pta-deleted mutant by homologous recombination. The fermentation kinetic suggested that the pta inactivation could enhance the butyrate productivity by 1.9-folder (Zhu et al. 2004). The ack deletion also shows similar result.

2.2.3.3 Hydrogenase

Hydrogenases catalyze the conversion between molecular hydrogen and two protons and two electrons (H₂ ↔2H⁺ + 2e⁻). The H₂ metabolism has been studied extensively in over sixty species. As at least 13 families of hydrogenase have been
identified, hydrogenases are a heterogeneous group of enzymes. Eleven out of the thirteen families of hydrogenase are metalloenzymes containing metal centers such as heme groups, [Fe-S] clusters, or Ni-Fe centers. The other two families contain non-metal redox groups such as FAD and FMN. The common Ni-Fe hydrogenases consist of a small subunit of 30 kDa and a large subunit of 60 kDa (Vignais et al. 2001; Frey 2002; Vignais and Colbeau 2004). The most intensively studied hydrogenase is the Fe-only hydrogenase called hydrogenase I encoded by hydA, which has been found in C. acetobutylicum (Gorwa et al. 1996) and C. pasteurinum (Meyer and Gagnod 1991). Most of hydrogenases are oxygen-sensitive, however, Venter team discovered a novel oxygen-insensitive hydrogenase (Lee et al. 2008).

The molecular biology tools can be used to direct the carbon source and shift the NADH to ferredoxin to increase the hydrogen production. Gene inactivation has been successful employed to improve the hydrogen molar yield from 0.56 to 1.17 in Enterobacter aerogenes (Rachman et al. 1997). Chemical inhibitor has also been used to block the ethanol production to increase the hydrogen production in Clostridium.

2.2.3.4 Aldehyde/alcohol dehydrogenase

The solvent-producing genes in C. acetobutylicum have been cloned, sequenced and characterized in C. acetobutylicum, including aad, ctfA/B, adc encoding aldehyde/alcohol dehydrogenase, CoA transferase A/B, acetoacetate decarboxylase respectively. These genes are located in the megaplasmid pSOL1 in C. acetobutylicum.
However, *C. tyrobutyricum* does not have the megaplasmid and the ability to produce solvent. Among these genes, *aad* is the key gene responsible for the alcohol production.

The *aad* gene has 2619 base-pair and codes for a 96,517 Dalton protein. The N-terminal section of *aad* shows homology to aldehyde dehydrogenase of bacteria while the C-terminal shows homology to alcohol dehydrogenase of bacteria. The entire amino acid sequence of *aad* exhibits 56% identity to the tri-functional protein *adhE* from *E. coli* (Nair and Papoutsakis 1994). The *aad*-expressing plasmid has been introduced into *C. acetobutylicum* and the butanol dehydrogenase, acetaldehyde dehydrogenase and butyraldehyde dehydrogenase activity show obvious increase and ethanol dehydrogenase shows a small increase in the mutant. The same plasmid has been also transformed into the M5 mutant which does not produce solvent and the transformation restored the butanol production without any acetone and ethanol detected (Nair and Papoutsakis 1994).

Fontaine *et al.* (Fontaine *et al.* 2002) characterized the second enzyme ADHE2 which also can catalyze the reduction of aldehyde and alcohol. The enzyme was coded by *adhE2* gene which has been cloned and over-expressed in the mutant DG1 which is not able to produce solvent. The transformed mutant resumed butanol production and shows higher selectivity on butanol than ethanol.

**2.3 Process development**

**2.3.1 Medium composition**

**2.3.1.1 Substrate selection and initial concentration**
Unlike recombinant protein fermentation, biofuels and bio-based chemicals fermentation values the substrate cost. Current commercial fermentation processes focus on starch based technologies. There is different opinion from both the scientific community and public arguing that the usage of food grains as feedstock does not possess environmental and economic benefits (Fargione et al. 2008). *Clostridium* has a broad substrate range including glucose, xylose, lactose, starch; some species such as *C. thermocelum* even can uptake cellulose directly. On the other hand, lignin-cellulose consisting of lignin, cellulose and hemicellulose attracts many attentions as its low price and sustainability. After pretreatment and hydrolysis, glucose and xylose are released and used as fermentation substrate. Corn fiber hydrolysate has been used in butyric acid production by *C. tyrobutyricum* fermentation. The medium was supplemented with corn steep liquor as nitrogen source and 0.47g/g butyric acid yield and 2.91g/l/h productivity was attained (Zhu et al. 2002).

Meanwhile, some industrial byproducts could be used as potential substrate in the bioprocess. Cheese whey is the byproduct from cheese production, containing whey protein and whey lactose. Whey protein concentrations (WPCs) is extracted from the cheese whey and the whey permeate containing lactose is left behind as byproduct, which can be obtained at low price. Whey permeate has been used for production of bio-based chemicals or biofuels through fermentation process.

Glycerol is another attractive substrate for fermentation process because its availability and low price as the byproduct from biodiesel production. In addition, it is more reduced compared to glucose or xylose and could benefit the production of
reductive chemicals such as hydrogen, ethanol, succinate, butanol, 1,3-propanediol (Biebl 2001; Ito et al. 2005; Dharmadi et al. 2006; Wang et al. 2007; Buelter et al. 2008).

The initial substrate concentration is another important factor in fermentation as it impacts microbial growth, yield and productivity of end products (Fabiano 2002...). Saccharide molecules cannot enter the cell membrane freely due to its high polarity. A complicated transportation mechanism exists to facilitate the transportation of glucose and other monosaccharide (Bisson 1988; Bisson et al. 1993). S. cerevisiae has 20 genes encoding a high efficient hexose transporter for hexose (Bisson et al. 1993; Lagunas 1993; Perez et al. 2005). Meanwhile, glucose displays different uptake mechanism depending on the glucose concentration in the environment (Fuhrmann and Volker 1992; Walsh et al. 1994). Consequently the fermentation will display different kinetics under different glucose concentration. In addition, high glucose concentration may cause the substrate inhibition. Anaerobic bacterium A. succinogenes shows tolerance to 165g/l glucose. However, the growth rate, succinate production was significantly reduced and long lag phase was observed when the initial glucose concentration exceeds 65g/l (Guettler et al. M. B. Institute 1996; Urbance et al. 2004). The similar inhibition pattern was observed in cane molasses fermentation (Liu et al. 2008). Lin et al. (Lin et al. 2008) developed a model to describe the glucose inhibition and confirmed the prediction using experimental data. Both model and experiment results showed a prolonged lag phase and reduction of specific growth rate with the higher initial glucose concentration (Lin et al. 2008). The main transport mechanism for carbohydrates in Clostridium involves the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) (Mitchell 1998),
which also takes part in gene expression and regulation (Tangney et al. 2003). It is also proposed that the uptake of sugars appears to be driven by ion gradients (H\(^+\) or Na\(^+\) symporters) or ATP hydrolysis (ABC transporters) (Mitchell 1998).

### 2.3.1.2 pH effect

The pH value is another important factor for fermentation by affecting the enzyme activity via changing the ionization state of enzyme’s components (Fabiano 2002). Consequently, pH value impacts the carbon source distribution (Zhu et al. 2004) and the product inhibition is also affected by the pH. The mechanism of short-chain organic acid inhibition was proposed as the acid form of the organic acid under the low pH in the extracellular space passes the cell membrane due to its non-polar property, then dissociated in the neutral environment in the intracellular space, the extra proton is released and causes pH drop in the intracellular space. In order to control the environment, the extra protons have to be pumped out of the intracellular space by H\(^+\)-ATPase at the price of ATP hydrolysis. In other words, under the same amount of acid radical existence, lower pH will result in more acid form and consumes more ATP to maintain the environment in the cells.

### 2.3.2 Fermentation mode

#### 2.3.2.1 Immobilized cell fermentation

The main problem associated with conventional carboxylic acid fermentation is the final product inhibition, which causes low product concentration, yield and
productivity, resulting in high cost in product recovery and decreased cost-effectiveness (Michel-Savin et al. 1990c). This problem can be partially addressed by the fibrous-bed bioreactor where cells are immobilized in fibrous matrix and high cell density could be achieved (Yang 1996). The productivity, final product concentration and production yield are also enhanced by FBB compared to free-cell fermentation (Yang et al. 1994). FBB fermentation has been successfully employed in the production of organic acids, including lactic acid, propionic acid, acetic acid (Silva and Yang 1995; Huang et al. 1998; Huang and Yang 1998; Zhu and Yang 2003). It was also carried out to adapt the C. tyrobutyricum wild type and pta/ack mutants. Both wild type and mutants were adapted in the FBB and showed a higher tolerance to butyric acid (Zhu and Yang 2003; Zhu et al. 2004; Liu et al. 2006; Liu and Yang 2006).

Zhu and Yang discovered that the adaptation mutant by FBB showed a higher tolerance to butyric acid and a higher H⁺-ATPase activity (Zhu and Yang 2003). It could be explained by the H⁺-ATPase functions as a proton transporter (Riebeling and Jungermann 1976). H⁺-ATPase is a membrane-bound enzyme essential for maintaining the transmembrane proton motive force required for ionic regulation and the active transport of nutrients (Riebeling and Jungermann 1976; Ivey and Ljungdahl 1986). The higher activity of H⁺-ATPase can assure the extra protons in the cytoplasm could be transported to the outside of cells, maintaining proper pH in cytoplasm. Additionally, the fatty acid composition ratio of cell membrane was changed in the adapted cells. More saturated and long-chain fatty acids were found, resulting in less membrane fluidity (Zhu and Yang 2003). It is consistent with the result obtained from S. cerevisiae (Casey and
Ingledew 1986) and *E. coli* (Ingram 1976). Both of them have the ability to regulate membrane lipid composition to increase their tolerance to organic solvents.

### 2.3.2.2 Continuous fermentation

Generally, Continuous fermentation has several advantages compared to batch fermentation, such as high productivity due to the continuous operation without lag phase, resistant to the foul and contamination (Van Groenestijn *et al.* 2002). It can significantly increase hydrogen production (Brosseau and Zajic 1982) and butanol production (Lee *et al.* 2008). Magnusson demonstrated the continuous hydrogen production using cellulose as substrate by *C. thermocellum* (Magnusson *et al.* 2008). The hydrogen production rate reached 24.8 ml/l·h while Collet *et al.* achieved 73 ml/l·h using soluble lactose as substrate (Collet *et al.* 2004). Both studies showed robust of the system and easy recovery from the pH failure or other disruption. Spores may form during the failure and the fermentation experienced some recovery time (Magnusson *et al.* 2008). Huang *et al.* used butyrate co-feeding strategy by continuous fermentation and investigated the relation between the dilution rate/pH and the butanol production (Huang *et al.* 2004). 4.6 g/l·h productivity and 0.42 g/g yield has been achieved under the optimized conditions by *C. acetobutylicum*. Lee *et al.* (Lee *et al.* 2008) studied the kinetics of butanol fermentation by *C. beijerinckii* using batch and continuous cultures containing suspension or immobilized cells. The highest butanol productivity and yields was obtained by continuous fermentation of immobilized cells with addition of butyrate or acetate in the medium.
2.2.2.3 Sparging and operational controls

Maeda (Maeda et al. 2007) found that a low partial pressure system increased hydrogen productivity in *E. coli*. This is probably due to the low partial pressure of hydrogen lowering the Gibb’s free energy. Gas sparging usually increases hydrogen production by decreasing the hydrogen solubility (Nath and Das 2004; Kraemer and Bagley 2007). In addition, sufficient mixing facilitates the release of hydrogen from the fermentation broth (Kraemer and Bagley 2007). Ceramic fittings has been used in continuous fermentation for hydrogen production from sucrose by *C. butyricum* and the hydrogen production rate achieved 307 ml/l/h without any additional energy input (Fritsch et al. 2008). The high surface area to volume ratio can help bubbles form to release the supersaturated hydrogen in the medium, which may induce inhibition on the cell (Jones et al. 1999).

2.4 Genome sequence and microarray

2.4.1 Genome sequence

Whole genome sequence is critical for metabolic engineering because it can provide the deep information of the gene and genome structure. The first whole genome sequenced is virus φX174 in 1977 (Sanger et al. 1977). Since then, the development of automation and the invention of PCR (Mullis et al. 1986) make the large-scale sequencing possible. These techniques have been incorporated into the genome sequencing and successfully sequenced the first bacterial genome (Fleischmann et al. 1995). Although the cost and time decreased dramatically, the principle technique
involved in genome sequence was still the Sanger method (Sanger et al. 1973). Recently, a new pyrosequencing technology commercialized by Roche was developed to sequence and generate many short pieces of DNA that could be assembled by the so-called 454 method. The first generation 454 method can read around 100 bp per read, and the read number was increased to ~500 bases recently. 454 methods could finish sequencing a bacterial genome within a few hours (Margulies et al. 2005) and at a relatively low cost. The results are many contigs with different size fragment and there are some gaps left behind. The quality of results mainly depends on the properties of the genome and the number of runs performed. Of course, from the same template sample, more runs can assure a better quality with less number of contigs at a higher price. The fosmid library could be constructed and used to order, direct and space the contigs to form a bigger “assembly” (Goldberg et al. 2006).

2.4.2 Microarray

DNA microarray or gene chips have been widely used for the functional genomics research. The availability of genome sequence is a prerequisite for the microarray construction. Therefore, most functional genomics studies are performed within eukaryotic species, especially for human medical research. Only several studies have been conducted with bacteria, such as *B. subtilis, C. acetobutyricum, C. glutamicum, E. coli* (Ikeda and Nakagawa 2003; Alsaker and Papoutsakis 2004; Gaertner et al. 2004). However, for most of important bacteria in industry, the genomic sequence information is
limited. In such case, a shot-gun DNA microarray may be employed to investigate the functional genomics.

In the shotgun microarray approach, the whole genome is represented by random DNA fragments of unknown sequence. These fragments are printed on a specially treated surface. The DNA fragments containing the target gene can be identified by the differential hybridization with dye-labeled cDNA from different conditions. Shotgun DNA microarray have been constructed and used to study the metabolism of archaeon *Haloferax volcanii* (Zaigler *et al.* 2003), gene function in environmental isolates of *Leptospirillum ferrooxidans* (Parro and Moreno-Paz 2003), and phylogenetic lineages of *Listeria monocytogenes* (Zhang *et al.* 2003).
2.5 References


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saccharoperbutylacetonicum sp. nov. and Clostridium saccharobutylicum sp. nov." Int. J. Syst. Evo. Microb. 51: 2095-2103.


Figure 2.1 The metabolic pathway in *Clostridium acetobutylicum* ATCC 824 (pfor: pyruvate: ferredoxin oxidoreductase; nfor: NADH: ferredoxin oxidoreductase; hyd: hydrogenase; pta: phosphotransacetylase; ack: acetate kinase; hbd: 3-hydroxybutyryl-CoA dehydrogenase; ptb: phosphotransbutyrylase; buk: butyrate kinase; ctf: CoA transferase; adc: acetoacetate decarboxylase; aad: aldehyde/alcohol dehydrogenase; bdh: butanol dehydrogenase; adh: alcohol dehydrogenase)
Figure 2.2 The metabolic pathway in Clostridium tyrobutyricum ATCC 25755 (pfor: pyruvate: ferredoxin oxidoreductase; nfor: NADH: ferredoxin oxidoreductase; hyd: hydrogenase; pta: phosphotransacetylase; ack: acetate kinase; hbd: 3-hydroxybutyryl CoA dehydrogenase; ptb: phosphotransbutyrylase; buk: butyrate kinase)
CHAPTER 3
CONSTRUCTION AND CHARACTERIZATION OF \textit{ptb} MUTANT OF \textit{CLOSTRIDUM TYROBUTYRICUM}

Summary

\textit{Clostridium tyrobutyricum} is a rod-shape, gram-positive bacterium that produces butyrate, acetate, hydrogen and carbon dioxide from various saccharides, such as glucose and xylose, under strict anaerobic conditions. Phosphotransbutyrylase (PTB) is a key enzyme in the butyric acid biosynthesis pathway. However, its role in affecting metabolic flux distribution and production of various metabolites is not well understood. In this work, a mutant with interrupted \textit{ptb} gene, encoding phosphotransbutyrylase, was created by homologous recombination with a non-replicative integrational plasmid carrying \textit{ptb} fragment disrupted by erythromycin resistant gene. Compared to the wild type, the activities of PTB and butyrate kinase (BK) in the mutant decreased 76\% and 42\%, respectively; meanwhile, phosphotransacetylase (PTA) and acetate kinase (AK) increased 7\% and 29\%. However, the disruption of \textit{ptb} did not reduce butyric acid production from glucose or xylose in batch fermentation. In addition, both acetic acid and hydrogen production increased significantly. The mutant also displayed a higher specific growth rate (0.20 h\textsuperscript{-1} vs. 0.15 h\textsuperscript{-1} for growth on glucose and 0.14 h\textsuperscript{-1} vs. 0.10 h\textsuperscript{-1} for growth on xylose) and higher tolerance to butyric acid ($K_p = 13.5$ vs. 9.0). Consequently,
fermentation in serum bottle gave a lower butyrate/acetate ratio and higher hydrogen yield by ptb mutant than wild type.

3.1 Introduction

*Clostridium tyrobutyricum* is a member of *clostridia* family and was discovered as the causative agent for the late-blowing defect in cheese production. It produces butyric acid, acetic acid, CO$_2$ and hydrogen from diverse carbon source including glucose and xylose (Klijn et al. 1995). Butyric acid is a 4-carbon carboxylic acid with many applications in food and perfume industries (Dziedzak 1986). Recent research suggested that butyrate have the ability to inhibit the histone deacetylases and is being evaluated as anti-neoplastic therapy (Pouillart 1998; Williams et al. 2003). The production of butyric acid from biomass became an alternative to the current petroleum chemical route due to the pollution and the depletion of the petroleum. Compared with other bacteria in butyric acid fermentation, *C. tyrobutyricum* has many advantages, including simple medium for cell growth and high productivity and yield (Wu and Yang 2003). Furthermore, hydrogen as gas by-product, is a clean, efficient and sustainable energy source with the highest energy content per unit weight (143 GJ/ton) among all known fuels (Boyles 1984). In addition, hydrogen does not contribute to any environmental problems since its oxidation product is H$_2$O only.

The suggested metabolic pathway in *C. tyrobutyricum* is shown in Figure 3.1. In this pathway, the substrate, hexose or pentose, is oxidized to acetyl-CoA, with the releasing of CO$_2$ and hydrogen. Then, acetyl-CoA is either converted to acetyl phosphate

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resulting in the excretion of acetate, or condensed and reduced to form butyryl-CoA resulting in the excretion of butyrate. The butyrate formation pathway is comprised of two consecutive enzymatic steps catalyzed by phosphotransbutyrylase (PTB) and butyrate kinase (BK), encoded by \textit{ptb} and \textit{buk}, respectively (Walter\textit{ et al.} 1993). Both enzymes have been purified and characterized from \textit{Clostridium acetobutylicum} strain ATCC 824 (Cary\textit{ et al.} 1988). PTB is an octamer of identical 31-kDa subunits consisting of 302 residues and BK was reported to be a dimer of identical 30-kDa subunits consisting of 356 residues. \textit{ptb} and \textit{buk} are located in the same operon with \textit{ptb} proceeding \textit{buk} 44 bp with two independent RBS (Hartimanis and Gatenbeck 1984; Wisenborn\textit{ et al.} 1989). They played critical roles in butyric acid biosynthesis in \textit{C. acetobutylicum} (Green\textit{ et al.} 1996; Green and Bennet 1997). In order to increase butyric acid production by \textit{C. tyrobutyricum}, mutants with inactivated phosphotransacetylase (PTA) and acetate kinase (AK), which catalyze the formation of acetate and also generate ATP for cell growth (Green\textit{ et al.} 1996), were developed and they showed increased butyrate and decreased acetate production in batch fermentations (Zhu\textit{ et al.} 2004; Liu\textit{ et al.} 2006; Liu and Yang 2006). However, no work has been done on \textit{ptb} and \textit{buk} in \textit{C. tyrobutyricum}.

The goal of this work was to understand the effects of \textit{ptb}-disruption on butyric acid fermentation by \textit{C. tyrobutyricum}. We were particularly interested in increasing hydrogen production in butyric acid fermentation by using \textit{ptb}-disrupted \textit{C. tyrobutyricum}. It was hypothesized that blocking the butyrate production pathway can redirect the carbon flux to acetate production and hence shift the reducing equivalent to
hydrogen production. The effects of *ptb*-disruption on butyric acid, acetic acid and hydrogen production from glucose fermentation in serum bottles were studied and the results are reported here. The potential of using *ptb*-disrupted mutant for butyrate and hydrogen production are also discussed in this chapter.

3.2 Materials and Methods

3.2.1 Cultures and media

The bacterial strains and plasmids used in this study are listed in Table 3.1. Unless otherwise noted, *C. tyrobutyricum* was maintained in RCM (Reinforced *Clostridium* Medium, Difco) in anaerobic conditions at 4°C. The fermentation medium was CGM (*Clostridium* growth medium) prepared as previously described by Huang *et al.* (Huang *et al.* 1998) and supplemented with glucose or xylose as the carbon source. *E. coli* was grown aerobically at 37°C in Luria-Bertani medium (LB) supplemented with 50 µg/ml ampicillin.

3.2.2 Cloning of *ptb*-disrupted mutant

The chromosomal DNA of wild type of ATCC 25755 was prepared using the QIAGEN genomic DNA kit. The amplification of the partial *ptb* sequence from *C. tyrobutyricum* chromosome DNA and the construction of the non-replicative plasmid used in the transformation to obtain *ptb*-disrupted mutant followed similar procedures described by Zhu *et al.* (Zhu *et al.* 2004).
3.2.2.1 PCR amplification for *ptb* fragment.

The whole genomes of several clostridia species, including *C. acetobutylicum*, *C. beijerinckii*, *C. tetani*, and *C. perfringens*, have been sequenced and annotated (Nolling *et al.* 2001; Shimizu *et al.* 2002; Bruggemann *et al.* 2003; Copeland *et al.* 2009). The amino acid sequence of PTB is highly conserved among those species (Figure 3.3). Degenerated primers were designed based on the highly conserved regions and the codon usage preference for *C. tyrobutyricum* (http://www.kazusa.or.jp/codon). The sequences of the degenerate primers were as follows:

- Forward primer: 5′- AATA(T)A(G)TAAAC(T)GAA(G)CCTAACG 
- Reverse primer: 5′- A(G)CTA(G)TCA(T)GCTCTA(T)GAAGTTA

PCR amplification of the partial *ptb* gene from the wild type *C. tyrobutyricum* chromosomal DNA was performed with optimized PCR buffer containing 2.5 mM MgCl₂, 0.5 mM dNTP, and 0.3 mM primers (each). The optimized program was as follows: 94°C for 3 min; 94°C for 50 s, 48°C for 50 s, 72°C for 1 min, 40 cycles, followed by 72°C for 10 min to allow the addition of deoxyadenosine (A) to the 3’ ends of PCR products. A 636 bp fragment was obtained and cloned into the pCR®4-TOPO vector (Invitrogen) to form plasmid pCR4ptb for sequencing and further manipulation.

3.2.2.2 Nested-PCR for the confirmation of *C. tyrobutyricum* chromosome

In order to confirm the chromosome template was from *C. tyrobutyricum* and no contamination from *C. acetobutylicum*, the 16S rRNA identification was performed by nested-PCR (Klijn *et al.* 1995). In the first step of nested PCR, part of 16S rRNA gene
was amplified by the *Clostridium*-specific primers at T = 55°C. The forward primer used was 5’-GCGGCGTGCCTAATACATGC-3’ and the reverse primer used was 5’-GGGTTGCGCTGTTGCGGGGA-3’. The second amplification was performed using the diluted product of the first step as template with species-specific reverse primers (GGACTTCATCCATTACGGACTAAC for *C. acetobutylicum* ATCC824 and CGCCTATCTCTCTAGTTATTCCCAG for *C. tyrobutyricum*) and a common forward primer (GGAATCTTCCACAATGGGG) in V6 region. The PCR result was examined on agarose gel and stained with EB.

### 3.2.2.3 Construction of non-replicative plasmid pPtbEm

The non-replicative plasmid pPtbEm was constructed by inserting a selection marker into the middle of the *ptb* fragment (Figure 3.4). Erythromycin has been successfully used as a selection marker in engineering *C. acetobutylicum* (Green and Bennet 1997) and *C. tyrobutyricum* (Zhu *et al.* 2004; Liu *et al.* 2006). The 1.6 kb erythromycin resistant gene cassette (*Em*) was obtained from plasmid pDG647 (Bacillus Genetic Stock Center). The pDG647 was digested by *Bam*HI (Guerout-Fleury *et al.* 1995) and the 1.6 kb *Em* fragment was purified by the gel extraction kit (Qiagen). The pCR4ptb was digested by *Bgl*II, dephosphorylated by CIAP and purified by Phenol: Chloroform extraction. *Em* fragment was ligated with the linearized pCR4ptb and the non-replicative plasmid pPtbEm was constructed. The pPtbEm was then transformed into *E. coli* DH5α and cells were cultured on LB plate supplemented with 250 μg/ml erythromycin and 100 μg/ml ampicillin. The colonies on the plate were selected and
cultured in LB with 50 \( \mu \)g/ml ampicillin, and then cells were harvested to extract the plasmid using QIAGEN plasmid MINIprep kit.

**3.2.2.4 Transformation**

Electroporation was used to introduce plasmid into *C. tyrobutyricum* following the procedures previously described by Zhu *et al.* (Zhu *et al.* 2004). All manipulations were operated in an anaerobic chamber (Thermo Fisher Scientific model 1025). Cells were grown in 50 ml of RCM supplemented with 40 mM DL-threonine and harvested at OD_{600}=0.6 (Zhu *et al.* 2004; Jiraskova *et al.* 2005), then washed with 30 ml of ice-cold electroporation SMP buffer twice and resuspended in 1 ml of SMP buffer in 0.4 cm electroporation cuvette (Bio-Rad). The SMP buffer (pH 7.4) consisted of 270 mM sucrose, 1 mM MgCl₂, and 7 mM sodium phosphate. The MgCl₂ solution was prepared and autoclaved separately before adding to the sucrose and phosphate solution to prevent precipitation. Two micrograms of ice-cold pPtbEm plasmid were added and the mixture was incubated on ice for 5 min. The transformation parameters were set as follows: 2.5 kV, 600 \( \Omega \), 25 AF. The transformed cells were transferred into pre-warmed RCM, incubated at 37°C for 2 hours, and then plated on RCM plates containing 40 \( \mu \)g/ml erythromycin. Plates were incubated in an anaerobic incubator at 37°C for 3–5 days to develop the mutant colonies.

**3.2.2.5 PCR characterization of ptb mutant**
PCR was used to confirm the insertion of $Em^r$ in the ptb gene in the mutant. The chromosome DNA of mutant was extracted using the QIAGEN genomic DNA kit. The \textit{ptb} primers and erythromycin resistant gene primers were designed to detect the length of the \textit{ptb} fragment and the existence of erythromycin resistant gene in the chromosome of the mutant. The \textit{ptb} primers were designed based on the sequence of the \textit{ptb} fragment and had the following sequences: 5'-ACAACTGGTGCAGAAGTTCC (\textit{ptb} forward) and 5’-GTAGAGCTTGTATCAACTGG (\textit{ptb} reverse). The erythromycin resistant gene primers were designed using the Invitrogen Vector NTI software and had the following sequences: 5’-AAGAAGATATGATAGTTTATGGCGG ($Em^r$ forward) and 5’-GCACAGTTCATTATCAACCAAAACA ($Em^r$ reverse).

3.2.3 Enzyme activity assays

Cells from 50 ml of wild type or ptb mutant culture were harvested by centrifugation and the cell pellets were stored at -80°C until use. Cells were washed with 25 ml of tris-HCl buffer (pH 7.4, 0.1 M) twice and resuspended in 5 ml buffer. The cell suspension was sonicated on ice using a sonic dismembrator (Fisher scientific model 100) at level 2 for 10 seconds with a 30 seconds interval, repeated 5 times. The cell debris was removed by centrifugation at 16,000 $\times$ g for 15 min at 4°C. The total protein concentration was measured by Biorad protein assay following the standard procedure for microtiter plates (Biorad).

The activities of AK and BK in cell extracts were measured by monitoring the formation of acyl phosphate from acetate and butyrate, respectively (Rose 1955).
Acylphosphate can react with hydroxylamine to give hydroxamic acid, which forms a complex with ferric ion giving yellow color with absorbance at 540 nm (Lipmann and Tuttle 1945; Cary et al. 1988). Enzyme activity was calculated based on the extinction coefficient of 0.169 mM$^{-1}$cm$^{-1}$ (Cary et al. 1988). One unit of AK or BK enzyme activity is defined as the amount of enzyme producing 1 μmol of hydroxamic acid per minute. PTA and PTB activities were measured by detecting the release of CoA from acetyl-CoA and butyryl-CoA, respectively (Andersch et al. 1983). The sulfyl group in CoA was quantified by the absorbance at 405 nm in the presence of Ellman’s reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman 1959). An extinction coefficient of 13.6 mM$^{-1}$cm$^{-1}$ was used for activity calculation. One activity unit of PTA or PTB is defined as the amount of enzyme converting 1 μmol of acyl-CoA or butyryl-CoA per minute under the reaction conditions. The specific activity of enzyme is defined as the unit of activity per mg of protein.

3.2.4 Butyrate tolerance

Butyrate tolerance was evaluated by investigating the inhibition effect of butyrate on cell growth in serum bottles containing media with various amounts of sodium butyrate (0, 5, 10, 15 g/l). Each serum bottle containing 100 ml of RCM was inoculated with 2 ml of freshly prepared *C. tyrobutyricum* culture in the exponential phase (OD$_{600}$ = 1.0) and incubated at 37°C. Samples were taken at regular intervals to measure the OD$_{600}$ with a spectrophotometer (Sequoia-Turner, model 340). The specific growth rates at various initial butyrate concentrations were determined from the growth curves.
3.2.5 Fermentation kinetics

The effect of *ptb*-disruption on fermentation profile was evaluated in serum bottles. Each bottle containing 50 ml of CGM with 10 g/l glucose was inoculated with 1 ml of fresh culture and incubated at 37°C. Samples were taken at regular time intervals and the final concentration of acetate and butyrate were analyzed with HPLC. Butyrate/acetate ratio was calculated by the mass ratio of butyrate and acetate to evaluate the effects of *ptb*-disruption on the metabolic flux distribution.

3.2.6 Analytical methods

Cell density was calculated based on the optical density of the cell suspension at 600 nm (OD$_{600}$) measured with a spectrophotometer (Sequoia-Turner, model 340). 0.68 g/l of cell dry weight corresponded to one unit of OD$_{600}$. The organic contents, including glucose, xylose, butyrate, and acetate, in fermentation broth samples were analyzed with high performance liquid chromatography (HPLC) with an organic acid analysis column (Aminex HPX-87H, Biorad) following the method described in appendix C (Zhu and Yang 2003).

3.3 Results

3.3.1 Cloning of *ptb* mutant

An estimated 650 bp of DNA fragment was amplified using the degenerate primers and the sequencing result showed a 636 bp fragment encoding a 212 amino acids
peptide. The partial *ptb* sequence isolated from *C. tyrobutyricum* genome had the identical DNA sequence to the *ptb* sequence previously reported for *C. acetobutylicum* ATCC 824 (gene ID 1119259, E.C. 2.1.3.19) (Walter *et al.* 1993).

Genus specific and species specific primers have been used to confirm that the chromosome DNA is from *C. tyrobutyricum*. The *Clostridium* genus specific primers gave a band of 614 kb. The nested PCR using the previous round PCR product as template and use *C. tyrobutyricum* and *C. acetobutylicum* specific primers as primer separately, only *C. tyrobutyricum* specific primers gave expected band of 614 bp (Figure 3.3).

The protoplast of *C. tyrobutyricum* was prepared and incubated with pPtbEm plasmid to check the existence of restriction system. The result showed that the pPtbEm were not digested by the protoplast of *C. tyrobutyricum* (data not shown). Due to the difficulty of transformation of *Clostridium*, 40 µg/ml of DL-threonine were added into the medium to weaken the cell wall formation. Five colonies were isolated under the 40 µg/ml erythromycin selective pressure. The transformation efficiency was 2.5 /µg DNA, which was similar to the previous *C. tyrobutyricum* transformation result (Zhu *et al.* 2004). As a negative control, *C. tyrobutyricum* was also transformed with pDG647 plasmid and no colony was obtained. Since both pDG647 and pPtbEm contained the same replicon *colE*, the results confirmed that the pPtbEm cannot replicate in *C. tyrobutyricum* without integration into the chromosome. Also, the existence of *ptb* fragment in the non-replicative plasmid was necessary for the homologous recombination.
The homologous recombination of the pPtbEm with chromosome of *C. tyrobutyricum* might happen in two ways (Figure 3.5). One possibility is that the whole plasmid was inserted into the chromosome which would add 6.2 kb to the *ptb* fragment (Zhu *et al.* 2004); while the other possibility was that only the *Emr*-inserted *ptb* fragment exchanged with the *ptb* gene in the chromosome and this would cause the *ptb* fragment 1.6 kb longer. Both of the recombination could result in the inactivation of *ptb* gene because a DNA fragment was inserted into the *ptb* gene. To confirm the insertion of the foreign DNA fragment in the *ptb* gene, *ptb* primers were used to identify the lengths of *ptb* fragment in the mutant and wild type. As expected, the wild type gave a 636 bp fragment while the mutant gave a 2.2 kb fragment, which is the length of the original *ptb* plus the erythromycin resistant gene cassette (Figure 3.6A). A pair of *Emr* primers was used to further confirm that the elongation of the *ptb* fragment was caused by the erythromycin resistant gene insertion (Figure 3.6B). The template was the PCR product of the mutant chromosome amplified by the *ptb* primers. It showed a single band with the same size as the positive control *Emr*-containing plasmid pDG647. As expected, there is no erythromycin resistant gene found in wild type chromosome. These PCR results confirmed that the *ptb* gene in the chromosome of the mutant has been disrupted by the insertion of *Emr*.

### 3.3.2 Effects of *ptb*-disruption on acid-forming enzyme activities

The effects of *ptb*-disruption on the activities of acid-forming enzymes (PTB, BK, PTA, and AK) in the mutant were investigated and the results are summarized in Figure
3.7. All enzyme activities are shown in the relative scale using wild type as 100%. PTB activity was reduced dramatically down to ~24% in the ptb-disrupted mutant. However, the ptb-disruption did not turn off the PTB activity completely suggesting the existence of isozyme such as PTA. Meanwhile, the BK activity also decreased ~42%, suggesting that buk is located at the downstream of ptb in the same operon (Walter et al. 1993). In contrast, AK activity increased ~29% in the mutant, but PTA activity was not significantly affected (p = 0.23).

3.3.3 Effect on fermentation profile

Three out of five clones obtained from the transformation grew well in the RCM containing 40 µg/ml erythromycin and all showed similar product profiles with a significantly (p = 0.007) reduced B/A ratio that was about 25.6% lower than that from the wild type fermentation (Figure 3.8). This result confirmed that the ptb-disruption caused a shift in the carbon flux toward acetate production. However, butyrate was still produced at a significant level, suggesting the existence of other butyrate biosynthesis pathway in this bacterium.

3.3.4 Butyrate tolerance

The butyrate tolerance was evaluated by investigating the effect of butyric acid on the specific growth rate. Cells were grown as suspension culture at various butyrate concentrations (0, 5, 10, 15 g/l). The relative specific growth rates with the rate without butyrate being 100% were plotted against the butyrate concentration (Figure 3.9). As
shown in Figure 9, butyrate strongly inhibited the wild type but not as strongly to the ptb mutant. At the highest butyrate concentration of 15 g/l, the ptb mutant displayed 53% of its maximum growth rate compared to 43% retained in the wild type. The growth inhibition by butyric acid followed the non-competitive product inhibition kinetics as follows (Liu et al. 2006):

\[
\mu = \frac{\mu_{\text{max}} K_P}{K_P + P}
\]

where \( \mu \) is the specific growth rate (h\(^{-1}\)), \( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\)), \( K_P \) is the inhibition rate constant (g/l), and \( P \) is the butyric acid concentration (g/l). The ptb mutant had a higher inhibition rate constant (\( K_P = 13.5 \) g/l vs. \( K_P = 9.0 \) g/l for the wild type), suggesting that the ptb mutant has a higher tolerance to butyrate inhibition. The higher growth rate and butyrate tolerance can be attributed to more ATP produced by the ptb mutant, which could help H\(^+\)-ATPase pumps more proton out of cells to maintain a suitable pH in cells (O'Sullivan and Condon 1999; Cotter and Hill 2003).

3.4 Discussion

The Clostridium is a heterogeneous genus of gram-positive, obligate anaerobic, rod-shaped bacteria which can form endospore under extreme conditions. Clostridium attracts intense interest due to its wide applications in medical research and the fermentation industry. To date 6 clostridia species, C. perfringens (Shimizu et al. 2002), C. difficile (Sebaihia et al. 2006), C. botulinum (Sebaihia et al. 2007), C. tetani (Bruggemann et al. 2003), C. acetobutylicum (Nolling et al. 2001), and C. beijerinckii

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Copeland et al. 2009) have been fully sequenced for their whole genomes. However, except for *C. acetobutylicum*, very little has been done in metabolic engineering of clostridia for fuels and chemicals production. *C. tyrobutyricum* was originally discovered as the causative agent for the late-blowing defect in cheese (Klijn et al. 1995). It produces butyric acid, acetic acid, carbon dioxide, and hydrogen under anaerobic conditions. Because of its many advantages in fermentation, its potential for industrial production of butyric acid and hydrogen from biomass has attracted intensive research interest (Michel-Savin et al. 1990a; Michel-Savin et al. 1990b; Michel-Savin et al. 1990c; Zigova et al. 1999; Zhu et al. 2002; Wu and Yang 2003; Zhu and Yang 2003; Liu et al. 2006; Jo et al. 2008; Jiang et al. 2009). To improve butyric acid production, metabolically engineered *C. tyrobutyricum* mutants with inactivated acetate formation pathway were obtained with integrational mutagenesis that selectively inactivated *ack* and *pta* genes from the host chromosome (Zhu et al. 2004; Liu et al. 2006). Fermentation kinetics with the mutants showed that *pta* and *ack* inactivation increased butyrate production, reduced cell growth, and decreased acetate production.

In this work, we were interested in cloning *C. tyrobutyricum* *ptb* gene for its important role in the butyrate synthesis pathway. This pathway is important in the energy metabolism of the organism, as ATP is produced during the conversion of butyryl-CoA to butyrate (Valentine and Wolfe 1960). The partial *ptb* sequence of *C. tyrobutyricum* obtained by PCR with degenerated primers has an identical DNA sequence to the one found in *C. acetobutylicum* ATCC 824, which also has the same butyrate synthesis pathway and produces butyric acid during its acidogenesis phase (Jones and Woods
Previous studies showed that the partial DNA sequences of *pta* and *ack* genes from *C. tyrobutyricum* had high similarity, but not identical, to *C. acetobutylicum* (Zhu et al. 2004; Liu et al. 2006). In order to exclude the possibility of contamination of the chromosome with *C. acetobutylicum* in our cloning experiment, the chromosome’s *C. tyrobutyricum* origin was checked by amplifying its 16S rRNA gene following the method previously reported (Klijn et al. 1995). Briefly, clostridia-specific primers P1 (GCGGCGTGCCTAATACATGC) and P2 (GGGTTGCGCTCGTTGCGGA) were used and the results showed its *Clostridium* origin. The PCR product was then used as the template for the next step of nested PCR with clostridia universal primer P5 (GGAATCTTCCACAATGGGCG) and *C. tyrobutyricum* specific primer Pty (CGCCTATCTCTAGGTTATTCAG), and the specific band of 660 bp was obtained, confirming the *C. tyrobutyricum* origin of the chromosome. As a control, *C. acetobutylicum* specific primer Pac (GGACTTCATCCATTACGGACTAAC) did not give the amplification product.

Compared to the wild type, the ptb mutant’s PTB activity was reduced by 76% and BK activity decreased 42%. Meanwhile, its AK activity increased 29%. However, butyrate production was not reduced in the fermentation with the ptb mutant although acetate production did increase significantly. A mutant of *C. acetobutylicum* ATCC 824 with disrupted *buk* gene showed fivefold lower BK activity, twofold higher PTA activity and threefold higher AK activity (Green et al. 1996). Consequently, fermentation with this mutant showed a 43% decrease in butyrate and 32% increase in acetate production. Meanwhile, the complementation of *buk*-inactivation strain with plasmid containing the
buk operon restored the butyrate kinase activity and butyrate production (Green and Bennet 1997). These experiments showed that buk played an important role in butyrate biosynthesis in C. acetobutylicum; however, its deletion could not eliminate butyrate production, suggesting the existence of a new pathway other than PTB-BK route also leading to butyrate biosynthesis in these clostridia species. Previous studies with ack and pta deleted mutants also suggested the existence of alternative pathways for acetate production in C. tyrobutyricum (Zhu et al. 2004; Liu and Yang 2006). Recently, Charrier et al. (Charrier et al. 2006) discovered a novel CoA transferase pathway in human colonic bacterium Roseburia sp. A2-183, which used acetate: butyryl-CoA CoA-transferase for butyrate formation in the human colon. The CoA transferase has also been located in the 454 sequencing database of C. tyrobutyricum (Chapter 4). It is very likely that this novel CoA-transferase provides alternative pathways for acetate and butyrate biosynthesis in clostridia. Future study will be conducted to investigate the CoA transferase function in C. tyrobutyricum.

Compared to the wild type, the ptb mutant had a higher acetate production, which stimulated the higher ATP formation and resulted in the enhanced specific growth rate in both glucose and xylose fermentations.

The ptb mutant showed an increased tolerance to butyric acid inhibition, which can be partially attributed to the increased production of acetate and ATP. The exact acid tolerance mechanism in C. tyrobutyricum is still unknown, but should be related to the ability of H⁺-ATPase to pump out the proton from its intracellular space at the expense of ATP consumption (O'Sullivan and Condon 1999; Cotter and Hill 2003). The increased
ATP production in the mutant thus can be used to pump the proton out of the cell and to maintain its suitable pH environment. End product inhibition is one of the limiting factors on the organic acid fermentation. It causes low product titer and hence high production cost. The ptb mutant with increased butyrate tolerance, growth rate, and productivity is thus a promising candidate for industrial fermentation to produce butyric acid and hydrogen from glucose and xylose.

3.5 Conclusion

The ptb-disrupted mutant of *C. tyrobutyricum* has been constructed and confirmed by PCR and reduced phosphotransbutyrylase and butyrate kinase activities. Compared to the parental strain, the mutant had a higher specific growth rate and significantly lower b/a ratio from both glucose and xylose in batch fermentations. However, butyrate production did not decrease significantly, suggesting the existence of another pathway leading to butyrate production.
3.6 References


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<table>
<thead>
<tr>
<th>Strains / Plasmids</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><em>Clostridium tyrobutyricum</em> ATCC 25755</td>
<td></td>
<td>ATCC</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td></td>
<td>Invitrogen</td>
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<tr>
<td>pCR4-TOPO</td>
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<td>pDG647</td>
<td>Em&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Guerout-Fleury <em>et al.</em> 1995</td>
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<td>This study</td>
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*Table 3.1* Strains and plasmids used in this study.
Figure 3.1 The metabolic pathway showing butyric acid, acetic acid, and hydrogen production from glucose in *C. tyrobutyricum.*
Figure 3.2 Alignment of amino acid sequences of PTB of several *Clostridium* species showing highly conserved regions. Arrows indicate the conserved sequences used in designing the forward and reverse primers for PCR amplification of *ptb* from *C. tyrobutyricum*.
Figure 3.3 The nested-PCR to confirm the chromosome from *C. tyrobutyricum* (3a: *Clostridium* specific primers; 3b: species specific primers)
Figure 3.4 Construction of the non-replicative integrational plasmid pPtbEm with one 0.64-kb *ptb* fragment cloned from *C. tyrobutyricum*. Abbreviations: *ptb*, partial *ptb* gene; Ap\(^r\), ampicillin resistance gene; Em\(^r\), erythromycin resistance gene; Kan\(^r\): kanamycin resistance gene; pUC ori: G(−) replication origin.
**Figure 3.5** Homologous recombination of pPtbEm containing an Em'inserted *ptb* fragment with the chromosome of wild type resulting in the disruption of the *ptb* gene.
Figure 3.6 PCR characterization of the ptb mutant. A) using *ptb* primers; B) using Em^r^ primers.
**Figure 3.7** Comparison of activities of PTA, PTB, AK, and BK in wild type (wt) and ptb mutant. (PTA: phosphotransacetylase; PTB: phosphotransbutyrylase; AK: acetate kinase; BK: butyrate kinase)
Figure 3.8 Effect of *ptb*-disruption on B/A ratio in batch fermentation of glucose by *C. tyrobutyricum* wild type and *ptb* mutants.
Figure 3.9 Noncompetitive inhibition of butyric acid on cell growth of *C. tyrobutyricum* wild type (wt) and ptb mutant (ptb).
CHAPTER 4

KINETICS OF BUTYRIC ACID FERMENTATION BY CLOSTRIDIUM TYROBUTYRICUM PTB MUTANT

Summary

Fermentation kinetics of glucose and xylose by Clostridium tyrobutyricum wild type and ptb mutant were studied. A simplified growth medium without vitamins and trace metals supplementation was developed based on a partial factorial experimental design and statistical analysis of the fermentation results. The optimal pH was found to be 6 and the optimal substrate concentration was 50 g/l by using one-factor-a-time screening. The impact of ptb-disruption on the fermentation kinetics was studied using glucose and xylose as substrates. Compared to the wild type, the B/A ratio decreased by 34.7% for glucose and 28.1% for xylose, which could be attributed to the inactivation of ptb. The productivity of hydrogen increased 38% for glucose and 46% for xylose, which could be partially attributed to the higher specific growth rate (33% increase for glucose and 40% increase for xylose). The butyric acid fermentation and hydrogen production were further improved by immobilizing cells in the fibrous-bed bioreactor to facilitate cell adaptation to attain a higher final organic acid concentration.
4.1 Introduction

Plant biomass is an abundant renewable energy resource and constantly being created by photosynthesis. A typical plant biomass has about 25% lignin and 75% cellulose containing glucose and hemicellulose containing glucose and xylose. The cellulose and hemicellulose can be broken down by acid or enzymatic hydrolysis to release its monomer glucose as fermentable sugar (Aristidou and Penttila 2000). While hydrolysis of cellulose has been greatly improved recently with new technologies in pretreatment and enzyme modification, many of current industrial microbial fermentations cannot use pentoses (e.g., xylose) as the substrate. *C. tyrobutyricum* has been demonstrated for its ability to convert xylose to butyric acid at the same rate comparable to glucose (Zhu *et al.* 2002).

Besides lignin-cellulose, bio-wastes, such as whey permeate and glycerol, are also regarded as economical carbon sources for bio-based chemicals and biofuels production. Whey permeate is the byproduct of whey protein production and has been used for lactic acid fermentation (Tejavadi and Cheryan 1988; Norton *et al.* 1994). Glycerol is the byproduct from biodiesel production and has been used for production of ethanol, succinic acid and 1,3-propanediol especially because of its highly reducing status (Ito *et al.* 2005; Dharmadi *et al.* 2006; Wang *et al.* 2007).

Medium composition affects the economics of a fermentation process. The major components of microbial fermentation medium include nitrogen sources, carbon sources
and buffers to control the pH value, ionic strength, and redox potential. The composition of the basal medium for *C. tyrobutyricum* is complicated and contains 27 components. As it contains expensive vitamins, the medium is uneconomical and its preparation is labor intensive. Traditional optimization method is one-factor-a-time by which one factor is optimized while the others are fixed. This single-dimensional optimization was time-consuming and not able to reach the optimal conditions because it ignores the interaction among variables (Chen and Lee 1997), especially when many factors need to be considered. Fractional factorial design was employed for screening multifactors to find the most significant independent factors at lower run numbers. In this work, seven medium components in the *Clostridium* Growth Medium (CGM) were screened to simplify the composition of the medium and free-cell fermentation was performed to verify that there was no negative effect using the simplified medium.

The initial sugar concentration and pH affect cell growth and product yield, productivity and titer. pH affects the metabolic pathway by changing the activities of key enzymes (Tanisho *et al.* 1987; Dabrock *et al.* 1992.; Lay 2000). It has been reported that pH could impact the ionization state of enzymes (Fabiano and Perego 2002). In the case of organic acid production, a high initial pH value can slow down the pH drop and thus alleviate proton inhibition. A lower pH is helpful to separate organic acid from the fermentation broth by extractive fermentation (Zigova *et al.* 1999; Wu 2003; Wu and Yang 2003). It has also been noticed that a low initial pH also led to a long lag phase (Khanal *et al.* 2004). On the other hand, high initial pH values reduced the length of the
lag phase, but led to a lower yield of hydrogen production (Zhang et al. 2003). The pH range for Clostridia growth has been determined to be 4.5-7.0 (Zigova et al. 1999).

The main problem associated with conventional carboxylic acid fermentation is the product inhibition, which causes low product concentration, and consequently, low yield and low productivity, resulting in high costs in product recovery (Michel-Savin et al. 1990). This problem can be partially addressed by using the fibrous-bed bioreactor (FBB) where bacteria are immobilized in a fibrous matrix to achieve a high cell density (US patent no. 5,563,069). This method has been successfully employed in the production of organic acids, including lactic acid, acetic acid, propionic acid, and butyric acid (Yang et al. 1994; Silva and Yang 1995; Yang et al. 1995; Yang 1996; Huang et al. 1998; Huang and Yang 1998; Zhu and Yang 2003).

In this work, CGM was simplified using fractional factorial experimental design. Different substrates were tested for the fermentation by the ptb mutant of C. tyrobutyricum and the fermentation conditions were optimized. The kinetics of free-cell batch fermentations was investigated using glucose and xylose as substrates to study the impact of ptb-disruption on the fermentation profile. Furthermore, cells were adapted by immobilization in the fibrous-bed bioreactor and higher final product concentrations were obtained in the FBB fermentation compared to free-cell fermentations.

4.2 Materials and methods

4.2.1 Culture and media
The stock culture of *C. tyrobutyricum* ATCC 25755 was stored in Difco™ Reinforced Clostridia Medium (RCM) in serum bottles under anaerobic conditions at 4 °C. Unless otherwise noted, the medium used for fermentation studies was the *Clostridia* Growth Medium (CGM) as previously described by Huang *et al.* (Huang *et al.* 1998) and supplemented with glucose or xylose as the carbon source. A Simplified *Clostridia* Growth Medium (SCGM) described later, was also developed and used in this study. The basal medium and the concentrated sugar solution, *i.e.* 500 g/l glucose or xylose, were autoclaved separately at 121°C and 15 psi for 30 min to avoid undesirable reactions. The concentrated sugar solution was then pumped into the fermenter aseptically.

4.2.2 Design of experiments for simplifying medium composition

JMP 7.0 was used to design the factor screening experiment. Seven factors were screened. Among them, Fe$^{2+}$ solution, Ni$^{2+}$ solution, vitamins solution and trace metals solution were categorical factors, while yeast extract, Trypticase™ soy broth, and Trypton were 2-level numerical factors, whose values were limited between 0 and 5 g/l. 2 center points and 1 duplicate were added and the total run number was 36 with a resolution of 4 as shown in Table 4.1. The experiments were performed in serum tubes each containing 10 ml of media with 10g/l glucose. Three responses which were cell growth, butyric acid final concentration and total gas production were measured. The OD$_{600}$ of each serum tube culture was monitored directly without sampling by using a
modified cuvette holder and a spectrophotometer (Sequoia-Turner, model 340). The total gas was collected and the gas volume was measured. The final butyric acid concentration was measured by HPLC

4.2.3 Effect of initial glucose concentration

The impact of initial glucose concentration on cell growth was evaluated in serum tubes each containing 10 ml RCM supplemented with various amounts of glucose to make the final glucose concentration in the range of 10 g/l – 90 g/l. Water was added to make the final volume to 12 ml. The culture was inoculated with 0.5 ml fresh inoculum. The OD\textsubscript{600} was measured with a spectrophotometer at 600 nm at regular time intervals. The specific growth rate was calculated based on the slope of the semi-logarithmic plot of OD\textsubscript{600} vs. time during the exponential phase.

4.2.4 Effect of pH

The pH influence on cell growth was evaluated in serum tubes each containing 10 ml RCM. The medium pH was adjusted to 4 ~ 9 by adding 1 N HCl or 1 N NaOH. The small difference in the liquid volume was ignored. A 0.5 ml fresh culture in the exponential phase was added into each tube as inoculum. The OD\textsubscript{600} was measured with a modified spectrophotometer at 600 nm at regular time intervals. The specific growth rate
was calculated based on the slope of the semi-logarithmic plot of OD$_{600}$ vs. time during the exponential phase.

4.2.5 Free-cell fermentations

The kinetics of free-cell fermentations of wild type and $ptb$-disrupted $C. tyrobutyricum$ were studied in 2-liter stirred bioreactor containing 1 l CGM supplemented with 50 g/l glucose or xylose. The sugar substrate was prepared in concentrated solutions (500 g/l) and pumped into the fermenter aseptically. The medium was sparged with filtered nitrogen gas for 2 hours to achieve anaerobiosis. Each reactor was inoculated with 50 ml of a fresh cell suspension in the exponential phase (OD$_{600}$ = 2.0) grown in serum bottles. Batch fermentations were performed at 37°C and pH 6.0 ± 0.1 adjusted with 6 N NaOH. CO$_2$ and hydrogen production was monitored by the water replacement method along with GC analysis. Liquid samples were taken at regular intervals (8-12 h) to analyze the cell density and concentrations of the substrate and products in the fermentation broth. For fed-batch fermentations, a concentrated substrate solution was added periodically when the glucose concentration was close to 0 g/l.

4.2.6 Immobilized-cell fermentations in FBB

The FBB fermentation system (Figure 4.1) consisted of a 5-l stirred-tank fermenter (Marubishi MD-300) and a 500 ml fibrous-bed bioreactor (FBB). The FBB
was made of a glass column with a water jacket to control the temperature. The inside of the bioreactor was packed with a spirally wound cotton towel supported by a stainless steel wire mesh. The working volume of the bioreactor was 500 ml. The FBB and the stirred-tank fermenter were connected to each other through a recirculation loop. The FBB system was operated under anaerobic conditions at pH 6 and 37°C. The anaerobic condition was maintained through N₂ sparging until gas was produced.

To start the fermentation, 100 ml of a fresh cell suspension were inoculated into the fermenter containing 2 l CGM and then grown at 37°C, 200 rpm and pH 6, which was controlled by adding 6N NaOH. The culture medium was circulated at 10 ml/min when cell optical density (OD₆₀₀) reached 5.0. The slow circulation rate allowed cells to attach to the matrix and to be stabilized. After 2 days of circulation, cells were mostly immobilized in the matrix and the OD₆₀₀ was stable again (~10). At this time, the circulation rate was increased to 50 ml/min for a better mixing. Additional carbon source was supplied when the glucose concentration was close to zero. The glucose feeding was repeated several times to allow the fermentation to continue and to increase the butyrate concentration until no glucose was consumed.

4.2.7 Analytical methods

The cell density was calculated based on OD₆₀₀ measured with a spectrophotometer (Sequoia-Turner, model 340). One unit of OD₆₀₀ was corresponding to 0.68 g/l of cell dry weight.
The composition of gas produced in the fermentation was analyzed with a gas chromatograph (Shimadzu GC 2014) equipped with Thermo Conductive Detector and RT-QPLOT column (Restek) with argon as the carrier gas at the flow rate of 4.38 ml/min. The injector temperature was 60°C, the column temperature was 30°C and the detector temperature was 60°C. The volume of produced gas was measured by the water replacement method.

High performance liquid chromatography (HPLC) with an organic acid analysis column (Aminex HPX-87H, Biorad) was used to analyze sugar and organic acid contents, including xylose, glucose, acetate, and butyrate in fermentation broth samples. The detailed method has been described in appendix C (Zhu and Yang 2003).

4.3 Results

4.3.1 Simplified medium composition

The medium composition plays a critical role in the economics of bio-based chemicals production. A medium using inexpensive materials and simplified preparation process can decrease the production cost. The basal *Clostridium* Growth Medium (CGM) used for *C. tyrobutyricum* fermentation consisted of the following components (per liter):

a) 40 ml of mineral #1 solution containing 7.86 g/l K$_2$HPO$_4$·3H$_2$O.

b) 40 ml of mineral #2 solution consisting of (per liter): 6 g KH$_2$PO$_4$; 6 g (NH$_4$)$_2$SO$_4$; 12 g NaCl; 2.5 g MgSO$_4$·7H$_2$O; 0.16 g CaCl$_2$·2H$_2$O.
c) 10 ml of the trace metals solution containing (per liter): 1.5 g nitrilotriacetic acid; 0.1 g FeSO₄·7H₂O; 0.5 g MnSO₄·2H₂O; 1.0 g NaCl; 0.1 g CoCl₂; 0.1 g CaCl₂·2H₂O; 0.1 g ZnSO₄·5H₂O; 0.01 g CuSO₄·5H₂O; 0.01 g AlK(SO₄)₂; 0.01 g H₃BO₃; 0.01 g Na₂MoO₄·3H₂O.

d) 10 ml of the vitamin solution containing (per liter): 5 mg thiamine-HCl; 5 mg riboflavin; 5 mg nicotinic acid; 5 mg pantothenate; 0.1 mg vitamin B₁₂; 5 mg p-aminobenzoic acid; 5 mg lipoic acid.

e) 10 ml of 0.005% (w/v) NiCl₂·6H₂O solution.

f) 2 ml of 0.2% (w/v) FeSO₄·7H₂O solution.

g) 5 g Trypticase™ soy broth (BD, catalog # 211768).

h) 5 g yeast extract.

The preparation of the CGM was time-consuming and labor intensive. As yeast extract and Trypticase™ soy broth, containing casein and soybean protein hydrolysates, were used as the nitrogen source, they could also provide vitamins and trace metals that are critical to cell growth, butyric acid production and hydrogen production. Furthermore, another nitrogen source Trypton containing casein hydrolysate was also tested.

A ¼ fractional factorial design with 1 duplicate and 2 center points was used to examine if the vitamin solution, trace metals solution, NiCl₂ solution, FeSO₄ solution, yeast extract, Trypticase™ soy broth, and Trypton are significant factors affecting C. tyrobutyricum ptb mutant fermentation. The result (figure 3.2) showed that only yeast extract and soy broth were significant factors with p = 0.0015 and p = 0.0016 for butyric
acid production and p = 0.0010 and p = 0.0001 for cell growth, respectively. In addition to these two nitrogen sources, the NiCl₂ solution showed a significant effect on hydrogen production with p = 0.0069, suggesting that the [NiFe]-hydrogenase was active in C. tyrobutyricum. It is well known that Fe plays a critical role in hydrogenase activity. FeSO₄ was thus included in the simplified medium although it did not show significant impact on any of the three responses (p=0.8915, 0.7458, 0.5971 for cell growth, hydrogen production and butyric acid production, respectively) due to factors interaction probably. It was apparent that the trace metals solution and the vitamin solution had no significant effects on the fermentation and thus could be excluded from the CGM.

Based on these findings, the medium was simplified to consist of only yeast extract (5 g/l), Trypton (5 g/l) and the following two solutions (per liter):

a) 40 ml of mineral #1 solution containing 7.86 g/l K₂HPO₄·3H₂O

b) 40 ml of mineral #2 solution consisting of (per liter): 6 g KH₂PO₄; 6 g (NH₄)₂SO₄; 12 g NaCl; 2.5 g MgSO₄·7H₂O; 0.16 g CaCl₂·2H₂O; 0.125 g NiCl₂ and 0.1 g FeSO₄ (In order to prevent Fe²⁺ oxidation and precipitation during autoclaving, the solution mineral #2 was sterilized by microfiltration).

Free-cell batch fermentation was performed to verify that the simplified medium did not have any negative impact on cell growth, butyric acid and hydrogen production. As shown in Figure 4.3, both the original and the modified medium gave similar specific growth rates (0.20 h⁻¹ vs. 0.21 h⁻¹) indicating that medium simplification did not change
cell growth. Similar results were also found for butyric acid yield (0.74 vs. 0.73) and hydrogen yield (1.6 vs. 1.62).

### 4.3.2 Substrate selection

The fermentation substrate is critical to the economy of bio-based chemicals production. Generally, low-cost substrates can be derived from agricultural residues containing starch, cellulose and hemicellulose, or from industrial wastes, such as lactose in whey permeate and glycerol in biodiesel wastewater. As shown in Figure 4.4, *C. tyrobutyricum* does not have the ability to use lactose and glycerol, although these two substrates have been used for propionic acid production and other fermentation processes. However, *C. tyrobutyricum* can uptake xylose, a major component of hemicellulose consisting of around 20% of the dry weight of renewable biomass. However, the specific growth rate and the product yield from xylose were slightly lower than those from glucose.

### 4.3.3 Optimized fermentation conditions

A high glucose concentration caused longer lag phase (Figure 4.5a) and inhibit cell growth (Figure 4.5b). In general, no significant effect on cell growth was observed when the glucose concentration was less than 70 g/l. At 70 g/l, the fermentation showed a longer lag phase, although the specific growth rate was not affected obviously. Further
increasing glucose concentration to 90 g/l showed significant inhibition on cell growth with p = 0.027. Considering the long lag phase would decrease productivity dramatically, 50 g/l of glucose appeared to be the optimal initial glucose concentration for the batch fermentation. Since xylose showed a similar metabolic rate as glucose (Zhu and Yang 2003), 50 g/l was also considered as the optimal initial concentration for xylose.

The pH of the medium is another important factor in fermentation affecting the enzyme activity by changing the ionization state of enzyme’s components (Fabiano and Perego 2002). Consequently, the pH affects the carbon source distribution (Zhu et al. 2004). For C. tyrobutyricum, the highest specific growth rate was observed at pH 6, which also had the shortest lag phase (Figure 4.6).

4.3.4 Effects of ptb-disruption on fermentation kinetics

Free cell batch fermentations with the wild type and ptb mutant were performed at pH 6.0 and 37 °C to examine the effects of ptb-disruption on the fermentation kinetics with glucose and xylose as the substrate. The batch fermentation results are shown in Figures 4.7 and 4.8, and the kinetic data are summarized in Table 4.2. Compared to the wild type, the B/A ratio of ptb mutant fermentation decreased 34.7% for glucose and 28.1% for xylose, which can be attributed to the inactivation of ptb. However the butyrate yield was still comparable to that of the wild type, suggesting a new pathway other than PTB-BK route could also produce butyrate. Charrier et al. (Charrier et al. 2006) discovered a novel CoA transferase pathway in human colonic bacterium Roseburia sp. A2-183,
which used butyryl-CoA: acetate CoA-transferase for butyrate formation in the human colon. It is very likely that this butyrate biosynthesis pathway is also used in *C. tyrobutyricum* since CoA transferase genes were also found in the 454 genome sequences of this bacterium.

The hydrogen yield by the ptb mutant increased 14% for glucose and 17% for xylose, supporting that the lower B/A ratio accompanied a higher hydrogen yield. In addition, the productivity of hydrogen increased 38% for glucose and 46% for xylose (Table 4.2), which could be partially attributed to a higher specific growth rate (33% increase for glucose and 40% increase for xylose). As shown in the metabolic pathway of *C. tyrobutyricum*, 2 mol of ATP are produced from 1 mol glucose if acetate is the only end product, while only 1 mol ATP is produced with butyrate formation. The increased acetate production in the ptb mutant caused more ATP production, which benefited cell growth and thus hydrogen productivity.

### 4.3.5 Free cell fed-batch fermentation kinetics

Figure 4.9 shows the fed-batch fermentation kinetics with free cells, which was performed to identify the butyrate inhibition level for the ptb mutant. In the first batch, 50 g/l glucose was completely consumed in 65 h and 16.6 g/l butyric acid and 9900 ml of hydrogen were produced. The butyric acid yield was 0.70 (mol/mol), the acetic acid yield was 0.137 (mol/mol), and the hydrogen yield was 1.67 (mol/mol). The second batch of 50 g/l glucose was added when the glucose concentration was close to zero and it was consumed at a slower rate due to the butyrate inhibition. The bacteria stopped producing
butyric acid and hydrogen when the butyric acid concentration reached 21.3 g/l, which was higher than that for the wild type (16.3 g/l) reported by Zhu and Yang (Zhu et al. 2002). This finding is consistent with the results from serum bottle fermentations (Chapter 3.3.4), which showed that the ptb mutant had a higher butyrate tolerance than the wild type with Kp = 13.5 g/l vs. Kp = 9.0 g/l. In the second batch, the butyrate yield was lower than the first batch due to the final product inhibition, which led to an overall butyric acid yield of 0.59 (mol/mol). The final (overall) hydrogen yield from the fed-batch fermentation was 1.68 mol/mol glucose.

4.3.6 FBB fed-batch fermentation kinetics

The maximum butyric acid concentration could be produced by the ptb mutant was also evaluated with cells immobilized and adapted to tolerate a higher butyrate concentration in fed-batch fermentation. Figure 4.10 shows the fermentation kinetics with glucose at pH 6.0 in the FBB by the ptb mutant. As shown in table 4.3, the maximum butyrate concentration was increased to 31.5 g/l, as compared to 21.3 g/l in the free-cell fermentation. Also noticed is the decreased B/A ratio (1.95 vs. 2.7), which again accompanied a higher hydrogen production (1.90 vs. 1.68 mol/mol).

4.4 Discussion

Generally, hydrogen production occurs in the transfer of reducing equivalents
produced during the pyruvate oxidation using the pyruvate-ferredoxin oxidoreductase (PFOR) coupling with the hydrogenase (Gray and Gest 1975; Cammack et al. 2001; Hallenbeck and Benemann 2002), as illustrated by the following equations:

\[
\text{Pyruvate} + \text{CoA} + \text{Ferredoxin (ox)} \rightarrow \text{Acetyl-CoA} + \text{CO}_2 + \text{Ferredoxin (red)}
\]

\[
\text{Ferredoxin (red)} \rightarrow \text{Ferredoxin (ox)} + \text{H}_2
\]

The theoretical molar yield of hydrogen is two per mol glucose if the reducing equivalent from pyruvate is the only reducing source of the hydrogen. However, some *clostridia* strains also contain NADH: ferredoxin oxidoreductase (NFPR), which can produce additional hydrogen using the reducing equivalent stored in NADH during the glycolysis (Saint-Amans et al. 2001):

\[
\text{NADH} + \text{Ferredoxin (ox)} \rightarrow \text{NAD}^+ + \text{Ferredoxin (red)}
\]

\[
\text{Ferredoxin (red)} \rightarrow \text{Ferredoxin (ox)} + \text{H}_2
\]

Although the reaction from NADH to hydrogen is energetically unfavorable under standard conditions (Hallenbeck 2005), it could be compensated by coupling with the ATP hydrolysis, which is highly energetically favorable. Compared to the wild type, the ptb mutant had a higher acetate production accompanied with a higher ATP production to facilitate the hydrogen production. The higher ATP yield can also explain the increased specific growth rate of ptb mutant.

It was noticed that in the ptb mutant fermentation, butyrate was still produced at a significant level as compared to the wild type, suggesting the existence of a new butyrate
formation pathway other than PTB-BK route. Charrier et al. (Charrier et al. 2006) discovered a novel CoA transferase pathway in human colonic bacterium Roseburia sp. A2-183, which used butyryl-CoA: acetate CoA-transferase for butyrate formation in the human colon. The genome of C. tyrobutyricum was sequenced with 454 technology and the results showed the existence of two genes for acetoacetylCoA: acetate/butyrate CoA transferase (EC# 2.8.3.9), one of them was located in the contig 64 starting at 129 bp and ending at 1682 bp and the other one was from 21339 bp to 22931 bp in the contig 22. The deduced amino acid sequences from these two genes show 57% identity to each other. The one located in contig 64 shows 80% identity to the CoA transferase from Clostridium Novyi (Gi: 4541513) and the other one shows 76% to the same protein. These CoA transferases may be responsible for transferring the CoA group from butyryl-CoA to acetate to form butyrate and acetyl-CoA. It is thus very likely that C. tyrobutyricum possesses both PTB-BK and CoA transferase pathways for butyrate biosynthesis, explaining why butyrate production was not eliminated in the ptb mutant. Similarly, the existence of CoA transferase also can explain acetate production in pta/ack knock-out mutants (Zhu et al. 2004; Liu et al. 2006).

It has been proposed that weak organic acids inhibit cell growth because they can disturb the pH gradient across the cell membrane (Gu et al. 1998). The mechanism of short-chain organic acid inhibition was that the undissociated acid form of the organic acid under the acidic pH condition in the extracellular space passes the cell membrane due to its non-polar property, and is then dissociated in the neutral environment in the intracellular space, releasing the proton ion and causing pH drop in the intracellular space.
In order to control the intracellular pH, extra protons have to be pumped out from the intracellular space by H\(^+\)-ATPase at the expense of ATP. In other words, with the same concentration of butyric acid, a lower pH value will result in more protonated butyric acid and consumes more ATP to maintain the pH environment in the cells. For *C. tyrobutyricum*, it has been noticed that the H\(^+\)-ATPase performance was related to the butyric acid tolerance. The adapted mutant with higher butyrate tolerance also showed a higher H\(^+\)-ATPase activity (Zhu and Yang 2003). H\(^+\)-ATPase is a membrane-bound enzyme essential for maintaining the transmembrane proton motive force required for ion regulation and transportation of certain nutrients (Riebeling and Jungermann 1976; Ivey and Ljungdahl 1986). The higher activity of H\(^+\)-ATPase can assure that more protons in the cytoplasm could be transported to the outside of cells, thus maintaining the proper pH in the cytoplasm. Additionally, the fatty acid composition of the cell membrane also changed in the adapted cells, which had more saturated long-chain fatty acids in their membrane, resulting in less membrane fluidity (Zhu and Yang 2003). Similar findings have also been reported for *S. cerevisiae* (Casey and Ingledew 1986) and *E. coli* (Ingram 1976), which have the ability to regulate membrane lipid composition to increase their tolerance to organic solvents.

4.5 Conclusion

Experimental design with statistical analysis was used to screen significant components of CGM. The preparation of the CGM was simplified and the medium cost
was decreased by removing vitamins and trace metals from the medium. Using one-factor-a-time screening, the optimal pH was found to be pH 6 and the optimal substrate concentration was 50 g/l for \textit{C. tyrobutyricum} batch fermentation. The effects of ptb-disruption on the fermentation profile were studied using glucose and xylose as substrates. Compared to the wild type, the B/A ratio decreased by 34.7% for glucose and 28.1% for xylose, which could be attributed to the inactivation of \textit{ptb}. In addition, the productivity of hydrogen increased 38% for glucose and 46% for xylose, which could be partially attributed to the higher specific growth rate (33% increase for glucose and 40% increase for xylose). Butyric acid and hydrogen production was further enhanced by immobilizing cells in the FBB to adapt cells to tolerate a higher butyrate tolerance, thus achieving a higher final product concentration in the fed-batch fermentation.
4.6 References


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**Table 4.1** Experimental Design and responses for screening medium components to simplify the medium composition (Y: Yes; N: No. Units for the factors and the responses: yeast, g/l; soy broth, g/l; trypton, g/l; butyric acid, g/l; hydrogen, ml; growth rate, /h.)
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<tr>
<td>Butyrate</td>
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<td>0.61±0.07</td>
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<tr>
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All data are based on the average from duplicate batch fermentations with the standard error indicated by the number after ±.

**Table 4.2** Comparison of product yields, productivities, and specific growth rates of wild type and ptb mutant in batch fermentations with glucose and xylose
Table 4.3 Comparison of product yields, final product concentration, B/A ratio from free-cell and FBB fermentations by ptb mutant.

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<td>B/A (mol)</td>
<td>2.7 ± 0.1</td>
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All data are based on the average from duplicate batch fermentations with the standard error indicated by the number after ±.
Figure 4.1 Construction of fibrous-bed bioreactor (A. Schematic drawing; B. Photograph)
Figure 4.2 Results of factor screening by design of experiments.
Figure 4.3 Comparison of the specific growth rate, butyric acid yield, and hydrogen yield from the simplified medium and the original CGM medium.
**Figure 4.4** Cell growth and organic acid production from different substrates.
Figure 4.5 Effect of the initial glucose concentration on cell growth in RCM. (A) The growth curve; (B) The specific growth rate in the exponential phase.
Figure 4.6 Effect of pH on cell growth: (A) The growth curve; (B) The specific growth rate in the exponential phase.
Figure 4.7 Fermentation kinetics of glucose by wild type and ptb mutant in free-cell fermentation.
Figure 4.8  Fermentation kinetics of xylose by wild type and ptb mutant in free cell fermentation
Figure 4.9 Fermentation kinetics of fed-batch free-cell fermentation of glucose by the ptb mutant at 37°C, pH 6.0.
Figure 4.10 Fermentation kinetics of fed-batch fermentation by ptb mutant from glucose in the FBB at 37°C, pH 6.0.
CHAPTER 5

GENOME SEQUENCING AND METABOLIC ENGINEERING OF
CLOSTRIDIUM TYROBUTYRICUM FOR BUTANOL PRODUCTION

Summary

The genome of Clostridium tyrobutyricum was sequenced using the 454 technology. Genes encoding enzymes involved in acid and solvent formation pathways were annotated. The only missing gene for a complete butanol biosynthesis route is butyrylaldehyde dehydrogenase. Moreover, the mutants of C. tyrobutyricum showed higher tolerance to butanol than the butanol-producing Clostridium acetobutylicum. Two expression plasmids carrying aldehyde/alcohol dehydrogenase gene aad and adhE2, respectively, from C. acetobutylicum ATCC 824 were constructed and introduced into C. tyrobutyricum. The engineered mutants showed significant butanol production in batch fermentation with glucose as the substrate. This work demonstrated that it is feasible to use C. tyrobutyricum to produce butanol.

5.1 Introduction

Recently, the crude oil price has increased and fluctuated dramatically. Consequently, biobutanol and other biofuels have become more attractive to the fuel industry. Butanol is regarded as a better fuel candidate than ethanol due to the following
advantages: (a) butanol has 25% more Btu per gallon; (b) butanol is less evaporative with a lower vapor pressure; (c) butanol is safer with a higher flash point; (d) butanol has a higher octane rating; (e) butanol is more miscible with gasoline and diesel but less miscible with water; (f) butanol can be dispersed through existing pipelines and filling stations and used as fuel without any modification to the modern automobile (Shapovalova and Ashkinazib 2008). In addition, butanol has many industrial applications as solvent, rubber monomer, break fluid, and extractant in food and cosmetics industry (Wackett 2008). Currently, butanol is almost exclusively produced via petrochemical routes and its current price as an industrial solvent is around $ 5.0 per gallon with a huge worldwide market of 1.4 billion gallons per year (http://www.icispricing.com/il_shared/Samples/SubPage153.asp). The market demand is expected to increase dramatically if butanol can be produced economically to be used as a gasoline substitute.

As an alternative to petroleum-based production, acetone-butanol-ethanol fermentation (ABE fermentation) by the strict anaerobic bacterium *C. acetobutylicum* was once (1917-1955) one of the largest fermentation processes ever developed in industry (Jones and Woods 1986). In a typical ABE fermentation, butyric and acetic acids are produced first by *C. acetobutylicum*; the culture then undergoes a metabolic shift and solvents (butanol, acetone, and ethanol) are formed. However, the actual fermentation is quite complicated and suffers from severe final product inhibition, resulting in low reactor productivity, yield and final butanol concentration. Consequently, biobutanol
produced from *clostridia* fermentation is uncompetitive to bioethanol and gasoline in the fuel market.

The product inhibition is the key issue for butanol fermentation. It is believed that the saturated/unsaturated fatty acid ratio of the cell membrane has significant impacts on the solvent tolerance (Bhupinder *et al.* 1987). The physiological response of bacteria to the environment with a high butanol concentration is to increase its saturated/unsaturated fatty acid ratio in order to maintain the rigidity of cell membrane (Lenaz *et al.* 1975; Moreira *et al.* 1981). It has been reported that *S. cerevisiae* with a higher ethanol tolerance also had a higher saturated/unsaturated fatty acid ratio in the cell membrane (Hilge-Rotmann and Rehm 1991). A similar finding was reported for *C. tyrobutyricum*, which acquired a higher saturated/unsaturated fatty acid ratio and became more tolerant to butyric acid after adaptation in the fibrous bed bioreactor (FBB) (Zhu *et al.* 2002). Furthermore, several *C. tyrobutyricum* mutants with disrupted acetate pathway also showed a higher tolerance to butyric acid (Zhu *et al.* 2004; Liu and Yang 2006) and butanol (in this study).

The genome of *C. tyrobutyricum* has been sequenced using the 454 technology. Some genes encoding the enzymes involved in solventogenesis were found to also exist in *C. tyrobutyricum* genome (data not published), although *C. tyrobutyricum* does not produce solvent. Those genes annotated in the 454 database include alcohol dehydrogenase, butanol dehydrogenase, and CoA transferase. In order to construct a complete butanol biosynthesis pathway in *C. tyrobutyricum*, aldehyde dehydrogenase needs to be expressed and functional in the bacterium. It is thus hypothesized that the
introduction of the *aad* gene encoding aldehyde/alcohol dehydrogenase into *C. tyrobutyricum* could yield a mutant producing butanol. Furthermore, partial blockage of the acetate formation pathway in *pta* and/or *ack* mutant could lead more carbon source to butyrylCoA and butanol production.

In this work, the 454 sequencing results were analyzed and the butanol production pathway was constructed by introducing either *aad* or *adhE2* gene of *C. acetobutylicum* ATCC 824. Two expression plasmids were constructed: pCAAD containing *aad* gene and its native promoter and pSOS-adhE2 containing *adhE2* gene and a constitutive *ptb* promoter. After introducing these plasmids into *C. tyrobutyricum* by electroporation, several mutants were selected and tested for butanol production in batch fermentation. The positive result from the fermentation kinetics study confirmed that introducing *aad* or *adhE2* into *C. tyrobutyricum* enabled the mutant to produce butanol from glucose.

### 5.2 Materials and Methods

#### 5.2.1 Strains, plasmids and media

*C. tyrobutyricum* ATCC 25755 was obtained from the American Type Culture Collection. The plasmids pCAAD and pSOS94 were obtained from Dr. Papoutsakis’ group at the University of Delaware (Nair and Papoutsakis 1994; Sillers *et al.* 2009). These two plasmids contained both gram-negative replicator *ColE1* and gram-positive replicator *RepL*, although not in the same arrangement, so they can be replicated in both *E. coli* and *C. tyrobutyricum*. They also carried two selective markers, erythromycin
resistant gene for *C. tyrobutyricum* and ampicillin resistant gene for *E. coli* (Table 5.1). The pSOS94 also contains the acetone operon including *ctfA*+*ctfB*+*adc* structure gene.

*C. tyrobutyricum* was maintained in RCM (Reinforced *Clostridium* Medium, Difco) under anaerobic conditions at 4°C. The mutant colonies were screened on RCM agar plate supplemented with 40 µg/ml erythromycin at 37°C in an anaerobic chamber. Unless otherwise noted, RCM supplemented with glucose as the carbon source was used in all studies. The P2 basal medium contains (per 100 ml): 0.5 g of K$_2$HPO$_4$, 0.5 g of KH$_2$PO$_4$, 2.2 g of CH$_3$COONH$_4$, 2.0 g of MgSO$_4$$\cdot$7H$_2$O, 0.1 g of MnSO$_4$$\cdot$H$_2$0, 0.1 g of NaCl, 0.1 g FeSO$_4$$\cdot$7H$_2$O, 100 mg of p-aminobenzoic acid, 100 mg of thiamine and 1 mg of biotin (Mermelstein and Papoutsakis 1993) was also used in fermentation kinetics study.

### 5.2.2 Genome DNA extraction

Chromosome DNA of *C. tyrobutyricum* was isolated using the QIAGEN genomic DNA kit (Qiagen, Valencia, CA). The DNA/protein ratio was checked by measuring O.D. 260/280. Agarose gel was also used to check the integrity of the chromosome DNA (Figure 5.2). Plasmid DNA in *E. coli* was isolated using QIAprep Miniprep plasmid purification kit (Qiagen, Valencia, CA) and used for transformation purposes.

### 5.2.3 454 sequencing and metabolic pathway reconstruction
454 sequencing was performed in Craig Venter Institute (Rockville, MD). The detailed sequencing process could be found in 454’s website (http://www.454.com/products-solutions/how-it-works/index.asp).

Genes encoding the enzymes involved in the metabolic pathway were annotated in 454 databases and Pairwise Sequence Alignment was performed between *C. tyrobutyricum* and *C. acetobutylicum* ATCC 824 (NC003030 & NC001988).

### 5.2.4 pCAAD and pSOS-adhE2 construction

The plasmid pCAAD (Figure 5.3) was used as received. It was constructed by inserting the *aad* gene with its own putative promoter into plasmid pIM13. The plasmid pSOS-adhE2 (Figure 5.4) was constructed by inserting *adhE2* gene into plasmid pSOS95 with the constitutive *ptb* promoter isolated from *C. acetobutylicum* ATCC 824.

The *adhE2* gene was amplified by PCR using primers PE1 and PE2 by *pfx* polymerase under standard conditions. The PCR amplification product and plasmid pSOS94 were digested with restriction enzymes *BamHI* and *SfoI* and ligated with T4 ligase to generate recombinant pSOS-adhE2.

**PE1(forward):** 5’- AT**GGATCC**TTTTTATATA**AAGGAG**GTATATATAATGAAAG -3’

*BamHI* RBS

**PE2-reverse:** 5’- TT**GGCGCC**ATAATGAAAGCAAAGACTATTTTACATTC -3’

*SfoI*
The recombinant plasmid pSOS-adhE2 was transformed into *Escherichia coli* DH5α using chemical transformation and selected on Luria–Bertani (LB) plates (1% (w/v) Trypton, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 2% (w/v) agar) containing 100 μg/ml ampicillin. Transformants were selected and screened by colony PCR using the above primers. The recombinant plasmids were isolated from positive transformants with a Maxi-Prep Kit (Qiagen). The gene *adhE2* with *ptb* promoter was sequenced completely, proving that no mutation occurred during the PCR.

### 5.2.5 Restriction endonuclease system identification

Protoplast and cell crude extract were prepared to detect the restriction endonuclease digestion system in *C. tyrobutyricum* ATCC 824, which has active restriction endonuclease system, was used as positive control (Mermelstein and Papoutsakis 1993). Protoplast were prepared according to the method previously described (Zhu 2003). Cell crude extracts were prepared from a 20 ml late-exponential phase culture using sonication. The supernatant was stored at -20 ºC.

For restriction assay using protoplast, 500 ng plasmid pCAAD was mixed with 0 μl, 1 μl, 2 μl, or 5 μl of protoplast and incubated for overnight at 37 ºC in reaction buffer prepared as previously described (Zhu 2003). The result was examined by electrophoresis with 0.8% agarose gel.

For restriction assay using cell crude extract, 500 ng plasmid pSOS-adhE2 was mixed with 2 μl of crude extract (approx. 2 μg protein) and incubated for 18 h at 37 ºC in
restriction enzyme buffers 2, 3 and 4 from New England Biolab. The products were analyzed by electrophoresis with 0.8% agarose gel.

5.2.6 Transformation

All manipulations were operated in an anaerobic chamber (Thermo Fisher Scientific). Cells were grown in 50 ml of RCM supplemented with 40 mM DL-threonine and harvested at OD_{600}=0.6 (Zhu et al. 2004; Jiraskova et al. 2005), then washed with 30 ml of ice-cold electroporation SMP buffer twice and resuspended in 1 ml of SMP buffer in 0.4 cm electroporation cuvette (Bio-Rad). The SMP buffer (pH 7.4) consisted of 270 mM sucrose, 1 mM MgCl₂, and 7 mM sodium phosphate. The MgCl₂ solution was prepared and autoclaved separately before adding to the sucrose and phosphate solution to prevent precipitation. Two micrograms of ice-cold plasmid pCAAD or pSOS-adhE2 were added into the cuvette containing 1 ml of cell and mixed. The mixture was incubated on ice for 5 min before the pulse applied. The transformation parameters were set as follows: 2.5 kV, 600 Ω, 25 AF. The transformed cells were transferred into pre-warmed RCM, incubated at 37°C for 2 hours, and then plated on RCM plates containing 40 µg/ml erythromycin. Plates were incubated in an anaerobic incubator at 37°C for 3–5 days to develop the mutant colonies.

5.2.7 Butanol and butyrate tolerance
Butanol tolerance was evaluated by the inhibition effect of butanol on cell growth in serum tubes. The experiments were performed on *C. tyrobutyricum* wild type and mutants (PTA, PAK, and HydEm). Butanol (0, 3, 6, 9, 12, 15 g/l) was added into 10 ml of RCM, which was then inoculated with 0.5 ml of freshly prepared *C. tyrobutyricum* culture in the exponential phase (OD$_{600}$=1.0) and maintained at 37°C. Samples were taken at regular intervals to measure the OD$_{600}$ with a spectrophotometer (Sequoia-Turner, model 340). The specific growth rates at various initial butanol concentrations were determined from the cell density.

Butyrate tolerance was also evaluated by the inhibition effect of butyrate on cell growth in serum tubes. Butyric acid, 0-80 g/l with 10 g/l intervals, was added into 10 ml of RCM and the pH was adjusted to 6. Each tube was then inoculated with 0.5 ml of freshly prepared *C. tyrobutyricum* culture in the exponential phase (OD$_{600}$=1.0) and maintained at 37°C. Samples were taken at regular intervals to measure the OD$_{600}$ with a spectrophotometer (Sequoia-Turner, model 340). The specific growth rates at various initial butyrate concentrations were determined from the OD$_{600}$.

### 5.2.8 Fermentation kinetics

The effect of introducing pCAAD and pSOS-adhE2 into *C. tyrobutyricum* on fermentation kinetics was evaluated in serum bottles. Each bottle containing 50 ml of RCM or P2 medium with 10 g/l glucose was inoculated with 0.5 ml of fresh culture and incubated at 37°C. Samples were taken at regular time intervals and the alcohol content
was analyzed with gas chromatography (GC) (Shimadzu, GC2014) as described previously.

Free-cell fermentation of pCAAD mutant was further studied in a 2-liter stirred bottle containing 1 L of RCM supplemented with 50 g/l glucose. The medium was sparged with nitrogen for 2 hours to achieve anaerobiosis. The inoculum was 50 ml of fresh cell suspension in the exponential phase. Batch fermentations were carried out at 37°C and started at pH 6.0, and the pH was maintained at 4.5 adjusted with 6 N NaOH. Liquid samples were taken at regular intervals to analyze the cell density (OD$_{600}$) and the concentrations of the substrate and products in the fermentation broth.

5.2.9 Analytical methods

The cell density was calculated based on OD$_{600}$ measured with a spectrophotometer (Sequoia-Turner, model 340). One unit of OD$_{600}$ was corresponding to 0.68 g/l of cell dry weight.

The composition of alcohol produced in the fermentation was analyzed with a gas chromatograph (Shimadzu GC 2014) equipped with Flame Ionization Detector and Stabilwax-DA column (Restek) with helium as the carrier gas at the flow rate of 2 ml/min. The injector temperature and the detector temperature were set to 200°C. The column temperature was programmed as the followings: 80°C hold for 3 min, and then increased to 150 °C at 30°C/min and hold at 150°C for 4 min.
5.3 Results

5.3.1 Genome sequencing

The 454 sequencing produced 211,099 good reads, containing 55,970,852 bp of raw sequence. Assembly produced 69 contigs, 3,016,691 bp.

The genes encoding the enzymes involved in the metabolic pathway (Figure 5.1) from pyruvate to various end products, including acidogenesis and solventogenesis according to ATCC 824, found and annotated in the 454 database are summarized in Table 5.2 with the gene name, enzyme name, EC number, contig number, and starting and ending positions in the 454 database. Among the 16 genes in the pathway, ptb and buk were missing, which are probably located in the gap due to the disadvantage of the 454 technology. BLAST has been performed against the corresponding gene from Clostridium. The amino acid sequences of these enzymes display high similarity (60%-80%) among the clostridium genus compared.

More interestingly, C. tyrobutyricum genome contains some genes necessary for solvent production, such as adc (acetoacetate decarboxylase), bdh (NADH dependent butanol dehydrogenase) and adh (NADH dependent alcohol dehydrogenase) (Figure 5.1). However, the genes encoding alcohol/aldehyde dehydrogenase (aad) cannot be located. The amino acid sequences from aad and adhE2 are available for C. acetobutylicum ATCC824. The deduced amino acid sequences from several bifunctional aldehyde alcohol dehydrogenase genes from GenBank database were compared. 66% identity was found between adhE2 and aad; 57% identity between adhE2 and adhE from E. coli K-12.
(Fontaine et al. 2002). It was thus proposed that *C. tyrobutyricum* will probably obtain the ability to produce butanol if the gene encoding alcohol/aldehyde dehydrogenase is introduced.

### 5.3.2 Restriction endonuclease system identification

The restriction enzyme activity has been demonstrated existing in *C. acetobutylicum* ATCC 824 with both protoplast and cell crude extract (Mermelstein and Papoutsakis 1993). However, no digestion of plasmid was observed in the incubation of plasmid pCAAD with the protoplast of *C. tyrobutyricum* (Figure 5.5A). Furthermore, the crude extract from *C. tyrobutyricum* did not show any digestion of the plasmid pSOS-adhE2 (Figure 5.5B). These results confirmed that there was no restriction enzyme activity in *C. tyrobutyricum*.

### 5.3.3 Transformation

*C. tyrobutyricum* is a gram-positive bacterium, which is difficult to transform due to its cell wall structure (Allen and Blaschek 1990; Young and Cole 1993). Electroporation has been demonstrated to be effective to introduce plasmids into gram-positive bacteria (Scott and Rood 1989; Phillips-Jones 1995; Jiraskova et al. 2005). Electroporation was thus used to transform the recombinant plasmids into *C. tyrobutyricum*. The results showed a low transformation efficiency of 2-10 transformants
per μg DNA. Nevertheless, several mutant colonies were obtained on the selection agar plates. They were selected and subcultured in CGM containing erythromycin before testing for their butanol production in batch fermentation kinetics study.

### 5.3.4 Butanol tolerance

All three *C. tyrobutyricum* mutants (PTA, PAK, HydEm) displayed a higher tolerance to butyrate than the wild type (Figure 5.6). At the same concentration of butyrate, the mutants had a higher specific growth rate and shorter lag phase. As expected, the mutants also showed higher tolerance to butanol than the wild type (Figure 5.7), mutants could tolerate 12 g/l butanol while wild type could not grow at 12 g/l butanol, which is similar to *C. acetobutylicum*’s butanol tolerance. Figure 5.8 shows all of the three mutants of *C. tyrobutyricum* shows higher specific growth rate than wild type under various amount of butanol, which supports our hypothesis that the rigid membrane acquired from high butyrate tolerance also contributed to the high solvent tolerance. It is thus reasonable to assume that if we can construct a butanol-producing *C. tyrobutyricum* mutant, a higher butanol titer probably can be obtained due to its ability to survive at higher butanol concentrations. This higher butanol tolerance was achieved under free-cell fermentation conditions in this study. Based on the experience with butyrate tolerance studies, FBB immobilization could increase the butanol tolerance further.

### 5.3.5 Fermentation kinetics
Butanol and ethanol production in *C. tyrobutyricum* wild type and two recombinant heterologous expression strains were studied in RCM and P2 medium in serum bottles and the results are summarized and compared in Table 5.3. Figure 5.9 shows the time course data on butanol production in batch fermentations by *C. tyrobutyricum* wild type and mutant pCAAD in serum bottles. It is clear that the mutant was able to produce a significant amount of butanol while no detectable butanol was produced by the wild type under the conditions studied. The final butanol concentration was 67 mg/l and 24 mg/l in RCM and P2 medium, respectively. Similarly, significant butanol production was also detected in the fermentations with the mutant pSOS-adhE2, which gave a final butanol concentration of 9 mg/l and 19 mg/l in RCM and P2 medium, respectively. It has been noticed that *Clostridium beijerincki* produced more butanol in P2 than in RCM (data not published), probably due to the nitrogen deficient condition in the P2 medium. However, the effect of different media on butanol production is species and strain dependent, and cannot be generalized without further studies. Although butanol production was low in these fermentations, both recombinant *C. tyrobutyricum* strains constructed in this study showed an ability to produce butanol. It is noted that both mutants also showed increased ethanol production, as compared to the wild type fermentation. This indicates that the cloned alcohol/aldehyde dehydrogenase has a broad substrate specificity and can work with both acetyl-CoA and butyryl-CoA. The relatively high ethanol background in the wild type fermentation broth was attributed to the ethanol added to the medium with erythromycin.
Free-cell batch fermentations in a pH-controlled fermenter were then performed to further evaluate butanol production in *C. tyrobutyricum* (pCAAD). The first batch was grown at 37°C in RCM and showed no butanol production. It has been reported that solvent production benefits from slower growth. The temperature was thus decreased to 30°C. Figure 5.10 shows that butanol was produced although at a low level and butyric and acetic acids remained the main fermentation products. The final butanol concentration was 19 mg/ml. Meanwhile ethanol was also produced and reached 60 mg/l. It’s probably due to the selectivity of AAD on ethanol than butanol, while ADHE2 showed a selectivity on butanol than ethanol (Fontaine *et al.* 2002). The results confirmed that the cloning of *aad* into *C. tyrobutyricum* enabled the mutant to produce both butanol and ethanol, but at a much lower level than those of the native acid products. It is thus necessary to further optimize the fermentation conditions and engineer the mutant to direct more carbon flux toward alcohol synthesis instead of acid production, which can be done by knocking-out *pta* and in the acid-forming pathways and using a stronger promoter for *aad* overexpression.

### 5.4 Discussion

Butanol production from biomass is a promising solution for the energy crisis. However, the low yield and low productivity due to the tightly regulated metabolic pathway and the final product inhibition make the biobutanol from the ABE fermentation process uncompetitive to gasoline and bioethanol. Many efforts have been made to
improve butanol production in ABE fermentation via metabolic engineering of \textit{C. acetobutylicum} and process engineering to alleviate inhibition caused by butanol. Despite all these efforts, there has been little progress towards making ABE fermentation economically competitive. A new process for the production of biobutanol is thus needed.

The solvent-producing genes have been cloned, sequenced and characterized in \textit{C. acetobutylicum}, including \textit{aad}, \textit{ctfA/B}, \textit{adc} encoding aldehyde/alcohol dehydrogenase, CoA transferase A/B, acetoacetate decarboxylase, respectively. These genes are located in the megaplasmid pSOL1 in \textit{C. acetobutylicum}. The \textit{aad} gene has 2619 base-pair and codes for a 96,517 Dalton protein. The N-terminal section of AAD shows homology to aldehyde dehydrogenase while the C-terminal shows homology to alcohol dehydrogenase. The entire amino acid sequence of AAD exhibits 56\% identity to the trifunctional protein ADHE from \textit{E. coli} (Nair et al. 1994). The introduction of \textit{aad}-containing plasmid into \textit{C. acetobutylicum} increased the activity of butanol dehydrogenase, acetaldehyde dehydrogenase and butyraldehyde dehydrogenase significantly and also slightly the ethanol dehydrogenase activity in the mutant. The same plasmid has also been transformed into the M5 mutant of \textit{C. acetobutylicum}, which does not produce solvent due to the lack of the megaplasmid. However, butanol production resumed upon the transformation without any detectable acetone and ethanol (Nair and Papoutsakis 1994). The other gene \textit{adhE2} also located on pSOL1 was cloned and characterized by Fontaine \textit{et al.} (Fontaine \textit{et al.} 2002). It has 2,577-bp encoding for a 94.4 kDa protein. The expression of \textit{adhE2} in the DG1 mutant of \textit{C. acetobutylicum} (BYDH', AAD') restored butanol production, and elevated activities of NADH-dependent butyraldehyde and
butanol dehydrogenases were observed. The recombinant ADHE2 protein expressed in *E. coli* also demonstrated NADH dependent butyraldehyde and butanol dehydrogenase activities. It is the first report that one bacterium has two *aad* genes. Comparing to *aad*, the *adhE2* shows selectivity on butanol over ethanol.

*C. tyrobutyricum* does not possess the megaplasmid and is not capable of producing butanol, ethanol and acetone. However, the 454 sequencing result revealed that *C. tyrobutyricum* genome contains butanol dehydrogenase. It was thus proposed that the introduction of butyraldehyde dehydrogenase could enable butanol production in *C. tyrobutyricum*. The engineered *C. tyrobutyricum* would have a relatively simple butanol fermentation pathway without any acetone production due to the lack of acetoacetate decarboxylase. Moreover, its native strong butyric acid production ability can lead more carbon source to butyryl CoA instead of acetate or ethanol. Thus, the engineered mutant is expected to have a more easily controlled metabolic pathway with high butanol production.

The final product inhibition is the key issue for butanol fermentation. The butanol inhibition is related to the cell membrane lipid composition (Weber and De Bont 1996). A higher saturated/unsaturated fatty acid ratio can better maintain the membrane’s rigidity and prevent the solvent from entering and disrupting the cell membrane, or even lysing the membrane (Bhupinder *et al.* 1987). Similarly, the tolerance to organic acid also relates to the lipid composition of cell membrane, in addition to ATPase activity. When the short-chain organic acid is produced, it may pass the cell membrane and enter the intracellular space. A higher saturated/unsaturated fatty acid ratio can make the
membrane not permeable to the acid. Once the acid enters the cell, it is disassociated and
the released proton is harmful to the cell because it lowers the intracellular pH from
neutral (O'Sullivan and Condon 1999; Cotter and Hill 2003). ATPase is associated with
the cell membrane and could pump extra proton ions from the intracellular space of the
cell to the outside to maintain a proper intracellular environment at the expense of ATP.
It has been reported that the higher tolerance to butyric acid accompanied a higher
saturated fatty acids content (Zhu and Yang 2003). Two gene knockout mutants with
disrupted acetate pathway and one FBB adaptation mutant from C. tyrobutyricum were
constructed previously in our group (Zhu et al. 2004; Liu et al. 2006) and all of them
showed higher butyrate tolerance than the wild type. It is expected that mutants with
higher butanol tolerance could be constructed upon the introduction of aad or adhE2 into
these acid-tolerant mutants.

In this research, butanol production has been demonstrated in C. tyrobutyricum
mutants, although at a low concentration. The low butanol productivity is probably due to
the promoter compatibility. Further studies should include the alcohol/aldehyde
dehydrogenase assay to detect the enzyme activity level and SDS-PAGE to confirm the
gene expression in the mutant. Meanwhile, as the genome sequence of C. tyrobutyricum
is available, efforts will be put on cloning the native constitutive thiolase promoter to
promote the aad/adhE2 overexpression. Furthermore, mutants with knock-out acetate and
butyrate pathways would serve as better hosts for butanol biosynthesis and should be
investigated.
5.5 Conclusion

The genome of *C. tyrobutyricum* has been sequenced by using the 454 technology. Most of genes encoding for the enzymes responsible for the metabolic pathway from glucose to acetate and butyrate have been identified and localized in the 454 database, except for *ptb* and *buk*. Several enzymes involved in solvent formation also exist in *C. tyrobutyricum*. The aldehyde/alcohol dehydrogenase genes *aad* and *adhE2* have been introduced into the wild type of *C. tyrobutyricum*. The mutant showed butanol production at 30°C in free cell fermentation. It is thus concluded that the introduction of *aad* gene and/or *adhE2* gene could complete the metabolic pathway needed for alcohol production and lead to the production of butanol in *C. tyrobutyricum*. 
5.6 References


<table>
<thead>
<tr>
<th>Strains / Plasmids</th>
<th>Characteristics</th>
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<tr>
<td><em>Clostridium tyrobutyricum</em></td>
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<td>Invitrogen</td>
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<td>pCAAD</td>
<td>Em&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;, <em>aad</em>, ColE1, RepL</td>
<td>Guerout-Fleury <em>et al.</em> 1995</td>
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<tr>
<td>pSOS94</td>
<td>Em&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;, ColE1, RepL, <em>ptb</em> promoter, acetone operon</td>
<td>Soucaille and Papoutsakis, unpublished</td>
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<td>pSOS-adhE2</td>
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<td>This study</td>
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*Table 5.1* Bacterial strains and plasmids used in this study.
<table>
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<th>Enzyme</th>
<th>Gene</th>
<th>EC number</th>
<th>454 sequence location</th>
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<td>Pyruvate ferredoxin oxidoreductase</td>
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<td>25 76288 77451</td>
<td>67.8%</td>
</tr>
<tr>
<td>Butanol dehydrogenase (NADPH dependent)</td>
<td>bdh</td>
<td>1.1.1.-</td>
<td>36 17262 18428</td>
<td>77.6%</td>
</tr>
<tr>
<td>Acetoacetyl-CoA: acetate/butyrate CoA transferase</td>
<td>ctf</td>
<td>2.8.3.9</td>
<td>64 129 1682</td>
<td>44.0%</td>
</tr>
<tr>
<td>Acetoacetyl-CoA: acetate/butyrate CoA transferase</td>
<td>ctf</td>
<td>2.8.3.9</td>
<td>22 21339 22931</td>
<td>42.0%</td>
</tr>
<tr>
<td>Ferredoxin hydrogenase</td>
<td>hyd</td>
<td>1.12.7.2</td>
<td>4 181223 182194</td>
<td>38.5%</td>
</tr>
<tr>
<td>Phosphate butyltransferase (phosphotransbutyrylase)</td>
<td>ptb</td>
<td>2.3.1.19</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Butyrate kinase</td>
<td>buk</td>
<td>2.7.2.7</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Alcohol/aldehydes dehydrogenase (NADH dependent)</td>
<td>adhE2</td>
<td>1.1.1.-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Alcohol/aldehydes dehydrogenase</td>
<td>aad</td>
<td>1.1.1.-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Acetoacetate decarboxylase</td>
<td>adc</td>
<td>4.1.1.4</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**Table 5.2** Location of genes and consensus against ATCC824
<table>
<thead>
<tr>
<th>Medium</th>
<th>Strain</th>
<th>$\text{OD}_{600}$</th>
<th>Ethanol (mg/l)</th>
<th>Butanol (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCM</td>
<td>Mutant pCAAD</td>
<td>15.4 ± 1.2</td>
<td>240 ± 22</td>
<td>67 ± 8</td>
</tr>
<tr>
<td></td>
<td>Mutant pSOS-adhE2</td>
<td>16.8 ± 1.0</td>
<td>190 ± 26</td>
<td>9 ± 1</td>
</tr>
<tr>
<td></td>
<td>Wild type</td>
<td>16.2 ± 0.8</td>
<td>210 ± 35</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>P2</td>
<td>Mutant pCAAD</td>
<td>3.2 ± 0.4</td>
<td>65 ± 8</td>
<td>24 ± 2</td>
</tr>
<tr>
<td></td>
<td>Mutant pSOS-adhE2</td>
<td>2.9 ± 0.2</td>
<td>56 ± 10</td>
<td>19 ± 3</td>
</tr>
<tr>
<td></td>
<td>Wild type</td>
<td>3.1 ± 0.5</td>
<td>32 ± 2</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

**Table 5.3** Butanol and ethanol production by *C. tyrobutyricum* wild type and mutants pCAAD and pSOS-adhE2 grown in RCM and P2 medium in serum bottles at $37^\circ C$, initial pH 6.
Figure 5.1 The enzymes involved in the pathway from pyruvate to the end products, highlighted enzyme presented in 454 database. (PFOR: pyruvate ferredoxin oxidoreductase, HYD: hydrogenase, AAD: alcohol/aldehyde dehydrogenase, ADH: alcohol dehydrogenase, iLDH: NADH dependent lactate dehydrogenase, PTA: phosphotransacetylase, AK: acetate kinase, THIL: thiolase, CoAT: CoA transferase, ADC: acetoacetate decarboxylase, HBD: 3-hydroxybutyryl CoA dehydrogenase, CRO: crotonase, BCD: butyryl-CoA decarboxylase, BDH: butanol dehydrogenase, PTB: phosphotransbutyrylase, BK: butyrate kinase)
Figure 5.2 The genomic DNA quality check for 454 sequencing (1: 100 bp-12 kb DNA ladded, 2: original genomic DNA, 3: 25 X dilution)
Figure 5.3 Structure of plasmid pCAAD. (RepL: gram-positive replicator; ColE1: Gram-negative replicator; *amp*<sup>R</sup>: ampicillin resistant gene; *mls*<sup>R</sup>: erythromycin resistant gene; *aad*: aad structure gene; *aad*-pro: aad native promoter)
Figure 5.4 Construction of plasmid pSOS-adhE2. (*RepL*: gram-positive replicator; *ColE1*: Gram-negative replicator; *amp*': ampicillin resistant gene; *mls*': erythromycin resistant gene; *adhE2*: adhE2 structure gene; *ptb-Pro*: ptb promoter)
Figure 5.5 The restriction endonuclease system check with protoplast and cell crude extracts. A. with protoplast (Lane 1, ladder; Lanes 2-5, pCAAD with *C. tyrobutyricum* protoplast in NEB buffers with different amounts of protoplast); B. with cell extracts (Lane 1, plasmid only; Lanes 2-4, plasmid with *C. tyrobutyricum* cell extract in NEB buffers; Lanes 5-7, plasmid with *C. acetobutylicum* cell extract in NEB buffers; Lane 8, DNA marker).
Figure 5.6 Growth kinetics of *Clostridium tyrobutyricum* wild type and mutants ACK, PTA, and HydEm under various amounts of butyrate at pH 6.
Figure 5.7 Growth kinetics of *Clostridium tyrobutyricum* wild type and mutants PTA, PAK, and HydEm under various amounts of butanol at pH6
Figure 5.8 Comparison of specific growth rate under various amount of butanol between wild type and mutants of C. tyrobutyricum.
Figure 5.9 Butanol production of *C. tyrobutyricum* (pCAAD) and wild type in RCM and P2 medium in serum bottles.
Figure 5.10 Free-cell fermentation kinetics of *C. tyrobutyricum* (pCAAD) at 30°C, pH4.5.
CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study explored the role of ptb in the metabolic pathway of Clostridium tyrobutyricum and demonstrated the feasibility of the integration of metabolic engineering and process development for enhanced hydrogen production. The advantages of the fibrous-bed bioreactor were also confirmed. It also verified the potential application of C. tyrobutyricum as a butanol-producing strain. The important results and conclusions obtained in this study are summarized below.

6.1.1 Metabolic engineering

- Partial ptb gene was amplified by PCR from the chromosome of C. tyrobutyricum by degenerated primers. The deduced amino acid sequence showed 100% identity to the PTB from Clostridium acetobutylicum.

- A non-replicative integrational plasmid containing ptb fragment disrupted by a erythromycin resistance gene (Em') was constructed. The ptb-disrupted mutant was obtained by homologous recombination. The insertion was confirmed by nested PCR.
PTB enzyme activity decreased by 76% in the mutant. However, the *ptb*
inactivation did not turn off the PTB activity completely. Meanwhile, the BK
activity also decreased by 42%, suggesting that the *buk* is located at the
downstream of the *ptb* in the same operon. In contrast, PTA activity in the mutant
increased by 7% and AK activity increased by 29%.

The specific growth rate of the mutant increased by 17%. The mutant also showed
50% less sensitive to butyric acid inhibition.

### 6.1.2 Fermentation kinetics

The composition of Clostridium Growth Medium was simplified based on statistic
experimental design. Trace metals solution and vitamin solution did not show
significant effects on cell growth and the production of butyric acid and hydrogen
thus were removed from the recipe. NiCl₂ and FeSO₄ were kept because they are
critical factors for hydrogenase. They were added into the buffer #2 solution and
the buffer was sterilized by microfiltration. Yeast extract and Trypticase™ soy
broth, were essential for cell growth and also kept.

*C. tyrobutyricum* ptb mutant can ferment xylose and glucose, but cannot uptake
lactose, glycerol, starch, or cellulose.

The initial glucose concentration of 50 g/l and pH 6 were determined to be
optimal for the ptb mutant fermentation.
The ptb mutant showed a lower B/A ratio, higher hydrogen yield, higher specific growth rate, higher productivity than the wild type in free-cell fermentations. But it still produced a significant amount of butyric acid, suggesting another pathway for butyrate formation.

Immobilized-cell fermentation in the fibrous-bed bioreactor increased the final butyric acid concentration from 21.3 g/l to 31.5 g/l. It demonstrated that the FBB could adapt the cells for an improved tolerance to butyric acid.

### 6.1.3 Genome sequence

The genome sequencing of *C. tyrobutyricum* by the 454 technology produced 211,099 good reads, containing 55,970,852 bp of raw sequence. Assembly produced 69 contigs, 3,016,691 bp.

*ptb* and *buk* were not found in the annotated open reading frames (ORF) in the 454 database. They probably fell into the gaps among various assembled contigs due to sequencing limitations of the 454 technology.

Two genes encoding for acetoacetyl-CoA acetate/butyrate CoA transferase were identified, suggesting the existence of CoA route in *C. tyrobutyricum* and explaining why the butyrate was still produced in the *ptb*-disrupted mutant.

Several genes involved in the clostridia solventogenesis pathway, such as *adc* (acetoacetate decarboxylase), *bdh* (NADH dependent butanol dehydrogenase) and *adh* (NADH dependent alcohol dehydrogenase), also exist in *C. tyrobutyricum,*
suggesting a possible butanol biosynthesis pathway that could be constructed with the cloning of an alcohol/aldehyde dehydrogenase gene in *C. tyrobutyricum*.

### 6.1.4 Butanol production by *C. tyrobutyricum*

- *C. tyrobutyricum* mutants pta, ack, hydem showed a higher butanol tolerance than the wild type and *C. acetobutylicum*. The tolerance to butanol and butyrate are strongly correlated.
- Two expression plasmids pCAAD and pSOS-adhE2 were constructed. pCAAD carried *aad* gene with its native promoter, and pSOS-adhE2 carried *adhE2* gene promoted by the constitutive *ptb* promoter of *C. acetobutylicum*. Both plasmids were transformed into *C. tyrobutyricum* and significant butanol production was verified in batch fermentations. This work demonstrated the potential application of *C. tyrobutyricum* as a butanol producing bacterium.

### 6.2 Recommendations

Several attempts have been explored to understand the metabolic pathway of *C. tyrobutyricum* and to improve butyric acid and hydrogen production. However, many problems remain unsolved and the exact metabolic pathway and its regulation system remain unknown. This section lists several areas suggested for future studies.

#### 6.2.1 Construction of novel butanol producers
Transformation of pCAAD and pSOS-adhe2 established the butanol production in *C. tyrobutyricum*. However, the butanol concentration in the fermentation broth was quite low, probably due to the low expression efficiency. Thus, the following studies are recommended:

- Construct a reporter gene plasmid to detect if *ptb* promoter from *C. acetobutylicum* works in *C. tyrobutyricum* efficiently.
- Thiolase is constitutively expressed in *Clostridia*. The thiolase structure gene is located in contig 37 from 46302 bp to 47483 bp and its upstream nucleotide sequence is also available in the *C. tyrobutyricum* 454 database. The suggested strategy is to clone the native thiolase promoter region and use it for the *aad* expression.
- Increase the copy number of plasmid by changing the current replicator repL from *Bacillus* to a native clostridia replicator. Several plasmids with replicator from *Clostridium* are available. The expression plasmid could be constructed based on those plasmids.
- Transformation is a key technique for the further study of genetic engineering on *C. tyrobutyricum*. The current cloning work is limited by the low transformation efficiency, which is only 10 colonies per microgram plasmid or even lower. Optimization study suggested that the limiting factor is not the cell viability after the electrical pulse shock, but the impermeability of the cell wall and cell membrane. More harsh conditions, such as higher voltage, pulse frequency, or
incubation with lysozyme, should be explored to improve the transformation efficiency.

- The butanol pathway needs to be established in the mutant (PTA, PAK, HydEm, PTB) of *C. tyrobutyricum* in order to acquire higher butanol tolerance.

- There are only two antibiotics (erythromycin and thiamphenicol) to which *C. tyrobutyricum* is known to be sensitive. For the purpose of constructing mutants with dual or multiple mutations, a selection marker rescue system needs to be developed in order to reuse the same antibiotic resistance gene repeatedly.

### 6.2.2 CoA transferase

It was noticed that in the ptb mutant fermentation, the butyrate was still produced at a comparable level to the wild type fermentation, suggesting the existence of a novel pathway other than PTB-BK route that could also produce butyrate. The genome of *C. tyrobutyricum* has recently been sequenced with the 454 technology and the results showed the existence of two genes for acetoacetyl-CoA: acetate/butyrate CoA transferase (EC# 2.8.3.9), one of them is located in the contig 64 starting at 129 bp and ending at 1682 bp and the other is from 21339 bp to 22931 bp in the contig 22. The deduced amino acid sequences from these two genes show 57% identity. The one located on contig 64 shows 80% identity to the CoA transferase from *Clostridium Novyi* (Gi: 4541513) and the other shows 76% identity to the same protein. The function of this enzyme is to transfer CoA group among butyryl-CoA, acetyl-CoA and acetoacetyl-CoA to form butyrate, acetate, and acetoacetate depending on the substrate availability. This pathway
has been identified as a major butyrate production pathway in several other species such as *Roseburia* sp. A2-183 (Charrier *et al.* 2006). It is possible that *C. tyrobutyricum* possesses both pathways for butyrate biosynthesis, which can explain why *ptb*-disruption cannot eliminate butyrate production and *pta/ack* knock out also cannot eliminate acetate production (Zhu *et al.* 2004; Liu *et al.* 2006). Further studies of the CoA transferase function in *C. tyrobutyricum* are thus recommended.

- Clone and sequence the two CoA transferases from the chromosome of *C. tyrobutyricum*.
- Overexpression of CoA transferase gene in *E. coli* and evaluate its expression and function by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), enzyme assay and fermentation kinetics.
- Construct mutants with overexpression or knock-out of CoA transferase to evaluate its effects on butyrate and acetate production.

### 6.2.3 Functional genomics and proteomics

DNA microarray or gene chips have been widely used for the functional analysis of the genome. The whole transcriptional response to gene mutations or environmental stimulus could be examined. This technique can offer the information of the network change compared to the traditional single gene mutation approach.

A shot-gun genome library of *C. tyrobutyricum* was constructed where the whole genome is represented by random DNA fragments. The library contains 1860 gene
fragments with size ranging from 1 kb to 5 kb. The probability of the gene of interest presenting in the library is 90%. Microarray chips have been fabricated onto the poly-lysine coated glass slide and the method for the target preparation and direct labeling has been validated (Appendix A). The microarray is ready to use for studying the global gene expression changes in the following situations:

- Transcriptome change in the 4 mutants of *C. tyrobutyricum*. Three different genetically engineered mutants, including PTA, PAK, PTB, were constructed and one adaptation mutant HydEm was obtained from the FBB fermentation. For the three single gene knock-out mutants, the transcriptome change will not be limited in the gene interrupted, some global change will happen due to the complicated regulation mechanism in *C. tyrobutyricum*. For the adaptation mutant, it is hypothesized that there were several genes mutated in the adaptation process, such as the H⁺-ATPase or genes responsible for the cell membrane composition and lipid biosynthesis. The transcriptome of each mutant and the wild type will be hybridized against microarray chip competitively and the fragments showing different expression levels will be identified and sequenced. The entire gene sequence will be obtained by BLASTing against the 454 database.

- Transcriptome change in different environments, such as pH, hydrogen partial pressure, and production concentration. All of these conditions have shown significantly impacts on the fermentation kinetics so they should have also induced global gene expression changes. One standard condition will be chosen as the baseline. The transcriptome from the target condition and the standard
condition will be competitively hybridized against microarray chip. The entire genes sequence will be obtained from the 454 database.

The purpose of the functional genomics analysis by microarray is to understand the metabolic pathway and the gene expression regulation. The results can be used to elucidate the function of key genes and to identify critical genes in the metabolic pathway as targets for further rational metabolic engineering to construct knock-out or overexpression mutants with higher yield, productivity and tolerance.

It’s also recommended to use two-dimensional electrophoresis to confirm the protein expression pattern change among the mutants and under various conditions. The results could support regulation mechanism obtained from functional genomics analysis. A precise understanding of metabolic pathway will facilitate the further manipulation of *C. tyrobutyricum* to improve butyric acid and hydrogen production.
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Appendix A Microarray Fabrication

A.1 Genomic library preparation

The chromosome DNA extracted by Qiagen Genomic-tip was partially digested with Sau3AI. The DNA fragments were separated on 0.7% agarose gel and the fragments of 1-5 kb were cut, purified and ligated to BamHI digested plasmid pXL. The ligated plasmids were transformed into Max E. coli DH5α competent cells (Invitrogen), and then plated on LB supplemented with 50 μg/ml kanamycin. More than 20,000 white colonies in one standard transformation reaction using E. coli DH5α competent cells have been obtained (Figure A.1).

The white colonies were picked from the agar plates and stored in 96-well plates at -80°C with 15% glycerol for further study. We prepared a 2.5× coverage library which containing 1920 fragments in 20 plates. The probability of the gene of interest presenting in the library is 85% according to \( n = \frac{\ln(1-p)}{\ln(1-f)} \), where \( f = 0.001 \) (fractional portion of genome present in a single DNA fragment or the average size of the insert (3 kb) divided by the genomic size 3 Mb) and \( n=1920 \). The cells were harvested, and the plasmid was extracted by Qiagen spin miniprep kit. After random quality check of the insert on agarose gel (Figure A.2), the insert genes were amplified by M13 forward primer (5’-GTAAAACGACGGCCAG-3’) and M13 reverse primer (5’-
CAGGAAACAGCTATGAC-3’) and the PCR products were randomly checked (Figure A.3). The PCR products were purified by isopropanol precipitation, checked by agarose gel again (Figure A.4), and then dissolved in 3XSSC buffer for printing.

A.2 Microarray slide preparation and quality control

The microarray slide printing is done in-house with a high-precision fast motion arrayer with 16 printing heads. For microarray printing, the widely used method is to print the DNA onto poly-lysine coated glass slides. Since the poly-lysine is positively charged, the negatively charged DNA fragment could easily bind to the poly-lysine on the slide surface. The slides are prepared following the standard protocols from Dr. DeRisi’s Lab in UCSF (http://derisilab.ucsf.edu/pdfs/PolylysineSlides.pdf). Salmon sperm DNA was spotted onto the slides for test printing. SpotQC kit (Integrated DNA Tech.) was used to hybridize the microarray slides to check the coating and printing quality (Figure A.5).

The random genomic library was printed on the slide with triplicate while controls are located on different parts of the chip. DNA fragments encoding PTA and 16S rRNA are used as positive control, while negative control is salmon sperm DNA which shows no homologies to the *C. tyrobutyricum*.

After spotting, UV cross-linking was used to immobilize the DNA fragments on the slide followed by post-processing which consists of re-hydration and blocking. Re-hydration allows the DNA to evenly distribute across the surface of the spot to eliminate the donut-shaped appearance of the spots and to increase the amount of total DNA bound.
Blocking is required to prevent the non-specific binding between the labeled sample and the exposed amine on the slide surface. Succinic anhydride reacts with amine and has the ability to cap the free amines.

Because all of the DNA fragments are PCR products amplified by M13 primers, the primers sequence exist in each spot with similar amount. The cy3 labeled M13 primer will be used to check the consistency of the slide as a quality control method after each batch of printing.

A.3 RNA extraction and cDNA preparation, dye labeling, and competitive hybridization

Standard procedures were followed to extract total RNA from *C. tyrobutyricum* using trizol reagent. Briefly, cells cultured under different conditions were harvested and the total RNA will be extracted using Trizol reagent (Invitrogen) following the manufacture’s standard protocol. The RNA concentration was be determined by spectrometry and the integrity was checked by agarose gel electrophoresis. Indirect labeling was employed by which aminoally-dUTP is firstly incorporated into the cDNA during reverse transcription, and then the dye is coupled to the function group amino-ally. The cDNA was synthesized using the tRNA samples from control and test sample (wild type and mutant) as the template. The reverse transcription was performed with reverse transcriptase (SuperScript III, Invitrogen), random hexamers and dNTP mixture containing 10% aa-dUTP. The cy3 and cy5 dyes was incubated with control and test
samples for labeling and the labeled cDNA will be pooled together for the following competitive hybridization.

Printed microarray slide was washed to remove unbound materials and the double-stranded DNA will be denatured by boiling. The microarray slide will be competitively hybridized in hybridization cassettes (Biorad) for 5 h at 42°C under a flat cover slips with 5.0 µl probe solution containing 5×SSC + 0.2% SDS + cye-labeled cDNA sample pool. Hybridized microarray will be washed twice for 5 min each in 2× SSC + 0.2% SDS at 25°C, once for 1 min in 2× SSC at 25°C and spun dry for 1 min at 500 ×g. The hybridization condition needs to be optimized to achieve satisfying results. The duplicated experiment will be performed.

The mRNA purification is an option to remove the ribosome RNA which counts for more than 90% of total RNA if the differentiation hybridization result is too weak to draw any conclusion.

A.4 Scanning and data analysis

Hybridized microarray will be scanned by a multi-wavelength laser microscope. The resulting fluorescence is measured at 570nm and 670 nm after excited at 550nm and 649nm for cy3 and cy5 respectively. The Cy3 and Cy5 signals will be quantified and the average of the measurements will be calculated. We will scatter plot the signal to examine the gene expression level. Most signals are supposed to be near the diagonal because of the constitutive expression. The gene fragments which have different transcript level will be spotted. The data from these spots will be clustered using the
standard correlation, and different clusters of co-regulated genes will be detected.

Furthermore, these gene fragments will be sequenced, and identified by comparison with the 454 database.

The whole process described above is illustrated by Figure A.6.
Figure A.1 White/blue screening for gene library construction
Figure A.2 Random quality check of plasmids.

Figure A.3 Random quality check of PCR product.

Figure A.4 Random quality check of purification.
Figure A.5 Photograph of printed microarray slide and the scanned results after hybridization with cy3-labeled oligomers.
Figure A.6 Scheme of shotgun DNA microarray construction. A. Construction of gene library; B. Construction of microarray gene chips; C. RNA labeling, chip hybridization, and data analysis. (adapted from Liu, 2005)
Appendix B Summary of CoA transferase and Genes involved in glycolysis.

The 454 sequencing produced 211,099 good reads, containing 55,970,852 bp of raw sequence. Assembly produced 69 contigs, 3,016,691 bp.

The genes encoding the enzymes involved in the metabolic pathway from pyruvate to various end products, including acidogenesis and solventogenesis according to ATCC 824, found and annotated in the 454 database are summarized in Table 5.2. Other genes responsible for enzymes in glycolysis are also summarized in Figure B.1 and Table B.1. Furthermore, two genes encoding for acetoacetyl-CoA: acetate/butyrate CoA transferase (EC# 2.8.3.9) are annotated in the database, one of them is located in the contig 64 starting at 129 bp and ending at 1682 bp and the other one is from 21339 bp to 22931 bp in the contig 22. The deduced amino acid sequences from these two genes show 58.4 % identity to each other. The one located in contig 64 shows 80% identity to the CoA transferase from Clostridium Novyi (Gi: 4541513) and the other one shows 76% to the same protein. The consensus against other bacterial CoA transferases is summarized in Table B.2.
Figure B.1 Genes annotated in the 454 sequencing database for the glycolysis pathway of C. tyrobutyricum.
Figure B.2 Alignment of two CoA transferases in *C. tyrobutyricum*
<table>
<thead>
<tr>
<th>Enzyme Name GLUCOSE/XYLOSE TO PYRUVATE</th>
<th>contig</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen Synthase</td>
<td>1</td>
<td>CDS 8</td>
</tr>
<tr>
<td>Triose Phosphate Isomerase</td>
<td>3</td>
<td>tpiA</td>
</tr>
<tr>
<td>Triose Phosphate Isomerase</td>
<td>13</td>
<td>tpiA</td>
</tr>
<tr>
<td>Glucose-6-Phosphate Isomerase (Phosphoglucose isomerase)</td>
<td>4</td>
<td>pgi</td>
</tr>
<tr>
<td>Fructose 1,6 bisphosphate aldolase class II</td>
<td>13</td>
<td>fba</td>
</tr>
<tr>
<td>fructose-bisphosphate aldolase</td>
<td>44</td>
<td>fbaA</td>
</tr>
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**Table B.1** Location of genes involved in glycolysis in the 454 sequencing database of *C. tyrobutyricum*. 
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**Table B.2** Comparison of amino acids sequences of two CoA transferases from *C. tyrobutyricum* against other bacteria.
Appendix C Chromatography methods and standard spectrum

C.1 High performance liquid chromatography

The high performance liquid chromatography (HPLC) system consisted of an automatic injector (Shimadzu SIL-10Ai), a pump (Shimadzu LC-10Ai), an organic acid analysis column (Bio-Rad HPX-87H), a column oven at 45°C (Shimadzu CTO-10A), and a reflective index detector (Shimadzu RID-10A). The eluent was 0.01 N H₂SO₄ at a flow rate of 0.6 ml/min. Samples, each with a volume of 15 µl, were injected by the autosampler. The running time for each sample was 25 min. Peak height was used to calculate the concentration of each component based on the analysis of the standard mixture containing all the compounds at 2 g/l. Samples of cell-free fermentation broth were diluted with distilled water at different ratios from 1/6 to 1/20 depending on the concentration of the compounds to be analyzed. A standard chromatogram is shown in Figure C.1.

C.2 Gas chromatography for gas production

An on-line gas chromatograph (Shimadzu GC 2014) was used for automatic measurement of gases (H₂ and CO₂) produced during the fermentation of C. tyrobutyricum. The fermenter was connected to GC and 100 µl of gas was collected and injected by an automatic six-head valve to analyze the composition of gas produced in the fermentation with a thermo conductivity detector and RT-QPLOT column (Restek).
Argon was used as the carrier gas at flow rate of 4.38 ml/min. The injector, column, and detector temperatures were maintained at 60°C, 30°C, and 60°C, respectively. A standard chromatogram is shown in Figure C.2.

**C.3 Gas chromatography for alcohol analysis**

The composition of alcohol produced in the fermentation was analyzed with a gas chromatograph (Shimadzu GC 2014) equipped with Flame Ionization Detector and Stabilwax-DA column (Restek) with helium as the carrier gas at the flow rate of 2 ml/min. 10 µl of sample in concentration range between 0.125 g/l to 2g/l was injected for the analysis. The injector temperature and the detector temperature were set to 200°C. The column temperature was programmed as the follows: 80°C hold for 3 min, and then increased to 150 °C at 30°C/min and hold at 150°C for 4 min. A standard chromatogram is shown in Figure C.3.
Figure C.1 HPLC chromatogram for standard sample containing glucose, xylose, lactose, acetic acid, lactic acid and butyric acid (2 g/l each).
Figure C.2 GC chromatogram for the standard sample containing hydrogen (80 %) and CO$_2$ (20 %).
Figure C.3 GC chromatogram for standard sample containing acetone, ethanol, butanol, acetic acid and butyric acid (0.5 g/l each).