METABOLIC ENGINEERING AND PROCESS DEVELOPMENT FOR ENHANCED PROPIONIC ACID PRODUCTION BY *PROPIONIBACTERIUM ACIDIPROPIONICI*

DISSERTATION

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ABSTRACT

Propionic acid is an important mold inhibitor. Its salt forms, such as ammonium, sodium, calcium, and potassium salts, are widely used as preservatives for animal feed and human foods. Moreover, propionic acid is an important chemical intermediate, widely used in the synthesis of cellulose fibers, herbicides, perfumes, and pharmaceuticals. Propionate esters are also used as flavors and fragrances. Currently, propionic acid is mainly produced via petrochemical processes. However, concerns over the sustainability of the crude oil supply have generated interest in investigating alternative way to produce propionic acid. Like most organic acid fermentations, the production of propionic acid suffers from low productivity, yield, and final concentration caused by end-product inhibition. The goal of this project was to develop an economical fermentation process for propionic acid production from glucose and processing wastes via integrated metabolic and process engineering approaches.

Propionibacterium acidipropionici has been extensively studied for propionic acid production, with acetic acid as the main byproduct. In order to eliminate or reduce acetate formation and increase propionic acid production, gene knock-out through homologous recombination was performed to inactivate acetate kinase gene (Ack) in the acetate formation pathway. Compared to the wild type strain, the mutant (ACK-Tet)
produced more propionate and less acetate, but the specific growth rate of the mutant was also decreased due to less ATP can be generated from the impaired acetic acid synthesis pathway. The mutant was used in a fibrous bed bioreactor (FBB), which has been shown to have many advantages in microbial fermentation, to further improve propionic acid production from glucose. The results showed that the maximum theoretical propionic acid yield of ~0.54 g/g glucose could be achieved. In fed-batch FBB fermentation, the final propionic acid concentration reached ~104 g/l, which was 43% higher than the highest concentration (~72 g/l) previously reported. Clearly, the bacteria in the FBB had adapted and acquired a higher tolerance to propionic acid. A growth kinetics experiment showed that the adapted mutant from the FBB was ~10 times less sensitive to propionic acid inhibition. The increased acid tolerance was partially attributed to increased expression of H⁺-ATPase, which plays a key role in proton pumping and maintaining the intracellular pH. Furthermore, after adaptation in the FBB, the ACK-Tet mutant recovered its specific growth rate to the same level as that of its parent wild-type strain.

Besides the final concentration of propionic acid in the fermentation broth, the P/A ratio (propionic acid vs. acetic acid) is another key factor affecting downstream purification and the overall production cost of propionic acid. Many prior studies have indicated that the oxidation level of the carbon source can determine the metabolic flux distribution in propionic acid fermentation. In general, the lower the oxidation level of the carbon source is, the higher the P/A ratio can be obtained due to the intracellular
NADH/NAD\(^+\) balance. Glycerol is thus an attractive substrate for the production of reductive chemicals (e.g., H\(_2\), ethanol, and propionic acid) because of its low oxidation state. There are also abundant supplies of low-cost glycerol as a waste product from the biodiesel industry. *P. acidipropionici* could use glycerol for growth and propionic acid production, with a high yield of 0.71 g/g glycerol, which was ~30% higher than that from glucose (0.55 g/g glucose). In addition, almost no acetic acid was produced from glycerol; the acetate yield was only 0.03 g/g glycerol (vs. 0.1 g/g glucose). Thus, glycerol fermentation produced a high-purity propionic acid with the propionic acid to acetic acid ratio of ~24 (vs. ~5 from glucose fermentation), facilitating the recovery and purification of propionic acid from the fermentation broth by simple solvent extraction. The highest propionic acid concentration obtained from glycerol fermentation was ~106 g/l, which was 2.5 times of the maximum concentration of ~42 g/l reported in the literature. Moreover, a stoichiometric metabolic model was set up based on the NADH/NAD\(^+\) balance and maximum ATP production. The trend predicted by the model fitted the experimental data very well.

The effects of CO\(_2\) (HCO\(_3^-\)) on cell growth and acids production from glycerol were studied. The productivity of propionic acid in glycerol fermentation with CO\(_2\) (HCO\(_3^-\)) reached 2.94 g/l/day, which was markedly higher than that without CO\(_2\) (HCO\(_3^-\)) (1.56 g/l/day). However, the propionic acid yield was decreased slightly from 0.77 to 0.67 g/g glycerol due to the higher biomass production when CO\(_2\) (HCO\(_3^-\)) was
supplemented in the media. Meanwhile, the yield and productivity of succinate increased 81% and 280%, respectively, suggesting a significant increase in the Wood-Werkman cycle rate that could be attributed to the increased activities of key enzymes (e.g. phosphoenolpyruvate carboxylase and propionyl CoA transferase) stimulated by CO2 (HCO3−).

Propionyl-CoA:succinate CoA transferase (CoA T, EC# 2.8.3.-) catalyzes the rate-limiting step in the propionic acid formation pathway. The genome of Propionibacterium acidipropionici ATCC 4875 was sequenced by 454 sequencing and annotated. The CoA transferase gene was identified in the 454 genome sequence database, and was obtained by PCR amplification and then inserted into an expression vector (pET-CoA). With IPTG induction, the CoA transferase gene was expressed in Escherichia coli BL21 (DE3) under both aerobic and anaerobic conditions. However, due to high sensitivity to oxygen, P. acidipropionici CoA transferase activity was only observed in the crude extract of BL21 (DE3) harboring pET-CoA grown anaerobically. In addition, a consensus Shine-Dalgarno sequence was found four bases upstream of the AUG codon and two inverted repeat regions were located at the downstream of the TGA stop codon. The amino acid alignment of the P. acidipropionici propionyl-CoA:succinate CoA transferase with other reported CoA transferases illustrated the presence of conserved sites in the amino acid sequence for CoA binding.
Dedicated to my parents
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CHAPTER 1
INTRODUCTION

Propionic acid is a clear, colorless liquid with a slightly sweet odor, and is soluble in water and alcohol. It has numerous applications in food and chemical industries. Propionic acid and its salts (ammonium, calcium and sodium propionates) are widely used as preservative in animal feed and dairy and bakery products. It is used in the chemical industry to produce pharmaceuticals, antifungal agents, agrochemicals, plastics, plasticizers, rubber chemicals, dyes, artificial flavors and perfumery synthetics. It is also used as a solvent and in nickel-electroplating solutions. About 23.5 % of the worldwide propionic acid is for animal feed; 33.8% for grain preservatives, and 21.8% for herbicides. Conventionally, propionic acid is synthesized from petroleum-based chemical process. The US market demand was ~370 million pounds in 2006, with an average annual growth rate of 2.5 percent to 2010. (www.icis.com).

Owing to the absence of toxicity and their valuable biotechnological properties, Propionibacteria have been granted the GRAS (generally recognized as safe) status by the United States Food and Drug Administration (Salminen et al., 1998). The probiotic potential of these bacteria is also attractive to the cheese industry. The demand for fermentation-produced propionic acid as a natural food preservative is high (Boyaval et
al., 1995; Huang et al., 2002; Yang et al., 1994). The future growth of human food preservatives, calcium and sodium salts, is projected to increase with population growth (www.chemicalmarketreporter.com). Meanwhile, there are abundant low-cost agricultural commodities and processing wastes available as renewable resources for fermentation process. For instance, the production of corn byproducts by the corn refinery industry is in the range of 10 million metric tons. The dairy industry generates another ~80 billion lbs of cheese whey annually. With the increased production of biodiesels, a large amount of glycerol is produced as a byproduct with limited use. These byproducts cause a significant environmental problem. It is thus desirable to use these wastes as renewable feedstock to produce industrial chemicals and fuels to replace fossil fuels and petrochemicals. Therefore, fermentation with Propionibacteria has a good potential for the production of natural propionic acid to satisfy the market demand. However, like most organic acid fermentations, conventional propionic acid fermentation processes suffer from low propionic acid yield, low final propionic acid concentration, and low reactor productivity caused by a strong inhibition of propionic acid. So, the current fermentation process is unable to compete with commercially used petrochemical routes. It is thus important to develop an effective fermentation technology for economical production of propionic acid.

Currently, there is a great interest in using biomass as the fermentation feedstock to produce propionic acid. New bioprocesses and mutant strains have been developed to improve propionic acid production in its yield, final product concentration, and productivity, but with limited success (Emde et al., 1990; Huang et al., 1998; Jin et al., 1998; Lewis et al., 1992b; Paik et al., 1994; Rickert et al., 1998; Solichien et al., 1995;
Woskow et al., 1991). One significant obstacle is the strong end-product inhibition caused by propionic acid even at a very low concentration of 10 g/L (Gu et al., 1998; Hsu et al., 1991). Higher product concentrations and propionic acid to acetic acid ratio (P/A ratio) in the fermentation broth are required to facilitate product separation and reduce the production cost (Van et al., 2003). However, higher propionic acid concentration and P/A ratio were usually obtained at the expense of lower productivity and specific growth rate, which makes the bioprocess of propionic acid production uncompetitive to current petrochemical method. A fibrous-bed bioreactor (FBB) has been developed to improve final product concentration, product yield, and volumetric productivity in several organic acid fermentations (Yang et al., 1994; Silva et al., 1995; Yang et al., 1995; Zhu et al., 2002; Zhu et al., 2003). Cells immobilized in the FBB experienced rapid adaptation that enabled them to tolerate higher concentrations of inhibitory fermentation products (Huang et al., 1998; Zhu et al., 2003). In this work, fed-batch fermentation using the FBB was performed to evaluate the potential to further enhance the final propionic acid concentration and yield, and to obtain adapted mutants with high tolerance to propionic acid.

The metabolic pathway shift and its effects on propionic and acetic acids production from different carbon sources can be predicted by using a stoichiometric metabolic model based on the assumptions of NADH/NAD$^+$ balance and maximum ATP production. Glycerol is an attractive feedstock for the production of reductive chemicals (e.g., H$_2$, ethanol, and propionic acid) because of its low oxidation state and low price (Barbirato et al., 1997; Himmi et al., 2000). $P$. acidipropionici can use glycerol for growth and propionic acid production, with a high yield of 0.71 g/g glycerol and high
propionic acid to acetic acid ratio of ~24 (vs. ~5 from glucose fermentation). The effects of CO$_2$(HCO$_3^-$) on cell growth and acids production from glycerol were also studied. The productivity of propionic acid with CO$_2$(HCO$_3^-$) in glycerol fermentation was increased under CO$_2$-enriched media. In addition, the yield and productivity of succinate were increased by 81% and 280%, respectively. The mechanism of CO$_2$ stimulation was investigated by activity assay of key enzymes (e.g., phosphoenolpyruvate carboxylase and propionyl CoA transferase) in the fermentation pathway.

Via genetic techniques, carbon source can be redistributed. Certain undesired genes can be knocked out in order to shift the metabolic pathway towards the desirable products. In addition, some key enzyme genes can be overexpressed to increase the synthesis rate. In this work, a rate limiting step enzyme, propionyl-CoA:succinate CoA transferase (CoA T, EC# 2.8.3.- ), was identified in the genome sequence database of *P. acidipropionici*. This gene was overexpressed in *Escherichia coli* BL21 (DE3) under both aerobic and anaerobic conditions. It was found that only under anaerobic fermentation conditions, CoA transferase activity was detectable in crude extracts of BL21 (DE3) harboring pET-CoA. In addition, the CoA transferase gene sequence and its amino acid sequence were studied by comparison with other reported CoA transferases. The results can then be used in genetic and metabolic engineering to create new mutants for industrial applications.
Objectives and Research Tasks

The goal of this project was to develop an economic fermentation process for propionic acid production from sugars and glycerol present in processing wastes. To achieve this goal, both metabolic engineering and conventional fermentation process engineering approaches were used in an integrated manner. Figure 1.1 provides an overview of the research objectives, approaches, and the scope of this study. The main experimental studies and tasks carried out in this work are presented in Chapters 3–6 and briefly described below.

Task 1. Engineering *Propionibacterium acidipropionici* for enhanced propionic acid production from glucose

Inactivation or over-expression of target genes is a powerful tool in metabolic engineering that has been widely used to redistribute the metabolic flux and enhance the production of desired metabolite in the fermentation pathway. One *Propionibacterium acidipropionici* mutant (ACK-Tet) was constructed by inactivating *ack* genes to eliminate or reduce acetate formation. However, the increase in the production of propionic acid was at the expense of reduced specific growth rate. The fibrous bed bioreactor (FBB) has previously been shown to have many advantages in microbial fermentation. Thus, the FBB and metabolically engineered mutant were used in the fermentation to further improve propionic acid production. The propionic acid yield from glucose in the FBB fermentation reached the maximum theoretical level of 0.55 g/g glucose. An adapted
ACK-Tet mutant was obtained from the FBB in long-term fed-batch fermentation. The adapted mutant showed significant changes in morphology and H^+-ATPase activity, which might have contributed to the higher propionic acid tolerance of the mutant.

**Task 2. Propionic acid production from glycerol by metabolically engineered**

*Propionibacterium acidipropionici*

Glycerol is the main byproduct of biodiesel fuel production. The purity of crude glycerol is around 80%. Owing to its low oxidation state and its low price (5-15 cents/lb), glycerol currently is an attractive feedstock for the production of reductive chemicals (e.g., H_2, ethanol, and propionic acid). It is thus desirable to use the waste glycerol as a renewable feedstock to produce industrial biochemicals and biofuels. Glycerol was demonstrated to be consumed by *P. acidipropionici* and converted to propionic acid with a high yield. The fermentation profiles with different carbon sources were compared and the metabolic pathway shift was confirmed. Although it was well known that the oxidation level of substrate could shift the pathway, no systematic stoichiometric metabolic analysis has been reported. Based on the NADH/NAD^+ balance and maximum ATP production, a stoichiometric metabolic model was established and used to predict fermentation performance with various substrates, which would allow us to explore more effective strategies to further enhance the utility of this organism in the manufacturing process.
Task 3. Effects of carbon dioxide on cell growth and propionic acid production from glycerol by *Propionibacterium acidipropionici*

The effects of CO$_2$ on cell growth and metabolism have been extensively studied. It was demonstrated that exogenous CO$_2$ stimulated the growth of *Actinomyces viscosus* (Howell et al., 1963 a, b) and succinate production (Brown et al., 1980). Dharmadi et al. (2006) reported that CO$_2$ had a positive impact on the anaerobic growth of *Escherichia coli* and the biosynthesis of small molecules, fatty acids, and central metabolism from glycerol under acidic conditions. On the other hand, inhibition by CO$_2$ has also been shown in cell growth and fermentation of yeast (Jones et al., 1982) and *Penicillium chrysogenum* (Ho et al., 1986). In contrast to the numerous studies of CO$_2$ effects shown above, the effects of CO$_2$ on *Propionibacteria* growth and end product formation have been minimally investigated. In this work, cell growth, carboxylic acid formation, and enzyme activities were studied in glucose and glycerol fermentations under CO$_2$-enriched and CO$_2$-limited conditions. The effect of CO$_2$ on metabolic pathway of *Wood-Werkman cycle* is also discussed.

Task 4. Molecular characterization of coenzyme A transferase gene from *Propionibacterium acidipropionici* and its expression in *E. coli*

Gene modification has been widely used in strain optimization and fundamental understanding of cell metabolism. Whole genome sequence of *P. acidipropionici* was obtained by 454 sequencing and the DNA sequence for the gene encoding propionyl-
CoA:succinate CoA transferase, which catalyzes the rate-limiting step in propionic acid synthesis was amplified by PCR. This gene was then inserted into the downstream of T7 promoter in a vector and expressed by IPTG induction in *E. coli*. The enzyme activities under aerobic and anaerobic growth conditions were also determined.
References

Barbirato F, Chedaille D, Bories A, 1997, Propionic acid fermentation from glycerol: comparison with conventional substrate, Appl Microbiol Biotechnol, 47: 441-446


Howell A., Jordan H.V., 1963b, A filamentous microorganism isolated from periodontal plaque in hamsters. II. Physiological and biochemical characteristics, Sabouraudia 3: 93-105


# Goal

To develop an economic fermentation process for propionic acid production from sugars and glycerol present in processing wastes

Propionibacterium acidipropionici

Modified strain ACK-Tet

Wild type ATCC 4875

## Process Development

- Medium optimization;
- Fermentation condition optimization;
- FBB fermentation;
- Batch fermentation;
- Fed-batch fermentation

## Metabolic Engineering

- Metabolic pathway analysis;
- Stoichiometric model of metabolic pathway;
- Genomics and Gene manipulation

<table>
<thead>
<tr>
<th>Ch. 3 - Propionate production from glucose and lactose</th>
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<tr>
<td>Ch. 4 - Propionate production from glycerol; Analysis of metabolic pathway shift due to oxidation level of substrate and modeling</td>
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<td>Ch. 5 - CO₂ effects on fermentation kinetics and enzyme activity</td>
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<tr>
<td>Ch. 6 - CoA transferase gene identification and expression in E. coli; Nucleotide and amino acid sequences analysis</td>
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**Figure 1.1** Research objectives, approaches, and the scope of this study.
CHAPTER 2

LITERATURE REVIEW

2.1 Propionic Acid Fermentation

2.1.1 Microorganisms

Propionibacteria are Gram-positive, catalase-positive, non-spore forming, non-motile, facultative anaerobic, rod-shaped bacteria. The genus of Propionibacterium is divided into two groups: the “cutaneous” and the “dairy” Propionibacteria, based on their habitat (Cummins et al., 1986). They are the sort of Actinobacteria with high GC content. The range of the percentage of G+C in their chromosome DNA is 53-68% (Meurice et al., 2004). The entire genome sequence of Propionibacterium acnes, one member of “cutaneous” Propionibacteria group, has been reported, facilitating the genetic studies of P. acnes associated diseases (Holger et al., 2004). “Dairy” Propionibacteria, commonly found in milk and cheese, are widely used in the production of Swiss cheese (Langsrud et al., 1973; Hettinga et al., 1972a), vitamin B12 (Gardner, 2005; Yongsmith, 1983) and propionic acid (Ozadali et al., 1996; Coronado et al., 2001; Jin et al., 1998; Lewis et al. 1992a;b; Yang et al., 1995). Owing to the absence of toxicity
and their valuable biotechnological properties, *Propionibacteria* have been granted GRAS (generally recognized as safe) status by the United States Food and Drug Administration. As probiotic bacteria, *Propionibacteria* currently attract more and more interests from the cheese industry (Jan et al., 2001a; b; Mantere-Alhonen, 1995; Leverrier et al., 2003). In addition to vitamin B12, the growth of these bacteria can also produce organic acids (e.g., propionic acid) to reduce amounts of detrimental and pathogenic bacteria (e.g., *Clostridia*, *Salmonella enteritidis*) (Fooks et al., 2002; Surawicz, 2003; Hopkins et al., 2003), to stimulate the population of *Bifidobacteria* (Rycroft, 2001), which are beneficial bacteria in intestinal tract, and to balance the intestinal ecosystem (Perez et al., 1999). In the Swiss-cheese production, the main end-products (propionic acid, acetic acid, and CO₂) fermented from lactic acid by dairy *Propionibacteria* contribute to the nutty and sweet flavour of the cheese (Hettinga et al., 1972b) and form the “eyes” of the cheese (Langsrud et al., 1973). Besides the conventional process of the vitamin B12 production by *Propionibacteria* fermentation under anaerobic environment (Hatanaka et al., 1988; Playne, 1985; Yongsmith, 1983), Ye et al. (1999) reported that *Propionibacterium freudenreichii* IFO 12424 growing with oxygen can be applied to produce vitamin B12 with periodic fermentation. Another important use of *Propionibacteria* is their ability to synthesize propionic acid. *P. acidipropionici* (Goswami et al., 2001; Hsu et al., 1991; Jin et al, 1998; Lewis et al., 1992a; b; c; Yang et al., 1994; Yang et al., 1995; Suwannakham et al., 2005), *P. shermanii* (Nanba et al., 1983; Elizabeth et al. 1987), *P. freudenreichii* (Balamurugan et al., 1999), and *P. freudenreichii* subsp. *Shermanii* (Emde et al., 1990; Himmi et al., 2000; Border et al., 1987) are widely used in propionic acid production. In addition to these “dairy” *Propionibacteria,*
Propionispora hippie sp. nov, a novel gram-negative, spore-forming anaerobe (Abou-Zeid et al., 2004) and Selenomonas ruminantium (Eaton et al., 1995) have also been applied for natural propionic acid production.

Since Propionibacteria have such wide applications in industry, the nutrient requirement and growth conditions for them have been extensively studied (Plyane, 1985). The main nitrogen source for Propionibacteria growth comes from yeast extract and trypticase. And a wide range of carbon sources, including glucose (Suwannakham et al., 2005), lactose (Goswami et al., 2001; Lewis et al., 1992a; Yang et al., 1995), lactate (Emde et al., 1990), glycerol (Barbirato et al., 1997; Himmi et al., 2000), and sorbitol (Suwannakham, 2005) can be consumed by Propionibacteria for cell growth and product synthesis. The optimal temperature for cell growth ranges from 30 to 32°C. Propionibacteria grow most rapidly at the pH range of 6.0 to 7.0 (Hsu et al., 1991). Practically, no obvious cell growth could be observed at pH below 4.5 (Plyane et al., 1985; Hsu et al., 1991). An anaerobic environment for propionibacteria culture can be obtained by purging nitrogen gas in the headspace of fermentors.

2.1.2 Propionic acid

Propionic acid, with the chemical formula CH₃CH₂COOH and systematically named propanoic acid (other name: ethanecarboxylic acid), is a clear, colorless liquid with a slightly sweet odor, and is soluble in water and alcohol. Like other weak organic acids, propionic acid is able to inhibit cell growth. The undissociated propionic acid can diffuse through cell membrane into cytoplasm due to the same hydrophobic nature of the
acid and cell membrane, and then release $H^+$ inside cells because of the alkaline environment (Gu et al. 1998). Therefore, the pH gradient across the cell membrane is disturbed and then the transport of nutrients and metabolites is limited (Gu et al. 1998).

Propionic acid has numerous applications in various food and chemical industries: (I) It can be used as a preservative for cheeses and baked goods directly or in salt forms (ammonium propionate, calcium and sodium propionate); (II) It is used to manufacture various propionates which are used in the production of pharmaceuticals, antifungal agents, agrochemicals, plastics, plasticizers, rubber chemicals, dyes, artificial flavors and perfumery synthetics; (III) It is used as a solvent and in nickel-electroplating solutions.

The worldwide market demand was ~770 million pounds in 2006, and the average annual growth is expected to be 2.5 percent to 2010. The price was $0.49 per pound (1997-2002), and increased up to $0.58-0.63 per pound in 2008 (www.icis.com). The globle utilization patterns are that about 33.8% is used in grain preservatives, 23.5% in animal feed preservatives, 21.8% in herbicides, 15.3% in cellulose acetate propionate, 1.6% in fungicides, 1.5% in flavor & fragrances, 0.6% in pharmaceuticals, and 0.3% in packaging (www.icis.com). Propionic acid and its salts (calcium, sodium, and potassium) have been listed in the summary of GRAS (generally recognized as safe) additives by the Food and Drug Administration (FDA) (Boyaval et al. 1995). The demand for fermentation-produced propionic acid as a natural food preservative is high (Boyaval et al. 1995; Huang et al., 2002; Yang et al., 1994). The future growth of human food preservatives is projected to increase with population growth.

Currently, propionic acid is mainly produced via petrochemical processes. The main producers are Dow Chemical, Eastman Chemical, Eastman Chemical, and Celanese
Generally, there are three chemical processes for propionic acid production (Boyaval et al. 1995). In industry, propionic acid is usually synthesized from the oxidation of propionaldehyde ($\text{CH}_3\text{CH}_2\text{CHO} + \frac{1}{2} \text{O}_2 \rightarrow \text{CH}_3\text{CH}_2\text{COOH}$) with cobalt or manganese ions as the catalyst. The reaction occurs at 40-50°C. Moreover, Reppe process and Larson process are also common processes which involve the reaction of ethylene, carbon monoxide, steam and the reaction of ethanol, carbon monoxide, respectively (Boyaval et al. 1995). Currently, industry has great interest in using biomass as the fermentation feedstock to produce propionic acid to replace petrochemical processes because of the high cost of crude oils. There are abundant low-cost agricultural commodities and processing wastes available as renewable resources. For instance, the production of corn byproducts by the corn refinery industry is in the range of 10 million metric tons, and the dairy industry generates another ~80 billion lbs of cheese whey annually. While these byproducts often pose significant environmental problems, a promising solution is to use these renewable resources to produce biochemicals and biofuels by fermentation to replace fossil fuels and petrochemicals. Therefore, fermentation by *Propionibacteria* has a good potential for the production of natural propionic acid to satisfy market demand. However, current propionic acid fermentation processes suffer from low propionic acid yield, low final concentration, and low reactor productivity caused by a strong inhibition of propionic acid. Thus, it is important to analyze cell metabolism and response mechanisms in high propionic acid concentration environment using genomics and proteomics technologies. With the knowledge in functional genomics, propionic acid-tolerant strains could be
developed to enhance production rate and product yield via genetic and metabolic engineering techniques.

2.1.3 Fermentation processes

2.1.3.1 State of the art

The dicarboxylic acid pathway is the main pathway related to the propionic acid fermentation with Propionibacteria (Playne et al., 1985; Boyaval et al., 1995). Propionic acid is the main product in this pathway with acetic acid, succinic acid, and CO₂ as byproducts. Fitz (1878) was the first to determine the stoichiometric equation of propionic acid formation by Propionibacteria, which is known as Fitz equation (Leaver et al., 1955; Boyaval et al., 1995):

\[
3 \text{lactic acid} \rightarrow 2 \text{propionic acid} + 1 \text{acetic acid} + 1 \text{CO}_2 + 1 \text{H}_2\text{O}
\]

\[
1.5 \text{glucose} \rightarrow 2 \text{propionic acid} + 1 \text{acetic acid} + 1 \text{CO}_2 + 1 \text{H}_2\text{O}
\]

According to these equations, the theoretical maximum yield from glucose is 54.8% (g/g) of propionic acid, 22% (g/g) of acetic acid, 17% (g/g) of CO₂, and 77% (g/g) as total acid. The propionic acid to acetic acid ratio is 2:1 (mol/mol) theoretically. The experimental yield of propionic acid, however, is much less than 50% (g/g) because of substrate consumption for cell growth.

Although most of carboxylic acids (e.g. butyric acid, propionic acid) can be produced by microorganisms from renewable resources, the fermentation routes usually cannot compete with petroleum-based chemical synthesis. Severe end-product inhibition
causes the low final product concentration, which increases the downstream purification cost owing to the small difference of volatility between acid and water, and low ratio of propionic acid to acetic acid.

Nevertheless, several attempts have been made to increase the production of propionic acid through several newly improved fermentation processes.

2.1.3.2 Improved fermentation processes with inexpensive media

The cost of media for propionic acid fermentation could significantly affect the end-product price. Although several carbon sources (e.g. sorbitol) was proved to benefit propionic acid production with higher propionic acid productivity, higher propionic acid yield, lower acetic acid yield, and higher P/A ratio (Suwannakham, 2005), the process is not feasible in the pilot level because the price of sorbitol is higher than propionic acid. Therefore, the optimization of media is a routine method to improve the fermentation process and make it economically competitive to chemical synthesis. As reported, Propionibacteria have a broad range of carbon sources, such as glucose (Suwannakham et al., 2005), lactose (Goswami et al., 2001; Lewis et al., 1992a; Yang et al., 1995), lactate (Emde et al., 1990), and glycerol (Barbirato et al., 1997; Himmi et al., 2000). Currently, several low-cost substrates as industrial by-products are available and can be applied in propionic acid fermentation.

Cheese whey is the main by-product of cheese manufacturing. The U.S. manufactured over 700,000 tons of whey per year according to the annual report of USDA (1998). Its main useful components are whey protein and whey lactose (98% of
total solid whey). Only half of the whey is used for whey derivatives; sweet whey powder, demineralized whey, delactosed whey, whey protein concentrates (WPCs), whey protein isolates (WPIs), and lactose. The rest of the whey and whey permeat, a byproduct of whey protein and whey lactose recovery process disposed into environment post serious pollution problems because of its high biological oxygen demand (BOD) (Yang et al., 1995). A promising solution is to use the whey as whey based media to produce value-added biochemicals through fermentation processes. High fermentation rate and conversions were obtained with the whey media in *Propionibacteria* fermentation. The highest propionic acid concentration reached 65 g/l in a recycle batch reactor (Yang et al., 1995; Boyaval et al., 1987).

Corn fiber and corn steep liquor (CSL), another two low-cost waste materials from the corn wet milling process, contain a relative low concentration of carbon sources as compared with cheese whey, which limits their application in fermentation processes. The accumulated corn fiber and corn steep liquor could cause an environment problem. It was found that corn fiber and CSL could be consumed by *Propionibacteria* to produce propionic acid (Paik et al., 1994).

To date, raw glycerol becomes a more attractive substrate in fermentation process not only because of its low price, but also because of its higher reduction level compared to glucose, xylose, etc. With the development of biodiesel production in current years, a large amount of crude glycerol generated in that process must find new applications. Using glycerol as an alternative carbon source is thus a desired choice. In the early 1950’s, glycerol was considered as a carbon source to produce propionic acid by *Propionibacteria* (Leaver et al., 1955; Stjernholm et al., 1960a). However, the relative
high price of glycerol limited the further development of glycerol-based propionic acid production until recent years when glycerol has become the main byproduct of biodiesel production process. In addition to the low-price of raw glycerol, the higher reduction level of glycerol obtains more attention from industries mainly producing some reductive biochemicals, such as H₂, ethanol, succinic acid, and propionic acid (Table 2.1). Glycerol as the sole carbon source in the fermentation process is capable of significantly increasing these reductive chemical yields by maintaining the redox balance in cells (Dharmadi et al., 2006; Menzel et al., 1997; Wang et al., 2003). In propionic acid fermentation with glycerol as the main substrate, the propionic acid concentration in the fermentation broth reached as high as 42 g/l and the P/A ratio was 37 (mol/mol) (Barbirato et al., 1997). The final acetic acid concentration was reduced two-fold in media containing 20 g/l of glycerol as the substrate (Himmi et al., 2000).

The broad utilization of these economic feedstocks in propionic acid fermentation processes significantly reduced the production cost of propionic acid, making it promising as the natural propionic acid generated from fermentation becomes price-comparable with that via chemical synthesis.
<table>
<thead>
<tr>
<th>Microbial Species</th>
<th>Products</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>1,3-propanediol</td>
<td>Boenigk et al., 1993</td>
</tr>
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<td><em>Klebsiella pneumoniae</em></td>
<td>1,3-propanediol</td>
<td>Menzel et al., 1997</td>
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<td>Biebl, 2001</td>
</tr>
<tr>
<td><em>Klebsiella planticola</em></td>
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<td>Jarvis et al., 1997</td>
</tr>
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<td><em>Klebsiella planticola</em></td>
<td>Formate</td>
<td>Jarvis et al., 1997</td>
</tr>
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<td>Propionic acid</td>
<td>Himmi et al., 2000</td>
</tr>
<tr>
<td><em>Propionibacterium freudenreichii ssp. Shermanii</em></td>
<td>Propionic acid</td>
<td>Himmi et al., 2000</td>
</tr>
<tr>
<td><em>Anaerobiospirillum succiniciproducens</em></td>
<td>Succinic acid</td>
<td>Lee et al., 2000</td>
</tr>
<tr>
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<td>Ethanol</td>
<td>Dharmadi Y. et al., 2006</td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>Succinic acid</td>
<td>Dharmadi Y. et al., 2006</td>
</tr>
</tbody>
</table>

Table 2.1 Microbial conversion of glycerol to biochemicals and biofuels

2.1.3.3 Process improvement in propionic acid yield

According to the Fitz equation (Fitz, 1878; Leaver et al., 1955; Boyaval et al., 1995), the theoretical yield of propionic acid on glucose via the EMP pathway of glycolysis is 0.548 (g/g) and the propionic acid to acetic acid ratio is 2:1 (mol/mol) without considering the consumption of carbon source for cell biomass. However, the
actual yield of propionic acid is less than 0.5 g/g since cell growth and production of some other acids (e.g. succinic acid and lactic acid) are inevitable (Suwannakham et al., 2005; Lewis et al., 1992c). The common range of the propionate yield is from 0.19 g/g to 0.40 g/g (Clausen et al., 1984; Obaya et al., 1994; Goswami et al., 2001). In order to increase the propionic acid yield in fermentation, the carbon source flux was redistributed in cell metabolic pathway by several fermentation process strategies. Starving bacteria by a proper feeding strategy without affecting cell activity is an effective way to limit cell growth and direct more carbon source to produce valuable products. The propionic acid yield obtained in continuous, immobilized cell bioreactor was ~46% with a minimal level of nutrients feeding (Yang et al., 1994). The application of carbon source with higher reductive level can also shift the metabolic pathway towards the production of propionic acid due to the redox balance. The yield of propionic acid was up to 0.79 mol/mol (0.64 g/g) with glycerol as the carbon source (Himmi et al., 2000). Acetic acid was almost eliminated when a high H2 pressure was applied during the fermentation of *Propionispira arboris* containing hydrogenase (Thompson et al., 1984). Emde and Schink (1990) reported that the maximal propionic acid yields of 0.973 and 0.900 g/g so far were obtained with the application of a three-electrode amperometric culture system in the fermentation of *P. freudenreichii* subsp. *freudenreichii*. Moreover, it was found that the propionic acid yield increased to ~ 0.63 g/g when the pH decreased from pH 6.1-7.1 to pH 4.5-5.0 (Hus et al., 1991). However, this increase was at the expense of lower productivity and more requirements for growth nutrients.
2.1.3.4 Process improvement in propionic acid productivity

The fermentation productivity depends on the cell growth rate and active cell density in fermentors. In conventional batch fermentation, the length of lag phase of cell growth directly affects the productivity. In propionic acid fermentation, end-product inhibition is another important factor that limits the productivity. High cell density fermentation is a common approach to increase the productivity. Higher concentrations of active cells in the fermentor can significantly reduce or even eliminate the lag phase of cell growth and reduce the sensitivity of Propionibacteria to propionic (Boyaval et al., 1987; Suwannakham et al., 2005). Cell recycle/cell retention systems are common methods for achieving high cell density in continuous fermentations. It was reported that the volumetric productivity of propionic acid reached 0.96 g/l/h with the cell density of $9.8 \times 10^9$ cells/g (wet weight) of beads in an immobilized cell fermentation (Paik et al., 1994). Continuous fermentation in an in situ cell retention bioreactor using spin filters obtained a propionic acid productivity of 0.9 g/l/h, about five times of that in conventional batch fermentation (Goswami et al., 2001). A continuous stirred tank reactor was conducted with an ultrafiltration unit to separate and recycle cells from the fermentation broth back to the fermentor. A high productivity of 14.3 g/l/h was attained in this system and the biomass reached 100 g/l (dry weight) (Boyaval et al., 1987). Furthermore, extractive fermentation, an integration of fermentation and separation processes, was used to eliminate end-product inhibition by removing propionic acid continuously from the media and improved the production of propionic acid significantly (Jin et al., 1998; Ozadali et al., 1996). A much higher propionic acid productivity of 1
g/l/h was observed, which was five times greater than the traditional batch fermentation (Jin et al., 1998).

2.1.3.5 Process improvement in final propionic acid concentration

Like most organic chemical fermentations, the strong end-product inhibition is the main obstacle in the propionic acid production. Blanc and Goma (1987) reported that the specific growth rate decreased 50% with 1% propionic acid in the medium. The strong inhibition causes low productivity (<1 g/l/h), low product yield (<50% g/g), and low final product concentration (<40 g/l) (Jin et al., 1998). An extractive fermentation process obtained 75 g/l final product concentration by removing propionic acid with an amine extractant and a hollow-fiber membrane extractor to maintain propionic acid at a low level in the fermentation broth (Jin et al., 1998). An adapted mutant of _Propionibactium acidipropionici_ with higher tolerance to propionic acid was obtained from a fibrous-bed bioreactor after several-month culturing. The final concentration was ~70 g/l (Suwannakham et al., 2005). Another propionic acid-tolerant strain of _Propionibactium acidipropionici_ was used in semicontinuous and immobilized cell fermentations, and the highest propionic acid concentrations produced in these processes were 47 g/l and 45.6 g/l, respectively (Woskow et al., 1991; Paik et al., 1994).
2.1.3.6 Process optimization by mathematical models

According to the achievements and promising future in biochemical engineering presented by Bailey (1998), mathematical modeling would be a very powerful tool in fermentation process optimization. The main functions of mathematical models are to organize experimental data, to discover new strategies to control and design processes, to understand complex systems in a rational way, to correct the conventional process, and to understand essential qualitative features of biological systems clearly described in prior research (Coronado et al., 2001, Bailey, 1998).

Several mathematical models have been set up to analyze the propionic acid production process in immobilized-cell fermentation (Crespo et al., 1990; Coronado et al., 2001). The main goal of these models was to study the effects of mass-transfer resistance of substrates and products on substrate consumption, cell growth and product formation ratew in immobilized cell reactors (ICRs).

2.2 Immobilized Cell Fermentation

2.2.1 Immobilized cell physiology

Immobilized cell fermentation, possessing distinct advantages with respect to obtaining high cell density, high tolerance to end product, and simplifying down-stream processing, has been extensively studied in industry. Generally, cells are immobilized mainly through entrapment, physical adsorption, and porous support colonization (Chun
et al., 1991; Hilge-Rotmann et al., 1991; Junter et al., 2002). With these technological properties, immobilized cell technology is capable of significantly improving the reactor volumetric productivity and final product concentration, avoiding cell wash-out in continuous fermentation, and protecting cells from strong shear stress. As reported by Paik and Galtz (1994), a propionic acid volumetric productivity of 0.96 g/l/h was obtained in continuous immobilized cell fermentation with a cell density of $9.8 \times 10^9$ cells/g (wet weight) of calcium alginate beads. Moreover, a high propionic acid concentration of 72 g/l was reached in a fibrous-bed bioreactor, immobilized cell fermentation, which was then the highest propionic acid concentration reported (Suwannakham et al., 2005).

However, although immobilized-cell technology has been broadly used in biochemical engineering in the past twenty years, no systematic studies have been conducted to investigate the important effects of immobilization on cell physiology. It is well known that responses of cells to immobilization are in a global range. 1) Morphological changes: *Lactobacillaceae plantarum* immobilized on the chitosan treated polypropylene (PP) matrix changed from normal rod shape to coccoid shape (Krishnan et al., 2001). 2) Growth rate changes: The effects of immobilization on cell growth are not consistent. In an immobilization system, some cells grow faster than others due to mass transfer limitation (Kiy et al., 1993), some remain the same growth rate, and some cell growth rates are enhanced owing to the protective effects of the support (Chun et al., 1991). 3) Enzyme expression changes: Immobilized cells often grow in an extreme environment (e.g., extreme pH or temperature), which induces existing protein over-expression or totally new protein synthesis as compared to suspended free cell
fermentations. For instance, H⁺-ATPase, a proton-pumping ATPase in cell membrane, was over-expressed when cells cultured in an immobilization fermentation system at low pH (O'Sullivan et al., 1999; Cotter et al., 2003). 4) Resistance to end product: The cells growing in immobilization system may more easily obtain higher tolerance to end products by adjusting the composition of cell wall and the plasma membrane. It was reported that the fatty acid composition of cell membrane shifted from unsaturated fatty acids to long-chain saturated fatty acids in cells isolated from an immobilized cell fermentation process (Suwannakham et al., 2005; Cotter et al., 2003; Hilge-Rotmann et al., 1991).

### 2.2.2 Immobilized cell bioreactor

Conventional immobilized cell bioreactors are of various types, including packed bed, hollow-fiber membrane, and fluidized bed, all of which provide a large amount of surface area for cell attachment or pores for cell entrapment. The immobilized, concentrated cells in bioreactors work as immobilized biocatalysts that consume substrates and excrete products into media with higher productivity compared with free-cell fermentation. However, these immobilization modes are subject to the loss of volumetric productivity in the long-term run owing to the loss of cell viability and mass transfer limitation of nutrients and products. Moreover, another common problem encountered in these conventional immobilization systems is clogging by cell biomass, which causes a high pressure drop through the reactor.
Yang et al. have developed a fibrous-bed bioreactor (FBB) (US patent no. 5,563,069) for various bioprocessing and biotechnology applications (Huang et al., 2002; Huang et al., 1998; Albayrak et al., 2002a; b;c). The FBB system contains a structured fibrous bed packed in a loose spiral form with sufficient space between layers. This structure facilitates multiphase flows through the reactor with a low pressure drop even at a high flow rate. In the FBB system, the dead or non-active cells could be sloughed off and replaced by new and active cells continuously to maintain the productivity at a high level. No contamination problem was encountered in long-term operation of the FBB due to high cell density (Yang et al., 1994). The design and construction of the fibrous-bed bioreactor are shown in Appendix C.2.

2.2.3 Immobilized cell fermentation

With the advantages of improving volumetric productivity, final product concentration, and resistance to inhibitors, immobilized cell technology has been extensively used in propionic acid fermentation to overcome the problems encountered in conventional free-cell fermentations, such as low specific growth rate, low productivity, and low P/A ratio.

Continuous production of propionic acid from whey permeate in the FBB system was studied by Yang et al. (1994). With the high cell density of ~50g/l, the yield of ~46% (w/w) and the productivity ranged from 0.35 to 0.78 g/l/h from lactose were obtained, which was over 10 times greater in productivity as compared to conventional batch fermentors (~0.045g/l/h) (Yang et al. 1994). The integration of immobilized cell
fermentation and extraction process was applied to eliminate the inhibition effect by continuously removing propionic acid from the fermentation broth. Whey lactose was used as the substrate for immobilized Propionibacterium acidipropionici to attain over 100% increase in the reactor productivity (Lewis et al., 1992a). In a recycle batch immobilized cell bioreactor, the highest propionic acid concentration of 65 g/l was attained from whey lactose without nutrient supplementation (Yang et al., 1995). In summary, various promising immobilized cell systems have been widely studied in various areas because of their practical features such as easy handiness, possible re-using, easier continuous operation and product recovery (Junter et al., 2002).

2.3 Metabolic Engineering of P. acidipropionici

2.3.1 State of the art

Metabolic engineering is defined as the practice of purposefully optimizing genetic and regulatory processes within cells, using genetic engineering techniques (recombinant DNA technique) to better understand metabolic mechanism or to increase the production from a certain substance. During the last thirty years, with the rapid progress in genetic techniques, screening techniques (e.g., microarray), and image analysis, metabolic engineering has been broadly used in many aspects of environmental, agricultural, and pharmaceutical industries. The common applications of metabolic engineering are: 1) to produce novel metabolites by introducing new pathways into microorganisms; e.g., production of recombinant human erythropoietin by Chinese
hamster ovary cells (Meyer et al., 1989), and isobutanol production in E. coli (Atsumi et al., 2008); 2) to improve existing processes by inactivating competing pathways of the desired product; e.g., succinate production by E. coli (Lin et al., 2005); 3) to improve existing processes by overexpressing key genes; e.g., enhanced solvent production in Clostridium acetobutylicum (Tomas et al., 2003). The techniques used in metabolic engineering include metabolic flux analysis (MFA), metabolic control analysis, physiological studies, thermodynamic analysis of pathways, and kinetic modeling (Nielsen, 1998). Although significant improvements have been obtained in metabolic engineering, the application of this approach is still limited in several organisms (e.g., E. coli, yeast, hybridoma cells) due to the lack of detailed information on the genetics.

2.3.2 Metabolic flux analysis of Propionibacteria

Metabolic flux analysis (MFA) is the most fundamental and crucial approach in metabolic engineering. In MFA, a metabolic network is established, containing the most important metabolites and reactions without considering the kinetic parameters of enzymes. The intracellular flux distribution can be calculated and analyzed by MFA.

The metabolism of glucose in Propionibacteria was studied by Stjernholm and Wood (1960b). Trehalose and fructose formed by P. arabinosum and P. shermanii were identified as the indicators in propionic acid fermentation on C\textsuperscript{14}-labeled glucose to determine the C\textsuperscript{14} distribution pattern. From the distribution pattern, an Embden-Meyerhof type of cleavage was proven to exist in propionic acid fermentation (Stjernholm et al., 1960b). Propionic acid and acetic acid were used as indicators to
investigate the metabolism of D-ribose-1-C\textsuperscript{14} and C\textsuperscript{14}-labeled potassium D-gluconate in an enzyme system of the genus \textit{Propionibacteria} (Stjernholm et al., 1962). Propionic acid and acetic acid were isolated and degraded to determine the C\textsuperscript{14} distribution. The substrates (D-ribose and potassium D-gluconate) might be metabolized in the Embden-Meyerhof pathway and the pentose cycle to trioses, which were converted to pyruvate. Pyruvate was then metabolized to acetate by decarboxylation, or to propionate via oxaloacetate and succinate (Stjernholm et al., 1962). In addition, the metabolism of three carbon substrates was studied (Leaver et al., 1955; Stjernholm et al., 1960a). Glycerol-1-C\textsuperscript{14} and glycerol-3-C\textsuperscript{14} were used to prove the existence of a symmetrical three-carbon compound (Stjernholm et al., 1960a). It was reported by Leaver et al. (1955) that succinate might be an intermediate in the propionic acid formation by \textit{Propionibacterium arabinosum} fermented on C\textsuperscript{14} labeled three carbon substrates (e.g. lactate, pyruvate, and glycerol). Although the study of metabolic flux analysis of \textit{Propionibacteria} started very early, no systematic studies have been reported due to the limitation of detection tools.

The Wood-Werkman cycle, which systematically described the metabolic map in dairy \textit{Propionibacteria}, was reported by Wood (1981), who reviewed all the achievements over the years. Currently, \textit{in vivo} \textsuperscript{13}C NMR spectroscopy and isotopic labels become powerful tools and have been used to elucidate metabolic pathway, confirm flux estimates obtained by material balance, and extract additional information about pathway fluxes (Deborde et al., 1999; Stephanopoulos, 1999). Kijeldstad and Johnsson (1987) were the first to use \textit{in vivo} NMR technique in studying \textit{Propionibacteria} fermentation. It was reported that the carboxylation reactions played an important role in the propionic acid metabolism by \textit{in vivo} \textsuperscript{13}C NMR (Houwen et al.,
The effect of lactate on the fluxes of pyruvate metabolism was studied by *in vivo* $^{13}$C NMR as well (Deborde et al., 2000). The propionate to acetate ratio increased to 1 in the cometabolism experiment as compared to the pyruvate experiment (P/A = 0.6) (Deborde et al., 2000). In addition, $^{13}$C-NMR of chloroform-methanol extracts was applied to establish the intracellular composition of dairy *Propionibacteria* (Rolin et al., 1995). Several metabolic pathways were proven to exist in the central metabolism of dairy *Propionibacteria* and many reactions in these pathways were bidirectional (Deborde et al., 1999). Moreover, an interesting observation was reported that no tricarboxylic acid cycle existed in *Propionibacteria* in the presence of oxygen and propionate was oxidized to pyruvate through the reverse direction (Ye et al., 1999).

### 2.3.3 Genetic engineering of *Propionibacteria*

#### 2.3.3.1 Dicarboxylic acid pathway in *Propionibacteria*

Normally, *Propionibacteria* synthesize propionic acid from carbon source using heterofermentative dicarboxylic acid pathway, which also leads to the production of acetate, succinate, and CO$_2$ as by-products in the fermentation (Figure 2.1, Playne, 1985). The propionic acid to acetic acid ratio is usually lower than the theoretical ratio (2:1) (Playne, 1985; Boyaval et al., 1995) owing to the generation of by-products and cell biomass. The lower P/A ratio causes the significantly higher cost in downstream purification. Many attempts have been conducted to increase P/A ratio with metabolic pathway shift. (1) To increase P/A ratio via the increase of propionic acid yield.
Suwannakham et al. (2005) reported that a P/A ratio of ~4 was obtained during the FBB fermentation with a high final propionic acid concentration of ~72 g/l. (2) To increase P/A ratio via the decrease of acetic acid yield. It was found that the media pH could significantly affect the metabolic pathway. A lower pH could shift more carbon flux into propionic acid production. The P/A ratio increased from 2 to 14 when the fermentation was operated under low pH conditions (Hsu et al., 1991; Seshadri et al., 1993). Moreover, a higher reductive level of substrate can cause significant increase in the P/A ratio due to the intracellular NADH/NAD⁺ balance. For example, with glycerol as the carbon source, the P/A ratio reached 37 (mol/mol) (Barbirato et al., 1997). The final concentration of acetic acid decreased two folds when 20 g/l of glycerol was used as the fermentation substrate (Himmi et al., 2000). A shift in the metabolic pathway from heterofermentative to homofermentative acid production was observed when a three-electrode amperometric culture system was used to provide external redox balance (Emde et al., 1990). However, these processes are not economically competitive to the chemical process because of the low productivity and high capital input required for the process.
2.3.3.2 Genetic manipulation for flux redistribution of in the formation pathway

As shown in Figure 2.1, there are two metabolic pathways involved in propionic acid fermentation. One is leading to the production of acetic acid; the other is to propionic acid. They share the same precursor, pyruvate. Theoretically, the decrease in
acetic acid production should increase propionic acid generation owing to the redistribution of pyruvate. When 0.3 mM o-iodosobenzoate was used in media to inhibit acetyl kinase, the last step in acetic acid formation, propionic acid production rate increased 2.4 fold but the final propionic acid concentration in the fermentation was extremely low (Morales et al., 2006).

Gene disruption in competing pathways of the desired product has been widely used in the study of protein function and gene expression regulation. As non-pathogenic and potential probiotic bacteria, Propionibacteria are attractive for use as host bacteria in genetic engineering. Recently, two Δack mutants of P. acidipropionici were obtained by gene disruption and integrational mutagenesis (Suwannakham et al., 2006). The specific growth rate of these two mutants was reduced by ~25% to 0.10 h⁻¹ (0.13 h⁻¹ for the wild type), probably because of reduced acetate and ATP production. Both mutants produced ~14% less acetate and 13% more propionate from glucose. Although ack deletion significantly reduced the activity of acetate kinase, it did not completely eliminate acetate production and the increase in propionic acid production by these Δack mutants was not as significant (Suwannakham et al., 2006). The possible reason is that there might be some unknown pathways that can also convert pyruvate to acetate. Also, because acetyl-phosphate is a labile compound, it can decompose automatically to form acetate (Kakuda et al., 1994). Thus, pyruvate would not be accumulated in the Δack mutant cells and no excess pyruvate was available for propionic acid production.
2.3.3.3 Genetics of *Propionibacteria*

Despite the prospect of obtaining some basic accomplishments in genetics and molecular biology of *Propionibacteria*, the genetic manipulation study is, however, still at an early stage. The high G+C content of *Propionibacteria*, which ranges from 53-68% (Guillaume et al., 2004), is a factor that inhibits the improvement in propionibacterium genetic operation. The strong interaction of G/C base pairs makes PCR and genome sequencing more difficult. So far, only two propionibacteria, *Propionibacterium acne* (Holger et al., 2004) and *Propionibacterium freudenreichii* ATCC 6207 (Klaenhammer T. et al., 2002) have been sequenced and annotated. However, the genome of *P. freudenreichii* is not publicly available. Recently, Meurice et al. (2004) reported that they are sequencing and annotating the *P. shermanii* genome. The lack of detailed information about the genome sequence hampers the progress in *Propionibacteria* genetic engineering.

In addition, low transformation efficiency also negatively affects the development of metabolically engineered mutants of *Propionibacteria*. Owing to the gram-positive cell wall, it is much more difficult to transform plasmids into *Propionibacteira* cells through the very thick peptidoglycan layer as compared with *E. coli*. Another reason causing the low transformation efficiency is the presence of a strong restriction modification system in *Propionibacteria* (Kiatpapan et al., 2000; 2002). Fortunately, many studies have been made to solve this problem and some considerable achievements have been obtained.
Gautier et al. (1995) reported that a high transformation efficiency of $7 \times 10^5$ transformants per $\mu$g of DNA was obtained by electrotansfection of *Propionibacterium freudenreichii* cells grown in the presence of glycine that was incorporated into the cell wall and weakened the cross-linking of cell wall components. Plasmids (pRGO1 through pRGO7) with the size ranging from 4.4 MDa to 119 MDa or higher were screened and characterized, which provided a platform for genetic manipulation of *Propionibacteria* (Rehberger et al. 1990). A shuttle vector, pPK705 which replicates in both *E.coli* and *Propionibacteria*, was constructed from pRGO1, pUC18, and hygB as a marker gene (Kiatpapan et al., 2000). Moreover, Jore et al. (2001) constructed another shuttle vector named pBRES1 from *E. coli* plasmid pBR322 and *P. freudereichii* LMG 16545 plasmid p545 with amp and ery as two selection marker genes. It was found that the electroporation with the shuttle vectors re-isolated from *Propionibacteria* could reach a much higher transformation efficiency of $\geq 10^8$ CFU/$\mu$g DNA, indicating that the extracted plasmids eliminated the effect of the restriction modification system in *Propionibacteria* (Jore et al. 2001).

Due to the high G+C content in the chromosome of *Propionibacteria*, few selective markers (antibiotics resistance gene) for mutant screening are suitable (Table 2.2) (Jore et al., 2001). It is tempting to speculate that genes with low G+C content may be poorly expressed in *Propionibacteria*, if they were expressed at all (Jore et al., 2001; Kiatpapan et al., 2000; Green et al., 1998). In order to get higher transformation efficiency, *ermE* (erythromycin resistance gene) was chosen as the selective marker for the integrational plasmid construction. In addition, *tet* was used by Huang (1998) to select *ack* inactivated mutant as well. Furthermore, expression vectors for
Propionibacteria, pKHEM01 and pKHEM04, were constructed using P138, P4 promoters, respectively, and shuttle vector pPK605 was successfully used in the over-expression of 5-aminolevulinic acid (ALA) in *P. freudenreichii* subsp. *shermanii* (Kiatpapan et al., 2001).

<table>
<thead>
<tr>
<th>Gene</th>
<th>GC content (%)</th>
<th>Origin</th>
<th>Propionibacterium transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>caf</em></td>
<td>29</td>
<td>pC194 (<em>Staphylococcus aureus</em>)</td>
<td>−</td>
</tr>
<tr>
<td><em>caf</em></td>
<td>53</td>
<td>pACYC184 (<em>Escherichia coli</em>)</td>
<td>−</td>
</tr>
<tr>
<td><em>cml</em></td>
<td>63</td>
<td>pEBM3 (<em>Corynebacterium striatum</em>)</td>
<td>(+)∧</td>
</tr>
<tr>
<td><em>ery</em></td>
<td>34</td>
<td>pAMB1 (<em>Enterococcus faecalis</em>)</td>
<td>−</td>
</tr>
<tr>
<td><em>ermE</em></td>
<td>71</td>
<td>pIJ488 (<em>Saccharopolyspora erythraea</em>)</td>
<td>+</td>
</tr>
<tr>
<td><em>hygB</em></td>
<td>71</td>
<td>pSMT3 (<em>Streptomyces hygroscopicus</em>)</td>
<td>+</td>
</tr>
</tbody>
</table>

∧. Only as a secondary selection marker

*caf*: chloramphenicol resistance gene
*ero*: erythromycin resistance gene
*ermE*: erythromycin resistance gene
*hygB*: hygromycin B resistance gene
*hygB*: hygromycin B resistance gene

Table 2.2 Antibiotic resistance genes used in propionibacteria

Meanwhile, promoter elements from *Propionibacterium freudenreichii* were analyzed by Piao et al. (2004). The putative consensus sequences of -35 and -10 hexamer were found in several promoter regions from *P. freudenreichii*. These investigations provided a deep insight into transcriptional studies in *Propionibacteria*.

454 sequencing is a novel DNA sequencing technology with unique mix of long reads, exceptional accuracy, and ultra-high throughput (Droege et al., 2008). The combination of 454 sequencing with conventional Sanger sequencing is markedly powerful in assembling the complete genome sequence, and has been widely utilized for
sequencing human, plant, and bacterial genomes (Pühler et al., 2008). Currently, this
technique is being adopted to sequence the whole genome of *P. acidipropionici*. The
genomic sequencing information would make it easier for the design and construction of
genetically modified *Propionicibacteria* strains for industrial applications.

### 2.3.4 Proteomics of *Propionibacteria*

Briefly, proteomics, defined as the study of proteins, is an analogy of genomics, the study of genes. However, proteomics is much more complicated than genomics because an organism could have different protein expressions in different phases of its life cycle and under different environmental conditions even though it has the constant genome. Proteomics is often considered the follow-up step in the study of response mechanism of organisms after genomics. Compared with genomics, proteomics could provide a much better understanding of the global regulation patterns of gene expression than genomics due to the important role of proteins in the life of organisms. Rapid development of proteomics in bioscience research has been facilitated by recent progress in protein separation (i.e., two-dimensional electrophoresis, 2-DE) and characterization (i.e., mass spectroscopy) techniques. The comparison of global regulation patterns of gene expression in wild type and mutants obtained from various environmental conditions can be conducted. The results can then be used in genetic and metabolic engineering of mutants for industrial applications.
Although extensive studies have been done in the optimization of propionic acid fermentation process, the understanding of the molecular level changes in Propionibacteria is poor, and no systematic studies have been reported. Most explanations still remain in the hypothesis level and need to prove by new techniques. It was reported that the acidic environment could induce changes in the morphology and protein synthesis of Propionibacteria (Jan et al., 2001b). Seventeen protein synthesis inductions were observed after the acid adaptation and five of them were identified as GroEL, GroES, RecR, RepB, and BCCP proteins, respectively (Jan et al., 2001b). This information gives important clues about the functions of the discovered proteins and allows scientists to elucidate the mechanism involved in the acid tolerance response of propionibacteria. One possible explanation was that the chaperon proteins (GroEL and GroES) may protect the existing fatal proteins (e.g., glycolytic enzymes, DNA repair proteins) from acid damage (Cotter et al., 2003) and replace the acid-sensitive proteins with acid-tolerant homology (Foster, 1995). Changes in cell morphology and enzyme activities were also observed in P. acidipropionici cultured in the FBB fermentation system for propionic acid production (Suwannakham et al., 2005). However, so far no protein level study has been done to investigate the mechanism of improved acid tolerance in the adapted mutant.

Gene deletion in competing pathways of the target metabolic product is a common strategy in metabolic engineering, which was utilized in propionic acid production by ack inactivated mutants of P. acidipropionici (Suwannakham et al., 2006). However, the responses of the organism to genetic and environmental disturbance were complicated and poorly understood at the molecular level. The initial response to the
gene deletion was to redistribute the flux at the branch point. And then, some silent genes or latent pathways might be activated or enzymes might be reassigned to complement the deleted reactions (Fong et al., 2006). This mechanism could well explain why small amounts of acetate were still produced in \textit{pta} deleted mutants of \textit{E. coli} (Chang et al., 1999) and in \textit{ack} deleted mutants of \textit{P. acidipropionici} (Suwannakham et al., 2006). In conclusion, the responses of cells to genetic or environmental perturbations are global and involve up/down regulation of existing proteins and/or synthesis of new proteins. The identification of these target proteins is necessary to better understand the metabolic mechanism in an organism. Coupled with genomics and metabolomics, global control of metabolic networks might be realized.
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CHAPTER 3

ENGINEERING *PROPIONIBACTERIUM ACIDIPROPIONICI* FOR ENHANCED PROPIONIC ACID PRODUCTION FROM SUGARS

Summary

*Propionibacterium acidipropionici*, which is a Gram-positive, anaerobic bacterium, has been the most used species for propionic acid production with acetic acid as the main byproduct. Genetic manipulations were performed to alter the metabolic pathways related to propionic acid and acetic acid production in *P. acidipropionici*. Mutants were constructed by inactivating genes associated with the acetate formation pathway to eliminate or reduce acetate formation so that more propionic acid can be produced from sugars. The mutant grew slower in batch fermentation, but produced more propionate and less acetate as compared to the wild type. Fermentation with mutants in a fibrous bed bioreactor (FBB) showed that the maximum theoretical propionic acid yield of ~0.54 g/g glucose could be achieved. The highest propionic acid concentration obtained from lactose fermentation was ~104 g/l, which was 43% higher than the highest concentration (~72 g/l) previously reported in the literature. In addition, the mutant
obtained from the FBB was less sensitive to propionic acid inhibition. SEM revealed a significant change in mutant morphology.

3.1 Introduction

Propionic acid is an important mold inhibitor. Its ammonium, sodium, calcium, and potassium salts are widely used as preservatives for animal feed and human foods. Propionic acid is also an important chemical intermediate widely used in the synthesis of cellulose fibers, herbicides, perfumes, and pharmaceuticals. Currently, propionic acid is mainly produced via petrochemical processes, with an annual production of ~770 million pounds worldwide in 2006 and an annual growth rate of 2.5 percent. Because of rising oil prices, public concerns about the environmental pollution caused by the petrochemical industry, and consumers’ preference for bio-based natural ingredients in foods, cosmetics, and pharmaceuticals, the production of propionic acid from biorenewable resources via propionic acid fermentation has become an increasingly attractive alternative to petroleum-based processes.

Members of the genus Propionibacteria are widely used in industry for production of vitamin B₁₂, tetrapyrrole compounds, propionic acid, as well as probiotic cultures and Swiss cheese (Coronado et al., 2001; Gardner et al., 2005; Hitchner, 1934; Kiatpapan et al., 2001; Langsurd et al., 1973; Ozadali et al., 1996; Yongsmith et al., 1983). Propionic acid is produced by propionibacteria in the dicarboxylic acid pathway, with acetate, succinate, and carbon dioxide as byproducts. Theoretically, one mol glucose produces 4/3 mol propionate and 2/3 mol acetate when glycolysis is through EMP
pathway. The actual propionate yield is much lower when there are significant cell growth and biomass formation. Propionate is a strong inhibitor to the fermentation even at a relatively low concentration of 10 g/L (Goswami et al., 2001; Hsu et al., 1991; Nanba et al., 1983). Typical batch propionic acid fermentation takes ~3 days to reach ~20 g/L propionic acid, with a propionic acid yield usually less than 0.4 g/g glucose. The low productivity, yield, and final product concentration render conventional propionic acid fermentation process difficult to compete with its petroleum counterparts. Although numerous attempts have been made to improve propionic acid fermentation (Bodie et al., 1987; Coronado et al., 2001; Goswami et al., 2001; Jin et al., 1998; Lewis et al., 1992a; 1992b; 1992c; Morales et al., 2006; Ozadali et al., 1996; Woskow et al., 1991; Yang et al., 1994; 1995), more work are still needed to improve propionic acid fermentation in order to make biobased propionic acid economically competitive.

One limitation in propionic acid fermentation is caused by the heterofermentation pathway that also produces acetic acid and thus significantly reduces the propionic acid yield from glucose. In order to eliminate or reduce acetate formation and increase propionic acid production, gene knock-out through homologous recombination was performed to inactivate acetate kinase gene ($Ack$) in the acetate formation pathway (Suwannakham et al., 2006). Compared to the wild type strain, the $ack$ knock-out mutant (ACK-Tet) produced more propionate and less acetate, but had a lower specific growth rate due to less ATP could be generated from the impaired acetic acid synthesis pathway. Another major limitation in propionic acid fermentation is its low final product concentration caused by strong product inhibition. How to enhance cellular tolerance to toxic product has been a major issue in many fermentation processes. The low acid
tolerance problem associated with conventional propionic acid fermentation can be partially addressed by cell immobilization (Huang et al., 2002). With cells immobilized in a fibrous-bed bioreactor (FBB), a high propionic acid concentration of ~72 g/L was produced from glucose by *P. acidipropionici* (Suwannakham and Yang, 2005). This improvement was achieved through not only the higher cell density maintained in the FBB, but also adaptation of cells to become more tolerant to propionic acid.

The main objective of this work was to further improve propionic acid production from glucose by using the metabolically engineered mutant ACK-Tet immobilized in the FBB. We expected to obtain much higher propionic acid concentration, yield and production rate by allowing the ACK-Tet mutant to acquire a high acid tolerance through adaptation and evolution in the FBB. After 6-month continuous operation under a fed-batch mode, the propionic acid concentration in the fermentation broth reached 104 g/L. The second objective of this study was to elucidate the mechanisms contributed to the increased acid tolerance of the mutants adapted in the FBB. Cells were harvested and characterized for their morphology, growth inhibition by propionic acid, protein expression profiles as observed in SDS-PAGE, and H⁺-ATPase activity, which is related to the proton pumping and cell’s ability to control its intracellular pH value. The results were compared with those from the parental strain and are reported in this paper.
3.2 Materials and Methods

3.2.1 Cultures and Media

*Propionibacterium acidipropionici* ATCC4875 wild type and ACK-Tet mutant were cultured in a synthetic medium containing (per liter) 10 g yeast extract (Difco Laboratories, Detroit, MI), 5 g Trypticase (BBL), 0.25 g K$_2$HPO$_4$, 0.05 g MnSO$_4$, and 50-100 g glucose or lactose as the sole carbon source. Unless otherwise noted, the medium was prepared in two parts: the basal medium (without the carbon source) and concentrated sugar solution. They were sterilized separately by autoclaving at 121°C and 15 psig for 30 min, and then mixed aseptically before inoculation.

3.2.2 Fermentation

3.2.2.1 Free-cell fermentation

Unless otherwise noted, all fermentations were conducted in a 5-L fermentor (Marubishi MD-300) containing 2 L of above medium at 32°C under controlled pH conditions. The fermentor pH was controlled at 6.5 (±0.02) by automatically adding 6 N NaOH. Anaerobiosis was maintained by sparging N$_2$ through the medium for 30 min before inoculation and once for 10 min each day afterwards. The fermentor was inoculated with 100 ml of exponential phase cells (OD$_{600}$ ≈ 2.0) grown in a serum bottle at 32°C. Liquid samples (3 ml each) were taken from the fermentor at proper time intervals.
3.2.2.2 Immobilized-cell fermentation

The immobilized cell bioreactor used in this study was made of a glass column fitted with a water jacket. A piece of cotton towel (32×22 cm) was spirally wound together with a stainless steel mesh and packed into the glass column (see Appendix C.1). The bioreactor working volume was ~600 ml. Detailed construction of the fibrous-bed bioreactor (FBB) has been described in Appendix C.2. The FBB was connected to a 5-L fermentor (Marubishi MD-300), with pH and temperature controls, through a recirculation loop. After inoculating 100 ml of a cell suspension into the fermentor, the fermentation was operated at the free-cell fermentation mode until the optical density (OD600) of the fermentation broth reached ~3.0. Then, the fermentation broth was circulated through the FBB at 30 ml/min to allow cells to attach to the fibrous matrix surface. This flow rate had been kept for ~48 hours to immobilize most of cells in the fibrous matrix. When no significant change in cell density was observed, the flow rate was increased to ~80 ml/min to increase mass transfer and mixing in the FBB. Fed-batch fermentation was carried out to study the fermentation kinetics and to gradually adapt the cells to tolerate and produce a high concentration of propionic acid. At the end of the fed-batch fermentation, adapted cells in the FBB were collected from the fibrous matrix and subcultured in serum bottles for further analyses.

3.2.3 Assay of cell tolerance to propionic acid

Wild type cells and adapted cells were grown in serum tubes containing 10 ml of synthetic media with 25 g/l glucose and varying amounts of propionic acid (0-20 g/l).
Cell growth was monitored by measuring the optical density (OD$_{600}$) using a spectrophotometer. The specific growth rates ($\mu$) under different initial propionic acid concentrations were determined from the slopes of semilogarithmic plots of OD versus time. The experiment was done in duplicate.

### 3.2.4 Cell viability assay

Cell viability assay was carried out by the method of Glenner (1977) with some modifications. Briefly, 1 ml of the culture broth was centrifuged at 16,000 rpm for 10 min. After discarding the supernatant, 1 ml of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) solution (1 g/l) was added. The tube was vortexed at the top speed to resuspend the cell pellet, and then incubated in dark at room temperature for 30 min for color development. After centrifugation at 16,000 rpm for 10 min, the cell pellet was collected and 1 ml of methanol was added to resuspend the pellet and to extract the pink color. After centrifuging at 16,000 rpm for 10 min, the absorbance of the supernatant at 485 nm was measured using a spectrophotometer (Shimadzu, UV-16-1) against a control (boiling cells before adding TTC solution). The viability of cells harvested from the exponential phase was assumed to be 100%.

### 3.2.5 Scanning Electron Microscopy

At the end of the FBB fermentation, small pieces (0.5 cm × 0.5 cm) were cut from different parts of the fibrous matrix as samples for scanning electron microscopy (SEM).
The samples were prepared as described previously (Suwannakham et al., 2005) and scanned and photographed with Nova 400 NanoSEM at 15 kV. The detailed protocol is given in Appendix B.8.

3.2.6 PCR amplification of \textit{tet}’ gene

Chromosome DNA of \textit{P. acidipropionici}, used as PCR template, was isolated using the QIAGEN genomic DNA kit (Qiagen, Valencia, CA). Based on the reported sequence of tetracycline resistant gene, the PCR primers were designed by using software Primer 3. The sequences of forward and reverse primers were 5’-tgcgagtacaaactgggtga-3’ and 5’-cgaaagcccacctaaacaa-3’, respectively. The reaction was carried out according to the description in Appendix D.10. The PCR products with an expected size of 1 kb were analyzed via 0.7% (w/v) agarose gel electrophoresis.

3.2.7 SDS-PAGE

The culture of \textit{P. acidipropionici} (100 ml) was harvested at the exponential phase and total proteins were extracted using ReadyPrep Protein Extraction Kit (Soluble and Insoluble) (Bio-Rad). The protein concentration was determined by Bio-Rad \textit{RC DC} protein assay. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted using 12% gel (Appendix D.13). The SDS-PAGE gels were scanned and analyzed by using TotalLab software and the intensity of each interested band was normalized to the sum of the total band intensities on the corresponding lane.
3.2.8 ATPase assay

ATPase activity in cells was determined based on the method of Belli and Marquis (1991). Cells were grown in the medium (50 ml) until the late exponential phase. About half of the cells were harvested from 25 ml of the culture broth by centrifugation at 8,000 rpm, 4°C for 10 min. The remaining cells in the other 25 ml of the broth were subjected to acid shock by adding 1 ml of pure propionic acid and incubating for 1.5 hours at 32°C before being harvested by centrifugation. Cells were made permeable by the method of Belli and Marquis (1991). Briefly, the cell pellets were resuspended in 2.5 ml of 75 mM Tris-HCl buffer (pH 7.0) with 10 mM MgSO₄. Toluene (250 μl) was added to each cell suspension followed by vigorous mixing and incubated at 37°C for 5 min. Each cell suspension was then treated with freezing at -80°C and thawing at 37°C for two times. The permeabilized cells were harvested by centrifugation and resuspended in 1.0 ml of 75 mM Tris-HCl buffer (pH 7.0) with 10 mM MgSO₄. ATPase activity was then determined by measuring the release of inorganic phosphate (Pi) from ATP. Unless otherwise mentioned, the reaction mixture contained 3.0 ml of 50 mM Tris-maleate buffer (pH 6.0) with 10 mM MgSO₄, 75 μl of permeabilized cell suspension and 30 μl of 0.5 M ATP (pH 6.0). The mixture was incubated at 37°C for 20 min. Samples (50 μl each) were removed for the Pi assay by the Fiske-SubbaRow method as modified by Weisman and Pileggi (1974). The standard unit of ATPase activity is defined as the amount of enzyme that releases 1 μmol of Pi per minute, and the specific activity of ATPase is defined as the unit of activity per milligram of cell dry weight.
3.2.9 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-ml cuvette (light path length: 1 cm) using a spectrophotometer (Shimadzu, UV-16-1). One unit of OD was equivalent to 0.435 g/l cell dry weight. The concentrations of carbon source (glucose or lactose) and main acid products (acetic, succinic, and propionic acids) in samples were analyzed by high-performance liquid chromatography (HPLC) with an organic acid column (Bio-Rad, HPX-87). HPLC was run at 45°C using 0.01 N H₂SO₄ as the eluant at a flow rate of 0.6 ml/min (Appendix B.3).

3.3 Results and Discussion

3.3.1 Kinetics and Fermentation Profiles of Glucose and Lactose

To obtain the adapted propionate-tolerant strain from the fibrous bed bioreactor (FBB) and the maximum propionic acid concentration, fed-batch fermentations with *P. acidipropionici* ACK-Tet mutant (Suwannakham et al., 2006) was carried out continuously for over 80 days until cells ceased to consume glucose or produce propionic acid. As shown in Figure 3.1 (A), the final propionic acid concentration reached 97 g/l, which was ~37% higher than the highest concentration (71 g/l) ever reported previously (Suwannakham et al., 2005). Lactose was also used as the sole carbon source in the FBB since lactose is the main component of cheese whey and it is desirable to use this waste lactose as a renewable feedstock to produce industrial chemicals and fuels to replace
fossil fuels and petrochemicals. In about 60 days, the propionic acid concentration in the fed-batch fermentation reached 104 g/l (Figure 3.1 B). The high final propionic acid concentration can facilitate downstream product recovery and significantly reduce the overall production cost.

In addition, the higher concentration of propionic acid indicated that the cells in the FBB obtained a higher tolerance to propionic acid. Figure 3.2 shows that the volumetric productivity of propionic acid of ACK-Tet mutant in the FBB fermentation was much higher than that of wild type at comparable propionic acid concentrations. The higher tolerance of ACK-Tet mutant may be in part attributed to the inactivation of ack gene in the chromosome. Further experiments were conducted to study the changes of the FBB adapted ACK-Tet strain in the ability of acid resistance, cell phenotype, and protein expression pattern.

Figure 3.3 shows the fermentation profiles of original ACK-Tet in free cell and FBB fermentations, and the FBB adapted ACK-Tet mutant in free cell fermentation. As expected, propionate, acetate, and succinate were the main products in the fermentation, the same as the fermentation of wild type of *P. acidipropionici*. It should be noted that the adapted ACK-Tet mutant acquired an approximate 2-fold specific growth rate of the original ACK-Tet mutant (0.21 vs. 0.1 h\(^{-1}\)) (Figure 3.4). About 50% higher of propionic acid was produced at the end of the FBB fermentation than the free cell fermentations (23.0 g/l vs. 15.6g/l and 15.1 g/l). These results suggested that more carbon source was used for cell growth in free-cell fermentation and the adapted ACK-Tet became less sensitive to propionic acid.
Comparing to free-cell fermentation of ACK-Tet, the immobilized-cell fermentation showed higher efficiency to convert glucose to propionate. The yield of propionic acid from immobilized fermentation achieved the maximum theoretical propionic acid yield of ~0.54 g/g glucose, which was 54% higher than the free cell fermentations (0.54 g/g vs. 0.35 g/g and 0.37 g/g). The increase in propionic acid yield could be attributed to the fact that more pyruvate, the common precursor of acetate and propionate, was redistributed to the propionic acid formation because the acetate production pathway was blocked (Suwannakham et al., 2006) in ACK-Tet strain and less carbon source was used for cell growth and energy production in immobilized cell fermentation. Furthermore, the propionate productivity in immobilized cell fermentation increased ~70% (0.41 vs. 0.22 g/L·h and 0.26 g/L·h). The increased productivity can be attributed to the high cell density and cell viability in the FBB. At the end of the fermentation, more than 90% of cells were immobilized in the fibrous matrix. The cell concentration (dry weight) in the fibrous matrix was more than 60 g/l. The viability of cells in the FBB was more than 80%.

In free cell fermentation (Table 3.1), both propionate and acetate yields from glucose were decreased slightly from 0.37 g/g and 0.045 g/g with the original ACK-Tet mutant to 0.35 g/g and 0.037g/g with the adapted ACK-Tet mutant ($p$ values > 0.05), respectively. In contrast, productivities of propionate and acetate with adapted ACK-Tet in free-cell fermentaiton increased significantly ($p$ values < 0.05) as compared to original ACK-Tet (0.26 vs. 0.22 g/l/h; 0.023 vs. 0.02 g/l/h), suggesting that adapted ACK-Tet obtained higher acid tolerance. The higher productivity might also be attributed to the
significantly higher specific growth rate of the adapted mutant (0.16 vs. 0.1 h⁻¹, \( p \) value < 0.05) (Figure 3.4)

It is worth noting that acetate production by ACK-Tet mutant in both free-cell and FBB fermentations were not eliminated. Similar results were also obtained in butyric acid fermentation (Zhu et al., 2005). The accumulation of acetate in the fermentation broth indicated that there might be some other pathways in \( P. \ acidipropionici \) to generate acetate, just like the low-affinity and high-affinity pathways in \( E. \ coli \) to produce acetate (Phue et al., 2005; Starai et al., 2005; Chang et al., 1999). Since 1 mol of NADH and ATP were generated in the acetic acid synthesis pathway, the blocked pathway could be equilibrated by other pathways to maintain the NADH/NAD⁺ balance and energy supplement in cells (Morales et al., 2006).

3.3.2 Propionic acid inhibition

Propionic acid, a weak organic acid, is a cell growth inhibitor by disturbing the pH gradient across cell membrane (Gu et al., 1998). 1 % (w/v) propionic acid in media can significantly reduce the specific growth rate of \( Propionibacteria \). Figure 3.5 compares the specific growth rates of the wild type, ACK-Tet, and adapted wild type and ACK-Tet from the FBB at various initial propionic acid concentrations (0-20 g/L). The specific growth rate in the absence of propionic acid was assumed as 1. The other specific growth rates were shown as relative values. With 20 g/L propionic acid in the media, the specific growth rates of wild type and ACK-Tet strains were reduced by 80% and 70%, respectively. However, the adapted ACK-Tet was much less sensitive to propionic acid
inhibition and maintained about 80% of its growth rate at 20 g/l propionic acid. The adapted wild type obtained by Suwannakham et al. (2005) also showed a better tolerance to propionic acid, but only maintained about 40% of its growth rate at 20 g/l propionic acid.

The inhibition curves of propionic acid on the cell growth could be simulated by the noncompetitive product inhibition model (Suwannakham et al., 2005).

\[
\mu = \frac{\mu_{\text{max}} K_i}{K_i + P} \quad \text{or} \quad \frac{1}{\mu} = \frac{1}{\mu_{\text{max}}} + \frac{1}{\mu_{\text{max}} K_i} P
\]

where \( \mu \) is the specific growth rate (h\(^{-1}\)), \( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\)), \( K_i \) is the inhibition rate constant (g/l), and \( P \) is the propionic acid concentration (g/l). The values of \( \mu_{\text{max}} \) and \( K_i \) were determined from intercepts and slopes of the linear plots of \( 1/\mu \) vs. \( P \) (Figure 3.6) and are listed in Table 3.2. Compared to the wild type and original ACK-Tet, the adapted culture had a slightly higher \( \mu_{\text{max}} \) (0.25 vs. 0.21 h\(^{-1}\) and 0.18 h\(^{-1}\)), and the \( K_i \) value was increased by ~10 times (59.5 vs. 4.3 and 7.6 g/L). It was obvious that the adapted ACK-Tet from the FBB acquired higher tolerance to propionic acid. Other remarkable findings were that original ACK-Tet obtained a higher \( K_i \) than wild type (7.6 vs. 4.3 g/l) and that adapted ACK-Tet obtained a much higher \( K_i \) than the adapted wild type (59.5 vs. 8.9 g/l). This result suggested that the knock-out of \( \text{ack} \) gene from the chromosome of \( P. \text{acidipropionic} \) not only affected the acetate production, but also decreased the sensitivity of the strain to propionic acid although the detailed mechanism is not clear.
3.3.3 Fermentation Long-term Stability

The reactor used in propionic acid production from glucose and lactose had been operated continuously for more than six months without encountering any degeneration or contamination problems, because of the inhibition effect of high propionic acid concentration in the fermentation broth (Yang et al., 1994). Several pieces of immobilized cell samples were cut from the fiber matrix at the end of the fermentation and used to take SEM photographs. As shown in Figure 3.7, the cells either attached to fiber surface or attached to each other to form large cell clumps entrapped within the void space, which caused the high cell density in the FBB. However, there still were enough gaps left for media passing through. No clogging or increased pressure drop was observed during the fermentation (Huang et al., 2002).

ACK-Tet mutant was constructed by inserting the tetracycline resistant gene (tet\(^r\)) into ack gene in P. acidipropionici chromosome to inactivate ack gene (Suwannakham et al., 2006). However, there was no tetracycline added in the media in the long-term fermentation concerning the possible inconvenience for downstream purification and production cost. It was possible that the tet\(^r\) gene might be lost in the absence of selective pressure after several generations. Thus, the adapted cells immobilized in the fibrous matrix were collected at the end of the fermentation and were detected the stability of tet\(^r\) gene in the chromosome. All wild-type, original ACK-Tet, and adapted ACK-Tet were cultured in media containing 10 \(\mu\)g/ml tetracycline for three days. The original ACK-Tet and adapted ACK-Tet could grow very well. In contrast, all wild-type cells died in the selective medium (Figure 3.8). Besides, the chromosomes were extracted from wild-type,
original ACK-Tet, and adapted ACK-Tet cultures and used as templates for \textit{tetr} gene amplification with PCR. The plasmid pTAT, containing \textit{tetr} gene, was used as positive control. The results are shown in Figure 3.9. The PCR products with the expected size were observed in both original ACK-Tet and adapted ACK-Tet samples (lanes 1 and 2). These results confirmed that \textit{tetr} gene was stable in chromosome without the requirement of tetracycline in media during the long term fermentation.

### 3.3.4 Morphological Changes of \textit{P. acidipropionici} Mutant from FBB

The changes of \textit{P. acidipropionici} mutant responding to the extreme environment occurred not only at the protein or gene expression level, but were also found in cell morphology. As can be seen in Figure 3.10, both the wild type and the original ACK-Tet cells had a short rod shape (~1.56 μm and ~1.41μm on average, respectively), while the adapted ACK-Tet mutant from the FBB had an significantly elongated rod shape with an average length of ~2.34 μm ($p$ value<0.05). Similar results were reported previously; the adapted \textit{P. acidipropionici} had a longer and slimmer rod shape, causing 10% increase in cell specific surface area, which may increase the efficiency of proton-pumping (Suwannakham et al., 2005). However, more work would be needed to study the mechanism of the response. It has been reported that \textit{Propionibacterium freudenreichii} cultured at pH 2.0 lost its original rod shape and became shorter, and that the changes at the morphology level might contribute to the increased tolerance to the acidic environment.
3.3.5 SDS-PAGE

Total proteins were extracted from wild type, original ACK-Tet, and adapted ACK-Tet, respectively, and analyzed for protein expression profiles with SDS-PAGE. At least triplicate samples were analyzed to obtain statistically reliable conclusion. Figure 3.11 A, B shows typical SDS-PAGE profiles of various samples. The expression of protein with ~56 kDa was significantly increased in the adapted ACK-Tet, while there was no difference in wild type and original ACK-Tet based on the t-test analysis with the significance level of $\alpha = 0.05$ ($P < 0.05$). In addition, the adapted ACK-Tet also over-expressed another protein with ~39 kDa molecular mass, comparing to the wild-type and original ACK-tet. The protein with ~56 kDa probably represents the B1 subunit of H$^+$-ATPase with a similar molecular weight and the other protein with 39 kDa might be the E subunit of H$^+$-ATPase although the molecular mass is slightly higher than the predicted 31 kDa of E subunit (Breton et al., 2000). H$^+$-ATPase, a proton-pumping ATPase in cell membrane, plays an important role in acid tolerance of cells (O’Sullivan et al., 1999; Cotter et al., 1999). Thus, the increased tolerance of adapted ACK-Tet to propionic acid might be relating to the H$^+$-ATPase protein expression level. Assuming that the level of B1 subunit represents the level of H$^+$-ATPase (Miwa et al., 2000), the result could, at least in part, explain the increase in propionic acid tolerance of the adapted ACK-Tet, although further work to identify these H$^+$-ATPase proteins on the gel would be needed.
3.3.6 H⁺-ATPase assay

The specific H⁺-ATPase activities in permeabilized cells of the wild-type, original ACK-Tet, and adapted ACK-Tet were assayed and are compared in Figure 3.12. The adapted ACK-Tet cells without acid shock (pH 7.0) contained the largest amount of H⁺-ATPase among the three strains, followed by the original ACK-Tet. The wild-type had the lowest value of the H⁺-ATPase activity. Similar results were observed from the permeabilized cells with acid shock (pH 3.5) by propionic acid. Despite the decrease in the activities of H⁺-ATPase in all the acid-shock treated cells, (Belli et al., 1991; Miwa et al., 1997), the permeabilized cells of adapted ACK-Tet still showed the highest activity of H⁺-ATPase. These results indicated that the adapted ACK-Tet not only obtained larger amount of H⁺-ATPase at normal pH but also maintained a relatively higher amount of H⁺-ATPase at lower pH and higher propionic acid concentration as compared to the wild-type and original mutant, which was consistent with the results obtained in the detection of propionic acid inhibition. In addition, these results could partially support the hypothesis of H⁺-ATPase over-expression in the adapted ACK-Tet discussed in the SDS-PAGE analysis.

3.4 Conclusions

The integration of metabolic engineering and the fibrous bed bioreactor fermentation was successfully carried out in this work to produce propionic acid with glucose or lactose as the sole carbon source. The particular structure of the FBB, spirally
wound fiber packed in a glass column, made it easier to get higher productivity, yield, and an adapted mutant with higher ability to survive in extreme environment. The interaction between cells and fiber surface was physical adsorption (Lewis et al., 1992b). The old cells could be replaced by active cells, maintaining a high cell viability (>80%) in the FBB. The gradually increased propionic acid concentration and high cell density in the FBB caused a fast adaptation of cells (Suwannakham et al., 2005). Although engineered \textit{P. acidipropionici} (ACK-Tet) could increase propionate production and decreased actate, a main byproduct, production in free cell fermentation (Suwannakham et al., 2006), immobilized cell fermentation was necessary for reaching higher propionate yield and final concentration. After 2-month fermentation, the maximum propionic acid concentration (104 g/l) was obtained with lactose as the substrate, which was the highest propionic acid concentration ever seen in the reported literature. An adapted ACK-Tet mutant was obtained from the FBB at the end of long-term fermentation. Further studies were conducted to determine the changes in the mutant. The morphology of mutant changed to a longer rod shape. Moreover, the tolerance to propionic acid was increased by ~10 times, possibly due to the over expression of H^+-ATPase in cells, which plays a key role in proton pumping. Similar results were obtained in the enzyme assay of H^+-ATPase activity. The \textit{tet}^\prime\ gene was stable in \textit{P.acidipropionici} recombinant chromosome in the absence of tetracycline during the fermentation. The higher final concentration of propionic acid in the FBB would facilitate the recovery and purification of propionic acid from the fermentation broth by simple solvent extraction.
3.5 Reference


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Morales J., Choi J.S., Kim D.S., 2006, Production rate of propionic acid in fermentation of cheese whey with enzyme inhibitors, Environmental Progress, 25, 228-234


Starai V.J., Garrity J., Escalante-Semerena J.C., 2005, Acetate excretion during growth of Salmonella enterica on ethanolamine requires phosphotransacetylase (EutD) activity, and acetate recapture requires acetyl-CoA synthetase (Acs) and phosphotransacetylase (Pta) activities, Microbiology, 151: 3793–3801.


Table 3.1 Comparison of batch fermentation kinetics by ACK-Tet and adapted ACK-Tet in free cell and immobilized cell FBB fermentations.

<table>
<thead>
<tr>
<th></th>
<th>ACK-Tet Free Cells</th>
<th>FBB</th>
<th>Adapted ACK-Tet (Free cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product yield (g/g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.37 ± 0.01</td>
<td>0.537 ± 0.004</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.045 ± 0.011</td>
<td>0.11 ± 0.03</td>
<td>0.037 ± 0.001</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.05 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>0.055 ± 0.004</td>
</tr>
<tr>
<td><strong>Productivity (g/L·h)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.22 ± 0.003</td>
<td>0.41 ± 0.02</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.02 ± 0.001</td>
<td>0.08 ± 0.002</td>
<td>0.023 ± 0.0002</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.022 ± 0.002</td>
<td>0.06 ± 0.01</td>
<td>0.029 ± 0.004</td>
</tr>
<tr>
<td><strong>Specific growth rate (h⁻¹)</strong></td>
<td>0.103 ± 0.003</td>
<td>N/A</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td><strong>Final propionic acid (g/L)</strong></td>
<td>15.6 ± 0.6</td>
<td>23.0 ± 0.6</td>
<td>15.1 ± 0.5</td>
</tr>
</tbody>
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Table 3.2 Comparison of inhibition rate constants, maximum specific growth rates of wild type, ACK-Tet, Adapted ACK-Tet, and Adapted wild type

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>ACK-Tet</th>
<th>Adapted Wild Type*</th>
<th>Adapted ACK-Tet</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_{\text{max}}$ (h$^{-1}$)</td>
<td>0.21 ± 0.02</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.03</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>$K_i$ (g/L)</td>
<td>4.26 ± 0.48</td>
<td>7.57 ± 0.62</td>
<td>8.93 ± 0.75</td>
<td>59.53 ± 4.4</td>
</tr>
</tbody>
</table>

*data from Suwannahkam et al., 2005
Figure 3.1 Long-term fed-batch fermentations by *P. acidipropionici* ACK-Tet cells immobilized in the FBB at pH 6.5, 32°C, with (A) glucose and (B) lactose as the substrate.
Figure 3.2 Effect of propionic acid on the volumetric productivity of propionic acid in FBB fermentations with ACK-Tet mutant and wild type (data from Suwannakham et al., 2005).
Figure 3.3 Kinetics of batch fermentations by *P. acidipropionici* at pH 6.5, 32°C (A) free cell fermentation with ACK-Tet; (B) immobilized cell fermentation with ACK-Tet; (C) free cell fermentation with adapted ACK-Tet obtained from the FBB.
Figure 3.4 Comparison of specific growth rates of *P. acidipropionici* ACK-Tet mutant and adapted ACK-Tet mutant in free cell, batch fermentations at pH 6.5, 32°C. The slope of the lines represent the specific growth rate of each strain.
Figure 3.5 Effect of propionic acid on the specific growth rates of *P. acidipropionici* wild type, adapted wild type from FBB, ACK-Tet, and Adapted ACK-Tet from FBB. The data for the adapted wild type were from Suwannakham *et al.*, 2005.
\[ y = 0.2275x + 0.9693 \quad R^2 = 0.9909 \]
\[ y = 0.1433x + 1.0854 \quad R^2 = 0.9883 \]
\[ y = 0.017x + 1.012 \quad R^2 = 0.9937 \]
\[ y = 0.1146x + 1.0232 \quad R^2 = 0.9951 \]

**Figure 3.6** Determination of $K_i$ and $\mu_{max}$ in the noncompetitive inhibition model (All data points are average of triplicate experiments)
Figure 3.7 Scanning electron micrographs of cells immobilized in the fibrous matrix in the FBB. (A), (B), (C) cells attached to fiber surface; (D), (E), (F) large cell clumps entrapped within the void space
Figure 3.8 Cultures grown in media containing 10 μg/ml tetracycline. (A) Wild type; (B) Adapted ACK-Tet; (C) Original ACK-Tet
Figure 3.9 PCR amplification of \textit{tet'} gene using chromosome genes as templates (1) Adapted ACK-Tet; (2) ACK-Tet; (3) Wild type; (4) pTAT plasmid; (5) dH$_2$O; (6) Marker
Figure 3.10 Scanning electron micrographs showing morphological changes after adaptation in FBB fermentation. (A) wild type with regular rod shape; (B) ACK-Tet with regular rod shape; (C) Adapted ACK-Tet with longer rod shape.
Figure 3.11 Comparison of total protein expressions (A) wild type (WT), ACK-Tet and adapted ACK-Tet mutant; (B) wild type (WT) and adapted ACK-Tet mutant
Figure 3.12 Comparison of intracellular H⁺-ATPase activities of \textit{P. acidipropionici} wild type, original ACK-Tet, and adapted ACK-Tet mutants at the exponential phase.
CHAPTER 4

PROPIONIC ACID PRODUCTION FROM GLYCEROL BY METABOLICALLY
ENGINEERED PROPIONIBACTERIUM ACIDIPROPIONICI

Summary

With the increased production of biodiesel, large amounts of glycerol are produced as a byproduct with limited use, causing a significant environmental problem. It is thus desirable to use this waste glycerol as a renewable feedstock to produce industrial chemicals and fuels to replace fossil fuels and petrochemicals. In addition to its low cost and abundance, glycerol has a higher reduced level than sugars (sucrose, glucose, xylose, etc.) and other carbon sources such as lactate, which is of benefit to reduced chemicals and biofuels production. In this work, glycerol as the carbon source in propionic acid fermentation by Propionibacterium acidipropionic (ACK-Tet) was studied. It was found that the adapted ACK-Tet mutant could use glycerol for its growth and produced propionic acid at a high yield of 0.54-0.71 g/g glycerol, which was much higher than that from glucose (0.35 g/g glucose). In addition, the production of acetic acid in glycerol fermentation was much less than that from glucose. Thus, glycerol fermentation produced a high purity propionic acid with the propionic acid to acetic acid ratio of 22.4 (vs. ~5
from glucose fermentation), facilitating the recovery and purification of propionic acid from the fermentation broth by simple solvent extraction. The highest propionic acid concentration obtained from glycerol fermentation was ~106 g/l, which is 2.5 times of the highest concentration (~42 g/l) previously reported in the literature.

4.1 Introduction

Propionic acid is a weak organic acid. The non-dissociated propionic acid can pass through cell membrane into cytoplasm and release protons due to the intracellular alkaline environment. As a result, the pH gradient across the cell membrane is disturbed, which affects the nutrients transfer and inhibits cell growth (Gu et al., 1998). Thus, propionic acid and its salts are widely used as food and feed preservatives. Moreover, propionic acid is an important chemical intermediate in the synthesis of cellulose fibers, herbicides, perfumes, and pharmaceuticals. Currently, propionic acid is mainly produced via petrochemical processes. However, the rising oil price encourages the chemical industry to find alternative approaches to produce the basic chemicals. Bioprocessing is a promising technology that can be used to produce various value added products from renewable carbon sources, such as agricultural commodities and processing wastes. For example, Du-Pont and Tate&Lyle opened a $100 million joint-venture plant in Loudon, Tenn., to make 1,3 propanediol from corn sugar and Dow Chemical is teaming up with Brazilian ethanol producer Crystalsev to produce polyethylene from sugarcane (Short, 2007).
Propionibacteria, such as Propionibacterium acidipropionici, have been studied extensively in the production of propionic acid from renewable feedstocks (Kiatpapan et al., 2001; Ozadali et al., 1996; Coronado et al., 2001; Jin et al., 1998; Lewis et al., 1992a;b; Yang et al., 1995; Yang et al., 1994; Goswami et al., 2001; Bodie et al., 1987; Morales et al., 2006; Woskow et al., 1991; Lewis et al., 1992c; Hsu et al., 1991; Suwannakham et al., 2005, 2006). However, like most organic acid fermentations, production of propionic acid has a big challenge ahead which is the low productivity, yield, and final concentration due to strong end-product inhibition (Goswami et al., 2001; Nanba et al., 1983; Hsu et al., 1991). The current bioprocess of propionic acid production is unable to compete with commercially used petrochemical routes.

Biodiesel is produced by the transesterification of vegetable oils or animal fats with an alcohol to produce fatty acid methyl or ethyl esters (Eggersdorfer et al., 1992; Chowdury et al., 1993; Marchetti et al., 2007). Glycerol is the main by-product of this process (Vicente et al., 2004; Du et al., 2003). As a promising alternative to traditional fossil fuels, biodiesel production has increased rapidly in recent years, resulting in significant increase in the availability of crude glycerol, which has a low value and poses significant environmental concern. It is thus desirable to use the waste glycerol as a renewable feedstock to produce industrial biochemicals and biofuels to replace fossil fuels and petrochemicals. Currently, glycerol bioconversion receives more and more attention in the fermentative production of reduced chemicals and biofuels such as hydrogen, ethanol by Enterobacter aerogenes (Ito et al., 2005), succinate by Escherichia coli (Dharmadi et al., 2006), and 1, 3 propanediol by Klebsiella pneumoniae (Wang et al., 2003) due to its low price and high reduction state.
In this work, propionic acid fermentation by metabolically engineered *Propionibacterium acidipropionici* ACK-Tet in a fibrous bed bioreactor (FBB) was investigated with glycerol (both pure and that present in biodiesel waste) as the carbon source. In addition, the morphology changes of ACK-Tet were studied and are discussed in this article as well.

### 4.2 Materials and Methods

#### 4.2.1 Culture and Media

A mutant strain of *Propionibacterium acidipropionici* ATCC 4875 with *ack* gene knock-out (ACK-Tet), which has been described elsewhere (Suwannakham et al., 2006), was used in this study. Unless otherwise noted, the bacteria were cultivated in a synthetic medium containing (per liter) 10 g yeast extract (Difco Laboratories, Detroit, MI), 5 g Trypticase (BBL), 0.25 g K$_2$HPO$_4$, 0.05 g MnSO$_4$, and 20-40 g glycerol or glucose as the carbon source. The basal medium (without the carbon source) and the concentrated carbon source solution were sterilized separately at 121°C and 15 psig for 30 min to avoid undesirable reactions. They were mixed aseptically before use in the fermentation study.
4.2.2 Fermentation kinetic studies

4.2.2.1 Free-cell fermentation

Unless otherwise noted, all fermentations were conducted in a 5-L fermentor (Marubishi MD-300) containing 2 L of the medium at 32°C and 7.0 (±0.02), which was controlled by automatically adding 6 N NaOH. Anaerobiosis was maintained by sparging N\textsubscript{2} through the medium for 30 min before inoculation and once for 10 min each day afterwards. The fermentor was inoculated with 100 ml of exponential phase cells (OD\textsubscript{600} ≈ 2.0) grown in a serum bottle at 32°C. Liquid samples (3 ml each) were taken from the fermentor at proper time intervals.

4.2.2.2 Immobilized-cell fermentation

Immobilized cell fermentations were studied in a fibrous bed bioreactor (FBB). The FBB was made of a glass column with a water jacket. A piece of cotton cloth was spirally wound with a stainless steel mesh and packed into the glass column. Detailed construction and operation of the fibrous-bed bioreactor (FBB) has been described elsewhere (see Appendix C). After seeding with ACK-Tet that had been adapted in a FBB with glucose as the carbon source (see Chapter 3), the FBB was operated under the repeated batch mode for several batches to increase the cell density in the reactor system. Then, fed-batch fermentation was carried out in a fibrous bed bioreactor (FBB) to study the fermentation kinetics and to gradually adapt the cells to tolerate and produce a high
concentration of propionic acid. At the end of the fed-batch fermentation, the adapted cells in the FBB were collected from the cotton cloth and subcultured in serum bottles for further analyses. Unless otherwise noted, all batch fermentation kinetic studies were performed in duplicate.

4.2.3 Scanning Electron Microscopy

At the end of the FBB fermentation, small pieces (0.5 cm × 0.5 cm) were cut from different parts of the cotton cloth as samples for scanning electron microscopy (SEM). The samples were prepared as described in Appendix B.8 and scanned and photographed with Nova 400 NanoSEM at 15 kV.

4.2.4 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-ml cuvette using a spectrophotometer (Shimadzu, UV-16-1). One unit of OD was equivalent to 0.435 g/l cell dry weight. The concentrations of carbon source (glycerol or glucose) and acid products (acetic, succinic, and propionic acids) in samples were analyzed by high-performance liquid chromatography (HPLC) with an organic acid column (Bio-Rad, HPX-87). HPLC was run at 45°C using 0.01 N H$_2$SO$_4$ as the eluant at a flow rate of 0.6 ml/min (Appendix B.3).
4.3 Results and Discussion

4.3.1 Fermentation in fibrous bed bioreactor

Glycerol as the sole carbon source in propionic acid fermentation can support cell growth and metabolism, with propionic acid as the main product and acetic and succinic acids as two byproducts, similar to the fermentation with glucose as the carbon source (see Chapter 3). However, cell growth on glycerol was much slower than that on glucose or lactose. In this work, the FBB was initially operated under the repeated batch mode for several batches to increase the cell density in the reactor system. Then, the reactor was operated under a fed-batch mode to gradually increase the propionic acid concentration produced in the glycerol fermentation. Figure 4.1 shows the fed-batch fermentation kinetics with P. acidipropionici ACK-Tet immobilized in the FBB and glycerol as the carbon source. The fed-batch fermentation continued for 4 months until cells ceased to consume glycerol or produce propionic acid. At this point, the propionic acid concentration had reached 106 g/l, which was 2.5 times of the highest concentration (~42 g/l) on glycerol fermentation ever reported in the literature (Barbirato et al., 1997). The high final concentration of propionic acid obtained in the fermentation indicated that the ACK-Tet culture had been adapted in the FBB and obtained a higher tolerance to propionic acid.

It is noted that only small amounts of acetic and succinic acids were produced in the fermentation. As can be seen in Figure 4.1B, the average product yields from glycerol in the fed-batch fermentation were 0.56 g/g for propionic acid, 0.061 g/g for succinic acid,
and 0.025 g/g for acetic acid. Therefore, even though the final propionic acid concentration was higher than 100 g/l, the final concentrations of acetic and succinic acids in the fed-batch fermentation were only 3.6 g/l and 9.7 g/l, respectively, which were much lower than those (22 g/l and 18 g/l, respectively) in the glucose fermentation (see Chapter 3). The final P/A ratio ([propionate]/[acetate], g/g) reached ~30, which was about 6.5 times of that on glucose (see Chapter 3). The higher P/A ratio would facilitate propionic acid recovery and reduce the overall production cost.

At the end of the fed-batch fermentation, the fermentation broth in the FBB system was drained and replaced with fresh medium. Several repeated batch fermentations were then conducted with the adapted culture. The batch fermentation kinetics before and after the long-term fed-batch fermentation period were compared to evaluate the effects of adaptation on culture performance in the FBB. Figures 4.2A and B show the fermentation kinetics with the original (before adaptation) and adapted cultures, respectively. Compared to the original culture, the adapted culture produced more propionic acid (23 g/l vs. 19 g/l) in a shorter time period (100 h vs. 160 h) from the same amount of glycerol (40 g/l). Consequently, the adapted culture gave a significantly higher propionic acid productivity (0.25 g/l/h vs. 0.17 g/l/h) and yield (0.59 g/g vs. 0.52 g/g). It is noted that the adapted culture also produced more acetic and succinic acids. The fermentation kinetic parameters (yields and productivities) for the original and adapted cultures are summarized in Table 4.1.

Figure 4.3 shows the effect of propionic acid on volumetric productivity of propionate in FBB fermentations with original and adapted cultures. At comparable propionic acid concentrations, the volumetric productivity of propionate with the adapted
culture was significantly higher than that from the original one. The higher propionate productivity can be partially attributed to the lower sensitivity of the adapted culture to propionic acid inhibition. It is noted that more acetic acid was produced in the adapted culture, which might have contributed to the higher tolerance to propionic acid. Acetic acid synthesis is coupled with ATP generation that supplies energy for cell growth as well as ATP-dependent H⁺-ATPase function, which requires one ATP for to pump out one proton from cells in order to reduce propionic acid toxic effect. Thus, the more ATP is supplied, the higher acid tolerance is obtained.

In order to study the influence of glycerol concentration, a lower initial concentration of glycerol (20 g/l) was performed in FBB fermentation with the adapted culture (Figure 4.2C). The fermentation kinetic parameters are also given in Table 4.1. In general, no significant difference in productivities and yields was found between batch fermentations with 40 g/l and 20 g/l, respectively, suggesting that the glycerol osmosis pressure in such concentration range (20-40 g/l) has negligible effect on cell growth and metabolism. A preliminary growth test in serum tubes also showed that glycerol concentrations up to 40 g/l had no significant effect on cell growth (data not shown). These findings are consistent with Barbirato’s results (1997).

4.3.2 Kinetics of free cell fermentation

The adapted culture was harvested from the FBB system and used in free-cell batch fermentation. Figure 4.4 shows the batch fermentation kinetics with free cells of original and FBB-adapted cultures. The fermentations were duplicated for each culture
and the results are summarized in Table 4.2. In general, the adapted culture grew much faster and completed the batch fermentation in a much shorter time period. As can be seen in Figure 4.4, the original culture still had 24% of the initial glycerol unused after 700 h, while the adapted culture fermented all glycerol in 300 h. Compared with the original culture, the adapted culture had a much higher specific growth rate (0.16 h\(^{-1}\) vs. 0.05 h\(^{-1}\)) with a higher biomass production (4.45g/l vs. 1.44g/l), and produced more propionic acid (26 g/l vs. 19 g/l) at a three-fold higher productivity (0.10 g/l/h vs. 0.03 g/l/h). However, the propionate yield from glycerol of the adapted culture was slightly lower than that of the original culture (0.55 g/g vs. 0.54 g/g) because more glycerol was used to produce cell biomass and acetate.

It should be noted that there was almost no acetic acid produced during the 700 h of fermentation by the original ACK-Tet culture. This should not be a surprise since ACK-Tet (with ack knock-out) had an impaired acetic acid synthesis pathway (Suwannakham et al., 2006). The negligible acetic acid production from glycerol might also be attributed to the higher reducing state of glycerol, which prefers the production of more reduced product (i.e., propionic acid) vs. more oxidized metabolite (i.e., acetic acid) in order to balance the intracellular redox potential (Himmi et al., 2000). However, the adapted culture increased its capability of producing acetic acid, as evidenced by the production of 1.54 g/l acetate in the batch fermentation. It is clear that culture adaption in the FBB might have activated certain unknown acetate formation pathways to allow the cells to grow and survive in a harsh environment. Adaptation occurred because acetic acid production pathway is an important route for ATP generation and NADH/NAD\(^+\) balance could not be completely eliminated (Morales et al., 2006).
Gene deletion in the competing pathways of the target product is a common strategy in metabolic engineering, which was used in the propionic acid production by *ack* inactivated mutant of *P. acidipropionici* (ACK-Tet) (Suwannakham et al., 2006). The initial response to gene deletion was to re-distribute the flux at the branch point which suggested that more propionic acid and less acetic acid were produced by the original ACK-Tet as compared to the wild type (Suwannakham et al., 2006). And then, some silent genes or latent pathways may be activated or enzymes may be reassigned to complement the deleted reactions (Fong et al., 2006). This mechanism could well explain why a small amount of acetate production was still detectable in the adapted ACK-Tet fermentation. Similar results were also obtained in a *pta* deleted mutant of *E. coli* (Chang et al., 1999).

Propionic acid production from crude glycerol discharged from a biodiesel manufacturing plant (biodiesel waste) was evaluated using the adapted ACK-Tet. The biodiesel waste water containing the crude glycerol was diluted with distilled water and added to the basal medium to a final glycerol concentration of about 17 g/l. The pH of the medium was adjusted to 7.0 with HCl before autoclaving. Figure 4.4C shows the fermentation kinetics with crude glycerol, with the product yields and productivities listed in Table 4.2. The propionate and acetate yields from crude glycerol appeared to be higher than those from pure glycerol (propionate, 0.71 g/g vs. 0.54 g/g; acetate, 0.035 g/g vs. 0.024 g/g), which might be attributed to the presence of other carbon sources (e.g., methanol) in biodiesel wastewater that can also be used to synthesize end products. However, the volumetric productivity of propionic acid from crude glycerol was 15% lower than that of using pure glycerol (0.085 g/l/h vs. 0.10 g/l/h). This could be attributed
to the high concentrations of salts such as sodium chloride in the biodiesel waste. A high salinity of the medium with crude glycerol would be one of the causal factors for the inhibition of product formation (Ito et al., 2005).

Although propionate productivity of adapted ACK-Tet in glycerol fermentation was much higher than that of original ACK-Tet, the productivity was still lower than that from glucose fermentation (see Table 4.2). To improve productivity, mixed substrates (10 g/l of glucose and 30 g/l of glycerol) were utilized to culture the adapted ACK-Tet. We expected that adapted mutant could use glucose first to produce cell biomass quickly, and then the cells would consume glycerol to produce propionic acid with higher yield and P/A ratio. However, the fermentation profiles (Figure 4.5) showed that adapted mutant consumed glucose and glycerol simultaneously. As can be seen in Table 4.2, the propionate yield and P/A ratio from the mixed substrates were higher than those of pure glucose fermentation, but still lower than those of pure glycerol fermentation. Some other fermentation kinetics (final products, cell biomass, or acetate yield) showed the same trend. However, the presence of glucose did not stimulate glycerol fermentation or the production rate of propionic acid in the fermentation, indicating that the glycerol influence on productivity was dominant in the mixed substrate fermentation. The ratio between glucose and glycerol might need to be optimized in order to obtain a better fermentation process with both high yield and productivity.
4.3.3 Morphological changes of *P. acidipropionici* mutant

To investigate the effect of glycerol on the cell morphology, fermentations of glucose and glycerol with the original ACK-Tet mutant were carried out. The cells were then harvested and immobilized on the filter paper by filtration for SEM photography, respectively, when reaching exponential phase. As can be seen in Figure 4.6, the original ACK-Tet cells on glucose had short rod shape (1.5 μm of length and 0.55 μm of diameter on average), while on glycerol the original ACK-Tet mutant elongated the length to 2.13 μm but the diameter did not change significantly. In addition, many nodes were observed in cells harvested from glycerol. The extension of cell size (length) in glycerol media could be attributed to the inhibition of glycerol due to the high osmotic stress. Cell cycle might be elongated in glycerol and most cells stayed at the stage between G2 phase and M phase. This indicated that the cells grew longer without division. The slower cell growth rate partially contributed to the higher yield of propionate from glycerol because more carbon source was used to produce acids instead of biomass. The SEM picture of the adapted ACK-Tet on glycerol from FBB is also shown in Figure 4.6. The length of the adapted mutant was even larger (4.3 μm on average) and the average diameter was reduced to 0.47 μm. The longer and slimmer morphology would be the global response of the cells to the high concentration of propionic acid in the FBB and contributed to the higher tolerance of the adapted mutant due to increased cell surface area that could facilitate mass transfer and proton pumping (Suwannakham et al., 2005a).
4.4 Conclusions

In this study, propionic acid was produced by the original *P. acidipropionici* mutant (ACK-Tet) and the adapted ACK-Tet from pure glycerol and crude glycerol from a biodiesel production plant with a high yield and a high P/A ratio which would facilitate the recovery and purification of propionic acid from the fermentation broth by simple solvent extraction. The adapted ACK-Tet mutant harvested from the FBB system could produce more propionic acid at a higher production rate compared to the original ACK-Tet, which strongly suggested that the adapted mutant obtained higher tolerance to the end product of propionate. Meanwhile, the adapted ACK-Tet partially recovered acetate production to supplement ATP required by the faster cell growth and higher tolerance. In addition, it has been demonstrated in this work that crude glycerol, a low cost feedstock, can be directly used for propionate production.
4.5 Reference

Barbirato F, Chedaille D, Bories A, 1997, Propionic acid fermentation from glycerol: comparison with conventional substrate, Appl Microbiol Biotechnol, 47: 441-446


Morales J., Choi J-S, Kim D-S, 2006, Production rate of propionic acid in fermentation of cheese whey with enzyme inhibitors, Environmental Progress, 25, 228-234


<table>
<thead>
<tr>
<th>Strain</th>
<th>Original ACK-Tet (40 g/l)</th>
<th>Adapted ACK-Tet (40 g/l)</th>
<th>Adapted ACK-Tet (20 g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate</td>
<td>0.52 ± 0.01</td>
<td>0.59 ± 0.02</td>
<td>0.56 ± 0.09</td>
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<tr>
<td>Acetate</td>
<td>0.020 ± 0.003</td>
<td>0.027 ± 0.003</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.056 ± 0.010</td>
<td>0.073 ± 0.002</td>
<td>0.078 ± 0.013</td>
</tr>
<tr>
<td>Product Yield (g/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/A (g/g)</td>
<td>26 ± 4</td>
<td>22 ± 2</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>Volumetric Productivity (g/l/h)</td>
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<tr>
<td>Propionate</td>
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<td>0.25 ± 0.02</td>
<td>0.24 ± 0.04</td>
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<td>Acetate</td>
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<td>0.012 ± 0.005</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.018 ± 0.006</td>
<td>0.032 ± 0.002</td>
<td>0.033 ± 0.007</td>
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**Table 4.1** Comparison of fermentation kinetics of glycerol in batch FBB fermentations by original and adapted cultures.
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<thead>
<tr>
<th>Strain Substrate</th>
<th>Original culture glycerol</th>
<th>Adapted culture glycerol</th>
<th>Adapted culture biodiesel waste</th>
<th>Adapted culture glucose</th>
<th>Adapted culture glucose + glycerol</th>
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<tr>
<td>Propionate</td>
<td>0.55 ± 0.01</td>
<td>0.54 ± 0.02</td>
<td>0.71 ± 0.03</td>
<td>0.35 ± 0.001</td>
<td>0.41 ± 0.06</td>
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<td>Acetate</td>
<td>0 ± 0.00</td>
<td>0.024 ± 0.015</td>
<td>0.035 ± 0.002</td>
<td>0.037 ± 0.014</td>
<td>0.030 ± 0.002</td>
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<tr>
<td>Succinate</td>
<td>0.028 ± 0.004</td>
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<td>0.05 ± 0.005</td>
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<tr>
<td>P/A (g/g)</td>
<td>N/A</td>
<td>29 ± 3</td>
<td>20 ± 1</td>
<td>9.5 ± 0.3</td>
<td>13.7 ± 0.5</td>
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<tr>
<td>Propionate</td>
<td>0.026 ± 0.002</td>
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<td>0.10 ± 0.02</td>
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<td>Acetate</td>
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<td>0.004 ± 0.001</td>
<td>0.023 ± 0.0002</td>
<td>0.004 ± 0.003</td>
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<td>Succinate</td>
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<td>0.007 ± 0.001</td>
<td>0.029 ± 0.004</td>
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<td>μ (h⁻¹)</td>
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<td>0.16 ± 0.02</td>
<td>N/A</td>
<td>0.21 ± 0.07</td>
<td>0.13 ± 0.03</td>
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<tr>
<td>Propionate</td>
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<td>15.2 ± 0.4</td>
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<td>1.1 ± 0.6</td>
<td>0.55 ± 0.02</td>
<td>2.57 ± 0.01</td>
<td>1.93 ± 0.01</td>
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<td>Biomass</td>
<td>1.44 ± 0.12</td>
<td>4.45 ± 1.25</td>
<td>3.64 ± 0.02</td>
<td>7.36 ± 0.31</td>
<td>6.1 ± 0.2</td>
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</table>

Table 4.2 Kinetics of batch free-cell fermentations by original and adapted ACK-Tet with various carbon sources (glycerol, crude glycerol, glucose, and the mixture of glucose/glycerol (1:3).
Figure 4.1 Long-term fed-batch immobilized-cell fermentation by \textit{P. acidipropionici} ACK-Tet at pH 7.0, 32°C in glycerol. A. Time course data; B. Determination of average product yields.
Figure 4.2 Kinetics of batch FBB fermentations by original ACK-Tet mutant, 40 g/l (A); adapted ACK-Tet mutant, 40 g/l (B); adapted ACK-Tet mutant, 20 g/l (C). Fermentation conditions: pH 7.0, 32°C.
Figure 4.3 Effects of propionic acid on the volumetric productivity of propionate in FBB fermentations with original (○ with dash line) and adapted (● with solid line) cultures.
Figure 4.4 Kinetics of batch free-cell fermentations by original ACK-Tet mutant in high grade glycerol (A); adapted ACK-Tet mutant in high grade glycerol (B); adapted ACK-Tet mutant in crude glycerol (C). Fermentation conditions: pH 7.0, 32°C.
Figure 4.5 Kinetics of batch free-cell fermentations by adapted ACK-Tet mutant in mixture of glucose and glycerol (1:3). Fermentation conditions: pH 7.0, 32°C
Figure 4.6 Differences in cell morphology of original ACK-Tet and adapted ACK-Tet in glucose and glycerol. (A) original ACK-Tet on glucose with regular rod shape (B) original ACK-Tet in glycerol with longer rod shape (C) adapted ACK-Tet in glycerol with longer and slimmer rod shape.
CHAPTER 5

EFFECTS OF CARBON DIOXIDE ON CELL GROWTH AND PROPIONIC ACID PRODUCTION FROM GYCEROL BY PROPIONIBACTERIUM ACIDIPROPIONICI

Summary

With the increased production of biodiesels, large amounts of glycerol are produced as a byproduct with limited use, causing a significant environmental problem. It is thus desirable to use this waste glycerol as a renewable feedstock to produce industrial chemicals and biofuels to replace fossil fuels and petrochemicals. Our previous study demonstrated that Propionibacterium acidipropionici can convert glycerol to propionic acid at high yield and high purity, but with a very low productivity. In this work, the effects of carbon dioxide on propionic acid productivity and cell growth were studied. Based on the metabolic analysis, CO₂(HCO₃⁻) is required in the Wood-Werkman cycle, which determines the propionic acid synthesis. Carbon dioxide and phosphoenolpyruvate (PEP) are catalyzed to form oxaloacetate by the enzyme phosphoenolpyruvate carboxylase. Through several sequential reactions, oxaloacetate is
converted to propionic acid. The volumetric productivity of propionic acid with CO$_2$ (HCO$_3^-$) in glycerol fermentation reached 2.94 g/l/day, which was much higher than that without CO$_2$ (HCO$_3^-$) (1.56 g/l/day). However, the propionic acid yield decreased slightly from 0.77 to 0.67 g/g glycerol due to the higher biomass production. In addition, the yield and productivity of succinate, the main intermediate in Wood-Werkman cycle, increased 81% and 280%, respectively. These results suggested that the increase in the Wood-Werkman cycle rate could be attributed to the effect of CO$_2$ (HCO$_3^-$). In addition, the effect of CO$_2$ (HCO$_3^-$) on the activities of several key enzymes (e.g. phosphoenolpyruvate carboxylase and propionyl CoA transferase) involved in the Wood-Werkman cycle were also studied in this work.

5.1 Introduction

Propionic acid is a weak organic acid and has wide applications. Its salt forms, such as ammonium, sodium, calcium, and potassium salts, are widely used as food and feed preservatives. Its derivatives, for example propionate esters, are applied in production of pharmaceuticals, fragrances, and artificial fruit flavors. Moreover, propionic acid is an important chemical intermediate, widely used in the synthesis of vitamin E, cellulose fibers, and perfumes. Currently, most of propionic acid is produced by petroleum-based chemical synthesis. However, concerns over the sustainability of the crude oil supply have generated interest in investigating alternative ways to produce propionic acid. Bioprocessing is a very promising technology that produces propionic acid from renewable carbon sources, such as agricultural commodities and processing
wastes (Boyaval et al., 1987; Colomban et al., 1993). Like most organic acid fermentations, the production of propionic acid shows low productivity, yield, and final concentration due to the strong end-product inhibition (Goswami et al., 2001; Nanba et al., 1983; Hsu et al., 1991). Several attempts have been tried to enhance the formation of propionic acid through newly improved fermentation processes (Ozadali et al., 1996; Coronado et al., 2001; Jin et al., 1998; Lewis et al., 1992a;b; Yang et al., 1995; Yang et al., 1994; Goswami et al., 2001; Bodie et al., 1987; Morales et al., 2006; Woskow et al., 1991; Lewis et al., 1992c; Hsu et al., 1991; Suwannakham et al., 2005).

According to the Wood-Werkman cycle (Deborde et al., 2000), CO$_2$ is released with acetic acid formation by Propionibacteria from glucose, lactose, or lactate fermentation. The theoretical molar ratio of propionate, acetate and CO$_2$ is 2:1:1 (Hettinga et al., 1972; Wood, 1981). Meanwhile, CO$_2$ can be fixed in Propionibacteria to form oxaloacetate from phosphoenolpyruvate (PEP) catalyzed by PEP carboxylase and then lead to succinate generation (Crow, 1986; Deborde et al., 2000).

With the increased production of biodiesels, large amounts of glycerol are produced as a byproduct with limited use, causing a significant environmental problem. It is thus desirable to use this waste glycerol as a renewable feedstock to produce industrial chemicals and fuels to replace fossil fuels and petrochemicals. Currently, glycerol bioconversion receives more and more attention in fermentative production of reduced biochemicals and biofuels such as hydrogen, ethanol by Enterobacter aerogenes (Ito et al., 2005), succinate by Escherichia coli (Dharmadi et al., 2006), propionic acid by propionibacteria (Chapter 4), and 1, 3 propanediol by Klebsiella pneumoniae (Wang et al., 2003) due to its low price and high reduction state. In contrast to glucose fermentation,
no acetic acid was formed by propionibacteria during the glycerol fermentation due to the redox potential balance in cells (Chapter 4). Therefore, no CO₂ was released in glycerol fermentation.

Extensive studies have been carried out regarding the effect of CO₂ on cell growth and metabolism. It has been reported that exogenous CO₂ serves to stimulate the growth of *Actinomyces viscosus* (Howell et al., 1963 a, b) and the production of succinate (Brown et al., 1980). Dharmadi et al. (2006) demonstrated that CO₂ had a positive impact on the anaerobic growth of *Escherichia coli* and the biosynthesis of small molecules, fatty acids, and central metabolism from glycerol under acidic conditions. On the other hand, inhibition by CO₂ has been shown in cell growth and fermentation of yeast (Jones et al., 1982) and *Penicillium chrysogenum* (Ho et al., 1986). It is worth noting that effects of CO₂ on microbial growth and metabolism are rather complicated and depend on the substrates and CO₂ concentration. The supplemental CO₂ could stimulate cell yields of *Peptostreptococcus productus* U-1 on fructose and decrease cell yields on xylose (Misoph et al., 1996). It was shown by Caldwell et al (1969) that maltose fermentation by *Bacteroides amylophilus* depended on the CO₂ concentration.

In contrast to the numerous studies of CO₂ effect shown above, the effects of CO₂ on *Propionibacteria* growth and end product formation have been minimally investigated. In this work, cell growth, carboxylic acid formation, and enzyme activities were studied in glucose and glycerol fermentations under CO₂-enriched and CO₂-limited conditions. The effect of CO₂ on metabolic pathway of *Wood-Werkman* cycle was also discussed in this paper.
5.2 Materials and Methods

5.2.1 Culture and Media

*Propionibacterium acidipropionici* ATCC 4875 was obtained from the American Type Culture Collection (Manassas, VA). Unless otherwise noted, the bacteria were cultivated in a synthetic medium containing (per liter) 10 g yeast extract (Difco Laboratories, Detroit, MI), 5 g Trypticase (BBL), 0.25 g K$_2$HPO$_4$, 0.05 g MnSO$_4$, and 50-100 g carbon source (glucose or glycerol). The basal medium (without the carbon source) and the concentrated carbon source solution were sterilized separately at 121°C and 15 psig for 30 min to avoid undesirable reactions. They were mixed aseptically before use in the fermentation study.

5.2.2 Fermentation

*Propionibacterium acidipropionici* was cultivated anaerobically at 32°C in 150-ml rubber-stoppered serum bottles containing 100 ml of above medium or in a 5-L fermentor (Marubishi MD-300) containing 2 L of above medium. The fermentor pH was controlled at 7.0 (±0.02) by automatically adding 6 N NaOH. CO$_2$-enriched medium was obtained by purging CO$_2$ in the headspace and adding filter sterilized sodium bicarbonate solution till reaching the saturated concentration at 32°C, pH 7.0, and 1 atm. The CO$_2$-limited medium was maintained in anaerobiosis by sparging N$_2$, instead of CO$_2$, through the medium for 10-30 min at the beginning of the fermentation, initiated by injecting 3.0
ml per bottle or 100 ml per fermentor of exponential-phase cells (OD$_{600}$ ≈ 2.0). Liquid samples (1 ml each) were taken from the fermentation broth at proper time intervals. Batch fermentation kinetics with glucose and glycerol as the carbon source were studied to evaluate the effects of CO$_2$ on cell growth and fermentation performance. Cells cultured with and without CO$_2$ supplementation were harvested at the end of each batch fermentation to examine the intracellular activities of phosphoenolpyruvate carboxylase and propionyl CoA transferase.

5.2.3 Preparation of Cell Extract

*P. acidipropionici* was grown at 32°C in CO$_2$-enriched or CO$_2$-limited medium (50 ml) in serum bottles. Cells were harvested in the exponential phase (OD$_{600}$ ~ 1.8) by centrifuging at 7,000 rpm for 10 min, washed three times and resuspended in 3 mL of ice cold Tris/HCl buffer (25 mM, pH 7.4). The cell suspension was then ultrasonicated using a sonic dismembrator (Fisher Scientific, Model 100). Samples were kept in an ice bath during cell disruption. Socication was conducted 5 seconds, followed by 25 seconds of resting to preventing overheating, for a total of 20 cycles, and then centrifuged at 15,000 rpm at 4°C for 1 h to remove cell debris (Appendix B.5). For phosphoenolpyruvate carboxylase assay, the supernatant was subjected to further centrifugation in an ultracentrifuge (Beckman, Optima TL) for 90 min at 45,000 rpm to remove the gelatinous sediment which contained most of the NADH oxidase activity. The cell extracts were kept cold on ice before they were used in the enzyme activity assays. The protein content
of the extracts was determined in triplicate by Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard protein.

5.2.4 Enzyme Assays

5.2.4.1 Phosphoenolpyruvate carboxylase (PEP C)

The activity of PEP C was assayed according to the method of Maeba and Sanwal (1969) with some modifications (Appendix B.7). Briefly, the assay mixture (300 μl) contained Tris-HCL (pH 9.0, 0.1 M), 0.2 ml; NADH (2 mM), 0.01 ml; MgCl₂ (0.6 M), 0.005 ml; NaHCO₃ (0.6 M, fresh), 0.005 ml; PEP (0.1 M), 0.01 ml; malate dehydrogenase (0.15 mg/ml), 0.01 ml; and the cell extract, 0.06 ml. The reaction was carried out at 25°C and the absorbance at 340 μm was followed throughout the reaction. A cuvette lacking PEP was used as a blank control. The standard unit of PEP C is defined as the amount of enzyme causing an absorbancy change of 1.0 unit per minute, and the specific activity is defined as units per milligram of protein.

5.2.4.2 Succinyl-CoA: Propionate CoA- Transferase (CoA T)

The activity of CoA T was assayed based on the method of Schulman and Wood (1975) with some modifications (Appendix B.7). The assay mixture (250 μl) contained 0.1 ml of Mixture 1 (1.0 M, pH 8.0 Tris/HCl buffer, 1 ml; 0.4 M sodium malate, 0.01 ml; 0.01 M NAD, 1.0 ml; and water to 4 ml), 0.01 ml of 1.5 M sodium acetate, 0.01 ml of
Mixture 2 (944 units/mg of malic dehydrogenase, 14 μl; 355 units/mg of citrate synthase, 11 μl; and 0.1 M, pH 6.8 phosphate buffer, 975 μl), 0.01 ml of 0.15 μmole succinyl-CoA, 0.05 ml of cell extract, and water to 0.25 ml. The reaction was conducted at 25°C followed by measuring the absorbance at 340 nm, which increased linearly with time for 3-5 minutes. A cuvette lacking CoA T was used as a blank control. The standard unit of CoA T is defined as the amount of enzyme causing an absorbancy change of 1.0 unit per minute, and the specific activity is defined as units per milligram of protein.

5.2.5 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-ml cuvette using a spectrophotometer (Shimadzu, UV-16-1). One unit of OD was equivalent to 0.435 g/l cell dry weight. The concentrations of carbon source (glycerol or glucose) and main acid products (acetic, succinic, and propionic acids) in samples were analyzed by high-performance liquid chromatography (HPLC) with an organic acid column (Bio-Rad, HPX-87). HPLC was run at 45°C using 0.01 N H₂SO₄ as the eluant at a flow rate of 0.6 ml/min (Appendix B.3).
5.3 Results

5.3.1 Effects of Carbon Source on Growth and Metabolism of *P. acidipropionici*

Figures 5.1A and B show the fermentation kinetics with glucose and glycerol as the sole carbon source, respectively. As shown in Figure 5.1A, about 13 g/l of propionic acid was synthesized from 40 g/l of glucose after 60-hours. The final cell density (OD600) reached 6.73. In glycerol fermentation, however, only 8.6 g/l of propionic acid was produced by consuming 10 g/l of glycerol after 150 hours (Figure 5.1B). Even though the fermentation was extended to 300 hours, glycerol was not depleted (data not shown). The cell density (OD600) in glycerol fermentation was about 1.0. In addition, different carbon sources affected the production of other organic acids (succinic and acetic acids) as well (Figure 5.1). Both succinic and acetic acids are important metabolites in the propionic acid synthesis process (Figure 5.2). Acetic acid is the main byproduct in the fermentation, and is converted from pyruvate through three consecutive enzyme reactions. Succinic acid is the precursor of propionic acid; its conversion to propionic acid is coupled with propionyl CoA and catalyzed by CoA transferase. This reaction is the rate limiting step of the overall propionic acid synthesis (Suwannakham et al., 2005). Compared with glucose fermentation, glycerol fermentation produced much less succinic acid and no acetic acid (Figure 5.1B). The metabolic pathway shift and its effect on biomass, and production of propionic, acetic, and succinic acids from different carbon sources can be partially attributed to the higher reduction state of glycerol. Because CO₂ release is accompanied by acetic acid synthesis, there should not be any CO₂ generated
when acetic acid pathway is blocked and no acetic acid is produced in the fermentation, as observed in the glycerol fermentation. Indeed, little or no gas production was observed, nor CO₂ could be detected in the headspace of the fermentor when using glycerol as the carbon source. In contrast, a large amount of gas (CO₂) production was observed in glucose fermentation.

5.3.2 Effect of CO₂ on Fermentation with Glucose

*P. acidipropionici* was cultured using glucose as the sole carbon source under both CO₂-riched and CO₂-limited conditions with pH control (at pH 7.0). As expected, CO₂ supplementation did not have significant effect on the main composition of final products from glucose fermentation. Propionic acid was the predominant product, followed by acetic acid and succinic acid. However, succinic acid yield and productivity were significantly enhanced by 33% and 24%, respectively, in CO₂-enriched medium. Also as a subsequent result, propionic acid yield also increased 11% (Table 5.1). In contrast, CO₂ supplementation did not show any effect on acetic acid production from glucose. Moreover, it was worth noting that cell growth rate was a little higher under CO₂-enriched cultivation compared with CO₂-limited cultivation (Table 5.1). This suggested that exogenous CO₂ could stimulate cell growth of *P. acidipropionici*, which was consistent with the previous observation reported by Misoph et al. (1996) and Brown et al. (1980).
To elucidate how propionic acid formation and cell growth were regulated by CO2 in glycerol fermentation, CO2-riched and CO2-limited cultivations were carried out at pH 7.0, respectively. Figure 5.3 shows the comparison of fermentation profiles under CO2-enriched and CO2-limited conditions. The cells cultured in the CO2-limited medium had a long lag phase, followed by a ~50 hours of exponential phase, and then reached the stationary phase after more than 100 hours after inoculation (Figure 5.3A). In contrast, there was no obvious lag phase when glycerol fermentation was supplemented with CO2 (Figure 5.3B). Only in about 50 hours, cell growth reached the stationary phase. Moreover, CO2 stimulated cell growth. Cells grew 3 times faster under CO2-enriched condition than under CO2-limited condition (Table 5.2). However, there was no remarkable difference in biomass production at the end of fermentation processes under both conditions. In addition, CO2 supplementation significantly affected glycerol metabolism. Cells consumed most glycerol in 300 hours of fermentation period when CO2 was added in the medium. About half of glycerol, however, was still in the fermentation broth under CO2-limited condition when cells ceased to grow. Apparently, exogenous CO2 may cause an enhancement in the cell’s capacity to ferment glycerol.

CO2 supplementation had substantial effects on organic acids formation as well. In the presence of exogenous CO2, more propionic and succinic acids were produced (Figure 5.3B). However, acetic acid was not formed from glycerol under both CO2-enriched and CO2-limited conditions (Figure 5.3A, B). The productivities of propionate and succinate were significantly increased, 100% and 200%, respectively, when CO2 was
added in the culture media (Table 5.2). The succinic acid yield also increased 80%, while the propionic acid yield decreased slightly in the presence of CO₂.

**5.3.4 Intracellular Enzyme Activities**

In order to understand the mechanism of regulation of organic acids synthesis associated with CO₂, intracellular activities of two key enzymes in the propionic acid synthesis pathway were determined for cells cultivated in the absence and presence of CO₂ and the results are compared in Figure 5.4. The specific activity of succinyl-CoA:propionate CoA- transferase (CoA T), which is considered as the rate-limiting step enzyme in the propionic acid synthesis pathway, was remarkably increased under the CO₂-enriched condition. In addition, phosphoenolpyruvate carboxylase (PEP C) activity, which catalyzes CO₂ fixation, also showed a considerable increase in the presence of CO₂. These enzyme activity assay results suggested that the observed increases in cell growth and propionic acid production rates in glycerol fermentation could be partially attributed to increases in the key enzyme activities.
5.4 Discussion

5.4.1 Comparison of CO₂ effects on Glucose and Glycerol Fermentations

It has been demonstrated in this work that CO₂ was able to influence cell growth, organic acids formation, and substrate utilization of *P. acidipropionici*. However, the effect extent changed significantly depending on different carbon sources. When using glucose as the sole carbon source, the supplementation of CO₂ had slight effects on cell growth and end product formation profiles, while glycerol fermentation was greatly influenced by CO₂ addition (Tables 5.1 and 5.2). To elucidate the mechanism of CO₂ regulation, the metabolic pathway of organic acid synthesis in *P. acidipropionici* was studied (Figure 5.2). One mole of CO₂ is released accompanying one mole of acetic acid generation. In glucose fermentation, acetic acid was the main byproduct so that CO₂ was produced at the same time. In remarkable contrast, there was no CO₂ released by *P. acidipropionici* in glycerol fermentation due to the high reduction state of glycerol. In glycolysis process, glycerol can generate two-fold more NADH than glucose (Figure 5.2). In order to maintain intracellular redox balance, cells must shift the metabolic pathway to produce more reduced chemicals, i.e., succinic acid and propionic acid (Dharmadi et al., 2006). Acetic acid production was inhibited in glycerol fermentation because one more mole of NADH was generated in that pathway (Figure 5.2). The excessive NADH could inhibit cell growth. No acetic acid formation caused the CO₂ deprivation in glycerol cultivation. These metabolic properties might explain differences in CO₂ effects on glucose and glycerol fermentations. In glucose culture, CO₂ was released from cells and
dissolved in the fermentation broth, which was sufficient to stimulate cell growth and end product formation. Therefore, the exogenous CO$_2$ effect was insignificant. In contrast, the exogenous CO$_2$ substantially enhanced cell growth and glycerol metabolism due to no CO$_2$ production from glycerol by *P. acidipropionici*.

In this work, 4 g/l (0.067mM) of acetic acid was formed from 40 g/l of glucose at the end of the batch fermentation with 2 liters of media. Therefore, 0.134 moles of CO$_2$ was released into the reactor, calculated according to the mole ratio between acetic acid and CO$_2$. The total amount of CO$_2$ included CO$_2$ in the gas phase, CO$_2$ (g), of 3 L headspace filled with N$_2$ originally (see Materials and Methods), CO$_2$ dissolved in the liquid phase, CO$_2$ (l), dissociated HCO$_3^-$ and CO$_3^{2-}$. For the pH range of 5-7, carbonate ion concentration (CO$_3^{2-}$) is negligible (Frick et al, 1999). Thus, the mass balance of total carbon dioxide can be described as follows:

$$\text{CO}_2(g) + \text{CO}_2(l) + \text{HCO}_3^- = 132 \text{ mmol} \quad (1)$$

To obtain CO$_2$ (g), the ideal gas law can be utilized:

$$\text{CO}_2(g) = \frac{p_{CO_2}V_{gas}}{RT} \quad (2)$$

where $V_{gas}$ is the headspace volume; $R$ is the universal gas constant; $T$ is the absolute temperature.

CO$_2$(l) can be determined by Henry’s law as follows:

$$\text{CO}_2(l) = p_{CO_2}S_{CO_2}V_{liquid} \quad (3)$$

where $V_{liquid}$ is the fermentation broth volume; $S_{CO_2} = 2.67E-2$ mmol/mmHg/L, which is the solubility of CO$_2$ in water (Austin et al, 1962).

In the liquid phase we also have: CO$_2$(l) + H$_2$O $\longrightarrow$ HCO$_3^-$ + H$^+$
Assume the reaction is very fast, that means they always at equilibrium, we have

$$K_a = \frac{[H^+][HCO_3^-]}{[CO_2(l)]}$$

or

$$[HCO_3^-] = \frac{[CO_2(l)]K_a}{[H^+]}$$

Thus,

$$HCO_3^- = \frac{p_{CO_2}S_{CO_2}K_a}{10^{-pH}}$$

(4)

where $K_a = 10^{-6.1}$ mol/l, which is the equilibrium constant at 32°C (Harned H. et al, 1945).

By substituting the items in equation (1) with equations (2), (3), and (4), the total amount of CO$_2$ in the reactor can be estimated as follows:

$$\frac{p_{CO_2}V_{gas}}{RT} + p_{CO_2}S_{CO_2}V_{liquid} + \frac{p_{CO_2}S_{CO_2}K_a}{10^{-pH}}V_{liquid} = 132 mmol$$

Therefore, the CO$_2$ partial pressure in the headspace can be calculated according to the fermentation conditions. At 32°C and pH 7.0, $P_{CO_2} = 208 \text{ mmHg}$. Thus, there were 5.6 mM of aqueous [CO$_2$] and 44.4 mM of [HCO$_3^-$] in glucose fermentation broth. On the other hand, under CO$_2$-enriched conditions, the CO$_2$ concentration in the fermentation broth reached saturated state (see Materials and Methods). Thus, in the gas phase $P_{CO_2} = 760 - 47 = 713 \text{ mmHg}$, where 47 mmHg is the saturated water vapor partial pressure. The corresponding aqueous [CO$_2$] in the liquid phase is 19.04 mM and [HCO$_3^-$] is 152 mM. Although there were marked differences in aqueous CO$_2$ and HCO$_3^-$ concentrations in glucose fermentation under CO$_2$-enriched and CO$_2$-limited conditions, the fermentation kinetics from both processes were similar (Table 5.1). These calculated results suggested that CO$_2$ saturated environment might not be necessary to improve propionic acid
production. Several levels of aqueous CO$_2$ concentration should be investigated to optimize the exogenous CO$_2$ supplementation in the future work.

An adapted ACK-Tet mutant of *P. acidipropionici* was obtained in fibrous-bed bioreactor (FBB) during long term glycerol fermentation (Chapter 4). The mutant obtained a higher tolerance to propionic acid and showed metabolic shifts in organic acid production. Acetic acid could be formed from glycerol by the adapted mutant and CO$_2$ production was recovered. The specific growth rate of adapted mutant under CO$_2$-limited condition was similar to that of the wild type under CO$_2$-enriched conditions (0.17 vs. 0.16 h$^{-1}$). This result can also partially support our hypothesis that CO$_2$ was able to stimulate cell growth and organic acid production by *P. acidipropionici*.

It was worth noting that CO$_2$ addition enhanced the productivity of propionic acid and biomass instead of the yield. Besides, more succinic acid was accumulated in the fermentation broth. These results suggested that the mechanism of CO$_2$ regulation might be attributed to the increase of reaction rate in the propionic acid synthesis pathway instead of the effect on the distribution of carbon source. The reaction below was catalyzed by succinyl-CoA: propionate CoA-transferase (E.C. 2.8.3.-):

\[
\text{Propionyl-CoA} + \text{Succinate} \rightarrow \text{Propionate} + \text{Succinyl-CoA}
\]

It is the rate-limiting step in the propionic acid synthesis pathway (Suwannakham et al., 2005). The excessive succinate formed under CO$_2$-enriched conditions may not be converted to succinyl-CoA instantly due to the low activity of CoA transferase and caused its accumulation in the fermentation broth. Therefore, the production of propionic acid might be improved further if CoA T activity can be increased in cells.
The pH effect on CO₂ regulation was also studied. *P. acidipropionici* was cultured in glycerol media under both CO₂-enriched and CO₂-limited conditions without pH control. Fermentation broth pH was gradually reduced with acid formation till cells ceased to grow. There was no obvious difference between with and without CO₂ supplementation (data not shown). Cell growth and end-product formation were inhibited by acidic pH. This suggested that at low pH, the inhibition effect of pH might be more obvious than the stimulation effect of CO₂.

**5.5 Conclusions**

In this work, the effects of carbon dioxide on cell growth and organic acids formation in propionic acid fermentation were investigated. We demonstrated that exogenous CO₂ supplementation can stimulate cell growth and utilization of glycerol. In the presence of CO₂, propionic acid productivity increased about 100% and cell growth increased 2 times. About 40g/l of initial glycerol was almost depleted and much more succinate was produced under CO₂-enriched condition, while only about half of initial glycerol was consumed under CO₂-limited condition. Compared with the CO₂ regulation in glycerol fermentation, exogenous CO₂ had small effects on cell growth and the metabolism of glucose except for the production of succinate. This might because *P. acidipropionici* has the capacity to produce CO₂ from glucose fermentation. The endogenous CO₂ had the similar function as exogenous CO₂ in stimulating the fermentation. The improved cell growth and fermentation rates could be attributed to the increased intracellular activities of CoA transferase and PEP carboxylase.
5.6 References


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Morales Jorge, Choi Joong-So, Kim Dong-Shik, 2006, Production rate of propionic acid in fermentation of cheese whey with enzyme inhibitors, Environmental Progress, 25, 228-234


### Table 5.1 Effects of CO₂ on fermentation kinetics of *P. acidipropionic* on glucose

<table>
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<tr>
<th></th>
<th>With CO₂</th>
<th>Without CO₂</th>
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<tbody>
<tr>
<td><strong>Product yield (g/g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.35 ± 0.002</td>
<td>0.316 ± 0.005</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.058 ± 0.003</td>
<td>0.0592 ± 0.0003</td>
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<tr>
<td>Succinic acid</td>
<td>0.08 ± 0.01</td>
<td>0.060 ± 0.002</td>
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<td><strong>Propionate:acetate ratio (g/g)</strong></td>
<td>6.0 ± 0.1</td>
<td>5.3 ± 0.1</td>
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<tr>
<td><strong>Productivity (g/L·h)</strong></td>
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</tr>
<tr>
<td>Propionic acid</td>
<td>0.2 ± 0.01</td>
<td>0.209 ± 0.001</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.033 ± 0.003</td>
<td>0.029 ± 0.004</td>
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<tr>
<td>Succinic acid</td>
<td>0.047 ± 0.004</td>
<td>0.038 ± 0.002</td>
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<tr>
<td><strong>Specific growth rate (h⁻¹)</strong></td>
<td>0.13 ± 0.01</td>
<td>0.11 ± 0.01</td>
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</table>
Table 5.2 Effects of CO$_2$ on fermentation kinetics of *P. acidipropionic* on glycerol.

<table>
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<tr>
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<th>With CO$_2$</th>
<th>Without CO$_2$</th>
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</thead>
<tbody>
<tr>
<td><strong>Product yield (g/g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.7 ± 0.04</td>
<td>0.77 ± 0.01</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.076±0.02</td>
<td>0.042±0.002</td>
</tr>
<tr>
<td><strong>Productivity (g/L·h)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.12 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.011±0.004</td>
<td>0.003±0.001</td>
</tr>
<tr>
<td><strong>Specific growth rate (h$^{-1}$)</strong></td>
<td>0.12 ± 0.03</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>
Figure 5.1 Kinetics of batch fermentations by *P. acidipropionici* from glucose and glycerol. Glucose fermentation (A); Glycerol fermentation (B).
Figure 5.2 Proposed metabolic pathways in propionic acid fermentation of glycerol by *P. acidipropionici*. The incorporation of CO₂ with PEP to produce oxaloacetate can produce additional energy (GTP) and thus increase cell growth and metabolic rates. (1) Propionyl CoA:succinyl CoA transferase; (2) PEP carboxylase.
Figure 5.3 Comparison of CO₂ effects on cell growth, glycerol utilization, and carboxylic acids production by *P. acidipropionici* at pH 7.0, 32°C in CO₂-limited (A) and CO₂-enriched media (B).
Figure 5.4 Comparison of CoA transferase (CoA T) and PEP Carboxylase (PEP C) activities of *P. acidipropionici* under CO₂-enriched and CO₂-limited conditions.
CHAPTER 6

MOLECULAR CHARACTERIZATION OF COENZYME A TRANSFERASE GENE FROM PROPIONIBACTERIUM ACIDIPROPIONICI AND ITS EXPRESSION IN ESCHERICHIA COLI

Summary

Propionyl-CoA:succinate CoA transferase (CoAT, EC# 2.8.3.-) of Propionibacteria acidipropionici ATCC 4875, which transfers a coenzyme A moiety from propionyl-CoA to succinic acid to form propionic acid and succinyl CoA, plays a very important role in propionic acid synthesis in this microbe. The reaction catalyzed by CoA transferase is the rate-limiting step in the propionic acid formation pathway. The whole genome of Propionibacteria acidipropionici ATCC 4875 was sequenced by 454 sequencing and annotated. A DNA sequence containing a single open reading frame of 1512 bp, which encodes a 504 amino acid protein, was identified to be the CoA transferase gene. This gene was then obtained by PCR amplification and inserted into an expression vector pET-CoA. With IPTG induction, the CoA transferase gene was overexpressed in Escherichia coli BL21 (DE3) under both aerobic and anaerobic conditions. However, CoA transferase activity was only observed in crude extracts of
BL21 (DE3) harboring pET-CoA grown anaerobically due to high sensitivity of CoA transferase of \textit{P. acidipropionici} to oxygen. In addition, a consensus Shine-Dalgarno sequence was found four bases upstream of the AUG codon and two inverted repeat regions were located at the downstream of the TGA stop codon. The amino acid alignment of \textit{P. acidipropionici} propionyl-CoA:succinate CoA transferase with other reported CoA transferases illustrated the presence of conserved sites in the amino acid sequence for CoA binding.

6.1 Introduction

\textit{Propionibacteria}, which were isolated from cheese and milk, are Gram-positive, non-spore forming, rod-shaped, anaerobic bacteria. They are widely used in the cheese industry and in the production of vitamin B\textsubscript{12} (Langsrud et al, 1973; Florent et al., 1979) due to their non-pathogenic property and GRAS (generally recognized as safe) status granted by FDA. \textit{Propionibacterium acidipropionici}, one of dairy \textit{Propionibacteria}, is the most used species for propionic acid production. Several attempts have been done to improve propionic acid production by overcoming the end product inhibition (Bodie et al., 1987; Coronado et al., 2001; Goswami et al., 2001; Hsu et al., 1991; Jin et al., 1998; Lewis et al., 1992a; b; Morales et al., 2006; Ozadali et al., 1996; Suwannakham et al., 2005; Woskow et al., 1991; Yang et al., 1995; Yang et al., 1994). However, most of the research has been focusing on the conventional fermentation process development. Currently, strain genetic modification is a promising technique extensively used to improve natural products production or to produce new products from organisms.
Suwannakham et al. (2006) constructed an *ack*-knock out mutant of *P. acidipropionici* which increased propionic acid yield by 13% and decreased acetic acid production by 14%. Due to the reduced acetic acid production in the fermentation broth, the fermentation of *ack*-knock out mutant can facilitate downstream processing and reduce purification costs significantly. Nevertheless, the lack of gene information in *Propionibacteria* has seriously hampered the utilization of genetic technique in this organism.

Recently, the whole genome of *P. acidipropionici* ATCC 4875 was sequenced by 454 sequencing and annotated. The gene sequence for the rate-limiting step enzyme propionyl-CoA:succinate CoA transferase (CoA T, EC# 2.8.3.-) in the propionic acid production pathway was identified. CoA transferase catalyzes a reversible transfer of CoA moiety from one carboxylic acid to another at the expense of CoA thioester. All organisms possess CoA transferases. However, the enzyme’s substrate specificities and activities are quite different (Cary et al., 1990; Wiesenborn et al., 1989; Yoo et al., 2001). These enzymes can be divided into three enzyme families (Heider, 2001). The CoA transferase (acetoacetyl-coenzyme A: acetate/butyrate-coenzyme A transferase, EC 2.8.3.9) of *Clostridium acetobutylicum* ATCC 824, belonging to family I, plays an important role in detoxifying by uptaking acetate and butyrate formed in the earlier fermentation phase (Cary et al., 1990). Most of CoA transferases in family I catalyze the reaction with succinyl-CoA or acetyl-CoA as CoA donors via the ping-pong mechanism (Heider, 2001; Lloyd et al., 2001). In contrast, the reactions catalyzed by CoA transferases of family II occurs via a ternary complex mechanism without covalently bound intermediates formation (Heider, 2001). Citrate lyase and citramalate lyase belong
to family II, which comprises the homodimeric α-subunits (Heider, 2001; Dimroth et al., 1975). Family III of CoA transferase was demonstrated recently, in which the members have completely different amino acid sequences from those in families I and II (Heider, 2001). The amino acid sequence alignment of CoA transferases of family III shows a 22-37% similarity (Heider, 2001). Members characterized in family III so far are all from anaerobic or facultative anaerobic bacteria, such as *Oxalobacter formigenes, Thauera aromatica, Clostridium difficile, and Escherichia coli*.

In this study, the DNA sequence of CoA transferase gene (1512 bp) of *P. acidipropionici* was identified and obtained by PCR amplification and then overexpressed in *E. coli*. The activity of CoA transferase was assayed under both aerobic and anaerobic conditions. In addition, the gene sequence and upstream/downstream nucleotide sequences were analyzed. Several conserved amino acid fragments were found as well by alignment of several reported CoA transferases. The genetic information obtained from this study can be used in metabolic engineering of this organism for enhanced propionic acid fermentation.

### 6.2 Materials and Methods

#### 6.2.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed with their characteristics and sources in Table 1.
6.2.2 Media and culture conditions

P. acidipropionici was cultured anaerobically at 32°C in the medium containing 1% yeast extract, 0.5% trypticase, 0.025% K₂HPO₄, 0.005% MnSO₄, and an appropriate amount of carbon source. All E. coli strains were grown at 37°C aerobically in 250 ml flasks or anaerobically in 150 ml serum bottles containing Luria broth (LB) (Miller, 1972) with 100 μg/ml of ampicillin when necessary. Both recombinant and wild-type strains were stored at -85°C in 15% (v/v) glycerol (Sambrook J et al., 1989).

6.2.3 DNA isolation and manipulation

Chromosome DNA of P. acidipropionici, used as PCR template, was isolated using the QIAGEN genomic DNA kit (Qiagen, Valencia, CA). Plasmid isolation from E. coli was performed using QIAprep MiniPrep plasmid purification kit. DNA fragments for subcloning were extracted from gel by QIAquick gel extraction kit. Restriction endonucleases and T4 DNA ligase were purchased from Invitrogen and used according to the manufacturer’s instructions (Appendix D).

6.2.4 PCR amplification

PCR primers were designed using the software Primer 3 based on the DNA sequence of CoA transferase gene obtained from 454 sequencing. Three nucleotides A, G, and T were substituted by C, C and G (italic) respectively to generate an internal NcoI
restriction site (underlined) in the forward primer (5’-AGGACCCATGGCAGATCGGATTGCAAC-3’). In the reverse primer (5’-GGTCCATAGCCTGGACGCAGAAC-3’), two nucleotides C and G were substituted by A and T respectively (italic) to generate an internal NdeI restriction site (underlined). The reaction system (50 µl) contained 5 µl of 10×PCR buffer, 1 µl of 10 mM dNTP, 1.5 µl of 50 mM MgCl₂, 1.5 µl of 10 µM forward and reverse primers, 2 µl of genomic DNA, 2.5 µl of PCR enhancer, and 1 µl of Platinum® Pfx DNA polymerase. The reaction parameters were set as: one cycle at 95°C for 10 min followed by 10 cycles of denaturation at 95°C for 1 min, annealing at 68°C for 30 s, and extension at 72°C for 1 min and then 20 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 30 s, and extension at 72°C for 1 min. Subsequently, 1 µl of Taq DNA polymerase was added in the reaction system and incubated for another 30 min, 4°C hold. The PCR products with an expected size of ~1.5 kb were purified via 0.7% (w/v) agarose gel electrophoresis. The gel extracted PCR fragment was ligated into pGEM-T vector and verified by restriction enzyme analysis and DNA sequencing.

**6.2.5 DNA sequencing and analysis**

DNA fragment sequence in plasmid was determined by the dideoxy chain termination method performed at the Plant-Microbe Genomics Facility (PMGF), the Ohio State University. The whole genome of *P. acidipropionici* ATCC 4875 was sequenced by using 454 sequencing technology and annotated at J. Craig Venter Institute (Maryland).
6.2.6 Cloning CoA transferase gene in \textit{E. coli}

The PCR product containing the open reading frame of CoA transferase was inserted into pGEM-T vector (Promega) to construct plasmid pGEM-CoA (Figure 6.1). Then, the CoA transferase gene was cut with \textit{NcoI}-\textit{NotI} and ligated into the same sites of pETDue-1, downstream of \textit{T7} promoter, to form plasmid pET-CoA (Figure 6.1). The recombinant plasmid, pET-CoA, was transformed into \textit{E. coli} BL21 (DE3). The recombinant mutants were selected by ampicillin resistance.

6.2.7 Gene expression

For protein overexpression experiments, 100 \(\mu\text{g/ml}\) of ampicillin and 30 \(\mu\text{g/ml}\) of chloroamphenicol were supplemented in LB medium when BL21 (DE3)pLysS harboring pET-CoA was cultivated under aerobic or anaerobic conditions. 0.5 ml of overnight culture was inoculated in 4.5 ml of fresh LB medium and incubated at 37°C until its OD600 reached 0.6. Then, 0.5 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) was added into the medium and cultured at 16°C for another 2 hours. For rifampin studies, rifampin was added according to the method described previously (Nair et al., 1994). The induced cells were harvested by centrifugation at 10,000 rpm for 5 min at 4°C.
6.2.8 Cell extract preparation and enzyme assays

About 50 ml of cells in the exponential phase were harvested by centrifuging at 7,000 rpm for 10 min and washed three times, and then resuspended in 3 mL of ice cold Tris/HCl buffer (25 mM, pH 7.4). The cell suspension was then ultrasonicated using a sonic dismembrator (Fisher Scientific, Model 100). Samples were kept in an ice bath during cell disruption. Sonication was conducted for 5 seconds followed by 25 seconds of resting to prevent overheating, total 20 cycles, and then centrifuged at 15,000 rpm, 4°C for 1 h to remove cell debris. Cell extracts were kept cold on ice before they were used in the enzyme activity assay. The protein content of the extracts was determined in triplicate by Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard protein.

The activity of CoA transferase was assayed based on the method of Schulman and Wood (1975) with some modifications. The assay mixture (250 μl) contained 0.1 ml of Mixture 1 (1.0 M, pH 8.0 Tris/HCl buffer, 1 ml; 0.4 M sodium malate, 0.01 ml; 0.01 M NAD, 1.0 ml; water to 4 ml), 0.01 ml of 1.5 M sodium acetate, 0.01 ml of Mixture 2 (944 unit/mg of malic dehydrogenase, 14 μl; 355 unit/mg of citrate synthase, 11 μl; 0.1M, pH 6.8 phosphate buffer, 975 μl), 0.15 μmole (in 0.01 ml) of succinyl-CoA, 0.05 ml of cell extract, and water to 0.25 ml. The reaction was conducted at 25°C, followed by measuring the absorbance at 340 nm, which increased linearly with time for 3-5 minutes. A cuvette lacking CoA T was used as a blank control. One standard unit of CoA T is defined as the amount of enzyme causing one micromole NADH increase per minute, and the specific activity is defined as units per milligram of protein.
6.2.9 SDS-PAGE

Protein samples or cell pellets were mixed with SDS-PAGE loading buffer and put in boiling water for 10 min. After 10 min centrifugation at 15,000 rpm, 4°C, the supernatant was used for SDS-polyacrylamide gel electrophoresis (Mini-PROTEAN® 3 Cell, Bio-Rad) according to the protocol of Bio-Rad. The gels were stained with Coomassie brilliant blue (Appendix D.13).

6.3 Results and Discussion

6.3.1 Sequencing and identification of CoA transferase gene from *P. acidipropionici*

The whole genome of *P. acidipropionici* ATCC 4875 was sequenced and assembled in 245 contigs using 454 Sequencing System, so-called second generation sequencing technologies (Droege et al., 2008). The estimated genome size is about 3.66 Mbp, which is slightly higher than the average genome size of *Propionibacteria* reported previously (Gautier et al., 1992; Klaenhammer et al., 2002; Rehberger, 1993; Brüggemann, 2004; Meurice et al., 2004). In the predicted and annotated putative genes, the CoA transferase gene was identified, which contains a single open reading frame of 1512 bp encoding a 504 amino acid protein. The complete nucleotide and deduced amino acid sequences of the CoA transferase gene, which begins with a methionine codon and ends with a TGA stop codon, are presented in Figure 6.2. With respect to the sequence data presented, the G + C content in the ORF of CoA transferase gene was 64%, which was in
the G + C content range of 53-67% previously reported for the genus *Propionibacteria* (Sneath et al., 1986). The distances between CoA transferase gene and the upstream or downstream adjacent gene ORFs are 161 bp and 186 bp, respectively, indicating that these three neighboring genes may not constitute an operon. In addition, the annotated up- and down-stream genes were putative aldose 1-epimerase subfamily and nucleoside 5'-monophosphatephosphohydrolase, respectively, which may not be related to CoA transferase or CoA hydrolase. These results suggest that CoA transferase in *P. acidipropionici* is a monomer, which is similar to the formyl-CoA: oxalate CoA transferase from *Oxalobacter formigenes* (Sidhu et al., 1997; Baetz et al., 1990) and propionate CoA transferase (Selmer et al., 2002).

The putative ribosomal binding site (Shine-Dalgarno sequence, AGGAGG) is located 5 nucleotides upstream of the start codon AUG (Figure 6.2). Two consensus sequences, CGCACA and CCGTTG, were found at the upstream of ORF corresponding to putative -35 and -10 regions, respectively, by comparison of the promoter sequences of *Propionibacterium freudenreichii* reported by Piao et al. (2004). The presence of putative -35 and -10 regions suggests that the upstream nucleotide sequence functions like promoter elements. Besides, two inverted repeats (11 bp and 12 bp) were found in the promoter region (Figure 6.2), which may form the potential stem-loop in the promoter element as a transcriptional enhancer (Piao et al., 2004). On the contrary, no consensus sequences of ACGCGCA (Piao et al., 2004) or 5’-TG-3’ (Graves et al., 1986) were observed, corresponding to putative -16 regions, in the promoter element. Since the -16 region plays an important role in compensating for weak promoters in gram-positive bacteria (Piao et al., 2004; Graves et al., 1986), the absence of consensus -16 region
indicates that the promoter for CoA transferase gene in *P. acidipropionici* may not be strong. This characteristic is consistent with the report of Suwannakham et al (2005) that CoA transferase catalyzed a rate-limiting step in propionic acid synthesis in *P. acidipropionici*. Several inverted repeats (agcttgttctgagct; gcgccccgccagccgcccc) were found in the downstream of the CoA transferase gene, 9 bp and 32 bp distances from the TGA stop codon (Figure 6.2), respectively, suggesting that a termination sequence (Yanofsky, 1981) is in the downstream.

### 6.3.2 Amino acid sequence comparison

The deduced amino acid sequence of putative *P. acidipropionici* CoA transferase indicates that this protein has a calculated molecular weight of 55.2 kDa and an isoelectric point (pI) of 5.62. The predicted amino acid sequence was used to search the NCBI data base using the NCBI BLAST 2 software. Significant homology could be observed between the query sequence of *P. acidipropionici* and the Coenzyme A transferase/hydrolase of several prokaryotic species. Except for the highest identity (83%) to the putative succinyl-CoA or butyryl-CoA:coenzyme A transferase from *Propionibacterium acne*, which is in the same genus with *P. acidipropionici*, the range of identity to other prokaryote CoA transferases was approximately 49-61%. However, no significant similarity was found with CoA transferases from eukaryotes such as pig heart succinate-CoA transferase (Bailey et al., 1993) or human succinyl CoA:3-oxoacid CoA transferase.
Figure 6.3 shows the alignment of putative *P. acidipropionici* CoA transferase with some reported analogous CoA transferases, such as *Propionibacterium acnes* (Brüggemann et al., 2004), *Saccharopolyspora erythraea* (strain NRRL 23338) (Oliynyk et al., 2007), *Corynebacterium diphtheriae* (Cerdeno-Tarraga et al, 2003), *Corynebacterium jeikeium* (strain K411) (Tauch et al., 2005), *Corynebacterium efficiens* (Nishio et al., 2003), *Azoarcus sp.* (strain BH72) (Krause et al., 2006) and *Pseudoalteromonas atlantica* T6c (Copeland et al., 2006). From the result, several highly conserved regions, which relate to the structure-function of CoA transferase during evolution, were located.

A glycine cluster \(\text{D-G-D-Q-I-G-F-G-F-T-G-S-G-Y-P}\) (Figure 6.3, region 1) was observed in the N-terminal amino acid sequence of putative propionyl-CoA: succinate CoA transferase of *P. acidipropionici*. This cluster is significantly identical to the consensus sequence (Prosite PS01273: [DN]-[GN]-x(2)-[LIVMFA](3)-G-G-F-x(3)-G-x-P) reported by Weirenga et al (1986). One more glycine was found in the *P. acidipropionici* glycine cluster (shaded letter). It has been demonstrated that the evolutionary conserved sequence is one of the CoA transferase signature patterns, which corresponds to a binding of CoA to the N-terminal amino acid sequence of CoA transferase (Parales et al., 1992). The same sequence was also identified in the CoA transferases of many prokaryotes and in mammalian tissues, such as acetate CoA-transferase subunit alpha of *Escherichia coli* (strain K12) (Itoh et al., 1996), butyrate--acetoacetate CoA-transferase subunit A of *Clostridium acetobutylicum* (Fischer et al, 1993), succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A of *Xanthomonas campestris pv. campestris* (strain B100) (Steinmann et al., 1997), succinyl-CoA:3-ketoacid-coenzyme A transferase 2, mitochondrial precursor of human (Tanaka et al., 1997).
2002), succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial precursor of mouse (Tanaka, 2003), and succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial precursor of rat (MGC Project Team, 2004). In addition, another conserved glycine-rich region (G-V/I-G-G-S-G/A) was found in the amino acid sequence (Figure 6.3, region 3), which was highly similar to the reported conserved motif (G-x-G-x-x-G) for nucleotide binding (Wierenga et al., 1986; Söhling et al., 1996).

In contrast, the second Coenzyme A transferase signature pattern (Prosite PS01274: [LF]-[HQ]-S-E-N-G-[LIVF](2)-[GA]), which contains a glutamate (E) that is involved in the catalytic mechanism in N-terminus (Rochet et al., 1994; Jencks, 1973; Parales et al., 1992), could not be observed in the sequence alignment of *P. acidipropionici* CoA transferase. Lin et al. (1992) reported that the conserved active site of glutamate was the probable CoA esterification site. CoA transferase of *P. acidipropionici* was not the only one missing the consensus motif S-E-N-G. In CatJ of *Pseudomonas* sp. Strain B13, this motif was also absent (Gobel et al., 2002). Meanwhile, Mack et al. (1994) reported that no S-E-N-G was detected in the glutaconate-CoA transferase of *Acid-aminococcus fermentans*. However, a similar motif (S-E/Q/H-x-G) could be found at position 126 (Figure 6.3, region 2), which might be responsible for the transferase catalytic site.

**6.3.3 Overexpression of the CoA transferase gene in *E. coli***

To overexpress the putative CoA transferase of *P. acidipropionici*, the PCR reaction was carried out using the genomic DNA of *P. acidipropionici* as template (see
Materials and Methods). The sequence of CoA transferase gene fragment from PCR amplification was confirmed by the dideoxy chain termination method. The recombinant cells were cultured at 16°C under both aerobic and anaerobic conditions with the presence of 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). Protein samples were prepared according to the protocol described in Materials and Methods. The supernatants of protein solution were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As can be seen in Figure 6.4, CoA transferase gene was overexpressed under both aerobic and anaerobic growth conditions after IPTG induction (Lanes 4 and 7). The distinct bands in Lanes 4 and 7 have the same molecular weight of approximately 55 kDa as that of P. acidipropionici CoA transferase.

6.3.4 Propionyl-CoA: succinate CoA transferase activity

Crude cell lysate of E. coli BL21 (DE3) harboring pET-CoA was assayed for CoA transferase activity. The propionyl-CoA: succinate CoA transferase activity was determined by measuring the formation rate of acetyl-CoA, which equals to the rate of formation of NADH in the presence of malate dehydrogenase and citrate synthase (Schulman et al., 1975). No CoA transferase enzyme activity was detected under aerobic culture conditions in the preliminary experiment, which indicated that either the protein itself did not function in E. coli or oxygen might affect the activity of the enzyme. Previous study by Yan et al. (1990) showed that an aldehyde dehydrogenase (ALDH) from Clostridium beijerinckii NRRL B592 was sensitive to oxygen since the gene was from an anaerobic organism. Thus, the enzyme assay was repeated with cells cultured
under anaerobic conditions. Meanwhile, to reduce the background activity of the host, rifampin was added to selectively inhibit the host RNA polymerase (Nair et al., 1994; Tabor et al., 1985). As shown in Figure 6.5, propionyl-CoA: succinate CoA transferase activity was detected in the mutant carrying the plasmid pET-CoA under these conditions, whereas no activity was detected in the wild type. This result confirmed that the cloned CoA transferase gene can be expressed in *E. coli*. These results also suggest that the CoA transferase of *P. acidipropionici* is very sensitive to oxygen.

6.4 Conclusions

The gene encoding propionyl-CoA:succinate CoA transferase (CoA T, EC# 2.8.3.-) of *Propionibacteria acidipropionici* ATCC 4875 was identified and characterized in this work. With IPTG induction, the CoA transferase gene was transcribed from *T7* promoter in *Escherichia coli* BL21 (DE3). The overexpressed enzyme of ~55 kDa was confirmed by SDS-PAGE. Only under anaerobic growth conditions, CoA transferase activity was observed in crude extracts of BL21 (DE3) harboring pET-CoA, which demonstrated that the CoA transferase of *P. acidipropionici* was very sensitive to oxygen. The amino acid alignment of the *P. acidipropionici* propionyl-CoA:succinate CoA transferase with some other reported CoA transferases illustrated the presence of conserved sites in the amino acid sequence for CoA binding.
6.5 References


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<td>Strains</td>
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</tr>
<tr>
<td><em>P. acidipropionici</em></td>
<td>Wild type</td>
<td>ATCC 4875</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>F- <em>ompT hsdS</em><em>{B}({r}</em>{B\cdot}m_{B}) dcm gal(DE3) pLysS Cm'</td>
<td>Novegen</td>
</tr>
<tr>
<td>DH5α</td>
<td>F- φ80lacZΔM<em>15</em> Δ(lacZYA-argF)<em>U169</em> <em>recA</em>1 endA1 <em>hsdR</em>17(rk-, mk+) phoA <em>supE</em>44 thi-1 <em>gyrA</em>96 <em>relA</em>1 λ-</td>
<td>Invitrogen</td>
</tr>
<tr>
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<td>Ap'; ColE1 replicon; T7 promoter</td>
<td>Novagen</td>
</tr>
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<td>This study</td>
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Table 6.1. Bacterial strains and plasmids
Figure 6.1 Construction of gene expression plasmid pET-CoA.
gacccctcccccgcggcggcggcgccgtcctctactcttt-121
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-35
gcccggaagggctaaggggagacatggtgagcaggctgggaag-31

--5' upstream primer ----->
atgtcagatcgatgatgcccaagacggctcagaggtgtaggtgtacgcatgagcaagcg

MSDRIANEALRQKVMSADDGACgtcctctaatcagagctgttcccgaacactctcccggttacggttcggctac
ASLIHDGDLQIGFGGFTGSGY

Ccagaaggttcgccgaggctcttccaagcgcataacgcccacgcagaagcggag
PKFPPPALAKRITAAHEKGE

Cacttcacccgtcagccttcaccggccgctttaccgagcaggtcgcaggggctctg
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AAGCDGIGMRSPYQSDPSTMRA

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VSPSTAK stain VPMVS
Gtcgaccacccaggacacagagatgtctcactcaccgagccagggtagctccggatctccac
VDHHEHTMVIITEQGIAIDL
Ctgtgcactggccccgccccggccgacgacgcgcggcagatcaggtctcaccddggcag
RGLAPRQRAPKIIDNCAHPD

(Continued on the next page)
Figure 6.2 Nucleotide and translated amino acid sequences of CoA transferase gene and flanking region from *P. acidipropionici* ATCC 4875. Bold letters represent the putative ribosome binding site. Single-underlined letters in the upstream of the open reading frame represent the inverted repeats. The putative -35 and -10 regions are indicated by double-underlined letters, respectively. The termination sequences are shown in color shaded.
Consensus

C.diphther
P.acnes
P.acidipro
S.erythrae
C.efficien
I

A.sp.
MSNL SNPAIVLS PRLRNKIMSA EAALIIPSC VNVGMGSGFTG AGYPKVFAA
Pseudoalte
MLFLSRVEC PILKRMIMA EEEAFTEDQ QTISMSGFTG AGYPASIIPAS
C.dipther MACTAADVVE EDLMSDRIAN AQAIQRKMSA EEAQYFHNG DKKWVSFTG AGYPKLAPT
C.jeikeium
MSERIAN EYFYSKVMSA EEAQYFHNG DKKWMSGFTG AGYPKLAPT
C.efficien
MSRIAS EKLRSKVMSA EEAQYFNAG DKKVMSGFTG AGYPKLAPT
Consensus .......... ...ms..R!an ..ir.K!msa #eAaaf/!...g d./GmsGFTG AGYPKa.P.a

1

P.acidipro
MSDRIAN EAALQKVMASA DDAASLIHDG DQIGEGGFSTG SGYPKEPPFA
P.acnes
MSRERIAN EAALQKVMASA DDAALIHDG DQIGEGGFSTG SGYPKEPPGA
S.erythrae
MRJRI ADKADKEEA EEAAAMIEP ANIGMSGFTG AGYPKVEPAA
A.sp.
MSNL SNPNADVLS PRLRNKIMSA EAALIIPSC VNVGMGSGFTG AGYPKVFAA
Pseudoalte
MLFLSRVEC PILKRMIMA EEEAFTEDQ QTISMSGFTG AGYPASIIPAS
C.dipther MACTAADVVE EDLMSDRIAN AQAIQRKMSA EEAQYFHNG DKKWVSFTG AGYPKLAPT
C.jeikeium
MSERIAN EYFYSKVMSA EEAQYFHNG DKKWMSGFTG AGYPKLAPT
C.efficien
MSRIAS EKLRSKVMSA EEAQYFNAG DKKVMSGFTG AGYPKLAPT
Consensus .......... ...ms..R!an ..ir.K!msa #eAaaf/!...g d./GmsGFTG AGYPKa.P.a

60

P.acidipro
LAKRITAAE KEHFTVNAF TGASTAPELD GLVAGVQIDG MRSPYQSDPT MRAKINDOTS
P.acnes
LAKRIQESHG RGEKTYTVNF GKLVAGVQIDG WMPYQSDPQ MRSKYINDOTS
S.erythrae
LAKRLAEAGQ ACQPAKVALM TGASTAPELD GLVAAADGID LMPYQSDPV SEKINSAGM
A.sp.
LAKRMDMNL NCKKFINQVW TGASTAPELD GLAMVDGVE LRKINAGEM
Pseudoalte
LEAKAKALHS NOQFPKINLQ TLAVASVQKIN MRLPYSQDP VLQCKINGIM
C.dipther IAEFAKAAH RCDMYIDLF TGASTAPCDL GLVAEADALR FRTVPQSDV LRKINDOTT
C.jeikeium
IAEKAKELKH RGKIVQFIDLQ TGASTAPCDL GLVAEADAVR YRTPYSQDPK MKKINAGEI
C.efficien
IANRAKAAH AGSEYILDPF TGASTAPCDL GLVAEADAVN FHPMPYSQDPK MRKINEGMK
Consensus lA.rak..h .G..f.!.f.1f TGASTAP#1D GvLa.aDg...R.PyqSDP...R.kIIn.Gm

120

P.acidipro
FYTDHLSQF GMVQREGFSG KLYAVIEAT KITAGDACNP TSSVGNNAVYVEKAKEIIIE
P.acnes
FYTDHLSHE GMVQREGFSG KLYAVIEAT RITACDVVL TSSVGNNAVYCDKAEVIIE
S.erythrae
DYTDHLSHV AGRQVQGGFF GLDVAQVEVS GITEDQRVLV TSSVGNNWTVW ICAKDVIL
A.sp.
EYLDMLHCVN AQFWVQGL NLDVAQEVA GILIDQRLP TSSVGNNWTVW LDAKDVIL
Pseudoalte
FYIVDHLSSG QSYQNGFGQ LNMDVIEIA GILADGRNPS TSSVGNNWTVW VEGAKDVE
C.dipther LYADHLSES SYIYEQGFGF QMNVAIEAV RITEGHIHP TSSVGNNWVY VNAKEIKEIE
C.jeikeium
KYQDIHLSHL GQVEQFSGF QLDVAIEAV RIDEAGNIP TSSVGNVNEY LMARKIKIE
C.efficien
FYSDHLSHQS SYQVENGFQ KLNVAIEAV RITEGHIHP TSSVGNNWVY VNAKEIKEIE
Consensus .Y.DIHLS. gg.v...GFQ k1#vA!!Es. .Ited.0.1ip sSvGNN...y 1#.A.K!IIE

180

P.acidipro
VNMQSESDELE GMHDIIYFYA LPPNVVIPIH THPDGRICET FLRVPQKIVV AIETIHPDOR
P.acnes
VNMQSESDELE GMHDIIYFYA LPPNVVIPIH THPDGRICKT FLIBVPQKIVV AIETIAGPDOR
S.erythrae
VNMQSESHELE GMHDIIYFTA LPPRERPITL THPDADICP YLEPCPEKVW AVRISIISOR
A.sp.
VNSKNQPALE GMHDIIYOTA LPPYKKLPL TKDLTDRQEP YLRCDDKKI AVETHTHOR
Pseudoalte
VNTQNNKMLE GMHDIYFAPA LHPRELKPLD NSPSRIRGK LSYLVPVEKV AIETSAIPOR
C.dipther
VNMQSESLEE GMADILRMKQ LPFNPQIPPI TEAQGRIIGT YIDYLDKVKV AVETPSRAPOR
C.jeikeium
VNMQSLDELE GMHDIIYKIQD LPFNPRTQIP NRPDRITGRT YIPVDVGKVNA AKTVNAEDR
C.efficien
VNMQSADLE GMHDIIYMPF LPNRAIIPK NAAQRIRIGA YIEPDYDJKV AVETNTDOPOR
Consensus VNSwQs.. Le GmHIYYQ.a LPPnR.pPip ..pg#RIG.p %i...d...K!! A!eT..pDOR

240

P.acidopro
NPFFKPLDED SHKAGYLLD FYANAEVHEGR MKPKNNLLQPS GVGNIPNAV LDDGLHLSDLH
P.acnes
NPFPKPLDED SHKAGYLLD FYANAEVHEGR MKPKNNLLQPS GVGNIPNAV LDDGLHLSDLH
S.erythrae
NTVPFDPPDV SRAIACHVFLD FLDELVAAGR LFPSLLQPS GVGNVNAV AGRIGEPFEG
A.sp.
NSAFASEP DEN SRJAHFFH LEHVEHVIGR LPPNLNLLQPS GVGNIANAV AGMNEGEFDN
Pseudoalte
NPFTPFKQSF NQIAEHHDLD FFSHAEIKGR LFKELPLLQPS GVGNIPNAV SLEGQQYGRN
C.dipther
NPPFKPLDET SKQIAHFFLD FLKGMVAGR LTYDGYVQPS GVGNIPNAV AGLLDSFKEN
C.jeikeium
NPPFKPLDET SKQIAHFFLD LLLRGEVDAGR LTYDGYVQPS GVGNIPNAV AGLLDSFKEN
C.efficien
NPPFKNVQED SQQJACHFNL FLKGVEVAGR LSYDGYVQPS GVGNIPNAV AGLLDSFKEN
Consensus N.pFk.Dev S..IAgh.L# fle.B!..agr lp..lip.QS GVGN!pNAV# aGL1.s.fen

(Continued on the next page)
Figure 6.3 Alignment of CoA transferases/hydrolases. Alignment of the gene product from Propionibacterium acidipropionici with the gene products from Propionibacterium acnes, Saccharopolyspora erythraea (strain NRRL 23338), Corynebacterium diptheriae, Corynebacterium jeikeium (strain K411), Corynebacterium efficiens, Azoarcus sp. (strain BH72), and Pseudoalteromonas atlantica T6c. High consensus in red, low consensus in blue. Region 1 represents the proposed CoA binding site, CoA transferase signature pattern 1; region 2 indicates the putative catalytic site of CoA transferase, signature pattern 2; region 3 indicates the putative nucleotide binding site.
Figure 6.4. SDS-PAGE analysis of cloned CoA transferase gene expression in *E. coli* BL21(DE3). Lanes 1-4: cells grown aerobically; Lanes 5-7: cells grown anaerobically. Wild type without IPTG induction (Lane 1); wild type with IPTG induction (Lanes 2, 5); Mutant harboring pET-CoA without IPTG induction (Lanes 3, 6); mutant harboring pET-CoA with IPTG induction (Lanes 4, 7). Arrows indicate the overexpressed CoA transferase. MW: molecular weight standards (kDa).
Figure 6.5. CoA transferase activity in crude extracts of BL21 (DE3) harboring pET-CoA grown anaerobically.
CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

This study demonstrated that the advantages of integration of metabolic engineering and fermentation process development for enhanced propionic acid production from renewable biomass. A higher yield, higher productivity, and higher final concentration of propionate can be obtained from the novel immobilized-cell fermentation system, Fibrous Bed Bioreactor (FBB). In addition, it was the first time to show that biodiesel waste containing glycerol can be used without any purification to produce propionic acid and the fermentation results were the same as those from pure glycerin. The important results and conclusions obtained in this study are summarized below.

7.1.1 Fermentation kinetics

- Fermentation with metabolically engineered \textit{P. acidipropionici} in the fibrous bed bioreactor (FBB) showed that the maximum theoretical propionic acid yield of \(\sim 0.56 \text{ g/g glucose} \) could be achieved. The highest propionic acid concentration
obtained from lactose fermentation was ~104 g/l, which was 43% higher than the highest concentration of ~72 g/l reported in a previous study using the wild type strain in the FBB.

- *P. acidipropionici* could use glycerol for its growth and produced propionic acid at a high yield of 0.71 g/g glycerol, which was much higher than that from glucose (0.55 g/g glucose). In addition, the production of acetic acid in glycerol fermentation was only 0.03 g/g glycerol, which was much less than that from glucose (0.1 g/g glucose). Thus, glycerol fermentation produced high purity of propionic acid with the propionic acid to acetic acid ratio of 20 (vs. ~5 from glucose fermentation), facilitating the recovery and purification of propionic acid from the fermentation broth by simple solvent extraction. The highest propionic acid concentration obtained from glycerol fermentation was ~106 g/l, which was 2.5 times of the maximum concentration of ~42 g/l reported in literature.

- CO₂ supplementation could significantly increase the productivity of propionate (0.12 vs. 0.07 g/l/h) and succinate (0.01 vs. 0.003 g/l/h), and the specific growth rate (0.17 vs. 0.06 h⁻¹) in glycerol fermentation. CO₂ has slight effects on the yield of propionic and succinic acids. Succinate had more accumulation with the addition of CO₂ in the fermentation broth.

### 7.1.2 Fibrous Bed Bioreactor

- A high cell density (> 60g/l cell biomass) could be obtained in the fibrous-bed bioreactor (FBB) system with 90% cell viability.
The maximum theoretical propionic acid yield from glucose can be reached in the FBB system with the genetically modified mutant due to the low substrate utilization for cell growth and by-product production.

The FBB system was very stable. An immobilized cell bioreactor had been run for more than one year for propionic acid production from glycerol without encountering any contamination issues.

Cells in the FBB system could be adapted effectively to obtain higher tolerance to end products. The adapted mutant from the FBB had 16-fold higher propionate tolerance as compared to the wild type.

The adapted mutant from the FBB system showed higher yield, productivity, final concentration of propionate, and specific growth rate in free cell fermentation as compared with wild type.

The morphology of adapted mutant changed significantly. The mutants became longer and slimmer, causing significant increase in the specific surface area. These changes were the global response of cells to extreme environment.

7.1.3 Metabolic Pathway Analysis

The higher tolerance to propionic acid of the adapted cells can be partially attributed to the over-expression of H\(^+\)-ATPase, which plays a key role in proton pumping. H\(^+\)-ATPase from mutants showed higher activity at both normal pH (pH 7.0) and acidic pH (pH 3.5) than that from wild type.

The CO\(_2\) effect on cell growth and carboxylic acid formation in glycerol fermentation can be attributed to its effects on the rate limiting step enzymes.
Both CoA transferase and PEP carboxylase activities were increased with CO₂ supplementation, which caused improvement in productivity of propionic acid.

- A variety of carbon sources (glucose, xylose, gluconate, sorbitol, and glycerol) was utilized to produce propionic acid by *P. acidipropionici*. Different substrates have different reducing states, which determine the ratios of end products.

- In glycerol fermentation, there was some NADH accumulation, which caused a decrease in NAD⁺ concentration. The cell growth rate could be inhibited due to the higher NADH/NAD⁺ ratio.

- The metabolic pathway shift and its effect on propionic and acetic acids production from different carbon sources can be predicted by a stoichiometric metabolic model.

### 7.1.4 Genetic engineering

- Tetracycline resistant gene (*tet*) was very stable in the chromosome of ACK-Tet mutant during long-term fermentation even though there was no any tetracycline in the media.

- CoA transferase gene was obtained by PCR amplification from *P. acidipropionici* chromosome and over-expressed in *E.coli* BL21 (DE3) host. The CoA transferase activity of *P. acidipropionici* was observed only with cell grown anaerobically due to the enzyme sensitivity to oxygen.
7.2 Recommendations

Several attempts were carried out in this research to develop an effective fermentation technology for economical production of propionic acid from low cost biomass. However, many problems still remain unsolved and the fundamental regulation mechanisms of cells still remain unknown. With the development of biotechnology and the sequencing of *P. acidipropionici* genome, it is feasible to study the fermentation nature at molecular level and improve the fermentation process further. The following are some suggestions for future work.

7.2.1 Fermentation process development

- The final goal of this project is to use low cost biomass to produce high value-added chemicals. Therefore, it is desirable to use raw materials completely as carbon and nitrogen sources. The current medium comprises low cost carbon sources (e.g. starch, cheese whey, and biodiesel waste) and high cost nitrogen sources (yeast extract). Corn steep liquor (CSL) is widely used as nitrogen source in the fermentation industry. To reduce the overall production cost, it is necessary to optimize the basal medium by replacing yeast extract with CSL completely or partially.

- In this study, glycerol showed high potential as main carbon source in propionic acid production with high yield and high P/A ratio. However, the productivity and cell growth rate was much lower than that in glucose fermentation due to high osmotic pressure of glycerol. In the future, two-phase fermentation process could
be performed with the mixture of glucose and glycerol as co-substrates. Glucose should be used to produce cell biomass and glycerol should be used to produce propionic acid.

- Several *P. acidipropionici* recombinants harboring CoA-transferase expression vectors were selected in this study. The characterization of these recombinants should be performed to screen the best candidate for propionic acid production. And then, the immobilized-cell fermentation system (FBB) could be applied for further improvement of acid production and propionate tolerance.

- Continuous fermentation process with the FBB system was studied in previous research. However, most work done to date has been mainly focusing on fermentation kinetics in Pseudo-steady state. This is a time consuming process. It would take more than ten days to reach stationary phase when changing operating parameters (dilution rate, medium initial concentration). It should be useful to establish a dynamic model to simulate the continuous fermentation process. The ideal model should be able to predict the end product concentration and pH at the outlet point, which could save operating time and money (Chapter 3).

### 7.2.2 Metabolic Flux Analysis (MFA)

- A stoichiometric model was set up in this work based on simplified metabolic pathway analysis. This model, however, can only simulate final product formation at the end of the fermentation process according to mass balance and energy balance assumptions. The current model cannot describe the production rate or the product concentration at certain time point. More information and parameters
about the enzyme reaction kinetics in metabolic pathway are required to establish a metabolic flux model to obtain more fundamental understanding regarding the metabolism and gene regulation in the cell.

- In this study, several attempts (genetic and metabolic engineering) have been tried to eliminate the formation of acetic acid, the main byproduct. However, acetic acid could always be observed in the fermentation broth in long-term fermentation. The whole genome sequencing result shows that there are many undetermined alternative pathways that can convert pyruvate to acetate as below (Figure 7.1).

![Alternative pathways converting pyruvate to acetate](image-url)

**Figure 7.1** Alternative pathways converting pyruvate to acetate (Dashed lines represent newly putative pathways; Solid lines represent default pathway)
(1) pyruvate dehydrogenase (cytochrome) EC 1.2.2.2

pyruvate + ferricytochrome b1 + H2O = acetate + CO2 + ferrocytochrome b1

(2) pyruvate dehydrogenase (acetyl-transferring) EC 1.2.4.1

pyruvate + lipoyllysine = S-acetyldihydrolipoyllysine + CO2

(3) dihydrolipoamide S-acetyltransferase EC 2.3.1.12

CoA + S-acetyldihydrolipoamide = acetyl-CoA + dihydrolipoamide

(4) pyruvate synthase EC 1.2.7.1

pyruvate + CoA + 2 oxidized ferredoxin = acetyl-CoA + CO2 + 2 reduced ferredoxin + 2 H+

(5) acetate-CoA ligase EC 6.2.1.1

AMP + diphosphate + acetyl-CoA = ATP + acetate + CoA

These enzyme functions in *P. acidipropionici* are still not clear. It should be helpful if we can demonstrate that whether these enzymes function at normal environment or only can be activated under extreme environment. A better understanding of the regulation mechanism of these enzymes would facilitate future metabolic engineering of *P. acidipropionici*.

- Since more than 90% of the whole genome sequence is known and annotated, it is highly recommended that in future work the annotated proteins should be sorted, analyzed, and compared with the reported genome of *Propionibacteria acne*. From the analysis, more detailed metabolic pathway could be set up, which could provide more strategies for process optimization (Appendix E).
7.2.3 Genetics and Proteomics

- In this research it was demonstrated that the increase in the activity of $\text{H}^+\text{-ATPase}$ contributed to the higher tolerance of adapted mutant to propionic acid due to the function of pumping out extra protons from cells. The cell tolerance will become a limiting factor when propionic acid production reaches a certain level. It is recommended that cell tolerance of end product be improved accompanying the increase in end product production. One objective could be to over-express $\text{H}^+\text{-ATPase}$ in *P. acidipropionici*. Since $\text{H}^+\text{-ATPase}$ is an ATP dependent enzyme, the ATP balance in cells should be considered as well.

- Aldehyde/Alcohol Dehydrogenase (*aad* gene) from *Clostridium acetobutylicum* ATCC 824 was identified and characterized (Nair et al., 1994). The enzyme can catalyze acetyl-CoA and butyryl-CoA to corresponding ethanol and butanol, respectively. Since the enzyme is not restricted to the substrate, it is feasible to express *aad* gene in *P. acidipropionici* to convert propionyl-CoA to propanol. Acid-tolerant mutants should have high alcohol-tolerance simultaneously because the mechanisms of acid/alcohol resistance are similar (cell membrane composition). So, the adapted mutants from the FBB could be good hosts for *aad* gene expression.

- In this research, SDS-PAGE electrophoresis revealed significant difference in the overall protein expression patterns between the adapted mutant and the wild type. The cells cultured in different fermentation processes (suspended, FBB) and different carbon sources (glucose, glycerol, and lactose) showed remarkable difference in fermentation profiles. To understand the fundamental responses of
cells under different conditions, two-dimensional electrophoresis should be performed to identify the interested proteins (Appendix D.14). With the whole genome sequence data base, the regulation mechanisms of cells could be investigated and the metabolic pathway map would be more precise. A better understanding of the fermentation nature would facilitate metabolic engineering of *P. acidipropionici* for further improving propionate production in the future.


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APPENDIX A

MEDIUM COMPOSITIONS
A.1 Medium Compositions for *Propionibacterium acidipropionici*

Production medium: the medium contained (per liter) 10 g yeast extract (Difco Laboratories, Detroit, MI), 5 g Trypticase (BBL), 0.25 g K$_2$HPO$_4$, 0.05 g MnSO$_4$, and 50-100 g carbon source. The pH of the medium was adjusted to 6.5 for glucose fermentation and 7.0 for glycerol fermentation.

Transformation medium: sodium lactate broth (NLB) medium contained (per liter) 10 g sodium lactate, 10 g yeast extract, and 10 g trypticase soy broth (Hofherr et al., 1983).

A.2 Medium Compositions for *Escherichia coli*

The Luria-Bertani (LB) medium contained (per liter) 10 g tryptone, 10 g NaCl, and 5 g yeast extract. The pH of the medium was adjusted to 7.0.

A.3 M9 Medium

The M9 medium contained (per liter) 200 ml of sterile 5×M9 salt solution, 2 ml of sterile 1M MgSO$_4$, 0.1 ml of sterile 1M CaCl$_2$, and 20 ml of sterile 20% carbon source solution.
B.1 Cell Concentration

Cell growth was monitored by measuring the optical density (OD) at 600nm in a 1.5-ml cuvette using a spectrophotometer (Shimadzu, UV-16-1). The value of OD was proportional to the cell dry weight when the value was less than 0.6. One unit of OD was equivalent to 0.435g/l cell dry weight. Samples were diluted with appropriate rate to bring down the OD below 0.6. To obtain the dry cell weight, cells in a known volume of fermentation broth were harvested by centrifuging at 10,000rpm, 15min and washed three times with distilled water and then the cell suspension was dried at 105°C overnight.

B.2 Cell Viability

Cell viability assay was carried out by the method of Glenner (1977) with some modifications. Briefly, 1 ml culture in centrifuge tube was centrifuged at 16,000 rpm for 10 min. Discard the supernatant, ensuring that all liquid is removed completely. 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) aquatic solution (1 g/l) was added into the tube and vertex at top speed to resuspend the cell pellet, and then incubated in dark environment at room temperature for 30 min for color development. After centrifugation at 16,000 rpm for 10 min, the cell pellet was collected and added 1ml methanol to resuspend the pellet to extract the pink color. After centrifuging for 10 min at 16,000 rpm the absorbance of the supernatant at 485 nm was measured using a spectrophotometer (Shimadzu, UV-16-1) against a control (boiling cells before add TTC solution). The viability of cells harvested from exponential phase was assumed to be 100% (Figure B.1). Thus,

\[
\text{sample viability} = \frac{(OD_{485}/\text{cell concentration})_{\text{sample}}}{(OD_{485}/\text{cell concentration})_{\text{standard}}}
\]
Figure B.1 100% viability determination.

B.3 High Performance Liquid Chromatography

The concentrations of carbon source (glycerol, lactose, or glucose) and main acid products (acetic, succinic, and propionic acids) in samples were analyzed by High Performance Liquid Chromatography (HPLC) with an organic acid column (Bio-Rad, HPX-87; ion exclusion organic acid column; 300mm-7.8mm). Samples were taken from fermentation broth with certain interval time, and then were centrifuged at top speed for 5 min. The supernatants were stored at -20°C. For HPLC analysis, the supernatants were diluted 15 times with distilled water.
HPLC was run at 45°C using 0.01 N $\text{H}_2\text{SO}_4$ as the eluant at a flow rate of 0.6 ml/min. 15 μL of each diluted cell-free sample were injected by an automatic injector (SIL-10Ai) and the running time was 25 min. A refractive index (RI) detector (Shimadzu-RID-10A) was set at the range of 200 to detect the organic compounds in samples. The HPLC column was installed in a column oven (CTO-10A) with temperature control at 45°C. Peak height and area were used to calculate a concentration of each component based on the peaks of standard sample. In the standard sample, concentration of each component is 2 g/L. The standard and experiment sample HPLC chromatograms are shown below (Figures B.2; B.3; B.4; B.5, and B.6).

**Figure B.2** The HPLC chromatogram for standard containing glucose, succinic acid, glycerol, acetic acid, and propionic acid.
Figure B.3 The HPLC chromatogram for standard containing lactose, glucose, succinic acid, acetic acid, and propionic acid.
Figure B.4 The sample HPLC chromatogram of propionic acid fermentation by *P. acidipropionici* using glycerol as the substrate.
Figure B.5 The sample HPLC chromatogram of propionic acid fermentation by \textit{P. acidipropionici} using glucose as the substrate.
Figure B.6 The sample HPLC chromatogram of propionic acid fermentation by *P. acidipropionici* using lactose as the substrate.

**B.4 Cell permeabilization**

Cells were made permeable by the method of Belli and Marquis (1991). Briefly, Cells were grown in medium (50 ml) until the late exponential phase of growth. 25ml of cultures were harvested by centrifugation at 8,000 rpm, 4°C for 10 min. 1 ml of pure propionic acid was added to the rest broth. The cells were cultured for another 1.5 hours at 32°C and harvest by centrifugation. The cell pellets from each sample were resuspended in 2.5ml of 75mM Tris-HCl buffer (pH7.0) with 10mM MgSO₄. Toluene (250 μl) was added to each cell suspension followed by vigorous mixing and incubated for 5 min at 37°C. Each cell suspension was then treated by freezing at -80°C and
thawing at 37°C for two times. The permeabilized cells were harvested by centrifugation and resuspended in 1.0 ml of 75mM Tris-HCl buffer (pH7.0) with 10mM MgSO4. The cell suspensions were immediately subjected to the enzyme assay or stored at -80°C.

### B.5 Preparation of Cell Extract

*P. acidipropionici* (ATCC 4875) was grown at 32°C in medium (50 ml) in serum bottle. The cells were harvested in the exponential phase (OD600 ~ 1.8) by centrifuging at 7,000 rpm for 10 min and washed three times, and resuspended in 3 mL of ice cold Tris/HCl buffer (25 mM, pH 7.4). The cell suspension was then ultrasonicated using a sonic dismembrator (Fisher Scientific, Model 100). Samples were kept in ice bath during cell disruption. Sonication was conducted 5 seconds followed by 25 seconds of resting to preventing overheating, total 20 cycles, and then centrifuged at 15,000 rpm, 4°C for 1 h to remove cell debris. For phosphoenolpyruvate carboxylase assay, the supernatant was subjected to further centrifugation in an ultra-centrifuge (Beckman, Optima TL) for 90 minutes at 45,000 rpm to remove the gelatinous sediment which contains most of the NADH oxidase activity. The cell extracts were kept cold on ice before they were used in the enzyme activity assays. The protein content of the extracts was determined in triplicate by Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard protein.

### B.6 Protein assay

Six dilutions of a protein standard (BSA in PBS buffer) were prepared with the concentration range from 0.05 to 0.5 mg/ml. 10 μl of each standard BSA and sample
were pipetted into separate 96-well microplate. And then, 200 μL of 5×diluted dye reagent, which was filtered to remove particulates, was added to each well. The mixture was incubated at room temperature for more than 15 min, less than 1 hour. The absorbance was measured at 595 nm (SpectraMax 250). The protein concentration of samples was determined based on the standard curve. Figure B.7 shows a typical protein (BSA) standard curve.

![Typical standard curve of protein assay using bovine serum albumin.](image)

**Figure B.7** Typical standard curve of protein assay using bovine serum albumin.

**B.7 Enzyme assays**

**H⁺-ATPase**

ATPase activity was determined based on the method of Belli and Marquis (1991) by measuring the release of inorganic phosphate (Pi) from ATP. Unless otherwise mentioned, the reaction mixture contained 3.0 ml of 50 mM Tris-maleate buffer (pH6.0) with 10mM MgSO₄, 75 μl of permeabilized cell suspension and 30 μl of 0.5 M ATP (pH6.0). The mixture was incubated at 37°C for 20 min. Samples of 50 μl were removed for the Pi assay by the Fiske-SubbaRow method as modified by Weisman and Pileggi.
(1974). The standard unit of ATPase activity is defined as the amount of enzyme that releases 1 μmol of Pi per minute, and the specific activity of ATPase is defined as the unit of activity per milligram of cell dry weight.

**Phosphoenolpyruvate carboxylase (PEP C)**

The activity of PEP C was assayed according to the method of Maeba and Sanwal (1969) with some modifications. In brief, the 300 μl of assay mixture contains Tris-HCL (pH 9.0, 0.1M), 0.2 ml; NADH (2mM), 0.01 ml; MgCl$_2$ (0.6M), 0.005ml; NaHCO$_3$ (0.6M, fresh), 0.005 ml; PEP (0.1M), 0.01 ml; malate dehydrogenase (0.15 mg/ml), 0.01 ml; and the cell extract, 0.06ml. The reaction was carried out at 25°C with measuring the absorbance at 340 μm (Figure B.8). A cuvette lacking PEP was used as a blank control. The standard unit of PEP C is defined as the amount of enzyme causing an absorbancy change of 1.0 per minute, and the specific activity is defined as units per milligram of protein.

![Figure B.8 Sample plot of PEP carboxylase activity determination](image-url)

$y = -0.0075x + 0.0118$

$R^2 = 0.9922$
Succinyl-CoA: Propionate CoA- Transferase (CoA T).

The activity of CoA T was assayed based on the method of Schulman and Wood (1975) with some modifications. The assay mixture (250 μl) contains 0.1 ml of Mixture 1 (1.0 M, pH 8.0 Tris/HCl buffer, 1 ml; 0.4 M sodium malate, 0.01 ml; 0.01 M NAD, 1.0 ml; and water to 4 ml), 0.01 ml of 1.5 M sodium acetate, 0.01 ml of Mixture 2 (944 unit/mg of malic dehydrogenase, 14 μl; 355 unit/mg of citrate synthase, 11 μl; and 0.1M, pH 6.8 phosphate buffer, 975 μl), 0.01 ml of 0.15 μmole succinyl-CoA, 0.05 ml of cell extract, and water to 0.25 ml. The reaction was conducted at 25°C followed by measuring the absorbance at 340 nm which increased linearly with time for 3-5 minutes (Figure B.9). A cuvette lacking CoA T was used as a blank control. The standard unit of CoA T is defined as the amount of enzyme causing an absorbancy change of 1.0 per minute, and the specific activity is defined as units per milligram of protein.

![CoA T assay graph](image)

**Figure B.9** Sample plot of CoA transferase activity determination
B.8 Scanning Electron Microscopy

At the end of the FBB fermentation, small pieces (0.5cm × 0.5cm) were cut from different part of the fibrous matrix as samples for scanning electron microscopy (SEM). The pieces were fixed in 2.5% glutaraldehyde solution at 4°C overnight and rinsed with distilled water twice for 15 min each. The samples were then dehydrated progressively with 20 to 70% ethanol at 10% increment for 30 min at each concentration. The samples were immersed in 70% ethanol for overnight at 4°C, and then dehydrated in 80%, 90%, and 100% ethanol for 30 min at each concentration. The samples were finally dried with hexamethyl disilazane (HMDS) and coated with gold/palladium using the spotter-coating machine in the presence of the medium containing argon gas. The samples were scanned and photographed with Nova 400 NanoSEM at 15 kV.
APPENDIX C

BIOREACTOR CONSTRUCTION AND OPERATION
C.1 Construction of Fibrous Bed Bioreactor

The immobilized cell bioreactor used in this study was made of a glass column fitted with a water jacket. A piece of cotton towel (32×22 cm) was spirally wound with a stainless steel mesh and packed into the glass column for cell immobilization (Figure C.1). The gap between the layers was about 5 mm. The bioreactor working volume was ~600ml. The packed glass column was sealed with rubber stoppers at both ends and was connected to a 5-L fermentor (Marubishi MD-300) through a recirculation loop (Figure C.1). The fermentation system was operated at set pH by pH controller (Cole Parmer 5997-20) and optimal temperature by circulating water with temperature control through the water jacket in the glass column.

C.2 Bioreactor Start-Up and Operation

The packed column was connected to a 5-L fermentor (Marubishi MD-300) through a recirculation loop and autoclaved for 30 min. The basal medium (without carbon source) and the concentrated carbon source solution were sterilized separately at 121°C and 15 psig for 30 min in order to avoid unexpected reactions and mix them together aseptically in fermentor. The total volume of 2 L of the medium was used for the entire system. After cooling down, N₂ gas was sparged into system for 30 min to reach an anaerobic environment. The fermentation was started by inoculating 100 ml of cell suspension, and then operated as free-cell fermentation mode. The system was maintained at 32°C, pH 6.5-7.0 by the addition of 6 M NaOH. When the cell optical density (OD₆₀₀) reached ~3.0, the fermentation broth was started to circulate at a flow rate of 30ml/min through the FBB to allow cells to attach to the fibrous matrix until
steady state was reached. And then, the medium circulation rate was increased to ~80 ml/min to enhance the mixing in system. Fed-batch fermentation was carried out to study the fermentation kinetics. Partial medium was replaced with concentrated fresh medium to replenish the nutrients. Samples were taken at regular time intervals throughout the fermentation for analyses. At the end of the fed-batch fermentation, adapted cells in FBB were collected from the fibrous matrix and sub-cultured in serum bottles for further analyses. In order to follow up the working process about chapters 3-5, the fermentation history is listed in table C.1.

Figure C.1 The fermentation system with a fibrous-bed bioreactor.
<table>
<thead>
<tr>
<th>Reactor</th>
<th>Operation</th>
<th>Date or time period</th>
<th>Data reported in</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBB 1</td>
<td>Seeded with ACK-Tet for start up</td>
<td>7/12/2005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Initial batch fermentation with glucose</td>
<td>7/23/2005 – 8/16/2005</td>
<td>Figure 3.3B</td>
</tr>
<tr>
<td></td>
<td>Fed batch fermentation for long-term operation to adapt the culture to produce high propionic acid from glucose</td>
<td>9/26/2005 – 1/11/2006</td>
<td>Figure 3.1A</td>
</tr>
<tr>
<td></td>
<td>Changed to lactose media</td>
<td>4/25/2006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fed batch fermentation for long-term operation to adapt the culture to produce high propionic acid from lactose</td>
<td>4/25/2006-8/08/2006</td>
<td>Figure 3.1B</td>
</tr>
<tr>
<td></td>
<td>Terminated; adapted cells were harvested from the reactor for further characterization</td>
<td>8/15/2006</td>
<td>Figure 3.3C</td>
</tr>
<tr>
<td>FBB 2</td>
<td>Seeded with ACK-Tet for start up</td>
<td>9/26/2005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fed batch fermentation for long-term operation to adapt the culture to produce high propionic acid from glycerol</td>
<td>10/02/2006-11/01/2006</td>
<td>Figure 4.2A</td>
</tr>
<tr>
<td></td>
<td>Changed the whole media to fresh glycerol media, batch fermentation</td>
<td>11/02/2006-3/03/07</td>
<td>Figure 4.1</td>
</tr>
<tr>
<td></td>
<td>Terminated; adapted cells were harvested from the reactor for further characterization</td>
<td>3/03/07-9/29/2007</td>
<td>Figure 4.2B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBB 3</td>
<td>Seeded with adapted ACK-Tet from FBB-2 for start up</td>
<td>10/02/2006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Initial batch fermentation with glycerol</td>
<td>10/02/2006-11/01/2006</td>
<td>Figure 4.2A</td>
</tr>
<tr>
<td></td>
<td>Fed batch fermentation for long-term operation to adapt the culture to produce high propionic acid from glycerol</td>
<td>11/02/2006-3/03/07</td>
<td>Figure 4.1</td>
</tr>
<tr>
<td></td>
<td>Changed the whole media to fresh glycerol media, batch fermentation</td>
<td>3/03/07-9/29/2007</td>
<td>Figure 4.2B</td>
</tr>
<tr>
<td></td>
<td>Terminated; adapted cells were harvested from the reactor for further characterization</td>
<td>4/11/2007</td>
<td>Figure 4.4 B,C; Figure 4.5</td>
</tr>
<tr>
<td>Free cell 1</td>
<td>WT in glycerol without CO₂</td>
<td>6/13/2007</td>
<td>Figure 5.3 A</td>
</tr>
<tr>
<td>Free cell 2</td>
<td>WT in glycerol with CO₂</td>
<td>11/15/2007</td>
<td>Figure 5.3 B</td>
</tr>
<tr>
<td>Free cell 3</td>
<td>WT in glucose without CO₂</td>
<td>1/24/2007</td>
<td>Table 5.2</td>
</tr>
<tr>
<td>Free cell 4</td>
<td>WT in glucose with CO₂</td>
<td>10/31/2007</td>
<td>Table 5.2</td>
</tr>
</tbody>
</table>

**Table C.1** Kinetics of batch free-cell fermentations by original and adapted ACK-Tet with various carbon sources. Fermentation history of the FBB and the corresponding data reported in the thesis.
APPENDIX D

GENETIC ENGINEERING PROTOCOLS
D.1 Preparation of Genomic DNA from *P. acidipropionici* with QIAGEN Genomic DNA Kit

1. The culture of *P. acidipropionici* (50 ml) was harvested at the exponential phase (OD600 = 2.0) by centrifugation for 15 min at 8,000 rpm. Discard the supernatant.
2. Rinse the cells with distilled water for 2 times and pellet cells by centrifugation for 15 min at 8,000 rpm. Discard the supernatant.
3. Resuspend the cells in 11 ml of lysis buffer (B1) containing 22 μl of RNaseA solution (100mg/ml) by vortexing at top speed
4. Add 300 μl of lysozyme solution (100mg/ml) and 500 μl of Invitrogen Proteinase K solution. Incubate at 37°C for at least 30 min.
5. Add 4 ml of lysis buffer (B2) and mix by inverting the tube several times.
6. Incubate at 50°C for 30 min or till the lysate becomes clear.
7. Lysate is centrifuged for 5 min at 5,000 rpm to remove the unsolved pellet.
8. Load sample onto the QIAGEN Genomic-tip 500/G equilibrated by 10 ml of equilibration buffer. Allow it to enter the resin by gravity flow.
9. Wash the column 2 times with 15 ml of wash buffer for each.
10. Elute the genomic DNA with 15 ml of elution buffer which is prewarmed to 50°C.
11. Add 0.7 volume isopropanol to the eluate to precipitate DNA.
12. Centrifuge at 10,000 rpm for 30 min at 4°C. Remove the supernatant.
13. Wash the DNA pellet with 4 ml of cold 70% ethanol. Remove the solution by centrifuging at 10,000 rpm for 10 min at 4°C.
14. Air dry for 10 min
15. Dissolve the DNA overnight in appropriate volume of TE buffer.
16. Determine the concentration and purity of the DNA by gel electrophoresis.

**D.2 Preparation of Plasmid DNA with QIAprep Spin Miniprep Kit**

1. Centrifuge 5 ml overnight *E. coli* culture at top speed for 5 min and remove the supernatant.

2. Resuspend the pellet with 250 μl of Buffer P1 in microcentrifuge tube.

3. Add 250 μl of Buffer P2 and pipette up and down the solution to thoroughly mix.

4. Add 350 μl of Buffer N3 and mix gently by inverting the tube 4-6 times.

5. Centrifuge for 30 min for 13,000 rpm.

6. Apply the supernatant to a QIAprep spin column by pipetting.

7. Centrifuge for 1 min and discard the flowthrough.

8. Add 500 μl of Buffer PB if necessary and centrifuge for 1 min. Discard the flowthrough.

9. Wash the column by adding 750 μl of Buffer PE. Centrifuge for 1 min.

10. Discard the flowthrough and centrifuge for an additional 1 min to remove residual wash buffer.

11. Place the column in a clean, sterile 1.5-ml microcentrifuge tube. Add 50 μl of Buffer EB or sterile distilled water to the center of each spin column to elute DNA. Let stand for 1 min and centrifuge for 1 min.
D.3 Preparation of Plasmid DNA with HiSpeed® Plasmid Maxi Kit

To obtain high concentrated plasmid for *P. acidipropionici* transformation, QIAGEN HiSpeed® Plasmid Maxi Kit is utilized.

1. Centrifuge 250 ml of overnight LB culture at 10,000 rpm, 4°C for 15 min.
2. Resuspend pellet in 10 ml of Buffer P1
3. Add 10 ml of Buffer P2, mix thoroughly, and incubate at room temperature for 5 min.
4. Add 10 ml of chilled Buffer P3. Mix thoroughly by inverting 4-6 times.
5. Centrifuge at 12,000 rpm, 4°C for 20 min.
6. Pour the supernatant into the QIAfilter Cartridge. Incubate at room temperature for 10 min.
7. Equilibrate a HiSpeed Maxi Tip by applying 10 ml of Buffer QBT and allow the column to empty by gravity flow.
8. Filter the cell lysate into the equilibrated Tip
9. Allow the cleared lysate to enter the resin by gravity flow.
10. Wash the QIAGEN Tip with 60 ml of Buffer QC.
11. Elute DNA with 15 ml of Buffer QF
12. Precipitate DNA by adding 10.5 ml of room-temperature isopropanol to the eluted DNA. Mix and incubate for 5 min.
13. Attach the QIApreficitator Maxi Module onto the outlet nozzle.
14. Place the QIApreficitator over the waste bottle, transfer the eluate/ isopropanol mixture into the 30-ml syringe, and insert the plunger. Filter the mixture through the QIApreficitator using constant pressure.
15. Remove the QIAprecipitator from the 30-mL syringe and pull out the plunger. Re-attach the QIAprecipitator and add 2 ml of 70% ethanol to the syringe. Wash the DNA by inserting the plunger and pressing the ethanol through the QIAprecipitator using constant pressure.

16. Remove the QIAprecipitator from the 30-mL syringe and pull out the plunger. Attach the QIAprecipitator again, insert the plunger and dry the membrane by pressing air through the QIAprecipitator quickly and forcefully. Repeat this step.

17. Dry the outlet nozzle of the QIAprecipitator with absorbent paper to prevent the ethanol carryover.

18. Remove the plunger from a new 5-mL syringe and attach the QIAprecipitator onto the outlet nozzle. Hold the outlet of the QIAprecipitator over a clean 1.5-ml microcentrifuge tube. Add 1 ml of Buffer TE or sterile distilled water to the 5-mL syringe. Insert the plunger and elute the DNA into the tube using constant pressure.

19. Remove the QIAprecipitator from the 5-mL syringe, pull out the plunger and reattach the QIAprecipitator to the 5-mL syringe.

20. Transfer the eluate from the previous step to the 5-mL syringe and eluate for a second time into the same tube.

D.4 Fast Preparation of Plasmid DNA by Boiling Water

1. Add 110 μl of STET buffer in each microcentrifuge tube

2. Transfer the cells from agar plates into with tubes and mix thoroughly.

4. Centrifuge at 13,000 rpm for 15 min at room temperature.

5. Remove the precipitate from tubes and collect the supernatant

6. Add 100 μl of isopropanol in each tube and vortex thoroughly.

7. Incubate at 4°C for 15 min

8. Centrifuge at 13,000 rpm for 15 min at room temperature.

9. Discard the supernatant.

10. Vacuum dry the pellet for 5 min

11. Dissolve the DNA with 50 μl of buffer EB or sterile distilled water.

D.5 DNA Electrophoresis

1. Weigh out appropriate agarose into a 250mL conical flask. Add 100 ml of 1×TAE to make 0.7% to 1.0% agarose gels, swirl to mix. The gel concentration depends on the DNA size.

2. Microwave for about 1.5 min to dissolve the agarose.

3. Cool down the agarose solution to ~50-60°C (just too hot to keep holding in bare hands), pour the gel slowly into a sealed gel casting platform. Push any bubbles away to the side using a disposable tip, and insert the gel comb.

4. Leave to set for more than 30 min till the gel solidifies.

5. Put the platform into the electrophoresis tank and then add 1×TAE buffer to submerge the gel completely and then remove the comb.

6. Load the DNA samples and marker mixing with loading buffer into wells.
7. Connect the Bio-Rad Mini Sub-Cell to the power supply (Bio-Rad PowerPac 300) and run at constant voltage of 90 V for 30 min or until the bromophenol blue dye is about 2/3 of the gel length.

8. Stain the gel with 0.5 μg/mL ethidium bromide solution for 20 min.

9. Rinse the gel with distill water 3 times

10. Visualize and photograph the DNA bands using gel documentation system (Bio-Rad Gel Doc 2000).

D.6 DNA Fragment Purification with QIAquick Gel Extraction Kit

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a 1.5-mL microcentrifuge tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg~100 μl).

3. Incubate at 50°C for 10 min. Mix by vortexing the tube every 2-3 min till the gel slice has dissolved completely.

4. Add 1 gel volume of isopropanol to the sample and mix.

5. Transfer the sample to a QIAquick column, centrifuge for 1 min at 13,200 rpm, 4°C, and discard flowthrough.

6. Add 500 μl of Buffer QG to the column, centrifuge for 1 min, and discard flowthrough.

7. Add 750 μl of Buffer PE to wash the column, centrifuge for 1 min, and discard flowthrough.

8. Centrifuge the column for an additional 1 min.

9. Place the column into a clean 1.5 ml microcentrifuge tube.
10. Add 30-50 μl of Buffer EB or sterile distilled water to the center of each column to elute DNA. Let the column stand for 1 min and then centrifuge for 1 min.

**D.7 DNA Digestion**

1. Set up the following reaction in a microcentrifuge tube: (total of 15 μl)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X digestion buffer</td>
<td>1.5</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>5</td>
</tr>
<tr>
<td>Sterile water</td>
<td>rest of volume</td>
</tr>
</tbody>
</table>

2. Add Restriction enzyme 0.5-1 μl into the tube

3. Incubate at recommended temperature (30°C or 37°C) for 1 h

4. Take 2 μl of the digested products to run on agarose gel to check the result

**D.8 DNA Ligation**

1. Set up the following reaction in a microcentrifuge tube: (total of 20 μl)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5× ligase reaction buffer</td>
<td>4</td>
</tr>
<tr>
<td>Insert:vector molar ratio</td>
<td>3:1</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>up to 20</td>
</tr>
</tbody>
</table>

2. Mix gently and spin for 2 seconds in a microcentrifuge.

3. Incubate at 14°C overnight.
**D.9 DNA Transformation in *P. acidipropionici***

This protocol is according to the method of Kiatpapan *et al* (2000) with some modifications. All transformation experiments were performed on the bench top without any special anaerobic conditions.

1. An appropriate volume of overnight culture of *P. acidipropionici* was inoculated into 50 ml of fresh NLB medium to reach OD$_{600} = \sim 0.05$

2. Culture the cells at 32°C until OD$_{600} = \sim 0.8$

3. Harvest the cells by centrifugation at 8,000 rpm for 15 min at 4°C. Discard the supernatant.

4. Wash the pellet with 0.5 volume of 1 mM HEPES buffer (pH7.0) and centrifuge at 8,000 rpm for 15 min at 4°C. Discard the supernatant.

5. Resuspend cells in 0.1 volume of prechilled 10% glycerol and incubate on ice for 30 min.

6. Centrifuge at 8,000 rpm for 15 min at 4°C. Discard the supernatant.

7. Resuspend cells in 0.02 volume of prechilled 10% glycerol.

8. 100μl of cell suspension is used for transformation. Store the rest of the competent cells at -80°C.

9. Add 1μg of plasmid DNA in the 100μl of competent cells and mix well by flick the tube.

10. Transfer the mixture into a pre-chilled 0.2-cm electroporation cuvette (Bio-Rad)

11. Incubate on ice for 5 min.
12. Put the cuvette into the safety chamber (Electrocell Manipulator 600, BTX Inc., San Diego, CA) and apply a pulse (25 μF, 100 Ω).

13. Add 1 ml of the fresh NLB medium.

14. Transfer the culture into a sterile microcentrifuge tube and incubate at 32°C for 9 hr.

15. Plate appropriate volume of culture on NLB agar plate containing 250 μg/ml hygromycin B.

16. Incubate the plates in anaerobic chamber at 32°C for 7-10 days.

17. Pick up colonies for analysis.

**D.10 PCR Amplification of tet and CoA T Genes from P. acidipropionici**

**D.10.1** Set up the following 50 μl PCR reaction for tet gene amplification in a clean, sterile 500-μL PCR tube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>5</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>1.5</td>
</tr>
<tr>
<td>10 mM dNTPs (each)</td>
<td>1</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>2</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>2</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>2</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μL)</td>
<td>1</td>
</tr>
<tr>
<td>Sterile water</td>
<td>up to 50</td>
</tr>
</tbody>
</table>
1. Amplify in a thermal cycler (MJ Research) using the following parameters:

   Step 1
   94°C ----- 1 min

   Step 2 (×30)
   94°C ----- 30 s
   60°C ----- 30 s
   72°C ----- 1 min

   Step 3
   72°C ----- 10 min

2. Remove 2 μl to analyze by agarose gel electrophoresis

**D.10.2** Set up the following 50 μl PCR reaction for CoA T gene amplification in a clean, sterile 500-μL PCR tube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>5</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>1</td>
</tr>
<tr>
<td>10 mM dNTPs (each)</td>
<td>1.5</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>1.5</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>1.5</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>2</td>
</tr>
<tr>
<td>PCR enhancer</td>
<td>2.5</td>
</tr>
<tr>
<td>Platinum® Pfx DNA polymerase</td>
<td>1</td>
</tr>
<tr>
<td>Sterile water</td>
<td>up to 50</td>
</tr>
</tbody>
</table>

3. Amplify in a thermal cycler (MJ Research) using the following parameters:
Step 1  
95°C ----- 10 min

Step 2 (×10)  
95°C ----- 1 min
68°C ----- 30 s
72°C ----- 1 min

Step 3 (×20)  
95°C ----- 1 min
62°C ----- 30 s
72°C ----- 1 min

Step 4  
72°C ----- 10 min

Step 5  
add *Taq* DNA polymerase 1μl

Step 6  
72°C ----- 30 min

4. Remove 2 μl to analyze by agarose gel electrophoresis

**D.11 Cloning of PCR products with pGEM®-T vector (Promega)**

1. Set up the following reaction in a microcentrifuge tube: (total of 10 μl)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2×rapid ligation buffer</td>
<td>5</td>
</tr>
<tr>
<td>pGEM®-T vector</td>
<td>1</td>
</tr>
<tr>
<td>PCR product</td>
<td>1-3</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>up to 10</td>
</tr>
</tbody>
</table>

2. Mix gently by pipetting and spin for 2 seconds in a microcentrifuge.

3. Incubate at 4°C overnight, and then centrifuge the tube to collect contents
4. Add 2 μl of ligation reaction to 50 μl of pre-thawed JM109 competent cells.
5. Mix gently by flick the tube and put on ice for 20 min
6. Heat-shock the cells for 45-50 sec at 42°C.
7. Immediately transfer the tube to ice for 2 min.
8. Add 950 μl of room temperature S.O.C. medium
9. Incubate for 1.5 hr at 37°C with shaking (200 rpm).
10. Spread 10-50 μl from each transformation on a prewarmed selective plate.
11. Incubate the plates at 37°C overnight.
12. Pick up ~ 10 colonies for analysis.

D.12 IPTG induction of recombinant protein production

The protocol is used to express proteins in BL21(DE3) host strains with plasmids containing T7 promoter (e.g. pETDuet™-1).

1. Incubate 5 ml of E.coli culture at 37°C overnight (200 rpm).
2. Subculture 0.5 ml of overnight culture in 4.5 ml of fresh LB medium
3. Incubate at 37°C for ~1 hr (200 rpm) until OD_{600}=0.6 (1cm light path).
4. Add appropriate IPTG and continue to culture at 30°C or 16°C for another 2 hr (200 rpm) until OD_{600}=1.8-2.0 (1cm light path).
5. Pipet 150 μl of induced cultures into clean microcentrifuge tubes and centrifuge at top speed for 5 min. Discard the supernatant.
6. Add 20 μl of 2× SDS-PAGE loading buffer and 20 μl of distilled water to each microcentrifuge tube.
7. Pipet up and down to mix thoroughly.

8. Put tubes in boiling water for 10 min and centrifuge at 15,000 rpm for 10 min.

9. The supernatant is used for SDS-PAGE.

**D.13 SDS-PAGE**

1. Clean and dry all glass plates and assemble Mini-PROTEAN® 3 cell (Bio-Rad) casting stand and frame following instruction manual.

2. Prepare the separating gel solution in beaker by combining all reagents except ammonium persulfate (AP) and TEMED. Mix well.

3. Add 10% AP and TEMED to the solution, mix thoroughly and pour to the plates smoothly.

4. Immediately overlay the gels with water to remove all bubbles

5. Allow the gel to polymerize for 1 hr. Rinse the top of gels with distilled water.

6. Dry the top of the separating gel with filter paper.

7. Prepare the stacking gel in beaker by combining all reagents except ammonium persulfate (AP) and TEMED. Mix well.

8. Add 10% AP and TEMED to the solution, mix thoroughly and pour to the plates smoothly.

9. Insert the desired comb

10. Allow the stacking gel to polymerize for 30-45 min.

11. Assemble the running unit following the instruction manual.

12. Gently remove the comb and rinse the wells with distilled water.
13. Load appropriate amount of samples into wells and then overlay samples with 1× running buffer.

14. Run gels at a constant voltage of 110 V until the tracking dye reached the gel bottom (~1.5 h).

15. Separate the SDS-PAGE gel from glass plates

16. Stain gels in coomassie brilliant blue or silver stain.


<table>
<thead>
<tr>
<th>Separating Gels (Mini-PROTEAN® / PROTEAN® II)</th>
<th>7%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O (ml)</td>
<td>6 / 40</td>
<td>5 / 32</td>
<td>4 / 27</td>
<td>3 / 19</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8 (ml)</td>
<td>3 / 20</td>
<td>3 / 20</td>
<td>3 / 20</td>
<td>3 / 20</td>
</tr>
<tr>
<td>10% (w/v) SDS (ml)</td>
<td>0.12 / 0.8</td>
<td>0.12 / 0.8</td>
<td>0.12 / 0.8</td>
<td>0.12 / 0.8</td>
</tr>
<tr>
<td>Acrylamide/Bis-acrylamide (30% w/v) (ml)</td>
<td>3 / 19</td>
<td>4 / 27</td>
<td>5 / 32</td>
<td>6 / 40</td>
</tr>
<tr>
<td>10% (w/v) Amp (μl)</td>
<td>60 / 400</td>
<td>60 / 400</td>
<td>60 / 400</td>
<td>60 / 400</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>6 / 40</td>
<td>6 / 40</td>
<td>6 / 40</td>
<td>6 / 40</td>
</tr>
<tr>
<td>Total (ml)</td>
<td>12 / 80</td>
<td>12 / 80</td>
<td>12 / 80</td>
<td>12 / 80</td>
</tr>
<tr>
<td>M.W. range (kDa)</td>
<td>50-500</td>
<td>20-300</td>
<td>10-200</td>
<td>3-100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stacking Gels (Mini-PROTEAN® / PROTEAN® II)</th>
<th>4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O (ml)</td>
<td>3.05 / 12.2</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8 (ml)</td>
<td>1.25 / 5</td>
</tr>
<tr>
<td>10% (w/v) SDS (μl)</td>
<td>50 / 200</td>
</tr>
<tr>
<td>Acrylamide/Bis-acrylamide (30% w/v) (ml)</td>
<td>0.65 / 2.6</td>
</tr>
<tr>
<td>10% (w/v) Amp (μl)</td>
<td>25 / 100</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>5 / 20</td>
</tr>
<tr>
<td>Total (ml)</td>
<td>5 / 20</td>
</tr>
</tbody>
</table>

Table D.1 SDS-PAGE gel compositions
D.14 Two-Dimensional Electrophoresis

D.14.1 Protein sample preparation

1. The culture of *P. acidipropionici* (100 ml) was harvested at the exponential phase (OD$_{600}$ = 2.0) by centrifugation for 15 min at 8,000 rpm at 4°C. Discard the supernatant.

2. Rinse the cells with 1×DPBS for 2 times and pellet cells by centrifugation at 8,000 rpm for 15 min at 4°C. Discard the supernatant.

3. Resuspend the cells in 1.5 ml of 1×DPBS. Mix thoroughly by pipetting up and down.

4. Freeze the cell suspension in a dry ice/ethanol bath or -80°C freezer and then thaw the material at room temperature or 37°C. Repeat 3 times.

5. Samples are treated using Sonic Dismembrator 100 (Fisher Scientific). Sonication treatments consisted of periods of 5 sec followed by 30 sec of resting, 6 rounds. To prevent over heating, samples are immersed in ice bath during the ultrasonic treatment.

6. Centrifuge at 15,000 rpm for 30 min at 4°C.

7. Collect the supernatant and store at -80°C.

D.14.2 Protein sample clean up with ReadyPrep™ 2-D Cleanup Kit

1. Transfer 100 μl of protein extract to a 1.5 ml microcentrifuge tube.

2. Add 300 μl of agent 1 to precipitate proteins. Incubate on ice for 15 min.

3. Add 300 μl of agent 1 to the mixture and mix well by vortexing.
4. To form a tight pellet, centrifuge the tube at top speed for 5 min.
5. Add 40 μl of wash reagent 1 on top of the pellet followed by centrifuging 5 min at top speed.
6. Discard the supernatant gently and completely.
7. Wash the pellet with proteomic grade water.
8. Add 1 ml of prechilled wash reagent 2 and 5 μl of wash 2 additive. Mix for 1 min by vortex.
9. Incubate the tube at -20°C for 30 min.
10. Centrifuge the tube at top speed for 5 min.
11. Discard the supernatant gently and completely.
12. Use 20 μl of rehydration buffer to resuspend the pellet.

D.14.3 First dimension isoelectric focusing (IEF)

1. Prewarm the DeStreak Rehydration Solution (GE Healthcare) at room temperature for 30 min.
2. Add 15 μl of the appropriate IPG buffer (GE Healthcare) to 3 ml of DeStreak Rehydration Solution.
3. For 7 cm strip, add 10 μl of protein sample to 115 μl of rehydration buffer and mix by pipette.
4. Carefully transfer the 125 μl of solution to IEF tray (BioRad).
5. Remove the cover of IPG strip and place the gel side down in the tray.
6. Remove all bubbles under the IPG.
7. Overlay the IPG strip with mineral oil to cover the strip completely.
8. Rehydrate the IEF strip under active conditions (50 V for 12 hr).

9. Place water hydrated wicks over both electrodes in IEF tray.

10. Run the IEF by preset method (voltage: 4,000V; v-hr: 20,000).

### D.14.4 Equilibrating IPG gels

1. Put the IPG strip in a tube.

2. Add appropriate volume of SDS equilibration buffer containing 10mg/ml of DTT (GE Healthcare) to the tube. Equilibrate for 15 min.

3. Pour off the previous buffer and the appropriate volume of SDS equilibration buffer containing 25 mg/ml of iodoacetamide (GE Healthcare) to the tube. Equilibrate for 15 min.

<table>
<thead>
<tr>
<th>SDS equilibration buffer solution (GE Healthcare)</th>
<th>Final concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>6 M</td>
<td>72.1 g</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>75mM</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>29.3% (v/v)</td>
<td>60 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>2% (w/v)</td>
<td>4.0 g</td>
</tr>
<tr>
<td>1% Bromophenol blue</td>
<td>0.002% (w/v)</td>
<td>400 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>To 200 ml</td>
<td></td>
</tr>
</tbody>
</table>

*Table D.2 SDS equilibration buffer solution*

### D.14.5 Second dimension SDS-PAGE

1. Rinse the equilibrated strip gels in SDS running buffer.

2. Load the strip on the top the SDS-PAGE gel and ensure that no air bubbles are between the strip and the gel surface.

3. Seal the strip with agarose to prevent moving or floating.
4. Run gels as standard SDS-PAGE electrophoresis protocol.

The standard *E. coli* protein sample and experiment sample are shown below (Figures D.1; D.2).

![Image of 2-D gel with silver stain](image)

**Figure D.1** The 2-D gel of standard *E. coli* protein sample with silver stain
Figure D.2 The 2-D gel of *P. acidipropionici* protein sample with coomassie brilliant blue stain.
D. 15 Gel Silver Staining

D.15.1 This protocol of Silver Staining is according to the method of Rabilloud et al (1988) with some modifications.

1. Let gels soak in Solution A overnight
2. The next day, or later, place gels in Solution B three times for 20 min each
3. Rinse gels in distilled H2O two times for 15 min each
4. *Place gels in Solution C for 1 min exactly
5. Rinse gels two times for 1 min each in distilled H2O
6. Place gels in Solution D for 20-30 min
7. Rinse gels in distilled H2O for 1 min
8. Place gels in solution E for stain development
9. When gels have reached desired intensity, add 8.75 ml of Glacial Acetic Acid to Solution E directly. Let mixture work for 10 min
10. Rinse with distilled H2O four times for 30 min
11. Store gels in a 20% Ethanol Solution until one is ready to dry gels

*Very important to use mixture immediately or sodium dithionite will oxidize.

**First of all, everything you use should be protein free, because this is a very sensitive method. All the containers for the solutions and handling of the gel should be clean. Always wear glove when you touch the gel. I found the gel staining box from Fisher (03-484-11B) is good for soaking the gel. Do not use glassware.
### D.15.2 Solutions for Silver Stain

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
</table>
| Solution A (soaking) | 20 mL Glacial Acetic Acid  
60 mL Ethanol  
120 mL Distilled H₂O  
200 mL Total Volume | 10%  
30%                             |
| Solution B (soaking) | 90 mL Ethanol  
210 mL Distilled H₂O  
300 mL Total Volume          | 30%                                      |
| Solution C (sensitizing) | 0.125 g Sodium Dithionite  
(sodium hydrosulfite, Na₂S₂O₄)  
into 500 ml Distilled H₂O  
Mix and Use Immediately |                                      |
| Solution D (silver) | 0.4 g AgNO₃ (0.2%)  
20μl Formaldehyde (1mM HOCH, 37%)  
Distilled H₂O  
200 ml Total Volume |                                      |
| Solution E (developing) | 10 g of K₂CO₃  
150 μL of 10 M Formaldehyde  
250 μL of 20 mM Na₂S₂O₃*  
Distilled H₂O  
250 mL Total Volume | 4%  
6 mM  
20 μM |
5x Running Buffer for SDS-PAGE:

- Tris base: 7.5 g
- Glycine: 36.0 g
- SDS: 2.5 g
- dH₂O: 500 ml

SDS-PAGE Gel Staining Buffer:

- Isopropanol: 60 ml
- Glacial acetic acid: 40 ml
- dH₂O: 100 ml
- Coomasie Blue: 0.4 g

Mix well and filter with filter paper

SDS-PAGE Gel Destaining Buffer:

- Glacial acetic acid: 10%
- Methanol: 20%
**4×Sample Protein Loading Buffer:**

- 0.5M Tris-HCl, pH 6.8 1ml
- Glycerol 1.6ml
- 10% SDS 1.6ml
- β-mercaptoethanol 0.4ml
- 0.5% (w/v) bromophenol blue (in water) 0.4ml
- Distilled H₂O 3 ml
- Total 8 ml

**TE Buffer**

- 10 mM Tris/HCl (pH 8.0)
- 1 mM EDTA (pH 8.0)

**TAE Electrophoresis Buffer, 50x**

- Tris Base 242 g
- Glacial Acetic acid 57.1 ml
- 0.5M EDTA (pH 8.0) 100 ml
- Distilled water up to 1000 ml
**STET-Buffer**

Sucrose 8%
Triton X-100 5%
Tris-HCl (pH 8.0) 50mM
EDTA (pH 8.0) 50mM

Autoclave at 121°C for 20 min. Before use, add lysozyme to 0.5 mg/ml.

**5×M9 Salt Solution (1 liter)**

Na$_2$HPO$_4$·7H$_2$O 64g
KH$_2$PO$_4$ 15g
NaCl 2.5g
NH$_4$Cl 5g
H$_2$O up to 1 liter

Autoclave at 121°C for 20 min.
APPENDIX F

SUMMARY OF GENES INVOLVED IN PROPIONIC ACID SYNTHESIS
There are 12 genes in *Wood-Werkman* cycle for propionic and acetic acids synthesis (Figure F.).

**Figure F.** Dicarboxylic acid pathway of *Propionibacterium acidipropionici*  
(1) Pyruvate kinase; (2) Pyruvate dehydrogenase or Pyruvate synthase; (3) Phosphate acetyltransferase; (4) Acetate kinase; (5) Phosphoenolpyruvate carboxykinase or Phosphoenolpyruvate carboxylase; (6) Methylmalonyl-CoA carboxytransferase; (7) Malate dehydrogenase; (8) Fumarate hydratase; (9) Succinate dehydrogenase; Propionyl-CoA:succinyl-CoA-transferase; (10) Methylmalonyl-CoA mutase; (12) Methylmalonyl-CoA epimerase

Some of them have been annotated in 454 sequencing data base of the whole genome sequence of *P. acidipropionici* and are listed in the following table.

---

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>PK</td>
<td>Glucose</td>
<td>ADP, NADH</td>
</tr>
<tr>
<td>(2)</td>
<td>PDH</td>
<td>Pyruvate</td>
<td>AcetylCoA, NADH, CO2</td>
</tr>
<tr>
<td>(3)</td>
<td>PAT</td>
<td>Acetyl phosphate</td>
<td>ATP</td>
</tr>
<tr>
<td>(4)</td>
<td>AK</td>
<td>AcetylCoA</td>
<td>Acetate, ADP</td>
</tr>
<tr>
<td>(5)</td>
<td>PC</td>
<td>CO2</td>
<td>NADH</td>
</tr>
<tr>
<td>(6)</td>
<td>MCA</td>
<td>Oxaloacetate</td>
<td>Malate, NADH</td>
</tr>
<tr>
<td>(7)</td>
<td>MDH</td>
<td>Malate</td>
<td>GTP</td>
</tr>
<tr>
<td>(8)</td>
<td>FDH</td>
<td>Succinate</td>
<td>NADH</td>
</tr>
<tr>
<td>(9)</td>
<td>SDH</td>
<td>Fumarate</td>
<td>SuccinylCoA</td>
</tr>
<tr>
<td>(10)</td>
<td>PSC</td>
<td>PropionylCoA</td>
<td>SuccinylCoA</td>
</tr>
<tr>
<td>(11)</td>
<td>MCM</td>
<td>Propionyl-CoA</td>
<td>Methylmalonyl-CoA</td>
</tr>
<tr>
<td>(12)</td>
<td>MCE</td>
<td>Methylmalonyl-CoA</td>
<td>Propionate</td>
</tr>
<tr>
<td>Rxn #</td>
<td>Enzyme</td>
<td>EC#</td>
<td>454 database ID</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------------</td>
<td>-------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Pyruvate kinase</td>
<td>EC# 2.7.1.40</td>
<td>Assembly 34 (195877..197289)</td>
</tr>
<tr>
<td>2</td>
<td>Pyruvate dehydrogenase</td>
<td>EC# 1.2.1.51</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Pyruvate synthase</td>
<td>EC# 1.2.7.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Phosphate acetyltransferase</td>
<td>EC# 2.3.1.8</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>Acetate kinase</td>
<td>EC# 2.7.2.1</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>EC# 4.1.1.49</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Phosphoenolpyruvate carboxylase</td>
<td>EC# 4.1.1.31</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Methylmalonyl-CoA carboxytransferase</td>
<td>EC# 2.1.3.1</td>
<td>Assembly 34 (722495..722860)</td>
</tr>
<tr>
<td>7</td>
<td>Malate dehydrogenase</td>
<td>EC# 1.1.1.37</td>
<td>Assembly 34 (2776753..2777709)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC# 1.1.1.82</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Fumarate hydratase</td>
<td>EC# 4.2.1.2</td>
<td>Assembly 34 (1778115..1779521)</td>
</tr>
<tr>
<td>9</td>
<td>Succinate dehydrogenase</td>
<td>EC# 1.3.99.1</td>
<td>Assembly 34 (1857732..1859078)</td>
</tr>
<tr>
<td>10</td>
<td>Propionyl-CoA: succinyl-CoA-transferase</td>
<td>EC# 2.8.3.-</td>
<td>Assembly 34 (848052..849563)</td>
</tr>
<tr>
<td>11</td>
<td>Methylmalonyl-CoA mutase</td>
<td>EC# 5.4.99.2</td>
<td>Assembly 34 (392607..394793)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Assembly 14 (3649..4110)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Methylmalonyl-CoA epimerase</td>
<td>EC# 5.1.99.1</td>
<td>Assembly 34 (392607..394793)</td>
</tr>
</tbody>
</table>

a. the numbers in parenthesis represent the start and end sites of the gene in genome sequence

**Table F.** The location of propionic and acetic acids synthesis genes in the whole genome sequence of *P. acidipropionici*
APPENDIX G

MATHEMATICAL MODEL ANALYSIS OF CARBON SOURCE EFFECT ON ACID PRODUCTION
A stoichiometric metabolic model was developed and used to analyze the effects of different carbon sources on the fermentation profile, metabolic pathway shift, and propionic and acetic acids production.

In addition to the low cost, another attractive property of glycerol as substrate is the higher reduction state compared to sugars. In order to maintain the intracellular redox potential balance, most glycerol will be converted to reductive chemicals and biofuels, such as propionic acid (Barbirato et al., 1997) and ethanol (Dharmadi et al., 2006). Besides glucose and glycerol, sorbitol and gluconate were also used as carbon sources in propionic acid production (Suwannakham, 2005b; Huang, 1998). These carbon sources with different oxidation states provided different fermentation profiles in propionic acid production. Although it was well known that the oxidation level of substrate could shift the metabolic pathway (Dharmadi et al., 2006; Barbirato et al., 1997; Himmi et al., 2000), the regulation mechanism was poorly understood. No systematic stoichiometric metabolic analysis has been reported.

To evaluate contributions of different carbon sources to propionate production, a stoichiometric metabolic model was set up based on the metabolic pathway analysis. In this model, all the carbons from substrate (glucose, sorbitol, gluconate, and glycerol) were assumed to be used for cell growth and acid production (acetate, lactate and propionate). Using different substrate, the ratios between final products were different. Since the concentrations of NADH and NAD\(^+\) were very low (μM) in cells, the NADH must be recycled to NAD\(^+\) to continue the metabolism. During the fermentation, assumptions were made that the NADH production and NADH consumption were equal and the ATP production should be maximum. The ATP was used for cell growth and
maintaining the basic cell function. There would also have a mass balance for ATP.
Furthermore, it was assumed that there was no accumulation of intermediates, such as
PEP and pyruvate, and there was an equilibrium coefficient between succinate and
propionate. The simplified metabolic pathway is shown in Figure G. Based on the
metabolic pathway analysis, the common reactions are as follows:

\[
\begin{align*}
\text{pyruvate} + NADH & \rightarrow \text{lactate} \\
\text{pyruvate} + 2NADH & \rightarrow \text{propionate} + ATP \\
\text{pyruvate} & \rightarrow \text{acetate} + NADH + ATP \\
4\text{pyruvate} + 5.75NADH + 33.7ATP & \rightarrow \text{biomass}
\end{align*}
\]

For different carbon sources, there were different reactions regarding the
conversion from the carbon source to pyruvate. To derive the production of propionate,
acetate and lactate, the model should satisfy the dynamic balances of NADH, ATP, and
pyruvate in cells, respectively, and the constraint (or objective function) of Max (ATP
production).

1) Use 1 mol glucose as substrate, 2 mol of pyruvate will be generated through glycolysis.

\[
glu\text{cose} \rightarrow 2NADH + 2\text{pyruvate} + 2ATP
\]

According to the reactions above, three equations are generated:
In which x mol of pyruvate is used for lactate production, y mol is used for propionate, z mol is for acetate, and w mol is used for biomass. The solution should satisfy: \( \max(y + z) \). By the calculation of MATLAB 7.0.1, the results are \( x = 0.989 \) mol, \( y = 0.586 \) mol, \( z = 0.4243 \) mol. Thus the yields of lactate, propionate, and acetate from glucose are \( 0 \text{ mol/mol (0 g/g)} \), \( 0.989 \text{ mol/mol (0.41 g/g)} \), and \( 0.586 \text{ mol/mol (0.20 g/g)} \), respectively. P/A mole ratio (propionate vs. acetate) = 1.7. Because 1 mole of glucose generates 2 moles of pyruvate, the percentage of glucose used for biomass is 21% (0.4243 divided by 2).

2) Use 1 mol of glycerol as substrate:

The equations are:

\[
\text{glycerol} \rightarrow 2\text{NADH} + \text{pyruvate} + \text{ATP}
\]

Pyruvate balance: \( x + y + z + w = 1.0 \)

NADH balance: \( 2 + z = 2y + x + \frac{5.75}{4}w \)

ATP balance: \( 1 + y + z = \frac{33.7}{4}w \)

max\((y + z)\)

However, there was no practical solution for these equations, because the NADH balance can not be satisfied. The most closed solution is \( x = 0 \text{ mol}, y = 1.0 \text{ mol}, z = 0 \text{ mol}, w = 0 \text{ mol} \). The yields of lactate, propionate, and acetate from glycerol are \( 0 \text{ mol/mol (0 g/g)} \).
g/g), 1.0 mol/mol (0.80 g/g), and 0 mol/mol (0 g/g), respectively. P/A mole ratio = ∞.

The theoretical ratio of glycerol used for biomass is 0.

For sorbitol, gluconate, xylose, and lactate, the reactions for pyruvate generation are as follows:

\[
\text{sorbitol} \rightarrow 3\text{NADH} + 2\text{pyruvate} + 2\text{ATP}
\]

\[
\text{gluconate} \rightarrow 8/3\text{NADH} + 5/3\text{pyruvate} + 5/3\text{ATP}
\]

\[
\text{xylose} \rightarrow 5/3\text{NADH} + 5/3\text{pyruvate} + 5/3\text{ATP}
\]

\[
\text{lactate} \rightarrow \text{NADH} + \text{pyruvate}
\]

Similar calculations were performed and the calculated results are listed in Table G with the experimental results. The results from this mathematic model using NADH, ATP and pyruvate balance and maximum of ATP production showed that glucose has the lowest P/A ratio and glycerol has the highest P/A ratio. Considering P/A ratio, the total comparison was glucose, lactate < xylose < sorbitol < gluconate < glycerol, which was consistent with the experimental results. In addition, the yields of propionic, acetic, and lactic acids from model well matched the fermentation data. Many fermentation profiles could be explained by this model simulation. Based on the model of culture on glycerol, no carbon source goes to synthesize biomass theoretically, which contributes the highest yield of propionate among the six substrates. This is because that a small amount of biomass generation may cause some NADH accumulation, which can cause a decrease in NAD\textsuperscript{+} concentration. The cell growth will be inhibited due to the higher NADH/NAD\textsuperscript{+} ratio (Chang et al., 1999). That may be able to explain that \textit{P. acidipropionici} showed a
smaller specific growth rate on glycerol fermentation as compared to other substrates. Also the reactions could accumulate ATP, and the propionate production would be inhibited by the higher ATP/ADP ratio. In order to maintain the ATP/ADP balance, cells need to find way to consume the extra ATP. The biomass’s basic maintenance energy consumption could convert ATP to ADP. Since this reaction (from ATP to ADP) is slow, the whole fermentation needs to last very long to generate a satisfied amount of propionate. This hypothesis has been justified by the 700 h of fermentation on 40 g/l-glycerol compared to the 150 h of fermentation on 40 g/l-glucose.

Moreover, the calculated lactate production is very low (x = 0) on most carbon sources (except lactate as substrate). In the real experiment, the lactate production was very low as well. This is because cells need to maximize the ATP production, but there is no ATP generated in the lactate production pathway (Figure G). Thus, the carbon flow will choose propionate production pathway in stead of lactate to generate one ATP even though the acetate pathway is blocked. This has been seen in the original ACK-Tet fermentation (Table 4.2 in Chapter 4).

The recovery of acetic acid production in the adapted ACK-Tet can also be explained by the model. Acetate production cannot be completely eliminated by gene blocking due to generation of ATP and NADH in the acetate pathway. To maintain the intracellular balance of ATP and NADH, mutants with gene knock-out in the acetate pathway might shift the metabolic pathway to recover the acetate synthesis (Chang et al., 1999; Fong et al., 2006).

However, it should be noted that the intermediates cannot be predicted by the stoichiometric model because the factor of time was not considered. In order to obtain
more information about the fermentation profiles, a dynamic model should be set up (Shinto et al., 2007).
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glucose&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sorbitol&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Gluconate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Xylose&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lactate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation state</td>
<td>0</td>
<td>-1/3</td>
<td>+1/3</td>
<td>0</td>
<td>0</td>
<td>-2/3</td>
</tr>
<tr>
<td>( Y_{\text{Propionate}} ) (g/g)</td>
<td>0.41 (0.40±0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54 (0.55±0.01)</td>
<td>0.44 (0.40±0.02)</td>
<td>0.47 (0.48±0.02)</td>
<td>0.48 (0.53±0.01)</td>
<td>0.80 (0.73±0.01)</td>
</tr>
<tr>
<td>( Y_{\text{Acetate}} ) (g/g)</td>
<td>0.20 (0.11±0.002)</td>
<td>0.08 (0.07±0.01)</td>
<td>0.05 (0.040±0.004)</td>
<td>0.14 (0.13±0.01)</td>
<td>0.21 (0.16±0.02)</td>
<td>0 (&lt;0.02)</td>
</tr>
<tr>
<td>( Y_{\text{Lactate}} ) (g/g)</td>
<td>0 (~0)</td>
<td>0 (~0)</td>
<td>0 (~0)</td>
<td>0 (~0)</td>
<td>0 (~0)</td>
<td>0 (~0)</td>
</tr>
<tr>
<td>( P/A ) (mol/mol)</td>
<td>1.7 (2.9±0.1)</td>
<td>5.2 (6.4±1.0)</td>
<td>7.4 (8.1±1.6)</td>
<td>2.63 (3.0±0.6)</td>
<td>1.85 (2.69±0.3)</td>
<td>( \infty ) (&gt;20)</td>
</tr>
<tr>
<td>% Substrate for biomass</td>
<td>21.2 (26.8±0.3)</td>
<td>21.2 (16.3±0.2)</td>
<td>21.2 (16.5±0.2)</td>
<td>21.2 (13.6±0.1)</td>
<td>6 (10±0.1)</td>
<td>0 (3±0.02)</td>
</tr>
</tbody>
</table>

<sup>a</sup> (#) represents the experimental data  
<sup>b</sup> The experimental data were cited from Suwannakham, 2005b  
<sup>c</sup> The experimental data were cited from Yan Huang, 1998

**Table G** Comparison of kinetic parameters from experiments and stoichiometric model analyses.
Figure G The simplified dicarboxylic acid pathway for the propionate production from glucose, glycerol, sorbitol, gluconate and lactate by *P. acidipropionici*. 