REALIZATION OF DIRECT BIOLOGICAL BUTANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS BY WILD-TYPE *CLOSTRIDIUM*

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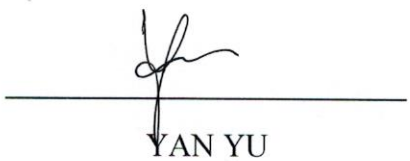
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



YAN YU

September 2016

I dedicate this thesis to

My grandparents

Yan Shenyan and Li Roufu

And my parents

Yan Xiaoshi and Yu Yuehui

For their love and encouragement.

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SUMMARY

Butanol is a four-carbon saturated alcohol that can be utilized not only as a chemical intermediate but also a fuel alternative due to its sufficient physicochemical similarities to gasoline. It can be produced through anaerobic fermentation, which is considered to be sustainable and carbon neutral. However, challenges still remain in achieving industrial-scale, economically friendly biological butanol production.

The development of biological butanol fermentation is impeded by low butanol production. Anaerobic acetone-butanol-ethanol (ABE) fermentation is a cofactor-dependent system, the availability of reducing cofactors such as NADH and NADPH, plays an important role in microbial catabolism and is thus expected to affect metabolic distribution. Results in this thesis showed that the addition of nicotinic acid, a precursor metabolite, to the fermentation medium, led to higher butanol production (18.7 g/L) by *Clostridium* species strain BOH3 from glucose, by increasing the availability of NADH and NADPH.

In addition to low butanol production, high costs associated with acquisition and pre-processing of raw materials for the fermentation process also affect the economic viability of biobutanol fermentation. Utilization of lignocellulosic biomass offers a promising option to reduce the cost of biological butanol production. Efficient conversion of lignocellulose-derived sugars to butanol is retarded by inherently inefficient pentose metabolism and glucose induced carbon catabolite repression (CCR). Pentoses (xylose and arabinose) are the second most abundant reducing sugar in lignocellulosic hydrolysate. Unless both pentose and hexose are utilized efficiently,

converting lignocellulosic biomass into solvents is unfavourable from an economic viewpoint, as yields are limited. *Clostridium* sp. strain MF28 shows the capability of co-fermenting xylose, arabinose and glucose to produce 14.40 ± 0.30 g/L butanol within 48 h.

Utilization of lignocellulosic hydrolysates as fermentation substrate is impeded by the lignocellulose-derived inhibitors generated during lignocellulose pretreatment and hydrolysis. These inhibitors will inhibit cell growth and the further fermentation process. Toxicity study on known solventogenic bacteria revealed that combination with furfural can cause severer synergistic effect on cell growth and solvent production. In this study, *Clostridium* sp. strain BOH3 produced 5.15 ± 0.52 g/L butanol from un-detoxified horticultural waste hydrolysate owing to its high resistance to the lignocellulose-derived inhibitors and the capability of converting furfural to its less toxic alcohol – furfuryl alcohol when furfural concentration is lower than 60 mmol/L.

Besides producing butanol from monosaccharides derived from lignocellulosic biomass and hydrolysates, direct conversion of lignocellulosic biomass such as xylan, which constitutes the principal hemicellulosic component of plant wastes and represents one third of all renewable organic carbon available on earth, is always desirable. *Clostridium* sp. strain G117 was able to generate 1.24 ± 0.37 g/L butanol directly from xylan that had undergone no prior enzymatic hydrolysis. More importantly, butanol was the only solvent produced from xylan by strain G117, which may greatly simplify downstream separation process and improve the economic viability of biological butanol production.

In summary, this doctoral study reported three wild-type *Clostridium* strains – *Clostridium* sp. strain BOH3 (fermenting 75.2 g/L glucose to 18.7 g/L butanol, producing 5.15 ± 0.52 g/L butanol from un-detoxified horticultural waste hydrolysate), *Clostridium* sp. strain MF28 (producing butanol from simultaneous fermentation of glucose, xylose and arabinose), and *Clostridium* sp. strain G117 (xylan can be utilized as the sole carbon source to produce 1.24 ± 0.37 g/L butanol as the only solvent product). These strains possess different abilities to overcome obstacles in direct conversion of lignocellulosic biomass to biobutanol. The discovery of these three strains should have laid a foundation to the industrial-scale cost-effective biological butanol production.

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ABBREVIATIONS

ABE	acetone-butanol-ethanol
qPCR	real-time polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
NA	nicotinic acid
adc	acetoacetate decarboxylase
adhE	acetaldehyde dehydrogenase
ctfAB	CoA-transferase
xylT	xylose transporter
xylA	xylose isomerase
xylB	xylulokinase
xylR	xylose repressor
PTS	glucose-specific phosphotransferase system
glcG	PTS permease
CCR	carbon catabolite repression
HPr	histone-containing protein
CcpA	catabolite control protein A
HMF	hydroxymethylfurfural
4-HBA	4-hydroxybenzoic acid
SA	syringaldehyde
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adnine dinucleotide phosphate hydrogen
NAD+	nicotinamide adnine dinucleotide

NADP+	nicotinamide adnine dinucleotide phosphate
buk	butyrate kinase
FBB	fibrous bed bioreactor
DCW	dry cell weight
GC	gas chromatography
FID	flame ionization detector
TCD	thermal conductivity detector
RID	refractive index detector
HPLC	high-performance liquid chromatography
μ_{cell}	specific cell growth rate
μ_{but}	specific butanol production rate
araA	L-arabinose transporter
araB	L-arabinose isomerase
XOS	xylo-oligosaccharides
pepT	peptidase T
GXA	glucose xylose arabinose
RSC	Residual sugar concentration

BIBLIOGRAPHY

Ms. Yan Yu was born in June 1988 in Xiangtan City, Hunan Province, People's Republic of China. She received her Bachelor of Engineering degree from Department of Environmental Science and Engineering (now School of Environment), Tsinghua Univeristy in July 2010. After that, she joined Division of Environmental Science and Engineering (now Department of Civil and Environmental Engineering), National University of Singapore for doctoral studies under the supervision of A/P He Jianzhong.

Researches described herein have been published or is under review in the following journals:

[1] **Yan Y**, Basu A, Li T, He J. Direct conversion of xylan to butanol by a wild-type *Clostridium* species strain G117. Published online: 15 Feb. 2016. Biotechnol. Bioeng.

[2] Li T*****, **Yan Y***, He J. 2015. Enhanced direct fermentation of cassava to butanol by *Clostridium* species strain BOH3 in cofactor-mediated medium. Biotechnol. Biofuels. 8: 12. ***** Contributed euqlly.

[3] Li T, **Yan Y**, He J. 2014. Reducing cofactors contribute to the increase of butanol production by a wild-type *Clostridium* sp. strain BOH3. Bioresour. Technol. 155:220-228.

[4] **Yan Y**, He J. *Clostridium* species strain BOH3 tolerates inhibitors present in horticultural waste hydrolysate. In preparation.

CHAPTER 1

Introduction

Biological butanol production is required due to the impending energy crunch and increasing attention towards environmental problems. The use of lignocellulosic biomass as a carbon source could greatly improve the economic viability of biobutanol production.

1.1 Background

Fossil fuels, which have been used extensively for the production of energy and chemicals, are formed by natural processes and include coal, natural gas and petroleum. The primary use of fossil fuels is for transportation, which also results in the largest proportion of greenhouse gas (GHG) emissions (Demirbas 2007). In 2006, more than 13 million barrels, representing 66% of total crude oil usage, was used for transportation each day in the United States alone. Additionally, the Energy Information Administration projects that use of transportation fuel will increase by up to 30% by the end of 2030 (Wu et al. 2008). The unsteady supply of fossil fuels often results in highly fluctuating market prices, and growing concerns of the negative impacts of fossil fuels on the environment have put great pressure on society to develop renewable fuel alternatives that are more environmentally friendly. The possibility of developing carbon neutral (no net GHG emissions) alternative fuels that can replace fossil fuels as energy sources is particularly attractive (Ranjan and Moholkar 2012). Biofuels create energy through microbial carbon fixation processes and are being considered to have the potential of counterbalancing CO_2 output, since all CO_2 released during combustion stems from organic material which could eventually be recaptured by bacteria and plants (Dürre 2007).

Several liquid fuel alternatives have been investigated over the past twenty years, including biodiesel, bioethanol and biobutanol. Compared to bioethanol and biodiesel, biobutanol is a more favourable fuel alternative since it has higher energy density and has sufficiently similar physical and chemical properties to gasoline to act as a gasoline replacement (Table 1.1) (Gu et al. 2011). Several studies have described the distinct merits of butanol over other biofuels (Alasfour 1997; Dürre 2007; Ranjan and Moholkar 2012). Generally, these are:

1) It has sufficiently similar characteristics to gasoline to be used as a direct replacement of gasoline or as a fuel additive without requiring engine modification and/or substitution.
2) It is easy to transport because of its low vapor pressure and its low corrosivity, which can extend the service life of pipelines through which it is transported.
3) It has a comparable heat of vaporization (0.43MJ/kg) to that of gasoline (0.36MJ/kg) and much lower than that of ethanol (0.92MJ/kg), making it less susceptible to cold weather issues associated with ethanol or methanol blended gasoline.
4) It is not hygroscopic, which allows blending of gasoline at the refinery well ahead of storage and distribution.
5) It has low water solubility, leading to less possibility of groundwater contamination.

Table 1.1 Comparison of properties of representative fuels.

Properties	Gasoline	Butanol	Ethanol
Energy density (MJ/L)	32	29.2	19.6
Air : fuel ratio	14.6	11.2	9
Heat of vaporization (MJ/kg)	0.36	0.43	0.92
Research octane number	91-99	96	129
Motor octane number	81-89	78	102

In addition to its potential as a biofuel, butanol is also an important bulk chemical precursor. Half of all butanol production is used as butyl acrylate and methacrylate esters in latex surface coating, enamels and lacquers (Kirschner 2006). Furthermore, butanol is an excellent diluent for brake fluid formulations and is used as a solvent for the manufacture of antibiotics, vitamins and hormones (Lee et al. 2008).

Biological production of butanol has a long history that can be traced back to 1862, and at one time was carried out on a world-wide scale as a chemical precursor (Jones and Woods 1986). However, the high cost of fermentation substrates, low concentration of produced butanol and generation of low-value byproducts have limited the development of biobutanol as an economically viable energy source. The following sections provide an overview of the limitations of and improvements made in butanol production. A more detailed discussion of previous and on-going research studies is presented in chapter 2.

1.2 Problem statement

The anaerobic production of butanol, typically through acetone-butanol-ethanol (ABE) fermentation by a number of *Clostridium* species, has received

renewed interest in recent years (Ezeji et al. 2007b). Biological butanol production is still not economically competitive compared to petrochemical-derived production due to a few major drawbacks: low butanol titer, high cost of fermentation feedstocks, and byproducts production.

In typical batch ABE fermentation, the butanol concentration is usually below 13 g/L (Jones and Woods 1986; Woods 1995). The low product concentration is associated with fermentation conditions such as pH, reducing driving forces, expression of butanol producing associated genes and butanol toxicity. It is estimated that if the final butanol titer of ABE fermentation can be increased to 19 g/L, the product recovery cost would be reduced by half as compared to 13 g/L (Gu et al. 2011). Various efforts have been made to improve butanol production, such as chemical mutagenesis (Qureshi and Blaschek 2001), serial enrichment procedures (Quratulain et al. 1995), and regulation of butanol-producing associated genes (Yu et al. 2011). Although some success have been obtained in genetically engineered strains or mutants, the unstable butanol production indicates difficulties and complexities in transferring related pathways to a host bacteria due to the inherent instability or inefficient expression compared to a wild-type strain (Alsaker et al. 2010; Antoni et al. 2007).

Utilization of lignocellulosic biomass provides a promising option to reduce the production cost of biological butanol production. Lignocellulosic biomass is not readily available for *Clostridium* strains to consume due to its lignin-cellulose-hemicellulose crystalline structure (Baral and Shah 2014). Pretreatment and hydrolysis is required to break down this structure and release reducing sugars before fermentation. One of the obstacles to utilize

lignocellulosic biomass by most *Clostridium* species is inefficient co-utilization of pentose and hexose elements that exist in the hydrolysates (Yu et al. 2015a), such as xylose, arabinose and glucose - all three of which present in lignocellulosic hydrolysates (Lu et al. 2013). In the presence of glucose, which is the most favored fermentation substrate for most *Clostridium* species, the uptake of less preferred carbon sources (e.g. xylose and arabinose) will be repressed due to carbon catabolite repression (CCR) (Fond et al. 1986a; Fond et al. 1986b). CCR can thereby cause technical problems in pentose utilization in fermentation of lignocellulosic hydrolysates, resulting in incomplete substrate utilization and low solvent titer (Heluane et al. 2011; Ounine et al. 1985).

Other difficulties in using lignocellulosic biomass for fermentation by *Clostridium* species are associated with the inhibitory effects on cell growth and butanol production by inhibitors, such as furfural, hydroxymethylfurfural (HMF) and phenolic compounds, that are generated during the pretreatment and hydrolysis processes (Baral and Shah 2014).

Apart from using lignocellulose-derived sugars and lignocellulosic hydrolysates as feedstocks, direct conversion of lignocellulsic biomass, such as cellulose and xylan, to value-added products in a consolidated bioprocess (CBP) is widely considered as an alternative approach for cost-effective biological conversion of lignocellulosic biomass (Lynd et al. 2005; Olson et al. 2012). Thus far, efforts to realize the direct conversion of cellulose or xylan to value-added products in one step fermentation have been unremitting; the reported yield and titer are still low and no wild-type strains have been reported to have the capability of generating butanol directly from cellulose or

xylan (Cao et al. 2014; Higashide et al. 2011; Sizova et al. 2011). The primary purpose of this thesis is to address the problems associated with low butanol production and fermentation of lignocellulosic biomass as stated above. Specific aims are discussed in the next section.

1.3 Aims and scope of the thesis

The primary goal of this doctoral study is to realize direct biological butanol production from lignocellulosic biomass. Since different strain exhibits advantages with different feedstocks, three interesting strains are used in this doctoral study. Studies are conducted using lignocellulosic biomass associated substrates, from simple to complex – monosaccharide, lignocellulose-derived sugars, lignocellulosic hydrolysates and lignocellulosic biomass.

The specific objectives are as follows:

1. To investigate the role of cofactor availability in ABE fermentation in order to increase the biobutanol titer and yield during biotuanol production from glucose by wild-type *Clostridim* sp. strain BOH3.
2. To characterize the lack of CCR and the role of *ccpA* in transition between acidogenesis and solventogenesis in a newly isolated *Clostridium* sp. strain MF28 via simultaneous fermentation of different sugars (pentose and hexose) that existing in lignocellulosic hydrolysates.
3. To investigate the effects of inhibitors present in the biomass of hydrolysates on ABE fermentation by *Clostridium* sp. strain BOH3 and identify conditions that allow efficient conversion of lignocellulosic biomass hydrolysates to butanol.

4. To convert xylan into butanol directly using a wild type *Clostridium* sp. strain G117.

A brief introduction of the thesis structure is provided as follows.

Chapter 1 provides a brief introduction of this study and states the significance and scope. Chapter 2 gives a detailed literature review of biological butanol fermentation and the utilization of different materials as fermentative carbon sources. Chapter 3 introduces butanol production from glucose by *Clostridium* sp. strain BOH3 and discusses improvements to butanol production and yield. Chapter 4 presents simultaneous fermentation of pentose and hexose sugars derived from lignocellulosic biomass without obvious acidogenic and solventogenic phases by a newly isolated *Clostridium* sp. strain MF28 that exhibits no CCR. Chapter 5 discusses the toxicity effects of inhibitors present in lignocellulosic hydrolysates on *Clostridium* sp. strain BOH3 and investigates butanol production from horticulture waste hydrolysates by this strain. Chapter 6 demonstrates the direct butanol production from xylan by *Clostridium* sp. strain G117 with butanol as the sole solventogenic product. Chapter 7 is a summary and conclusion of this study and provides recommendations for future work.

CHAPTER 2

Literature Review

Butanol, also butyl alcohol, is a four-carbon primary alcohol with the molecular formula C_4H_9OH. A colorless, flammable liquid, it is considered toxic due to its irritant effect on mucous membranes and has narcotic effects in higher concentrations. It has four isomers including *n*-butanol (1-butanol), *sec*-butanol, isobutanol and *tert*-butanol. The butanol discussed in this thesis is *n*-butanol, which has a straight chain with the hydroxyl group at the terminal carbon, unless specified otherwise. Butanol is an important chemical intermediate and is considered to be a promising fuel alternative. In recent years, biological butanol production has received renewed interest due to fluctuating crude oil market prices and growing concerns over global warming (Lee et al. 2008). Development of environmentally friendly fuel alternatives like butanol will substantially help to extend crude oil availability and to reduce greenhouse gas emissions (Dürre 2007). This review is thus focused on characteristics of biological butanol production, factors that may affect the biobutanol production process and methods that have been employed to improve the economics of biobutanol production at industrial scales.

2.1 Chemical synthesis of butanol

Chemical synthesis of butanol is achieved mainly through three pathways: oxo synthesis, Reppe synthesis and crotonaldehyde hydrogenation (Figure 2.1). In oxo synthesis, aldehyde mixtures are obtained from propylene in the first step by adding CO and H_2 to the carbon-carbon double bond, an addition catalyzed by Co, Rh or Ru substituted hydrocarbonyls (Falbe 1970). Following this, butanol is produced from hydrogenation and different ratios of butanol isomers are obtained depending on the reaction conditions (pressure,

temperature) and catalysts used. In the Reppe process, butanol is synthesised directly from propylene by reaction with CO and H_2O in the presence of catalysts; however, this process has not been successfully commercialized due to the high cost of process technology (Bochman et al. 1999). In addition to these syntheses, butanol can also be synthesized from acetaldehyde using crotonaldehyde hydrogenation, which consists of sequential aldol condensation, dehydration and hydrogenation. The crotonaldehyde hydrogenation process was widely used for butanol production until a few decades ago and may receive renewed interests since it utilizes acetaldehyde - a petroleum independent source - for butanol synthesis. Acetaldehyde can be generated from ethanol, which in turn can be produced from biomass, potentially making crotonaldehyde hydrogenation a sustainable, economically friendly method for butanol synthesis (Swodenk 1983).

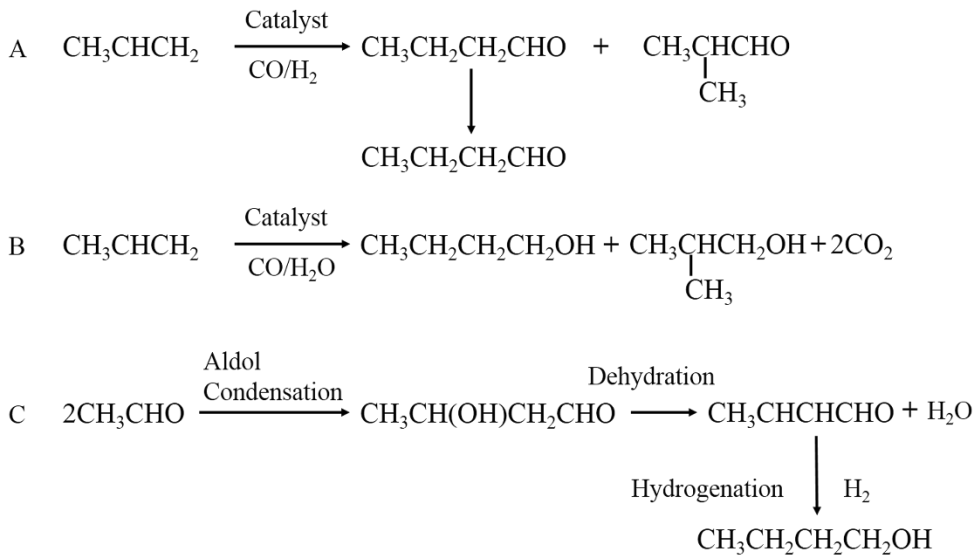


Figure 2.1 Chemical synthesis of butanol. A: Oxo synthesis; B: Reppe process; C: Crotonaldehyde hydrogenation.

2.2 General aspects of biological butanol production

2.2.1 History of biological butanol production

Biological butanol formation has a long history that can be traced back to 1861 (Jones and Woods 1986), when it was first reported by famous French scientist Louis Pasteur as a fermentation product of "Vibrion butyrique" (Pasteur 1862). The fermentation was found to be performed, not by a pure culture, but by a mixed culture, presumably containing *Clostridium butyricum* or *Clostridium acetobutylicum* (Sauer 1993). The first pure culture able to ferment glycerol, mannitol and sucrose to butanol, butyrate, carbon dioxide and hydrogen together with minor amounts of ethanol, acetate, lactate and propanediol was probably isolated by Albert Fitz in Strasbourg between 1876 and 1882 (Fitz 1876; Fitz 1877; Fitz 1878; Fitz 1882).

Conventional ABE fermentation was once the second largest biotechnological industry in the world, after ethanol (Dürre 2007). Around the turn of the twentieth century, a shortage of natural rubber inspired interest in butanol synthesis. This interest was due to the fact that butanol is a precursor of butadiene which can be used to synthesize artificial rubber through polymerization (Gabriel 1928b; Gabriel and Crawford 1930; Killeffer 1927). Around that time, Fernbach and Strange isolated a mixed culture that can ferment potatoes to butanol in 1911 and filed two English patent applications (Fernbach and Strange 1911a; Fernbach and Strange 1911b). After that, Weizmann isolated and characterized several cultures between the year of 1912 and 1914, one of which he called BY. This culture BY was later re-named *Clostridium acetobutylicum*. *Clostridium acetobutylicum* is able to produce a mixture of butanol and acetone from a variety of starchy materials

(Gabriel 1928b; McCoy et al. 1926). However, due to increasing production from plantations in Asia, the price of natural rubber started to decrease resulting in a decrease in biological butanol production.

However, the development of biological butanol production was dramatically altered attributable to acetone by the outbreak of World War I in 1914 (Dürre 2007). During the war the British army required a large amount of acetone for the preparation of cordite, a kind of smokeless powder used for the manufacture of munitions (Killeffer 1927). Before the war acetone had been chemically synthesized from calcium acetate, which was imported from Germany, Austria and the United States in small amounts (Gabriel 1928b). With the outbreak of war, most of these supply routes were cut off and the limited import from the United States was far from adequate to meet demand in the UK. This emergency prompted the British War Office to find alternative acetone suources, and the firm of Strange and Graham Ltd. which had previously produced butanol through anaerobic fermentation with the solventogenic *Clostridium acetobutylicum* attracted interest and began supplying acetone for the British government in 1915, storing the produced butanol (considered as a low-value byproduct at this time) in large vats.

After the war, all bioacetone production operations were closed. However, new value was found in butanol with the rapid development of the automobile industry (Gabriel and Crawford 1930; Killeffer 1927). Butanol and its ester butyl acetate are suitable solvents for nitrocellulose lacquer manufacture, a quick-drying lacquer which would give a good finish to car bodies.

Further expansion of biological butanol production was nevertheless impeded by the high cost of starchy fermentation substrates, which lower the competitiveness of biological production processes with synthetic production processes. Attempts were therefore made to isolate new microbial strains that could produce solvents from molasses, an alternative fermentation substrate that was cheaper than starch. At the end of 1935, acetone and butanol fermentation from molasses became operational using a *Clostridium saccharoacetobutylicum* isolated in the laboratory at Terre Haute (Hastings 1971).

In 1939, World War II broke out and the demand for acetone in munitions manufacture again rose. Successful efforts were made to develop continuous distillation and semicontinuous fermentation processes for further development of acetone and butanol production. However, the industry of acetone and butanol fermentation started to decline during the 1950s and had virtually stopped in the US and Britain by 1960, in South Africa by 1980s and in China by 2004 (Chiao and Sun 2007; Rose 1961; Zverlov et al. 2006). There are two main reasons for the decline of this industry: i) expansion of the petrochemical industry and low crude oil prices resulting in intense competition between fermentation and chemical process, and ii) the increase in cost of common fermentable substrates.

In 2005, high crude oil prices and growing concerns over global warming have renewed research interests in biological production of butanol, especially from renewable lignocellulosic biomass. BP and DuPont declared a joint effort in 2006 to develop biological butanol production process. BP

Biofuels, a subsidiary of BP, is also trying to commercialize butanol production by fermentation (Lee et al. 2008).

2.2.2 Microorganisms

Biological butanol production typically proceeds through an ABE fermentation pathway by a number of *Clostridium* species. *Clostridium* are rod-shaped, spore-forming Gram positive bacteria and typically strict anaerobes (Rogers 1986). Before the name *Clostridium* was introduced by Adam Prazmowski (Prazmowski 1880), many other names have been applied to these butanol-producing organisms in the past. After Louis Pasteur isolated "Vibrion butyrique" from his anaerobic cultivation in 1861 (Pasteur 1862), Albert Fitz isolated butanol- and butyrate- producing strains named as *Bacillus butylicus* (Fitz 1876; Fitz 1882). Around same time, cultures producing butanol and isopropanol were isolated by Dutch microbiologist and named as *Granulobacter butylicus* and *Granulobacter saccharobutyricum* (Beijerinck 1893). Official publication of the name *Clostridium acetobutylicum* was made by McCoy in 1926 (McCoy et al. 1926).

Solvent-producing *Clostridium* have relatively simple growth requirements and can utilize a large variety of substrates, ranging from sugars, beet molasses, glycerol, whey permeate, starch (corn, cassava, rye, etc.), lignocellulosic hydrolysates, to lignocellulosic biomass (Ranjan and Moholkar 2012). Additional nutrients, including complex nitrogen sources, such as yeast extract, and trace elements, such as ferrous, are also generally required for good growth and solvent production (Lee et al. 2008). Moreover, supply of additional reducing power to increase NAD(P)H availability can lead to

higher butanol production since *Clostridium* strains require a high redox potential to generate butanol (Mitchell 1998).

Other than *C. acetobutylicum*, a number of other primary solvent producers including *C. beijerinckii, C. saccharobutylicum,* and *C. saccharoperbutylacetonicum* are also being identified based mainly on differences in the type and ratio of the solvents produced (Dürre 2005; Jones and Woods 1986). Comparative studies of ABE fermentation by representative strains of these four *Clostridium* species using molasses and maize as fermentation substrates were conducted (Shaheen et al. 2000). Result indicates the fermentation performance of different strains of these four *Clostridium* species was highly correlated with the substrates used (Table 2.1) (Ranjan and Moholkar 2012). It could be concluded from this study that *C. acetobutylicum* was more suitable for fermenting starch materials, whereas *C. saccharobutylicum* was suitable for fermenting molasses. Strains of *C. saccharoperbutylacetonicum* were considered to be versatile in fermenting mixed substrates since they showed moderate yet consistent ABE yield in sugar and starch based fermentations.

The choice of strains for use in industrial fermentation depends on the nature of the raw material used, the ratio of end products required, the need for additional nutrients, and phage resistance (Jones and Woods 1986). In majority cases, strains that exhibiting characteristics such as higher butanol yield, simultaneous fermentation of pentose and hexose derived from lignocellulosic biomass and direct utilization of lignocellulose without byproducts production are desired.

Table 2.1 Comparative fermentation analysis of representative strains of four *Clostridium* species[a].

Strains	**ABE titer [g/L]**		**ABE yield (%)**	
	Molasses medium	Maize medium	Molasses medium	Maize medium
C. acetobutylicum				
NCIMB 619	7.8	19.6	13.0	24.5
ATCC824	9.5	19.2	15.8	24
DSM 1732 or NCIMB 2951	4.1	17.9	6.8	22.4
C. beijerinckii				
NRRL B592	11.1	16.2	18.5	20.8
NRRL B593	11.5	14.1	19.2	17.6
NCP P260	18.9	11.3	31.5	14.0
C. saccharobutylicum				
NCP P258	18.3	10.8	30.5	13.5
NCP P262	17.9	11.3	29.8	14.1
C. saccharoperbutylacetonicum				
N 1-4	4.9	14.2	8.2	17.8
N 1-504	18.3	10.8	30.5	13.5

[a]Data taken from (Shaheen et al. 2000).

2.2.3 Metabolic pathways in biological butanol production

In ABE fermentation, pyruvate is a key intermediate as it links the transportation and fermentation of carbohydrates to solvents (acetone, butanol and ethanol), acids (acetic acid and butyric acid) and gases (hydrogen and carbon dioxide). The metabolic pathways that transform pyruvate to end products are similar in different *Clostridium* species while the pathways breaking down carbohydrates to pyruvate are different depending on the fermentation feedstock. Hexoses (glucose, galactose, and fructose) are catabolized through the Embden-Meyerhof-Parnas (EMP) pathway (Figure 2.2), while pentoses (xylose and arabinose) are catabolized through the pentose phosphate pathway (PPP) (Figure 2.3), to generate pyruvate (Jones and Woods 1986). Pyruvate is then converted into acetyl-CoA by pyruvate-ferredoxin oxidoreductase (*pfor*) before it branches into different pathways thereafter (Dürre 2007).

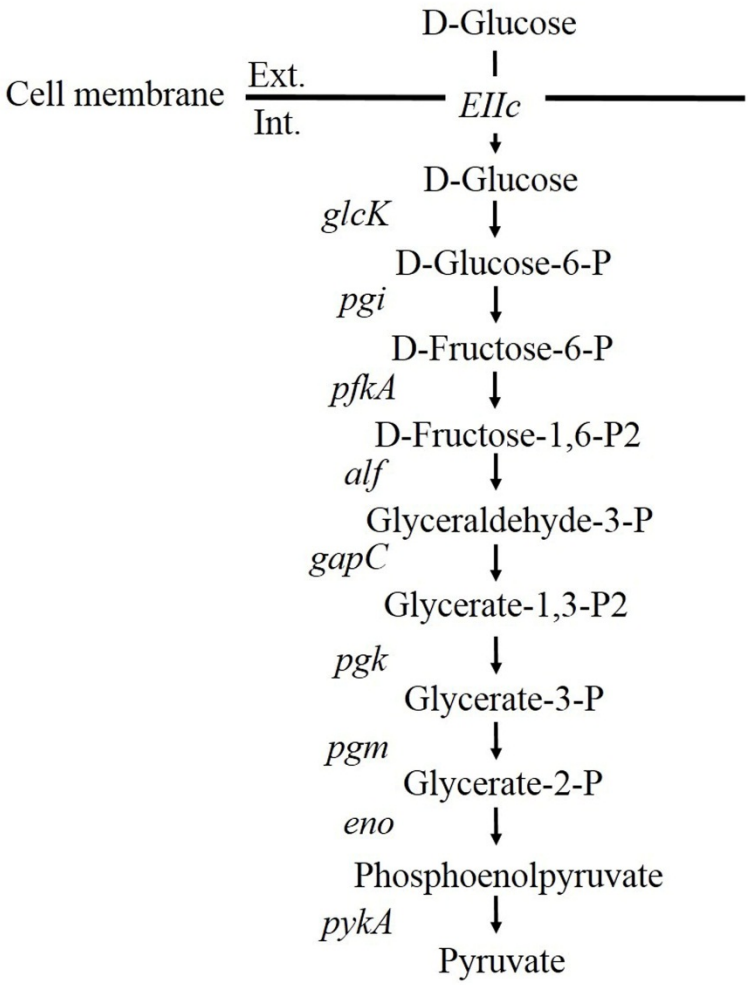


Figure 2.2 Schematic representation of the glucose catabolic pathway in *Clostridium acetobutylicum*. *EIIc*: phosphoenolpyruvate-dependent phosphotransferase system (PTS) enzyme II; *glcK*: hexokinase; pgi: isomerase; *pfkA*: phosphofructokinase; *alf*: aldolase; *gapC*: glyceraldehyde-3-P dehydrogenase; *pgk*: phophoglycerokinase; pgm:probable phosphoglycerate mutase ; *eno*: enolase; *pykA*: pyruvate kinase.

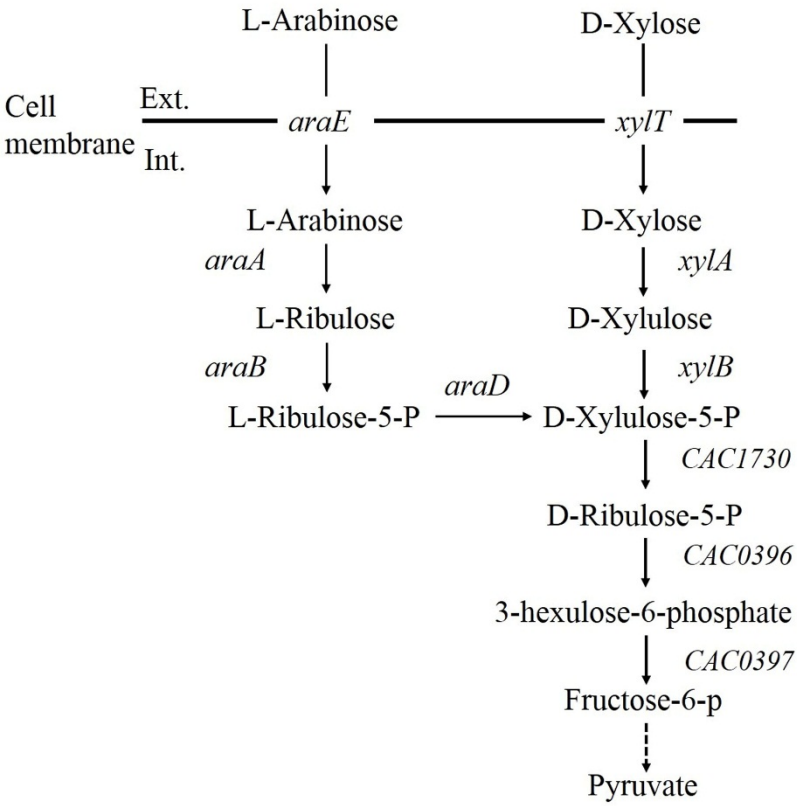


Figure 2.3 Schematic representation of xylose and arabinose catabolic pathway in *Clostridium acetobutylicum*. *araE*: arabinose transporter; *araA*: arabinose isomerase; *araB*: ribulokinase; *araD*: ribulose-5-phosphate 4-epimerase; *xylT*: xylose transporter; *xylA*: xylose isomerase; *xylB*: xylulokinase; *cac1730*: ribulose-phosphate 3-epimerase; *cac0396*: 3-hexulose-6-phosphate synthase; *cac0397*: 6-phospho-3-hexuloisomerase.

The carbon flow after acetyl-CoA through the main branch of the pathway lead to the production of acids and solvents (Figure 2.4)(Lee et al. 2008). A typical feature of ABE fermentation is biphasic fermentation (Figure 2.5). The first phase is the acidogenic phase, during which acetate and butyrate are produced from acetyl-CoA and butyryl-CoA by means of two analogous steps which lead to the production of the corresponding acyl-phosphate, followed by the generation of ATP (Jones and Woods 1986) (Figure 2.4). Since ATP production supports rapid growth of *Clostridium* during this phase, the acidogenic phase usually occurs during exponential cell growth (Lee et al. 2008). Acid accumulation in the acidogenic phase causes

decrease in pH, triggering onset of the second, solventogenic phase, when the pH reaches a critical point (Dürre 2007); therefore, pH is an important factor in ABE fermentation. During the solventogenic phase acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA function as the key intermediates for ethanol, acetone, and butanol production. The branch pathways leading to ABE formation generate acetoacetate, butyraldehyde and acetaldehyde as intermediates, respectively, and the pathway requires the function of two sets of dehydrogenases to achieve the necessary reductions for ethanol and butanol production (Figure 2.4).

In the solventogenic phase, the acid end products produced during the first fermentation phase are reassimilated, and the uptake of acetate and butyrate will only occur when sugars are metabolized concurrently (Davies 1942). It has been suggested that the uptake of acids (normally accompanied by an increase in pH) during the solventogenic phase is a detoxification process initiated in response to the accumulation of acids, which results in conditions unfavourable for growth (Hartmanis et al. 1984). In addition, one previous study proposed that *abrB310* might act as a regulator in shifting acidogenic phase to solventogenic phase (Scotcher et al. 2005). Hence, control of growth conditions (e.g. pH) and manipulation of related genes (e.g. *abrB*) may make it possible to control the transition between acidogenic and solventogenic phases in solvent-producing *Clostridium*.

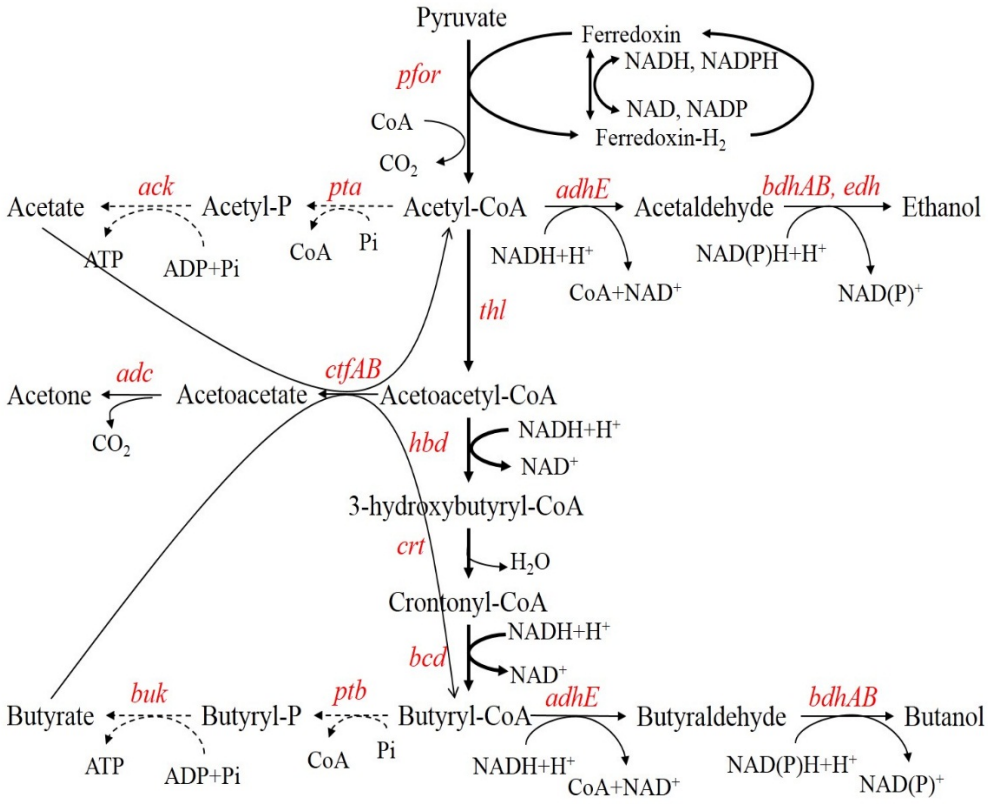


Figure 2.4 Metabolic pathways in *C. acetobutylicum*. Reactions predominate during acidogenesis and solventogensis are indicated by dotted and solid arrows, respectively. Thick arrows indicate reactions which activate the whole fermentative metabolism. Italic letters indicate genes for the reactions. *pfor*: pyruvate-ferredoxin oxidoreductase; *thl*: thiolase; *hbd*: 3-hydroxybutyryl-CoA dehydrogenase; *crt*: crotonase; *bcd*: butyryl-CoA dehydrogenase; *pta*: phosphotransacetylase; *ack*: acetate kinase; *adhE*: acetaldehyde dehydrogenase; *bdhAB*: butanol dehydrogenase; *edh*: ethanol dehydrogenase; *ctfAB*: CoA-transferase; *adc*: acetoacetate decarboxylase; *ptb*: phosphotransbutyrylase; *buk*: butyrate kinase.

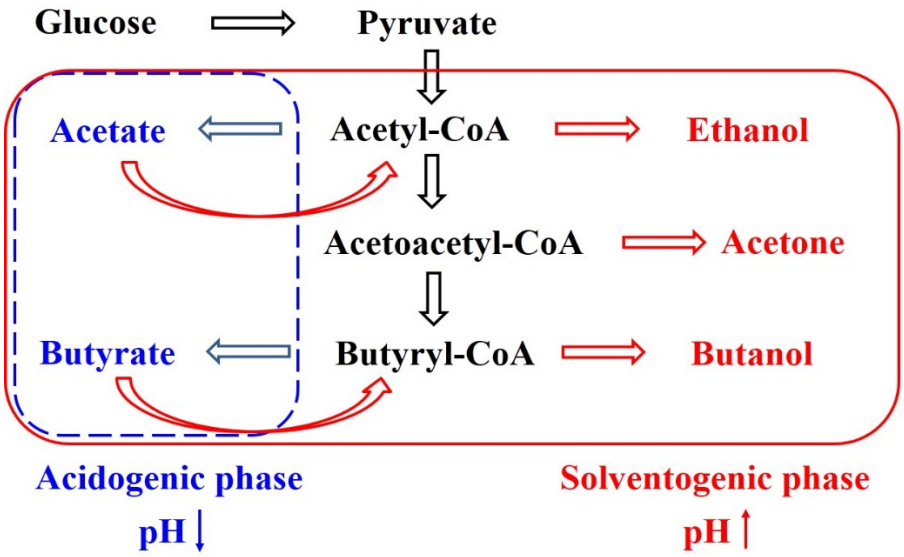


Figure 2.5 Acidogenic (dotted line) and solventogenic (solid line) phases of ABE fermentation.

Notably, addition of either exogenous acetate or butyrate can enhance solvent production and eliminate strain degeneration (Chen and Blaschek 1999; Lin et al. 2015a). RNA and enzyme analysis indicated that in the presence of exogenous sodium acetate, *C. beijerinckii* NCIMB 8052 exhibited more CoA transferase expression, as well as higher acetate kinase- and butyrate kinase-specific activity.

In *Clostridium*, solvent production is also closely coupled to sporulation since the transcription factor responsible for initiation of sporulation (Spo0A) also initiates solvent production (Bahl et al. 1995). In *Clostridium acetobutylicum*, Spo0A controls solvent production by activating transcription of the acetoacetate decarboxylase (*adc*), alcohol dehydrogenase (*adhE*), and CoA transferase (*ctfAB*) genes (Sullivan and Bennett 2006). Due to its diversity of function, the manipulation of Spo0A sometimes lead to unwanted morphological changes. For example, strains with amplified *spo0A*

overexpress solventogenic genes, but fail to generate more solvent due to an accelerated sporulation process (Harris et al. 2002).

2.3 Selection of fermentation substrates

2.3.1 Non-cellulosic substrates

Sugar and starch based feedstocks such as corn, molasses, wheat, millet, and rye are conventional substrates that being widely used for biological butanol production (Ranjan and Moholkar 2012). Corn mash was the earliest substrate for ABE fermentation industry that can be utilized directly without additional nutrients (Jones and Woods 1986). However, in 1930s, substrate for ABE fermentation industry switched to molasses which is much cheaper than corn. Different with corn mash, additional nutrients supplying nitrogen and phosphorous were necessary when using molasses as fermentation substrate (Beesch 1952; Gabriel 1928a; Killeffer 1927). After World War II, most of the conventional substrates were increasingly used for food supplies or main integrates of cattle feed, which is not sustainable for industrial-scale ABE fermentation. Hence, a large variety of alternative substrates including rice, bajra, apple pomace, jawari, cassava, lactose and cheese whey were investigated for economical butanol production (Table 2.2).

At present, starchy materials from various sources including maize, wheat, rice, tapioca, and corn etc. are still widely utilized as a substrate for butanol production with varying degrees of success. The high cost of fermentation feedstock has been identified as one of the major factors affecting the economic viability of the ABE fermentation. An economic assessment of butanol production from corn was conducted. Based on an

average industrial ABE plant from China in 2008, production of each ton ABE solvents required 4.0-4.5 ton corn, 13-25 ton steam, 20-30 ton water and 700-1000kWh electricity (Ni and Sun 2009). The corn price was around 2000 Yuan/ton in the first half of 2011, which shows that the cost of feedstock represents over 70 % of the total production cost (Gu et al. 2011). The price of other feedstocks, such as glucose, cassava and molasses, has also been rapidly pushed up in recent years, it is essential to find other low-cost renewable substrates for sustainable development of ABE fermentation.

Table 2.2 Butanol production from non-cellulosic fermentation substrates.

Strain	Substrate	Product	Reference
C. pasteurianum	Algal biomass and 4 % glycerol	butanol, 1,3-propanediol, and ethanol (16 g/L in total)	(Nakas et al. 1983)
C. acetobutylicum NCIB 2951	Whey filtrate	butanol (13 g/L)	(Maddox 1980)
C. acetobutylicum ATCC 824	Acid whey and lactose	Acetone and butanol (9.2 g/L in total)	(Welsh and Veliky 1984)
Clostridium sp. strain G117	glucose	butanol (13.50 g/L)	(Chua et al. 2013)
Clostridium sp. strain BOH3	cassava	butanol (7.41 g/L)	(Bramono et al. 2011)

2.3.2 Lignocellulosic biomass

Lignocellulose, being the most abundant renewable resource in the world, is a promising candidate substrate for economical biobutanol production, consisting of cellulose and hemicellulose components that can be hydrolyzed into hexose and pentose for butanol generation by solventogenic *Clostridium* (Annous and Blaschek 1991). Though lots of researchers have put effort towards utilization of lignocellulosic material as a fermentation feedstock, efficient conversion of lignocellulose into butanol still encounters some challenges. Since lignocellulosic material cannot be efficiently hydrolyzed by most of the *Clostridium* strains, current process mainly consists of feedstock pretreatment, detoxification, cellulase/xylanase-based saccharification, fermentation and product recovery.

2.3.2.1 Fermentation of sugars derived from lignocellulose

Glucose, xylose and arabinose are three important reducing sugars derived from lignocellulosic biomass (Xiao et al. 2011). However, inefficient pentose utilization is a common phenomenon in solventogenic *Clostridium*. The efficiency of xylose uptake and transportation is closely related to the level and activity of specific proteins including xylose transporter (*xylT*), xylose isomerase (*xylA*), xylulokinase (*xylB*), xylose repressor (*xylR*) and xylose proton-symporter. Among these proteins, XylT, XylA and XylB are rather important since the initial steps of xylose utilization are considered to be the rate-limiting steps of xylose metabolic pathway (Figure 2.3) (Xiao et al. 2011; Xiao et al. 2012). Besides these proteins, glucose utilization related enzymes such as glucose-specific phosphotransferase system (PTS) permease

(*glcG*) also affects xylose utilization. Hence, manipulating these enzymes is a possible solution to improve xylose utilization by *Clostridium* species. The industrial strain, *Clostridium acetobutylicum* EA 2018 with *glcG* disruption and expression of the xylose proton-symporter, *xylA* and *xylB* was able to produce 13.19 g/L butanol with a total ABE yield of 0.33 g/g from 65 g/L reducing sugars, which is 50 % higher compared to that of the untreated EA 2018 (Li et al. 2013). Similarly, overexpression of *xylT* plus *xylR* inactivation, butanol production by *C. beijerinckii* NCIMB 8052 from xylose improved from 9.67 g/L to 11.56 g/L compared to *xylR* inactivation alone (Xiao et al. 2012).

In addition to the inherently inefficient pentose utilization, the glucose-induced carbon catabolite repression (CCR) also inhibits pentose metabolism (Ren et al. 2010). In Gram-positive bacteria, CCR is facilitated through the histone-containing protein (HPr). The HPr is a phosphor-carrier protein and a component of the phosphoenolpyruvate-dependent PTS, which is the major carbohydrate transport system in *Clostridium*. Normally, HPr is phosphorylated at a conserved histidine residue, however in the presence of glycolytic intermediates expression of an HPr kinase, which competitively phosphorylate HPr at a conserved serine residue, is induced. HPr that phosphorylated at serine residue forms a complex with catabolite control protein A (CcpA) which binds at catabolite responsive element sites within the promoter regions or coding sequences of transcriptional units to inhibit transcription of genes related to transport and catabolism of less-preferred carbon sources (Bruder et al. 2015). Hence, some carbon sources, such as xylose and arabinose, are not utilized until the supply of glucose is exhausted.

Pentoses (xylose and arabinose), the second most abundant reducing sugar representing 20-60 % of the total reducing sugars in lignocellulosic hydrolysate (Zaldivar et al. 2001), are generated simultaneously with glucose from lignocellulose hydrolysis. Unless both glucose and pentose are utilized efficiently, converting lignocellulosic biomass into solvents is unfavourable from an economic viewpoint, as yields are limited. Hence, it is desirable to develop novel strains that efficiently utilize pentoses to make fermentation from lignocellulosic biomass an industrialization feasible process. The disruption of *ccpA* in *Clostridium acetobutylicum* allowed for co-fermentation of xylose and glucose to butanol (Ren et al. 2010), however a concomitant accumulation of butyric acid was observed. This accumulation occurred because CcpA is a multifunctional regulator that controls not only genes involved in carbon metabolism, but also genes responsible for solvent production and sporulation in *C. acetobutylicum* (Ren et al. 2012). Wild-type strains that not affected by CCR are thus expected due to the difficulties in manipulating *ccpA*. *Clostridium* sp. strain BOH3 was able to simultaneously ferment glucose and xylose to butanol with 13 g/L butanol obtained from a mixture of glucose and xylose in 2:1 ratio (total 60 g/L) (Xin et al. 2014).

2.3.2.2 Fermentation of lignocellulosic hydrolysate

Lignocellulosic hydrolysates can be prepared through various methods including dilute acid, alkali, and/or enzymes hydrolysis (cellulases/hemicellulases) (Chandel et al. 2013). The degradation of lignocellulosic structure consists of pretreatment and hydrolysis steps (Olsson and Hahn-Hägerdal 1996). The goals of pretreatment of lignocellulosic

biomass are to better expose cellulose for downstream hydrolysis, to convert hemicellulose to pentoses, and to remove lignin (Mills et al. 2009). Pretreatment of lignocellulose also generates a group of inhibitors (Mills et al. 2009). These inhibitors can be divided into three groups: weak acids (mainly acetic acid, formic acid and levulinic acid), furan derivatives (furfural and HMF), and phenolic compounds (Figure 2.6) (Olsson and Hahn-Hägerdal 1996; Palmqvist and Hahn-Hägerdal 2000b).

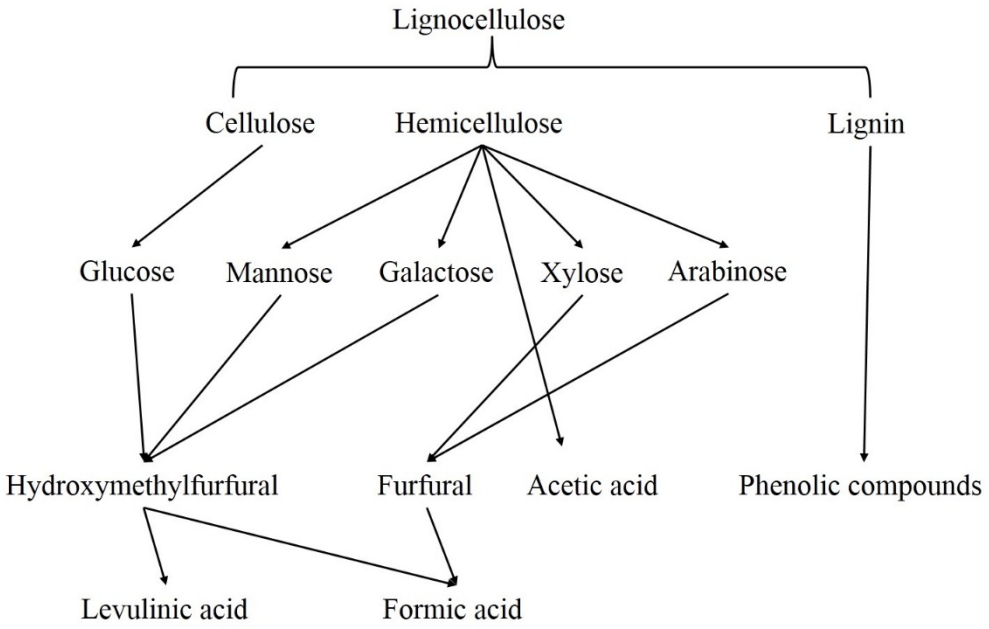


Figure 2.6 Generation of inhibitors during hydrolysis of lignocellulosic biomass. The furan derivatives and phenolic compounds will react further to form some polymeric materials.

The composition of inhibitors differs from different sources of lignocellulosic biomass and also depends on the pretreatment methods (Olsson and Hahn-Hägerdal 1996). At high temperature and pressure, furan derivatives include furfural and HMF are formed from the dehydration of pentose (Dunlop 1948) and hexose (Ulbricht et al. 1984), respectively. Formic

acid is formed during furfural and HMF degradation and levulinic acid is formed by HMF degradation (Ulbricht et al. 1984). Acetic acid is released from acetylxylan decomposition (Mills et al. 2009). Phenolic compounds such as ferulic acid, 4-hydroxybenzoic acid, vanillic acid, vanillin, and syringaldehyde are generated from partial breakdown of lignin (Cho et al. 2009). Hydrolysates from willow (Jönsson et al. 1998), poplar (Ando et al. 1986), red oak (Tran and Chambers 1985), and pine (Clark and Mackie 1984) have been reported to contain vanillic acid and vanillin, while syringaldehyde and syringic acid, formed in the degradation of syringyl propane units, have been detected in hardwood hydrolysates (Ibraheem and Ndimba 2013; Jönsson et al. 1998; Tran and Chambers 1985).

The presence of inhibitors in lignocellulosic hydrolysates inhibits both cell growth and solvent production by affecting essential fermentative enzymes, disrupting extensive membrane, damaging polynucleotides, inducting oxidative stress, and diminishing nutrient transport (Abdehagh et al. 2014; Ibraheem and Ndimba 2013; Mills et al. 2009; Zhang et al. 2012). Combination of different kinds of inhibitors can be synergistic (Ezeji et al. 2007a; Martinez et al. 2000).

Undissociated weak acids are liposoluble and can diffuse across the cell membrane. The inflow of weak acids into the cytosol decreases intracellular pH and affects cell growth (Pampulha and Loureirodias 1989). The concentration of undissociated weak acids in lignocellulosic hydrolysates depends on pH. The inhibitory effect of weak acid is more pronounced in ethanol generation by yeast. Under pH control, low concentrations of exogenous acids can even enhance biobutanol production (Bramono et al.

2011; Lin et al. 2015a). Therefore, weak acids are not selected for toxicity study in chapter 5.

Furan derivatives are toxic to microorganisms by reducing the specific growth rate, the solvent productivity and the cell-mass yield on ATP (Allen et al. 2010; Wilson et al. 2013; Zhang and Ezeji 2013). Bioconversion of furan derivatives to their less toxic alcohol is a NAD(P)H dependent process (Ask et al. 2013; Horvath et al. 2003; Zhang and Ezeji 2013). Butanol production is also closely related to the level and proportion of the activity forms of NAD(P)H. The presence of furan derivatives affects redox equilibrium and inhibits the shift of redox flux towards butanol production (Baral and Shah 2014).

Phenolic compounds affect cell membrane permeability and cause leakage of cellular contents by interfere with the hydrophobic sites of cell membrane (Heipieper et al. 1991; Heipieper et al. 1994). The toxicity of phenolic compounds is thus correlated with their degree of hydrophobicity (Zaldivar et al. 1999). Notably, phenolic compounds such as syringaldehyde, ferulate, and vanillin have been shown to play a key role in ATP decrease (Cho et al. 2009).

Various methods have been developed to reduce inhibitors from lignocellulosic hydrolysates, including physical (evaporation, membrane-mediated detoxification), chemical (anion exchange resins, neutralization, overliming, activated charcoal column, calcium hydroxide and extraction with ethyl acetate), and biological (enzymatic and microbial) (Chandel et al. 2011; Gupta et al. 2011; Parawira and Tekere 2011). The different detoxification methods cannot be strictly compared since the composition of hydrolysates

varies and different fermentation microorganisms have been used (Palmqvist and Hahn-Hägerdal 2000a). It should be noted that all of these methods would more or less lead to fermentable sugars reduction which results in inefficient sugar utilization, and increase in production cost which affects the economic viability of lignocellulosic hydrolysates fermentation (Chandel et al. 2013; Stoutenburg et al. 2011).

Some of the recently reported studies using lignocellulosic hydrolysates as fermentation substrate to generate butanol are listed in Table 2.3. It can be concluded that without detoxification, dilute acid pretreated lignocellulosic hydrolysates are more difficult for *Clostridium* to ferment. However, dilute acid hydrolysis is one of the most commonly used methods of chemical pretreatment since it is fast and easy to perform (de Vasconcelos et al. 2013; Palmqvist and Hahn-Hägerdal 2000b). Hence, it is rather important to develop solventogenic strains that can resist in high lignocellulose-derived inhibitor concentrations for large-scale butanol generation from undetoxified lignocellulosic hydrolysates.

Table 2.3 Utilization of lignocellulosic hydrolysates for butanol production.

Strain	Substrate	Pretreatment method	Reducing sugar, [g/L]	Detoxification method	Butanol titer, [g/L]	Reference
C. beijerinckii DSM 6422	wheat straw hydrolysate	Steam-exploded	33.88	None	7.21	(Ding et al. 2016)
C. beijerinckii P260	Barley straw hydrolysate	Dilute acid	60	None	4.75	(Qureshi et al. 2010)
C. saccharoperbutylacetonicum N1-4	Palm kernel cake hydrolysate	Dilute acid	17.99	XAD-4 resin extraction	3.59	(Shukor et al. 2014a)
C. acetobutylicum ATCC 824	Hardwood hydrolysate	Dilute acid	72.0 ± 1.1	None	0.4	(Mechmech et al. 2015)
C. beijerinckii BA101	Corn fiber hydrolysate	Dilute acid	29.8 46.3	None XAD-4 resin extraction	1.4 6.4	(Qureshi et al. 2008)
C. saccharobutylicum DSM 13864	Corn stover hydrolysates	Recycled ionic liquid	18.7	None	7.9	(Ding et al. 2016)

2.3.2.3 Direct utilization of lignocellulosic biomass

Besides producing butanol from monosaccharides derived from lignocellulosic biomass and hydrolysates, direct conversion of cellulose and xylan into butanol using a one-step fermentation strategy is always desirable. Numerous studies have been conducted with this goal using various methods, such as introducing cellulolytic genes into solventogenic strains and constructing an ABE fermentation pathway in microbes that exhibit high cellulase/xylanase activities.

Several wild-type and engineered *Clostridium cellulyticum* strains have been reported to generate value-added products directly from cellulose, but no wild-type strains have been found to be capable of producing butanol directly from cellulose (Higashide et al. 2011; Sizova et al. 2011). For direct utilization of xylan, most of studies were focused on ethanol or hydrogen production using metabolically engineered microorganisms (la Grange et al. 2010; Lynd et al. 2005; Olson et al. 2012; Tolonen et al. 2011). A wild-type *C. phytofermentans* ATCC 700394 has been reported of successfully producing 2.9 g/L ethanol from 10 g/L filter paper, and 0.46 g/L ethanol from 3 g/L birchwood xylan (Tolonen et al. 2011). Another wild-type strain *C. acetobutylicum* 7 has been reported to produce 4.1 g/L butanol from a mixture of 30 g/L of flour and 30 g/L of grass (Berezina et al. 2008). More studies using cellulose and xylan as substrate to produce value added products by *Clostridium* species are listed in Table 6.3.

In addition to fermentation condition optimization and metabolically engineered strain development, approaches toward genetically modified energy crops for higher biomass yield and less recalcitrant for degradation

were also made for realizing the one-step fermentation from lignocellulose to biofuels (Taylor et al. 2008). Energy crops such as perennial C4 grasses, not only require less inputs and energy consumption than annual C3 grain crops, but also capture solar energy more efficiently (Heaton et al. 2008). Key enzymes for lignin biosynthesis can also be manipulated to reduce the recalcitrance of lignin carbohydrate complex (Saathoff et al. 2011).

Despite the above mentioned success, challenges still need to be met to move biobutanol towards commercialization, and to compete with more conventional fuels (Peralta-Yahya et al. 2012).

2.4 Limitations and possible solutions for biological butanol production

2.4.1 Low butanol production

2.4.1.1 Problem statement

In typical batch ABE fermentation, the total ABE production is normally around 20g/L from glucose and the butanol concentration is usually below 13 g/L (Jones and Woods 1986; Woods 1995). The low product concentration is associated with fermentation conditions such as pH, reducing driving forces and expression of butanol producing associated genes. It is estimated that if the final butanol titer of ABE fermentation can be increased to 19 g/L, the product recovery cost would be reduced by half as compared to 13 g/L (Gu et al. 2011).

The pH of fermentation medium is a key factor in determining the performance of ABE fermentation since it is closely related to the initiation of solvent production (Andersch et al. 1983; Jones and Woods 1986). Generally,

cultures at high pH mainly produce acids whereas solvent production usually predominates when cultures are maintained at a low pH. However, the pH range over which solvent formation may occur appears to vary quite widely among particular strains and to depend upon the fermentation conditions (Lee et al. 2008). Another issue associated with the pH is so called “acid crash”, which refers to the phenomenon that undissociated acetate and butyrate accumulated and eventually lead to cessation of all metabolic activities in the cell by collapse of the proton gradient across the membrane (Dürre 2007). Hence, it is rather important to identify the optimal pH for cell growth and solvent production of strains used for ABE fermentation.

In addition to pH, the availability of reducing cofactors such as NADH and NADPH play a major role in cellular metabolism and product synthesis since biological butanol production is a cofactor-dependent system (Berrios-Rivera et al. 2002a; San et al. 2002; Yu et al. 2011).

Moreover, expression level of some butanol producing associated genes is crucial for butanol generation such as *adc*, *adhE*, *ctfAB*, butyrate kinase (*buk*) (Figure 2.4) and *solR*, a putative *sol* locus (*aad*, *ctfAB* and *adc*) transcriptional repressor (Nair et al. 1999). For example, *ctfAB* is responsible for reassimilating acetate and butyrate to acetyl-CoA and butyryl-CoA for the following ethanol and butanol production, respectively. The overexpression level of *ctfAB* directly affects the onset of solvent production and the final solvent titers (Yu et al. 2015b).

2.4.1.2 Potential solutions

Since the range of pH allows solvent formation to occur is quite wide, optimization of pH for solvent production is always conducted for newly isolated strains (Bramono et al. 2011; Chua et al. 2013). Regulation of pH was also reported to be effective in shorten the lag phase for butanol production (Li et al. 2016).

Increase the availability of reducing cofactors is a possible solution for butanol production enhancement. Through the artificial driving forces created by NADH and acetyl-CoA accumulation in metabolically engineered *Escherichia coli*, a titer of 15 g/L butanol was obtained (Shen et al. 2011).

Overexpression or disruption of target genes was also applied to enhance butanol production (Table 2.4). One of the successful metabolic engineering examples was the overexpression of *groESL*, an operon in heat shock genes, in *C.acetobutylicum* ATCC 824, which resulted in an increased final solvent production compared to both wild-type (40% higher) and plasmid control strains (33 %) (Tomas et al. 2003).

Table 2.4 Metabolic engineering of *Clostridium* species for butanol production enhancement. *adc*: acetoacetate decarboxylase, *groESL*: an operon in heat shock genes, *adhE*: acetaldehyde dehydrogenase, *ctfAB*: CoA-transferase, *buk*: butyrate kinase, *solR*: *sol* locus (*aad, ctfAB* and *adc*) transcriptional repressor, *cac3319*: histidine kinase; *fdh*: formate dehydrogenase.

Strain	**Up-regulated**	**Down-regulated**	**Acetone, [g/L]**	**Butanol, [g/L]**	**Ethanol, [g/L]**	**Reference**
C. acetobutylicum ATCC 824	Control		3	11	1.1	(Tomas et al. 2003)
	groESL	-	8	17	1.1	
C. acetobutylicum ATCC 824	Control		4.5	9.5	0.74	(Mermelstein et al. 1993)
	adc, ctfAB		8.7	13	1.4	
C. acetobutylicum ATCC 824	Control		3.8	5.5	0.7	(Nair et al. 1999)
	-	*solR*	8.1	17.8	1.0	
C. acetobutylicum ATCC 824	Control		3.8	5.5	0.7	(Harris et al. 2000)
	adhE	*buk*	3.8	16.7	4.5	
C. acetobutylicum ATCC 824	Control		5	11.7	0.73	(Harris et al. 2001)
	-	*solR*	5.6	14.6	4.4	
	adhE	*solR*	8.2	17.6	2.1	
C. acetobutylicum ATCC 824	Control		4.07	8.89	0.69	(Harris et al. 2002)

	Spo0A	-	5.52	10.01	0.55	
C. tyrobutyricum ATCC 25755	Control		0	10.4	0.8	(Yu et al. 2015b)
	adhE2, ctfAB		5.0	13.9	0.8	
C. acetobutylicum ATCC 55025	Control		8.1	12.6	0.9	(Xu et al. 2015)
	-	*cac3319*	9.1	18.2	3.7	
C. tyrobutyricum ATCC 25755	Control		-	6.14	0.25	(Ma et al. 2016)
		fdh	-	12.34	0.28	

2.4.2 Byproducts formation

2.4.2.1 Problem statement

In typical ABE fermentation, acetone, butanol and ethanol are produced in a ratio of 3:6:1. As a consequence, separation processes are needed to recover pure butanol, resulting in higher production cost (Ranjan and Moholkar 2012).

2.4.2.2 Potential solutions

Approaches towards converting ABE fermentation into a butanol-only fermentation have received general interests (Table 2.5). Increasing butanol percentage in ABE fermentation products can greatly simplify the downstream separation process and thus make industrial-scale biobutanol production more feasible. Most recently, disruption of *adc* in the hyperbutanol-producing strain *C. acetobutylicum* EA 2018 using TargeTron technology was shown to increase the butanol ratio from 71 % to 82 % compared to the wild-type strain (Jiang et al. 2009a). However, due to relatively poor understanding of the complex metabolic pathways in *Clostridium*, great challenges still remain in achieving a butanol-only process through metabolic engineering. For example, blocking the branch pathways always lead to undesired phenotypes such as acids accumulation and deficient growth rate.

Table 2.5 Metabolic engineering of *C. acetobutylicum* to increase butanol ratio. pSOL1: a 210-kb plasmid in *C. acetobutylicum* ATCC 824.

Strain	**Up-regulation**	**Down-regulation**	**Butanol percentage**	**References**
C. acetobutylicum ATCC 824	control		66 %	(Cornillot et al. 1997)
	-	pSOL1	0	
	aad	pSOL1	94 %	
C. acetobutylicum ATCC 824	control		33.5 %	(Nair and Papoutsakis
	adhE	-	58.8 %	1994)
C. acetobutylicum ATCC 824	control		55 %	(Nair et al. 1999)
	-	*solR*	66 %	
C. acetobutylicum ATCC 824	control		67 %	(Harris et al. 2001)
	-	*solR*	69 %	
C. acetobutylicum EA 2018	control		71 %	(Jiang et al. 2009a)
	-	*adc*	82 %	

In addition to the use of metabolic engineering tools, isolation of novel wild-type strains which produce less or no byproducts is an alternative option to achieve butanol-only fermentation. Recently, a newly isolated *Clostridium* sp. strain G117 was found to exhibit a novel acetone-butanol fermentation profile, producing 13.5 g/L butanol from 60 g/L glucose with a butanol/acetone ratio of 2.14 and no ethanol (Chua et al. 2013). Another solvent producing strain *C. sporogenes* BE01 that makes only butanol and ethanol has been isolated (Gottumukkala et al. 2013), the butanol concentration produced was 5.52 g/L.

2.4.3 Solvent toxicity

2.4.3.1 Problem statement

Butanol is a lipophilic solvent, which can change membrane structures and interfere with normal functions of the cell membranes (Liu and Qureshi 2009). Membrane fluidity has been shown to increase 20-30% in response to 1% butanol exposure, which would result in disruption of membrane associated functions particularly transport processes, substrate uptake and membrane-bound ATPase activity (Bowles and Ellefson 1985). Higher butanol concentrations also severely affect cellular energy status by decreasing intracellular pH and ATP concentrations which lead to a disruption of the membrane ΔpH and $\Delta\Psi$ (Tomas et al. 2003). Most butanol-producing bacterial can tolerate not more than 20 g/L butanol (Lin and Blaschek 1983), with cell growth reduced by 50 % in the presence of 7-13 g/L of butanol (Jones and Woods 1986). The addition of acetone and ethanol reduce cell

growth by 50 % only after 40 g/L (Jones and Woods 1986), thus butanol is the only solvent that can reach toxic levels during ABE fermentation.

2.4.3.2 Potential solutions

In order to achieve higher butanol tolerance, methods such as random mutagenesis and antisense RNA were applied to solvent-producing *Clostridium* strains. As an example, strain *C. beijerinckii* BA101 is a solvent tolerant strain derived from *C. beijerinckii* NCIMB 8052 by random mutagenesis (Qureshi and Blaschek 2001), yet the mechanism underlying the increased butanol tolerance of strain BA101 is still unclear. Another success of mutagenesis is *C. acetobutylicum* RH8 which can withstand 18 g/L butanol (increased 23 % as compared to the wild-type strain) after chemical mutagenesis and genome shuffling (Mao et al. 2010). Comparative proteomic analysis of *C. acetobutylicum* RH8 demonstrated a different expression profile of proteins involved in ATP synthesis, solvent formation, and protein folding compared to the wild-type strain, indicating the observed tolerance towards butanol is a complex global response (Bao et al. 2014).

Additionally, physical methods of process optimization, such as *in situ* product recovery and immobilized fermentation system, also have been used as a means to overcome solvent toxicity by lowing the solvent concentration before it becomes toxic. Immobilized fermentation systems have several distinct advantages over free cell systems (Jones and Woods 1986), namely:

1) Ease of separation of cells from products;
2) High cell density per reactor volume;
3) High cell concentration;

4) Smaller reactor volumes;
5) Greater productivity;
6) Flexibility of reactor design (such as fixed bed, trickle bed, and fluidized bed) for continuous operation;
7) Maximum reaction rates;
8) Minimum nutrient depletion and product inhibition;
9) Better mass transfer characteristics.

Shang-tian Yang investigated fed-batch fermentation for butanol production from cassava bagasse hydrolysate in a fibrous bed bioreactor (FBB) with continuous gas stripping using a hyper butanol-producing *C. acetobutylicum* strain JB200 derived from ATCC 55025 through mutagenesis and adaptation (Lu et al. 2012). In this study, the immobilized cells in the FBB were used as seed culture to produce butanol over 581h, demonstrating the long-term operation stability of the immobilized FBB system. As shown in Table 2.6, higher yield and productivity were obtained through fed-batch fermentation with gas stripping.

Table 2.6 ABE production from cassava bagasse hydrolysate by JB200 in batch and fed-batch fermentation with gas stripping

	Batch fermentation	**Fed-batch fermentation**
Glucose utilization rate (g/L·h)	1.12	1.46
Butanol produced (g/l)	9.71	59.81
Total ABE production (g/l)	15.41	90.31
Butanol yield (g/g)	0.22	0.25
Butanol productivity (g/L·h)	0.24	0.35
ABE productivity (g/L·h)	0.39	0.53

Although some success have been obtained in overcoming these limitations through microbial engineering and process optimization, the unstable butanol production or undesired phenotypes implies difficulties and complexities in manipulating related pathways due to the inherent instability or inefficient expression compared to wild-type strain (Alsaker et al. 2010; Antoni et al. 2007).

CHAPTER 3

Improved Butanol Production from Glucose Triggered by Reducing Cofactors Using a Wild-type *Clostridium* Species Strain BOH3

The ultimate goal for this doctoral study is to realize direct biobutanol production from lignocellulosic biomass. Before direct utilization of lignocellulose polymer, monosaccharide such as glucose was used as carbon source for butanol generation. This chapter is aimed to achieve high butanol titer, yield and productivity from glucose.

3.1 Abstract

In cofactor-dependent anaerobic acetone-butanol-ethanol (ABE) fermentation systems the availability of reducing factors, such as NADH and NADPH, plays an important role in microbial catabolism and is thus expected to affect metabolic distribution. In this study, the effect of nicotinic acid, a precursor metabolite, on intracellular accumulation of NADH and NADPH and the concomitant changes in metabolite distribution in a wild-type *Clostridium* species strain BOH3 was studied. The addition of nicotinic acid to the fermentation medium led to a significant increase in the availability of NADH and NADPH as indicated by an increase in the ratio of $NADH/NAD^+$ and $NADPH/NADP^+$ of 2.96 and 3.42 fold, respectively. Additionally, a pH shift strategy was developed to improve cell growth and butanol production. Strain BOH3 achieved high butanol titer (18.7 g/L), yield (24.6 %) and productivity (0.26 g/L·h) in batch fermentation conducted in a bioreactor. The metabolic pattern shifted towards more reduced metabolites, reflected by a higher butanol/acetone ratio (11 %) and butanol/acids ratio (292 %). This study demonstrates the importance of reducing cofactors on metabolite distribution and proposes an approach to achieve desired metabolic engineering goals via manipulation of reducing cofactors and pH.

3.2 Introduction

With growing concerns about environment pollution and the fluctuation of fossil fuel prices, development of alternative and environmentally friendly biofuels has attracted increasing attention (Antoni et al. 2007; Dürre 2007; Fortman et al. 2008). Biobutanol, produced by a number of *Clostridium* species through conventional acetone-butanol-ethanol (ABE) fermentation, is not only an important intermediate chemical but is also considered to be a promising renewable biofuel due to the comparability of its physical and chemical properties to gasoline (Dürre 2007; Gu et al. 2011).

One of the main bottlenecks impeding the progress of biobutanol becoming an economically viable fuel alternative is the low butanol concentration which can currently be realized by ABE fermentation (Gu et al. 2011). Numerous attempts have been made to improve the butanol titer, including: construction of a solvent production pathway in more genetically tractable organisms (Bond-Watts et al. 2011; Shen et al. 2011), inactivation or overexpression of corresponding genes in butanol producing strains (Harris et al. 2001; Tomas et al. 2003) and abiotic process optimization (Lu et al. 2012). However, the unstable butanol production apparent in these strains reflects the difficulty and complexity involved in constructing an artificial solvent producing pathway in modified strains or hosts (Alsaker et al. 2010; Antoni et al. 2007).

In contrast to genetic engineering strategies, metabolic engineering strives to manipulate the throughput of certain pathways as an alternative solution to stably enhance process productivity and yield. The availability and

proportion of cofactors in active forms play an important role in cellular metabolism and may be a major determinant of the overall process yield and product distribution in cofactor-dependent production systems (Berrios-Rivera et al. 2002a; San et al. 2002; Yu et al. 2011). For example, a lack of or inefficient regeneration of NADH and NADPH cofactors required for butanol biosynthesis could lower the intracellular reducing power leading to cessation of the butanol dehydrogenase reaction (Berrios-Rivera et al. 2002b; Knepper et al. 2008). This suggests a crucial role of constant NADH and NADPH availability in the solventogenic phase during fermentation for purposes of butanol production.

In this study, nicotinic acid, which is the metabolic precursor of NADH and NADPH, was supplemented to culture medium to increase the intracellular availability of these cofactors. Moreover, a pH shift strategy which uncouples cell growth and solvent production was developed in a batch bioreactor to improve the butanol production of strain BOH3.

3.3 Materials and methods

All chemicals in this study were purchased at least analytical-grade purity from Sigma Aldrich, USA unless specified otherwise.

3.3.1 Culture medium and cultivation

A wild-type *Clostridium* species strain BOH3 isolated from a paddy field was used in this study (Bramono et al. 2011). The fermentation medium in the bioreactor consisted of (per liter): glucose 90 g; KH_2PO_4 0.5g; K_2HPO_4 0.5 g; $MgSO_4$ 0.2 g; CH_3COONH_4 2.2 g; $MnSO_4$ 0.05 g; $FeSO_4 \cdot 7H_2O$ 0.01 g;

NaCl 1 g; yeast extract 3 g; plus 1 ml of trace element solution at a concentration of (per liter): H_3BO_3 0.006 mg; $NiCl_2 \cdot 6H_2O$ 0.024 mg; $ZnCl_2$ 0.1 mg; $CoCl_2 \cdot 6H_2O$ 1.9 mg; $Na_2MoO_4 \cdot 2H_2O$ 0.036 mg; and $CuCl_2 \cdot 2H_2O$ 0.05 mg.

For microbial seed cultivation the medium was amended with 0.25 ml resazurin solution (1%), boiled, and cooled to room temperature under continuous nitrogen flow. Anaerobic medium (pH 6.5) with 30 mmol/L 2-(*N*-morpholino) ethanesulfonic acid (MES) as a buffering agent was prepared by the addition of the reducing agents L-cysteine, 0.0242 g/L, and $Na_2S \cdot 6H_2O$, 0.048 g/L (He et al. 2003). Medium aliquots of 42 ml were distributed to 160 ml serum bottles and sealed with butyl stoppers and aluminum caps before autoclave. Before inoculation, 5 ml sterile glucose stock solution (300 g/L) and 1 ml sterile yeast extract stock solution (150 g/L) were added to each bottle. Actively growing cells [4%, v/v] were inoculated to bottles and cultures were incubated in an orbital shaker at 150 rpm at 37 ˚C for 24-30 h.

The bioreactor fermentation was conducted in a 3 L bioreactor (BIOSTAT® B plus, Sartorius, Germany) equipped with probes measuring redox potential and pH. Experiments were conducted with a working volume of 1.5 L at an agitation speed of 150 rpm at 37 ˚C. Nicotinic acid was added at a final concentration of 10 mg/L (an optimized concentration based on butanol production) in the medium before fermentation. Seed culture was inoculated to the bioreactor with a percentage of 6% [v/v]. pH was controlled by automatic addition of 9 mol/L NaOH or 3 mol/L H_2SO_4.

Studies of metabolic cofactors and regulators were conducted in serum bottles. Cultures grown in serum bottles were incubated in an orbital shaker at

150 rpm at 35 °C with an initial pH of 6.5 and maintained at the desired pH levels using 9 M sodium hydroxide solution until no further gas was produced. Samples were taken at regular intervals and analyzed for the presence of acetone, butanol, ethanol, acetic acid and butyric acid on a GC-FID as described later. All experiments were done in triplicate.

3.3.2 Cell extract preparation

Samples (16 mL) were taken from the bioreactor at various time intervals. 1 ml of the samples were used for GC and HPLC analysis, 5 ml of the samples were used for dry cell weight determination and 10 ml of the samples were used to test enzyme activity. Anaerobic conditions were maintained throughout the entire sampling procedure. Cells were harvested by centrifugation at 14,000 rpm at 4 °C for 15 min. Cell pellets were resuspended in 0.5 ml ice-cold TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.5). Cells were lysed by ultrasonication on ice for 15 min using a 20 kHz ultrasonicator (VCX 130, Sonics & Materials Inc., CT, USA) set at 50 % amplitude in pulses of 5 s of sonication with a 10 s intervals. The resulting lysate was collected and centrifuged at 14,000 rpm at 4 °C for 20 min to remove cell debris. The supernatant was topped up to 1 ml using TE buffer (10 x concentrated to original sample) for subsequent enzyme assays. The protein concentration of cell extracts was measured using the DC protein assay kit (BioRad, USA). All values of enzyme assays are averaged values of at least three independent extract procedures.

3.3.3 NAD^+/NADH and $NADP^+$/NADPH assay

NAD^+/NADH or $NADP^+$/NADPH levels were measured with Amplite™ Fluorimetric NAD^+/NADH or $NADP^+$/NADPH ratio assay kits (ATT Bioquest, CA). The traditional NAD^+/NADH or $NADP^+$/NADPH assay were done by monitoring the changes in NADH or NADPH absorption at 340 nm. The assay methods of this study used enzymes that specifically recognize NAD^+/NADH or $NADP^+$/NADPH in an enzyme recycling reaction which significantly increased detection sensitivity.

Standards (0, 0.01, 0.03, 0.1, 0.3, 1 and 3 µM) were prepared through serial dilutions of NADH (10 µM) or NADPH (10 µM) stock solutions provided in the kit. PBS buffer was used as blank control, and NADH or NADPH reactions were used as positive control. Cells were harvested by centrifugation at 14,000 rpm at 4 ˚C for 15 min. Pellets were resuspended in 0.2 ml PBS (pH 7.4) and 0.2 ml NAD^+/NADH or $NADP^+$/NADPH lysis buffer that provided in the kit. Resuspended pellets were then used for NAD(P), NAD(P)H, and total NAD(P), NAD(P)H assay in a solid black 96-well microplate.

For NAD or NADP assay, 25 µL NAD or NADP extraction solution was added into the wells of test samples. After incubated at room for 10 minutes, 25 µL NADH or NADPH extraction solution was added into the wells to neutralize the NAD or NADP extracts.

For NADH or NADPH assay, 25 µL NADH or NADPH extraction solution was added into the wells of test samples. After incubated at room for 10 minutes, 25 µL NAD or NADP extraction solution was added into the wells to neutralize the NADH or NADPH extracts.

For total NAD and NADH or NADP and NADPH assay, 25 µL NAD/NADH or NADP/NADPH control solution was added into the wells of test samples and NADH or NADPH standards. After incubated at room for 10 minutes, 25 µL control solution was added into the corresponding wells.

After adding above solutions to the corresponding wells, 75 µL of NADH or NADPH reaction mixture was added into each well of NADH or NADPH standard, blank control, and test samples for NAD^+/NADH or $NADP^+$/NADPH assay. After incubated at room temperature for 1 hour in dark, readings could be monitored by running a 96-well plate on a fluorescence microplate reader (Infinite 200 pro, Tecan, Switzerland) at Ex/Em = 530-570/590-600nm (maximum Ex/Em = 540/590 nm) (Ikegami et al. 2007; Kimura et al. 2006; O'Donnell et al. 2004; Ziegenhorn et al. 1976).

3.3.4 Analytical methods

Dry cell weight (DCW) was determined by measuring the weight difference of membrane filter before and after filtration of 5 ml sample taken from the bioreactor at various time intervals. Nylon membrane filters (0.22 nm, Whatman, US) were dried at 105 ˚C for 4 h until constant weight before and after filtration.

Volatile fatty acids (i.e., acetic and butyric acids) and biosolvents (i.e., acetone, ethanol and butanol) were measured using gas chromatography (GC) (model 7890A, Agilent Technologies, U.S.A.) equipped with a Duranbond (DB)-WAXetr column (30m x 0.25mm x 0.25µm; J&W, U.S.A) and a flame ionization detector (FID), while residual glucose concentrations were measured using high-performance liquid chromatography (HPLC) (model

1260 Infinity, Agilent Technologies, USA) equipped with an Agilent Zorbax Carbohydrate Analysis column (4.6 × 150 mm, 0.5 μm) and a refractive index detector (RID). 1 ml of samples were centrifuged at 4 °C, 14000 rpm for 15 min. 475 μL of the obtained supernatant was then mixed with 25 μL 2 M HCL in 2 ml GC vials for fatty acids and biosolvent analysis; 400 μL of the obtained supernatant was pipetted into a separate vial for HPLC analysis of residual glucose.

GC analysis of the samples was carried out by injecting 1 μL of sample (prepared as above) into the GC column. Concentrations of target metabolites were determined from a five-point calibration curve prepared by running standard solutions containing known quantities of acetone, butanol, ethanol, acetic acid, and butyric acid. Data analysis was done using Agilent ChemStation.

HPLC analysis was carried out by injecting 3 μL of sample into a column equilibrated with mobile phase (acetonitrile and water (75:25, [v/v])) and maintained at a flow rate of 1ml/min and 40 °C oven temperature. Glucose was used for preparation of corresponding calibration curves for identification and quantification of residual sugars in samples. Data analysis was done using Agilent ChemStation.

3.4 Results and discussion

3.4.1 A pH-shift regulation for improved cell growth and butanol production

A typical feature of traditional ABE fermentation processes is biphasic fermentation wherein the pH of the medium plays a key role in shifting

fermentation from the acidogenic phase to the solventogenic phase (Lee et al. 2008). Typically, acids (acetic acid and butyric acid) are produced as major products at higher pH (pH 5.7) while solvents are dominant products at lower pH (4.5). However, the pH range for solvent production appears to vary quite widely among different strains and culture conditions (Jones and Woods 1986). To study the effect of pH on cell growth and butanol production, different pH values were maintained throughout the fermentation process in a bioreactor (Figure 3.1).

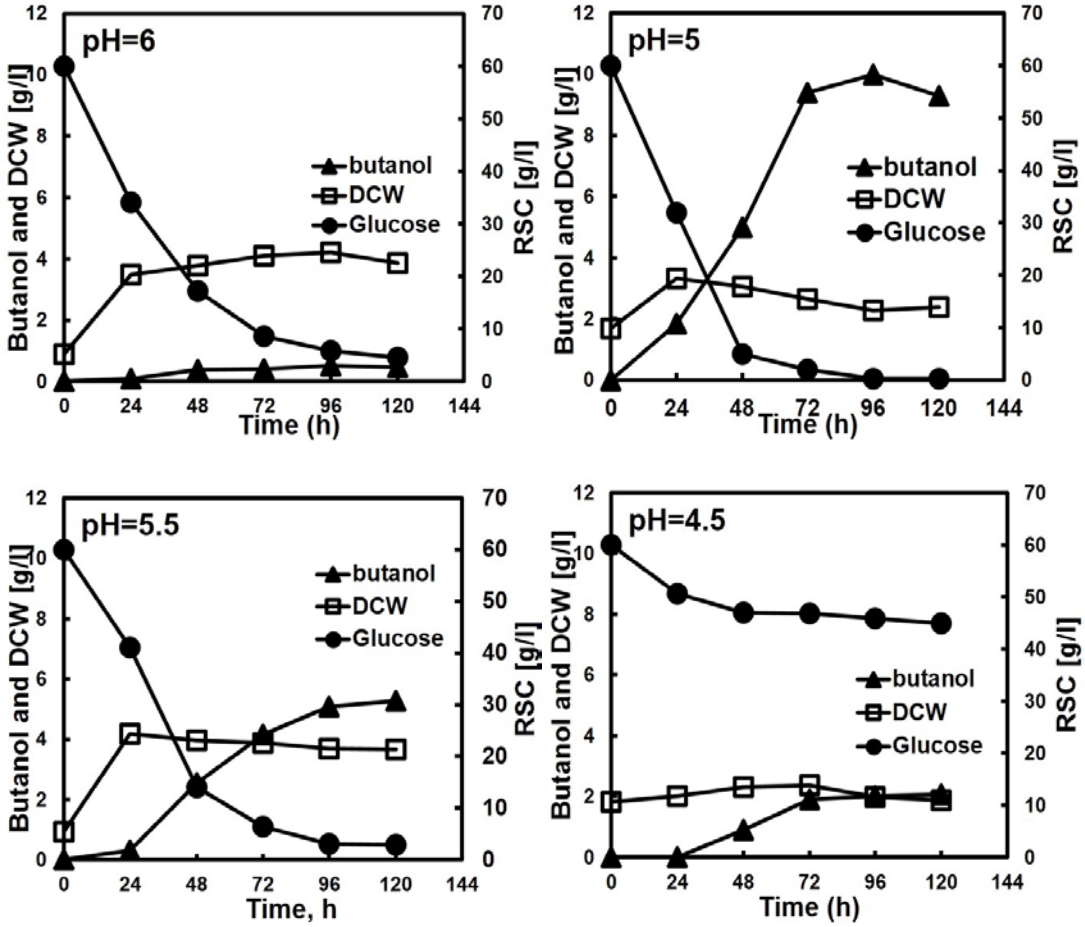


Figure 3.1 Dry cell weight, residue glucose concentration and butanol production of strain BOH3 at various pH values. RSC: residual sugar concentration.

Results indicated that little butanol was produced at pH of either 4.5 or 6.0, but cell density at pH 6.0 was similar with that of pH 5.5, which is the highest, ~4.2 g/L. The highest butanol concentration was obtained at pH 5.0, which coincided with only trace amounts of residual glucose. Together, these results suggest that the optimum pH for cell growth or butanol production is different. This conclusion was confirmed during development of a pH shift strategy to increase butanol titer in which more pH values were tested (Figure 3.2). Specific cell growth rate and specific butanol production rate at different pH were calculated using the following equations:

$$\mu_{cell} = \frac{1}{x}\frac{dx}{dt} = \frac{\ln(x_2/x_1)}{t_2 - t_1}$$

Equation 3.1 Specific cell growth rate, where x (g/L) denotes biomass concentration and t (h) denotes time.

$$\mu_{But} = \frac{1}{x}\frac{dp}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta p}{\Delta t}$$

Equation 3.2 Specific butanol production rate, where p (g/L) denotes butanol concentration and t (h) denotes time.

Where specific cell growth rate (μ_{cell}) refers to the increase in cell mass per unit time (expressed in gram cells per gram cells per hour), and specific butanol production rate (μ_{but}) refers to the increase in butanol concentration per unit time by unit cell mass (expressed in grams per liter butanol per gram cells per hour), x represents cell mass, t represents time, and p represents butanol concentration. By comparing specific rates effects of varying cell mass can be excluded, resulting in a more precise evaluation of the influence of pH on butanol production.

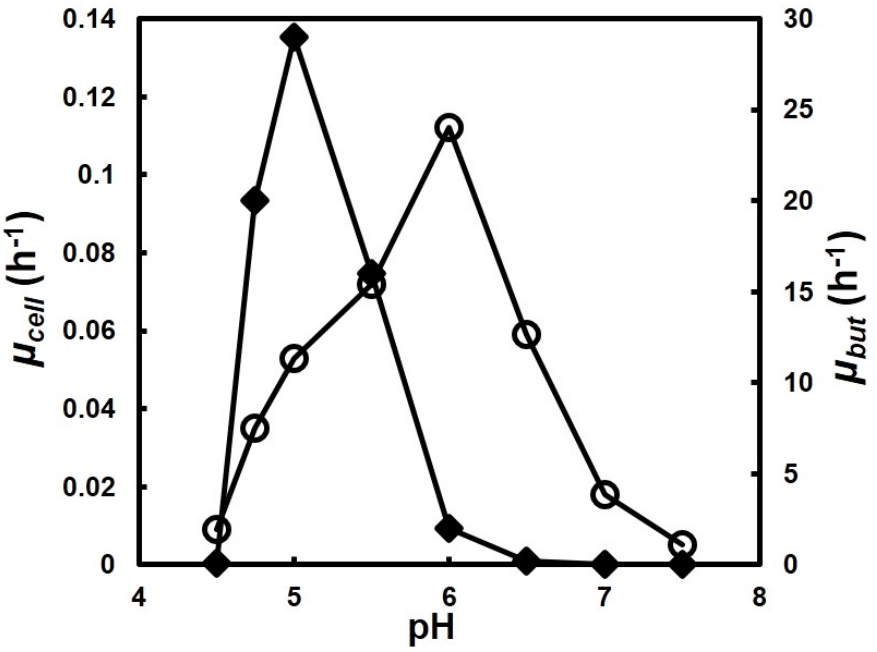


Figure 3.2 Effects of pH on specific cell growth rate and specific butanol production rate. Cell μmax: open circle symbol; butanol μmax: solid square symbol.

As shown in Figure 3.2, μ_{but} increased almost linearly from pH 4.5 to 5.0 and reached the highest value (~ 30 h^{-1}) at pH 5.0, then decreased sharply. No butanol production occurred at pH values lower than 4.5 or higher than 6.5, indicating a very narrow pH range (4.8-5.3) suitable for butanol production by strain BOH3. On the other hand, the optimum pH for cell growth was 6.0, where μ_{cell} reached 0.112 h^{-1}. Cell growth was severely inhibited at pH values lower than 4.5 or higher than 8.0. Hence, in order to increase butanol production by strain BOH3 it would seem to be beneficial to maintain the pH at a relatively higher value (e.g., pH 5.5-6.0) to maximize cell growth during the early phases of fermentation, while a relative lower pH value (e.g., pH 5.0) would be more appropriate at mid- to late- fermentation phases. The optimal pH-shift strategy can therefore be set up as the following: pH is controlled at 6.0 during the first 6 h (excluding lag phase), after which the pH is allowed to drop to 5.0 as the culture progresses and maintained at 5.0.

3.4.2 Effect of metabolic cofactors and regulators on butanol production by strain BOH3

To further improve butanol production and glucose utilization, various concentrations of related factors were tested. The roles of metabolic cofactors and regulators affecting sugar utilization and butanol production are listed in Table 3.1. Experiments were conducted in serum bottles without pH control and results are shown in Table 3.2. Compared with controls, in some cases cofactors improved butanol production up to 500% (2 g/L L-Asparagine), while some had negative effects on fermentation processes (neutral red). To provide optimum conditions for cell growth and butanol production, different nitrogen sources, including NH_4Cl, $(NH_4)_2SO_4$ and NH_4AC, were also studied. As shown in Table 3.3, NH_4AC was found to be the optimum nitrogen source for butanol production among tested options. Medium composition of batch bioreactor fermentation was revised based on optimal values obtained through these experiments.

Table 3.1 Related functions of metabolic cofactors and regulator, affecting sugar utilization and butanol production.

Factor	Related functions
Mg^{2+}	Pyruvate kinase, Hexokinase, etc.
Cu^{2+}	Enzyme cofactor
Zn^{2+}	Aldolase & dehydrogenase
L-Cysteine	Reductant
L-Asparagine	Regulate redox
L-Glutathione	Regulate redox, resistant to acid stress
Neutral red	Use as electron transport carrier

Table 3.2 Relative effectiveness and optimal value of metal cofactor and regulator affecting sugar utilization and butanol production. Optimal values refers to the cofactor concentrations that strain can reach the highest butanol titer.

Cofactor	Effectiveness	Optimal values
$MgSO_4$ (g/l)	high	0.2
$CuCl_2$ (μg/l)	high	50
$ZnCl_2$ (μg/l)	high	100
L-Cysteine (g/L)	high	0.1
L-Asparagine (g/L)	very high	2
L-Glutathione (g/L)	high	0.1
Neutral red (g/L)	low	N.A.

Table 3.3 Relative effect of nitrogen source on butanol production

Nitrogen sources	Effectiveness	Optimal values (g/L)
NH_4CL	high	2.0
$(NH_4)_2SO_4$	high	2.0
NH_4AC	very high	2.2

3.4.3 Addition of nicotinic acid increased reducing cofactor availabilities

Nicotinic acid (soluble and transportable) is a precursor of NADH and NADPH formation (Figure 3.3) (Li et al. 2014). Addition of nicotinic acid may enhance butanol production by increasing the availability of the reducing

cofactors (NADH and NADPH). To study the effects of nicotinic acid on cell growth and butanol production in strain BOH3, varying amounts of nicotinic acid (0-20 mg/L) were added to the fermentation medium of serum bottles. As shown in Figure 3.4, DCW and butanol production reached a maximum value of 4.8 g/L and 12.3 g/L, respectively, with the addition of 10 mg/L nicotinic acid. This is a 50% and 84% increase compared to the control (absence of nicotinic acid) DCW and butanol production, respectively.

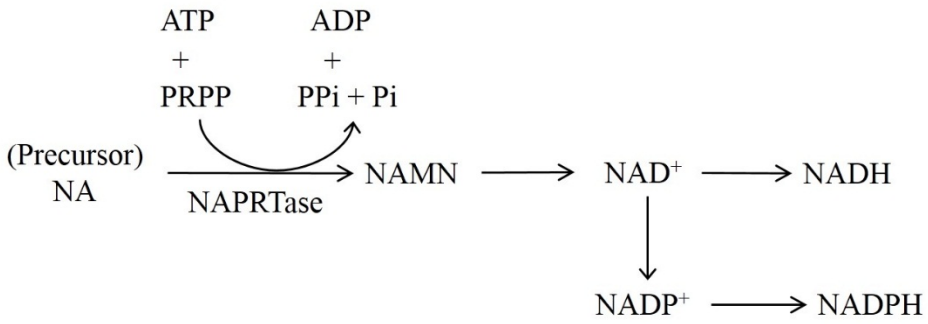


Figure 3.3 Salvaging synthesis of NADH and NADPH from precursor nicotinic acid (NA). NAPRTase: Nicotinic acid phosphoribosyl transferase; NAMN: Nicotinic acid mononucleotide; NAD+: Nicotinamide adnine dinucleotide; NADH: Nicotinamide adnine dinucleotide hydrogen; NADP+: Nicotinamide adnine dinucleotide phosphate; NADPH: Nicotinamide adnine dinucleotide phosphate hydrogen.

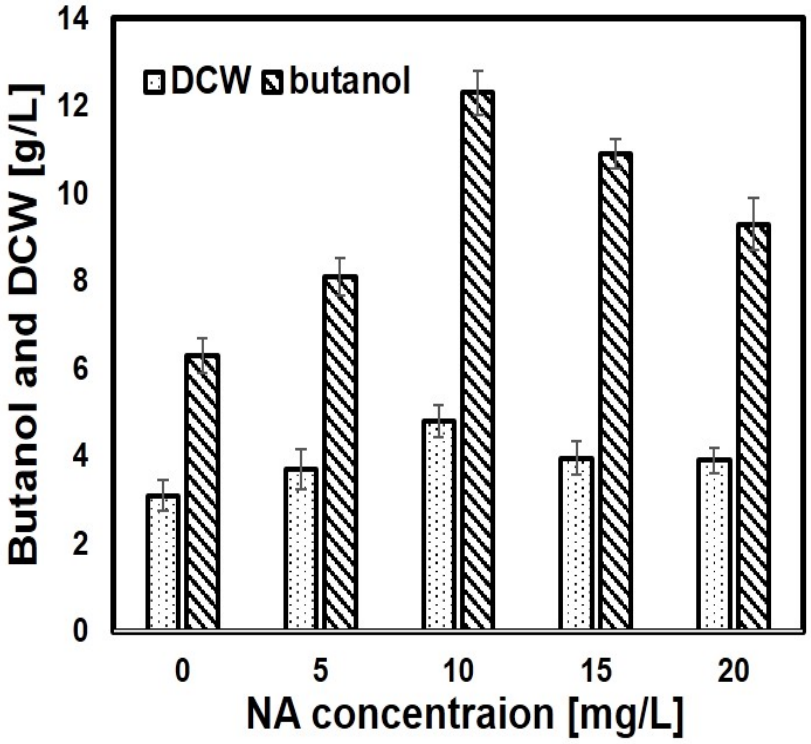


Figure 3.4 Effects of nicotinic acid (NA) on cell growth (dry cell weight) and butanol production of *Clostridium* sp. strain BOH3. Experiments were conducted in biological triplicate, samples were taken every 24 h and the maximum value for DCW and butanol under each NA concentration are presented. Error bars are calculated from independent fermentation runs.

Cell growth was not much different between treatment and non-treatment, but NADH and NADPH were quite different (Table 3.4). Compared to control cultures, the total NADH and NADPH levels increased 1.95 and 2.27 times, respectively, after addition of nicotinic acid to the medium. The ratio of cofactors in active forms also increased, from 0.78 to 2.31 ($NADH/NAD^+$) and from 0.36 to 1.23 ($NADPH/NADP^+$) (Table 3.4), indicating more reducing equivalents were produced in the form of NADH and NADPH with the addition of the nicotinic acid. This may due to the lack of NADH or NADPH in the reducing cofactor-dependent production system stimulated more reduction of NAD^+ and $NADP^+$.

Table 3.4 Variation of cofactors to grow strain BOH3 in a bioreactor.

Parameters	**Control**[a]	**With NA**[b]
DCW (g/L)	5.4 ± 0.3	6.6 ± 0.4
Total NADH (µmol/g DCW)	82.6 ± 5.0	244.1 ± 13.2
Total NAD^+ (µmol/g DCW)	105.8 ± 5.7	105.6 ± 6.3
Total NADPH (µmol/g DCW)	17.5 ± 0.9	57.3 ± 3.7
Total $NADP^+$ (µmol/g DCW)	48.9 ± 2.9	46.5 ± 2.8
NADH / NAD^+ ratio	0.78 ± 0.06	2.31 ± 0.12
NADPH / $NADP^+$ ratio	0.36 ± 0.01	1.23 ± 0.07
Sum of NAD(H/$^+$) (µmol/g DCW)	188.4 ± 9.5	454.1 ± 25.1
Sum of NADP(H/$^+$) (µmol/g DCW)	66.4 ± 3.8	103.8 ± 6.2

[a] Grown in a two-stage pH-shift bioreactor without addition of NA

[b] Grown in a two-stage pH-shift bioreactor with addition of 10 mg/L NA

The *in vitro* physiological profiles of NADH and NADPH were also monitored in crude cell extracts from cells in the bioreactor. While similar patterns were exhibited for both cofactors, the NADPH level was found to be lower and peaked 18 h earlier than NADH levels, regardless of nicotinic acid addition. The different peak time of NADH and NADPH may be due to their different cellular functions. NADPH provides energy in anabolic biological reactions, such as fatty acid and nucleic acid synthesis, while NADH provides energy for catabolic reactions (Ying 2008). The levels of NADH and NADPH were 96 % and 27 % higher in the presence of nicotinic acid than in controls, respectively (Figure 3.5).

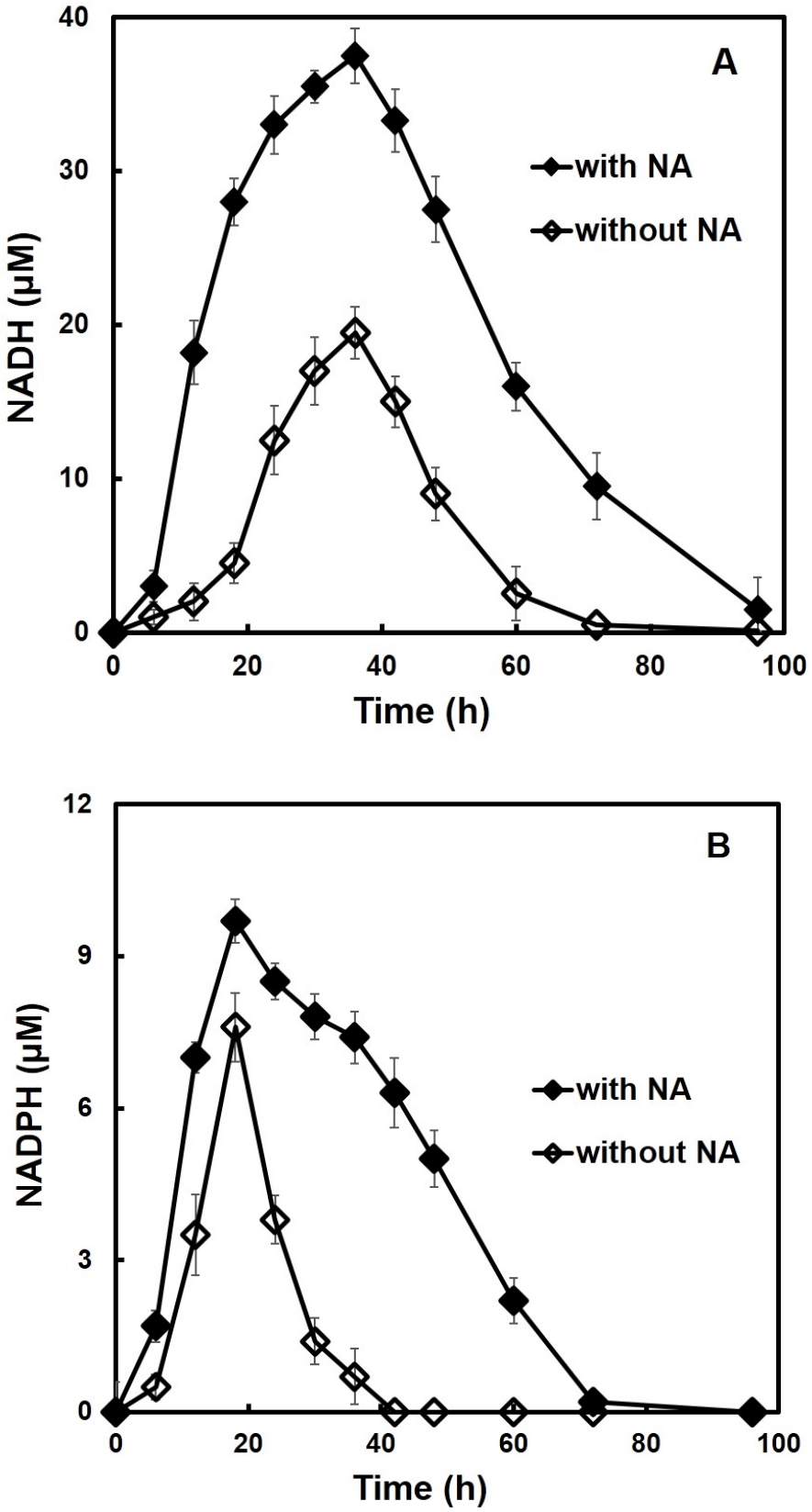


Figure 3.5 In vitro physiological profiles of reducing cofactors NADH and NADPH with or without nicotinic acid addition under a pH shift strategy. A: NADH profiles; B: NADPH profiles. Error bars are calculated from analytical errors.

3.4.4 Enhanced butanol production triggered by elevated availabilities of reducing cofactors

The addition of nicotinic acid to the fermentation medium was found to increase NADH and NADPH availability, thereby increasing butanol yield from ABE fermentation by strain BOH3. In order to gain more insight into the physiological changes of strain BOH3, batch fermentations utilizing the pH shift strategy were carried out with or without nicotinic acid addition in the bioreactor. The addition of 10 mg/L nicotinic acid increased cell growth of strain BOH3 by 11 % and reduced the doubling time by ~40 % (Figure 3.6). More importantly, butanol production by strain BOH3 reached 18.7 g/L (27.8 g/L total ABE production), a 16 % increase as compared to cultures without the addition of NA (Figure 3.7) and 179 % greater than cultures grown without the pH shift (6.3 g/L). Less acid were produced in the presence of nicotinic acid as well, indicated by the higher biosolvent/bioacid ratio (Figure 3.8). As precursors of biosolvent formation, bioacids are normally produced in the acidogenic phase and subsequently reassimilated in the solventogenic phase. The highest value of butyric acid (4.3 g/L) was reached at 30 h of fermentation in the presence of nicotinic acid, 18 h sooner than in the control (6.3 g/L) without nicotinic acid addition (Figure 3.7A and B). Although butyric acid was reassimilated at the end of fermentation in both conditions, more acetic acid was accumulated in the control batch, resulting in inhibition of cell growth and an increase in residual carbon source. The glucose consumption rate, butanol yield and butanol productivity were 9 %, 7% and 14 % higher, respectively, compared to the corresponding values of the control without nicotinic addition (Figure 3.9).

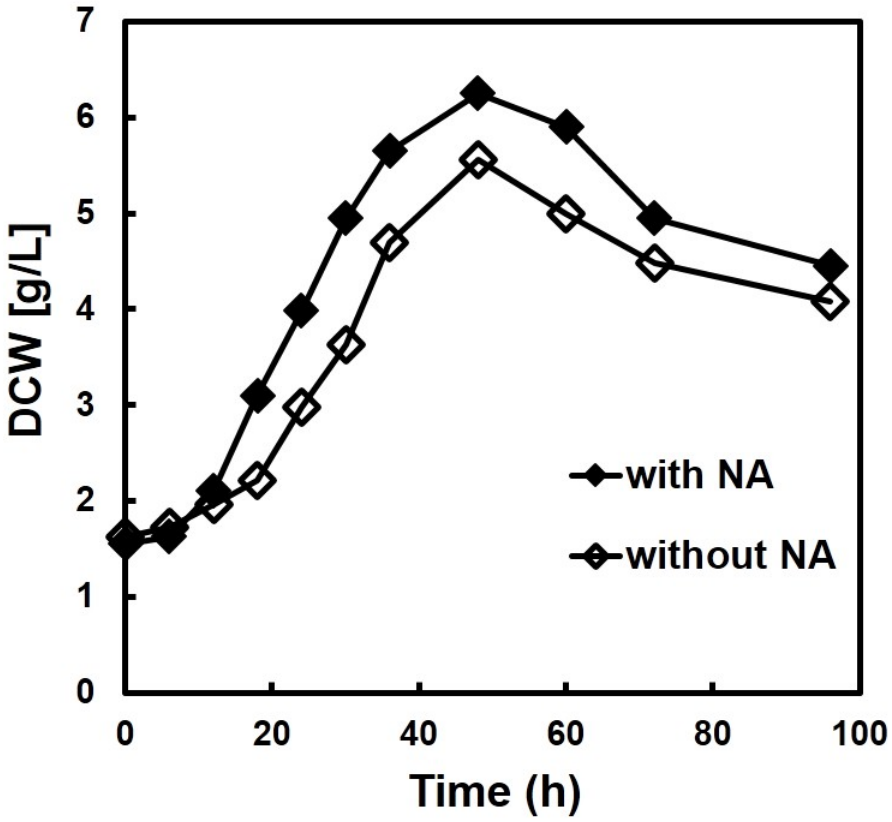


Figure 3.6 Cell growth of batch fermentation in a bioreactor by *Clostridium* sp. strain BOH3 with or without and addition of NA.

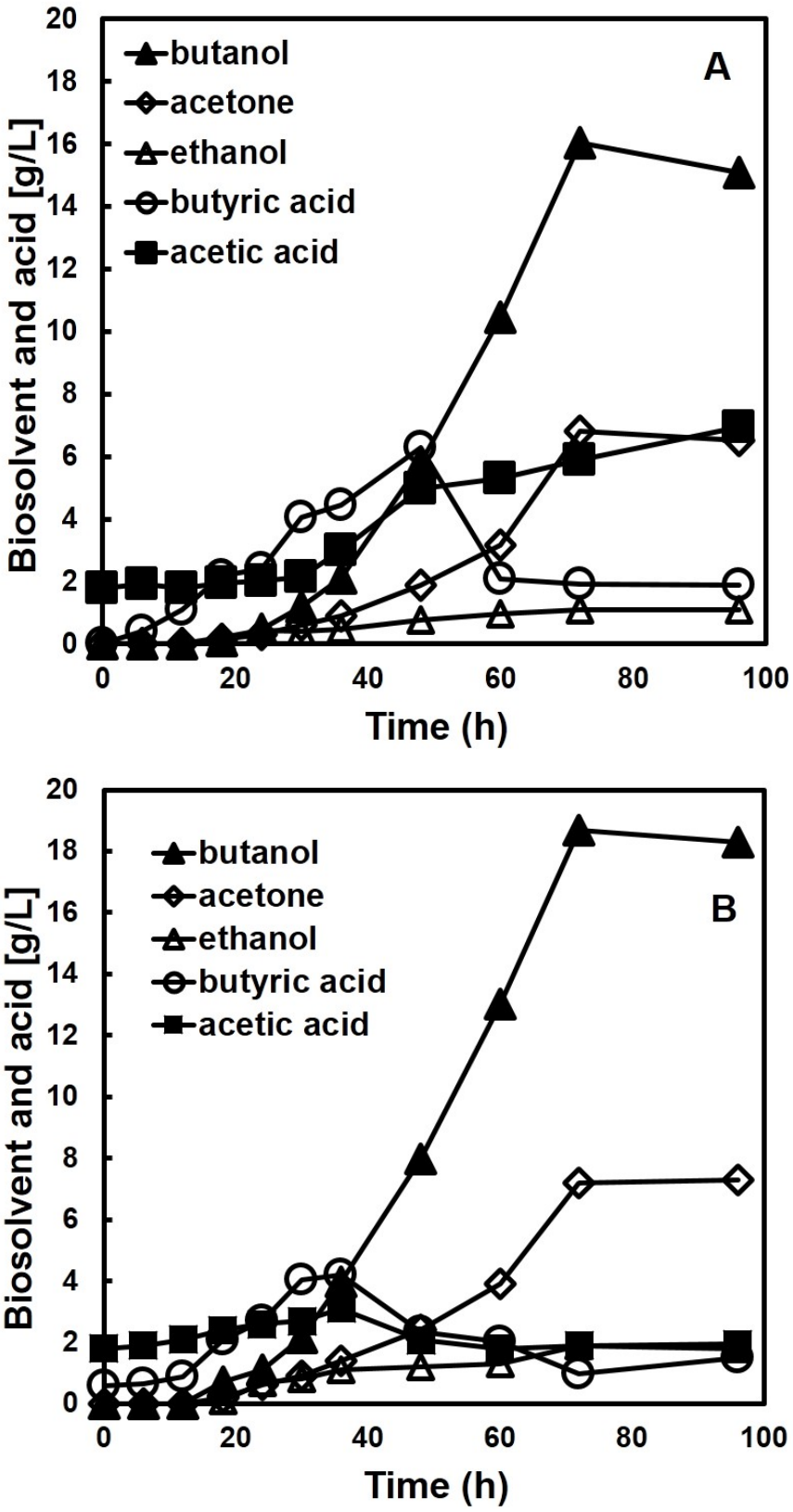


Figure 3.7 Biosolvent and volatile fatty acids production (A) without NA addition and (B) with NA addition of batch fermentation in a bioreactor by *Clostridium* sp. strain BOH3.

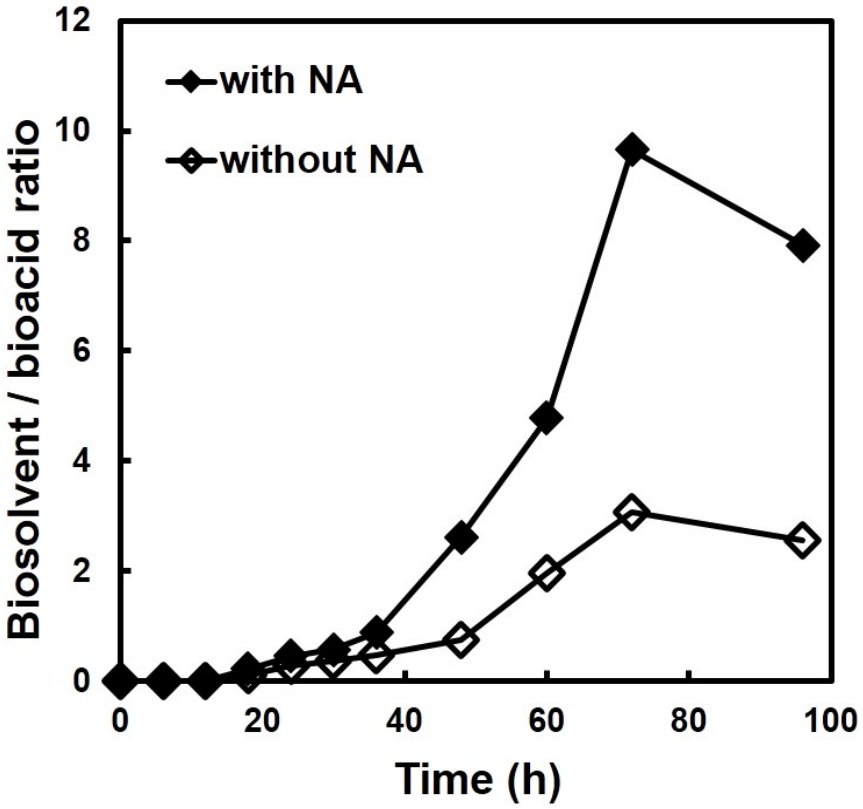
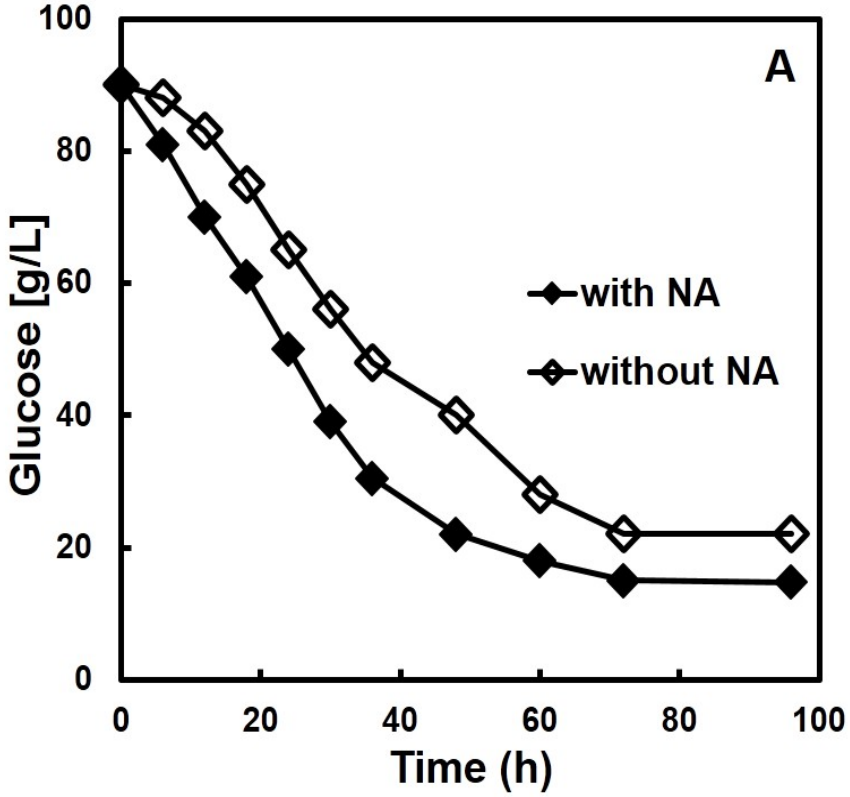


Figure 3.8 Ratios of total biosolvent to bioacid concentration of batch fermentation in a bioreactor by *Clostridium* sp. strain BOH3 with or without and addition of NA.



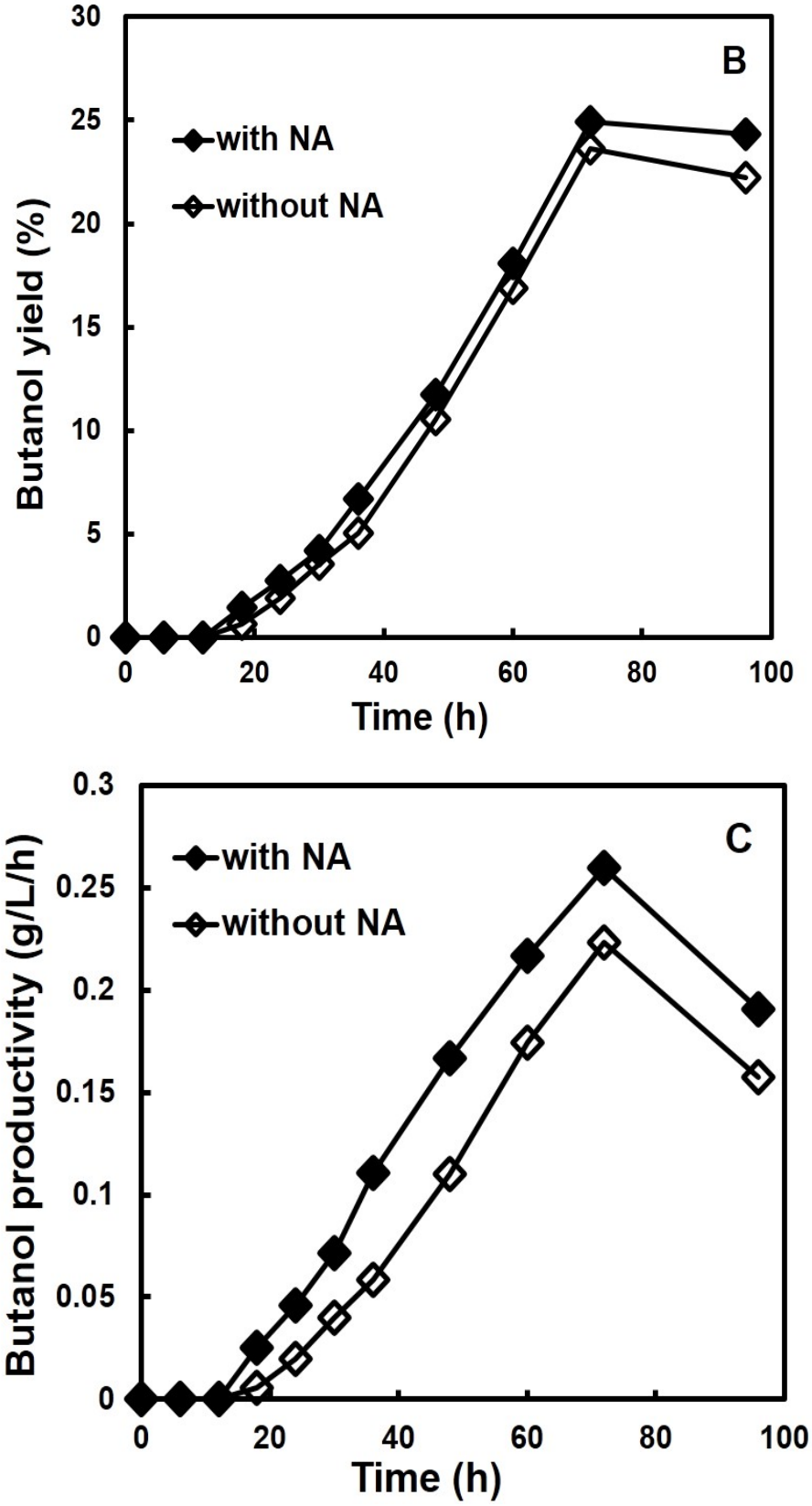


Figure 3.9 (A) Glucose utilization, (B) average butanol yield and (C) average butanol productivity of batch fermentation in a bioreactor by *Clostridium* sp. strain BOH3 with or without and addition of NA.

With increased availability of NADH and NADPH in a 3-L bioreactor operating under a pH shift strategy, the wild-type strain BOH3 achieved 18.7 g/L butanol with a yield of 24.6 % and a productivity of 0.26 g/L·h, which is at least 25% higher than previously reported wild-type strains and comparable to genetically-modified strains (Table 3.5). In order to achieve high butanol yield and productivity, most studies focus on the utilization of metabolic engineering tools such as gene overexpression (Harris et al. 2002; Tomas et al. 2003; Yu et al. 2011), target gene disruption (Jiang et al. 2009b; Shao et al. 2007), genome shuffling (Mao et al. 2010) and regulation of corresponding genes (Harris et al. 2001; Sillers et al. 2008). Among these studies, *Clostridium acetobutylicum* mutant Rh8 was able to produce 15.3 g/L butanol with ~21% butanol yield and 0.26 g/L·h butanol productivity in a 4-L bioreactor after chemical mutagenesis and genome shuffling (Mao et al. 2010). Another study reported that 15 g/L butanol could be achieved through the artificial driving forces created by NADH and acetyl-CoA accumulation in metabolically engineered *Escherichia coli* (Shen et al. 2011). In our study, butanol production was improved by increased intracellular levels of NADH and NADPH as a result of the addition of soluble nicotinic acid that can be transported to the cells. Our study demonstrated that the availability of reducing cofactors (e.g., NADH and NADPH) determines the amount of butanol generated by a wild-type *Clostridium* sp. strain BOH3 possessing a native cofactor-dependent enzymatic system.

Table 3.5 Comparison of butanol production by wild type and engineered strains from glucose.

Strain	**Relevant genotype**	**Butanol titer, g/L**	**Butanol yield, g/g**	**Butanol productivity, g/L·h**	**References**
C. acetobutylicum DSM 1731	Wild type	12.4	0.170	0.269	(Mao et al. 2010)
C. acetobutylicum Rh8	Genome-shuffled	15.3	0.217	0.254	(Mao et al. 2010)
C. sp. strain G117	Wild type	13.5	0.304	0.141	(Chua et al. 2013)
C. acetobutylicum JB200	Evolutionary engineered	19.1	0.21	0.24	(Xue et al. 2012)
		~21	~0.21	~0.5	(Xu et al. 2015)
C. tyrobutyricum ATCC 25755	Metabolic engineered	10.0	0.27	0.026	(Yu et al. 2011)
C. acetobutylicum M5	Metabolic engineered	11.1	~0.141	0.292	(Sillers et al. 2008)
C. sp. strain BOH3	Wild type	18.7	0.246	0.260	This study

Under optimized conditions (10 mg/L NA, pH shift and optimal concentrations of metabolic cofactors and regulators), *Clostridium* sp. strain BOH3 has been maintained to produce over 18 g/L of butanol stably for more than one year. Improved butanol production can likely be attributed to changes in metabolite flux caused by the increased availability of reducing cofactors and the concomitant shift in metabolic direction towards the production of more reduced metabolites (e.g., butanol) as evidenced by the improved butanol to acetone ratio (11%) and butanol to acid ratio (292%) at the end of the fermentation (Figure 3.7 and Figure 3.8). These results suggest that adjusting the levels of NAD(P)H could be an efficient approach to improve butanol production. Manipulating cofactors may provide an additional means to determine final cellular metabolites distribution.

3.5 Conclusions

The addition of the precursor nicotinic acid to the medium led to an increased availability of NADH and NADPH allowing a higher butanol production and yield by the wild-type *Clostridium* sp. strain BOH3. The improved butanol production suggests that redistributing metabolic flux to butanol via manipulations of reducing cofactors and pH could be an alternative approach to reach the same goals that can be achieved through metabolic engineering.

CHAPTER 4

Simultaneous Fermentation of Pentose and Hexose without Carbon Catabolite Repression by *Clostridium* Species Strain MF28

Strain BOH3 shows its capability of achieving high butanol titer from glucose. Besides glucose, pentoses such as xylose and arabinose are also generated during pretreatment and hydrolysis of lignocellulose. One of the obstacles to utilize lignocellulosic biomass by most *Clostridium* species is inefficient co-utilization of pentose and hexose elements that exist in the hydrolysates. So far, strain BOH3 was the only wild-type strain that reported to simultaneous ferment glucose and xylose to butanol (Xin et al. 2014). To broaden our knowledge, the capability of a newly isolated strain MF28 in simultaneously fermenting pentose and hexose is investigated in this chapter.

4.1 Abstract

Efficient conversion of lignocellulose-derived sugars to butanol by solventogenic *Clostridium* is impeded by inherently inefficient pentose metabolism and glucose induced carbon catabolite repression (CCR). In contrast to most solventogenic *Clostridium*, strain MF28 is capable of simultaneously fermenting glucose, xylose and arabinose to produce butanol without observable CCR. Compared to single carbon sources, strain MF28 produced a higher amount of butanol (14.40 ± 0.30 g/L) with higher butanol yield (0.30 g/g) and productivity (0.30 g/L·h) when fed with a mixture of glucose, xylose and arabinose. Moreover, strain MF28 can finish majority of the fermentation within 48 h, resulting in a high productivity. These observations offer a better solution for economical biobutanol production.

4.2 Introduction

Fermentative biological butanol production by solventogenic *Clostridium* was once a world-wide industry, but was almost completely replaced by more economically viable chemical synthesis processes since 1950s (Jones and Woods 1986). However, biological butanol production through the ABE fermentation pathway, especially from renewable lignocellulosic biomass, has attracted increasing interest in recent years due to highly fluctuating prices in the petrochemical market (Ezeji et al. 2007b; Fatehi 2013; Olson et al. 2012; Ranjan and Moholkar 2012).

The high substrate cost, which represents over 70 % of the total production cost of biobutanol, is one of the major obstacles in biological butanol production (Gu et al. 2011). Utilization of lignocellulosic biomass as a fermentation substrate provides a promising solution for cost-effective biobutanol production since it is abundant, renewable and cheap (Jang et al. 2012b; Jurgens et al. 2012; Kumar et al. 2012). However, inefficient co-fermentation of the three major reducing sugars derived from liglocellulosic biomass, glucose, xylose and arabinose, typically leads to incomplete sugar consumption and poor butanol production (Yu et al. 2015a). The inefficiency of simultaneous fermentation of pentose and hexose is due to carbon catabolite repression (CCR), the phenomenon that utilization of alternate carbon sources (e.g. xylose, arabinose) will be inhibited in the presence of preferred carbon sources, e.g. glucose (Aristidou and Penttilä 2000; Goerke and Stulke 2008; Mitchell 1998; Yao and Shimizu 2013). To most efficiently convert lignocellulosic biomass to butanol, it is hence essential to eliminate CCR. CCR is mediated by a catabolite control protein A (CcpA) which is a

conserved regulator in Gram-positive bacteria, not only involved in carbon metabolism but also solvent production and sporulation (Henkin et al. 1991; Warner and Lolkema 2003). Several metabolically engineered *Clostridium* strains have been reported to simultaneously ferment xylose and glucose to butanol (Xiao et al. 2011; Xiao et al. 2012; Yu et al. 2015a). For example, Ren et al. disrupted *ccpA* in *Clostridium acetobutylicum* and a co-fermentation of xylose and glucose to butanol was achieved. However, a concomitant accumulation of butyric acid was observed, which could result in a reduced substrate metabolism and defective cell growth (Ren et al. 2010). The inactivation of *ccpA* may also result in down-regulation of *abrB310*, which is likely associated with the transition from acidogenic phase to solventogenic phase in *C. acetobutylicum* ATCC 824 (Scotcher et al. 2005). The manipulation of *ccpA* may induce a cascade of changes in solventogenic metabolic activity and lead to undesirable phenotypes, such as butyric acid accumulation, a prolonged acidogenic phase as well as delayed solvent production (Scotcher et al. 2005). For wild-type strain, although the butanol production from xylose is comparable to that of glucose, a lower butanol titer and yield was obtained when using a mixture of xylose and glucose as fermentation substrate, indicating inefficient sugar consumption (Xin et al. 2014). As such, there is need for development of a robust strain that can simultaneously ferment pentose and hexose with high butanol titer, yield and productivity.

This study reports the development of a process for simultaneous fermentation of glucose, xylose and arabinose by a wild-type *Clostridium* sp. strain MF28, which was isolated from a two year old spent mushroom

substrate in Singapore (Li and He 2016). The role of *ccpA* in the regulation of transition from acidogenic phase to solventogenic phase was also studied through the investigation of *abrB* expression levels in strain MF 28.

4.3 Materials and methods

4.3.1 Culture medium and cultivation

Clostridium sp. strain MF28, a generous gift from Dr. Li Tinggang (Li and He 2016), was used in this study. Batch cultures were cultivated under anaerobic conditions in 160 ml serum bottles containing 50 ml defined mineral salts medium. Media for fermentation experiments was composed as described in chapter three. D-(+)-glucose, D-(+)-xylose and L-(+)-arabinose (hereinafter called glucose, xylose and arabinose, respectively) were used as carbon sources in mixtures for fermentation. Sterile butyric acid stock solution was injected separately into sealed and autoclaved culture bottles to a concentration of 3 g/L prior to inoculation. All experiments were carried out using a total carbon source concentration of 60 g/L, unless specified otherwise. Concentrated glucose/ xylose/ arabinose stock solutions were sterilized separately and injected to autoclaved cultivation medium at different concentrations as indicated. Seed cultures were grown in 50 ml defined medium amended with carbon sources for ~20 h at 35 °C and sub-cultured 4 % [v/v] into batch cultures for further fermentation study. Cultures were incubated in an orbital shaker at 150 rpm at 35 °C. Initial pH was adjusted to 6.5 and maintained at the desired pH using 9 M sodium hydroxide solution thereafter until cessation gas production. Samples were taken at regular intervals and analyzed for cell growth using a UV-visible spectrophotometer

and the presence of acetone, butanol, ethanol, acetic acid and butyric acid were quantified using GC-FID as described below.

4.3.2 Butanol inhibition on cell growth of glucose and xylose fed cultures

Duplicate cultures were cultivated for 10 h (exponential phase) at 35 °C in defined mineral salt medium amended with either 60 g/L glucose or 60 g/L xylose prior to amendment with butanol to specified concentrations (0, 2, 4, 6, 8, 10, 12 and 15 g/L). Samples were taken at regular intervals and cell growth was measured by optical density. Optical density of samples was normalized to control samples (no exogenous butanol added) at each time point.

4.3.3 Relative transcription levels of *abrB*

There is one *abrB* in strain MF28 shares 85 % similarity with *abrB310* in *C. acetobutylicum* ATCC 824. The relative transcription level of this *abrB* was thus analyzed to illustrate the mechanism of the one-phase fermentation profile in strain MF28. Culture of strain MF28 was harvested at desired points by centrifuging at 140,000 rpm, 4 °C for 10 min (in duplicate). Supernatant was discarded and cell pellets resuspended in 300 µL trizol and stored at -50 ˚C until further processing. Total RNA was extracted from samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Residual genomic DNA was removed using the RNase-free DNase Kit (Qiagen, Hilden, Germany). cDNA was generated from extracted RNA using the QIAGEN Sensiscript Kit according to the manufacturer's protocol. cDNA samples were used as template for PCR of the transition from acidogenic phase to solventogenic phase associated gene *abrB* and the

peptidase T (*pepT*) gene. The *pepT* gene is a housekeeping gene used as an internal control of cDNA synthesis efficiency and PCR amplification, while luciferase was used as an internal control of RNA extraction efficiency. The specific primer used for *abrB* and *pepT* were listed in Table 4.1. Final concentration of primer was 0.4 µM in each qPCR reaction. After activating the enzymes in master mix at 98˚C for 3 min, qPCR reaction entered a thermal cycle consisting 40 cycles of 95 ˚C for 20 s and annealing temperature for 30 s. Melting curve was also measured for each sample.

Table 4.1 Specific primer for gene *abrB* and *pepT* in strain MF28

Gene	**Forward (5'-3')**	**Reverse (5'-3')**	**Annealing temperature**
abrB	TGTAGACGGAGAGC AAATAATCT	TTGCATCTCCACAG AAGATACAA	49˚C
pepT	TGATGGAGGCGAGG AAGGTG	CATTGTATTCTTTGC AGACCCTGG	51˚C

To test the transcription level of *abrB* in strain MF28, cDNA generated from RNA extracted from cultures of both strains was used as template for qPCR. qPCR was performed on an ABI 7500 Fast real-time PCR system (ABI, Foster City, CA) using QuantiTect SYBR green (Qiagen, GmBH, Germany). Data was analyzed using iQ5. Relative abundance of transcripts was calculated based on cycle threshold value (Ct value) of target genes and normalized to the abundance of *pepT*.

4.3.4 Analytical methods

Volatile fatty acids (i.e., acetic and butyric acids) and biosolvents (i.e., acetone, ethanol and butanol) were measured on a GC using the same method as described in chapter three, while residual glucose, xylose or arabinose

concentrations were measured using a HPLC (model 1260 Infinity, Agilent Technologies, USA) equipped with an Aminex HPX-87P column (1,300 × 7.8 mm, 9 μm) (Bio-Rad, U.S.) and a refractive index detector (RID). Compositions of gaseous products were measured using an Agilent GC7890 equipped with a thermal conductivity detector (TCD) as described previously (Bramono et al. 2011). 1.3 ml samples were withdrawn from cultures at pre-determined time points under sterile conditions. Samples were centrifuged at 4 °C at 14000 rpm for 15 min and 475 μL of supernatant was taken out and mixed with 2 M HCL (25 μL) in 2 ml GC vials for fatty acids and biosolvents analysis, while another 400 μL of the supernatant was transferred into a separate vial for HPLC.

GC analysis was carried out as described in chapter three.

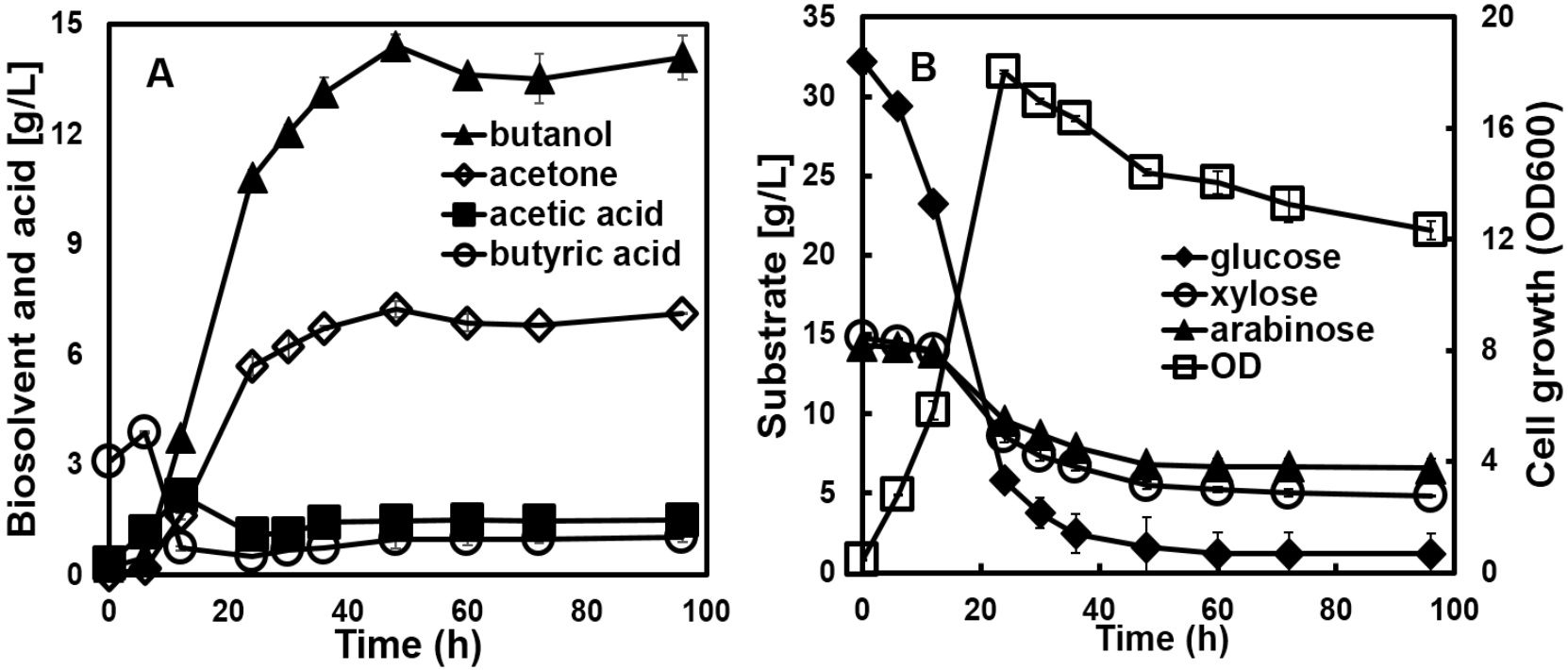
HPLC analysis of samples was carried out by injecting 20 μL of samples into the column equilibrated with deionized water as the mobile phase maintained at a flow rate of 0.6 ml/min and 80 °C oven temperature. Standard solutions containing known quantities of glucose, xylose and arabinose were used for preparation of corresponding calibration curves for identification and quantification of residual sugars in samples. Data analysis was done using Agilent ChemStation.

Cell density in the fermentation broth was determined by measuring optical density on a UV-visible spectrophotometer at 600 nm (V-660; UV-VIS Spectrophotometer, Jasco, Japan).

4.4 Results and discussion

4.4.1 Butanol and hydrogen production from mixed carbon sources by strain MF28

To evaluate the performance of pentose utilization in the presence of glucose by strain MF28, batch fermentations with different glucose-xylose-arabinose (GXA) ratios (2:1:1, 1:2:1 and 1:1:1) were carried out in serum bottles. Results show that these three sugars were simultaneously utilized by strain MF28 at all tested ratios (Figures 4.1B, D, and F). However, during early fermentation (0-12 h), the utilization of xylose and arabinose was found to be less efficient than glucose no matter in what ratios (Table 4.2). A possible explanation for this is that the initial steps in pentose metabolism may be rate-limiting. The initial steps of xylose uptake, including transport and isomerization of xylose, and phosphorylation of xylulose into xylulose-5-phosphate, have been suggested as putative rate-liming steps in xylose catabolism in *Clostridium acetobutylicum* and many other microbes (Jojima et al. 2010; Ren et al. 2010; Xiao et al. 2011).


A
B
Biosolvent and acid [g/L]
Substrate [g/L]
Cell growth (OD600)
Time (h)
Time (h)
butanol
acetone
acetic acid
butyric acid
glucose
xylose
arabinose
OD
0
3
6
9
12
15
0
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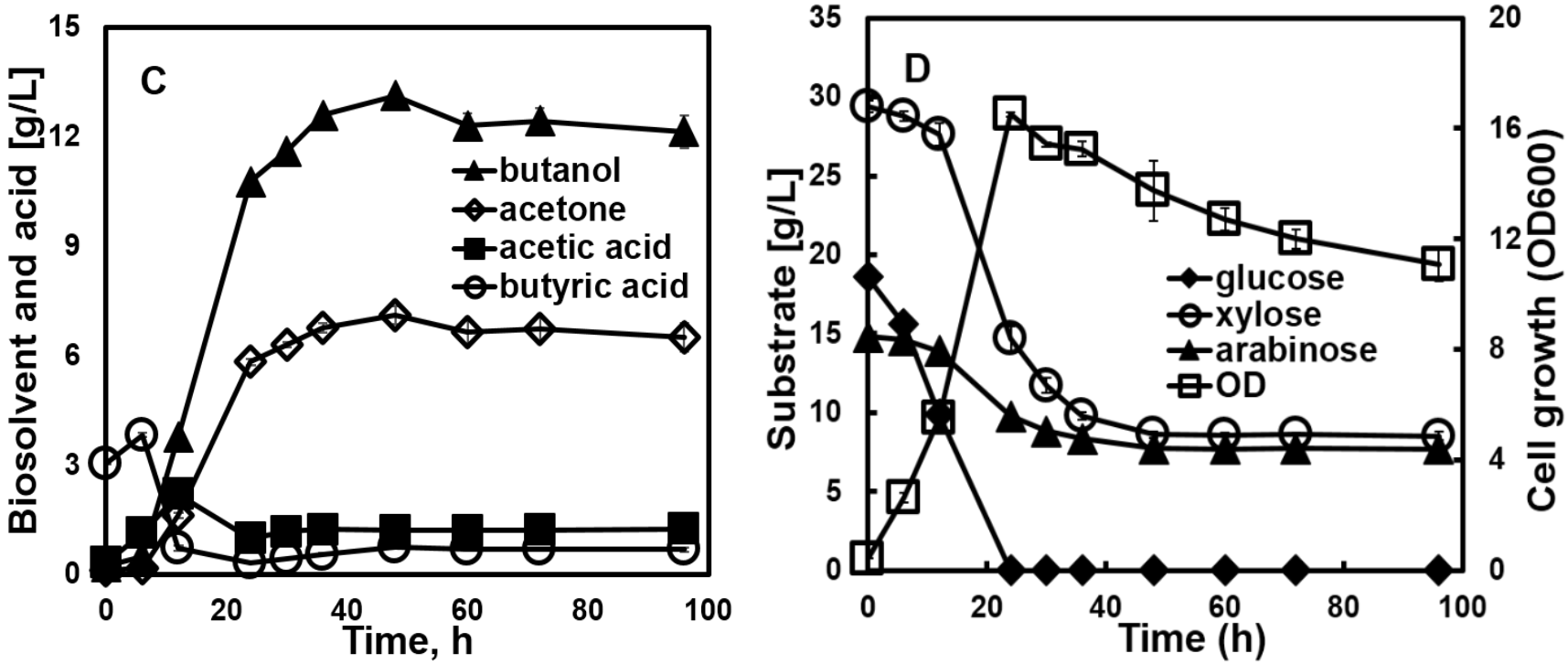
C
Biosolvent and acid [g/L]
Time, h
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Substrate [g/L]
Cell growth (OD600)
Time (h)
glucose
xylose
arabinose
OD

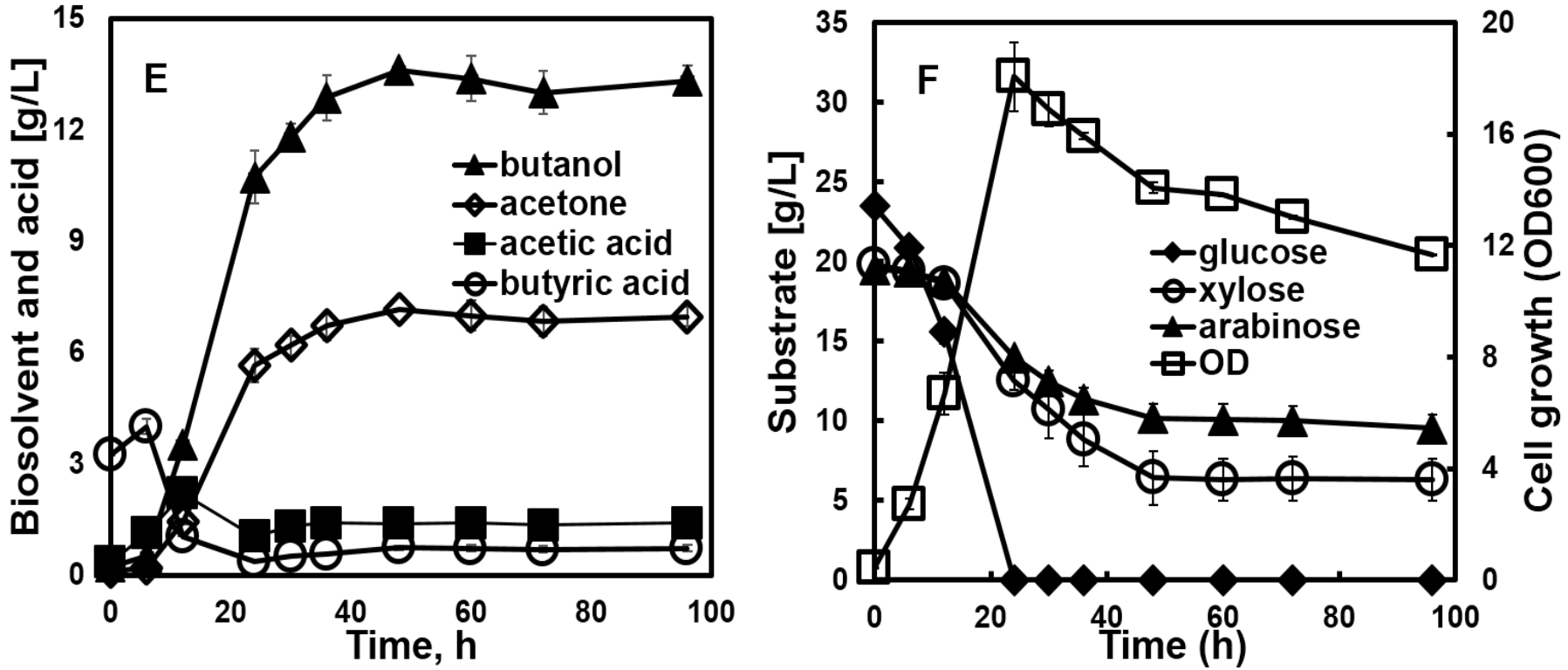


Figure 4.1 Biosolvent and acid production, cell growth and carbon source utilization by *Clostridium* sp. strain MF28 fed with glucose, xylose and arabinose in different ratios: (A & B) 2:1:1, (C & D) 1:2:1, (E & F) 1:1:1. All experiments were conducted in duplicate and error bars are calculated from independent fermentation runs.

Table 4.2 Average carbon source consumption rate of *Clostridium* sp. strain MF28 fed with single and mixed carbon sources.

Carbon source	**12 h Consumption rate (g/L·h)**			**48 h Consumption rate (g/L·h)**			
	Glucose	Xylose	Arabinose	Glucose	Xylose	Arabinose	Total
2:1:1*	0.75±0.02	0.07±0.003	0.03±0.007	0.64±0.02	0.19±0.001	0.16±0.01	0.99±0.03
1:2:1*	0.73±0.03	0.15±0.03	0.07±0.005	0.39±0.01	0.43±0.003	0.15±0.006	0.97±0.01
1:1:1*	0.66±0.05	0.10±0.007	0.06±0.005	0.49±0.002	0.28±0.03	0.20±0.03	0.96±0.054

*: ratio of initial concentration of glucose: xylose: arabinose.

Table 4.3 Fermentation kinetics of *Clostridium* sp. strain MF28 fed with single and mixed carbon sources.

Carbon source	**Butanol (48h)**			**Butanol/Acetone ratio**	**H_2 yield [ml/g]**	**H_2 : CO_2**
	Titer [g/L]	Yield [g/g]	Productivity (g/L·h)			
2:1:1*	14.40±0.30	0.30±0.002	0.30±0.01	1.99±0.10	7.78±0.20	0.87±0.03
1:2:1*	13.13±0.19	0.28±0.002	0.27±0.004	1.85±0.037	8.05±0.02	0.90±0.02
1:1:1*	13.61±0.07	0.30±0.02	0.28±0.001	1.90±0.01	8.35±0.03	0.88±0.01

*: ratio of initial concentration of glucose: xylose: arabinose.

It is notable that the xylose consumption rate (12 h) increased from 0.069 ± 0.003 g/L·h to 0.149 ± 0.028 g/L·h as the ratio of xylose increased from 1:2 to 2:1 (Table 4.2), indicating that xylose uptake was positively related to the extracellular concentration of xylose. On the contrary, glucose consumption rate did not increase along with the increase in the extracellular glucose concentration. This is likely because different systems are employed to uptake these two substrates when they are supplied together. Xylose uptake by the xylose proton-symporter is a facilitated diffusion process driven by an electrochemical gradient, and would depend not only on the enzyme activities, but also on the extracellular xylose level, while glucose uptake is mediated by ATP-dependent transport systems that is independent of extracellular concentration (Cook et al. 1993; Hu et al. 2011; Xiao et al. 2011).

The highest butanol titers were reached after 48 h of incubation without lag phase in all scenarios (Figure 4.1A, C and D). Along with butanol, a cumulative yield of 7.778 ± 0.20, 8.049 ± 0.02 and 8.351 ± 0.03 ml/g substrate gaseous hydrogen was obtained from cultures fed with 2:1:1, 1:2:1 and 1:1:1 GXA ratios over the entire fermentation cycle, respectively (Table 4.4). The highest butanol concentration (14.398 ± 0.30 g/L) was obtained when strain MF28 was fed with 2:1:1 GXA, with a productivity of 0.304 ± 0.002 g/L·h. The butanol titer, yield and productivity obtained from this experimental set-up were even higher than single carbon source fermentations (Table 4.4) (Li and He 2016). Carbon balance when using 2:1:1 GXA as fermentation carbon source was calculated as shown in Table 4.5. Despite carbon that utilized for cell growth, the total carbon output is 70.3 % of total carbon input.

In strain MF28, co-fermentation of glucose, xylose and arabinose, the three major sugars derived from lignocellulose biomass (Xiao et al. 2011), provides a possible strategy for cost-effective utilization of lignocellulosic biomass. Several studies also reported successful co-fermentation of glucose and xylose into butanol by wild-type or genetically engineered strains (Table 4.4). Among these reports, wild-type *Clostridium* sp. strain BOH3 could be considered as the most promising one since it was found to produce the highest titer of butanol (13.0 g/L) from simultaneous fermentation of glucose and xylose, with a butanol yield of 0.25 g/g and a productivity of 0.18 g/L·h (Xin et al. 2014). In addition to wild-type strains, numerous genetically modified strains have also been developed to improve the butanol production from simultaneous fermentation of pentose and hexose. *Clostridium tyrobutyricum* ATCC 25755 with Δ*ack* and inducible overexpression of *xylT, xylA, xylB* and *adhE2* can achieve a butanol titer of 12.0 g/L with a butanol yield and productivity of 0.24 g/g and 0.29 g/L·h (Yu et al. 2015a). With the integration of *glcG* disruption and genetic overexpression of *xyl T, xylA* and *xylB, Clostridium acetobutylicum* ATCC 824 which is putatively suffered from CCR was able to produce 9.11 g/L butanol from mixtures of glucose and xylose (Xiao et al. 2011). However, the butanol titer, yield and productivity obtained by these strains were lower than reported here. Based on metrics in Table 4.4, strain MF28 shows advantages in several aspects including simultaneous fermentation of pentose and glucose to higher amount of butanol with higher butanol yield and productivity.

Table 4.4 Comparison of butanol production through co-fermentation of pentose and hexose.

Organism	**Relevant genotype**	**Initial substrate concentration [g/L]**			**Titer [g/L]**	**Yield [g/g]**	**Productivity (g/L·h)**	**Reference**
		Glucose	**Xylose**	**Arabinose**				
Ct(Δ*ack*)-pM2	Engineered	45	60	0	3.2±0.2	0.07±0.01	0.07±0.01	(Yu et al. 2015a)
Ct(Δ*ack*)-pTBA	Engineered	45	60	0	12.0±0.2	0.12±0.00	0.17±0.01	(Yu et al. 2015a)
C. sp. strain BOH3	Wild type	20	40	0	13.0	0.25	0.18	(Xin et al. 2014)
C. acetobutylicum 824glcG-TBA	Engineered	38.54	14.92	3.51	9.11	0.16	0.31	(Xiao et al. 2011)
C. acetobutylicum 824ccpA-c	Engineered	39.01±0.04	19.19±0.11	0	9.00±0.04	0.24	0.125	(Ren et al. 2010)
C. sp. strain MF28	Wild type	32.22±0.78	14.79±0.25	14.3±0.16	14.4±0.3	0.30±0.00	0.3±0.01	This study

Table 4.5 Carbon balance when using a mixture of GXA (2:1:1) as fermentation substrate.

	Carbon source			**Products**				
	Glucose	Xylose	Arabinose	Butanol	Acetone	Butyric acid	Acetic acid	CO_2
Quality, g	30.62	9.3	7.49	14.4	7.23	0.96	1.47	316.9 ml
MW, g/mol	180.16	150.13	150.13	74.12	58.08	88.11	60.05	22.4 L/mol
mole	0.17	0.06	0.05	0.19	0.12	0.01	0.02	0.01
Total carbon (mole)	1.55			1.09				

4.4.2 Butanol inhibition on cell growth of glucose and xylose fed cultures

Butanol toxicity is one of the most crucial problems in biobutanol production, and it has been reported to be more pronounced when cell growth occurred on xylose (Lee et al. 2008; Ounine et al. 1985; Xiao et al. 2011). To compare the effects of butanol on cell growth in xylose and glucose fed cultures of strain MF28, different concentrations of butanol were added to cultures after 10 h of incubation. Strain MF28 exhibited comparable cell growth in xylose and glucose fed cultures under same conditions (Figure 4.2), which is in contrast with previous studies. A possible explanation for this difference may be that *Clostridium acetobutylicum* possesses an inherently inefficient xylose metabolism while strain MF28 does not (Grimmler et al. 2010; Xiao et al. 2011; Xiao et al. 2012).

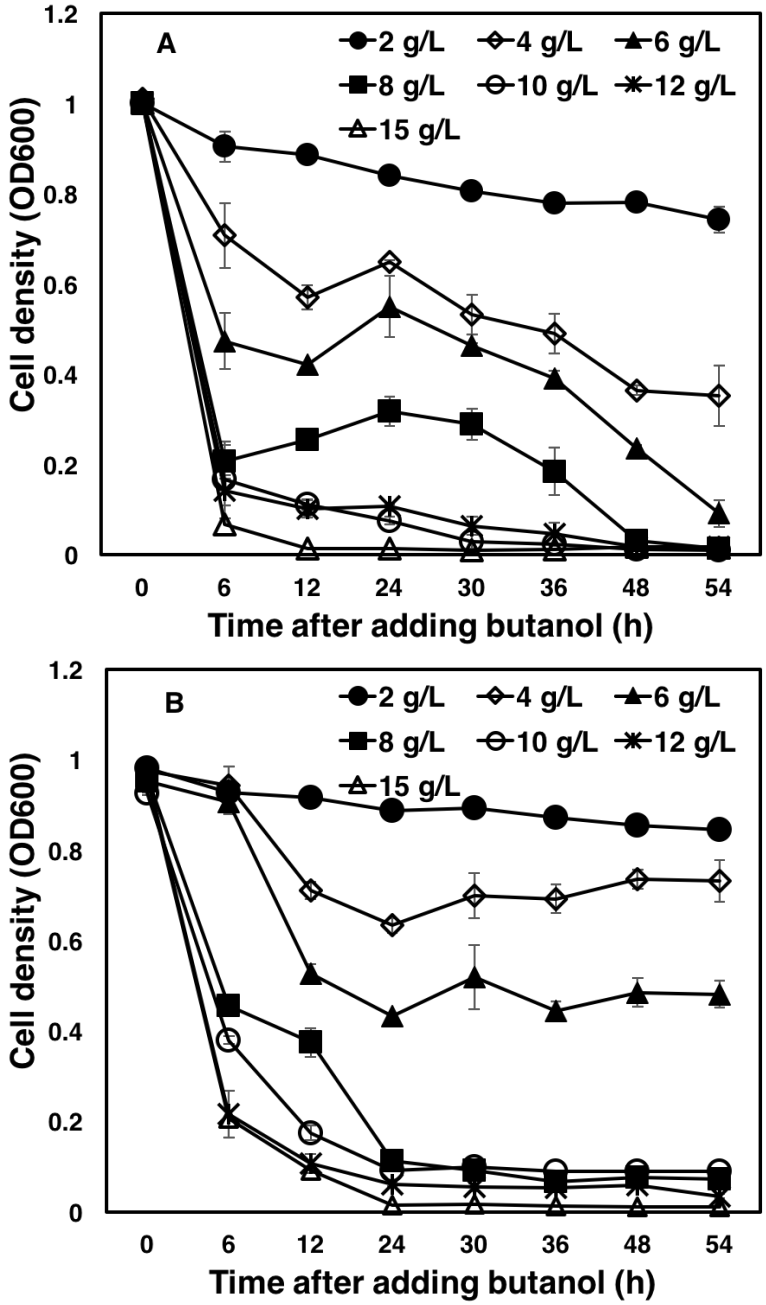


Figure 4.2 Cell growth of *Clostridium* sp. strain MF28 fed with 60 g/L (A) glucose or (B) xylose and amended with butanol to final concentrations as indicated. Cultures without exogenous butanol were used as control. Cell growth of cultures with exogenous butanol was normalized to the control. All experiments were conducted in duplicate and error bars are calculated from independent fermentation runs.

4.4.3 Lack of CCR and one-phase fermentation profile of strain MF28

Simultaneous fermentation of glucose, xylose and arabinose by wild-type strain MF28 without glucose-mediated CCR was observed in this study.

Negligible transcription of *ccpA* was observed in both acidogenic and solventogenic phases (Li and He 2016), indicating the transcription of genes that related to transport and metabolism of xylose and arabinose such as *xylA*, *xylB*, *araA* and *araB* were not repressed by CcpA, which is constant with fermentation kinetics data (Figure 4.1). In addition to carbon metabolism, expression of *ccpA* also relates to efficient sporulation (e.g. *sigE sigG* and *sigK*) and solvent production in *Clostridium acetobutylicum* (Ren et al. 2012; Varga et al. 2004). Strain MF28 has been shown to be non-sporulating due to the expression of a sporulation inhibition gene – *spoih* (Li and He 2016). The low transcription level of gene *ccpA* may provide an additional explanation to the absence of sporulation in strain MF28.

Typically, ABE fermentation by solventogenic *Clostridium* is biphasic. The acidogenic phase, which usually occurs during exponential growth, produces acids as major products. The subsequent solventogenic phase generates biosolvents through acids reassimilation (Jones and Woods 1986). It has been proposed that transcription factor *abrB* may act as a regulator at the transition between acidogenenic and solventogenic phases (Scotcher et al. 2005). The expression of an antisense RNA target against *abrB310* led to an acid accumulation as well as a delay and decrease in solvent production in *C. acetobutylicum* ATCC 824. qPCR result showed that the *abrB* gene in MF28 which shares 85 % similarity with *abrB310* was experienced a transient elevation of expression at 24 h of fermentation (Figure 4.3). The short and obscure acidogenic phase of strain MF28 may due to the up-regulation of this *abrB* gene during the early fermentation stage.

Meanwhile, it has been found that *ccpA* inactivation resulted in a significant down-regulation of *abrB310,* indicating a regulation role of CcpA in *abrB* expression (Ren et al. 2012). The relationship between CcpA and *abrB* remains unclear in strain MF28. Further genomic and proteomic studies will be conducted to illustrate their relationships.

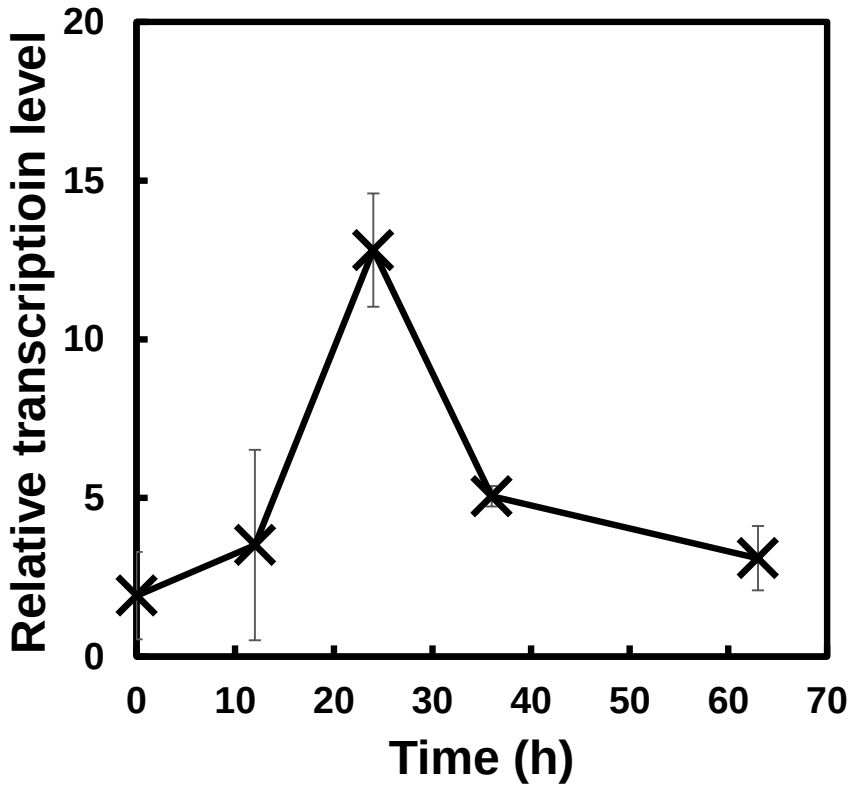


Figure 4.3 Relative transcription level of *abrB* in cultures fed with glucose, xylose and arabinose in a ratio of 2:1:1. Results were normalized to the expression level of housekeeping gene *pepT*. All experiments were conducted in duplicate and error bars are calculated from independent fermentation runs.

4.5 Conclusions

Strain MF28 was found to be capable of simultaneously fermenting hexose and pentose sugars commonly found in lignocellulose hydrolysates, to butanol without carbon catabolite repression. Notably, the majority of the fermentation by strain MF28 can be finished within 48 h leading to high

butanol productivity. Strain MF28 could thus be considered as a highly promising butanol producing candidate from a process commercialization perspective, where carbon catabolite repression often has a significant impact on solvent yields.

CHAPTER 5

Clostridium Species Strain BOH3 Tolerates Inhibitors Present in Horticulture Waste Hydrolysate

After achieving simultaneous fermentation of pentose and hexose elements that present in lignocellulosic hydrolysates, effort was made towards direct butanol production from lignocellulosic hydrolysates. Difficulties in using lignocellulosic hydrolysates for fermentation by *Clostridium* species are associated with the inhibitory effects on cell growth and butanol production by inhibitors that generated during the pretreatment and hydrolysis of lignocellulosic biomass. The ability of strain BOH3 to tolerate furan derivatives as well as phenolic compounds and to ferment lignocellulosic hydrolysate will be investigated in this chapter.

5.1 Abstract

Efficient conversion of lignocellulosic hydrolysate to biofuels is impeded by the toxicity effects of inhibitors that generated during the pretreatment and hydrolysis processes. Here we describe a wild-type *Clostridium* sp. strain BOH3 with high resistance to the lignocellulose-derived inhibitors. Strain BOH3 is found to be capable of resisting over 60 mM furfural, 60 mM HMF and 6.6 mM vanillin, respectively. Moreover, this strain is able to convert 53.74 ± 0.37 mM furfural into furfuryl alcohol within 90 h. Toxicity study revealed that combination of different inhibitors with furfural lead to a severer synergistic effect on cell growth and butanol production of strain BOH3, suggesting an important role of furfural in the hydrolysates to affect butanol generation process. The high furfural resistance is closely related to the high transcription levels of two short chain dehydrogenase/reductases. High toxic resistance also enables strain BOH3 to produce butanol from dilute sulfuric acid pretreated horticultural waste

hydrolysate (HWH) that had undergone no prior detoxification process. After applying to the HWH, which contained 27.58 ± 1.08 g/L reducing sugars, 5.15 ± 0.52 g/L butanol was generated by strain BOH3. The capability of strain BOH3 to produce butanol from un-detoxified HWH lays the foundation of cost-effective biofuels production from lignocellulosic materials.

5.2 Introduction

Lignocellulosic material is abundantly available, sustainable, carbon neutral and inexpensive (Jang et al. 2012a). Utilization of lignocellulosic biomass other than traditional cereal materials as fermentation substrate offers a promising solution for economical biobutanol production (Gu et al. 2014). Before fermentation, lignocellulosic biomass needs to be pretreated and hydrolyzed to liberate the fermentable sugars such as glucose and xylose (Blanch et al. 2011). However, besides sugars, a range of inhibitors also generated during the processes. These inhibiting compounds can be divided into three groups: weak acids, phenolic compounds, and furan derivatives (Palmqvist and Hahn-Hägerdal 2000a). These compounds are considered as inhibitors since they will inhibit cell growth and the further fermentation process by affecting cell membrane, damaging nucleic acids, inducting oxidative stress and inhibiting key metabolic enzymes (Allen et al. 2010; Almeida et al. 2009; Ezeji et al. 2007a). Numbers of physical, chemical and biological methods have been developed to remove these inhibitors. Treatments with enzymes like laccase and microorganisms detoxification are representative biological detoxification methods. However, laccase can only remove phenolic monomers and it is very difficult to isolate or develop

microorganisms that can detoxify all the toxic compounds without consuming the sugars at the same time (Chandel et al. 2013; Jönsson et al. 1998). Typical physical detoxification methods are filtration, evaporation, and extraction. Although considerable decrease of volatile inhibitors can be achieved by evaporation, the non-volatile fraction still remains in the hydrolysates and was found to be more toxic to microorganisms (Palmqvist and Hahn-Hägerdal 2000a). Overliming is one of the prevalent chemical detoxification method, yet, it is not that efficient without the combination with other detoxification methods (Arslan and Eken-Saraçoğlu 2010; Stoutenburg et al. 2011). Moreover, all of these methods would more or less lead to fermentable sugars reduction and increase in production cost (Arslan and Eken-Saraçoğlu 2010). To conclude, every detoxification method has its limitations and drawbacks. Consequently, it is favorable to omit the detoxification step by finding a butanol producing strain with high resistance to lignocellulose-derived inhibitors.

Horticultural waste refers to tree trunks and branches, plant parts and trimmings generated during the maintenance and pruning of trees and plants (Eugene Tay, 2008). In Singapore, agriculture is not well developed and horticultural waste is one of the abundantly available lignocellulosic material. According to the waste statistics and overall recycling data of National Environment Agency of Singapore, 149,800 tons of horticultural waste was generated and only 37% has been recycled in 2011. Therefore, horticultural waste can be considered as one of the promising lignocellulosic source for biofuels generation in Singapore.

This study investigates effects of selected lignocellulose-derived inhibitors on cell growth and butanol production of wild-type *Clostridium* species strain BOH3. This strain produces considerable amount of butanol directly from un-detoxified horticultural waste hydrolysate owing to its capability of resisting high inhibitor concentrations and thereby provides a promising solution for cost-effective biological butanol production.

5.3 Material and methods

5.3.1 Culture medium and cultivation

Clostridium sp. strain BOH3 was cultivated under anaerobic conditions in 160 ml serum bottles containing 42 ml defined mineral salt medium. The media composition used for the fermentation experiments was the same as described in chapter three. Concentrated glucose (600 g/L), xylose (600 g/L) and yeast extract (150 g/L) stock solutions were sterilized separately and injected into the cultivation medium after autoclaving. Final concentration of reducing sugar and yeast extract is 60 g/L and 5 g/L, respectively, unless specified otherwise. When horticultural waste hydrolysate was used as fermentation substrate, additional glucose, xylose and yeast extract were added to the fermentation medium to reach a final concentration of 40 g/L, 20 g/L and 5 g/L, respectively. Active cells (cultivated for 24 h) were inoculated with 4 % [v/v] to the bottles and cultures were incubated in an orbital shaker at 150 rpm at 35 °C with an initial pH of 6.5 and maintained at the desired pH using 9 mol/L NaOH until no further gas was produced from the serum bottles. All experiments were done in duplicate.

5.3.2 Toxicity effects of selected hydrolysate inhibitors on cell growth and butanol production

To investigate the toxicity effects of inhibitors in lignocellulosic hydrolysates, selected inhibitors of different concentrations (furfural, HMF and vanillin) were added to the culture medium before inoculation. Samples were taken at regular intervals for cell growth and butanol production analysis.

Synergistic effects of inhibitors on cell growth of strain BOH3 were studied by adding binary combinations of inhibitors to the culture medium before inoculation. Cultures grown in the medium without additional inhibitors were used as control. Two inhibitors from furan derivatives, furfural (40 mM) and HMF (40 mM), and two from phenolic compounds, syringaldehyde (4 mM) and 4-hydroxybenzoic acid (4 mM) were selected for this study. Samples were taken at 60 h of fermentation to measure cell growth and relative cell growth was calculated by normalizing to the cell growth of control cultures.

5.3.3 Transcription of furfural biotransformation related genes

To perform gene expression studies related to furfural biotransformation, 40 mM furfural was added to the culture medium containing 60 g/L glucose before fermentation. At regular intervals, 1 ml of cultures was harvested by centrifuging at 140,000 rpm and 4 °C for 10 min. RNA extraction and cDNA generation were conducted as described in chapter four. cDNA samples were used as a DNA template for PCR amplification of the aldo/keto reductase (*akr),* short chain dehydrogenase/reductase (*sdr*), alcohol dehydrogenase (*ad*) and (3R)-hydroxymyristoyl-ACP dehydratase (*fabZ*) genes. Aldo/keto

reductase, short chain dehydrogenase and alcohol dehydrogenase were genes related to furfural biotransformation in *Clostridium* and yeast (Zhang and Ezeji 2013). The *fabZ* gene is a housekeeping gene for strain BOH3 (Nolling et al. 2001; Xin et al. 2014) and was used as an internal control to monitor efficiency of cDNA synthesis and PCR amplification. Details of the primers used for the experiments are shown in Table 5.1. The obtained PCR products were analyzed by 1 % agarose gel electrophoresis. Final concentration of primer was 0.4 μM in each qPCR reaction. After activating the enzymes in master mix at 98˚C for 3 min, qPCR reaction entered a thermal cycle consisting 40 cycles of 95˚C for 20 s and annealing temperature for 30 s.

Table 5.1 Specific primers for gene *akr, sdr, adg* and *fabZ* in strain BOH3.

Gene	Forward 5'-3'	Reverse 5'-3'	Annealing temperature
akr	GGAAAGCACTAGAAAAGC	TGAGGAAATTGAGGATGA	42˚C
sdr1	CTTGAGCTTAAAGGTGCTAT	TTTACCCATCATATTTCTCG	42˚C
sdr2	AATTGGGAAACGAAGTAATC	TGCCACATCGCAAACTA	43˚C
sdr3	GGCCCTATAAAGACCAACT	GATCAGCCTGCTTCTGC	47˚C
sdr4	AGAGTCGTCTGCGATTTATG	TCTCGTCCAAGTCCCGTA	47˚C
adg	GGTGGTTCAAAGTCCGCAATA	CGTAAACGCCGACAACAGATA	50˚C
fabZ	AAATAGAACCAGGGAAAAGAGCA	GCAACACCACCAAGTTGAGC	50˚C

To compare the transcription ratio of gene *akr, sdr* and *ad,* cDNA samples obtained from cultures at regular intervals of fermentation were used

as a DNA template for qPCR amplification. The method for qPCR amplification was the same as described in chapter four. The relative abundance was calculated based on cycle to threshold value (Ct value) of target genes (*akr*, *sdr*, and *ad*) and was normalized to the abundance of housekeeping gene *fabZ* for comparison.

5.3.4 Horticultural waste hydrolysate preparation

Horticultural waste collected from a horticultural waste treatment plant (ecoWise Solution Pte. Ltd.) in Singapore was used in this study. Before pretreatment, horticultural waste was washed, dried and milled to a size between 200 to 500 μm. Pretreatment was carried out by adding the milled horticultural waste to 2 % [v/v] H_2SO_4 (5 % [w/w]) and heated at 121 ˚C for 20 min. After centrifugation and filtration, supernatant of the pretreated horticultural waste was collected, and pH was adjusted to 6.5 using NaOH. Sugar concentration was topped up to 60 g/L with a glucose/xylose ratio of 2:1.

5.3.5 Analytical methods

Methods to measure biomass, volatile fatty acids (i.e., acetic and butyric acids), biosolvents (i.e., acetone, ethanol and butanol) and residual sugar concentrations were the same as described in chapter four. Furfural and furfuryl alcohol concentrations were measured using the HPLC equipped with Waters Atlantis dc18 column (4.6 × 150 mm, 0.5 μm) and a UV detector set at 540 nm.

5.4 Results and discussion

5.4.1 Toxicity effect of single hydrolysate inhibitors on cell growth and butanol production

Wild-type *Clostridium* species strain BOH3 is able to produce butanol from simultaneous fermentation of xylose and glucose (Xin et al. 2014), which are two of the most abundant reducing sugars derived from lignocellulosic material. This ability thus offers possibilities of efficient lignocellulosic hydrolysate utilization by strain BOH3. However, inhibitors generated during the pretreatment and hydrolysis of lignocellulosic material can inhibit cell growth and butanol production by solventogenic *Clostridium* (Ezeji et al. 2007a). To analyze toxicity effects of lignocellulose-derived inhibitors on strain BOH3, selected inhibitors including furfural, HMF and vanillin were added to the fermentation medium at different concentrations using the "one factor at a time" method. Cultures without additional inhibitors were used as control. Result indicates that strain BOH3 can withstand up to 60 mM of furfural and produces butanol after experienced a 66 h lag phase as compared to the control (Figure 5.1). No obvious inhibitory effects were observed when furfural concentration was lower than 30 mM. Cell growth and butanol production were being totally inhibited when 80 mM furfural was added to the fermentation medium. However, the presence of furfural seems to stimulate cell growth of strain BOH3 when its concentration was lower than 40 mM (Figure 5.1A). After experienced a ~10 h lag phase, the highest OD600 value in the presence of 40 mM furfural was 10 ± 0.11 % higher as compared to the control cultures. Correspondingly, there is a ~12 h lag phase on the onset of butanol production with 40 mM furfural, while the butanol titer and

productivity was comparable with the control cultures (Figure 5.1B). Similar result was found in HMF challenged cultures (Figure 5.2A). In the presence of 50 mM HMF, although a ~ 24 h lag phase on cell growth was occurred, the final OD600 value was 5 ± 0.21 % higher as compared to the control. However, butanol titer and productivity were both lower than control cultures when 50 mM HMF was added to the fermentation medium. When the concentration of HMF was lower than 40 mM, no significant inhibitory effect on butanol production was observed (Figure 5.2B). Previous studies also reported the stimulate effect of furan derivatives on cell growth of solventogenic *Clostridium* (Ezeji et al. 2007a; Zhang et al. 2012), but the reported concentrations were lower than this study. For *Clostridium beijerinckii* BA101, cell growth and ABE production were elevated when furfural and HMF concentration was 20 mM and 16 mM, respectively (Ezeji et al. 2007a). Under this furfural or HMF concentration, growth of *C. acetobutylicum* ATCC 824 was also being stimulated, while ABE production by strain ATCC 824 decreased when furfural and HMF concentration increased to 31 mM and 24 mM, respectively (Zhang et al. 2012). The concentration of furan derivatives in lignocellulosic hydrolysates can range from 0.5 – 11 g/L (equals to 5 – 115 mM furfural or 4 – 87 mM HMF) (Almeida et al. 2007). The concentration of furan derivatives in lignocellulosic hydrolysates may exceed the tolerance capacity of *Clostridium* strains. Hence, it is desirable to cultivate solventogenic strains with higher furan derivative tolerance.

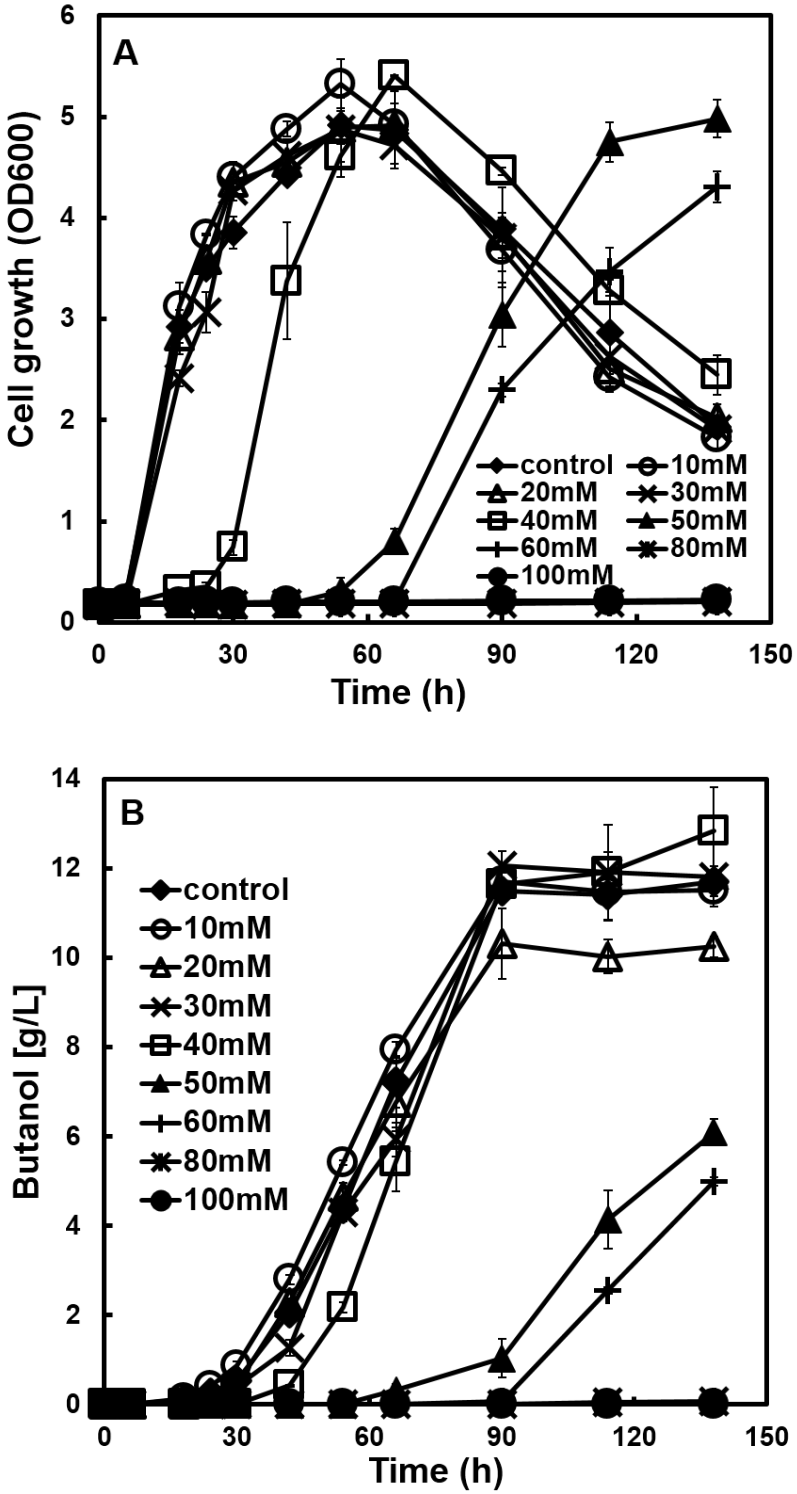


Figure 5.1 (A) Cell growth and (B) butanol production of *Clostridium* sp. strain BOH3 in the presence of furfural. All experiments were conducted in biological duplicate and error bars are from independent fermentation runs.

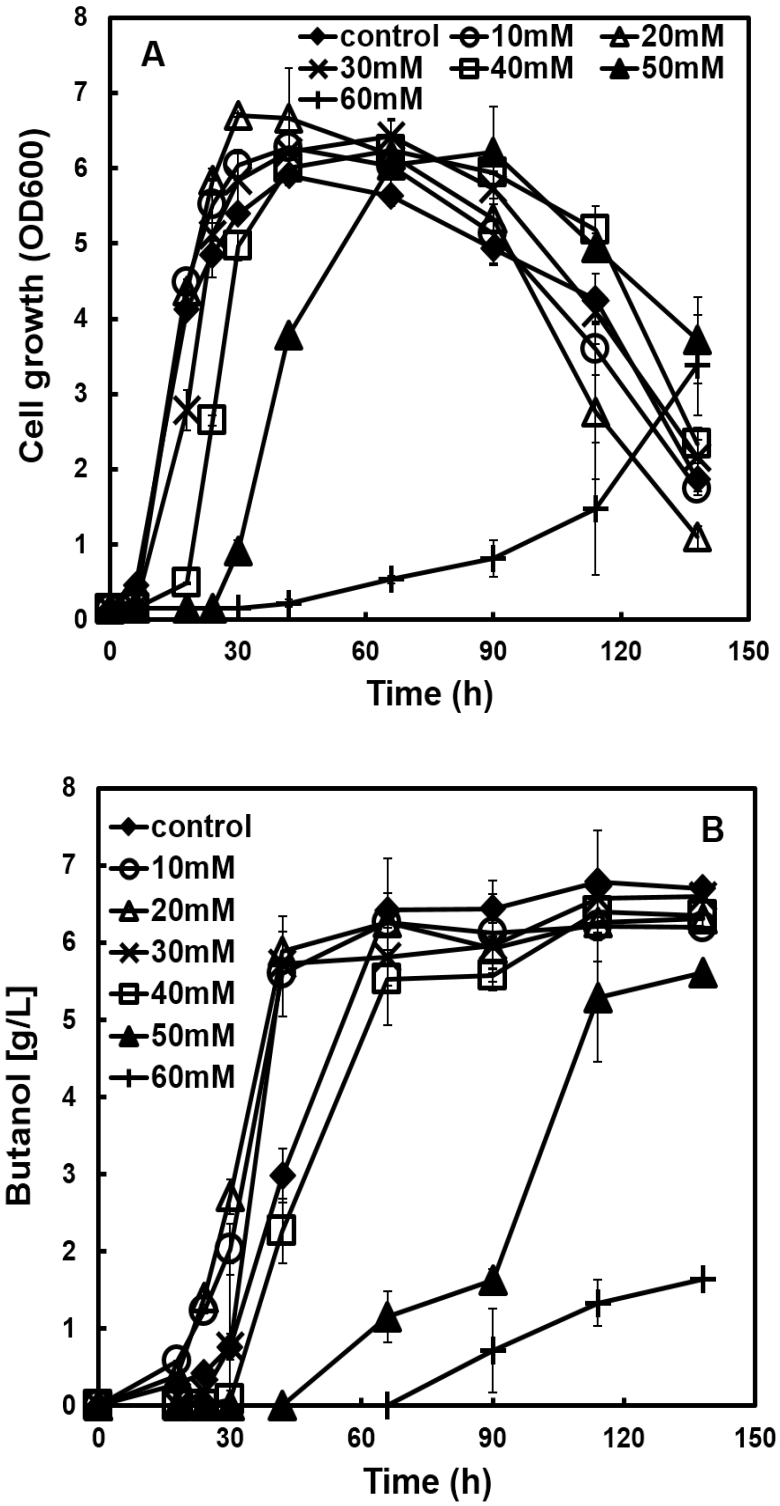


Figure 5.2 (A) Cell growth and (B) butanol production of *Clostridium* sp. strain BOH3 in the presence of hydroxymethylfurfural. All experiments were conducted in biological duplicate and error bars are from independent fermentation runs.

In addition to furan derivatives, toxicity effects of phenolic compounds, such as vanillin, on cell growth and butanol production of strain BOH3 was also investigated. Interestingly, self-aggregation was observed in the presence of vanillin. Optical density cannot indicate the condition of cell growth, and butanol production was thus occupied as the only dependent variable to analyze toxicity effect of vanillin on strain BOH. No obvious inhibitory effect on butanol production occurred when vanillin concentration was lower than 2 mM (Figure 5.3). Butanol titer decreased with increasing the vanillin concentration. When vanillin concentration exceeded 3.3 mM (0.3 g/L), a lag phase of 32 h was observed, and butanol production reduced 15.58 ± 0.66 % with 6.6 mM vanillin compared to the control. Even though, strain BOH3 shows higher resistance to phenolic compounds compared to previously reported strains including *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052. The threshold of phenol compounds for butanol production by strain ATCC 824 is 0.3 g/L in un-detoxified hydrolysate (Mechmech et al. 2015). For strain NCIMB 8052, butanol production can be completely inhibited in the presence of 1 g/L vanillin (6.6mM) as well as *p*-coumaric acid, ferulic acid, 4-hydroxibenzoic acid, vanillic acid and syringaldehyde (Cho et al. 2009). Phenolic compounds are considered to be toxic to cells as they can affect the cell membrane permeability and cause leakage of cellular contents by damaging the hydrophobic sites of cell membrane (Heipieper et al. 1991; Heipieper et al. 1994). The toxicity of phenolic compounds is thus correlated with their degree of hydrophobicity (Zaldivar et al. 1999). However, solubility in water of most phenolic compounds are very poor, for example, ferulic acid is insoluble in water at room temperature. Consequently, the

amount of phenolic compounds in lignocellulosic hydrolysates was lower than furan derivative, which was reported to be 2.5 g/L in the willow hydrolysate (Jönsson et al. 1998).

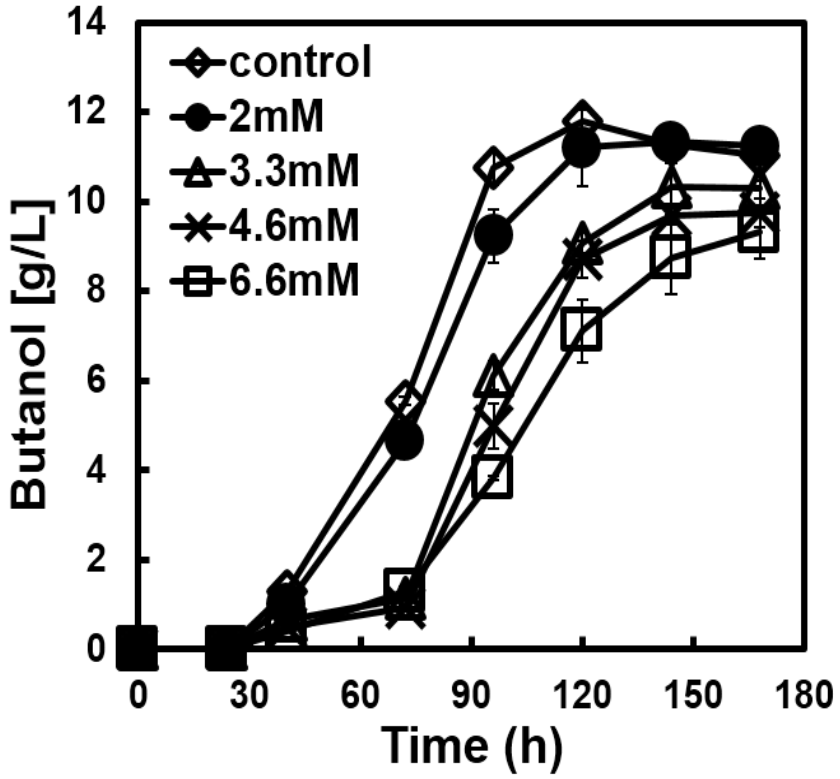


Figure 5.3 Butanol production by *Clostridium* sp. strain BOH3 in the presence of vanillin. All experiments were conducted in biological duplicate and error bars are from independent fermentation runs.

Clostridium sp. strain BOH3 reported in this study, which can resist higher furan derivative and vanillin concentrations compared to previously reported strains (Cho et al. 2009; Mechmech et al. 2015; Zhang et al. 2012), showed its great potential in direct conversion of un-detoxified lignocellulosic hydrolysates into butanol.

5.4.2 Synergistic effect of inhibitors on cell growth of strain BOH3

Strain BOH3 showed its capability of resisting high concentrations of single lignocellulose-derived inhibitors. However, the inhibitors were generated together during pretreatment and hydrolysis processes. Although single inhibitor did not cause significant reduction in cell growth or butanol production, mixture of different inhibitors may have pronounced inhibitory effects. To study the synergistic effect, selected inhibitors including furfural, HMF, 4-hydroxybenzoic acid (4-HBA) and syringaldehyde were added to the fermentation medium before fermentation in binary combinations. The relative cell growth at 48 h of cultures with two inhibitors were lower than cultures with single inhibitor (Figure 5.4), suggesting a synergistic effect by combination of two inhibitors. For example, when furfural, 4-HBA and syringaldehyde was added to the fermentation medium together with HMF, respectively, the cell growth was reduced by 59.8 %, 33.9 % and 26.2 % compared to the cultures where these inhibitors were tested individually. Notably, binary combination with furfural caused severer inhibitory effect on cell growth than other conditions. Cell growth was reduced by 61.2 %, 63.2 % and 43.0 % when HMF, 4-HBA and syringaldehyde was added to the fermentation medium together with furfural, respectively, as compared to the experiments where these inhibitors were tested alone. Previous studies also reported the synergistic effect of hydrolysate inhibitors. Ezeji et al. observed a stimulate effect of furfural and HMF on cell growth of *C. beijerinckii* BA101 when their concentrations were 2 g/L, respectively. However, when a mixture of furfural (1 g/L) and HMF (1 g/L) was added to the fermentation medium, cell growth reduced by 7 % as compared to the control (without inhibitor) (Ezeji et al. 2007a). Our study demonstrated that combination with furfural

led to severer synergistic effect on ABE fermentation by *Clostridium* although similar result has been reported in ethanol fermentation by *E. coli* (Zaldivar et al. 1999).

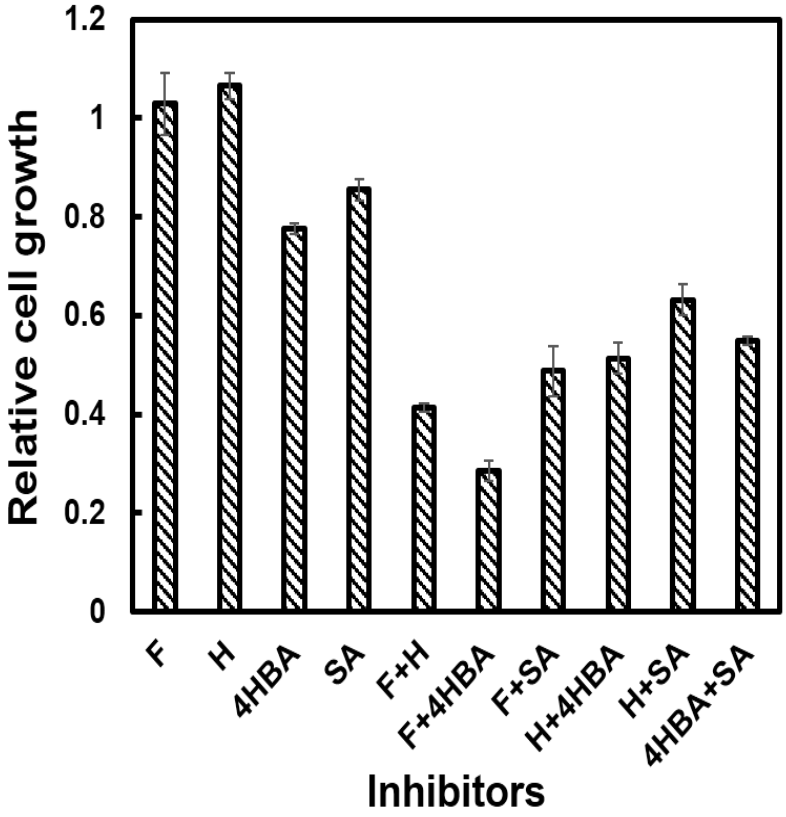


Figure 5.4 Relative cell growth of strain BOH3 in the presence of single and two hydrolysate inhibitors. F: furfural, H: hydroxymethylfurfural, 4-HBA: 4-hydroxybenzoic acid, SA: syringaldehyde. Results were normalized to cell growth of the control culture without presence of hydrolysate inhibitor. All experiments were conducted in biological duplicate and error bars are calculated from independent fermentation runs.

5.4.3 Bioconversion of furfural to furfuryl alcohol by strain BOH3

To investigate the feasibility of furfural bioconversion by strain BOH3, different concentrations of furfural were added to the fermentation medium before fermentation. Strain BOH3 was found to be capable of converting

furfural to furfuryl alcohol, a less toxic compound and cannot be further utilized (Figure 5.5). Interestingly, the time of furfural depletion was closely correlated with the lag phase on butanol production by strain BOH3, suggesting a relationship between solvent production and furfural detoxification. For instance, a ~42 h lag phase has been observed when strain BOH3 was challenged with 40 mM furfural. On the other hand, ~36 mM furfural can be totally converted to furfuryl alcohol within 42 h (Figure 5.1B & Figure 5.5C).

The delay of butanol production in the presence of furfural may be due to the competition of reducing power between butanol generation and furfural bioconversion. Conversion of furfural affects the reducing equivalents by consuming two electrons from NAD(P)H to convert the aldehyde group on the furan ring to the hydroxymethyl group on furfuryl alcohol (Ask et al. 2013; Horvath et al. 2003; Zhang and Ezeji 2013). Reducing cofactors such as NAD(P)H are also essential for butanol generation (Mitchell 1998). However, no significant lag phase was observed on cell growth and butanol production when furfural concentrations were lower than 30 mM (Figure 5.1). There are two possible explanations, i) the time for strain BOH3 to convert furfural to furfuryl alcohol is within its inherent lag phase of butanol production, ii) low concentration of furfural may stimulate glycolysis via regeneration of NAD^+ (Zhang et al. 2012).

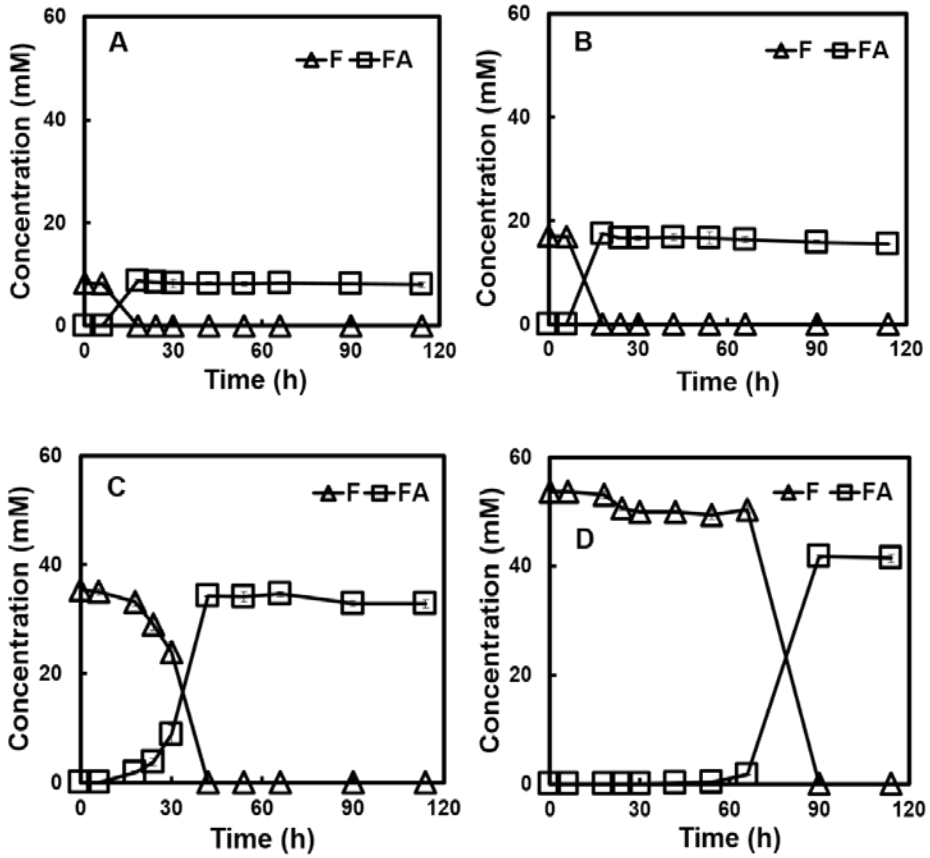


Figure 5.5 Bioconversion of furfural to furfuryl alcohol by strain BOH3 under different furfural concentrations. A: 10 mM; B: 20 mM; C: 40 mM; D: 55 mM. All experiments were conducted in biological duplicate and error bars are calculated from independent fermentation runs.

5.4.4 Transcription of genes related to furfural bioconversion

Bioconversion of furfural and HMF in ethanologenic microorganisms is a NAD(P)H-dependent aldehyde reduction that catalyzed by also/keto reductases (*akr*), short chain dehydrogenase/reductase (*sdr*), and alcohol dehydrogenases (*ad*) (Liu 2011; Liu et al. 2008; Miller et al. 2009). In butanol generating *Clostridium* strains, an AKR (Cbei_3974) and a SDR (Cbei_3904) was found to be significantly up-regulated in furfural-challenged *Clostridium beijerinckii* NCIMB 8052 (Zhang and Ezeji 2013). Draft genome sequence analysis of strain BOH3 revealed the presence of one *akr*, four *sdr* (*sdr1 to sdr4*), and one *ad* genes. However, they have a low similarity of less than 50 % with Cbei_3974 or Cbei_3904.

Relative transcription levels of these genes were compared between cultures with 40 mM exogenous furfural and without furfural to illustrate the relationship between these genes and furfural bioconversion in strain BOH3 (Figure 5.6). Among these genes, only *sdr2* and *sdr4* were up-regulated in furfural-challenged cultures, which peaked at 18 h and 6 h of fermentation, respectively (Figure 5.6B). No significant transcription level changes of these two genes were observed in the control (Figure 5.6A). These results are consistent with furfural bioconversion profiles (Figure 5.5C), wherein the conversion of furfural was started after 6 h of fermentation and the conversion rate increased after 18 h of fermentation, suggesting the conversion of furfural to furfuryl alcohol in strain BOH3 is closely related to *sdr2* and *sdr4*. Interestingly, *sdr3* and *ad* showed a transient elevation at 24 h of fermentation in the control, their transcription levels also increased after 24 h of fermentation in furfural-challenged cultures. Correlate this gene transcription

profile with the fermentation kinetics data (Figure 5.1B), it could be conjectured that these two genes may relate to solvent generation during the fermentation process.

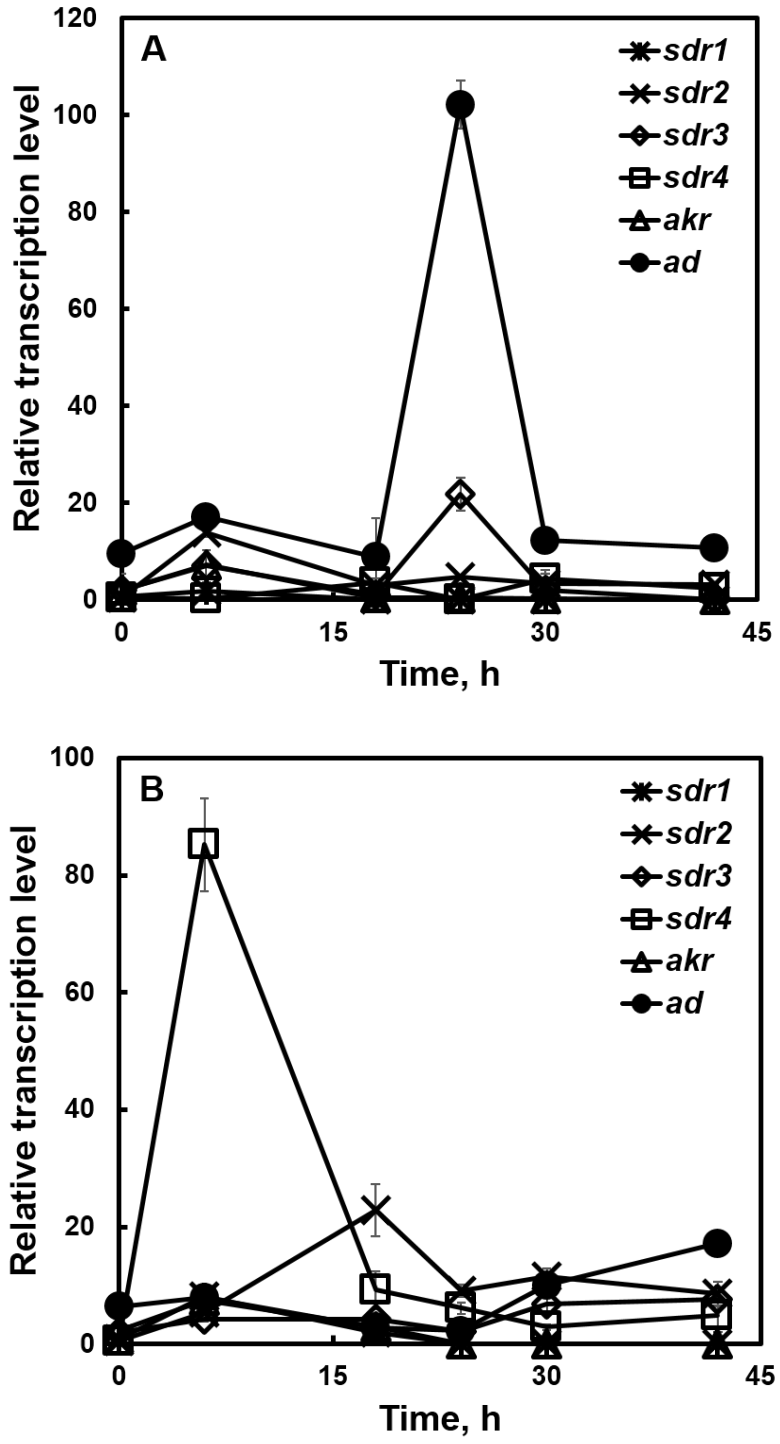


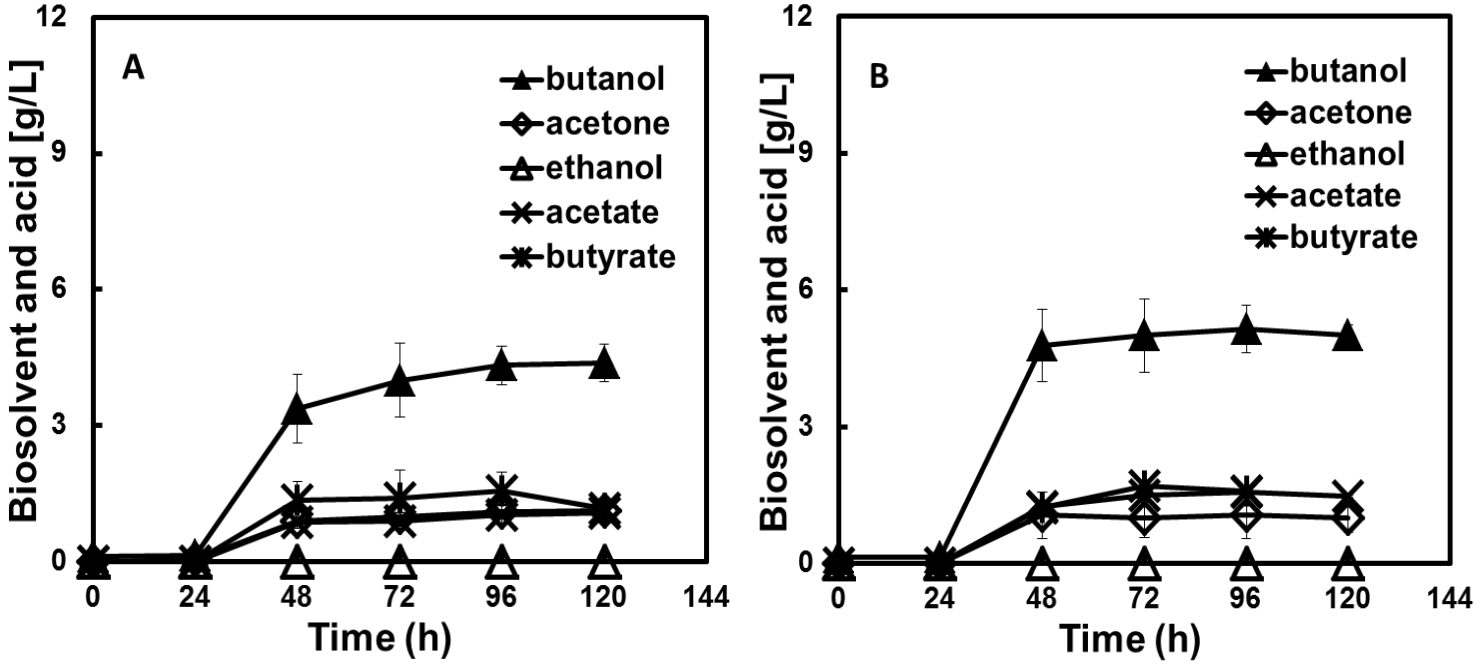
Figure 5.6 Comparison of relative *akr*, *sdr* and *ad* transcription levels between (A) control and (B) furfural (40 mM) challenged cultures. Results normalized to *fabZ*, error bars are calculated from analytical errors.

5.4.5 Butanol production from horticultural waste hydrolysate

To further investigate strain BOH3's capability of fermenting lignocellulosic hydrolysates, dilute sulfuric acid pretreated horticultural waste hydrolysate (HWH) was used as carbon source for fermentation. After pretreatment, 27.58 ± 1.08 g/L reducing sugar with a glucose/xylose ratio of 1:2 was obtained in HWH. Additional glucose and xylose were added to the fermentation medium to reach a concentration of 40 and 20 g/L, respectively. Cultures that fed with glucose (40 g/L) and xylose (20 g/L) were used as controls. Direct fermentation of HWH resulted in 4.38 ± 0.42 g/L ABE, consisting of 1.13 ± 0.11 g/L acetone and 4.38 ± 0.42 g/L butanol. Negligible ethanol production was observed during the fermentation (Figure 5.7A). The solvent production from HWH was much lower compared to the control (Figure 5.7D), implying an inhibitory effect of hydrolysate inhibitors on solvent production by strain BOH3. When HWH was 1.5 times diluted with sterilized mineral salt medium, slightly higher butanol titer was achieved (5.15 ± 0.52 g/L) (Figure 5.7B). The higher butanol yield and productivity achieved after dilution suggest the rate-limiting factor may not be sugar concentration, but the concentration of inhibitors (Table 5.2). To substantiate this hypothesis, HWH treated with $Ca(OH)_2$ (overliming) was applied to strain BOH3 for ABE fermentation (Figure 5.7C). After detoxification, a significant increase in butanol production (8.11 ± 0.34 g/L) was achieved with increased sugar consumption (Table 5.2), which well demonstrated our previous conjecture.

Since the constitution of hydrolysate inhibitors varies from different pretreatment methods, butanol production in this study was compared with those fermentation processes using dilute sulfuric acid pretreated hydrolysates as substrate (Table 5.3). Among these reports, *C. beijerinckii* P260 was found

to be capable of producing 4.75 g/L butanol from un-detoxified barley straw hydrolysate which contains 60 g/L reducing sugar (Qureshi et al. 2010). Apart from wild type strains, metabolic engineering approaches have also been applied to increase butanol production from lignocellulosic hydrolysates. An adapted strain of *C. beijerinckii* BA101 was reported to be capable of generating 1.4 g/L and 6.4 g/L butanol from un-detoxified and XAD-4 resin extracted corn fiber hydrolysate, respectively (Qureshi et al. 2008). Compared to theses previous studies, strain BOH3 shows comparable butanol production from un-detoxified hydrolysate with those fermentation strategies applied with detoxification processes. This may due to the higher furfural resistance and higher furfural conversion capability of strain BOH3.


A
B
butanol
acetone
ethanol
acetate
butyrate
Biosolvent and acid [g/L]
Time (h)
0
3
6
9
12
0
24
48
72
96
120
144

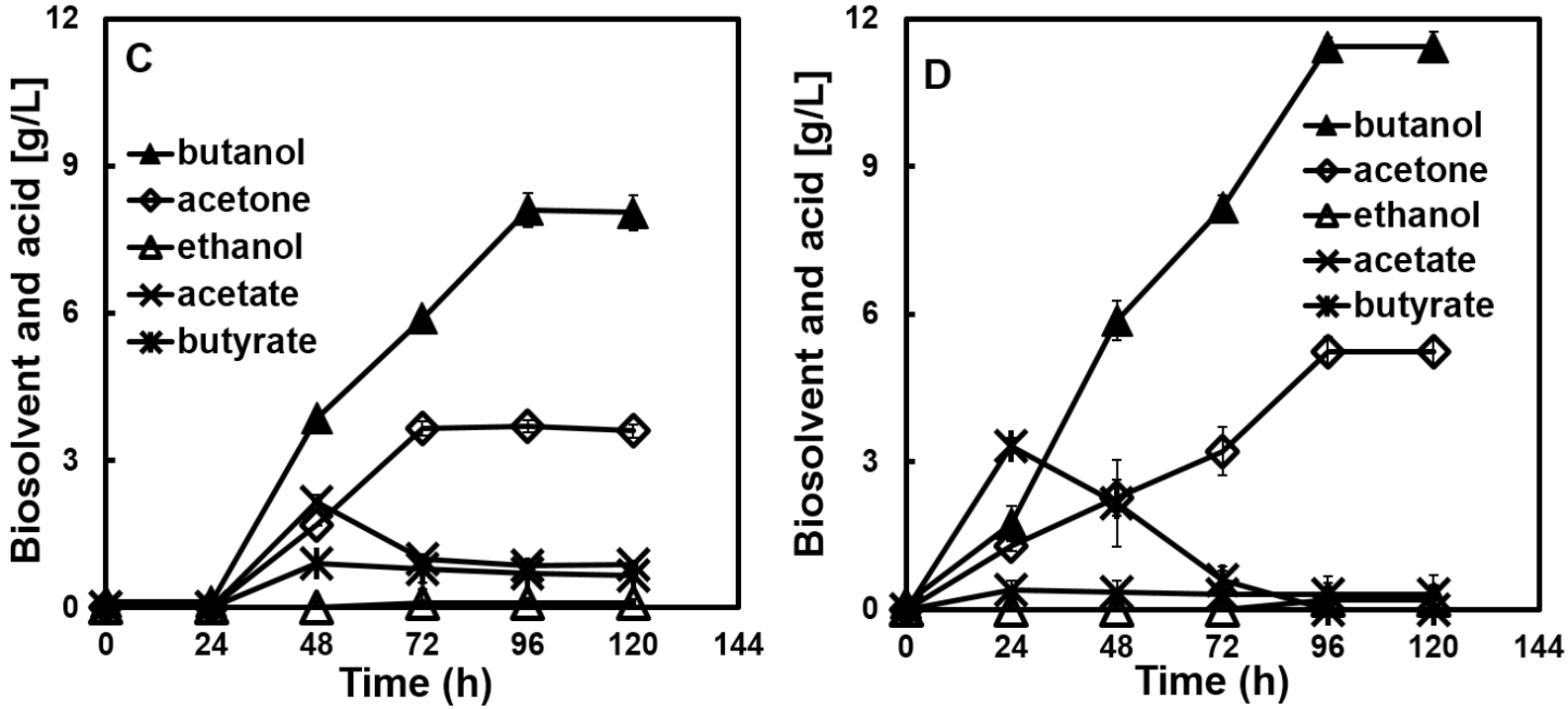


Figure 5.7 Butanol production from (A) original, (B) 1.5 times diluted and (C) overliming treated horticultural waste hydrolysate. Butanol production from cultures that grown in defined mineral salt medium with glucose (32 g/L) and xylose (16 g/L) were occupied as control (D). All experiments were conducted in biological duplicate and error bars are calculated from independent fermentation runs.

Table 5.2 Horticultural waste hydrolysate fermentation by strain BOH3. Cultures grown in a mixture of glucose (40 g/L) and xylose (20 g/L) were used as controls. All experiments were conducted in biological duplicate and standard divisions are from independent fermentation runs.

Carbon source	**Sugar consumption [g/L]**	**Yield [g/g]**	**Productivity**
Hydrolysate	16.16 ± 0.92	0.27 ± 0.02	0.037 ± 0.002
1.5 times diluted hydrolysate	14.95 ± 0.73	0.34 ± 0.03	0.054 ± 0.003
Overliming treated hydrolysate	35.44 ± 0.56	0.23 ± 0.01	0.084 ± 0.001
Glucose and xylose	39.13 ± 0.28	0.29 ± 0.01	0.119 ± 0.001

Table 5.3 Comparison of butanol production from dilute sulfuric acid pretreated lignocellulosic hydrolysates in mono-cultures.

Organism	**Relevant genotype**	**Substrate**	**Pretreatment method**	**Initial reducing sugar [g/L]**	**Detoxification**	**Butanol titer [g/L]**	**Reference**
C. saccharoperbutylacetonicum N1-4	Wild-type	Palm kernel cake hydrolysate	Dilute sulfuric acid	17.99	XAD-4 resin extraction	3.59	(Shukor et al. 2014b)
C. acetobutylicum ATCC 824	Wild-type	Hardwood hydrolysate	Dilute sulfuric acid	72.0 ± 1.1	None	0.4	(Mechmech et al. 2015)
				63.2 ± 1	Flocculation	3.9	
C. beijerinckii BA101	Adapted	Corn fiber hydrolysate	Dilute sulfuric acid	29.8	None	1.4	(Qureshi et al. 2008)
				46.3	XAD-4 resin extraction	6.4	
C.beijerinckii P260	Wild-type	Barley straw hydrolysate	Dilute sulfuric acid	60	None	4.75	(Qureshi et al. 2010)
C. sp. strain BOH3	Wild-type	Horticultural waste hydrolysate	Dilute sulfuric acid	40	None	5.15	This study

5.5 Conclusions

Clostridium sp. strain BOH3 shows high resistance to the inhibitors generated during the pretreatment and hydrolysis processes of lignocellulosic hydrolysate. Strain BOH3 can convert high concentration of furfural to its less toxic alcohol-furfuryl alcohol with the up-regulation of two short chain dehydrogenases/reductases. Correspondingly, strain BOH3 was able to produce 5.15 ± 0.52 g/L butanol with higher yield (0.34 ± 0.03 g/g) from un-detoxified dilute acid pretreated horticultural waste hydrolysate as compared with the control (0.29 ± 0.01 g/g) that fed with glucose and xylose. Strain BOH3 can be considered as a potential candidate for large scale butanol production from lignocellulosic hydrolysate.

CHAPTER 6

Direct Conversion of Xylan to Butanol by a Wild-type *Clostridium* Species Strain G117

Apart from using lignocellulose-derived sugars and lignocellulosic hydrolysates as feedstocks, direct conversion of lignocellulsic biomass, such as cellulose and xylan, to value-added products in a consolidated bioprocess (CBP) is always desirable. Studies in this chapter are focused on realization of direct butanol production from xylan by wild-type *Clostridium* strain.

6.1 Abstract

Lignocellulosic biomass has great potential for use as a carbon source for the production of second-generation biofuels by solventogenic bacteria. Here we describe the production of butanol by a newly discovered wild-type *Clostridium* sp. strain G117 with xylan as the sole carbon source for fermentation. Strain G117 produced 0.86 ± 0.07 g/L butanol and 53.4 ± 0.05 mL hydrogen directly from 60 g/L xylan provided that had undergone no prior enzymatic hydrolysis. After process optimization, the amount of butanol produced from xylan was increased to 1.24 ± 0.37 g/L. In contrast to traditional acetone-butanol-ethanol (ABE) solventogenic fermentation, xylan supported fermentation in strain G117 and negligible amount of acetone was produced. The expression of genes normally associated with acetone production (*adc* and *ctfB2*) were downregulated compared to xylose fed cultures. This lack of acetone production may greatly simplify downstream separation process. Moreover, higher amount of butanol (2.94 g/L) was produced from 16.99 g/L xylo-oligosaccharides, suggesting a major role for strain G117 in butanol production from xylan and its oligosaccharides. The unique ability of strain G117 to produce a considerable amount of butanol directly from xylan without also producing undesirable fermentation

byproducts opens the door to the possibility of cost-effective biofuels production in a single step.

6.2 Introduction

The four carbon saturated alcohol butanol is considered to be one of the most promising alternative fuels due to its physicochemical similarities with gasoline (Cascone 2008; Ranjan and Moholkar 2012). Importantly, butanol can be generated as a product of anaerobic fermentation by a number of solventogenic *Clostridium* species (Dürre 2008; Lütke-Eversloh 2014). Butanol produced from a biological source is often termed "biobutanol". Sustainable biobutanol production is impeded by (i) the high costs associated with acquisition and pre-processing of substrates for the fermentation process and (ii) the complexities of downstream purification of biobutanol (Gu et al. 2011).

Many traditional fermentation substrates, such as corn starch, are also human foods and so the price of these substrates fluctuates wildly and has been generally increasing in recent years (Gu et al. 2011). One strategy available for circumvention of high substrate costs is use of inexpensive and abundant feedstock materials as the fermentative substrate. Consequently, over the past decade considerable research efforts have been focused on cost-effective utilization of lignocellulosic biomass such as xylan, which constitutes the principal hemicellulosic component of plant wastes and represents one third of all renewable organic carbon available on earth (Bastawde 1992; Prade 1996). However, the β-1,4-D-xylopyranose bonds that join xylose monomers in xylan must first be enzymatically hydrolyzed by a

group of xylanases for effective fermentation to occur (Bajpai 1997; Beg et al. 2001; Juturu and Wu 2014). Current research efforts are focused on developing consolidated bioprocessing (CBP) strategies for biofuels production wherein microorganisms are used to hydrolyze and ferment inexpensive lignocellulosic materials directly into desired products without additional enzymes (Lynd et al. 2005). CBP is widely considered to be the best solution for cost-effective hydrolysis and fermentation of lignocellulosic biomass (Olson et al. 2012). Though several strains of *Clostridium cellulyticum*, including metabolically engineered and wild-type strains, have been reported to generate value-added products directly from cellulose (Higashide et al. 2011; Sizova et al. 2011; Yang et al. 2015), most of the reported studies using hemicellulose compounds of lignocellulose as a fermentation substrate have been focused on ethanol or hydrogen production rather than on butanol (la Grange et al. 2010; Lynd et al. 2005; Olson et al. 2012; Tolonen et al. 2011). However, no wild-type strains are known to produce butanol directly from cellulose or xylan, leaving a need for development of one-step strategies for biobutanol production from lignocellulosic materials.

Difficulties in downstream separation of butanol from the acetone and ethanol produced as byproducts through traditional ABE fermentation are another barrier to large scale production of butanol (Gu et al. 2011). To simplify the downstream separation process, approaches seeking to redirect carbon flow into fermentative pathways that generate only butanol through the use of metabolic engineering have received general interest. However, several reports suggest that elimination of acetone producing metabolic pathways is

often associated with an undesirable decrease in overall butanol titer (Cornillot et al. 1997; Janatiidrissi et al. 1987; Nair and Papoutsakis 1994; Tummala et al. 2003a; Tummala et al. 2003b). Despite this, Jiang et al. (2009) achieved significant success in engineering acetone production out of a solventogenic *Clostridium* by disrupting the acetoacetate decarboxylase gene (*adc*) in *C. acetobutylicum* EA 2018. This engineered strain produced a butanol concentration comparable to its wild-type strain while generating a dramatically decreased amount of acetone in the presence of the exogenous electron acceptor methyl viologen, which can alter the carbon flow towards butanol in ABE fermentation (Jiang et al. 2009a). However, butanol production through such strategies has been conducted with expensive reducing sugars, like glucose, as the fermentative substrate. Hence, to obtain an economical yet simple process, it is desirable to use bacterial strains that can directly ferment lignocellulosic materials, such as xylan, to butanol as a principal product.

This study reports direct butanol production from xylan and xylo-oligosaccharides by a wild-type *Clostridium* sp. strain G117 isolated from grassland soil in Singapore. Compared to the traditional ABE fermentation process, this strain has the capability to produce acetone and butanol as the only products from fermenting glucose (Chua et al. 2013). Interestingly, acetone production was suppressed when strain G117 was fed solely with xylan, thereby laying the foundations for a butanol-only fermentation strategy using xylan as the sole carbon source.

6.3 Material and methods

6.3.1 Culture medium and cultivation

A wild-type *Clostridium* sp. strain G117 was cultivated under anaerobic conditions in 60 ml serum bottles containing 30 ml defined medium. The media composition used for the fermentation experiments was the same as described in chapter three. Xylan from beechwood (X4252), D-(+)-xylose (hereinafter called xylan and xylose, respectively) and xylo-oligosaccharides (XOS) were used as the carbon sources for fermentation. All experiments were carried out using a single carbon source, unless specified otherwise. For experiments involving xylan as the sole carbon source, xylan powder was weighed into serum bottles before addition of the fermentation medium under continuous nitrogen flushing, bottles were then sealed and autoclaved. For other cases, concentrated xylose / XOS stock solutions were sterilized separately and injected into the cultivation medium after autoclaving. Sterile yeast extract and vitamin stock solutions (Wolin et al. 1963) (used as additional nutrients) were injected separately into the sealed autoclaved culture bottles in selected experimental setups prior to inoculation. Fermentation studies conducted with xylose / XOS as the carbon source were inoculated with 6 % [v/v] inoculum. Cultures grown in serum bottles were incubated in an orbital shaker under the same conditions as described in chapter four.

6.3.2 Comparison of relative transcription level of acetone production related genes in xylose and xylan fed cultures

To perform gene expression studies related to acetone production, control cultures of strain G117 were fed with both xylan and xylose. At 48 h of fermentation, 1 ml of cultures was harvested by centrifuging at 140,000 rpm and 4 °C for 10 min. Total RNA was extracted using the same method as described in chapter four. Extracted RNA concentration was quantified using a NanoDrop such that equal quantities (242.75 ± 0.03 ng RNA from each sample) would be used to generate corresponding cDNA. cDNA samples were used as a DNA template for PCR amplification of the *adc* and Peptidase T (*pepT*) genes. The *pepT* gene (Wu et al. 2012) is a housekeeping gene for strain G117 and was used as an internal control to monitor efficiency of cDNA synthesis and PCR amplification. Details of the primers used for the experiments are shown in Table 6.1. The obtained PCR products were analyzed by 1 % agarose gel electrophoresis. Final concentration of primer was 0.4 µM in each qPCR reaction. After activating the enzymes in master mix at 98˚C for 3 min, qPCR reaction entered a thermal cycle consisting 40 cycles of 95˚C for 20 s and annealing temperature for 30 s.

Table 6.1 Specific primers for gene *adc, adhE1, adhE2* and *pepT* in strain G117.

Gene	Primer-forward 5'-3'	Primer-reverse 5'-3'	Annealing temperature
adc	CTTGCTGCTCCAGCG TTTC	GGCATAGCCATCATC TCAAATC	50˚C
ctfA	ATCTGGACTCGGAGG TGT	CATCGGCTGATAAAG GAA	45˚C
ctfB1	TCTTTCGCACTAATA AGAGG	GTATGTTGCATTGCC ACTA	45˚C
ctfB2	GATAGGAGAATCTGA CCCAG	TGCCCACCTCTTATTA TTG	45˚C
pepT	TGATGGAGGCGAGGA AGGTG	CATTGTATTCTTTGCA GACCCTGG	51˚C
adhE1	GATAGAATAGATAAG TTCGGAGTA	CCCAAGATCCACAGC CAAG	52˚C
adhE2	TGGAGTTGGAGCGGG AAATAC	TAGCGATTATGCTTT GTTCTGATGC	52˚C

The functional gene acetaldehyde dehydrogenase (*adhE*, G117 has two *adhE* genes named *adhE1* and *adhE2*) is a butanol production related gene which is responsible for converting butyrl-CoA into butyraldehyde (Figure 6.3). CoA transferase (*ctfAB*, G117 has three *ctfAB* genes: *ctfA, ctfB1* and *ctfB2*) is another gene associated with acetone production. To compare the transcription ratio of gene acetoacetate decarboxylase (*adc*), *ctfAB* and *adhE12*, cDNA samples obtained from cultures at 48 h of fermentation were used as a DNA template for qPCR amplification using the same method as described in chapter four. The relative abundance was calculated based on cycle to threshold value (Ct value) of target genes (*adc, ctfA, ctfB1, ctfB2 adhE1 and adhE2*) and was normalized to the abundance of housekeeping gene *pepT* for comparison.

6.3.3 Optimization of butanol production from xylan

To further improve butanol production through xylan fermentation, the impact of different parameters on target product output was evaluated.

Diverse fermentation parameters like inoculum size, substrate concentration, along with levels of vitamin, trace elements, yeast extract, metal cofactors (Fe^{2+}, Cu^{2+}, Ca^{2+}), and redox regulators (nicotinic acid (NA), L-asparagine (LA)) were tested within the experiments using the method of "one factor at a time" and the butanol output was monitored. All experiments were carried out at least twice and the corresponding butanol concentrations achieved were determined after 120 h of incubation when gas production from the fermentation broth had ceased (fermentation completed).

Among the different parameters tested, four different factors: Fe^{2+}, Cu^{2+}, vitamin and yeast extract were found to impact achieved butanol concentrations. To further optimize butanol production, interactive effects of these four factors were determined using response surface methodology (RSM) (Merrill 1994), wherein the fermentation parameters were used as the independent variable and the butanol concentration achieved within the experiments as the dependent variable.

6.3.4 Analytical methods

Methods for volatile fatty acids, biosolvents and gaseous products measurement were the same as described in chapter four.

Xylanase activity in the culture supernatants (obtained by centrifuging at 14000 rpm, 4 °C for 15 min) was measured according to the method described previously by Bailey *et al*. (Bailey et al. 1992) with slight modifications. Briefly, culture supernatants were amended with 1 ml 1 % [w/v] beechwood xylan and incubated at 55 °C, pH 5.5 for 10 min. The liberated reducing sugars were measured according to the 3,5-dinitrosalicylic acid (DNS) assay

method (Miller 1959) using a Micro Plate Reader (Infinite 200 PRO, Tecan, Switzerland) with absorbance measured at 540 nm. The reducing sugar concentrations were then read-off by a calibration curve prepared by standard xylose (0-1 g/L). One international unit (U) was defined as the enzymatic activity required for the release of 1 µmol of xylose equivalents per unit volume and per minute of reaction.

6.4 Results and discussion

6.4.1 Butanol production from pentose

To ascertain the ability of strain G117 to utilize pentose, it was first grown in presence of sugars like xylose and XOS. Strain G117 was found to efficiently utilize the pentose substrates under anaerobic conditions and butanol was observed in both xylose and XOS fed cultures within 24 h of inoculation (Figure 6.1). Although the initial substrate concentration for both cases was kept constant at 60 g/L, the final substrate utilization was found to be higher in the case of xylose (66.67 %) compared to that of XOS (28.3 %) with a corresponding butanol concentration of 6.03 and 2.94 g/L, respectively. Interestingly, despite the disparity in the substrate utilization, the butanol yields for both substrates were found to be similar i.e., 0.16 ± 0.1 g butanol / g substrate.

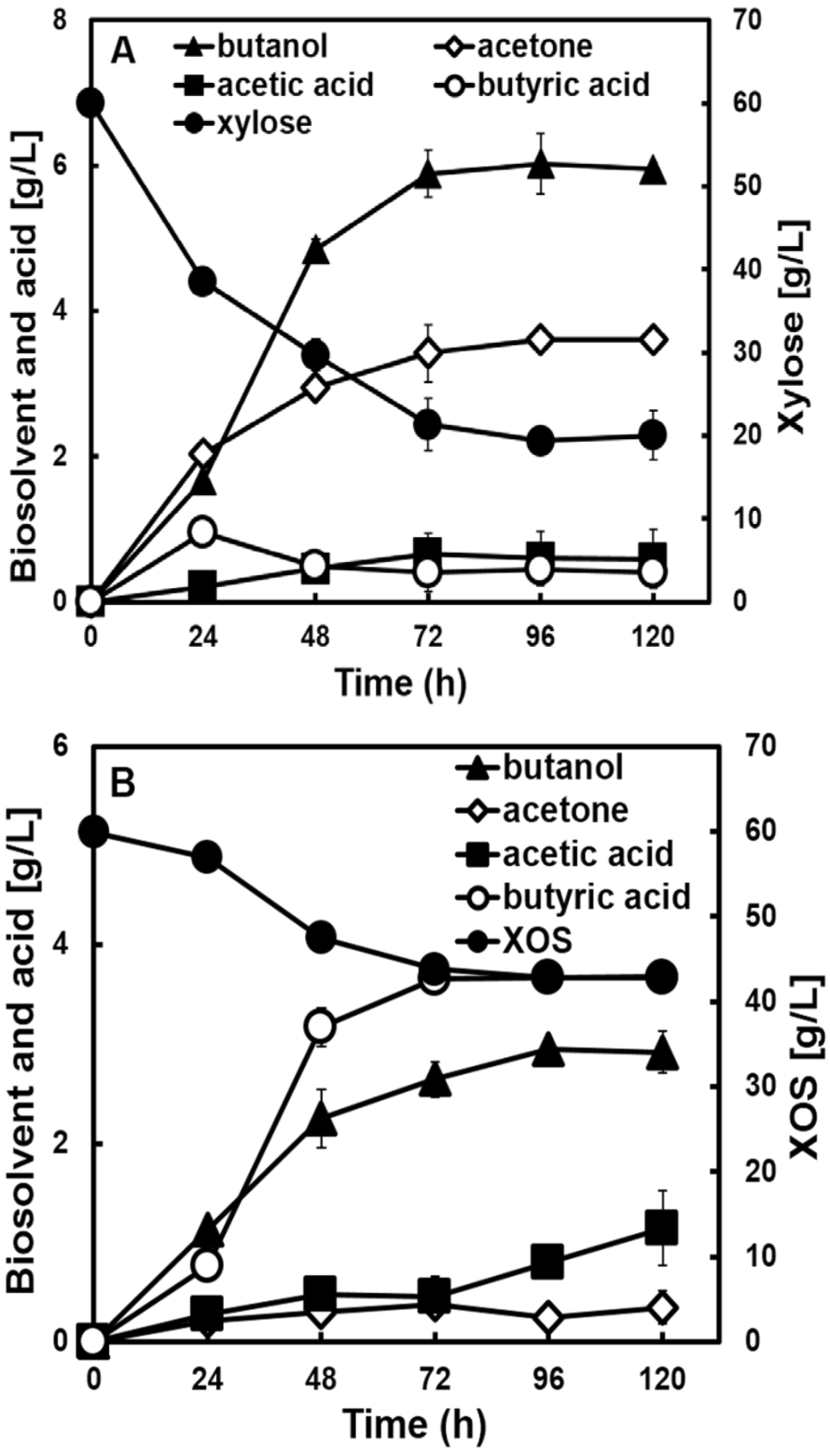


Figure 6.1 Biosolvent and acid production by *Clostridium* sp. strain G117 fed with (A) 60 g/L xylose, and (B) 60 g/L xylo-oligosaccharides. All experiments were conducted in biological duplicate and error bars are from independent fermentation runs.

As previously reported, solvent production by strain G117 is advantaged by its capability to produce only butanol and acetone compared to the conventional ABE fermentation pathway (Chua et al. 2013). In this study the observed butanol to acetone ratio from xylose fermentation was found to be similar to that of conventional ABE processes (1.67 ± 0.53 ≈ 2:1), however, the butanol to acetone ratio was significantly higher in the XOS fermentation (11.76 ± 3.58). Such a high butanol to acetone ratio from XOS fermentation is considered to be highly advantageous from a process commercialization perspective due to the complexities involved in the downstream separation of the solvent components from the fermentation broth. This ability to generate a higher ratio of butanol to acetone makes strain G117 a promising candidate for converting complex pentose polymers into butanol or similar value added products.

6.4.2 Direct butanol and hydrogen production from xylan by strain G117

The breakdown of xylan polymers into monomers can be achieved through a group of enzymes collectively called xylanases. Draft genome sequence analysis of strain G117 revealed the presence of several xylanase related genes (Wu et al. 2012), indicating the genetic potential to breakdown xylan into more metabolically tractable monomers (Table 6.2). Direct fermentation strategies were therefore attempted using strain G117 with 60 g/L xylan as the sole carbon source. The fermentation profile shown in Figure 6.2 indicates that strain G117 can produce butanol directly from xylan within 48 h with the concentration of butanol reached ~ 0.85 ± 0.07 g/L after 72 h of

incubation. Along with butanol, a cumulative yield of 53.4 ± 0.05 ml gaseous hydrogen was directly produced from xylan over the entire fermentation cycle. The proportion of hydrogen within the total gas produced was 42.4 ± 1.4 % with the balance being CO_2.

Table 6.2 Xylanase related genes presented in *Clostridium* sp. strain G117.

Enzyme	**Enzyme function**	**GI No.**
endo-1,4-β-xylanase	Glycosidic bonds (backbone)	515776638, 150017882
β-D-xylosidase	xylobiose; attack the non-reducing ends of short xylooligosaccharides to liberate xylose	515776646, 515779697, 150017888
α-glucuronidase	Alpha-1,2 bonds between the glucuronic acid residues and backbond	
acetylxylan esterase	O-acetyl group	515777783
α-arabinofuranosidase	Alpha-arabinose	515776700, 515776853, 515779698, 150017222, 150018938, 150019780

As expected from anaerobic fermentation operations, butyric acid and acetic acid were also produced from xylan along with butanol. In contrast to xylose fermentation, XOS utilization by strain G117 was also accompanied with a considerable amount of butyric acid accumulation (Figure 6.1), implying that complex sugars may have the potential to trigger systemic changes within the bacterial cell and prevent re-assimilation of the acids. Moreover, the butyric acid production from xylan fermentation was found to

monotonically increase, reaching 4.4 g/L after 96 h as shown in Figure 6.2, though butanol production had stopped within 72 h. The accumulation of butyric acid from fermenting xylan, particularly in the later fermentation stage may further indicate the impact of complex sugars on triggering reduced assimilation of acids by the bacteria. Compared to simple sugars, xylan is a more complex carbon source that requires the action of xylanases and xylosidases to degrade it into xylose before fermentation. In typical ABE fermentation butyric acid is the precursor of butanol production (Lee et al. 2008), however the accumulation of butyric acid during fermentation of xylan may be due to i) the low level of enzymatic xylan hydrolysis leading to insufficient formation of the reducing sugar necessary for butanol production (Jones and Woods 1986); ii) two more NAD(P)H are needed for butanol formation by re-assimilation of butyric acid (Lütke-Eversloh and Bahl 2011); iii) the low expression level of *ctfAB* which is responsible for acid re-assimilation to biosolvents. Hence, an increase in enzymatic hydrolysis leading to higher xylose accumulation, overexpression of *ctfAB* in xylan fed cultures, or an increase the reducing potential may increase acid re-assimilation and subsequent butanol production.

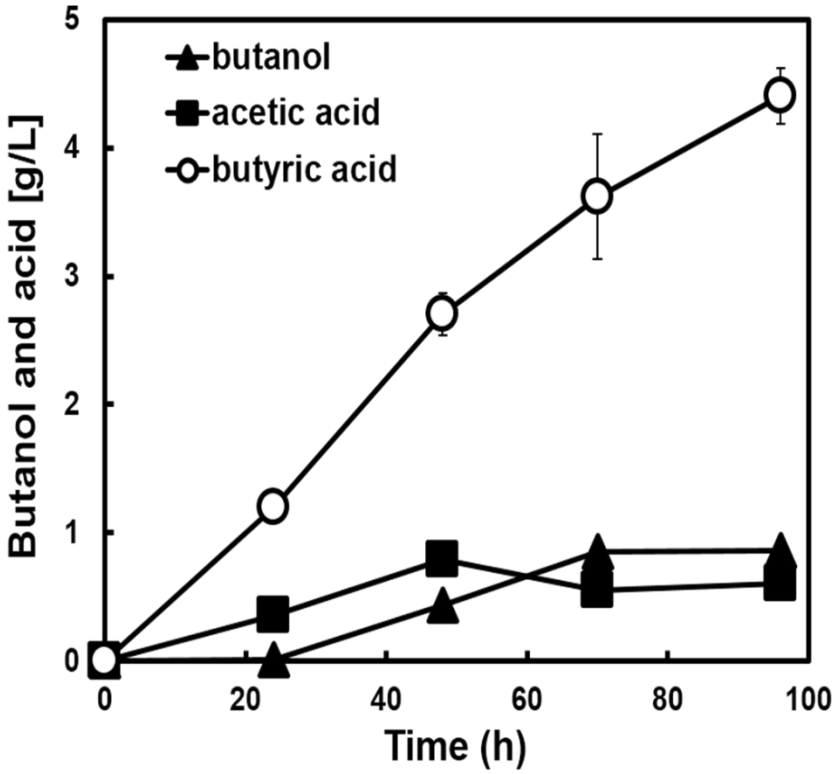


Figure 6.2 Biosolvent and acid production by *Clostridium* sp. strain G117 fed with 60 g/L xylan. Experiments were conducted in biological duplicate and error bars are from independent fermentation runs.

6.4.3 Negligible acetone production from xylan by strain G117

Xylan fermentation by strain G117 was accompanied by an interesting phenomenon, wherein no acetone production was observed and butanol was the sole solventogenic product (Figure 6.2). This observation implies that downstream butanol purification can be greatly simplified and thereby improve the overall process economics of butanol production (Ranjan and Moholkar 2012). To the best of our knowledge, this is the first instance where butanol was the only solvent produced in the solventogenic stage (without acetone and ethanol production). To confirm whether acetone production was systemically repressed, expression levels of the *adc* gene were compared during xylan and xylose fermentation. As shown in Figure 6.3 (Lee et al.

2008), the acetoacetate decarboxylase encoding *adc* gene is responsible for converting acetoacetate to acetone (Petersen and Bennett 1990) and disruption of *adc* in *Clostridium acetobutylicum* has been shown to result in reduced acetone production and a corresponding increase in the butanol to acetone ratio (Jiang et al. 2009a). A comparison of the band intensity of *adc* PCR amplicons (Figure 6.4A) indicates that *adc* expression in xylan fed G117 cultures was significantly reduced. Since the same quantity of RNA was used for synthesis of the cDNA used as a template in the PCR cycle of each sample, the concentration of corresponding PCR amplified products is indicative of gene expression within the samples. In contrast, the expression of the house-keeping gene *pepT* was similar in the two growth conditions.

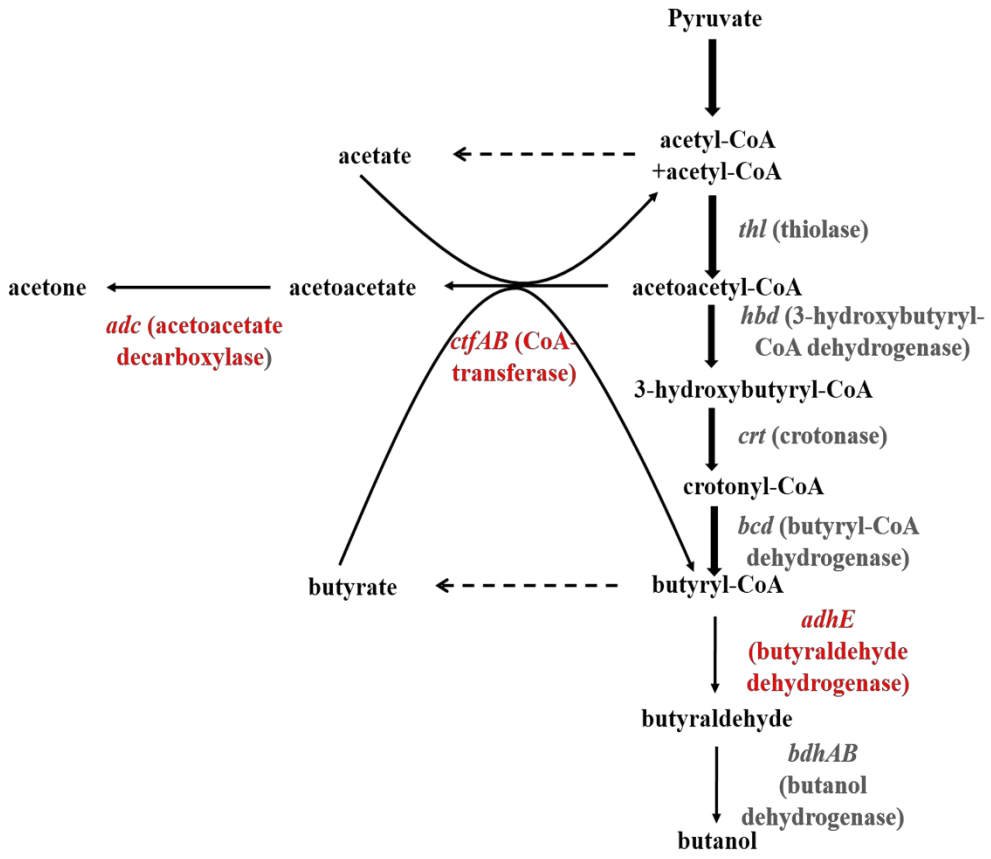


Figure 6.3 Typical metabolic pathway for acetone production by *Clostridium* species. Italic letters indicate genes and enzymes for the reaction. Dotted and solid arrows indicate reactions during acidogenic and solventogenic phase, respectively.

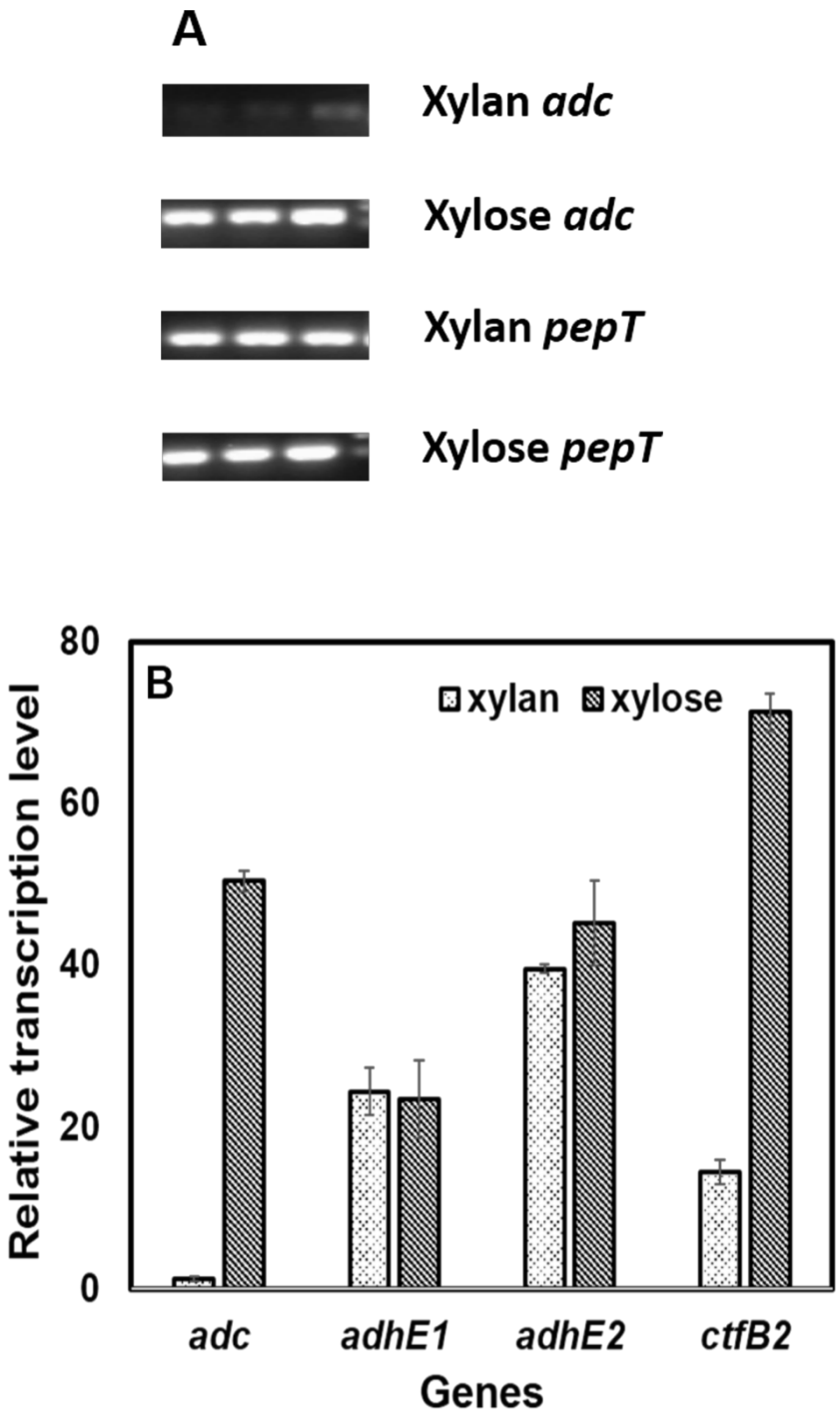


Figure 6.4 A: Band intensities obtained from the PCR amplified products using specific primers of gene *adc* and *pepT*. Expression of housekeeping gene *pepT* remained constant in both xylan (60 g/L) and xylose (60 g/L) fed cultures while gene *adc* was expressed less with xylan than that with xylose. B: Comparison of relative *adc*, *ctfB2* and *adhE12* transcription levels between xylan (60 g/L) and xylose (60 g/L) fed cultures. Results normalized to *pepT*, error bars are calculated from analytical errors.

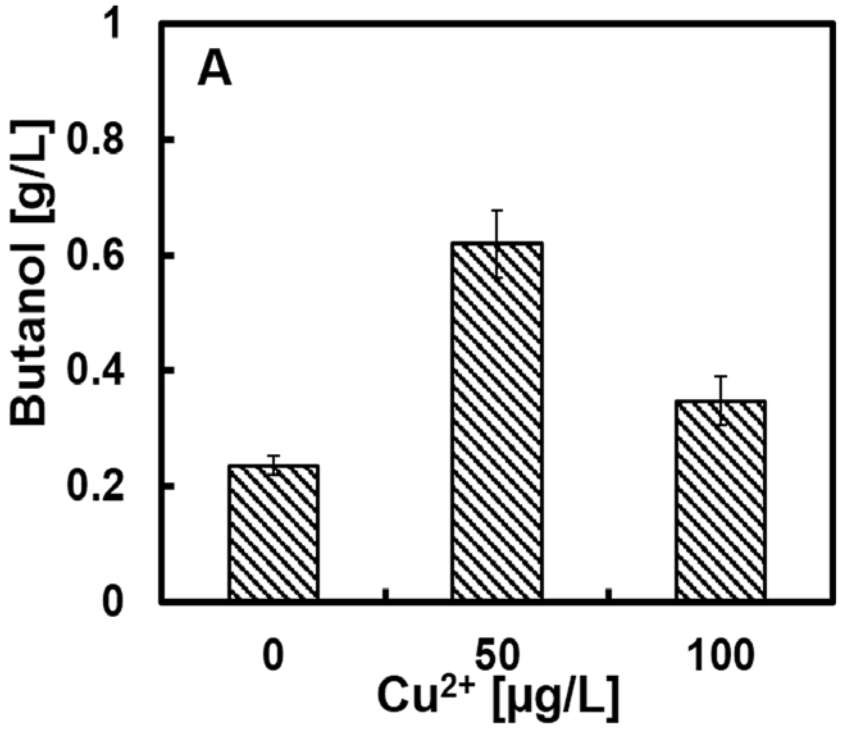
To confirm whether the lowered expression levels of the *adc* gene corresponds to the lowered acetone-production, the *adhE* (butanol generation associated gene) and *adc* (acetone generation associated gene) transcript levels were also quantified in both xylose and xylan fed cultures through quantitative real-time PCR (RT-qPCR). Figure 6.4B shows that the transcription levels of gene *adhE12* were comparable in xylose and xylan fed cultures while transcription of *adc* was lower in cultures fed with xylan. The disparity of the expression levels of *adc* accompanied with the comparable expression of *adhE* in xylan and xylose fed cultures suggests that the reduction of acetone production may result from inhibition of *adc*, possibly by electron flux balancing (Nakayama et al. 2011).

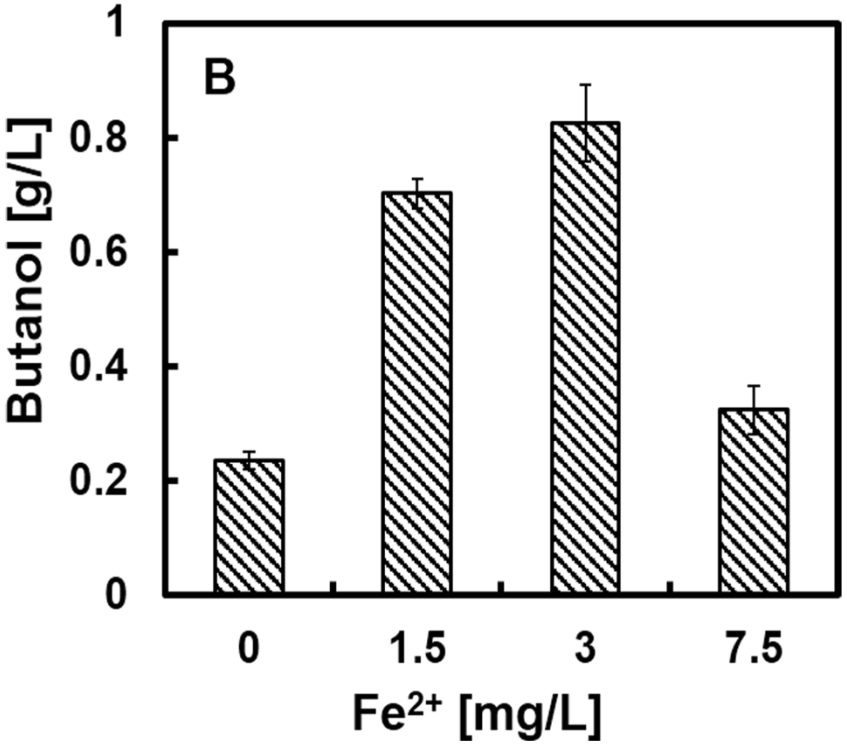
Additionally, it has been reported that acetone can be produced through non-enzymatic breakdown of acetoacetate, suggesting that *ctfAB*, converting acetoacetyl-CoA to acetoacetate, plays an important role in acetone generation (Han et al. 2011; Yu et al. 2015b). Hence, transcription levels of *ctfA, ctfB1* and *ctfB2* were compared in both xylose and xylan fed cultures through RT-qPCR. Results show that transcription levels of *ctfA* and *ctfB1* were comparable in both conditions; while *ctfB2* was transcribed at lower levels in xylan than xylose fed cultures (Figure 6.4B). This suggests that the negligible acetone production in strain G117 may result from the lower transcription levels of either or both *adc* and *ctfB2*. The lower expression level of *ctfB2* also provides a possible explanation for butyric acid accumulation in xylan fed cultures. Regarding the comparable transcription levels of *ctfA* and *ctfB1,* strain G117 may possess some unique metabolic properties accounting for its difference in *ctfA* and *ctfB1* expression from strains used in previous reports in

Clostridium tyrobutyricum and *C. beijerinckii* NCIMB 8052. For example, unlike *C. tyrobutyricum* and *C. beijerinckii* NCIMB 8052, strain G117 produces negligible ethanol from reducing sugars such as glucose and xylose and there is no obvious acidogenic phase and solventogenic phase during the whole fermentation process (Chua et al. 2013). In addition, it also has been reported that disruption of *adc* resulted in a decrease of acetone production in *C. acetobutylicum* ATCC 824 as well as in other solventogenic *Clostridium* species (Jiang et al. 2009a). This supports our finding that downregulation of *adc* is associated with a decrease of acetone production by solventogenic *Clostridium*.

6.4.4 Optimization of butanol production from xylan

For efficient xylan utilization solventogenic bacteria need to degrade xylan in the surrounding environment before metabolizing the degraded substrates to butanol. The role of xylanase in this extracellular xylan hydrolysis suggests a correlation between enzyme activity and butanol production from xylan. However, our results demonstrate that higher xylanase activities did not correspond to increased butanol yields (data not shown). Interestingly, among the factors tested for increasing butanol production, four independent parameters (Cu^{2+}, Fe^{2+}, vitamin and yeast extract) seemed to improve butanol titer (Figure 6.5). The interactive effects of these four variables were analyzed using RSM to obtain optimum conditions for butanol production from xylan by strain G117.


A
Butanol [g/L]
1
0.8
0.6
0.4
0.2
0
0
50
100
Cu2+ [µg/L]


B
Butanol [g/L]
1
0.8
0.6
0.4
0.2
0
0
1.5
3
7.5
Fe2+ [mg/L]

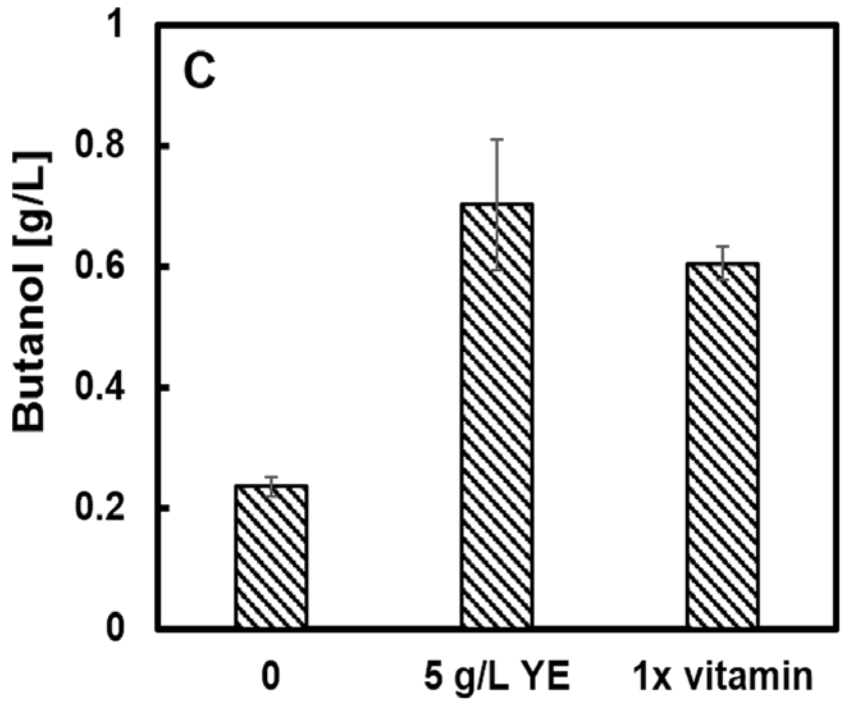


Figure 6.5 Effect of individual factors on butanol production from xylan. YE: yeast extract. All experiments were conducted in biological duplicate and error bars are from independent fermentation runs.

A total of 54 trials were conducted and the butanol concentration obtained from each trial was analyzed and fitted to a second-order polynomial equation which was used to draw a response surface plot as shown in Figure 6.6. This response surface plot indicates that maximum butanol production should be 1.25 g/l when Fe^{2+}, Cu^{2+} and yeast extract concentrations are 1500 μg/L, 50 μg/L and 5 g/L, respectively and the vitamin concentration is maintained as 1X. This analysis indicates that the impact of the yeast extract on butanol production significantly overshadow the effects of other individual factors. In the presence of yeast extract, factors like Fe^{2+}, Cu^{2+} and vitamin concentrations did not seem to have a significant impact on the butanol production. Interestingly, even under such conditions the interactive effects of Fe^{2+} and vitamin solution showed significant contribution to butanol

production. The less significant effects of individual factors may be due to the elemental composition of the yeast extract itself which is known to contain around 70 µg/g Cu^{2+}, 150 µg/g Fe^{2+} and traces of vitamin B complex (Grant and Pramer 1962). However, the Fe^{2+} concentration used in the RSM study was much higher than that present within yeast extract (ranging from 750 to 3750 µg/L) and the vitamin solution used consisted of various vitamins in addition to B-complex. Hence, although the effects of Fe^{2+} and vitamin were not as obvious as yeast extract individually, the interaction of these two factors significantly affected the butanol production from xylan by strain G117.

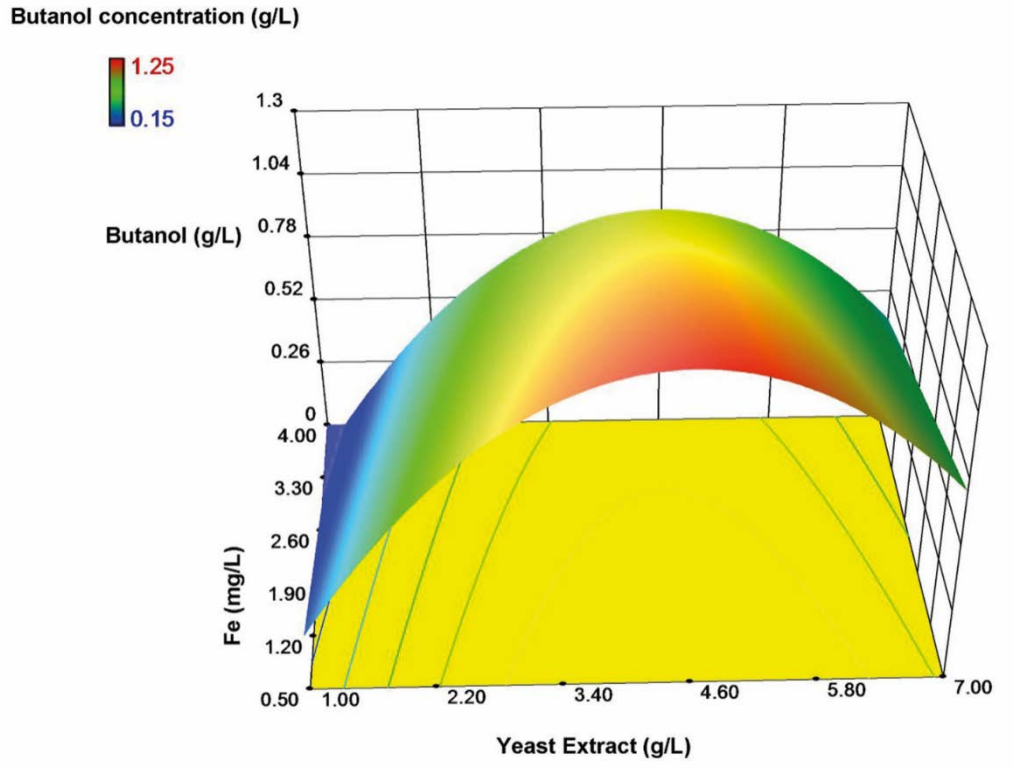


Figure 6.6 Response surface plot for butanol production from strain G117 using xylan as a substrate.

Carbon balance was calculated as shown in Table 6.3. Total carbon source was 1.8 g xylan (60 g/L in 30 ml). The xylan used in this study is from

beechwood purchased from Sigma. Beechwood is a kind of hardwood and the substitution of acetyl groups and 4-*O*-methylglucuronic acid to xylose backbone are 70-80% and 10%, respectively (Beg et al. 2001; Polizeli et al. 2005). Hence, based on the molecular weight, quantity percentage of xylose in xylan should be ~70.75% [g/g] and then the total carbon source is 1.26 g xylose. The amount of carbon in the products is less than the equivalent amount of xylose contained in xylan.

Table 6.3 Calculation of carbon balance when using 60 g/L xylan as substrate for butanol production by strain G117.

	Total carbon source	**Products**			
	Xylose	Butanol	Butyric acid	Acetic acid	CO_2
Quality, g	1.26	0.0255	0.132	0.018	72.5 ml
mole	0.0084	0.000344	0.001498	0.0003	0.003237
Mole of carbon in total	0.042	0.011			

Table 6.4 presents a comparison of the results obtained through this study with those obtained from the limited avaiable literature reporting direct conversion of lignocellulosic biomass into butanol or other similar value-added products. Among these reports, *C. acetobutylicm* 7 was found to be capable of producing 1-butanol (4.1 g/L) from 30 g/L grass with a butanol/acetone ratio of 1.78 only when the medium was amended with 30 g/L flour (Berezina et al. 2008). Apart from wild type strains, numerous genetically modified strains have also been developed for the direct bioconversion of lignocellulosic biomass to biofuels. Among the previously reported metabolically engineered strains, *C. cellulolyticum* H10, engineered

to extrachromosomally express *kivd yqhD alsS ilvCD* which generates 0.66 g/L iso-butanol from cellulose within 7 to 9 days can probably be considered as the best butanol producing strain (Higashide et al. 2011). Compared to theses previous studies, strain G117 shows significant advantages in several aspects including direct conversion of xylan to a higher amount of butanol as the only solventogenic product. This metabolic property of G117 can greatly improve the economic viability of biobutanol production both in terms of the associated substrate costs and the downstream separation complexities.

Table 6.4 Comparison of biofuel production from lignocellulosic biomass in mono-cultures.

Organism	Relevant genotype	Substrate (g/L)	Product	Titer (g/L)	Butanol/Acetone ratio	Reference
C. japonicus Ueda 107	Metabolic engineered	Avicel (10)	Ethanol	< 0.5		(Gardner and Keating 2010)
C. phytofermentans ATCC700394	Wild type	Filter paper (10)	Ethanol	2.9		(Tolonen et al. 2011)
C. thermocellum	Metabolic engineered	Avicel	Ethanol	1.7		(Deng et al. 2013)
C. thermocellum	Metabolic engineered	Switchgrass	Ethanol	1.7		(Yee et al. 2014)
C. thermocellum	Metabolic engineered	Avicel	Ethanol	5.61		(Argyros et al. 2011)
C. cellulolyticum pWH320	Metabolic engineered	Crystalline cellulose (10)	Isobutanol	0.66		(Higashide et al. 2011)
C. thermocellum	Metabolic engineered	Avicel	Isobutanol	5.4		(Lin et al. 2015b)
C. acetobutylicum 7	Wild type	Grass (30) and flour (30)	1-Butanol	4.1	1.78	(Berezina et al. 2008)
C. cellulovorans	Metabolic engineered	Crystalline cellulose	Butanol	1.42		(Yang et al. 2015)

C. phytofermentans ATCC700394	Wild type	Birchwood xylan (3)	Ethanol	0.46		(Tolonen et al. 2011)
C. sp. strain G117	Wild type	Beechwood xylan	Butanol	1.25	N.A.	This study

6.5 Conclusions

This study presents how a wild type *Clostridium* sp. strain G117 can be used as a potential candidate for direct conversion of xylan into butanol. Strain G117 was found to be particularly advantageous due to its capability to repress acetone production while fermenting xylan (fed with 60 g/L) thereby enabling it to produce butanol (1.25 g/L) as the sole solventogenic product within 5 days. The findings of this study thus offer fundamental knowledge for the future development of economically viable alternative fuel production strategies.

CHAPTER 7

Conclusions and Recommendations

This doctoral study was initiated to realize direct conversion of lignocellulosic biomass to biobutanol. Three newly isolated *Clostridium* strains exhibiting unique fermentation capabilities were subjected to detailed investigation in order to gain insights. The discovery of these three strains has added promising candidates for direct biobutanol production from lignocellulosic biomass, and should have profound impacts on the future development of industrial-scale cost-effective biobutanol production.

7.1 Conclusions

Through a series of studies using lignocellilosic biomass associated substrates for fermentation, direct biological butanol production from xylan by wild-type *Clostridium* strain was achieved. The key conclusions made in each section of this doctoral study are listed below.

Chapter three introduced an enhancement on butanol production from glucose by adding nicotinic acid, a metabolic precursor of NADH and NADPH, to the fermentation medium in bioreactor using *Clostridium* species strain BOH3.

1. A pH-shift strategy for butanol production improvement based on specific cell growth rate and specific butanol production rate was set up in a bioreactor as following: pH is controlled at 6.0 during the first 6 h (excluding lag phase), after which the pH is allowed to drop to 5.0 as the culture progresses and maintained at 5.0.
2. The addition of nicotinic acid to the fermentation medium led to a significant increase in the availability of NADH and NADPH as

indicated by an increase in the ratio of NADH/NAD^+ and NADPH/$NADP^+$ of 2.96 and 3.42 fold, respectively.

3. With pH-shift and additional nicotinic acid (10 mg/L), cell growth of strain BOH3 increased by 11 % and the doubling time reduced by ~40 %. Butanol production reached 18.7 g/L (27.8 g/L total ABE production), a 16 % increase as compared to cultures without the addition of nicotinic acid. The glucose consumption rate, butanol yield, butanol productivity and biosolvent to bioacid ratio were 9 %, 7%, 14 % and 214 % higher, respectively, compared to the corresponding values of the control without nicotinic acid addition.
4. These results describs the increase of intracellular NADH and NADPH availability to enhance the salvaging pathway on butanol production through introduction of nicotinic acid. Results suggest that adjusting the levels of NAD(P)H could be an efficient approach to improve butanol production, and manipulating cofactors may provide an additional means to determine final cellular metabolites distribution.

Chapter four discussed a simultaneous fermentation of glucose, xylose and arabinose by wild-type *Clostridium* sp. strain MF28.

5. The lack of carbon catabolite repression in strain MF28 was approved by its simultaneous utilization of glucose, xylose and arabinose.
6. Strain MF28 can finish butanol production within 48 h without obvious acidogenic and solventogenic phases, leading to high

butanol productivity which is favorable from a process commercialization perspective.

Chapter five investigated the toxicity effects of lignocellulose-derived inhibitors on cell growth and butanol production from undetoxified lignocellulosic hydrolysate by *Clostridium* sp. strain BOH3.

7. Strain BOH3 was found to be capable of resisting over 60 mM furfural, 60 mM HMF and 6.6 mM vanillin, respectively. Cell growth of strain BOH3 was stimulated by furfural and HMF after a lag phase, when their concentrations were lower than 40 mM and 50 mM, respectively.
8. Combination of inhibitors with furfural led to a severer synergistic effect on cell growth and butanol production of strain BOH3. Strain BOH3 is able to convert ~60 mM furfural into furfuryl alcohol within 90 h, which is related to the up-regulation of short chain dehydrogenase/reductases.
9. Strain BOH3 was shown to generate considerable butanol (5.15 ± 0.52 g/L) from un-detoxified horticultural waste hydrolysate. This observation can be considered highly promising from economical view, where detoxifications need to be directed towards removal of hydrolysate inhibitors.

Chapter six reported direct butanol production from xylan by *Clostridium* sp. strain G117.

10. Strain G117 produced 0.86 ± 0.07 g/L butanol and 53.4 ± 0.05 mL hydrogen directly from 60 g/L xylan provided that had undergone no

prior enzymatic hydrolysis. After process optimization, the amount of butanol produced from xylan was increased to 1.24 ± 0.37 g/L.

11. Butanol is the only solvent produced by strain G117 when xylan was used as the sole carbon source.
12. The lack of acetone production from xylan was associated with downregulated transcription levels of *adc* and *ctfB2* as compared to xylose fed cultures.

7.2 Recommendations for future studies

The findings in this doctoral study broadens our understanding in cultures specialized in butanol production from lignocellulose associated substrates. Future studies will strive to obtain more in-depth knowledge about these cultures, including regulators involved in transition between acidogenic and solventogenic phases, mechanisms for self-aggregation, as well as elimination of byproducts production. Specifically, the areas that require continued research work include:

1. More genes involved in sporulation and transition between acidogenic and solventogenic phases will be identified and characterized in strain MF28 to figure out reasons for the one-phase fermentation by this strain. Up- or down-regulation of the responsible genes could offer an alternative option to improve butanol productivity in other solventogenic strains.
2. Stimulate effect of vanillin on self-aggregation in strain BOH3 will be studied. Microarray analysis will be conducted to investigate expression fold changes of genes in cultures with or without self-

aggregation. Through analysis of gene transcription level changes in cultures with or without self-aggregation, mechanism of self-aggregation in strain BOH3 is expected to be illustrated. Self-aggregated culture is superior to other immobilized fermentation since no materials are needed to support its immobilization.

3. The butanol-only fermentation by strain G117 only occurred when xylan was utilized as the sole carbon source. Manipulation of *adc* and *ctfAB* in strain G117 will be conducted to achieve butanol-only fermentation from other substrates such as glucose and xylose.

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