Acetone-Butanol-Ethanol Fermentation by Engineered *Clostridium beijerinckii* and *Clostridium tyrobutyricum*

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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Graduate Program in Food Science and Technology

The Ohio State University

2010

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Abstract

Recently, biofuel production is a popular topic with the rising concerns about the limited supply of fossil fuel and the fluctuating price of crude oil. Biofuels can be categorized into different groups based on their characteristics and applications, including biodiesel, bioalcohols, biogas, and solid biofuels. Bioalcohols, such as methanol, ethanol, and butanol, have been considered to replace transportation fuels. Among the bioalcohols, butanol attracted more and more attentions because of its superiority over other bioalcohol candidates with its similar properties to automobile gasoline, such as octane numbers, energy content, and energy density. Other advantages for using butanol as a biofuel are its low hygroscopicity and low vapor pressure which prevent increase in moisture content in butanol and make butanol a safe and environmentally friendly biofuel. Butanol, a butyl alcohol, can be produced from sugars and starch by solvent-producing clostridia through acetone-butanol-ethanol (ABE) fermentation. ABE fermentation was once a large industrial fermentation for solvent production from starch-based substrates and molasses during the World Wars in the early twentieth century. However, this practice gradually disappeared because its products could not compete with solvents produced through petro-chemical syntheses. Another reason that butanol production from ABE fermentation declined was because it suffered from low titer, yield and productivity, which made it not economically feasible for commercialization with the
high cost in downstream product recovery. In order to improve butanol production from ABE fermentation, extensive studies have been investigated in the aspects of metabolic engineering and process development. The objectives of this study were to develop enhanced butanol production from ABE fermentation in continuous FBB by mutants of \textit{C. beijerinckii}, and to explore the possibility of producing butanol by metabolic engineered hosts.

ABE fermentation has two phases, acidogenesis and solventogenesis. In acidogenesis, acetic acid and butyric acid are produced, while acetone, ethanol, and butanol are produced in solventogenesis. A stoichiometric model was developed for the analysis of the metabolic pathway in solvent producing clostridia that carry out ABE fermentation. The model predicted the maximum butanol yield based on mass and energy balance at steady state in continuous process. This helped process development without redundant experiments. In the stoichiometric analysis, the supplement of butyric acid in the medium was shown to benefit butanol production from ABE fermentation by reducing required energy to produce butanol (NADH) and increasing butyrate phosphate production which upregulates solventogenesis.

Two different mutants of \textit{C. beijerinckii} were cultured in continuous fermentation to study the effect of using butyric acid as a supplement in the medium under different conditions. At a high dilution rate of 1.88 h\(^{-1}\), asporogenic \textit{C. beijerinckii} ATCC 55025 in the fibrous bed bioreactor (FBB) was able to utilize glucose and butyrate and steadily give a high butanol productivity of 17.29 g/L\cdot h, which was 170 folds higher than that in conventional free-cell batch fermentation. A new strain isolated from \textit{C. beijerinckii}
ATCC 55025 in repeated-batch culture in the FBB, *C. beijerinckii* JB200 was studied on its performance in continuous fermentation. *C. beijerinckii* JB200 is superior to other industrial strains with its better tolerance of solvent, acid and feeding substrate. In continuous fermentation, butanol titer was increased to 12 g/L at lower dilution rate by *C. beijerinckii* JB200.

In the studies of continuous fermentation by two *C. beijerinckii* mutants, fibrous bed bioreactor (FBB) was used. It is a novel technology that can help cell immobilization at high cell density for adaptation. In continuous FBB, *C. beijerinckii* mutants were able to be retained at high cell density (>100 g dried weight/L) in the bioreactor and steadily produce butanol without contamination or degeneration. With incorporation of FBB, *C. beijerinckii* was able to effectively use both glucose and butyric acid to produce butanol.

Besides solvent producing clostridia, metabolically engineered *Clostridium tyrobutyricum* was studied for its potential to produce butanol. The metabolic engineered mutant of *C. tyrobutyricum* ATCC 25755 was able to actively produce butanol over six repeated batches in the FBB using glucose and/or xylose. In batches with only xylose as the carbon source, the mutant was able to produce more butanol at 7.12 g/L with a higher yield because of the slower cell growth that made the foreign aldehyde/alcohol dehydrogenase competitive to the native enzyme for butyryl CoA.

A pilot scale FBB was constructed for the evaluation of the feasibility of large scale butanol production in the FBB. Two batches of batch fermentation were operated and the results of ABE fermentation were similar to those in batch fermentation at lab scale. ABE fermentation at pilot scale was able to produce butanol at 8.7 g/L and 9.4 g/L
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In summary, continuous butanol production with improved productivity from ABE fermentation with supplement of butyric acid as additional carbon source in the FBB was demonstrated. The supplement of butyric acid was able to maintain the process in solventogenesis and produce higher ratio of butanol to total produced solvents. The FBB was able to stabilize butanol production in continuous process. More studies are necessary to improve butanol production by metabolically engineered strains. At pilot scale, the FBB was able to produce butanol with a result that was comparable to that in free cell batch reactor at lab scale. The pilot scale FBB can be further operated under different modes of fermentation for process optimization.
Dedication

Dedicated to my parents and my sisters
Acknowledgments

First of all, I would like to thank my advisor, Dr. Shang-Tian Yang, for his guidance, encouragement, patience, and full support during my graduate study. I am eternally grateful for his help academically and financially throughout the completion of my Ph.D study. I have benefited a lot from his expertise in science and his great personality.

I wish to thank Dr. Ahmed Yousef and Dr. Jeffrey Chalmers for their time serving on my committee and their valuable suggestions and advices to my research project.

I would like to acknowledge Dr. An Zhang for his help on preliminary experiment setup and sincere thanks to all the previous and current laboratory members, especially Dr. Yali Zhang, Dr. Mingrui Yu, Ching-suei Hsu, Jianxin Sun, and Congcong Lu for their encouragement and support. Special thanks to Mr. Bingxu Song for his help on the establishment of the foundation of pilot plant, valuable discussion with Dr. Yali Zhang on the study of stoichiometric analysis, and Dr. Mingrui Yu for his construction of C. tyrobutyricum mutants.

Financial supports from the Ohio Department of Development Third Frontier Advanced Energy Program, the Consortium for Plant Biotechnology Research, Inc., and EnerGenetics International during the course of this research are appreciated.
Finally I would like to thank my parents Mr. Tien-Yang Chang and Mrs. Chao-Yu Chang Yu, my sisters, and my boyfriend for their unconditional love and support, and all of my family members and friends for their having faith in me.
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Chapter 1: Introduction

Biofuels have become more and more popular because of the concern of limited supply of fossil fuels and unpredictable prices of crude oil (Qureshi et al., 2008). Generally, biofuels are considered as combustible materials in the forms of gas, liquid, or solid derived from biomass. Biofuels, including bioalcohols, biodiesel, biogas, and solid biofuels, are categorized into different groups based on differences in physical and chemical characteristics.

Recently, butanol has received much attention with its potential to be an alternative fuel for petroleum gasoline. Butanol is a four-carbon alcohol with a wide variety of applications in cosmetics, drugs, and household cleaners. Traditionally, it is used as solvent, diluent, plasticizer, and raw material for chemical production. As a biofuel, butanol can be used directly in automobiles without any modification in the engine (Alasfour, 1995). The advantages of using butanol as a biofuel include its high energy content, lower vapor pressure and low hygroscopicity as compared to bioethanol. To produce butanol biologically, acetone-butanol-ethanol (ABE) fermentation is usually involved. By producing butanol through ABE fermentation, renewable biomass can be used to relieve the burden of waste disposal from industries and agriculture.

Acetone-butanol-ethanol fermentation is usually carried out by solvent-producing clostridia. It was ranked second in industrial fermentation in the early 20th century.
During the two World Wars, butanol was in high demand for explosive production and transportation fuel (Jones and Woods, 1986). The practice declined quickly with its inability to compete with petroleum-based butanol. Figure 1.1 shows the overall metabolic pathway of ABE fermentation carried out by solventogenic bacteria. Two distinctive stages can be defined in ABE fermentation: acidogenesis and solventogenesis. During the exponential phase, the bacteria follow the route in acidogenesis and produce acetic acid and butyric acid, which decrease the pH in the environment. With accumulated acids, acidogenesis shifts to solventogenesis and acids are used to produce acetone, butanol and ethanol. Because of the complexity of the metabolism, butanol production from ABE fermentation suffers from low titer (<15 g/L), yield (<0.25 g/g sugar fermented) and productivity (<0.2 g/L-h) (Qureshi and Blaschek, 2001).

Extensive studies have been done on process development to reduce the overall production cost of butanol, including substrate pretreatments, fermentation processes, and product recoveries. While the price of starch-based substrates became too high for butanol production, lignocellulosic substrates were put into consideration with many different methods for their hydrolysis to release fermentable sugars (Qureshi et al., 2008a; Qureshi et al., 2008b; Ezeji et al., 2003). Fermentation processes, including batch, fed-batch, and continuous processes, were also investigated for improved butanol fermentation.

Cell immobilization is a technology that can be integrated into fermentation processes to improve performance. Many different media have been studied for cell immobilization (Zhang et al., 2009). The fibrous bed bioreactor (FBB) is a novel
technology that can be used in many different biotechnology applications (Yang, 1996). With a very high surface area for cell immobilization, the FBB can greatly improve chemical and solvent productions with significantly increased cell density (Yang et al., 1994; Yang et al., 1996; Zhu and Yang, 2003; Cho, et al., 1996; Huang, et al., 2004).

In addition to process development, strain development is also very important in improving butanol production from ABE fermentation, as well. Many different strains have been studied for butanol production through ABE fermentation, including Clostridium acetobutylicum ATCC 824, C. beijerinckii ATCC 55025, and C. beijerinckii BA101 (Bahl et al., 1982; Huang et al., 2004; Maddox et al., 1995; Parekh et al., 1998; Soni et al., 1987). Strain development can be done either by spontaneous mutation with mutagenic chemicals or by metabolic engineering (Jain, et al., 1993; Papoutsakis, 2008).

1.1 Objectives and Research Tasks

The goal of this project was to develop an economic fermentation process for butanol production from sugars in ABE fermentation. To achieve this goal, various approaches were used in an integrated manner, including the development of a mathematical model for stoichiometric analysis of the fermentation pathway and fermentation process engineering with different Clostridia species with enhanced butanol tolerance and productivity. Figure 1.2 provides an overview of the research objectives, approaches, and the scope of this study. The experimental studies and results are presented in Chapters 3-7 and briefly described below.
Task 1. Stoichiometric analysis of continuous ABE fermentation by butanol producing clostridia

In order to effectively develop the ABE fermentation process for butanol production from sugars, a stoichiometric model based on the metabolic pathway of ABE fermentation during continuous process was constructed. At the steady state of continuous fermentation, the metabolic flux is steady with constant inputs and outputs. It was found that butyrate as a cosubstrate with glucose could improve butanol production in the continuous ABE fermentation. With more butyrate uptake during the fermentation, more butyryl phosphate (BuP), a putative regulator for solventogenesis (Desai and Papoutsakis, 1999), can be produced to upregulate solventogenesis. The effect of butyrate uptake on butanol production was evaluated using the model. The theoretical butanol yield was calculated by energy and mass balances under different circumstances of biomass formation, acetate production, and butyrate uptake. The results of this study are presented in Chapter 3.

Task 2. Biobutanol production by Clostridium beijerinckii ATCC 55025 in a single pass continuous fibrous bed bioreactor

While conventional ABE fermentation in batch reactors suffered from low titer, yield, and productivity, ABE fermentation in a continuous FBB by Clostridium beijerinckii ATCC 55025 was studied to increase butanol productivity. C. beijerinckii ATCC 55025 is a mutant without the ability to form spores, which can help maintain a steady continuous fermentation. Different dilution rates were applied in the continuous
fermentation with continuous feed with glucose and butyric acid as carbon sources. Butanol productivities, titers, and yields under different dilution rates and butyrate concentrations in the feed medium were evaluated and the results are reported in Chapter 4.

**Task 3. Continuous butanol production by *Clostridium beijerinckii* JB200**

In order to improve butanol production from ABE fermentation, a mutant with higher tolerance to final products obtained from adaptation in the FBB was used in continuous fermentation with controlled pH. Several other industrial strains for butanol production were also cultured in media with different concentrations of butyric acid to compare with *C. beijerinckii* JB200. The performance of butanol production in five different strains was examined based on the butanol yield and productivity in media with increasing concentration of butyric acid. Continuous fermentation by *C. beijerinckii* JB200 was operated in a different setup with controlled pH. The performance of *C. beijerinckii* JB200 in free cell chemostat and continuous FBB fermentations were studied and the results are reported in Chapter 5.

**Task 4. Kinetics of butanol fermentation by metabolically engineered *Clostridium tyrobutyricum***

In order to improve butanol yield and productivity, a metabolically engineered mutant of *Clostridium tyrobutyricum* was constructed and cultured in repeated batch FBB. The strain was selected because of its metabolic pathway partially similar to that in
butanol-producing clostridia and its high tolerance to butanol and butyric acid, which are the main products and inhibitors in ABE fermentation. The mutant with a partially disrupted acetate biosynthesis pathway was cloned with butyaldehyde/butanol dehydrogenase to produce butanol from butyryl-CoA. Without the acetone producing pathway in *C. tyrobutyricum*, butanol yield from sugar can be higher than that in conventional ABE fermentation. Six repeated batches were performed in the FBB with different combinations of glucose and xylose as substrates, and the results are discussed in Chapter 6.

**Task 5. Scale-up of butanol production by *Clostridium beijerinckii* ATCC 55025 in a fibrous bed bioreactor**

Because the FBB is not a common reactor for commercialized systems, an intermediate level experimental setup is necessary for evaluating butanol production in the FBB at a larger scale. An 150-liter stirred tank bioreactor with fibrous bed packings was built for the pilot scale fermentation. Butanol production in two separate ABE fermentation batches was studied and the results are discussed in Chapter 7.
1.2 Reference


Figure 1.1 Metabolic pathway of ABE fermentation by *Clostridium acetobutylicum* and *C. beijerinckii*. 
Figure 1.2 Overview of research objectives and major tasks.

**Goal**
Enhanced butanol production through ABE fermentation

**Process Development**
Ch. 3 – Stoichiometric analysis of metabolic pathway

**Metabolic Engineering**
Ch. 6 – Repeated batch fermentation kinetics with different sugars by engineered *C. tyrobutyricum*

Ch. 4 – Continuous fermentation in FBB by *C. beijerinckii* ATCC 55025

Ch. 5 – Continuous fermentation in FBB by *C. beijerinckii* JB 200

Ch. 7 – Scale up of ABE fermentation in FBB by *C. beijerinckii* ATCC 55025
Chapter 2: Literature Review

2.1 Biofuels

Many current studies are focused on biofuel production because of the increasing concerns on the limited supply of fossil fuel and the unpredictable price of crude oil (Qureshi et al., 2008). As shown in Figure 2.1, renewable energy occupied 18% of global final energy consumption and biofuels shared 75% of consumed renewable energy in 2006 (Martinot, 2007). Biofuels are combustible materials derived from biomass, which can be plants, animals, and microorganisms. Served as energetic purposes, biofuels may be in the forms of gas, liquid, or solid. Nowadays biofuels are categorized into three generations based on their current and future availabilities. First generation biofuels are produced using traditional technology from basic feedstocks such as corn, grains, sugar cane, seeds, etc. Second generation biofuels are produced through either fermentation or thermochemical conversion from non-food sources, such as corn stover, corn stalks, and wood. With lack of production experience, third generation biofuels, including biopropanol and biobutanol, are likely to be important biofuels on the market after 2050 (Bringezu, 2009). Several biofuels will be discussed in the following paragraphs based on their physical and chemical characteristics.
2.1.1 Bioalcohols – Bioethanol

Bioalcohol is produced from biological sources by microorganisms. Several primary alcohols, such as methanol, ethanol, butanol, etc., have been considered to be biofuel candidates because of their similar physical and chemical properties to gasoline (Table 2.1) (Lee et al., 2008). With the advantages of high octane numbers and lower concerns of air pollution, bioalcohols can be used as a blend for gasoline or direct replacement of gasoline.

Among the three candidates just mentioned, bioethanol production was the most developed process. Large scale productions of bioethanol can be found in Brazil, the United States, China, etc. In 2008, global bioethanol production was 67 billion liters per year, which was twice higher than that in 2004 (Martinot and Sawin, 2009). Being one of the first generation biofuels, bioethanol was produced using food based substrates, such as corn starch and sugarcane. This raised an issue about competition between bioethanol industry and food industry. The substrate source was soon changed to non-food based biomass such as corn stover, corn cob, wheat straw, etc. These lignocellulosic substrates were treated with dilute acid at high temperature and/or cellulose hydrolyzing enzymes to release monosaccharides from the substrate (Himmel et al., 2007). Many different species of microorganisms were able to produce bioethanol from biomass, including *Saccharomyces cerevisiae, Clostridium* sp., *Escherichia coli*, and *Zymomonas mobilis*. *Saccharomyces cerevisiae*, commonly known as yeast, has been used in ethanol production in wine and beer industries. However, wild type *S. cerevisiae* does not have the ability to utilize pentose released from cellulosic substrates. By several approaches of
metabolic engineering, pentose utilization pathways were integrated into *S. cerevisiae* for bioethanol production (Weber *et al.*, 2010). *Escherichia coli* was another microorganism commonly used in bioethanol fermentation utilizing pentose from cellulosic biomass. For example, the metabolically engineered *E. coli* KO11 with genes encoding *Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase was able to utilize both pentose and hexose to produce bioethanol faster than yeast at 90% theoretical yield (Ingram *et al.*, 1997). Fermentation technologies for bioethanol production have been well developed and improved to make bioethanol commercially available. In order to reduce the overall cost of bioethanol production, process integration from pretreatments of lignocellulosic biomass, fermentation, to downstream separation was commonly found in the industrial setup. Simultaneous saccharification and fermentation (SSF) was the combination of biomass pretreatment and bioethanol fermentation. Reactors needed for bioethanol production in SSF were reduced with the combined process of biomass hydrolysis and fermentation. Another advantage of SSF was the better performance of enzymatic hydrolysis with glucose steadily removed during fermentation. Sequential hydrolysis and fermentation (SHF) included substrate pretreatment, fermentation, and partial downstream separation. The lignocellulosic biomass is treated with acid hydrolysis and enzymatic hydrolysis to release both pentose and hexose. Hexose in the hydrolysate was first fermented and the produced ethanol was removed through distillation. The remaining pentose was transferred into the next fermentation reactor and the produced ethanol was then distilled again (Hamelinck *et al.*, 2005).
Although bioethanol has been investigated for decades, more research is still required for bioethanol production from lignocellulosic biomass to be competitive. As an example, enzyme hydrolysis needs to be improved with more efficient enzymes at lower cost. Microorganisms with better substrate tolerance and performance are required for increased productivity and titer of bioethanol produced from hydrolysate (Hahn-Hägerdal et al., 2006).

2.1.2 Biodiesel

Biodiesel (fatty acid alkyl esters) is derived from transesterification of alcohols and triglycerides, the main constituents in vegetable oils and animal fats (Janaun and Ellis, 2010). The process produces biodiesel with similar properties compared to diesel fuels by reducing the molecular weight to one-third and viscosity to one-eighth of the substrates. Biodiesel is superior to petroleum diesel because it is biodegradable, non-toxic, free of sulfur, and has lower exhaust emissions (Singh and Singh, 2010).

The raw materials for biodiesel production are vegetable oils, animal fats and waste oils from restaurants. The decision on which substrate to be processed is based on its cost and availability in the region. In order not to compete with other applications (e.g. food and pharmaceutical sources) and increase cost, non-edible and/or processed oils can also be used to produce biodiesel. The most commonly used oils for the production of biodiesel are soybean, sunflower, palm, rapeseed, canola, cotton seed and Jatropha (Atadashi et al., 2010). The quality of the raw materials plays a very important role in making high quality biodiesel. Free fatty acids in the substrates can affect the efficiency
of the transesterification process and further increase the cost for downstream purification. Water content in the feedstock was also reported to be a factor that influences the process of biodiesel production (Sheedlo, 2008).

In the process of the transesterification for biodiesel production, triglycerides react with alcohols to produce ester and glycerin with addition of catalyst to accelerate the process. The process happens in three consecutive steps. The triglyceride first becomes diglyceride, then monoglyceride, and glycerin in the end, while an ester molecule is released in each of the three steps. Catalysts used to facilitate the process can be alkalis, acids, or biological enzymes. For both alkali and acid catalysis, reasonable yield of esters could be obtained and cost of catalysts was relatively low. However, downstream separation of glycerol and ester after alkali catalysis could be costly if water content in the feedstock was high and resulted in the formation of an emulsion. Lipase, a biological enzyme, was also studied for possibilities of biodiesel production. It could be fixed in a reactor and steadily produce esters at higher yield without being affected by the water content in the feedstock. Although lipase catalysis was able to give higher yield and cheaper downstream separation, its disadvantages were the relatively expensive catalyst and the risk of losing enzyme activity during the process. Other than using catalysts for biodiesel production, use of supercritical alcohols was also under investigation. This process was able to convert free fatty acids in the feedstock to esters and gave a higher reaction rate. On the other hand, the reaction temperature used was extremely high (239~385 ºC) compared to other processes (30~80 ºC) and might increase the overall cost (Marchetti et al., 2007)
Biodiesel can be used directly in current diesel engines with qualified chemical and physical properties that are similar to those of petroleum diesels. As a supplement to other energy supply, biodiesel could be very useful as it reduces the concern of air pollution and green house gas effects. It could also be blended with petroleum diesel to minimize the carbon deposition in the combustion chamber or solve other problems that might occur when using straight biodiesel in diesel engines. As a result, more research is still needed to produce biodiesel with better quality.

2.1.3 Biogas and solid biofuels

Biogas usually refers to a gas produced by the biological breakdown of biodegradable materials in anaerobic environment. The main components in biogas are methane (CH$_4$) and carbon dioxide (CO$_2$) and some other compounds depend on the source of biogas. Biogas was considered as another renewable energy with its high energy content (15~30 MJ/Nm$^3$). Another reason to collect and utilize biogas was to reduce its contribution to the increasing greenhouse gas concentration. Biogas could be recovered from landfill, wastewater sewage, manure from animal farms, etc. Besides the major components in biogas, there are also some minor compounds, such as hydrogen sulfide (H$_2$S), ammonia (NH$_3$), and siloxanes. These compounds are considered as contaminants when biogas is used as a biofuel. They could release more pollution into the environment or corrode the pipeline used to transport the fermented biogas without purification. Currently, many technologies have been developed for biogas purification, such as H$_2$S and CO$_2$ removal by microorganisms and siloxane removal by gas
permeation or adsorption (Abatzoglou and Boivin, 2009). Biogas could be introduced to biogas burners in household appliances directly or further converted to synthetic gas which is composed of carbon monoxide (CO) and hydrogen (H₂) (Kossman et al., 1990; Wang et al., 2005).

While the name is quite self-explanatory, solid biofuels are biological matter that can be burned in a furnace for heat. The main components of solid biofuels are C, H, and O. Wood chips, sawdust, straw, grasses, etc. are all considered to be solid biofuels. For better transportation, raw materials of solid biofuels are usually dried and compressed into briquettes with uniform size and condition (i.e., chemical and physical characteristics) (Chen et al., 2009; Belbo, 2006). Solid biofuel can be used as a substitute for charcoal and incinerated at power plants for energy production. However, solid biofuel of different raw materials could behave differently during combustion. After combustion solid biofuel from wood materials could produce a great amount of ash containing high concentrations of heavy metals. The ash could cause pollution problem with improper disposal as well as aerosol formation. Solid biofuel from crop wastes rich in nitrogen (N), chlorine (Cl), and sulfur (S) might give NOₓ, HCl, and SOₓ emissions and corrosion. Therefore, combustion conditions should be carefully controlled according to the content of solid biofuels (Obernberger et al. 2006).
2.2 Production of biobutanol

2.2.1 Butanol

Butanol is a butyl alcohol (MW 74.12) with a wide variety of applications. It is a flammable, clear, and colorless liquid with a banana-like odor. Butanol can be used as solvent, diluent, plasticizer, and raw material for chemical production. It can be found in cosmetics, drugs, and household cleaners. As a biofuel candidate, butanol has the advantage of being directly used in normal combustion engine built in automobiles (Alasfour, 1995). With its low vapor pressure (2.3 kPa), butanol is more environmentally friendly than gasoline (60-90 kPa). Another important advantage is that butanol does not absorb moisture from air because it is less hygroscopic than ethanol. The common problem of corrosion when transporting ethanol through existing pipelines does not occur to butanol transportation as less moisture will be captured by butanol. As a result, butanol is superior to other biofuels. It is more gasoline-like and can be blended easier with gasoline or directly used in the conventional internal combustion engine without any modification.

Butanol is traditionally produced via chemical processes from propylene (CH₃CHCH₂), carbon monoxide (CO), and hydrogen (H₂) as shown in Eq. 2.1.

\[
\text{CH}_3\text{CHCH}_2 + \text{CO} + \text{H}_2 \xrightarrow{\text{Co or Rh}} \text{CH}_3\text{CH}_2\text{CHO} \xrightarrow{\text{H}_2} \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} \quad \text{Eq. 2.1}
\]

Besides chemical syntheses with raw materials from petroleum, butanol can also be produced from microbial fermentation, acetone-butanol-ethanol (ABE) fermentation,
with many advantages. Biological production processes are environmentally friendly since they can use renewable biomass as feedstock, including industrial and agricultural wastes. The establishment of the biorefinery industry also favors energy independence and economic security of a country.

2.2.2 ABE fermentation

Microbial production of butanol was first reported by Pasteur in 1861 and later found that not only butanol was produced from the fermentation but also acetone and ethanol. This fermentation is thus named acetone-butanol-ethanol fermentation. The microbial production of acetone and butanol was rapidly developed during the two World Wars with high demands for acetone and butanol for explosive production and as transportation fuel (Jones and Woods, 1986). During the 1920s, acetone-butanol-ethanol (ABE) fermentation ranked second in industrial fermentation using molasses as the fermentation substrate. With its increasing raw material cost and expensive downstream separation, this practice declined rapidly in the 1950s because it was unable to compete with the petro-chemical industry.

The metabolic pathway of solventogenic bacteria that carry out ABE fermentation is shown in Figure 2.2. Acetic and butyric acids are produced in the early stage of fermentation (exponential phase) called acidogenesis (left part of Figure 2.2. from the mid line). A metabolic shift to solventogenesis (right part of Figure 2.2. from the mid line) is then induced with accumulated acids and decreased pH to < 5.0. During solventogenesis, bacteria use the accumulated acids as an additional carbon source to
produce solvents, including acetone, ethanol and butanol. With acids present in the environment, cell growth is slowed down or inhibited (entry of stationary phase). With accumulated metabolites of both acids and solvents, sporulation is then induced (Long et al., 1984; Papoutsakis, 2008). Although ABE fermentation has been studied over several decades, the mechanism of the metabolic shift from acidogenesis to solventogenesis is still unclear.

One major reason that the practice of ABE fermentation in the early 20th century stopped was its final product inhibition, which causes the low product concentration (<20 g/L). Low product concentration results in low yield (~0.30 g/g) and productivity (<0.3 g/L·h), thus incurs high cost in product recovery during downstream processing (Qureshi and Blaschek, 2001).

2.3 Process Development

In order to improve butanol production from ABE fermentation and reduce the overall production cost, extensive studies have been done on substrate pretreatments, fermentation processes, and product recoveries.

2.3.1 Substrate pretreatment

Before the controversy of using food-based substrates for biofuel production, butanol was produced by microorganisms utilizing whey permeate, corn and molasses (Jones and Woods, 1986). For amylolytic microorganisms with the ability to break down starch and release sugars, substrates were treated with hot water to gelatinize and
liquefy starch before being fed to fermentation. There are several approaches to acquire sugars from starch based substrates for microorganisms that are unable to break down starch. The gelatinized starch could be further treated with amylolytic enzymes, or dilute acid to release sugars for microorganisms to consume and produce butanol. Amylases, the enzymes that break down starch, include α-amylase, β-amylase and pullulanase. While α-amylase and β-amylase target α-1,4 glycosidic bond and release glucose and oligosaccharides from starch, pullulanase cleaves α-1,6 glycosidic bond and remove branches from starch. Among the three enzymes, α-amylase is commonly used to break down starch in the process of pretreatment (Ezeji et al., 2005).

Table 2.2 summarizes the non cellulosic feedstocks used in ABE fermentation. Total ABE titer achieved was at least 19 g/L (Campos et al., 2002; Ezeji et al., 2003; Parekh and Blaschek, 1999; Qureshi et al., 2001). By direct fermentation of casasava starch, 16.9 g/L butanol and 21.0 g/L total ABE were produced (Thang et al., 2010). As a common by-product of biodiesel production process, the cost and availability of glycerol became economic for butanol production from ABE fermentation. Butanol was successfully produced at the titer of 16 g/L from pure glycerol by C. pasteurianum DSM 525 (Bielb, 2001).

When the demand of food based substrates increased from the agricultural industry, the cost for raw materials increased and became less economical for butanol production. Possibilities of using lignocellulosic biomass as feeding materials were thus investigated, such as corn fiber, corn cob, wheat straw, wood chips, and agricultural wastes (Qureshi et al., 2008a; Qureshi et al., 2008b; Ezeji et al., 2003). Pretreatments for
lignocellulosic substrates to release sugars were similar. Substrates were first dried and physical breakdown through milling. The milled substrates were then treated with dilute acid at high temperature and high pressure. Xylose was the major product after the acid hydrolysis. Enzymetic hydrolysis was then carried out with cellulase to release both glucose and xylose after acid hydrolysis. The typical yield of sugar from dried lignocellulosic biomass was around 70% (Saha et al., 2005). The common problem of using acid pretreated substrate in fermentation is the inhibition of microbial growth caused by the by-product of acid hydrolysis, such as fufurals, and hydroxymethyl furfural (HMF). The toxic by-products could be removed by passing through a column packed with absorptive resin. Table 2.3 summarizes ABE fermentation using lignocellulosic biomass, including corn fiber, corn stover, wheat straw, distillers dried grains, and pine wood. More studies were still required to optimize acid hydrolysis and decrease formation of microbial inhibitors for improved butanol production.

### 2.3.2 Mode of Fermentation

The majority of studies on butanol fermentation were operated in batch reactors with the advantages of simplicity, easy manipulation, and low risk of contamination. Minor or no modification of the reactor setup was required when different types of feeding materials were used. In a batch reactor, however, productivity was usually low due to the lag phase in the beginning and the end product inhibition at the end of fermentation. Extra time for cleaning, sterilization, and medium preparation was also a factor that affected the performance of butanol production. In batch fermentation, the
average butanol productivity was 0.2 g/L∙h, which was not economical enough for large scale production (Evans and Wang, 1988).

Fed-batch fermentation was commonly used in industrial production with its advantage of easier maintenance over batch reactors. After the reactor was started in batch mode, condensed substrate was fed into the fermentation reactor slowly to maintain optimal concentration of substrate in the fermentation broth. For butanol production in fed-batch fermentation, the reactor was usually integrated with product recovery techniques to keep steady butanol production by removing toxic butanol *in situ* (Qureshi and Maddox, 1991; Groot *et al.*, 1984). The butanol productivity was improved to 0.4~0.6 g/L∙h, depending on the type of product recovery techniques used.

Besides batch and fed-batch fermentations, continuous (perfusion) fermentation was also studied for butanol production with the advantage of continuous removal of toxic end-product during the process. With continuous feeding of medium and removal of product, the fermentation was maintained at steady state with a higher butanol productivity (1.47 g/L∙h) compared to that in batch fermentation. However, continuous fermentation for butanol production was not operated at large scale because of the physiological complexity of the butanol producing clostridia. When butanol concentration was too high and the bacteria entered stationary phase, the spore forming clostridia started to form spores and stopped producing butanol.

In order to further improve productivity, techniques of cell immobilization were commonly integrated into fermentation processes. In the immobilized cell reactor, the higher cell density helped the microorganisms to tolerate higher concentrations of end
products. Many materials have been studied as cell immobilizing media, such as brick, bonechar, chitosan, corn stalk (Zhang et al., 2009). Cells are generally immobilized by entrapment, adsorption, and porous support colonization (Chun et al., 1991; Hilge-Rotmann et al., 1991; Junter et al., 2002). While higher cell density gave higher productivity, the buildup of cells in the reactor could also produce problems such as clogging by cell mass and mass transfer limitation of nutrient and products.

Yang has developed a fibrous-bed bioreactor (FBB) (Yang, 1996) for various bioprocessing and biotechnology applications. The FBB has been successfully employed in the production of organic acids, enzymes, and organic solvents (Yang et al., 1994, 1996, Zhu and Yang, 2003, Cho et al., 1996, Huang et al., 2004). The FBB contains a fibrous bed packed together with a stainless steel mesh. During the fermentation process, dead cells would be detached from the fibrous bed and replaced by new cells to maintain steady productivity. With the high cell density in the FBB, common concerns of contamination problems were not significant (Yang et al., 1994). In the production of butyric acid from Clostridium tyrobutyricum, it has been noticed that the immobilized cells adapted to higher concentration of butyric acid with modified fatty acid composition of their cell membrane, which resulted in less membrane fluidity and better acid tolerance (Zhu and Yang, 2003).

Recent studies on process development of fermentation and optimization of butanol production are summarized in Table 2.4. A novel continuous two-stage, dual-path fermentation using two different strains was reported to give a stable process with high butanol productivity of 8 g/L·h (Ramey, 1998). Continuous fermentation with cell
immobilization on brick gave a high ABE productivity of 15.8 g/L·h (Qureshi et al., 2000). Increased specific butanol production rate of 0.42 g/g cell·h and butanol titer of 16 g/L were achieved with a fed-batch culture with pH-stat continuous butyric acid and glucose feeding (Tashiro et al., 2004). Recently, our research group developed a new strain for butanol production which gave a high butanol titer of 24.1 g/L in the FBB (Zhao and Yang, 2009).

2.3.3 Product recovery

Distillation was the main butanol recovery method from the fermentation process. While the average butanol concentration at the end of fermentation was around 10 g/L, it was not considered economically feasible to recover butanol from the fermentation broth using distillation. In order to produce butanol at a lower cost, the final concentration of butanol needs to be increased to reduce the separation cost. Several technologies have been incorporated into butanol fermentation process to alleviate fermentation pressure by removing butanol from the reactor and obtain concentrated butanol from product recovery device. Figure 2.3 shows some butanol recovery systems used in butanol fermentation process, including gas stripping, liquid-liquid extraction, and pervaporation (Lee et al., 2008).

Gas stripping removed butanol by bubbling fermentation gas through the fermentation broth and passing the butanol containing gas through a condenser to collect condensed butanol. By removing butanol from the fermentation broth, the final butanol yield could be increased because the inhibition of toxic end product was reduced. The
butanol concentration collected from the condenser was also four to five times higher than that in the fermentation broth (Qureshi et al., 2001).

In liquid-liquid extraction, an extractant insoluble in the fermentation broth is mixed with the fermentation broth. Butanol is more soluble in the extractant and thus separated from the fermentation broth. The extracted butanol can be further recovered from the extractant by distillation. In order to use liquid-liquid extraction, the extractant must be non-toxic to the fermentation, able to extract solvent selectively, immiscible with the fermentation broth and inexpensive (Ezeji et al., 2004).

Pervaporation selectively removes volatile solvents from the fermentation broth through a membrane. The volatile compounds diffuse through the membrane and condense when passing through a condenser. The separating membrane can be in the form of solid or liquid with supporting material (Matsumura et al., 1988; Qureshi et al., 2001).

Each of the three separation technologies discussed above exhibited its own advantages and disadvantages. By combining together with fermentation systems, the overall performance can be improved with better butanol production and less end-product inhibition. Table 2.5 gives some examples of ABE fermentation with product recovery in situ. Although there have been some improvements in productivity and product titer, the butanol and total solvent yields were still not significantly improved by the incorporated process. Integration of product recovery might also affect the fermentation process. The bubbles in gas stripping could produce excessive shear stress which would damage or kill the bacterial cells during fermentation. For both liquid-liquid extraction and
pervaporation, the qualified extractant or materials with the required chemical and physical characteristics can be difficult to find for large scale production.

2.4 Strain Development and Metabolic Engineering

Solventogenic clostridia are a group of Gram-positive anaerobic bacteria that form spores and produce solvents. Many different strains have been studied for butanol production via ABE fermentation, such as *Clostridium acetobutylicum* ATCC 824, *C. beijerinckii* ATCC 55025, *C. acetobutylicum* P262, *C. acetobutylicum* DSM 1731, *C. beijerinckii* NCIMB 8052, *C. beijerinckii* BA101, etc. (Bahl et al., 1982; Huang et al., 2004; Maddox et al., 1995; Parekh et al., 1998; Soni et al., 1987). Currently, genomes of only two strains of solventogenic clostridia are known: *C. acetobutylicum* 824 and *C. beijerinckii* NCIMB 8052. In *C. acetobutylicum* ATCC 824, the *sol* operon, which resides in plasmid pSOL, regulates both solvent production and sporulation during the stationary phase (Papoutsakis, 2008). With genes that are responsible for solvent production and sporulation in the same cluster, bacteria are not able to produce solvents steadily over a long period of time because solvent production and sporulation are likely to occur together.

Butanol-producing clostridia have been selected and improved with both traditional methods, such as selection and mutation, and novel techniques of gene insertion and deletion through metabolic engineering. Table 2.6 summarizes the studies that have been done on strain improvement for butanol production by traditional methods of mutation and screening. The conventional methods to isolate mutants with increased
butanol tolerance are direct selection on agar plates containing butanol and serial transfer of fermentation culture with increased butanol concentration in the medium (Liyanage et al., 2000; Soucaille et al., 1987). However, the obtained mutants with better butanol tolerance did not give better butanol production. Another conventional method used for strain improvement is to induce mutation with treatment of mutagen (Jain, 1993; Blaschek, 2002). In Jain’s work, a mutant, C. acetobutylicum ATCC 55025, was isolated with better butanol production, increased butyric acid uptake rate, and non-spore forming trait. Recently, Zhao and Yang have isolated a mutant strain that gives a high butanol titer using a FBB-based adaptation system as a rapid strain improvement tool (Zhao and Yang, 2009).

There have been many attempts to improve butanol production in ABE fermentation through metabolic engineering of butanol-producing Clostridia. Table 2.7 summarizes the genes that have been cloned and manipulated in C. acetobutylicum ATCC 824, a butanol producing strain with known genome. However, the improvement was limited mainly due to the difficulty in cloning Clostridia, the complicated metabolic pathway, and the unresolved gene regulation control. The low butanol titer in ABE fermentation is the major obstacle.

Among butanol-producing clostridia mutants, Clostridium beijerinckii ATCC 55025 and JB200 were studied. ATCC 55025 is an asporogenic mutants derived from ATCC 4259 with higher glucose and butyrate uptake rates (Jain et al., 1993) and JB200 was selected with higher butanol tolerance and higher butanol titer. The reason of choosing these strains over other alternatives is their inability to form spores during
fermentation, which facilitates continuous fermentation and gives better productivity compared with batch fermentation. However, the genomic sequence for *C. beijerinckii* ATCC 55025 and JB200 has not been resolved completely. Comparison of their ancestor strain with other strains would help to connect similarities among strains. While the ancestor strain *C. acetobutylicum* ATCC 4259 carries its solventogenic genes in a plasmid (Cornillot and Soucaille, 1996), it is reasonable to assume *Clostridium beijerinckii* ATCC 55025 and JB200 also carry the solventogenic genes in a 210-kb plasmid. In *Clostridium acetobutylicum* ATCC 824 (Papoutsakis, 2008) and *Clostridium saccharoperbutylacetonicum* strain N1-4 (Kosaka *et al.* 2007), solventogenic genes are also carried in a plasmid with a similar size (210 kb) while *C. beijerinckii* NCIMB 8052 carries its solventogenic genes in the chromosome. Although the physiologies of ATCC 4259, 824 and *Clostridium saccharoperbutylacetonicum* strain N1-4 are not identical, it was reported that they exhibited similar solvent metabolism (Cornillot *et al.*, 1997). As a result, *C. acetobutylicum* ATCC 824 and *C. saccharoperbutylacetonicum* strain N1-4 can be used as models for metabolism construction of *C. beijerinckii* ATCC 55025.
2.5 Reference


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Tummala SB, Junne SG, Papoutsakis ET. 2003. Antisense RNA downregulation of coenzyme A transferase combined with alcohol-aldehyde dehydrogenase
overexpression leads to predominantly alcohogenic *Clostridium acetobutylicum* fermentations. J Bacteriol 185:3644-3653.


<table>
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<th>Gasoline</th>
<th>Methanol</th>
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<th>Butanol</th>
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<td>102</td>
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Table 2.1 Properties of gasoline and some biofuels (Lee et al., 2008).
<table>
<thead>
<tr>
<th>Feedstock</th>
<th>Other conditions</th>
<th>Best results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degermed corn with corn steep liquor</td>
<td><em>C. beijerinckii</em> BA101, batch fermentation, 35°C, 84 h</td>
<td>n-butanol 14.4 g/L, ABE 19.3 g/L, productivity 0.23 g/L/h</td>
<td>Campos <em>et al.</em>, 2002</td>
</tr>
<tr>
<td>Packing peanuts</td>
<td><em>C. beijerinckii</em> BA101, batch fermentation, 35°C, 110 h</td>
<td>ABE 18.9 g/L, yield 0.32 g/g, productivity 0.17 g/L/h</td>
<td>Ezeji <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>Maltodextrin plus corn steep water</td>
<td><em>C. beijerinckii</em> BA101, batch fermentation, 34°C, 96 h</td>
<td>n-butanol 14.5 g/L, ABE 19.3 g/L</td>
<td>Parekh and Blaschek., 1999</td>
</tr>
<tr>
<td>Soy molasses with glucose</td>
<td><em>C. beijerinckii</em> BA101, batch fermentation, medium with tri-calcium phosphate, 35°C</td>
<td>ABE 30.1 g/L</td>
<td>Qureshi <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>Cassava starch</td>
<td><em>C. saccharoperbutylacetonicum</em> N1-4, medium with 3g/L ammonium acetate, 2 g/L yeast extract and 6 g/L tryptone, batch fermentation, 30°C, 48 h</td>
<td>n-butanol 16.9 g/L, ABE 21.0 g/L</td>
<td>Thang <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>Glycerol</td>
<td><em>C. pasteurianum</em> DSM525, fed-batch fermentation, 35°C, pH 6.0</td>
<td>n-butanol 17.0 g/L</td>
<td>Bielb, 2001</td>
</tr>
</tbody>
</table>

Table 2.2 ABE fermentation with non-cellulosic feedstocks.
<table>
<thead>
<tr>
<th>Feedstock</th>
<th>Process conditions</th>
<th>ABE production</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn fiber</td>
<td>pretreated with sulfuric acid, inhibitor removal with XAD-4 resin, batch fermentation with <em>C. beijerinckii</em> BA101</td>
<td>ABE 9.3 g/L, yield 0.39 g/g</td>
<td>Qureshi et al, 2008</td>
</tr>
<tr>
<td>Corn fiber, arabininoxylan and xylose</td>
<td>batch fermentation integrated with enzyme hydrolysis and gas stripping, with <em>C. acetobutylicum</em> P260</td>
<td>ABE 24.7 g/L, yield 0.44 g/g, 0.47 g/L/h</td>
<td>Qureshi et al, 2006</td>
</tr>
<tr>
<td>Corn stover</td>
<td>SO\textsubscript{2}-prehydrolysed, enzyme hydrolysis, extractive batch fermentation with <em>C. acetobutylicum</em> P262</td>
<td>ABE 25.8 g/L, yield 0.34 g/g, 1.08 g/L/h</td>
<td>Parekh et al, 1988</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>pretreated with alkaline peroxide, enzyme hydrolysis with salt removal, batch fermentation with <em>C. acetobutylicum</em> P260</td>
<td>ABE 22.2 g/L, 0.55 g/L/h</td>
<td>Qureshi et al, 2008</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>pretreated with dilute sulfuric acid; simultaneous enzyme hydrolysis and fermentation integrated with gas stripping, with <em>C. acetobutylicum</em> P260</td>
<td>ABE 21.4 g/L, yield 0.41 g/g, 0.31 g/L/h</td>
<td>Qureshi et al, 2008</td>
</tr>
<tr>
<td>Wheat straw plus glucose</td>
<td>pretreated with dilute sulfuric acid, enzyme hydrolysis, batch fermentation with <em>C. acetobutylicum</em> P260</td>
<td>ABE 28.2 g/L, yield 0.42 g/g, 0.63 g/L/h</td>
<td>Qureshi et al, 2007</td>
</tr>
<tr>
<td>Distillers dried grains and solubles</td>
<td>pretreated with liquid hot water, enzyme hydrolysis, batch fermentation with <em>C. butylicum</em> 592</td>
<td>ABE 12.9 g/L, yield 0.32 g/g,</td>
<td>Ezeji et al, 2008</td>
</tr>
<tr>
<td>Pine wood</td>
<td>SO\textsubscript{2}-prehydrolysed, enzyme hydrolysis, extractive fermentation with <em>C. acetobutylicum</em> P262</td>
<td>ABE 17.6 g/L, yield 0.36 g/g, 0.73 g/L/h</td>
<td>Parekh et al, 1988</td>
</tr>
</tbody>
</table>

Table 2.3 ABE fermentations with lignocellulosic biomass.
<table>
<thead>
<tr>
<th>Processes</th>
<th>Other conditions</th>
<th>Best results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous two stage, dual path fermentation using two different strains</td>
<td><em>C. tyrobutyricum</em> ATCC 25722, <em>C. acetobutylicum</em> ATCC 4259</td>
<td>n-butanol productivity 8 g/L/h</td>
<td>Ramey, 1998</td>
</tr>
<tr>
<td>Continuous fermentation by cells immobilized on brick</td>
<td><em>C. beijerinckii</em> BA101, P2 glucose medium, dilution rate 2 h(^{-1}), 35 °C</td>
<td>ABE 7.9 g/L, yield 0.38 g/g, productivity 15.8 g/L/h</td>
<td>Qureshi <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Fed-batch culture with pH-stat continuous butyric acid and glucose feeding</td>
<td><em>C. saccharoperbutylacetonicum</em> N1-4, glucose and butyric acid medium, 30 °C</td>
<td>Increased specific n-butanol production rate (0.42 g/g cell/h) and titer (16 g/L)</td>
<td>Tashiro <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Batch fermentation in fibrous bed bioreactor (FBB)-connected fermentor</td>
<td><em>C. beijerinckii</em> JB 200, P2 glucose medium, 37 °C</td>
<td>n-butanol 24.1 g/L, ABE 40.3 g/L, yield 0.43 g/g, productivity 0.4 g/L/h</td>
<td>Zhao and Yang, 2009</td>
</tr>
</tbody>
</table>

Table 2.4 Process development and optimization in ABE fermentation.
<table>
<thead>
<tr>
<th>Recovery processes</th>
<th>Fermentation conditions</th>
<th>ABE production</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Titer (g/L)</td>
<td>Yield (g/g)</td>
</tr>
<tr>
<td>Gas stripping</td>
<td><em>C. beijerinckii</em> BA101</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P2 glucose medium, batch</td>
<td>75.9</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>fed-batch</td>
<td>233</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>continuous</td>
<td>460</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>liquefied corn starch, fed-batch</td>
<td>56.2</td>
<td>0.41</td>
</tr>
<tr>
<td>Pervaporation with silicon membrane</td>
<td>batch</td>
<td>51.5</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>fed-batch</td>
<td>165</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>continuous immobilized biofilm reactor</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. acetobutylicum</em> ATCC 824</td>
<td>glucose, continuous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>supported ionic liquid membrane</td>
<td>glucose, continuous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adsorption</td>
<td>glucose, batch</td>
<td>butanol</td>
<td>22.2 g/L</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em> ATCC 4259</td>
<td>glucose and butyric acid medium, batch</td>
<td>butanol</td>
<td>16.3 g/L</td>
</tr>
</tbody>
</table>

Table 2.5 ABE fermentation with in situ solvent recovery.
<table>
<thead>
<tr>
<th>Mutant phenotype</th>
<th>Original strain</th>
<th>Mutation method</th>
<th>Screening method</th>
<th>References</th>
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<tbody>
<tr>
<td>G-1 strain with increased n-butanol tolerance and production (13 g/L)</td>
<td><em>C. acetobutylicum</em> ATCC 824</td>
<td>Spontaneous mutation</td>
<td>Serial transfer in serum tubes with increased n-butanol concentration</td>
<td>Soucaille <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>Asporogenic ATCC 55025 with high butyrate uptake and n-butanol titer (20.2 g/L)</td>
<td><em>C. acetobutylicum</em> ATCC 4259</td>
<td>Mutagen EMS</td>
<td>Screening after growth in CAB medium</td>
<td>Jain, 1993</td>
</tr>
<tr>
<td>Increased n-butanol tolerance</td>
<td><em>C. beijerinckii</em> NCIMB 8052</td>
<td>Tn 1545</td>
<td>Agar plate containing erythromycin and n-butanol</td>
<td>Liyanage <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Hyperamylolytic BA101 with increased n-butanol tolerance and production (20.9 g/L)</td>
<td><em>C. beijerinckii</em> NCIMB 8052</td>
<td>Mutagen NTG</td>
<td>Agar plate with starch and 2-dexoyglucose, and exposed to iodine vapor</td>
<td>Blaschek, 2002</td>
</tr>
<tr>
<td>JB 200 with increased n-butanol tolerance and production (24.1 g/L)</td>
<td><em>C. beijerinckii</em> ATCC 55025</td>
<td>Spontaneous mutation</td>
<td>Fibrous bed bioreactor-based adaptation</td>
<td>Zhao and Yang, 2009</td>
</tr>
</tbody>
</table>

Table 2.6 Strain improvement for ABE fermentation by mutation and screening.
<table>
<thead>
<tr>
<th>Strain with genetic changes</th>
<th>Effects on ABE production</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pFNK6) with adc(↑), ctfAB(↑)</td>
<td>Increased n-butanol (13 g/L) and ABE (23.1 g/L) production</td>
<td>Mermelstein et al, 1993</td>
</tr>
<tr>
<td>PJC4BK with buk(↓)</td>
<td>Increased n-butanol (16.7 g/L) and ABE (23.7 g/L) production</td>
<td>Harris et al, 2000</td>
</tr>
<tr>
<td>solR::pO1X with solR(↓)</td>
<td>Deregulated, prolonged and increased n-butanol (17.8 g/L) and ABE (26.9 g/L) production</td>
<td>Nair et al, 1999</td>
</tr>
<tr>
<td>SolRH(pTAAD) with solR(↓), adhE(↑)</td>
<td>Increased n-butanol (17.6 g/L) and ABE (27.9 g/L) production</td>
<td>Harris et al, 2001</td>
</tr>
<tr>
<td>(pGROE1) with groESL(↑)</td>
<td>Increased n-butanol tolerance and production (17 g/L)</td>
<td>Tomas et al, 2003</td>
</tr>
<tr>
<td>EA2018 (adc) with adc(↓)</td>
<td>Increased n-butanol ratio by 82% and yield by 70.8%</td>
<td>Jiang et al, 2009</td>
</tr>
<tr>
<td>with buk(↓), ctfAB(↓) ldh(↓), pta(↓) ack(↓), hydA(↓)</td>
<td>to increase n-butanol yield to 0.4 g/g glucose with no or little byproducts</td>
<td>Soucaille, 2008</td>
</tr>
<tr>
<td>(pAADB1) with adhE(↑), ctfB(↓)</td>
<td>Decreased acetone (1.4 g/L), increased ethanol (8.8 g/L), n-butanol unchanged (10 g/L)</td>
<td>Tummala et al, 2003</td>
</tr>
<tr>
<td>(pASspo) with SpoIIE(↓)</td>
<td>No spore formed in solventogenic phase</td>
<td>Bennett and Scotcher, 2008</td>
</tr>
</tbody>
</table>

Table 2.7 Metabolic engineering of *C. acetobutylicum* ATCC 824.
Figure 2.1 Renewable energy share of global energy consumption in 2006 (Martinot, 2007).
Figure 2.2 Metabolic pathway of ABE fermentation by *Clostridium acetobutylicum* (Papoutsakis, 2008).
Figure 2.3 Integrated systems of fermentation and solvent recovery: fermentation combined with (a) gas stripping, (b) liquid-liquid extraction, (c) pervaporation (Lee et al., 2008).
Chapter 3: Stoichiometric Analysis of Continuous ABE Fermentation by Butanol Producing Clostridia

Summary

Acetone-butanol-ethanol (ABE) fermentation has two distinctive phases – acidogenesis and solventogenesis. Acetic acid and butyric acid are produced in acidogenesis and then the fermentation switches to solventogenesis with sugars and acids as carbon sources for the production of acetone, butanol and ethanol. It was found that co-feeding butyric acid with glucose could increase butanol yield. In order to better understand the effect of butyrate uptake on butanol production in ABE fermentation, a stoichiometric model was developed for the analysis of the metabolic pathway in solventogenic Clostridia. The stoichiometric model can predict the maximum butanol yield based on mass and energy balance and thus can be used to help process development without requiring excessive experiments. In this study, a stoichiometric model was constructed and used to evaluate the effect of butyrate uptake on butanol production. In the stoichiometric model, NADH balance and ATP sufficiency are set as the only two necessary conditions for cells to grow and maintain in the fermentation. In general, more butanol can be produced when less glucose is used for cell growth or biomass formation due to lower ATP requirement. Since butyrate inhibits cell growth, co-feeding butyrate with glucose increases butanol yield. In addition, butyrate uptake is
beneficial to butanol production because more butyrate phosphate, which upregulates solventogenesis, can be produced.

3.1 Introduction

In order to make biobutanol production economically feasible, many approaches have been taken, including gene manipulation, optimization of fermentation condition, and strains adaptations by different methods to increase solvent production. Cell immobilization to increase butanol production and prolong the fermentation lifetime is also widely studied (Larger et al., 1985; Lienhardt et al., 2002; Ezeji et al., 2003).

Biobutanol is commonly produced in ABE fermentation by strictly anaerobic clostridia. The metabolic pathway of ABE fermentation has been discussed in Chapter 2. Although the genome sequence for C. beijerinckii ATCC 55025 is not resolved completely, comparison of its parental strain with other strains would help to connect similarities among strains. Since its parental strain C. acetobutylicum ATCC 4259 carries its solventogenic genes in a 210-kb megaplasmid, pSOL1 (Cornillot and Soucaille, 1996), it is reasonable to assume C. beijerinckii ATCC 55025 also carries its solventogenic genes in the megaplasmid. In C. acetobutylicum ATCC 824 (Papoutsakis, 2008) and C. saccharoperbutylacetonicum strain N1-4 (Kosaka, et al. 2007), solventogenic genes are also carried in a plasmid with a similar size (192 kb) while C. beijerinckii NCIMB 8052 carries its solventogenic genes in the chromosome. Although the physiologies of C. acetobutylicum ATCC 4259, ATCC 824 and C. saccharoperbutylacetonicum strain N1-4 are not identical, it was reported that they exhibited similar solvent metabolism
As a result, *C. acetobutylicum* ATCC 824 can be used as model for stoichiometric studies of *C. beijerinckii* ATCC 55025.

*C. acetobutylicum* ATCC 824 contains a 3.94 Mb chromosome and a 192 kb megaplasmid pSOL1 possessing genes involved in sporulation and solvent formation, including *aad, ctfA, ctfB, and adc* (Cornillot *et al.*, 1997; Tomas *et al.*, 2003). Several genes involved in acids production, solvents production and sporulation have been cloned, and mutants with inactivated genes or overexpressed genes were constructed to examine each function and to explore gene expression regulation. The mutant losing the megaplasmid displaying an asporogenous and non-solventogenic phenomenon confirmed that the megaplasmid played an important role in solvent production (Tomas *et al.*, 2003). In the butyrate kinase (*buk*) inactivation mutant, a higher level of butyryl phosphate (BuP) was detected, and consistently, solvents were produced earlier at higher concentrations than those in the wild type (Desai and Papoutsakis, 1999). The results suggested that the intracellular BuP was a putative regulator of solventogenesis in *C. acetobutylicum*, which probably acts as a phosphodonor of transcriptional factor. DNA array-based transcriptional analysis confirmed that the higher BuP level in the buk mutant was related to the upregulation of solvent formation genes (Zhao *et al.*, 2005). In an alcohol/aldehyde dehydrogenase (*aad*) mutant, overexpression combining antisense RNA downregulation of CoA transferase B (*ctfb*) mutant, *aad* was overexpressed and resulted in the highest reported ethanol production. On the other hand, the butyrate depletion in the fermentation suggested that butyryl CoA might be the limiting factor on butanol production. The addition of butyric acid increased the butanol/ethanol ratio confirming
this hypothesis (Harris et al., 2000; Tummala et al., 2003). Moreover, the enzyme phosphotransbutyrylase (PTB) and butyrate kinase (BK) were able to uptake butyrate without acetone production (Desai et al., 1999).

Considering the important roles of butyrate, BuP and butyryl CoA in solvent production, butyrate co-feeding with glucose could probably increase the butanol yield since both BuP and butyryl CoA concentrations were higher during the butyrate/glucose cofeeding than the glucose solo feeding. Both experiments and stoichiometric modeling indicate that butanol production can be increased by co-feeding sugar with butyric acid (Tashiro et al., 2004; Shinto et al., 2007). A two-step fermentation process has been developed (Ramey, 1998) which increased the butanol production by feeding C. acetobutylicum with sugar and butyric acid produced from saccharide by C. tyrobutyricum (Figure 3.1).

Herein we proposed a stoichiometric model to evaluate the effect of butyrate uptake on butanol production in ABE fermentation.

3.2. Construction of Metabolic Stoichiometric Model

The following equations are obtained based on the metabolic pathway (Figure 3.2). Only the reactions related to calculation are detailed. Ethanol production was observed close to zero when butyrate was co-fed with glucose during the fermentation (Huang et al., 2004). Hydrogen production is also assumed to be zero to assure the maximum butanol yield. When 1 mol glucose is uptaken, $a$ mol is used to produce acids and solvents while $(1-a)$ mol is consumed for biomass formation and cell biological
activity. The coefficients in equation 2 for the biomass formation were calculated by Papoutsakis (Papoutsakis, 1984).

\[ a \text{ Glucose} + 2a \text{ADP} + 2a \text{NAD}^+ \rightarrow 2a \text{pyruvate} + 2a \text{ATP} + 2a \text{NADH} \quad \text{Eq. 1} \]

\[ (1 - a)\text{Glucose} + 0.875 (1 - a)\text{NADH} + 14.85 (1 - a)\text{ATP} \rightarrow \text{Biomass} \quad \text{Eq. 2} \]

\[ 2a \text{Pyruvate} + 2a \text{CoA} + 2a \text{Fd} \rightarrow 2a \text{AcetylCoA} + 2a \text{CO}_2 + 2a \text{FdH}_2 \quad \text{Eq. 3} \]

\[ 2a \text{FdH}_2 + 2a \text{NAD}^+ \rightarrow 2a \text{NADH} + 2a \text{Fd} \quad \text{Eq. 4} \]

\[ b \text{AcetylCoA} + b \text{ADP} + b \text{Pi} \rightarrow b \text{Acetate} + b \text{ATP} + b \text{CoA} \quad \text{Eq. 5} \]

\[ 2c \text{AcetylCoA} + 2c \text{NADH} \rightarrow c \text{ButyrylCoA} + c \text{CoA} + 2c \text{NAD}^+ \quad \text{Eq. 6} \]

Equations 3, 5 and 6 are for reactions involving CoA transfer. Since no acetoactylCoA was detected in the fermentation broth (Boynton et al., 1994), it is reasonable to combine reactions 7(a) through 7(d) to form the acetone formation from acetylCoA as illustrated in Eq. 7.

\[ \text{AcetylCoA} + \text{ADP} + \text{Pi} \rightarrow \text{Acetate} + \text{ATP} + \text{CoA} \quad \text{Eq. 7(a)} \]

\[ 2 \text{AcetylCoA} \rightarrow \text{AcetoacetylCoA} + \text{CoA} \quad \text{Eq. 7(b)} \]

\[ \text{Acetate} + \text{AcetoacetylCoA} \rightarrow \text{AcetylCoA} + \text{acetoacetate} \quad \text{Eq. 7(c)} \]

\[ \text{Acetoacetate} \rightarrow \text{Acetone} + \text{CO}_2 \quad \text{Eq. 7(d)} \]

\[ 2d \text{AcetylCoA} + d \text{ADP} + d \text{Pi} \rightarrow d \text{Acetone} + d \text{CO}_2 + 2d \text{CoA} + d \text{ATP} \quad \text{Eq. 7} \]
To conclude the acetone formation from butyrate through the butyrate/acetoacetylCoA CoA transfer reaction, Eq. 8(a), 8(b), 8(c) are combined to form Eq. 8.

\[
\begin{align*}
2 \text{AcetylCoA} & \rightarrow \text{AcetoacetylCoA} + \text{CoA} & \text{Eq. 8(a)} \\
\text{Butyrate} + \text{AcetoacetylCoA} & \rightarrow \text{ButyrylCoA} + \text{Acetoacetate} & \text{Eq. 8(b)} \\
\text{Acetoacetate} & \rightarrow \text{Acetone} + \text{CO}_2 & \text{Eq. 8(c)}
\end{align*}
\]

\[
2e \text{AcetylCoA} + e \text{Butyrate} \rightarrow e \text{Acetone} + e \text{CO}_2 + e \text{ButyrylCoA} + e \text{CoA} & \text{Eq. 8}
\]

Besides conversion to butyrylCoA through CoA transfer reaction, butyric acid also can be uptaken through the reverse butyrate formation reaction (Desai et al. 1999).

\[
\begin{align*}
f \text{Butyrate} + f \text{ATP} + f \text{CoA} & \rightarrow f \text{ButyrylCoA} + f \text{ADP} + f \text{Pi} & \text{Eq. 9} \\
g \text{ButyrylCoA} + 2g \text{NADH} & \rightarrow g \text{Butanol} + 2g \text{NAD}^+ + g \text{CoA} & \text{Eq. 10}
\end{align*}
\]

The following conditions need to be met for the continuous fermentation at steady-state.

1. NADH balance: \(2c + 2g + 0.875(1-a) = 2a + 2a\)
2. ATP sufficient: \(f + 14.85(1-a) \leq 2a + b + d\)
3. AcetylCoA balance: \(b + 2c + 2d + 2e = 2a\)
4. butanol production: \(g = c + e + f\)
5. butyrate uptake: \( h = e + f \)

6. acetone production: \( i = d + e \)

The acetate/acetoacetylCoA CoA transfer reaction and the butyrate/acetoacetylCoA CoA transfer reaction are catalyzed by the same enzyme, CoA transferase, which has been purified and characterized kinetically (Wiesenborn et al. 1989). The ratio of butyrate uptake to acetate uptake has been determined \textit{in vitro} to be 0.315 when both acids were at 200 mM, which was in the range of reported intracellular concentrations of butyrate and acetate (Desai 1998; Husemann and Papoutsakis 1988; Gottwald and Gottschalk 1985; Terracciano and Kashket, 1986)

7. CoAT selectivity: \( e = 0.315 \times d \)

All equations and conditions are summarized in Table 3.1 and Table 3.2. Firstly, a value was assigned to \( a \), which meant \( a \) glucose enters the production pathway while \((1-a)\) was used for biomass formation. The amount of glucose used for biomass formation should be less than 20%; otherwise, the ATP would be insufficient. The results would be displayed with \( a = 1, 0.95, 0.9, 0.85 \), where 0, 0.05, 0.1, 0.15 glucose was consumed for biomass formation, respectively. Then, acetate production \( b \) was assumed as the net production of acetate and did not include the acetate reentering the pathway via the CoA transfer reaction. Thirdly, \( c \) value was assumed to stand for the amount of glucose converted to butyryl CoA. In terms of the butanol production, there are 2 constraints:
NADH balance and ATP sufficiency. When biomass formation was low, NADH was the only constraint since ATP produced during the glycolysis would be enough for the ATP consumption for biomass formation and butyrate uptake. Thus, butanol production is \( g = \frac{(4a - 0.875(1-a) - 2c)}{2} \) and the butanol yield is \( g/(1+g-c) \). However, with increased biomass formation, the amount of ATP available for butyrate uptake decreased, and the constraint condition would be shifted to ATP sufficiency. The factor determining butanol production was the amount of uptake butyrate at the cost of ATP. The uptake butyrate via ATP consumption is \( 2a + b + d - 14.85(1-a) \) and the butanol production \( g \) is \( 2a + b + d - 14.85(1-a) + 0.315d + c \) where \( d = \frac{(2a-b-2c)}{2.63} \). The butanol yield is \( g/(1+g-c) \).

3.3 Results and Discussion

The maximum theoretical butanol yield against the by-product production was plotted in Figure 3.3 under different biomass formation conditions without acetate formation. When 0% or 5% glucose was used for biomass formation, the determining factor of butanol theoretical yield was NADH balance and the maximum theoretical butanol yield was only related to the carbon flow going to acetate, acetone and carbon dioxide, \( b+2*i \), no matter what the by-products were. When biomass formation consumed more glucose, ATP supply began to play an important role in the butanol yield. For instance, if 10% glucose is consumed for biomass formation, the butanol yield would be determined by only NADH when the by-product formation is low. With more by-product formation, the supply of ATP becomes critical because ATP is required for the formation of butyryl CoA from butyrate. When 15% glucose is consumed for biomass,
the butanol yield would be controlled by ATP only. As shown in Figure 3.4, with 15% glucose consumed for biomass formation, only 2.45 mol of ATP were produced with one mol of glucose uptaken by the cell and 0.75 mol of acetone production. With 2.227 mol of ATP used for biomass formation, butanol yield would remain at 0.323 g/g glucose due to insufficient ATP for the cell to uptake butyrate and produce butanol with by-product production lower than 1 mol/mol glucose uptaken. With higher by-product formation, the theoretical butanol yield would be dependent on the byproduct distribution. In Figure 3.5, the theoretical butanol yield is plotted against by-product formation with different amounts of acetate production when 10% glucose is utilized for biomass formation. With less by-product formation (0.2~0.4 mol/mol), the theoretical butanol yield is determined by NADH balance. With more by-product formation, supply of ATP would become insufficient and acetate production is necessary to give enough ATP for butanol production. According to the stoichiometric model, one mol of ATP is produced with two mol of acetyl CoA used for production of one mol acetone, while two mol of ATP are produced with two mol of acetyl CoA used for two mol of acetate production. As a result, with a higher portion of acetate in the by-product formation, the theoretical butanol yield would become higher with more ATP available for butyryl-CoA formation from uptaken butyrate.

When 15% glucose is consumed for biomass, the butanol yield is controlled by ATP only. The butanol yield is also determined by both the total amount and the distribution of the byproduct. The theoretical butanol yield against the by-product formation when 15% glucose is used for biomass formation, as shown in Figure 3.6.
Noticeably, under the situation of no acetate formation, the butanol yield remains the same when the total byproduct amount is less than 1 mol because ATP is insufficient for butyrate uptake. In this case, butyrate would actually be produced, rather than uptaken, to supply ATP. Generally speaking, the less biomass is formed, the more butanol can be produced; the lower the byproduct is produced, the higher the butanol yield. However, under the ATP controlling situation, the more acetate is formed, the higher butanol yield can be obtained since more ATP required for butyrate uptake can be produced through acetate formation.

Figures 3.7 and 3.8 present two extreme scenarios in *C. acetobutylicum*. The highest theoretical butanol yield is 1 mol/mol when no glucose was used for biomass formation (Figure 3.7). Under this circumstance, no butyrate uptake was needed and all carbon from glucose was converted to butanol and carbon dioxide without any production of biomass, acetate, acetone, and ethanol. This suggests that blocking acetate and acetone production would increase the butanol yield. On the other hand, butyric acid in the broth inhibited the biomass formation, as shown in our previous experiments; thus the butyrate cofeeding could enhance the butanol yield by lowering biomass formation. Another extreme condition is the lowest theoretical butanol yield of 66.7% occurs when all carbon from glucose goes to acetate and acetone production without biomass formation (Figure 3.8). The butanol yield was 66.7% if 2 mol of butyric acid were uptaken with 1 mol of glucose. Moreover, acetate production had more significant effects on the butanol yield than acetone production, and acetate production was more preferred than acetone production because acetate production produced more ATP that benefits butyrate uptake.
The maximum butanol yield could be achieved only if enough butyrate were uptaken. Figure 3.9 shows that the butanol yield depends on the amount of butyrate uptake when 5% of glucose is consumed for biomass and NADH is the determining factor under this circumstance. With less biomass formation, more ATP is available for butanol production from uptaken butyrate.

Similar result is obtained when ATP is the determining factor, as shown in Figure 3.10. When 15% of glucose is consumed for biomass formation, butanol yield is improved with more butyrate uptake. With more glucose used for biomass production, the theoretical yield of butanol decreases with less ATP provided from by-product production and less butyrate uptake. It can be concluded that the butanol yield would increase with the enhanced butyrate uptake no matter which determining factor is considered significant.

3.4 Conclusion

Butyrate uptake was found to be beneficial to butanol production. It can be explained in the following two ways. Firstly, butyrate uptake could produce more BuP, the putative regulator of solventogenesis in C. acetobutylicum, which probably acts as a phosphodonor for a transcription factor (Desai and Papoutsakis 1999; Zhao et al. 2005). Secondly, butyrate could inhibit cell growth and thus cause less glucose to be used for biomass formation. Both of these two facts contributed to the increased butanol yield. In addition, butyrate uptake promotes butanol production by its direct conversion to butyryl-CoA, with ATP generated from acetate production, which reduces the amount of NADH
required for butanol production. Since both butyrate uptake and biomass formation require ATP, more biomass formation results in less butyrate uptake and thus less butanol production. For more butanol production, excess biomass formation should be prevented.
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acetobutylicum and their implications for solvent formation. Appl Environ
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<table>
<thead>
<tr>
<th>Equation</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a$ Glucose + $2a$ ADP + $2a$ NAD$^+$ $\rightarrow$ $2a$ pyruvate + $2a$ ATP + $2a$ NADH</td>
<td>Eq. 1</td>
</tr>
<tr>
<td>$(1 - a)$ Glucose + 0.875 $(1 - a)$ NADH + 14.85 $(1 - a)$ ATP $\rightarrow$ Biomass</td>
<td>Eq. 2</td>
</tr>
<tr>
<td>$2a$ Pyruvate + $2a$ CoA + $2a$ Fd $\rightarrow$ $2a$ AcetylCoA + $2a$ CO$_2$ + $2a$ FdH$_2$</td>
<td>Eq. 3</td>
</tr>
<tr>
<td>$2a$ FdH$_2$ + $2a$ NAD$^+$ $\rightarrow$ $2a$ NADH + $2a$ Fd</td>
<td>Eq. 4</td>
</tr>
<tr>
<td>$b$ AcetylCoA + $b$ ADP + $b$ Pi $\rightarrow$ $b$ Acetate + $b$ ATP + $b$ CoA</td>
<td>Eq. 5</td>
</tr>
<tr>
<td>$2c$ AcetylCoA + $2c$ NADH $\rightarrow$ $c$ ButyrylCoA + $c$ CoA + $2c$ NAD$^+$</td>
<td>Eq. 6</td>
</tr>
<tr>
<td>$2d$ AcetylCoA + $d$ ADP + $d$ Pi $\rightarrow$ $d$ Acetone + $d$ CO$_2$ + $2d$ CoA + $d$ ATP</td>
<td>Eq. 7</td>
</tr>
<tr>
<td>$2e$ AcetylCoA + $e$ Butyrate $\rightarrow$ $e$ Acetone + $e$ CO$_2$ + $e$ ButyrylCoA + $e$ CoA</td>
<td>Eq. 8</td>
</tr>
<tr>
<td>$f$ Butyrate + $f$ ATP + $f$ CoA $\rightarrow$ $f$ ButyrylCoA + $f$ ADP + $f$ Pi</td>
<td>Eq. 9</td>
</tr>
<tr>
<td>$g$ ButyrylCoA + $2g$ NADH $\rightarrow$ Butanol + $2g$ NAD$^+$ + $g$ CoA</td>
<td>Eq. 10</td>
</tr>
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</table>

Table 3.1 Summary of equations for stoichiometric analysis.
<table>
<thead>
<tr>
<th>Energy Balance</th>
<th>NADH balance</th>
<th>$2c + 2g + 0.875(1-a) = 2a+2a$</th>
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<tbody>
<tr>
<td></td>
<td>ATP sufficiency</td>
<td>$f + 14.85(1-a) \leq 2a + b + d$</td>
</tr>
<tr>
<td>Mass Balance</td>
<td>AcetylCoA balance</td>
<td>$b + 2c +2d + 2e = 2a$</td>
</tr>
<tr>
<td></td>
<td>Butanol production</td>
<td>$g = c + e+ f$</td>
</tr>
<tr>
<td></td>
<td>Butyrate uptake</td>
<td>$h = e + f$</td>
</tr>
<tr>
<td></td>
<td>Acetone production</td>
<td>$i = d + e$</td>
</tr>
<tr>
<td></td>
<td>CoAT selectivity</td>
<td>$e =0.315 * d$</td>
</tr>
</tbody>
</table>

Table 3.2 Conditions at steady-state during continuous fermentation.
Figure 3.1 A two-step fermentation process involving butyric acid production from glucose by *C. tyrobutyricum* and butanol production from glucose and butyrate by *C. acetobutylicum*. 
Figure 3.2 Major biochemical reactions involved in the metabolic pathway when butyrate is used as co-substrate with glucose. Ethanol production is not included in this model because of its relatively small amount produced in the fermentation.
Figure 3.3 Effects of biomass and byproduct (acetone and CO₂) formation on the theoretical butanol yield from glucose with no acetate production.
Figure 3.4 The illustration of carbon flow without acetate formation when butyrate cannot be uptaken due to the ATP insufficiency.
Figure 3.5 Effects of acetate formation and byproduct (acetate, acetone and CO₂) formation on the theoretical butanol yield from glucose when 10% glucose is used for biomass formation.
Figure 3.6 Effects of acetate formation and byproduct (acetate, acetone and CO₂) formation on the theoretical butanol yield from glucose when 15% glucose is used for biomass formation.
Figure 3.7 The illustration of carbon flow when 100% glucose goes to butanol formation.
Figure 3.8 The illustration of carbon flow when all carbon from glucose forming various byproduct without biomass formation.
Figure 3.9 The effect of butyrate uptake on the butanol yield with different by-product formation (acetate, acetone, and CO$_2$) when 5% glucose is consumed for biomass formation.
Figure 3.10 The effect of butyrate uptake on the butanol yield with different by-product formation (acetate, acetone, and CO₂) when 15% glucose is consumed for biomass formation.
Chapter 4: Biobutanol Production by *Clostridium beijerinckii* ATCC 55025 in a Single Pass Continuous Fibrous Bed Bioreactor

Summary

Biobutanol can be produced by microbes through acetone-butanol-ethanol (ABE) fermentation. However, conventional ABE fermentation is usually operated in batch reactors and suffers from low butanol titer, yield and productivity. In order for biobutanol to be produced economically, these limitations must be resolved. In this work, a single-pass continuous fibrous bed bioreactor (FBB) was developed for butanol production from glucose by asporogenic *Clostridium beijerinckii* ATCC 55025. In this process, butyric acid was also included in the feed medium as an additional carbon source to keep cells in the solventogenic phase so that stable long-term continuous production of butanol can be achieved. In this study, the effects of feed butyrate concentration and dilution rate on continuous butanol production were studied. In the continuous FBB, the butanol productivity, titer, and yield were 1.24 g/L·h, 10.37 g/L, and 0.24 g/g at a low dilution rate of 0.12 h⁻¹ and 17.29 g/L·h, 9.18 g/L, and 0.22 g/g at a high dilution rate of 1.88 h⁻¹, respectively. Compared to free-cell fermentation with the same bacterium and medium in a batch reactor, which typically gave a low butanol productivity of ~0.1 g/L·h with a final butanol titer of 13.84 g/L and yield of 0.21 g/g, the butanol productivity in the single-pass continuous FBB was 12 to 170-fold higher while maintaining comparable butanol titer and yield. The productivity of total solvents (acetone, butanol, and ethanol)
from the continuous FBB operated at 1.88 h⁻¹ was 25.55 g/L·h, which was also much higher than those (1.45 to 15.8 g/L·h) reported for other continuous ABE fermentation processes. The superior performance of the continuous FBB can be attributed to the high viable cell density (~100 g/L with >70% viability) and adaptation of cells immobilized in the fibrous matrix to tolerate butyric acid and butanol.

4.1 Introduction

Biobutanol can be produced by microorganisms that carry out acetone-butanol-ethanol (ABE) fermentation in different process modes, including batch, fed-batch, chemostat, and perfusion fermentations. As one of the oldest industrial fermentations, ABE fermentation has been extensively studied on different process modes. However, practices of industrial ABE fermentation gradually vanished after World War II due to increased costs of feedstock and inability to compete with cheaper solvents produced through petrochemical syntheses (Jones and Woods, 1986). In the 1980’s, ABE fermentation in batch reactor suffered from its low productivity because of the lag phase at the start of every batch fermentation as well as the toxic end products that inhibited the metabolism of the solvent producing bacteria and extended fermentation time required to reach higher final concentrations of produced solvents. A typical ABE fermentation in batch reactors gives a final butanol concentration of 12 g/L and productivity of 0.1 g/L·h after 100 hours of cultivation (Welsh, 1986). For ABE fermentation in a continuous reactor, solvent productivity could be greatly improved to 4 g/L·h with toxic end-products removed continuously and steady supply of nutrients. However, the final solvent titer
was also greatly decreased to around 5 g/L, which significantly increased the cost for
downstream purification (Qureshi and Maddox, 1991) (Pierrot, Fick, and Engasser,
1986). Other than the higher cost in downstream purification, continuous fermentation
was considered not feasible because of the complicated metabolism of ABE fermentation
and the higher risk of contamination during extended continuous operation.

In order to alleviate the problem of low titer, yield and productivity in ABE
fermentation, different techniques have been incorporated into fermentation processes to
increase cell density and improve overall productivity (Lee et al., 2008). Cell
immobilization was commonly used to increase the cell density in the reactor and give
stable fermentation. Bacterial cells can be retained in the reactor by either adsorption or
entrapment (Qureshi, et al., 2000). Many materials have been studied as cell
immobilizing matrix in ABE fermentation, including brick, bonechar, chitosan, corn
stalk, etc (Qureshi et al., 1988; Frick and schugerl, 1986; Zhang et al. 2009). Most of the
materials gave moderate improvement in productivity of total solvents from 1 g/L∙h to 10
g/L∙h on average. Fibrous bed bioreactor is a versatile cell immobilizing system that can
be incorporated into most of the current fermentation processes. The low cost fibrous
bed has been reported to support higher cell densities and help bacterial cells to quickly
adapt and tolerate higher concentrations of inhibitory end products (Yang, et al., 1994;
Huang, Ramey, and Yang, 2004).

Many different strains have been studied for butanol production via ABE
fermentation, including *Clostridium acetobutylicum* ATCC 824, *C. beijerinckii* ATCC
55025, *C. acetobutylicum* P262, *C. acetobutylicum* DSM 1731, *C. beijerinckii* NCIMB
8052, *C. beijerinckii* BA101 etc. (Bahl, Andersch et al. 1982; Soni, Soucaille et al. 1987;
In this study, *C. beijerinckii* ATCC 55025 was used with its better butanol productivity, its higher butyrate uptake rate and its deficiency in forming spores to facilitate stable continuous fermentation.

The objective of this study was to improve and stabilize butanol production from glucose and butyric acid in a single-pass continuous FBB using asporogenic *C. beijerinckii* ATCC 55025. By adding butyric acid in the feed medium, the excess butyric acid stabilizes the cells to stay in solventogenesis and serves as additional carbon source. While the main problem for continuous fermentation is uncontrollable contamination during the process, continuous butanol fermentation would not be problematic because the end products are toxic to most commensal bacteria and fungi are not able to grow anaerobically. We expect to acquire better butanol production by optimizing continuous fermentation conditions and allowing bacteria to have better product tolerance through adaptation in the FBB.

4.2 Materials and Methods

4.2.1 Cultures and Media

Asporogenic *Clostridium beijerinckii* ATCC 55025 was used in this study. The bacterium was cultured in a minimal medium modified from P2 medium with glucose and butyric acid as carbon sources. The modified P2 medium was composed of the following separately prepared solutions: 0.5 g KH$_2$PO$_4$ and 0.5 g K$_2$HPO$_4$-45 mL distilled water (solution I), 1 g yeast extract and 0.5 g tryptone-45 mL distilled water (solution II), 2 g MgSO$_4$·7H$_2$O, 0.1 g of MnSO$_4$·H$_2$O, 0.1 g of NaCl, and 0.1 g of FeSO$_4$·7H$_2$O in 5 mL
distilled water (solution III), 100 mg of \( p \)-aminobenzoic acid, 100 mg of thiamine, and 1 mg of biotin in 500 mL distilled water (solution IV). Fifty grams of glucose were dissolved in 800 mL distilled water and sterilized through autoclave. Solution I and II were autoclaved separately and mixed into sterile glucose solution subsequently. Five milliliters of solutions III and IV were filter-sterilized with 0.2 \( \mu \)m pore size filter and aseptically transferred to the medium. Various amounts of butyric acid and \( \text{NH}_4\text{OH} \) with the final volume of 100 mL were added into the medium to make final pH 5.5. The medium was made anaerobic by purging with \( \text{N}_2 \) for 30 minutes in sterile air-tight containers.

4.2.2 Experimental Set-Up

Figure 3.1(a) is the overview of the continuous process and Figure 3.1(b) shows the actual experiment setup used in this study. The FBB was made of a glass column with a water jacket packed with spiral-wound cotton and stainless-steel wire mesh, with a working volume of 150 and 500 mL. Before use, the bioreactor was autoclaved at 121°C for 30 minutes, held overnight at room temperature to allow spore germination, and then autoclaved at 121°C for one hour for complete sterilization. The bioreactor was then aseptically connected to the 10-L feed medium bottle.

4.2.3 Cell Tolerance to Butyric Acid

Media at pH 5.0 with 20 g/L of glucose and different concentrations of butyric acid were prepared and sterilized in serum bottles. Each 100 mL media was inoculated with 3 mL of fresh culture of \( \text{Clostridium beijerinckii} \) ATCC 55025 and incubated at
35°C. Samples were taken over time and the microbial growth was monitored by measuring the optical density at 600 nm in a cuvette with a light path length of 1 cm using a spectrophotometer. The specific growth rates ($\mu$) under different initial butyric acid concentrations were determined from the slopes of semilogarithmic plots of OD versus time.

4.2.4 Continuous Fermentation Kinetics

The fibrous bed bioreactor was filled with feed medium and made anaerobic by sparging N$_2$ from the bottom of the bioreactor. The bioreactor was inoculated with 60–100 mL of exponential phase cells grown in a serum bottle at 37°C. Temperature was maintained at 35°C with a water jacket surrounding the bioreactor. After inoculation, the FBB was held static for 12–24 h to increase cell density and to immobilize cells onto the fibrous bed. When OD$_{600nm}$ of the fermentation broth reached 1.0 and gas production was observed, continuous fermentation was started by pumping feed medium into the bottom of the FBB. Fermentation broth was sampled (1.5 mL) at proper time intervals throughout the fermentation.

4.2.5 Cell Viability Assay

Cell viability was determined using the method of Glenner (1977) with some modifications. One milliliter 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 to the cell pellet. The cell pellet was resuspended in TTC solution by vortexing at the maximum speed, and then incubated in the dark at room
temperature for 30 min for color development. The cells were collected by centrifugation at 16,000 rpm for 10 min and 1 ml of methanol was added to the cell pellet 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 (Shimazu, Columbia, MD, UV-16-1) against a control of boiled cell culture before adding TTC solution. The viability of cells from the exponential phase was determined to be 100%.

4.2.6 Scanning Electron Microscopy

At the end of the continuous FBB fermentation, small pieces of the fibrous matrix were cut from different locations for scanning electron microscopy (SEM). The samples were fixed in 2.5% glutaraldehyde solution at 4°C overnight and dehydrated progressively with 10 to 100% ethanol at 20% increment for 30 min at each concentration. The samples were finally dried with hexamethyl disilazane (HMDS) and coated with gold using a spotter-coating machine in the presence of the medium containing argon gas. The samples were scanned and photographed with FEI Quanta 200 SEM at 20kV.

4.2.7 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-mL cuvette (1 cm light path length) using a spectrophotometer (Shimazu, Columbia, MD, UV-16-1). One unit of OD was found to be equivalent to 0.534 g/L cell dry weight (Appendix C.1). The glucose concentration in each fermentation broth sample was analyzed using a glucose and lactate analyzer (YSI 2300). For analyses of solvents and
acids in the fermentation broth, samples were centrifuged and filtered with a 0.2 µm syringe filter (if necessary). In order to accurately measure concentrations of organic acids, samples were diluted and acidified with buffer containing 10 mL/L phosphoric acid to a final volume of at least 600 µL in the vial. The acidified samples were analyzed with a GC-2014 Shimadzu gas chromatograph (GC) (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (0.25 µm film thickness and 0.25 mm ID, Stabilwax-DA). The GC was operated at an injection temperature of 200 ºC with 1 µL of the acidified sample injected by the AOC-20i Shimadzu auto injector. Column temperature was held at 80 ºC for 3 min, raised to 150 ºC at a rate of 30 ºC/min, and held at 150 ºC for 3.7 min.

4.3 Results and Discussion

4.3.1 Cell Tolerance to Butyric Acid

Figure 4.2(a) shows the cell growth with different initial concentrations of butyric acid in the medium. Both the lag phase and exponential phase were decreased with increased concentration of butyric acid in the medium. Bacteria entered the stationary phase within 15 hours of incubation without butyric acid in the medium. With 5 g/L butyric acid in the medium, cell growth was greatly inhibited with a longer lag phase of ten hours and a lower cell density at the stationary phase. At the butyric acid concentration of 10 g/L in the medium, cell growth was completely stopped without any change in cell density. A linear trend between cell specific growth rates and butyric acid concentrations is shown in Figure 4.2(b). To start up a continuous fermentation, the
medium with 2 g/L butyric acid can help bacteria first adapt to an environment with a presence of butyric acid and would not drastically decrease cell growth. Butyric acid concentration can be slowly increased to 5 g/L to serve as an additional carbon source and help cells focus on solvent production from biomass production. At the butyric acid concentration of 4 g/L in the medium for continuous fermentation, bacteria could remain in solventogenesis stage without shifting back and forth to acidogenesis.

4.3.2 Butanol Production in Continuous Fermentation

Figure 4.3 is the typical result of free cell batch process of ABE fermentation in a spinner flask with the overall butanol productivity of 0.05 g/L·h. The shift from acidogenesis to solventogenesis could be observed after 20 hours of cultivation. Butanol productivity in the batch reactor was inhibited by the produced end products and only gave 0.05 g/L·h. Figure 4.4(a) shows the kinetics of a continuous fermentation at the dilution rate of 0.06 h⁻¹ in a bioreactor with a working volume of 500 mL at 35°C. Carbon sources in the medium include 50 g/L glucose and 4 g/L butyric acid. In this fermentation, butanol production initiated after four days of fermentation. However, butanol concentration stopped increasing after it reached 5 g/L in the first 250 hours of fermentation. The bioreactor was drained aseptically and refilled with a fresh medium at 290 hour and butanol concentration increased to 10 g/L in 200 hours of fermentation after the medium refill. In this fermentation, cells could not steadily utilize butyric acid at a concentration lower than 2 g/L which means only 2 g/L of butyric acid was uptaken by cells for butanol production. After 450 hours of fermentation, averaged butanol productivity was 0.6 g/L·h and yield was 0.29 g/g glucose (shown in Figure 4.4(b)). In
order to study the effect of different concentrations of butyric acid in the feed medium on butanol production, butyric acid concentration in the feed medium was increased to 8 g/L from fermentation time of 700 hours. With increased concentration of butyric acid in the FBB, bacteria completely halted the solvent production with no glucose consumption (Figure 4.5). At the low dilution rate, continuous fermentation behaved more like batch fermentation. Cell growth and activities were slowed down with increased concentrations of secondary metabolites and wastes. As a result, butanol production was not stable and predictable in continuous fermentation at low dilution rate.

With the same FBB, continuous fermentation was conducted at a higher dilution rate of 0.12 h\(^{-1}\), which was two times higher than that in Figure 4.4 and Figure 4.5. Due to the insufficient feed of butyric acid in the earlier study, the butyric acid concentration was increased to 6.8 g/L while feed glucose concentration remained 50 g/L in the feed medium. According to Figure 4.6(a), bacterial cells switched to solventogenesis within 24 hours after continuous fermentation was started. A sudden drop of butanol concentration at 100 hours indicated the induction of lyase and short term cell lysis. Butanol production continued to increase after the cells reached a balance of growth and death with no obvious change in cell density. At the end of the fermentation, the final butanol concentration reached 10.5 g/L while 58.8% of butyric acid in the feed medium was consumed. As shown in Figure 4.6(b), the average butanol productivity was 1.2 g/L\(\cdot\)h and butanol yield was 0.26 g/g.

In order to further increase the dilution rate of the fermentation, a FBB with a working volume of 150 mL was made. In this fermentation, the dilution rate was increased to 1.88 h\(^{-1}\) with a feed medium containing carbon sources of 50 g/L glucose and
5 g/L butyric acid. As can be seen in Figure 4.7(a), the butanol concentration rapidly increased to 11.5 g/L within 120 hours of fermentation. The sudden drop of butanol concentration around 160 hours of fermentation also indicated the cell lysis due to starvation of carbon source. However, the high dilution rate helped speed up the metabolism in the bacteria and maintain the butanol production after starvation. At the high dilution rate, butanol was steadily produced at a high productivity of 17.29 g/L·h with an average yield of 0.22 g/g glucose (Figure 4.7(b)).

Table 4.1 summarizes the results from different modes of fermentation processes in this study. While yields of solvent products were not greatly affected by different processes, the productivities of solvent and acid products were significantly improved from batch (0.1 g/L·h butanol) to continuous fermentation (17.29 g/L·h butanol). It was also noted that the yields and titers of butyric acid and acetic acid decreased with increased dilution rate in continuous fermentation. Continuous process at high dilution rate favored butanol production from ABE fermentation since solvent titers were not significantly affected by increased dilution rate, which did affect the final acid titers and yields. The butyric acid supplemented in the feed medium was utilized by the bacteria to produce butanol to give higher ratio of butanol to total solvents produced.

4.3.3 Butanol production in FBB

Fibrous bed bioreactors with different working volumes (500 mL and 150 mL) were used in different dilution rates. For dilution rates at 0.06 and 0.12 h⁻¹, FBB with 500 mL working volume was used while that with 150 mL was used for dilution rate at 1.88. For continuous fermentation, it was easier to monitor the fermentation condition
with smaller volume than with larger volume. For continuous fermentation with FBB volume of 500 mL, it was possible that only half part of the reactor was actively producing solvents while the bacteria in the rest of the reactor were inhibited with the lower pH value caused by the acid production. For processes require steady conditions (i.e. pH value), smaller reactor was preferred for precise control.

4.3.4 Effect of Butyric Acid on Butanol Production

For butanol production in 100 mL serum bottles, addition of butyric acids in the media mainly inhibited the bacterial growth. Bacteria growth was completely ceased in the medium with butyric acid concentration higher than 10 g/L. Since butanol production depended on the cell density in the medium, the final titer, yield, and productivity of solvents were low in serum bottles with supplement of butyric acid, which inhibited cell growth at concentration over 5 g/L in the medium.

In continuous FBB with high cell density, butanol production was increased with addition of butyric acid because bacteria were able to convert butyric acid to produce more butanol. Butanol production was 69% of total produced solvents in the FBB with medium containing 4 g/L butyric acid at dilution rate of 0.06 h\(^{-1}\) and with medium containing 6.8 g/L butyric acid at dilution rate of 0.12 h\(^{-1}\), while it was 66% of total produced solvent in free cell batch process of ABE fermentation. At high dilution rate of 1.88 h\(^{-1}\), butanol production occupied 68% of total produced solvents with 4 g/L butyric acid in the medium. As a result, supplementing butyric acid in the medium helped increase the ratio of butanol to total produced solvents in continuous fermentation, regardless of the dilution rate. However, butyric acid could still inhibit butanol
production at high concentration. At the dilution rate of 0.12 h\(^{-1}\), butanol production was completely stopped with medium containing 8 g/L butyric acid.

4.3.5 Cell Density and Morphology in FBB

Cell immobilization onto the fibrous matrix can be observed in Figure 4.8 at different magnifications. An overview of the fiber used in the fibrous bed bioreactor is shown in Figure 4.8(a) at the magnification of 200 fold. Figure 4.8(b) shows the biofilm formation in the fibrous bed. The sample in Figure 4.8(b) was collected from the bottom of the fibrous bed, which was the entry for the medium into the FBB. At the bottom of the fibrous bed bioreactor, the cell metabolism was faster with higher concentration of nutrients and lower concentration of inhibiting end products. As a result, cells aggregated into a thin film covering the fibrous bed at higher cell density. In Figure 4.8(c), rod shaped bacteria in the biofilm covering the fiber could be observed with magnification of 1500 folds. At magnification of 4000 fold (Figure 4.8(d)), the cell immobilization at a very high cell density could be observed. The sample in Figure 4.8(d), (e), and (f) was collected from the top of the reactor, where the metabolism of the bacteria was slower with less nutrient and higher concentration of toxic end products. Since it is very easy to observe the cell division in Figure 4.8(e) and (f), the bacteria were considered very active at the end of process. The ratio of cell length to cell width seemed to be higher than usual clostridia. With longer and thinner shape of bacterial cells in the FBB, bacteria were able to possess increased uptake of substrate and metabolite excretion rates (Suwannakham and Yang, 2005).
In the FBB, old bacterial cells could be replaced by new cells with a great amount of bacterial cells attached to the fibers through physical adsorption (Lewis and Yang, 1992) at a concentration of 100 ± 15 g cell dry weight/L with at least 70% cell viability. Table 4.2 summarizes the solvent titer, productivity and yield of ABE fermentation utilizing different immobilization techniques. Among the reported results with different immobilization techniques, the highest solvent titer and productivity were obtained in continuous FBB using \textit{C. beijerinckii} ATCC 55025 at a high dilution rate of 1.88 h\(^{-1}\).

4.4 Conclusion

Acetone-butanol-ethanol fermentation in continuous FBB produced butanol at a high productivity of 17.29 g/L\cdot h by the non-spore forming \textit{C. beijerinckii} ATCC 55025. With the combination of continuous fermentation at a high dilution rate and cell immobilization in the FBB, butanol productivity increased 170 fold compared to batch free-cell fermentation. In batch fermentation, bacteria suffered from accumulated solvents in the environment and gave low productivity. Butanol productivity was greatly improved in continuous fermentation at high dilution rates, which rapidly removed inhibitory end products and prevented or minimized their inhibition on cells. Continuous fermentation in the FBB for butanol production was further improved by adding optimized concentration of butyric acid in the feed medium. Although higher concentration of butyric acid stalled the fermentation process in batch reactor, butyric acid with a proper concentration could help stabilize butanol production in continuous FBB and improve butanol yield by reducing cell growth in the reactor. The risk of
contamination by other commensal bacteria could be reduced as well with butyric acid which was toxic to many bacteria.
4.6 Reference


89
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Table 4.1 Comparison of fermentation kinetics of batch and continuous processes at different dilution rates.
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<td><strong>0.22</strong></td>
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Table 4.2 Results of solvent titer, productivity and yield using different immobilization techniques.
Figure 4.1 (a) Schematic diagram and (b) a photograph showing the experimental set-up for continuous fermentation used in this study.
Figure 4.2 (a) Cell growth in media with different concentration of butyric acid; (b) A trend showing how specific growth rate affected by butyric acid concentration in the medium.
Figure 4.3 Typical kinetics of ABE fermentation in free-cell batch reactor in P2 medium with 55 g/L glucose and 4 g/L butyric acid.
Figure 4.4 (a) Kinetics, and (b) butanol yield and productivity in continuous fermentation fed with 50 g/L glucose and 4 g/L butyric acid at dilution rate of 0.06 h\(^{-1}\).
Figure 4.5 Continuous fermentation fed with P2 medium with 60 g/L glucose and 8 g/L butyric acid at dilution rate of 0.06 h$^{-1}$. 
Figure 4.6 (a) Kinetics, and (b) butanol yield and productivity in continuous fermentation fed with P2 medium with 60 g/L glucose and 6.8 g/L butyric acid with dilution rate of 0.12 h⁻¹.
Figure 4.7 (a) Kinetics, and (b) butanol yield and productivity in continuous fermentation fed with P2 medium with 60 g/L glucose and 5 g/L butyric acid with dilution rate of 1.88 h⁻¹.
Figure 4.8 Scanning electron micrographs of cells immobilized in the fibrous matrix in the FBB at magnification of (a) 200x, (b) 600x, (c) 1500x, (d) 4000x, (e) 10000x, (f) 20000x.
Chapter 5: Continuous Butanol Production by *Clostridium beijerinckii* JB200

**Summary**

There are many different approaches to improve butanol production from acetone-butanol-ethanol (ABE) fermentation, such as optimization of fermentation process, media formulation, and integration of fermentation with product recovery. However, it is crucial to select a host that continuously produces butanol at a high titer, yield and productivity. While one of the natural characteristics of solvent-producing clostridia is spore-forming ability, this ability is not preferred in continuous bioprocess. *Clostridium beijerinckii* JB200, a mutant obtained from *C. beijerinckii* ATCC 55025 does not produce spores in a fibrous bed bioreactor (FBB), was used in this study. Several other industrial butanol producing strains were compared together with *C. beijerinckii* JB200 for evaluating their capability of efficiently producing butanol in media with different concentrations of butyric acid. Continuous ABE fermentation in the FBB from *C. beijerinckii* JB200 was also conducted. Compared with its parental strain and other industrial strains, *C. beijerinckii* JB200 was superior with its high butanol productivity and ability to give a high butanol titer. *Clostridium beijerinckii* JB200 was able to produce butanol in the medium containing 8 g/L butyric acid without any inhibition effect. In pH-controlled continuous fermentation, *C. beijerinckii* JB200 was able to utilize more glucose in the medium to produce butanol at a higher yield. In the FBB, *C.*
beijerinckii JB200 was able to continuous produce butanol at 12 g/L for an extended period without noticeable culture degeneration.

5.1 Introduction

In order to improve biobutanol production and make it economically feasible, many studies have been done in the quest for the optimal strain for industrial use. There are a number of solvent producing clostridia that naturally produce butanol. Butanol producing clostridia are classified into different species based on their biotyping and DNA fingerprint analysis, including C. acetobutylicum, C. beijerinckii, C. saccharobutylicum, and C. saccharoperbutylacetoniu (Ezeji et al., 2007). Because butanol production by native butanol-producing clostridia was not efficient enough for a industrial process, much effort has been put into developing strains with desired characteristics.

For industrial applications, it is desired to operate the bioprocess continuously with constant production at a high titer, yield, and productivity. However, conventional solventogenic fermentation process using Clostridia is limited by several problems that must be resolved first. Clostridia are spore-forming bacteria, and thus the process of butanol production could be disrupted if sporulation is induced in the solventogenic phase and cells entered the dormant phase and stopped producing butanol. It has been reported that solvent-producing clostridia readily degenerated and lost their ability to produce solvents after repeated subculture or continuous fermentation (Kashket and Cao, 1995). Another problem detrimental to butanol production is infection by bacteriophage. Upon
contamination by lysogenic bacteriophage, the continuous fermentation process had to be halted and the affected process would be difficult to restore without a significant loss (Jones et al., 2000). In order to resolve these problems, the desired strain for industrial butanol production needs to be non-spore forming, degeneration-deficient, and phage resistant.

The traditional methods to acquire strains with the desired characteristics are strain isolation, mutation, and screening (Jones and Woods, 1986). Most of the studies for strain improvement focused on improving cell’s butanol tolerance since high butanol titer requires better butanol tolerance. Strains with better butanol tolerance were commonly first treated with mutagenic chemicals and selected from agar plates containing butanol or selected by serial transfers in media with increased butanol concentration. The strains were usually selected by using cell growth rate as an index for the selection of improved strain. However, the selected strains with better butanol tolerance do not necessarily give higher butanol titer because there is no direct relationship between butanol tolerance and butanol production in the bacteria.

Recently, our research group has obtained a mutant strain with improved butanol tolerance and titer through culture adaptation and evolution in a fibrous bed bioreactor (FBB). The parental strain, *C. beijerinckii* ATCC 55025, a non-spore forming mutant originally developed from *C. acetobutylicum* ATCC 4259 (Jain, 1993), was cultured in the FBB to a high cell density. Cells in the FBB were then exposed to multiple stresses, including high concentrations of butanol and butyric acid. After adaptation in the reactor environment for an extended period, cells evolved with better butanol tolerance and
production capability were screened and isolated. The evolved strain, *C. beijerinckii* JB200, was able to give a high butanol titer of 24.1 g/L.

This study was first to demonstrate the superiority of *C. beijerinckii* JB200 over its parental strain *C. beijerinckii* ATCC 55025 and other well studied strains, including *C. beijerinckii* BA101, *C. acetobutylicum* ATCC 824, and *C. acetobutylicum* NCIMB 8052. Effects of butyric acid on butanol yield and productivity of these five strains were studied. Then, *C. beijerinckii* JB200 was chosen based on its better butyric acid tolerance and superior performance and was used in continuous ABE fermentation to improve the process with steady butanol production without degeneration.

5.2 Materials and Methods

5.2.1 Culture and media

*C. beijerinckii* JB200 was stored in reinforced clostridial medium (RCM) with 25% glycerol in a deep freezer at -80 °C. The culture was reactivated from glycerol stock by incubating in RCM at 37°C the day before inoculation to the bioreactor. During continuous fermentation, the bacterium was fed with a minimal medium modified from P2 medium with glucose and butyric acid as carbon sources, unless otherwise mentioned. The modified P2 medium (per liter) was composed of the following separately prepared solutions: 45 mL Solution I containing 0.5 g KH$_2$PO$_4$ and 0.5 g K$_2$HPO$_4$ in distilled water, 45 mL Solution II containing 1 g yeast extract and 0.5 g tryptone in distilled water, 5 mL Solution III containing 2 g MgSO$_4$$\cdot$7H$_2$O, 0.1 g MnSO$_4$$\cdot$H$_2$O, 0.1 g NaCl, and 0.1 g FeSO$_4$$\cdot$7H$_2$O in distilled water, 500 mL Solution IV containing 100 mg of *p*
aminobenzoic acid, 100 mg of thiamine, and 1 mg of biotin in distilled water. Fifty grams of glucose were dissolved in 800 mL distilled water and sterilized through autoclave. Solutions I and II were autoclaved separately and mixed into sterile glucose solution subsequently. Five milliliters of solutions III and IV were filter-sterilized with 0.2 μm pore size filter and aseptically transferred to the medium. Various amounts of butyric acid and NH₄OH with the final volume of 100 mL were added into the medium to make final pH 5.5. The medium was made anaerobic by purging with N₂ for 30 minutes in a sterile air-tight container.

5.2.2 Experimental Set-Up

Figure 5.1 shows the process and apparatus used in this study. Fresh medium was fed to the first FBB, then entered a free cell buffering spinner flask. The fermentation broth with controlled pH was continuously pumped into the bottom of the second FBB. The final fermentation broth was collected in the product tank at the far right in the scheme. Each FBB was made of a water-jacketed glass column (diameter: 6 cm, length: 8 cm, working volume: 150 mL) packed with a spiral-wound cotton towel and stainless-steel wire mesh as the spacer and matrix support. The free cell buffering spinner flask had a working volume of 1.5 L and was equipped with a pH probe (Mettler Toledo) for pH monitoring and control at a desired value. Before use, the complete system was autoclaved at 121°C for 30 minutes, held overnight at room temperature to allow spore germination, and then autoclaved at 121°C for one hour for complete sterilization.
5.2.3 Effects of Butyric Acid on Butanol Production

Enhanced P2 media (see appendice) with 50 g/L glucose and various concentrations of butyric acid at pH 6.0 were prepared in serum bottles. Before sterilization, each bottle was made anaerobic by sparging N₂ for 10 min. Fresh cultures of *C. acetobutylicum* ATCC824, *C. beijerinckii* ATCC 55025, *C. beijerinckii* JB200, *C. beijerinckii* BA101, and *C. acetobutylicum* NCIMB 8052 were prepared the day before inoculation. Each serum bottle was inoculated with 3 mL of a fresh culture and placed in a 37°C incubator. Samples were taken periodically for analyses of cell growth and fermentation products.

5.2.4 Continuous Fermentation Kinetics

The fibrous bed bioreactors and the spinner flask were filled with feed medium and made anaerobic by sparging N₂ from the bottom of the bioreactors. Each bioreactor was inoculated with 50 mL of exponential-phase cells grown in serum bottles at 37°C. Temperature in the system was maintained at 35°C with a water jacket for each FBB while the spinner flask was agitated to maintain a well mixed condition with its temperature controlled with a water bath. After inoculation, cells in the reactors were allowed to grow for 12~24 h to increase cell density and to immobilize cells onto the fibrous bed. When OD₆₀₀nm of the fermentation broth in the spinner flask reached 1.0 and gas production was observed, continuous fermentation was started by pumping the feed medium into the bottom of the first FBB. Unless otherwise noted, the feed medium contained ~80 g/L glucose as the main substrate and 4 g/L butyric acid. Fermentation
broth samples (1.5 mL each) were taken from various points in the process at proper time intervals throughout the fermentation.

5.2.5 Cell Viability Assay

Cell viability was determined using the method of Glenner (Glenner, 1977) with some modifications. One milliliter 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 to the cell pellet. The cell pellet was resuspended in the TTC solution by vortexing at the maximum speed, and then incubated in the dark at room temperature for 30 min for color development. The cells were collected by centrifugation at 16,000 rpm for 10 min and 1 ml of methanol was added to the cell pellet 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, Columbia, MD, UV-16-1) against a negative control of dead cells boiled before adding the TTC solution. Cells from the exponential phase were used as the positive control with its viability being 100%.

5.2.6 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-mL cuvette (1 cm light path length) using a spectrophotometer (Shimazu, Columbia, MD, UV-16-1). One unit of OD was found to be equivalent to 0.562 g/L cell dry weight of *C. beijerinckii* JB200. The glucose concentration in each fermentation broth sample was
analyzed using a glucose analyzer (YSI 2300, Yellow Spring, Ohio). For analyses of solvents and acids in the fermentation broth, samples were centrifuged and filtered with a 0.2 µm syringe filter (if necessary). In order to accurately measure concentrations of organic acids, samples were diluted and acidified with buffer containing 10 mL/L phosphoric acid to a final volume of at least 600 µL in the vial. The acidified samples were analyzed with a GC-2014 Shimadzu gas chromatograph (GC) (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (0.25 µm film thickness and 0.25 mm ID, Stabilwax-DA). The GC was operated at an injection temperature of 200ºC with 1 µL of the acidified sample injected by the AOC-20i Shimadzu auto injector. For each sample injection, the column temperature was initially held at 80ºC for 3 min, gradually raised to 150ºC at a constant rate of 30 ºC/min, and held at 150 ºC for 3.7 min.

5.3 Results and Discussion

5.3.1 Effects of Butyric Acid on Butanol Production

Five butanol producing strains (C. beijerinckii JB200 C. acetobutylicum ATCC 824, C. beijerinckii ATCC 55025, C. beijerinckii BA101, and C. beijerinckii NCIMB 8052) were compared with their butanol productivity, titer, and the ratio of butanol to total solvent produced in media with different concentrations of butyric acid at pH 6.0. Figure 5.2 shows the effect of butyric acid on the butanol titer from the five strains studied. In general, the butanol titer increased with increasing the butyric acid concentration to 4 g/L and then decreased at higher butyric acid concentrations. This
trend was observed with four strains (C. beijerinckii ATCC 55025, C. beijerinckii BA101, C. beijerinckii NCIMB 8052, and C. acetobutylicum ATCC 824) studied. C. acetobutylicum ATCC 824 produced the highest butanol titer of ~10 g/L in media with 2 g/L and 4 g/L of butyric acid. Surprisingly, little butanol was produced by these four strains in serum bottles when no butyric acid was added in the medium. This might have been caused by acid crash without a proper buffering capacity and pH control in serum bottles. Butyric acid could inhibit cell growth and provide some buffering capacity, thus slowing down cell growth and preventing acid crash under uncontrolled pH conditions. For C. beijerinckii JB200, the final butanol titer was the second highest and not significantly affected by the concentration of butyric acid.

Similar effects on butanol productivity were observed (Figure 5.3), with C. beijerinckii JB200 giving the highest butanol productivity of >0.12 g/L·h at all concentrations of butyric acid studied. Figure 5.4 shows the effect of butyric acid on the ratio of butanol to total solvents produced by these five strains. In general, the ratio of butanol to total produced solvents increased with increasing the butyric acid concentration in the medium, suggesting that these clostridia strains can uptake butyric acid to produce more butanol. This finding also confirmed the results from the stoichiometric analysis presented in Chapter 3.

In summary, adding butyric acid in the medium improved butanol production. Among the five strains tested, C. beijerinckii JB200 was superior to the other four strains with its ability to produce butanol at a higher productivity and titer at all butyrate concentrations, up to 8 g/L, studied. Although C. acetobutylicum ATCC 824 also gave a
high butanol titer and productivity at 4 g/L butyric acid, it was not considered a good candidate for continuous butanol fermentation because its spore-forming characteristics would disrupt butanol production.

5.3.2 Continuous Fermentation in the First Stage FBB

The FBB was initially operated as a batch reactor with the medium containing 2 g/L yeast extract and 0.5 g/L tryptone as the nitrogen sources for the bacteria to reach the desired cell density until vigorous cell growth and gas production were observed. Continuous fermentation was then started when the reactor was switched from the batch mode to the continuous mode with continuous feeding of the medium containing 80 g/L glucose and 4 g/L butyric acid at a steady feed rate of 156 mL/h (dilution rate: 1.04 h\(^{-1}\)). Figure 5.5 shows the continuous fermentation kinetics of the first-stage FBB over 600 hours. A shift from acidogenesis to solventogenesis could be observed with a rapid increase in the butanol concentration and a decrease in the concentration of butyric acid at 100 hours after continuous feeding started. The nitrogen source in the feed medium was reduced gradually to 1 g/L yeast extract and 0.5 g/L tryptone after 120 hours of continuous fermentation. Butanol production decreased slowly from 7 g/L to 3.5 g/L due to the decreased nutrients in the feed medium. It was recommended that ABE fermentation should be operated with phosphate limitation (Bahl et al., 1982). However, the concentration of phosphate buffer at 0.5 g/L was ineffective to maintain the fermentation pH at the desirable level of ~5.0. The pH in the effluent of the first FBB was ~4.73, although the feed medium pH was 5.5. Consequently, only about 25% of
glucose in the feed medium was consumed and the effluent with ~60 g/L glucose left the first FBB. The butanol titer fluctuated between 3.5 to 5.5 g/L with an average butanol productivity of 4.08 g/L·h and yield of ~0.20 g/g glucose.

5.3.3 Continuous Fermentation in the Second Stage Chemostat

The effluent from the first-stage FBB was continuously fed into the second-stage chemostat (the 1-liter spinner flask) with its pH maintained at 5.5 by adding ammonium hydroxide. Figure 5.6 shows the fermentation kinetics of the chemostat operated over 600 hours. During the first 120 hours (start-up period), butanol production gradually increased to ~9.5 g/L. A gradual drop in the butanol titer after 120 hours was caused by the reduced nutrients (yeast extract and tryptone) in the feed medium. However, the butanol titer increased gradually from 7.5 g/L to >10.5 g/L in the final 350 hours. With the working volume of 1 liter in the chemostat, the average dilution rate was 0.156 h\(^{-1}\) and the average butanol productivity was ~0.77 g/L·h, with an overall butanol yield of ~0.19 g/g glucose consumed. The effluent from this chemostat stage contained ~35 g/L glucose, which was fed into the final stage of the fermentation with a FBB discussed below.

5.3.4 Continuous Fermentation in the Final Stage FBB

During the startup of the continuous fermentation, the second FBB in the process was not inoculated initially. After continuous feeding started, this final-stage bioreactor was gradually filled up with the fermentation broth from the second-stage chemostat.
Figure 5.7 shows the kinetics of continuous fermentation in the final-stage FBB. The sampling point for the final-stage FBB was at the outlet of the reactor, and no sample was collected until after 40 hours. Since the pH condition of the fermentation broth fed into this FBB was adjusted with ammonium hydroxide, the fermentation was relatively stable as compared to the first-stage FBB without pH control. As expected, the concentration profiles in this stage followed similar trends in the second-stage chemostat, but with more products and less glucose in the effluent, which contained ~11.0 g/L butanol and ~21.8 g/L glucose. On average, this stage FBB fermentation gave a butanol productivity of ~3.39 g/L·h and yield of ~0.25 g/g glucose.

Table 5.2 summarizes the performance of the continuous fermentation in the three-stage process. While titer indicates the product concentration from each stage of the three reactors, the yield and productivity were calculated based on the production in each reactor. As a result, when butyric acid was uptaken by the bacteria in the bioreactor, butyrate yield and productivity could not be calculated. No matter the FBB was pH controlled or not, the productivities were much higher than that in free-cell chemostat. With more cells immobilized in the FBB, acids were uptaken more easily than in free-cell fermentation. It was also clear that product yields were greatly affected by the reactor pH. The butanol yield was higher in the second FBB with pH at ~5.0 than that in the first FBB with pH dropping to below 4.8. Between the two FBBs, the productivity was similar although the second reactor was more affected by butanol at higher concentrations. In the second FBB with its pH maintained at >5.0, the bacteria were able
to pick up the produced acids and produced more solvents and resulted in a higher butanol yield of ~0.25 g/g glucose.

5.3.5 Cell Density and Morphology in FBB

Cell immobilization in the fibrous matrix was examined with scanning electron microscopy and is illustrated in Figure 5.8 at different magnifications. Figures 5.8(a) and (b) show the structure of the fiber used in the fibrous bed bioreactor. The cells forming a thin biofilm that was partially attached to the fiber can be seen in Figure 5.8(c). In Figures 5.8 (d), (e) and (f), how cells were attached to the fiber could be observed. Although the samples were collected from the reactor with higher concentrations of end products, the cells were still active with observed cell division. From the cell viability assay, ~75% of cells were still viable at the end of the 600 hours continuous operation.

5.4 Conclusion

The effect of butyric acid on butanol production was tested with five solventogenic clostridia strains in serum bottles. *C. beijerinckii* JB200 was found to be superior to the other four strains with a high butanol titer of 7.1 g/L and high butanol productivity of >0.12 g/L·h. Continuous fermentation with free cell reactor in the middle for pH adjustment greatly helped maintaining cell productivity without inhibition by the acid products. Although butanol productivity was not as high as that from *C. beijerinckii* ATCC 55025 reported in Chapter 4, the percentage of sugar consumption from the feed medium was greatly improved with the newly developed *C. beijerinckii* JB200. While
cell growth of *C. beijerinckii* ATCC 55025 was inhibited with glucose concentration over 65 g/L, *C. beijerinckii* JB200 was able to tolerate glucose concentration over 90 g/L without any negative effect. The continuous fermentation was operated over three weeks and gave a steady final butanol titer of ~12 g/L without any sign of degeneration or contamination.
5.5 Reference


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Table 5.1 Summary of process performance in the three-stage continuous fermentation with *C. beijerinckii* JB200.
Figure 5.1 Experimental set-up for continuous fermentation in this study.
Figure 5.2 The effect of butyric acid on butanol titer for five strains.
Figure 5.3 The effect of butyric acid on butanol productivity for five strains.
Figure 5.4 The effect of butyric acid on the ratio of butanol over total solvent production for five strains.
Figure 5.5 Continuous fermentation kinetics of the first-stage FBB fed with 80 g/L glucose and 4 g/L butyric acid in P2 medium at a dilution rate of 1.05 h\(^{-1}\).
Figure 5.6 Continuous fermentation kinetics of the second-stage chemostat fed with the effluent from the first-stage FBB.
Figure 5.5 Continuous fermentation kinetics of the final-stage FBB.
Figure 5.6 Scanning electron micrographs of cells immobilized in the fibrous matrix in the FBB at different magnifications: (a) 200x, (b) 600x, (c) 1200x, (d) 1500x, (e) 4000x, (f) 10000x.
Chapter 6: Kinetics of Butanol Fermentation by Metabolically Engineered Clostridium tyrobutyricum

Summary

Butanol production from acetone-butanol-ethanol (ABE) fermentation was the second largest industrial fermentation in the early 20th century. The practice decreased dramatically because it suffered from low titer, low yield and low productivity. The low titer, yield and productivity were caused by the low cell tolerance to the fermentation products. Therefore, a host with better tolerance for the produced acids and solvents is desirable for improved butanol production. In this study, butyraldehyde/butanol dehydrogenase was cloned and overexpressed in Clostridium tyrobutyricum mutant with a disrupted acetate pathway to produce butanol from glucose and xylose. The mutants were cultured in free-cell reactor and fibrous bed bioreactor (FBB) to evaluate their butanol productivities and yields in batch fermentation. The mutant with pM2-thladhE2 grew faster and gave higher productivities than the mutant with pMTL-thladhE2. In the FBB with the medium containing only xylose as the carbon source, the mutant with pM2-thladhE2 was able to produce more butanol at 7.12 g/L with a higher yield of 0.172 g/g xylose. By maintaining the pH >6.1 in the reactor, the mutant was able to produce more butanol compared to at pH <6.0.
6.1 Introduction

Extensive studies have been done for improving butanol production in acetone-
butanol-ethanol (ABE) fermentation. Besides process developments on different modes
of fermentation, media optimization and integration of fermentation process with in situ
product recovery, a great numbers of studies have also investigated strain improvement
through metabolic engineering. Many genes have been cloned and manipulated in C. 
acetobutylicum ATCC824, a butanol-producing strain with known genome sequence.
However, limited improvement on butanol production was achieved because of the
difficulty in cloning clostridia, complicated metabolic pathway, and intricate gene
regulation (Mermelstein et al, 1993; Harris et al, 2000; Harris et al, 2001; Jiang et al,
2009). Recently, the focus of metabolic engineering studies has been shifted to non-
clostridia hosts for butanol production, including E. coli (Atsumi et al, 2008; Bramucci,
2008; Gunawardena, 2008; Lee et al, 2008; Nielsen et al, 2009; Papoutsakis et al, 2008;
Shen et al, 2008), P. pudida (Nielsen et al, 2009), S. cerevisiae (Raamsdonk, 2008; Steen
et al, 2008), and B. subtilis (Nielsen et al, 2009). However, butanol production by these
metabolically engineered strains was low as compared to native butanol-producing
clostridia strains.

In solvent-producing clostridia, the major bottleneck to improve butanol
production is the low tolerance to produced butanol. In this study, a different approach
for improved butanol production was attempted: producing butanol in metabolically
engineered C. tyrobutyricum ATCC 25755, which naturally has higher butanol tolerance.
C. tyrobutyricum is an anaerobic, spore-forming, rod-shape bacterium with a metabolic
pathway that partially resembles the metabolic pathway in clostridia carrying out ABE
fermentation. *C. tyrobutyricum* acquires energy by producing acetic acid and butyric acid from carbon sources, including glucose, glycerol, lactose, and xylose from wheat straw (Zigová and Šturdík, 2000). Figure 6.1 shows the metabolic pathway of *C. tyrobutyricum*, which does not have the solvents (acetone, butanol and ethanol) producing pathways present in solventogenic *C. acetobutylicum* and *C. beijerinckii*. However, by cloning a butyraldehyde/butanol dehydrogenase gene into *C. tyrobutyricum*, the new mutant strain can produce butanol with better butanol tolerance and without producing any acetone, potentially increasing the butanol yield from sugar and making the down-stream purification easier than the conventional ABE fermentation process.

One physiological characteristic that determines the solvent tolerance of a bacterium is the ratio of saturated and unsaturated fatty acid in the cell membrane (Bhupinder et al., 1987). With a higher ratio of saturated fatty acids, the cell membrane is more rigid and thus more resistant to solvent toxicity. The bacteria response to the environment with a high butanol concentration is to increase its membrane saturated/unsaturated fatty acid ratio in order to maintain the integrity of the cell membrane (Moreira et al., 1981). In *C. tyrobutyricum*, a higher ratio of saturated/unsaturated fatty acids and higher butyric acid tolerance were observed after adaptation in a fibrous bed bioreactor (FBB) (Zhu et al., 2002). In addition, mutants of *C. tyrobutyricum* with a knocked-out acetate kinase gene (*ack*) showed the partially disrupted acetate biosynthesis pathway and higher tolerance to butyric acid and butanol (Zhu et al., 2004; Liu and Yang, 2006; Zhang 2009). The *C. tyrobutyricum* mutant with a partially disrupted acetate pathway tolerated higher concentrations of butyrate (60 g/L) and butanol (12 g/L) than wild type did (30 g/L and 6 g/L, respectively) (Zhang, 2009).
Our group has recently cloned an *ack* knocked-out mutant of *C. tyrobutyricum* that overexpresses aldehyde/alcohol dehydrogenase (AAD), enabling the mutant to produce butanol from butyryl-CoA. In this work, butanol production from glucose and xylose by *C. tyrobutyricum* mutants expressing AAD was evaluated in batch fermentation in the FBB.

6.2 Materials and Methods

6.2.1 Culture and Media

Two different plasmids, pMTL-thladhE2 from pMTL007 (Heap et al. 2007) and pM2-thladhE2 from pMTL82151 (Heap et al., 2009) were used for gene expression. These plasmids were inserted with the *C. acetobutylicum adhE2* gene and the native thiolase promoter from *C. tyrobutyricum*. The constructed plasmids were transformed into *E. coli* as donor cells, and then transferred into *C. tyrobutyricum* through conjugation. Detailed descriptions of the construction of these two plasmids are given in Appendix D.

The metabolically engineered mutants were maintained in reinforced *Clostridium* medium (RCM) (Difco) with 25% glycerol at -80 ºC. The fermentation medium was simplified *Clostridium* growth medium (CGM) as previously described by Zhang (Zhang, 2009), with the supplementation of 20 μg/ml thiamphenicol, which was the selection marker for the mutants. Unless otherwise noted, the fermentation medium contained either glucose, xylose, or both as the carbon source.
6.2.2 FBB Fermentation System

Figure 6.2 shows the FBB fermentation system used in this study. The FBB, which was made of a glass column (working volume: 80 mL) with a water jacket and packed with a spiral-wound cotton towel and stainless-steel wire mesh, was connected to a spinner flask (working volume: 1.5 L) with pH and temperature controls. Before use, the bioreactor system was autoclaved at 121°C for 30 minutes, held overnight at room temperature to allow spore germination, and then autoclaved again at 121°C for one hour for complete sterilization. The system containing the fermentation medium was then prepared for repeated-batch fermentations described below.

6.2.3 Repeated-Batch Fermentation

The fermentation was first operated in the spinner flask as free-cell fermentation. After sterilization, the spinner flask filled with the medium was made anaerobic by sparging N₂ from the bottom of the bioreactor. The bioreactor was then inoculated with 50 mL of exponential-phase cells grown in a serum bottle at 37°C. Temperature was maintained at 35°C with a water jacket surrounding the bioreactor. The free-cell batch fermentation kinetics was first studied with mutants pMTL-thladhE2 and pM2-thladhE2. Then, repeated batch fermentations with mutant pM2-thladhE2 were carried out with the FBB system. After inoculation, the spinner flask was held static for at least 24 h to allow cells to grow. When OD₆₀₀nm of the fermentation broth reached 1.0 and gas production was observed, the fermentation broth in the spinner flask was pumped into the bottom of the FBB and recirculated back to the spinner flask at a flow rate of 40 mL/min. At the end of each batch fermentation, the spinner flask was disconnected from the FBB, 129
cleaned and sterilized with fresh medium. The antibiotic, thiamphenicol, was added to the fresh medium in each batch before the spinner flask was reconnected to the FBB. Unless otherwise noted, the fermentation broth in the spinner flask was sampled (1.5 mL) at proper time intervals throughout the fermentation.

6.2.4 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-mL cuvette (1 cm light path length) using a spectrophotometer (Shimazu, Columbia, MD, UV-16-1). The glucose concentration in each fermentation broth sample was analyzed using a glucose and lactate analyzer (YSI 2300). For analyses of solvents and acids in the fermentation broth, samples were centrifuged and filtered with a 0.2 μm syringe filter (if necessary). In order to accurately measure the concentrations of organic acids, samples were diluted and acidified with a buffer containing 10 mL/L phosphoric acid to a final volume of at least 600 μL in the vial. The acidified samples were analyzed with a GC-2014 Shimadzu gas chromatograph (GC) (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (0.25 μm film thickness and 0.25 mm ID, Stabilwax-DA). The GC was operated at an injection temperature of 200 ºC with 1 μL of the acidified sample injected by the AOC-20i Shimadzu auto injector. The column temperature was initially held at 80 ºC for 3 min, raised to 150 ºC at a constant rate of 30 ºC/min, and held at 150 ºC for 3.7 min.

For the xylose concentration in the fermentation broth, samples were analyzed with a high performance liquid chromatograph (HPLC, Shimadzu) with an organic acid
analysis column (Aminex HPX-87H, BioRad) following the method described in appendix (Zhu and Yang, 2003)

6.3 Results and Discussion

Table 6.1 summarizes all the fermentation results, which are discussed below.

6.3.1 Free-Cell Batch Fermentation Kinetics

The two mutants were first evaluated for their abilities to produce butanol from glucose in free-cell fermentations carried out in the spinner flask (without the FBB column in Figure 6.2). Two batch fermentations were operated with the mutant pMTL-thladhE2 at pH 6.5 (Figure 6.3) and 6.0 (Figure 6.4), respectively. At the higher pH of 6.5, cell growth was faster than that at the lower pH of 6.0. The final butanol concentration was ~4.5 g/L when glucose was depleted in the fermentation broth at both pHs studied. However, butyric acid production was significantly higher at pH 6.5 than at pH 6.0. Figure 6.5 shows the batch fermentation kinetics with the mutant pM2-thladhE2 at pH 6.5. More butanol production was obtained with the mutant pM2-thladhE2 (~6.46 g/L vs. ~4.5 g/L for the mutant pMTL-thladhE2). The mutant pM2-thladhE2 also produced significantly more ethanol (~3.0 g/L), more acetic acid (~7.4 g/L) and less butyric acid (~4.6 g/L) as compared to the other mutant (~0.5 g/L ethanol, ~6.5 g/L acetic acid, >9.5 g/L butyric acid). It is clear that pM2-thladhE2 had a higher expression level of the AAD enzyme that allowed more butyryl-CoA and acetyl-CoA to be converted to butanol and ethanol, respectively. The increased acetic acid production in the mutant pM2-thladhE2 might be attributed to the reduced production of butyric acid, which is
known to inhibit the enzymes in the acetic acid biosynthesis pathway. In addition, cell
growth was much faster with pM2-thladhE2, which reached a high optical density of 7.0
within 50 hours of fermentation. With higher and faster butanol production, the mutant
pM2-thladhE2 was chosen for further fermentation study in the FBB.

6.3.2 Repeated Batches in FBB

The first two batches in the FBB fermentation were with glucose (~65 g/L) as the
substrate and at pH 5.6 (Figure 6.6) and 5.7 (Figure 6.7), respectively. In the first batch,
cells inoculated in the fermentation broth were allowed to be immobilized in the FBB
with the medium recirculation. There was a lag phase of ~10 hours. The first batch ended
with a butanol titer of 6.46 g/L and yield of 0.09 g/g glucose. In the second batch, the
fermentation ended within 100 hours and gave a butanol titer of 5.12 g/L and yield of
0.07 g/g glucose.

In order to investigate if the mutant can utilize xylose, the feed medium in the
third batch was changed to 30 g/L glucose and 30 g/L xylose as the carbon sources. As
can be seen in Figure 6.8, xylose utilization occurred simultaneously with glucose
utilization. However, glucose was consumed within 50 hours while xylose was not
completely used even after 140 hours. Clearly, cell uptake of glucose (0.82 g/L·h) was
much faster than xylose (0.15 g/L·h). The consumption of xylose appeared to be
accelerated after glucose depletion. These results suggested that xylose uptake by the cell
was not as efficient and could be inhibited by glucose. In general, both acids (acetic and
butyric) and solvents (butanol and ethanol) were produced steadily throughout the
fermentation. The final butanol titer was 4.71 g/L with an overall butanol yield of 0.092 g/g sugar and productivity of 0.033 g/L·h in this batch fermentation.

Sixty grams per liter of xylose was prepared in the medium for the fourth batch. The fermentation kinetics is shown in Figure 6.9. After 12 hours of lag phase, butanol was steadily produced in the reactor and reached a higher titer of 7.55 g/L with the butanol yield of 0.18 g/g xylose and productivity of 0.046 g/L·h at the end of the batch. The xylose uptake rate was higher at ~0.36 g/L·h with only xylose as the carbon source. The fifth batch was conducted with the medium containing 30 g/L glucose and 30 g/L xylose to evaluate if the bacteria would utilize both sugars at the same rate after induction of xylose utilization in the previous two batches. As shown in Figure 6.10, the final butanol titer was 4.73 g/L with the yield of 0.106 g/g sugar and productivity of 0.03 g/L·h. The glucose uptake rate was 0.44 g/L·h and the xylose uptake rate was 0.13 g/L·h. Another batch with 60 g/L xylose as the sole carbon source was conducted. After 180 hours of fermentation, 6.68 g/L butanol was produced with the yield of 0.164 g/g xylose and productivity of 0.037 g/L·h. These results were similar to those obtained in the previous batches.

In the FBB, the bacteria were able to eliminate the lag phase in every new batch with a high cell density maintained in the fibrous bed and gave reproducible results over six batches. Among them, the first two batches with glucose as the carbon source were operated at pH below 6.0, while the pH values of the later four batches were all higher than 6.0. Both butanol and butyric acid yields were higher at pH >6.0 because C. tyrobutyricum could produce more butyrate and less acetate at ~pH 6.3 as reported in the literature (Zhu and Yang, 2004).
It is clear that *C. tyrobutyricum* can simultaneously utilize glucose and xylose but with glucose as the preferred substrate at a much higher uptake rate. However, more butanol can be produced in the fermentation with xylose as the sole carbon source, indicating that the slower cell growth and fermentation on xylose would allow the AAD enzyme to convert more butyryl-CoA to butanol. In batches with glucose as the carbon source, cell growth and fermentation were faster than those without glucose in the medium. Among the six batches performed in the FBB, higher butanol concentration and yield were obtained with xylose as the sole carbon source. Because aldehyde/alcohol dehydrogenase expressed from the foreign plasmid needed to compete with phosphobutyrylase for butyryl-CoA, slower cell growth would favor butanol production as less phosphobutyrylase would be available for butyrate production.

While the acetate biosynthesis pathway in *C. tyrobutyricum* was disrupted before the plasmid insertion, acetic acid production was not completely eliminated in these mutants. This is believed to be attributed to the existence of CoA transferases that can transfer the CoA between acetyl-CoA and butyrate and vice versa. Therefore, further engineering of the mutants with knock-out butyrate biosynthesis would be necessary to produce mainly butanol (and some ethanol) as the main fermentation product by the mutant of *C. tyrobutyricum*. It is also interesting to see if more butanol can be produced at a higher titer by the mutant in fed-batch fermentation, which is currently being investigated.
6.4 Conclusion

A difference in the performance of two plasmids on butanol production in *C. tyrobutyricum* ATCC 25755 with a partially disrupted acetate biosynthesis pathway was demonstrated in free cell fermentations. More butanol was produced with a shorter fermentation time by the mutant with pM2-thladhE2. For mutant with pM2-thladhE2, glucose was used much faster than that for mutant with pMTL-thladhE2. In the FBB fermentation, butanol was steadily produced in all repeated batches. Media with different carbon sources could affect butanol production by *C. tyrobutyricum* mutants. With glucose as the carbon source, cell growth was faster than that with xylose as the carbon source. The butanol-producing aldehyde/alcohol dehydrogenase expressed from the foreign plasmid competed for butyryl-CoA with phosphobutyrylase, whose activity greatly depended on cell growth. As a result, bacteria produced more butanol (7.12 g/L on average) and gave higher butanol yield (0.150 g/g xylose on average) with xylose as the sole carbon source. At a pH value higher than 6.1, more butanol and butyric acid were produced.
6.5 Reference


Mermelstein LD, Papoutsakis ET, Petersen DJ, Bennett GN. 1993. Metabolic engineering of Clostridium acetobutylicum ATCC 824 for increased solvent production by


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<td>Titer (g/L)</td>
<td>4.43</td>
<td>4.49</td>
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<tr>
<td>Yield (g/g)</td>
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<td>0.07</td>
</tr>
<tr>
<td>Productivity (g/L·h)</td>
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<td>0.037</td>
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<tr>
<td>Acetic acid</td>
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<td></td>
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<tr>
<td>Titer (g/L)</td>
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<td>6.43</td>
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<tr>
<td>Yield (g/g)</td>
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<td>0.11</td>
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<td>0.053</td>
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<td></td>
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<tr>
<td>Yield (g/g)</td>
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</tr>
<tr>
<td>Productivity (g/L·h)</td>
<td>0.132</td>
<td>0.078</td>
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</table>

Table 6.1 Summary of batch fermentations with mutants pMTL-thladhE2 and pM2-thladhE2 in free-cell and FBB fermentations with glucose and/or xylose as substrates.
Figure 6.1 The metabolic pathway showing acid production from glucose by *C. tyrobutyricum* and butanol production from butyryl-CoA with a heterologous *aad* gene.
Figure 6.2 FBB batch fermentation set-up with medium recirculation for butanol production from glucose and/or xylose by metabolically engineered *C. tyrobutyricum*. 
Figure 6.3 Free cell fermentation of *C. tyrobutyricum* mutant pMTL-thldhE2 with 60 g/L glucose at pH 6.5.
Figure 6.4 Free cell fermentation of *C. tyrobutyricum* mutant pMTL-thladhE2 with 65 g/L glucose at pH 6.0.
Figure 6.5 Free cell fermentation of *C. tyrobutyricum* mutant pM2-thladhE2 with 65 g/L glucose at pH 6.5.
Figure 6.6 Fermentation kinetics of *C. tyrobutyricum* mutant pM2-thladhE2 in the FBB with 65 g/L glucose at pH 5.6.
Figure 6.7 Fermentation kinetics of *C. tyrobutyricum* mutant pM2-thldhE2 in the FBB with 65 g/L glucose at pH 5.7.
Figure 6.8 Fermentation kinetics of *C. tyrobutyricum* mutant pM2-thladhE2 in the FBB with 30 g/L glucose and 30 g/L xylose at pH 6.3.
Figure 6.9 Fermentation kinetics of *C. tyrobutyricum* mutant pM2-thlAdhE2 in the FBB with 55 g/L xylose at pH 6.1.
Figure 6.10 Fermentation kinetics of *C. tyrobutyricum* mutant pM2-thladhE2 in the FBB with 30 g/L glucose and 30 g/L xylose at pH 6.4.
Figure 6.11 Fermentation kinetics of *C. tyrobutyricum* mutant pM2-thladhE2 in the FBB with 60 g/L xylose at pH 6.1.
Summary

While the demand for butanol as a transportation fuel from bioprocesses increases with the increasing price of gasoline, it is necessary to investigate the feasibility of butanol fermentation at a large scale. Although there have been many large-scale ABE fermentations, fermentation conditions are usually kept confidential and not available for the public. However, several problems with large-scale ABE fermentation have been reported, including contamination and strain instability. In order to evaluate the feasibility and scalability of the fibrous bed bioreactor for large-scale ABE fermentation, a pilot-scale fermentation system consisting of a 150-L FBB, a 220-L feed tank, a 20-L preparation tank and a 200-L product tank was constructed to investigate the fermentation process. Two separate batches of ABE fermentation in the 150-L FBB were conducted. The final butanol titers from the two batches were 8.7 g/L and 9.4 g/L, respectively. With the addition of butyric acid in the medium, the bacteria utilized butyric acid to produce more butanol and the ratio of butanol over total produced solvents increased from 0.60 to 0.69. The results were comparable to those from the fermentation at the bench scale, suggesting that the FBB with further scale up can be used for ABE fermentation at an industrial scale.
7.1 Introduction

Butanol production from ABE fermentation was the second largest industrial fermentation during the World Wars, with many large-scale biobutanol plants all over the world, including the United States, former Union of Soviet Socialist Republics, France, and Lower Austria (Nimcevic and Gapes, 2000). Due to the impact of cheaper butanol production from the petroleum industry, large scale butanol production from ABE fermentation in most countries ceased in the late twentieth century. Also, the fermentation conditions at large scales usually remained confidential within the companies. As reported in the literature, potential problems in industrial ABE fermentation included contamination during the fermentation process and poor stability of solvent producing strains (Zverlov et al., 2006). These problems potentially can be alleviated with the fibrous bed bioreactor (FBB) (Yang, 1996). By immobilizing solvent producing bacteria in the FBB, the bacteria were retained in the reactor at a high cell density that can better resist contamination and stabilize the process with improved tolerance to the environment (Yang et al., 1994, 1996, Zhu and Yang, 2003, Cho, et al., 1996, Huang, et al., 2004). However, the fibrous-bed bioreactor has not been scaled up and tested for large-scale operation. The purpose of this study was to evaluate the feasibility of using the FBB for large-scale ABE fermentation.

There are several important considerations during the scale up process (Schmidt, 2005). Mixing quality is usually reduced with increasing the vessel size. With improper mixing, localized nutrient depletion can occur in a large-scale fermentor. Different impeller designs can provide different mixing patterns with different hydrodynamics in
Aeration is an important factor during the scale up of an aerobic fermentation process. However, aeration is not considered significant in this study because ABE fermentation is an anaerobic process. Sterility is another consideration in designing a large-scale fermentor. Pilot-scale and large-scale industrial fermentors are usually sterilized by introducing pressurized steam for in-place sterilization. Clean-in-place (CIP) technology with highly alkaline detergents is usually also used. Heat transfer is also an important consideration in fermentation process scale up. For temperature control in an aerobic fermentation process, cooling is usually required because aerobic cell growth is accompanied with metabolic heat generation. Solid deposition is commonly found on the surface of cooling coils and decreases the efficiency of temperature control.

For anaerobic fermentation, solid deposition from cooling water is not a concern because anaerobic fermentation does not generate much heat and heating may be required in some processes.

Besides aforementioned considerations during the scale up process, there are several other concerns that need to be considered for scaling up ABE fermentation. Because Clostridia are strictly anaerobic microorganisms, the fermentation process needs to be oxygen-free to maintain viable cells in the bioreactor. Anaerobic fermentation processes are usually kept anaerobic by purging nitrogen in the system to sweep away the oxygen in the reactor. Catalyst such as palladium can also be used together with H₂ to remove oxygen (Aranki et al., 1972). For large-scale fermentation, serial inoculations are necessary to assure effective operation. However, serial inoculations can result in degeneration in solvent-producing Clostridia (Kosaka et al. 2007). Conventional solution
for preventing degeneration was to maintain the culture pH at ~6.0. Excessive acids production could reduce the pH to a low level that was suspected as the cause of degeneration (Kashket et al., 1995).

The objective of this study was to demonstrate ABE fermentation in the FBB at a pilot scale. Two batches of ABE fermentation were operated in a 150-L FBB to evaluate the feasibility of butanol fermentation and the results are compared to that from a laboratory-scale batch fermentation.

7.2 Materials and Methods
7.2.1 Cultures and Media

Asporogenic \textit{Clostridium beijerinckii} ATCC 55025 (Jain et al., 1993) was used in this study. The bacterium was cultured in a minimal medium modified from P2 medium with glucose and butyric acid as carbon sources (see Appendix A). Modified P2 medium was sterilized in a 20-L bioreactor (Chemap) separately and aseptically transferred to the feed tank through \( \frac{1}{4} \)" stainless-steel tube. Sixteen liters of liquid were sterilized for 45 minutes in each batch of sterilization and the averaged time to complete one batch is four hours. The materials required for preparing 160-L P2 medium and the order of material sterilization are listed in Table 7.1. Glucose, potassium phosphate, and yeast extract were first dissolved in distilled water and then sterilized in the 20-L bioreactor. Butyric acid with a proper concentration was neutralized with sodium hydroxide (NaOH) and sterilized before transferred to the feed tank. The medium was made anaerobic by purging with sterile \( \text{N}_2 \) for 120 minutes in the feed tank.
7.2.2 Experimental Set-Up

A 150-liter stirred-tank bioreactor was used for the optimization of pilot-scale fermentation. As shown in Figure 7.1, two fibrous bed packings were installed in the bioreactor with one marine agitator in between the packing and another one at the bottom of the reactor. The agitation speed of the agitator can be controlled automatically with an AC micro drive (GS1) ranging from 0 to 800 rpm. The bioreactor can be aseptically sampled from the bottom, center, or the top of the reactor to monitor the difference in the three points. To ensure effective mass transfer, four baffles were fixed on the inside wall of the bioreactor without contact with the fibrous-bed packings. The bioreactor temperature was monitored with a thermocouple inserted in the middle of the reactor and maintained at 35°C with a temperature controller (Dwyer, Michigan City, IN). The water in the water jacket was heated by an inline immersion heater (TruHeat, Allegan, MI).

Beside the bioreactor, the complete pilot plant also included one 20-liter fermentor for medium preparation, one 220-liter tank for medium storage, and one 200-liter tank for product storage. An overall flow diagram for butanol production in 150-liter FBB is shown in Figure 7.2. All major parts used in the construction of the pilot scale FBB are listed in Table 7.2. High pressure steam can be introduced into these tanks and all connecting pipes to ensure aseptic process. Nitrogen can aseptically purge the reactors through an on-line 0.22 μm filter (Satorius, Goettingen, Germany). A pressure gauge was installed at the top of the bioreactor to monitor the change of pressure inside the vessel due to gas production during fermentation.
7.2.3 Fermentation Kinetics

The 150-L FBB was sterilized with high pressure steam, filled with 140 liters medium and made anaerobic by sparging N\textsubscript{2} from the bottom of the reactor for 2 hours. Temperature was maintained at 35°C with a water jacket surrounding the bioreactor. The bioreactor was inoculated with 10 liters of the culture at the exponential phase grown in a 20-L fermentor at 37°C overnight. Samples were collected at proper time intervals throughout the fermentation.

7.2.4 Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-mL cuvette (1 cm light path length) using a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1). One unit of OD was found to be equivalent to 0.534 g/L cell dry weight. The glucose concentration in each fermentation broth sample was analyzed using a glucose and lactate analyzer (YSI 2300). For analyses of solvents and acids in the fermentation broth, samples were centrifuged and filtered with a 0.2 μm syringe filter (if necessary). In order to accurately measure concentrations of organic acids, samples were diluted and acidified with buffer containing 10 mL/L phosphoric acid to a final volume of at least 600 μL in the vial. The acidified samples were analyzed with a GC-2014 Shimadzu gas chromatograph (GC) (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (0.25 μm film thickness and 0.25 mm ID, Stabilwax-DA). The GC was operated at an injection temperature of
200 °C with 1 μL of the acidified sample injected by the AOC-20i Shimadzu auto injector. Column temperature was held at 80 °C for 3 min, increased to 150 °C at the rate of 30 °C/min, and held at 150 °C for 3.7 min.

7.3 Results and Discussion

After the pilot plant was set up, steam was introduced in the pipes and reactors for sterilization. After the 150-L FBB was filled with sterile P2 medium with 2 g/L butyric acid and 50 g/L glucose and made anaerobic by purging N₂ into the reactor, batch fermentation was performed. Samples were aseptically taken from the top of the vessel during the fermentation period. The changes in substrate and product concentrations in the FBB over time are shown in Figure 7.3. Solventogenesis started in ~10 hours after inoculation with decreasing butyric acid in the bioreactor. About 43.5 g/L glucose and 1 g/L butyric acid were consumed within three days, and the butanol concentration reached 8.7 g/L with the yield of 0.19 g/g substrate consumed. A total of ~13.8 g/L of solvents was produced, giving a total solvents yield of 0.30 g/g.

Another batch fermentation was performed after the reactor was cleaned and packed with new fibrous bed packings. The reactor was filled with P2 medium containing 42 g/L glucose and 5 g/L butyric acid. Ammonium hydroxide was pumped into the reactor for pH adjustment. As shown in Figure 7.4, acidogenesis occurred initially with steady production of butyric acid and acetic acid. At ~40 hours, the fermentation switched to solventogenesis and produced solvents by utilizing both glucose and butyric acid at the same time. At the end of the fermentation, 9.4 g/L butanol were
produced with the productivity of 0.09 g/L·h and yield of 0.19 g/g substrate (glucose + butyric acid).

In the first batch, the process was operated at the pH value of ~4.6, while it was maintained at pH 5.0 with the addition of ammonium hydroxide in the second batch. Although the results from the two batches were similar, butanol productivity at the higher pH was slightly higher. It is noted that in the second batch, the feed butyric acid was also utilized to produce butanol by the bacteria. With 5 g/L of butyric acid in the medium, the ratio of butanol to total produced solvents was 0.69, while it was 0.60 with 2 g/L butyric acid in the medium.

Table 7.3 summarizes and compares the production of solvents and acids from the 150-liter FBB and free-cell batch fermentation in a 1-liter fermentor (see Chapter 4). Although the final butanol titers and yields were lower in the two FBB batches than in the free-cell fermentation, the results demonstrated the feasibility of producing butanol in the 150-liter FBB. Further work should be conducted in the continuous fermentation mode in order to have a direct comparison to the bench-scale FBB fermentation reported in Chapters 4 and 5.

7.4 Conclusion

Two batches of ABE fermentation were carried out in a 150-L FBB by Clostridium beijerinckii ATCC 55025. Similar results were obtained from two separate batches of ABE fermentation in the FBB. Final butanol titers in the two batches were similar and comparable to that from batch fermentation at the bench scale. In addition, C.
beijerinckii ATCC 55025 immobilized in the FBB was able to utilize butyric acid in the medium to produce butanol and gave a higher ratio of butanol over total produced solvents. More studies are needed to optimize the fermentation condition for the pilot-scale FBB and to operate it at a continuous mode for better assessment of its feasibility and scalability for industrial operation.
7.5 Reference


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<tr>
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<td>Glucose</td>
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<td>Yeast extract</td>
<td>Nitrogen source</td>
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<td>480</td>
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<td>1</td>
<td>4000 g Glucose</td>
<td>dissolved in 16 L distilled water and steam sterilized</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>4000 g Glucose</td>
<td>dissolved in 16 L distilled water and steam sterilized</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>16 L of distilled water were sterilized</td>
</tr>
<tr>
<td>5</td>
<td>480 g Yeast extract</td>
<td>dissolved in 16 L distilled water and steam sterilized</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>16 L of distilled water were sterilized</td>
</tr>
<tr>
<td>7</td>
<td>Phosphate buffer</td>
<td>dissolved in 16 L distilled water and steam sterilized</td>
</tr>
<tr>
<td>8</td>
<td>Minerals</td>
<td>dissolved in distilled water and filter-sterilized; transferred to 16 L of steam-sterilized water after cool down</td>
</tr>
<tr>
<td>9</td>
<td>Vitamins</td>
<td>dissolved in distilled water and filter-sterilized; transferred to 16 L of steam-sterilized water after cool down</td>
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<tr>
<td>10</td>
<td>None</td>
<td>16 L of distilled water were sterilized</td>
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Table 7.1 Composition and preparation of 160 L of P2 medium in 20-L preparation tank.
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<th>Model number</th>
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</tr>
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<td>STFT-1500-120</td>
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<tr>
<td>Water pump</td>
<td>March Pumps</td>
<td>809-HS</td>
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<tr>
<td>Ammonium gas pump</td>
<td>Masterflex</td>
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<td>Medium pump</td>
<td>Fluid Metering Inc.</td>
<td>QDX</td>
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Table 7.2 List of equipment used in the construction of pilot scale fibrous-bed bioreactor
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<th>Yield (g/g)</th>
<th>Productivity (g/L-h)</th>
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<tr>
<td><strong>Acetone</strong></td>
<td>5.52 ± 0.14</td>
<td>0.097 ± 0.002</td>
<td>0.02 ± 0.002</td>
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<td><strong>Butanol</strong></td>
<td>12.84 ± 0.23</td>
<td>0.23 ± 0.01</td>
<td>0.05 ± 0.004</td>
</tr>
<tr>
<td><strong>Ethanol</strong></td>
<td>0.87 ± 0.02</td>
<td>0.015 ± 0.0002</td>
<td>0.003 ± 0.0002</td>
</tr>
<tr>
<td><strong>Acetic acid</strong></td>
<td>1.83 ± 0.076</td>
<td>0.032 ± 0.002</td>
<td>0.007 ± 0.001</td>
</tr>
<tr>
<td><strong>Butyric acid</strong></td>
<td>2.35 ± 0.11</td>
<td>0.04 ± 0.001</td>
<td>0.009 ± 0.0005</td>
</tr>
<tr>
<td><strong>ABE</strong></td>
<td>19.23 ± 0.38</td>
<td>0.34 ± 0.01</td>
<td>0.07 ± 0.01</td>
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<tr>
<td>Titer (g/L)</td>
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<td>0.068</td>
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<td>Titer (g/L)</td>
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<tr>
<td>Yield (g/g)</td>
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<td>0.010</td>
</tr>
<tr>
<td>Productivity (g/L-h)</td>
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<td>0.005</td>
</tr>
<tr>
<td>Titer (g/L)</td>
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<tr>
<td>Yield (g/g)</td>
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</tr>
<tr>
<td>Productivity (g/L-h)</td>
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<td>0.035</td>
</tr>
<tr>
<td>Titer (g/L)</td>
<td>0.30</td>
<td>2.38</td>
</tr>
<tr>
<td>Yield (g/g)</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Productivity (g/L-h)</td>
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<td>N/A</td>
</tr>
<tr>
<td>Titer (g/L)</td>
<td>14.74</td>
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<tr>
<td>Yield (g/g)</td>
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</tr>
<tr>
<td>Productivity (g/L-h)</td>
<td>0.124</td>
<td>0.118</td>
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Table 7.3 Comparison of fermentation kinetics of 1-L free-cell reactor and 150-L FBB.
Figure 7.1 (a) Schematic diagram and (b) photograph of the 150-liter fibrous bed bioreactor used in this study.
Figure 7.2 (a) Schematic flow diagram and (b) photograph of the fermentation pilot plant including a 20-L fermentor, a 220-L feed tank, a 150-L FBB, and a 200-L product tank.
Figure 7.3 Batch ABE fermentation kinetics in 150-liter bioreactor in P2 medium with 50 g/L glucose and 2 g/L butyric acid at pH 4.6.
Figure 7.4 Batch ABE fermentation kinetics in 150-liter bioreactor in P2 medium with 50 g/L glucose and 5 g/L butyric acid at pH 5.0.
Chapter 8: Conclusions and Recommendations

8.1 Conclusions

This study demonstrated the advantages of continuous butanol production in the fibrous bed bioreactor (FBB) and examined the feasibility of butanol production by metabolic engineered hosts. A higher productivity and higher final concentration of butanol can be obtained from continuous fermentation in the FBB. The important results and conclusions obtained in this study are summarized below.

8.1.1 Stoichiometric analysis of continuous ABE fermentation

Butanol production is benefited from the supplementation of butyric acid in the medium. With more butyrate uptaken by cells, more butyrate phosphate can be produced for butanol production. NADH balance and ATP sufficiency are two key factors affecting butanol production from glucose. With low biomass production, NADH balance is more important in determining the carbon flow for the maximum butanol yield, while ATP sufficiency plays a critical role in controlling butanol production when there is active biomass production in the fermentation.
8.1.2 Continuous butanol production from ABE fermentation

Butanol production benefited from the addition of butyric acid in the feed medium during continuous fermentation. Butyric acid at an appropriate concentration in the medium can stabilize butanol production and improve butanol yield by reducing bacterial growth in the fermentation. A higher ratio of butanol to total solvents produced in ABE fermentation can also be obtained with a proper concentration of butyric acid in the feed medium. In the continuous process of ABE fermentation by *C. beijerinckii* ATCC 55025 in the FBB at a high dilution rate of 1.88 h\(^{-1}\), a high butanol productivity of 17.29 g/L·h was achieved with a butanol titer of 9.18 g/L and yield of 0.22 g/g. At the high dilution rate, the inhibition effect of toxic solvents on ABE fermentation is alleviated because of the continuous removal of the fermentation products.

For the continuous fermentation process with a newly developed mutant strain *C. beijerinckii* JB200 operated at a lower dilution rate of ~1.05 h\(^{-1}\), the final butanol titer increased to ~12 g/L and the process was stable without encountering any contamination or degeneration over the 25 days continuous operation period. *C. beijerinckii* JB200 is able to tolerate higher concentrations of glucose (>90 g/L) and butyrate (>8 g/L), and can utilize both glucose and butyric acid for butanol production, and is thus an ideal candidate for industrial ABE fermentation.

8.1.3 Fibrous Bed Bioreactor (FBB)

A high cell density (>100 g dried weight/L) with at least 70% cell viability was achieved in continuous FBB fermentation. ABE fermentation in the FBB was stable over
600 hours of continuous operation. A new strain with high solvent tolerance, *C. beijerinckii* JB200, was isolated from the FBB. The superiority of *C. beijerinckii* JB200 has been demonstrated in both serum bottles and continuous FBB. Stable butanol production in the medium with different concentrations of butyric acid was observed. In continuous FBB, *C. beijerinckii* JB200 was able to tolerate higher concentrations of glucose and utilize more glucose than *C. beijerinckii* ATCC 55025 did.

8.1.4 Butanol production by metabolically engineered *C. tyrobutiricum*

Metabolically engineered *C. tyrobutyricum* with a partially knockout acetic acid biosynthesis pathway was cloned to overexpress a heterologous *aad* gene that enabled the host mutant to produce butanol from glucose and xylose. Higher butanol yield (0.172 g/g xylose) and titer (7.12 g/L) were obtained with xylose as the carbon source due to slower cell growth allowing more butyryl-CoA to be converted to butanol.

8.1.5 Scale up of ABE fermentation in FBB

ABE fermentation was successfully carried out with a pilot-scale fibrous bed bioreactor with a working volume of 150-L. Butanol production in the pilot-scale FBB at a batch fermentation mode was comparable to that from small bench-scale batch fermentation under otherwise similar conditions.
8.2 Recommendations

While attempts to develop an economical process for butanol production have made significant progress, there are still many challenges to overcome. Although the metabolism and the gene regulation system in the solvent-producing clostridia are largely unknown, it is possible to study the ABE fermentation at the molecular level and to further improve the fermentation process. The following lists several suggestions for future work.

8.2.1 Process development of ABE fermentation

At the high dilution rate of 1.88 h\(^{-1}\), the bacteria did not consume all glucose present in the feed medium. Twenty percents of feed glucose still remained in the fermentation broth at the end of the operation. The residual glucose not only reduced the overall process yield but also could cause problem in the downstream process for product recovery. The fermentation process can be further optimized in order for the bacteria to fully utilize all glucose in the medium. Since pH was also an important factor in ABE fermentation, an online pH monitor and controller could also be incorporated in the process to maintain a steady fermentation pH at the optimal level of ~5.0.

With a steady output of 12 g/L butanol from the process carried out with \textit{C. beijerinckii} JB200, a process for in situ product recovery could be considered and incorporated into the continuous fermentation process. By incorporating a product recovery process, such as gas stripping, into the fermentation process, residual sugars in the fermentation broth can be recycled back to the fermentation process, allowing more
complete utilization of sugar substrate for a better substrate economy. More studies could also be done on the medium formulation for enhanced butanol production from ABE fermentation. For example, the buffering salts in the current medium were not able to maintain the pH of the fermentation broth within a stable, desirable range conducive for solventogenesis without acid crash. Inexpensive nitrogen sources and additional nutrients should be evaluated for their potential benefits of increasing fermentation productivity and reducing raw material costs.

8.2.2 Butanol production by *C. tyrobutyricum*

*C. tyrobutyricum* mutants used for butanol production were still able to produce acetic acid even though key genes in the acetate pathway have been disrupted. In order to produce more butanol with *C. tyrobutyricum*, it may be necessary to completely block the acetate biosynthesis in the cells. It was found that more butanol was produced with xylose as the carbon source. In other words, butanol production was preferred with slower cell growth. Butanol production can be further enhanced by optimizing the growth medium that gives slower cell growth but faster butanol production. Fed-batch fermentation has the potential to increase butanol titer and yield, and thus should be investigated as well.

8.2.3 Scale up for butanol production

More studies are needed to optimize the fermentation condition for the pilot-scale FBB fermentation. A more extensive fermentation pilot plant will be needed for better
assessment of large-scale butanol fermentation. Different modes of fermentation process should be carried out in the pilot-scale FBB to explore its full potential in an economical butanol production process. Finally, alternative and inexpensive feedstocks, including food processing wastes and agricultural residues such as corn fiber, sugarcane bagasse and wheat straw should be investigated. \textit{C. beijerinckii} and \textit{C. tyrobutyricum} can use both glucose and xylose present in lignocellulosic biomass, the most abundant organic resource on the earth that is nowadays considered as the second generation feedstock for biofuels production.
Bibliography


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Appendix A. Medium Compositions

A.1 Reinforced clostridial medium (RCM) for clostridia

The medium (Difco) contained (per liter): 5.0 g pancreatic digest of casein, 5.0 g proteose peptone No. 3, 10.0 g beef extract, 3.0 g yeast extract, 5.0 g dextrose, 5.0 g sodium chloride, 1.0 g soluble starch, 0.5 g cysteine hydrochloride, 3.0 g sodium acetate, 0.5 g Agar.

A.2 P2 medium for *Clostridium beijerinckii*

P2 medium was composed of the following separately prepared solutions (per liter):

(a) Various amount of glucose in 790 mL distilled water

(b) 0.5 g of K$_2$HPO$_4$, 0.5 g of KH$_2$PO$_4$, 2.2 g of CH$_3$COONH$_4$, 0.92 g yeast extract in 100 mL distilled water.

(c) 2.0 g of MgSO$_4$·7H$_2$O, 0.1 g of MnSO$_4$·H$_2$O, 0.1 g of NaCl, 0.1 g of FeSO$_4$·7H$_2$O in 100 mL distilled water.

(d) 100 mg of *p*-aminobenzoic acid, 100 mg of thiamine, 1 mg of biotin in 100 mL distilled water.
Solutions I and II were autoclaved separately and cooled to room temperature. Solutions III and Solution IV were filter sterilized with a 0.2 μm pore size filter. Solution I, II, III and 10 mL of solution IV were mixed aseptically.

A.3 Simplified *Clostridium* growth medium (Simplified CGM)

The medium contained yeast extract (5 g/l), Trypton (5 g/l) and the following two solutions (per liter):

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

b) 40 ml of mineral #2 solution consisting of (per liter): 6 g KH$_2$PO$_4$, 6 g (NH$_4$)$_2$SO$_4$, 12 g NaCl, 2.5 g MgSO$_4$·7H$_2$O, 0.16 g CaCl$_2$·2H$_2$O, 0.125 g NiCl$_2$ and 0.1 g FeSO$_4$ (In order to prevent Fe$^{2+}$ oxidation and precipitation during autoclaving, the solution mineral #2 was filter-sterilized).

A.4 Modified P2 medium for *C. beijerinckii* and *C. acetobutylicum*

The modified P2 medium (per liter) was composed of the following separately prepared solutions: 0.5 g KH$_2$PO$_4$ and 0.5 g K$_2$HPO$_4$-40 mL distilled water (solution I), 1 g yeast extract and 0.5 g tryptone-40 mL distilled water (solution II), 2 g MgSO$_4$·7H$_2$O, 0.1 g of MnSO$_4$·H$_2$O, 0.1 g of NaCl, and 0.1 g of FeSO$_4$·7H$_2$O in 5 mL distilled water (solution III), 100 mg of p-aminobenzoic acid, 100 mg of thiamine, and 1 mg of biotin in 50 mL distilled water (solution IV). Solution I and II were autoclaved separately and mixed into sterile glucose solution subsequently. Solutions III and IV were filter-sterilized with 0.2 μm pore size filter and aseptically transferred to the medium.
Appendix B. P2 Medium Optimization

In order to screen for suitable supplement in growth medium, different proteinacious nitrogen sources (yeast extract, beef extract, and peptone), varied concentration of oleic acid (0, 10, 20, 40 mg/L) and pH values (4~7) in P2 medium sealed in air-tight serum bottles were used for desirability evaluation. In Figure B.2 (a), medium supplemented with beef extract promote butanol production by ~30% compared to that supplemented with yeast extract, while addition of peptone in medium did not help butanol production. Oleic oil, where unsaturated fatty acids are rich in, was reported to help enhance butanol production by increasing butanol tolerance of microorganism (Jones and Woods, 1986). However, while supplement of oleic oil at low concentration in the medium did help faster bacterial growth, it did not promote butanol production. The effect of different pH in the medium was inconclusive and needed more studies.

In order to be more efficient and effective in process development, valid experimental designs for enhanced butanol production are necessary. Table B.1 shows a fractional factorial experimental design with one duplicate for medium ingredient screening created by JMP. Ingredients were transferred into 10-mL serum tubes according to the created table and purged with N₂ before autoclave. The media were then inoculated with primary culture of *C. beijerinckii* ATCC 55025. The microbial growth is monitored by measuring optical density using spectrophotometer at 600 nm. The
dependent variables for analyses were specific growth rate (h\(^{-1}\)) and final concentration of butanol.

The analyzed results for specific growth rate and for butanol production are shown in Figure B.3 (a) and (b), respectively. The interactions between variables are considered significant in the process when t ratio is over two (probability > |t| is lower than 0.05). According to the result, addition of tryptone in growth medium is significant for improve butanol production and increase specific growth rate, while urea significantly decrease microbial growth in the medium without adverse effect on butanol production. Addition of glycerol alone in the medium may inhibit microbial growth of \textit{C. beijerinckii} ATCC 55025.

However, addition of both ammonium chloride and glycerol in the medium can positively affect the specific growth rate.

With the preliminary screening data, more ingredients commonly used for microbial growth can be used to screen for other beneficial supplement in the medium including minerals, vitamins, and some low cost agricultural biomass for low cost medium development.
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Table B.1 Fractional factorial experimental design of medium ingredient screening for *C. beijerinckii* ATCC 55025.
Figure B.1 (a) Specific growth rate and (b) butanol production in serum bottle supplemented with different nitrogen sources (yeast extract, beef extract, and peptone), different concentration of oleic oil (0, 10, 20, and 40 mg/L), and under different pH (4, 5, 6, and 7).
Figure B.2 Sorted parameter estimates for responses of (a) specific growth rate, and (b) butanol production.
Appendix C. Analytical Methods

C.1  Cell concentration

Cell concentrations were described as values of optical density (OD) or biomass dry weight. The OD value was measured with a spectrophotometer (Shimadzu, UV-16-1) and the value was proportional to the cell dry weight when OD was less than 0.7 (Figure C.1). Cell concentrations of five strains were determined, including Clostridium beijerinckii ATCC 55025, Clostridium acetobutylicum ATCC 824, Clostridium beijerinckii BA101, Clostridium beijerinckii NCIMB 8052, and Clostridium beijerinckii JB 200. A known volume of cell from fermentation broth were centrifuged, washed by resuspension with water, and centrifuged again. The pellet was dried by freeze-dryer overnight to obtain the dry cell weight. Table C.1 summarizes the correlation between OD value and cell dry weight for the five strains in P2 medium.

C.2  Cell viability

Cell viability was determined using the method of Glenner (1977) with some modifications. One milliliter 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 to the cell pellet. The cell pellet was resuspended in TTC solution by vortexing at the maximum speed, and then incubated in the dark at room temperature for 30 min for color development. The cells were collected by centrifugation
at 16,000 rpm for 10 min and 1 ml of methanol was added to the cell pellet 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 (Shimazu, Columbia, MD, UV-16-1) against a control of boiled cell culture before adding TTC solution. The viability of cells from the exponential phase was determined to be 100%. Sample viability can be obtained through the following equation:

\[
\text{sample viability} = \frac{(\text{OD}_{485\text{nm}}/\text{cell concentration})_{\text{sample}}}{(\text{OD}_{485\text{nm}}/\text{cell concentration})_{\text{standard}}}
\]

C.3 Gas Chromatography

For analyses of solvents and acids in the fermentation broth, samples were centrifuged and filtered with a 0.2 µm syringe filter (if necessary). In order to accurately measure concentrations of organic acids, samples were diluted and acidified with buffer containing 10 mL/L phosphoric acid to a final volume of at least 600 µL in the vial. The acidified samples were analyzed with a GC-2014 Shimadzu gas chromatograph (GC) (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (0.25 µm film thickness and 0.25 mm ID, Stabilwax-DA). The GC was operated at an injection temperature of 200 ºC with 1 µL of the acidified sample injected by the AOC-20i Shimadzu auto injector. Column temperature was held at 80 ºC for 3 min, raised to 150 ºC at increasing rate of 30 ºC/min, and held at 150 ºC for 3.7 min. Figure C.3 is the GC chromatogram with 1 g/L acetone, ethanol, butanol, acetic
acid, and butyric acid. Figure C.4 and C.5 show the typical chromatograms of samples from acidogenesis and solventogenesis in ABE fermentation.

C.4 High Performance Liquid Chromatography
The concentrations of carbon sources (glucose and xylose), acids (acetic acid and butyric acids), and solvents (acetone, ethanol, and butanol) in samples were analyzed by high performance liquid chromatography (HPLC) with an organic acid column (Bio-Rad, HPX-87; ion exclusion organic acid column; 300 mm x 7.8mm). Samples were centrifuged at top speed for 5 minutes and diluted 10 times with distilled water. HPLC was run at 45°C using 0.01 N H₂SO₄ as the eluant at a flow rate of 0.6 ml/min. Fifteen μL of each diluted cell-free sample were injected by an automatic injector (SIL-10Ai) and the running time was 38 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 HPLC chromatogram of the standard is shown in Figure C.6. Figure C.7 and C.8, are the typical chromatograms for samples in acidogenesis, solventogenesis in ABE fermentation. Figure C.9 shows HPLC chromatogram for fermentation carried out by metabolic engineered C. tyroputryicum for butanol production.
Table C.1 Correlation between optical density at 600 nm and cell dry weight (g/L) for five butanol producing strains.

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Figure C.1 Cell dry-weight determination for 5 butanol producing strains.
Figure C.2 100% viability determination for 5 butanol producing strains.

\[ y = 0.3735x - 0.0435 \]

\[ R^2 = 0.9995 \]
Figure C.3 The GC chromatogram for standard containing acetone, ethanol, butanol, acetic acid and butyric acid (1 g/L each).
Figure C.4 GC chromatogram of sample at acidogenesis with 10 fold dilution in ABE fermentation with 0.3 g/L acetone, 0.1 g/L ethanol, 1.1 g/L butanol, 0.9 g/L acetic acid and 3.5 g/L butyric acid.
Figure C.5 GC chromatogram of sample at solventogenesis with 10 fold dilution in ABE fermentation with 4.2 g/L acetone, 0.5 g/L ethanol, 11.7 g/L butanol, 3.3 g/L acetic acid and 2.9 g/L butyric acid.
Figure C.6 GC chromatogram of sample with 10 fold dilution from butanol production by *C. tyrobutyricum* from glucose and xylose with 1.26 g/L ethanol, 6.01 g/L butanol, 5.26 g/L acetic acid and 6.00 g/L butyric acid.
Figure C.7 HPLC chromatogram for standard containing glucose, xylose, acetic acid, ethanol, acetone, butyric acid and butanol (2 g/L each).
Figure C.8 Typical HPLC chromatogram of sample at acidogenesis with 10 fold dilution in ABE fermentation with 0.5 g/L acetone, 1.7 g/L butanol, 1.7 g/L acetic acid and 3.6 g/L butyric acid.
Figure C.9 HPLC chromatogram of sample at solventogenesis with 10 fold dilution in ABE fermentation with 3.8 g/L acetone, 0.6 g/L ethanol, 10.55 g/L butanol, 3.8 g/L acetic acid and 3.3 g/L butyric acid.
Figure C.10 HPLC chromatogram of sample from butanol production with 10 fold dilution by *C. tyrobutyricum* from glucose and xylose with 12.4 g/L glucose, 23.7 g/L xylose, 1.8 g/L ethanol, 2.1 g/L butanol, 3.9 g/L acetic acid and 2.6 g/L butyric acid.
Appendix D. Plasmid Construction in Mutants of C. tyrobutyricum

The plasmid maps of pMTL-thladhE2 and pM2-thladhE2 are shown in Figure D.1 and D.2. The plasmid pMTL-thladhE2 was constructed from pMTL007 with the size of 10.9 kilobase-pair. The key elements in pMTL-thladhE2 include genes for conjugation, TraJ and oriT, chloramphenicol/thiamphenicol resistant gene, catP, pCB102 replicon from C. butyricum, ORFH, E. coli replicon, ColE1, aldehyde/alcohol dehydrogenase 2 from C. acetobutylicum, adhE2, and thiolase promoter from C. tyrobutyricum, Pthl. The plasmid pM2-thladhE2 was constructed from pMTL82151 with the size of 7.9 kilobase-pair. The key components in pM2-thladhE2 are similar to those in pMTL-thladhE2. In pM2-thladhE2, the E. coli replicon, ORFH, was replaced with a replicon from C. botulinum, pBP1.
Figure D.1 Structure of pMTL-thladhE2. (TraJ and oriT: genes for conjugation; catP: chloramphenicol/thiamphenicol resistant gene; ORFH: pCB102 replicon from C. butyricum; ColE1: E. coli replicon; adhE2: aldehyde/alcohol dehydrogenase 2 from C. acetobutylicum; Pthl: thiolase promoter from C. tyrobutyricum)
Figure D.2 Structure of pM2-thladhE2. (TraJ and oriT: genes for conjugation; catP: chloramphenicol/thiamphenicol resistant gene; pBP1: replicon from C. botulinum; ColE1: E. coli replicon; adhE2: aldehyde/alcohol dehydrogenase 2 from C. acetobutylicum; Pthl: thiolase promoter from C. tyrobutyricum)