Butanol Production from Lignocellulosic Biomass and Agriculture Residues by Acetone-Butanol-Ethanol Fermentation

Dissertation

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

By

Jie Dong, M.S.

Graduate Program in Chemical and Biomolecular Engineering

The Ohio State University
2014

Dissertation Committee:
Professor Shang-Tian Yang, Advisor
Professor David Wood
Professor Aravind Asthagiri
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Abstract

Butanol is an important intermediate and solvent in the chemical industry. Moreover, compared to ethanol, butanol has higher energy density and lower vapor pressure, so butanol is also considered a preferred fuel additive or even a potential replacement for gasoline. As the oil price rises, the cost of producing butanol from petrochemical processes increases dramatically. Therefore, more and more research is being done on ways to produce butanol from fermentation of renewable resources. It is necessary to reduce the cost of fermentation-derived butanol in order to make it competitive with petrochemically produced butanol. One of the most influential economy factors in producing butanol by fermentation is the cost of substrate. In this research, in order to reduce the cost of substrate, different types of lignocellulosic biomass, such as sugarcane bagasse, soybean hull, cotton stalk and corn fibers were utilized as substrates in ABE (Acetone-Butanol-Ethanol) fermentation. However, lignocellulosic biomass cannot be used by the solventogenic *clostridia* directly and needs to be hydrolyzed first. The hydrolysis process usually produces some inhibitory compounds that could severely inhibit bacteria growth and butanol production. Therefore, the inhibitors in the hydrolysate must be reduced or removed by certain detoxification processes before fermentation. To make the detoxification process more efficient, a better understanding of these inhibitors is needed. In this research, the effects of 9 inhibitors derived from the degradation of carbohydrate and lignin on the fermentation kinetics of several
solventogenic Clostridia strains were investigated at various concentration levels. The inhibitors' effects on butanol and butyraldehyde dehydrogenase activities were also investigated. Among the 9 inhibitors studied, four lignin derived inhibitors (syringaldehyde, ferulic acid, vanillin, and p-coumaric acid) were found to strongly inhibit cell growth and butanol and butyraldehyde dehydrogenases. These four phenolic inhibitors were further tested in C. tyrobutyricum mutants. They were very toxic to one mutant overexpressing ctfAB and adhE2 genes, but they had no effects on the mutant overexpressing only adhE2 gene. Therefore, the phenolic compounds are inhibitors to CoA-transferase expressed by ctfAB. This was further confirmed by their toxicity to C. beijerinckii and C. acetobutylicum. This also explained the toxicity of corn steep liquor as nitrogen source in the butanol fermentation.

Then four lignocellulosic biomass, cotton stalk, corn fiber, soybean hull and sugarcane bagasse, were pretreated with acid and then hydrolyzed by enzymes. The obtained hydrolysates were further detoxified to remove the inhibitors in them. The detoxified hydrolysates were then used as carbon source in ABE fermentation of Clostridium. All hydrolysates gave very high butanol production. The acid and alkali pretreatments followed with removing the supernatant and washing before enzymatic hydrolysis were studied, and their effects on biomass compositions and subsequent fermentation were also studied and compared.
Other than lignocellulosic biomass, another possibility for substrates is some other agriculture residues generated during the bioprocessing. In this study, four of these agriculture residues were investigated: cassava bagasse, Jerusalem artichoke, soy molasses and soybean meal. Starch based cassava bagasse only needs enzyme (glucoamylase) hydrolysis. Its hydrolysate contained no toxic inhibitors and performed very well in ABE fermentation. Inulin based Jerusalem artichoke was hydrolyzed only by dilute acid. But its hydrolysate contained high concentration of ferulic acid which is an inhibitor to ABE fermentation. Soy molasses contain mainly oligosaccharides and can be hydrolyzed by α-galactosidase. But in the ABE fermentation using soy molasses hydrolysate, the butanol production was partly inhibited. Soybean meal was also proved to be a good nitrogen source after the acid hydrolysis. The detailed capital and operating cost of large scale butanol plants were also analyzed.
Dedication

Dedicated to my wife Yao Nie,
my son Felix
and my parents
Acknowledgements

First of all, I would like to give my greatest thanks to my advisor, Dr. Shang-Tian Yang, for his academic and financial support during my four years Ph.D. study. His great personalities, his patience, his insights have greatly influenced me, both on academic research and personal life. I'm really grateful that he gave me the opportunity to enter the field of biotechnology and bioengineering. It's a great honor to do research under his guidance and encouragement. As an excellent scientist and admirable person, he sets a brilliant example that someday I wish I can be. I gained really a lot from him.

I would also thank Dr. Aravind Asthagiri and Dr. David Wood for taking time to be on my committee, as well as their valuable advice to my research.

Then I would like to thank Dr. Congcong Lu. She taught me everything about microbiology at the beginning, all the basic knowledge and techniques. She taught me how to use every piece of equipment in the lab and every detailed experiment skills. I'm really grateful. I am thankful to Dr. Jingbo Zhao for teaching me about the fibrous bed bioreactor and giving me great suggestions on my research. My thanks also go to all the previous and current group members, especially Dr. Chuang Xue, Dr. Mingrui Yu, Dr. Zhongqiang Wang, Dr. Yiming Du, Dr. Chih-Chin Chen, Fangfang Liu, Le Yu, Mengmeng Xu, for their helps and suggestions. I also thank Dr. Dong Wei from South China University of Technology for providing the biomass used in my work. I would also thank ARPA-E, NSF-STTR, and USB for their financial support during my Ph.D. research.
Finally, I would like to thank my wife Yao Nie who accompanies me and sacrifices her time taking care of our son Felix at home so I have more time doing research. Thank my parents and friends for their supports.
Vita

June 2003 .......................................................... Rizhao No. 1 high school

2003 – 2007 ........................................... B.S. Chemical Engineering & Technology,

Zhejiang University

2007-2010 ........................................................... M.S. Chemical Engineering,

Zhejiang University

2010-2011 ........................................... Graduate Fellowship, Department of Chemical &

Biomolecular Engineering

The Ohio State University

2011 – present ................................ Graduate Research Associate, Department of Chemical &

Biomolecular Engineering

The Ohio State University

Publications

Lu, Congcong; Dong, Jie; Yang, Shang-Tian, Butanol production from wood pulping
hydrolysate in an integrated fermentation-gas stripping process, Bioresource Technology
(2013), 143, 467-475.

Dong, Jie; Wu, Linbo; An, Dong; Li, Bo-Geng; Zhu, Shiping, Stability study of inverse
suspension copolymerization of 1,1,3,3-tetramethylguandium acrylate and N,N'-methylene

Fields of Study

Major Field: Chemical & Biomolecular Engineering

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Chapter 1: Introduction

The growing scarcity and increasing price of fossil fuels are driving researchers to find viable substitutes. Butanol is a saturated primary alcohol (CH$_3$CH$_2$CH$_2$CH$_2$OH) and also a promising biofuel. Compared to ethanol, butanol has a higher energy density and lower vapor pressure, so it is more convenient and safer for utilization and storage. More importantly, butanol’s energy density, air-fuel ratio and heat of vaporization are similar to those of gasoline. Besides being a potential replacement for fossil fuels, butanol is an important solvent used in the production of antibiotics, vitamins and hormones. Butanol is also an intermediate in the manufacturing of butyl acrylate and methacrylate esters (Jones et al., 1986).

Acetone-Butanol-Ethanol (ABE) fermentation is an important bioprocess that produces butanol. It was first discovered by Pasteur in 1861 (Jones et al., 1986). During 1900s, ABE fermentation developed very rapidly both in the research field and in industrial process due to the large demand for acetone in the synthesis of rubber, especially during World War I (Gabriel, 1928). However, after 1950s, several petrochemical processes were developed for butanol production, and ABE fermentation was no longer economically competitive due to the high cost of substrates (Kumar et al., 2011). Recently, because the oil price is increasing very quickly and the crude oil is depleting, more and more research is returning to ABE fermentation. In order to reduce
the cost of ABE fermentation and make it competitive to petrochemical process, a lot of research is being done on increasing the butanol production by metabolic engineering and reducing the substrate cost by using cheaper lignocellulosic biomass or agriculture residues as feedstocks.

Solventogenic *Clostridia* species are usually the microorganisms in ABE fermentation. There are mainly 5 metabolic products in ABE fermentation, two acids (acetic acid and butyric acid) and three solvents (acetone, ethanol and butanol). In most solventogenic *Clostridia*, the solvent (acetone/butanol/ethanol) ratio is 3:6:1. The total ABE titer is 15-18 g/L with 10-13 g/L butanol. ABE fermentation by *Clostridia* usually has two phases, acidogenesis and solventogenesis (Lee et al., 2008). In the acidogenesis phase, glucose or other sugars are converted into acids such as acetate acid and butyrate acid, which leads to rapid decrease in pH. Under certain critical pH, *Clostridia* shifts to the solventogenesis phase during which it consumes acids to produce solvents (acetone, ethanol and butanol). The most important factor that triggers the shift from acidogenesis to solventogenesis is external and internal pHs (Jones et al., 1986). Some metabolically engineered *Clostridia* do not have this shift mechanism (Yu et al., 2011). Extensive metabolic engineering work has been done to enhance butanol production in Clostridia (Dürre, 1998; Ezeji et al., 2004; Qureshi et al., 2008). This is one way to reduce the cost of ABE fermentation.

Another way to reduce the cost of ABE fermentation is looking for cheaper substrates. Over 50% of ABE fermentation cost is due to the cost of substrates. Maize and molasses, which are the current substrates used in ABE fermentation, make up a
large percentage of the processing costs. Based on maize, butanol production cost was projected to be $0.55/kg and substrate cost accounted for over 56% ($0.31/kg butanol) of the production cost (Ezeji et al., 2008). It is therefore necessary to find other, more economical substrates in order to reduce the cost of producing butanol by ABE fermentation. Lignocellulosic biomass, including wheat straw, barley straw, maize stover, switchgrass, etc., is generally much cheaper than starch and sugar based substrates. Their prices ranged from $26.5 to $66.1/ton as opposed to $256.3/ton for maize in 2009 (Zverlov et al., 2006). So they are more economical substrates for ABE fermentation.

The main components of lignocellulosic biomass are cellulose (30%-60%), hemicellulose (5%-30%) and lignin (5%-30%). Most solventogenic *Clostridium* species cannot use these carbon sources directly. Therefore, the lignocellulosic biomass needs to be hydrolyzed into sugars before ABE fermentation. The hydrolysis of lignocellulosic biomass usually contains three steps: pretreatment, enzymatic hydrolysis and detoxification. The most important part is the enzymatic hydrolysis, in which cellulose is hydrolyzed by cellulase enzymes. Cellulase is being investigated and combined with other enzymes to increase its activities and efficiencies in lignocellulosic biomass (Henrissat, 1994; Duarte, 2012). In order to facilitate the contact between cellulose and cellulase, some pretreatments to break down the rigid structure of lignocellulosic biomass are necessary. Extensive research has been done on different pretreatment methods to increase the sugar yield from lignocellulosic biomass (Kumar et al., 2011). The acid pretreatment was investigated in detail in this research because its process is relatively simple and cheap and it can hydrolyze the hemicellulose part of lignocellulosic biomass.
During the pretreatment, some inhibitory compounds are produced due to the degradation of sugars and lignin. These inhibitory compounds can inhibit the growth of Clostridia severely and also block butanol production in ABE fermentation. There are three main groups: furan derivatives (Furfural and 5-hydroxymethyl furfural (HMF)), weak acids (formic acid, and levulinic acid) and phenolics (p-coumaric acid, ferulic acid, vanillin, hydroquinone, and syringaldehyde). Furan derivatives and weak acids come mainly from the degradation of monosaccharides, while phenolics are generated from lignin degradation. Although the inhibition mechanism is still unclear, their inhibition effects to Clostridia are obvious (Ezeji et al., 2008; Lee et al., 2011). Therefore, these inhibitors must be removed or at least reduced by some detoxification methods before ABE fermentation. Various methods of detoxification have been studied, such as overliming (Mussatto et al., 2010), electrodialysis (Martinez et al., 2010), adsorption (Qureshi et al., 2007), filtration (Qureshi et al., 2008) and enzymatic reactions (Larsson et al., 1999).

Lignocellulosic biomass needs a long treatment process before being used in ABE fermentation, including pretreatment, enzymatic hydrolysis and detoxification. These treatments increase the operating cost of ABE fermentation. Therefore, other non-lignocellulosic biomass has also been studied as feedstocks for ABE fermentation (Qureshi et al., 1995; Ezeji et al., 2007; Cho et al., 2009; Larsson et al., 1999). Non-lignocellulosic biomass includes agriculture residues and byproducts from bioprocessing, which usually do not need the three steps of treatment as for lignocellulosic biomass.
1.1 Project goal and specific tasks:

The goals of this research were to find economical substrates for butanol production and to investigate the effects of these substrates on ABE fermentation. The most economical substrates for ABE fermentation are lignocellulosic biomass, such as cotton stalk, sugarcane bagasse, soybean hull and corn fiber, which are composed mainly of cellulose, hemicellulose, and lignin. In general, no solventogenic *Clostridium* can utilize these polysaccharides directly. So for ABE fermentation to take place, one or more hydrolysis processes are necessary to decompose cellulose and hemicellulose into single sugars. The hydrolysis processes were also studied and optimized in this research. An overview of this study is provided in Figure 1.1. The specific objectives and major tasks are described below.

**Task 1: Effects of lignocellulosic biomass hydrolysate derived inhibitors on ABE fermentation**

The effects of 9 inhibitors (furfural, HMF, formic acid, levulinic acid, hydroquinone, syringaldehyde, p-coumaric acid, vanillin and ferulic acid) derived from the degradation of carbohydrate and lignin on the fermentation kinetics of several solventogenic *C. beijerinckii*, *C. acetobutylicum* and *C. tyrobutyricum* strains were investigated at various concentration levels. The effects of these inhibitors on butanol and butyraldehyde dehydrogenase activities were also studied. Among the 9 inhibitors studied, four lignin-derived inhibitors (syringaldehyde, ferulic acid, vanillin, and *p*-coumaric acid) were found to strongly inhibit cell growth and butanol and butyraldehyde dehydrogenases (Chapter 3).
Task 2: Butanol production from lignocellulosic biomass

Four different kinds of lignocellulosic biomass, cotton stalk, corn fiber, soybean hull and sugarcane bagasse, were pretreated with acid and then hydrolyzed by enzymes. The effects of different acid concentrations on sugar yields and inhibitors production were studied. The main inhibitors after acid pretreatment were formic acid and ferulic acid. The obtained hydrolysates were further detoxified by adsorption with activated carbon to remove the inhibitors. The detoxified hydrolysates were then used as carbon sources in ABE fermentation of *Clostridium*. All hydrolysates gave high butanol production. The acid and alkali pretreatments followed with removing the liquors containing the dissolved inhibitors were also studied and compared for their effects on biomass compositions and subsequent fermentation (Chapter 4).

Task 3: The inhibition mechanism of lignin-derived phenolic compounds

The inhibition mechanism of four lignin-derived phenolic compounds was investigated. Four phenolic inhibitors, syringaldehyde, ferulic acid, vanillin, and *p*-coumaric acid, were tested in *C. tyrobutyricum* mutants. They were found to be very toxic to the mutant overexpressing *ctfAB* and *adhE2* genes, but not to the mutant overexpressing only *adhE2* gene. Therefore, the phenolic compounds are inhibitors to CoA-transferase expressed by *ctfAB*. This was further confirmed by their toxicity to *C. beijerinckii* and *C. acetobutylicum*. This inhibition mechanism was also found to exist when corn steep liquor was used as nitrogen source in ABE fermentation (Chapter 5).

Task 4: Butanol production from non-lignocellulosic biomass
Four agriculture residues were investigated: cassava bagasse, Jerusalem artichoke, soy molasses and soybean meal. The main composition of cassava bagasse is starch. It did not need the acid pretreatment. After hot water pretreatment and enzymatic (glucoamylase) hydrolysis, a high sugar yield was obtained. Its hydrolysate contained no toxic inhibitors and performed very well in the ABE fermentation. Jerusalem artichoke contains plenty of inulin. It did not need enzymatic hydrolysis. After acid pretreatment, most sugars were released. Its hydrolysate contained a high concentration of ferulic acid. Soy molasses contain mainly oligosaccharides, which can be hydrolyzed by α-galactosidase. But in the ABE fermentation using soy molasses hydrolysate, butanol production was partly inhibited. Soybean meal was also proved to be a good nitrogen source, after acid hydrolysis, for ABE fermentation (Chapter 6).

1.2 Significance and major impacts

n-Butanol is currently an important chemical and solvent in industry. The main application of n-butanol currently is to synthesize butyl acrylate and butyl methacrylate. Butyl acrylate and butyl methacrylate are further polymerized to esters, which are widely used for latex paints, coatings, binders and adhesives (Mascal, 2011). In 2011, the global market size for n-butanol was 2.8 million metric tons. In USA, the consumption of butanol is 740,000 metric tons in 2011 with 2.2% growth rate (Mascal, 2011). So there is already a big market for n-butanol. Moreover, as a potential biofuel, the market would be much larger. The Energy Independence and Security Act of 2007 (EISA) set a revised Renewable Fuels Standard (RFS). The revised RFS mandates the volume of renewable
fuels sold in the USA must be 36 billion gallons by 2022, of which 16 billion gallons must be cellulosic biofuels.

This study provided a better understanding of the inhibitors present in the biomass hydrolysates and their effects on solventogenic clostridia. The inhibition mechanism of phenolic compounds on CoA-transferase was revealed, and this finding can guide the detoxification process in the hydrolysis of lignocellulosic biomass. This study also showed the diversity and feasibility of feedstocks in butanol production. Four different kinds of lignocellulosic biomass and three different agricultural residues were proved to be good substrates for ABE fermentation. With proper treatments, they all could be converted to butanol at a high concentration suitable for industrial application. The hydrolysis of lignocellulosic biomass was successfully optimized. The acid pretreatment was demonstrated to be an efficient pretreatment for lignocellulosic biomass. With activated carbon detoxification, the yield of butanol from all four lignocellulosic biomass can be as high as that from pure glucose. The detailed capital and operating costs of large scale butanol plants were also analyzed. The large scale cellulosic butanol was proved to be profitable and competitive with petrochemically synthesized butanol. This also means that a promising sustainable fuel, butanol, can be produced from economical renewable resources.
1.3 References


Henrissat, B. Cellulose, 1994, 1:169–196


Mascal M. Chemicals from biobutanol: technologies and markets, 2011, 6: 483-493


Meinita M.N., Yong-Ki Hong, Gwi-Taek Jeong, Detoxification of acidic catalyzed hydrolysate of Kappaphycus alvarezi (cottonii), Bioprocess Biosyst Eng 2012, 35:93–98


Qureshi N. Agricultural residues and energy crops as potentially economical and novel substrates for microbial production of butanol (a biofuel), Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2010, 5: 1-8


Yu, M.-R.; Zhang, Ya-Li; Tang, I.-Ching; Yang, Shang-Tian, Metabolic engineering of Clostridium tyrobutyricum for n-butanol production, Metabolic Engineering, 2011, 13(4), 373-382.

Figure 1.1 Overview of project goal and major tasks carried out in this study
Chapter 2: Literature Review

2.1 Acetone-Butanol-Ethanol (ABE) Fermentation

The production of butanol in fermentation was first discovered by Pasteur in 1861 (Jones et al., 1986). In 1905, Schardinger found the fermentation could also produce acetone. Due to the shortage of natural rubber and the research of synthetic rubber, butanol fermentation was further studied as butanol can be converted into butadiene. Butadiene is the monomer to synthesize rubber. Between 1912 and 1914, Chaim Weizmann (Hastings et al., 1978) isolated a bacterium which could produce a large amount of butanol and acetone. This strain was named as *Clostridium acetobutylicum* by McCoy et al. (1926). During World war I, the large demand of acetone further promoted the development of ABE fermentation. Weizmann developed an ABE fermentation process to produce acetone and directed a large pilot-scale work (Dürre, 1998). From 1920 to 1980, many ABE fermentation plants were built all over the world. Acetone and butanol were the major products. The main application of acetone and butanol was to synthesize rubber. However, after 1950s, several petrochemical processes were developed for butanol production, and ABE fermentation was no longer economically competitive due to the high cost of substrates (Kumar et al., 2011).

Over the past few decades, because the oil price is increasing very quickly and the crude oil is depleting, more and more research is returning to ABE fermentation because
butanol is a promising biofuel (Demain, 2009; Dürre, 1998; 2007; Lee et al., 2008; Qureshi et al., 2008; Swana et al., 2011; Weber et al., 2010; Zheng et al., 2009). Compared to ethanol, butanol has a higher energy density and lower vapor pressure, so it is more convenient and safer for utilization and storage. More importantly, butanol’s energy density, air-fuel ratio and heat of vaporization are similar to those of gasoline (Table 2.1).

2.1.1 Microorganisms and strain improvement

Clostridia species are usually the microorganisms used in ABE fermentation. Clostridia are rod-shaped, spore-forming, gram-positive and obligate anaerobic bacteria, belonging to the Firmicutes. Besides C. acetobutylicum, three other species, C. beijerinckii, C. saccharoperbutylacetonicum and C. saccharobutylicum (Lee et al., 2008), were also identified as butanol producers and they gave high butanol production and yields. Some other Clostridia strains including C. carboxidivorans, C.butylicum, C. aurantibutyricum and C. pasteurianum were also investigated (Bruant et al., 2010; Ezeji et al., 2008; Somrutai et al., 1996; Ahn et al., 2011). Their substrates, products, fermentation pH and temperature were summarized in Table 2.2. Almost all strains can use a variety of hexose and pentose sugars as substrates. Most strains can use disaccharides such as sucrose and cellobiose and even polysaccharides such as starch. Some strains can even use novel substrates. C. carboxidivorans can use syngas and C. pasteurianum can ferment glycerol.
Among the solventogenic Clostridia, *C. acetobutylicum* and *C. beijerinckii* are the most widely studied butanol producing strains. Researchers studied a large variety of mutants from these strains, including *C. acetobutylicum* P262, *C. beijerinckii* P260, 8052, BA101, and LMD 27.6 (Huang et al., 2004; Parekh et al., 1998; Qureshi et al., 2006, 2010; Kumar et al., 2011). All of these strains have similar characteristics for ABE fermentation. For example, they produce total ABE in the range of 10 g/L-30 g/L and the typical ratio of acetone, butanol and ethanol is about 3:6:1.

ABE fermentation by *C. acetobutylicum* and *C. beijerinckii* has two phases, acidogenesis and solventogenesis (Lee et al., 2008) (Figure 2.1). At first, the bacterium will convert glucose into acids such as acetate acid and butyrate acid. This is called the acidogenesis phase and leads to rapid decrease of pH. Under certain critical pH, the bacterium will shift to the solventogenesis phase during which it will consume acids to produce ethanol and butanol. The most important factor that triggers the shift from acidogenesis to solventogenesis is external and internal pH (Jones et al., 1986). One shortage of *Clostridia* is degeneration phenomenon. They gradually lose the ability to produce solvent during the cultivation. In *C. acetobutylicum*, this degeneration phenomenon is due to the loss of megaplasmid pSOL1 (Lee et al., 2008). Another problem lies that butanol is high toxic to *Clostridia* itself. 0.1–0.15M butanol caused 50% inhibition of both cell growth and sugar uptake rate by negatively affecting the ATPase activity (Jones et al., 1986). So the production of butanol ceases when the concentration of solvent reaches around 20g/L. This solvent toxicity highly inhibits the butanol yields of *Clostridia*. 
C. tyrobutyricum was studied in our group (Yu et al., 2011). Different from solventogenic Clostridia, the Wild type C. tyrobutyricum only produces acetate acid and butyric acid, so its metabolic pathway doesn’t contain the right side in Figure 2.1. Our lab has engineered C. tyrobutyricum ATCC 25755 to overexpress aldehyde/alcohol dehydrogenase 2 (adhE2) and CoA-transferase (ctfAB). Then this mutant can convert acetate/butyrate into ethanol/butanol, but it doesn’t have the shift mechanism from acidogenesis to solventogenesis. Butanol production from glucose by these mutants was high with 20 g/L butanol titer. Moreover, C. tyrobutyricum showed good butanol tolerance. It still can reach >80% and ~60% relative growth rate at 1.0% and 1.5% (v/v) butanol, respectively. C. tyrobutyricum is a promising butanol production strain that may be used in the future.

2.1.2 Renewable feedstocks

Over 50% of ABE fermentation cost is due to the cost of substrates. Maize and molasses, which are the current substrates used in ABE fermentation, make up a large percentage of the processing costs. Based on maize, butanol production cost was projected to be $0.55/kg and substrate cost accounted for over 56% ($0.31/kg butanol) of the production cost (Ezeji et al., 2008). It is therefore necessary to find other, more economical substrates in order to reduce the cost of producing butanol by ABE fermentation. Lignocellulosic biomass, including wheat straw, barley straw, maize stover, switchgrass, etc., is generally much cheaper than starch and sugar based substrates. Their prices ranged from $26.5 to $66.1/ton as opposed to $256.3/ton for maize in 2009 (Zverlov et al., 2006). So they are more economical substrates for ABE fermentation.
ABE fermentation using traditional substrates and lignocellulosic feedstocks was listed in Table 2.3.

Lignocellulose is the main component of plant cell walls. Lignocellulose biomass is mainly composed of cellulose, hemicellulose, and lignin. The composition of these three constituents can vary from one plant to another. For one plant species, it can even vary from one season to another. For instance, wood usually contains more cellulose, whereas wheat straw and corn cobs have more hemicelluloses. The composition and current use of some lignocellulosic biomass were listed in Table 2.4. Most lignocellulosic biomass are just burnt or landfilled. Some of them can be used as animal feed. So they are waste materials from industrial bioprocessing with extremely low price. If they can be used as feedstocks in ABE fermentation, the cost of ABE fermentation will be reduced.

The cellulose and hemicellulose contents in lignocellulosic biomass can be converted into sugars and used as substrates in ABE fermentation. Cellulose which is usually in a fibrous structure in cell walls is a linear polymer consists of D-glucose subunits linked to each other by β-(1,4)-glycosidic bonds. Hydrogen and van der Waals bonds link those cellulose long chains together to form microfibrils. Hemicellulose is also a long chain polysaccharide but has branches with short chains consisting of some other monosaccharides, such as pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose). The backbone of hemicellulose is usually linked by β-(1,4)-glycosidic bonds and occasionally by β-(1,3)-glycosidic bonds. Lignin is a complex polymer of phenolic monomers which is cross-linked with each other. It is the safeguard of plant cell wall, which is impermeable and resistant against most microbial attack. The
monomers of lignin are coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol) which are linked by alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds. Usually, woods have the highest lignin contents, whereas grasses have the lowest (Howard et al., 2003; Saha, 2003; Reddy and Yang, 2005; Qureshi et al., 2007; Kumar et al., 2009; Sun and Cheng, 2002).

*C. acetobutylicum* and *C. beijerinckii* are capable of fermenting a variety of sugars, including hexoses (glucose, galactose and mannose) and pentoses (xylose, arabinose). They can even utilize more complex carbohydrates such as dextrin, starch and inulin. This flexibility makes a lot of biomass as potential substrates to produce butanol (Table 2.2). Cane molasses has been used in the production of butanol (Qureshi et al., 2005). Researchers have also investigated whey permeate (Maddox et al., 1993), maize starch and soy molasses (Qureshi et al., 2001) as substrates. For these substrates, the yield is high because these substrates contain a large quantity of carbohydrates that can be utilized by the solventogenic *clostridia* mentioned above. However, some of these substrates are also used as animal feed, which leads to a higher price. Improved food processes also reduce the sugar concentrations in those molasses.

Other, less expensive substrates have also been studied (Table 2.2). These substrates can be divided into two main categories: lignocelluloses and starch. Lignocelluloses, like barley straw, wheat straw, corn fiber and corn stover, are mainly composed of cellulose, hemicelluloses, lignin and a little ash. The solventogenic *clostridia* cannot utilize these polysaccharides directly, so lignocelluloses must be hydrolyzed into single sugars before fermentation. On the other hand, starch, such as sago,
extruded corn and cassava can be directly used as carbon source by the solventogenic *clostridia*; therefore no hydrolysis is needed before fermentation (Table 2.2). Corn fiber is a by-product of the maize wet milling process. Qureshi et al studied it as a substrate to produce butanol after hydrolysis (Qureshi et al., 2007), 9.3 g/L total ABE (Acetone, butanol and ethanol) was produced. Qureshi et al also used wheat straw as feedstock to produce butanol, 25.0g/L ABE was obtained (Qureshi et al., 2010a). Moreover, Qureshi et al investigated barley straw (Qureshi et al., 2010b), maize stover and switchgrass (Zverlov et al., 2006). Thaddeus et al chose distillers dry grains and soluble (DDGS) as a substrate and compared it with different mixed streams of pure sugars (Ezeji et al., 2008). The highest ABE yield in Thaddeus et al’s research is 12.1 g/L. Instead of feed-grade biomass, Zverlov et al investigated agriculture wastes, such as hemp waste, corn cobs and sunflower shells (Qureshi et al., 2008).

Although all of these substrates can be utilized by solventogenic *clostridia*, the yield of butanol is much lower than the control which uses pure glucose as substrates. Most maximum ABE concentration is only around 25 g/L and ABE yield ranged from 0.24-0.44. One possible reason for the low yield is that the total sugar concentration is relative low after hydrolysis; another possible reason is that some fermentation inhibitors that are toxic to strains may be contained in those hydrolysates. Therefore, further research is needed to analyze the optimized hydrolysis conditions to get more sugars and proper methods to remove inhibitors in hydrolysates, in order to get high yields of ABE, especially butanol.
2.2 Hydrolysis of lignocellulosic biomass

2.2.1 Pretreatments

Most solventogenic *clostridia* can’t use lignocellulosic biomasses directly. Therefore, hydrolysis is always needed to decompose the lignocelluloses. Before the enzymatic hydrolysis, some pretreatments are usually used to break up the rigid structure of lignocelluloses so that the enzymes can easily get contact with them in the following process.

The main goal of the pretreatment process is to reduce the crystallinity of cellulose and increase the porosity of these materials. Some methods can even remove some parts of lignin and degrade hemicelluloses. Pretreatment can improve the formation of monosaccharides or the abilities to form sugars by hydrolysis. Pretreatment also needs to avoid the further degradation monosaccharides and the formation of byproducts that are inhibitory to the subsequent fermentation processes. Table 2.5 listed those processes used to pre-treat lignocellulosic biomass. They can be roughly divided into five categories: physical (mechanical comminution, pyrolysis); physicochemical (steam explosion, AFEX, CO₂ explosion); chemical (ozonolysis, acid hydrolysis, alkaline hydrolysis, organosolv); biological; other methods (pulsed electrical field).

Mechanical comminution is usually combined with other pretreatments and used to reduce the size of biomass particles before further pretreatments. It is used to reduce cellulose crystallinity. After milling or grinding, the biomass particle size can be reduced to 0.2-2 mm (Sun et al., 2002). The energy consumption in milling is the major concern
in large scale process. Smaller particle size means much higher energy input. If the final particle size is 3-6 mm, the energy consumption can be kept below 30 kWh per tons of biomass (Cadoche et al., 1989).

Steam explosion is the most common method to pretreat lignocellulosic biomass. Biomass is immersed in high pressure saturated steam (0.7-5.0 MPa, 160-260 °C) for several minutes, and then the pressure is suddenly reduce to atmospheric pressure (McMillan et al., 1994). The explosive decompression causes fragmentation of cellulose fiber which increase the surface area to contact with cellulase enzymes. The high temperature also hydrolyzes hemicellulose and partly removes lignin which also increase the contact surface between cellulose and cellulase. The efficiency of enzymatic hydrolysis of polar chips increased to 90% after steam explosion, compared to 15% in untreated polar chips (Grous et al., 1986). Ammonia fiber explosion (AFEX) is very similar to steam explosion. It only replaces the saturated steam with liquid ammonia. The temperature is much lower than steam explosion (~90 °C) and the residence time is much longer (0.5-1.5 hr). Almost no hemicellulose and lignin are removed during AFEX. But the biomass structure becomes more porous and water holding capacity is increased. After AFEX, 90% of cellulose and hemicellulose in switchgrass and bagasse was hydrolyzed into sugars (Holtzapple et al., 1991). CO₂ explosion is to replace ammonia in AFEX with supercritical CO₂. Supercritical CO₂ needs lower temperature than ammonia and reduces the cost further. CO₂ can form carbonic acid with water in the biomass and the carbonic acid can hydrolyze hemicellulose. CO₂ molecule has similar size with water and ammonia. So it can penetrate the biomass structure as well as water or ammonia. For
pretreatment of recycled paper mix and sugarcane bagasse, CO₂ explosion was more cost-effective compared with steam and ammonia explosion (Zheng et al., 1998).

Dilute acid pretreatment is also a common method to pretreat lignocellulosic biomass. The most obvious advantage of dilute acid pretreatment is that it can hydrolyze and recover almost all hemicellulose as dissolved sugars in the solution. High temperature is favorable (>120 °C) to increase the hydrolysis efficiency of hemicellulose. Dilute H₂SO₄ (below 4 wt %) is most commonly used in the dilute acid pretreatment (Mosier et al., 2005). HNO₃, HCl and H₃PO₄ were also tested (Brink et al., 1993; Israilides et al., 1978; Goldstein et al., 1983). Plenty of biomass were examined by dilute acid pretreatment, such as legume byproducts, reed canary grass, corn cobs and stover, hardwood bark from aspen, polar and sweet gum (Kumar et al., 2009). Pilot-scale dilute H₂SO₄ pretreatment of corn stover was also tested in a vertical reactor. The temperature was 180-200 °C, the solid loading was 25%-35%, the acid loading was 0.03-0.06 g/g dry biomass and the residence time is ~1 min (Ishizawa et al., 2007).

Bases can also be used for pretreatment of lignocellulosic biomass which is called alkali pretreatment. The temperatures and pressures used in alkali pretreatment are much lower than the explosion and dilute acid pretreatment. It can be carried out at room temperature and atmospheric pressure, but takes longer time from several hours to days. NaOH, KOH, Ca(OH)₂ and NH₃ · H₂O are all suitable bases for alkali pretreatment. Among them, NaOH was studied the most (Elshafei et al., 1991; Soto et al., 1994; Fox et al., 1989). But Ca(OH)₂ pretreatment is more cost effective because it is less expensive and easy to be precipitated by CO₂ from solution and recovered. Alkali pretreatment
induces less sugar degradation than dilute acid pretreatment. It only removes part of lignin and hemicellulose which increases the crystallinity index of cellulose. So after alkali pretreatment, the initial enzymatic hydrolysis rate will be slow. But it has no effects on the final sugar yields (Chang et al., 2000). Lime was successfully used to pretreat corn stover (Kim et al., 2006), wheat straw (Chang et al., 1998), poplar wood (Chang et al., 2001) and switchgrass (Chang et al., 2001).

Other than the above three common pretreatment methods, there are some other novel pretreatments that show promising characters. Ozone can be used to reduce the lignin and hemicellulose contents in many lignocellulosic biomass and doesn't produce toxic inhibitors (Quesada et al., 1999). Organic solvents mixed with acids can also be used to break lignin and hemicellulose in biomass. Methanol, ethanol, acetone and ethylene glycol are the common solvent used in this organosolvation pretreatment (Thring et al., 1990). It involves both the hydrolysis of hemicellulose and delignification of biomass supported by organic solvents. In biological pretreatment, fungi such as brown, white and soft rots are used to degrade lignin and hemicellulose in biomass. White-rot fungi are the most effective to degrade lignin because it has lignin degrading enzymes such as peroxidase and laccase (Lee et al., 2007).

Each process has its own advantages and disadvantages (Table 2.5). Therefore, sometimes two or more pretreatment processes are combined together to use their advantages and make up their disadvantages. It’s hard to say which process is the best. It depends on the lignocellulosic biomass, like their compositions and possible products produced in the pretreatment process. We are inclined to use dilute acid pretreatment.

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because it can hydrolyze hemicelluloses sugars and its cost is not very high. However, some components that are inhibitory to the fermentation process may be generated in the acid hydrolysis process. How to minimize these inhibitors is one of our tasks.

2.2.2 Enzymatic hydrolysis

After the pretreatment, enzymes such as cellulase and xylanase are used to further degrade the lignocelluloses. The main work of hydrolysis is to break the β-(1,4)-glycosidic bonds existed either in cellulose or hemicelluloses. These bonds can be broken either by acids or by enzymes. However, concentrated acids are usually needed to degrade cellulose due to its microfibril structure, which can induce a lot of side reactions and may further degrade those monosaccharides. Those byproducts produced in the concentrated acid hydrolysis are usually very toxic to the ABE fermentation process. So enzymes such as cellulase and xylanase are usually preferred to be used to degrade celluloses and hemicelluloses into monosaccharides.

Cellulase is a class of enzymes that catalyze cellulysis which is the hydrolysis of cellulose. Many organisms can produce cellulase, such as fungi, bacteria, protozoans and even some termites. Cellulase breaks the 1,4-β-D-glycosidic linkages in cellulose. There are mainly three types of cellulases: endocellulase, exocellulase and β-glucosidase. Although three types all work on the hydrolysis of 1,4-β-D-glycosidic linkages, their mechanisms are a little different from each other. Endocellulases (EGs) randomly cleave glycosidic linkages at amorphous sites and chains with different length (molecular weight) are generated in this process. Exocellulase cleaves mainly two glucose units from one end
of cellulose chains and produces cellubiose. So there are two main types of exocellulases (celllobiohydrolases, CBHs): CBHI works from the reducing end, and CBHII works from the nonreducing end. β-glucosidase breaks cellubiose into single sugars. When lignocellulosic biomass are hydrolyzed, all these three celluloses can work together in a synergistic mechanism to get a better production of monosaccharides (Figure 2.2). *Trichoderma reesei* which is the Industrial strain of cellulase production can produce up to five types of EGs and two types of CBHs (CBH I and CBH II). There are other two types of cellulases that are not very common, oxidative cellulases and cellulose phosphorylases.

Although EGs, CBHs and β-glucosidase have different functions in the breakdown of cellulose, they share very similar molecular structures. They mainly have two functional domains: a cellulose binding domain (CBD) and a catalytic domain (CD). CBD binds to the cellulose substrate while CD cleaves the glycosidic bonds (Henrissat, 1994). These two domains are linked together by a peptide sequencer (Figure 2.3).

The hydrolysis of celluloses and hemicelluloses is a crucial step in the fermentation to produce biofuels because lignocellulosic biomasses are more promising and economical substrates. But most yeast or bacteria strains cannot use celluloses or hemicelluloses directly. So they must be hydrolyzed before fermentation. Cellulases catalyze this key hydrolysis process. However, it’s hard to use cellulases directly to hydrolyze celluloses due to the rigid structure of plant cell walls. There are mainly three components in the plant cell wall: cellulose, hemicelluloses and lignin. Lignin has a three dimensional network of *p*-hydroxyphenylpropane units and its main function is to protect
celluloses from breakdown or hydrolysis. It is entangled with or even chemically linked to celluloses (Siqueira et al., 2010). Cellulose crystallization is another resistance to the hydrolysis. So before cellulase hydrolysis, biomasses need to be treated in some way that destroy the rigid structure between celluloses and lignin and also reduce the degree of cellulose crystallinity. Different methods and materials have been used in this pretreatment process as described in the previous section, such as liquid hot water, steam explosion, dilute acid, etc. Although these pretreatments can facilitate the contact between celluloses and cellulases, they may generate some toxic components that can inhibit the cellulase activity. The cellulose inhibitors derived in the biomass treatment processes need to be removed before enzymatic hydrolysis.

2.3 Detoxification of lignocellulosic hydrolysates

2.3.1 Inhibitors to enzymatic hydrolysis

Although the pretreatments of lignocellulosic biomass can facilitate the contact between cellulose and cellulase, they can also generate or release some components that can inhibit the cellulase activity.

First inhibitor group comes from the partly degradation of lignin in the pretreatment process. The partly degradation of lignin can generate some phenolic compounds that may be very toxic to cellulas. Table 2.6 lists some of these lignin derived phenolic compounds and their effects on cellulas. Tannic acid is the most toxic inhibitor to cellulas. 300 μg condensed tannins/mL can completed inhibit the activity of endoglucanases (Bae et al., 1993). Tannic acid can precipitate β-glucosidases, which is
one reason for its high toxicity. Sinapic acid is another very toxic compound. The production of cellulases was completely inhibited in the presence of 3 μmol cm$^{-3}$ of sinapic acid (Sineiro et al., 1997). Simple and oligomeric phenolics can absorb onto cellulose and thus prevent the contact between cellulase and its substrate. Simple phenolics can also form complex with cellulases and deactivate them (Tejirian et al., 2011). Insoluble lignin also has some negative effects on the hydrolysis process because it can absorb cellulases. Therefore, it’s better to use some separation methods to remove those inhibitors before the hydrolysis using cellulases.

Second inhibitor group comes from the products-monosaccharides. Table 2.7 lists the main monosaccharides and cellobiose produced in the hydrolysis process and their inhibition effects on cellulases. Glucose has a significant inhibition effect on cellulases. Glucose strongly inhibited the cellulase activity from *T. reesei* QM 9414, in which 2.5% glucose inhibited 50% of the reaction. *Trichoderma* β-glucosidase activity was inhibited by 50% in the presence of 0.2–0.4 g/L glucose (Takagi, 1984). Cellobiose, which is the main product of exocellulases, is considered to be a powerful inhibitor of cellulases and inhibits this enzyme 14 times more than glucose (Duarte, 2012). Therefore, it’s better to continuously remove these monosaccharides in the hydrolysis process so that this kind of production inhibition can be limited.

There are some other components that may be generated in the hydrolysis process and inhibit cellulase activity. Acetic acid, formic acid and sodium gluconate are all strong inhibitors to cellulase activities (Takagi, 1984). Acetic acid and formic acid are products from the degradation of glucose or other pentose sugars. So they may exist in the medium
after pretreatment. Sodium gluconate can be produced in the pH adjustment process, especially after dilute acid pretreatment when pH adjustment is very necessary.

2.3.2 Inhibitors to ABE fermentation

During hydrolysis process, some inhibitors that could inhibit fermentation process may be generated due to the complex components of lignocelluloses (celluloses, hemicelluloses and lignin). Table 2.8 shows the most common potential inhibitors after hydrolysis process (Ezeji et al., 2008; Lee et al., 2011). There are three main groups: furan derivatives (Furfural and 5-hydroxymethyl furfural (HMF)), weak acids (glucuronic acid, formic acid, acetic acid and levulinic acid) and phenolics (p-coumaric acid, ferulic acid, vanillin, hydroquinone, and syringaldehyde). Furan derivatives come mainly from degradation of monosaccharide molecules, while phenolics are generated by lignin degradation or monosaccharide degradation during acid hydrolysis. HMF’s further breakdown could form some weak acids like formic acid and levulinic acid (Ezeji et al., 2008; Lee et al., 2011).

Their toxicities vary with different mechanisms. Furfural and HMF can even simulate the growth of C. beijerinckii BA101 (Cho et al., 2009). Ferulic acid and p-coumaric acid are the most toxic compounds to most solventogenic clostridia. The growth of C. beijerinckii BA101 can be totally inhibited when their concentration reaches 1.0g/L. Ferulic and p-coumaric acid can damage the hydrophobic sites of bacterial cells and increase membrane permeability, which causes leakage of cellular contents. Syringaldehyde don’t inhibit the growth of cells but decrease the production of ABE dramatically by affecting in the glycolytic pathway and alcohol dehydrogenase enzymes.
secretion (Ezeji et al., 2008). Moreover, at the same concentration, combinations of these inhibitors may become even more toxic than single one due to synergistic effects. For example, furfural and HMF can severely block the xylose metabolic pathway (Vogel-Lowmeier et al., 1998).

Therefore, these inhibitors must be removed before fermentation. Some other inhibitors can be brought in by pretreatment (Qureshi et al., 2007; Martinez et al., 2001). One such inhibitor is sodium sulfate, which is produced by dilute sulfuric acid pretreatment (Cho et al., 2009). So proper pretreatment methods must be chosen to reduce the generation of inhibitors. For example, dilute hydrochloric acid pretreatment can avoid the production of sodium sulfate in the hydrolysis process. Proper choose of lignocellulosic biomass can also reduce the concentrations of inhibitors. In the three main components of lignocelluloses: celluloses, hemicelluloses and lignin. Only lignin contains these phenolic structures in the inhibitors (Table 2.8). Possibly, these inhibitors come from partial hydrolysis of lignin. Hence, utilizing lignocelluloses with less lignin could reduce the possibility of producing inhibitors.

2.3.2 Detoxification methods

During hydrolysis process, some inhibitory components may be produced which can severely inhibit the butanol production. So after hydrolysis, different methods of removing inhibitors which are called detoxification will be employed, such as overliming (Mussatto et al., 2010), electrodialysis (Martinez et al., 2010), and adsorption (Qureshi et al., 2007), filtration (Qureshi et al., 2008) and enzymatic reactions (Larsson et al., 1999). The effectiveness of some detoxification methods are shown in Table 2.9.
Evaporation, usually under vacuum, can reduce the volatiles in the hydrolysate, such as furfural, formic acid, acetic acid and vanillin (Larsson et al., 1999; Rodrigues et al., 2001) while the sugar was concentrated. But other non-volatile inhibitors can also be concentrated during evaporation. Therefore the detoxification effects of evaporation largely depends on the types of inhibitors in the hydrolysate. The concentration of green liquor wood extracts resulted in increased inhibition to *E. coli* KO11 due to the concentration of sodium (Walton et al., 2010).

pH adjustment, often called overliming because Ca(OH)\(_2\) is used in the process, is the most widely studied in the detoxification of hydrolysate (Mussatto et al., 2010). The pH is usually raised to ~10 by some base, usually Ca(OH)\(_2\), and then reduced to the fermentation level. Although the mechanism of overliming is still unclear, it's already an effective way to detoxify hydrolysate, especially spruce hydrolysate (Persson et al., 2002). pH=10 tends to be a optimized condition for Ca(OH)\(_2\). lower pH gives little improvement in detoxification and higher pH increases the degradation of lignin and induce higher concentration of phenolic inhibitors. For corn stover hydrolysate, the detoxification of NH\(_3\) • H\(_2\)O adjustment was more effective than Ca(OH)\(_2\) (Jennings et al., 2011). Ammonia treatment resulted in the change of phenolic inhibitors.

Adsorption is another effective detoxification method. Activated carbon is a sorbent with broad spectrum, so it can remove almost all inhibitors but only partly (Qureshi et al., 2007a). And activated carbon is also very cheap. Control of pH during the adsorption can minimize the sugar reduction (Berson et al., 2005). After activated carbon treatment, furfural, acetic acid and phenolic in soybean hull hydrolysate were reduced by
95%, 37% and 75% respectively (Schirmer-Michel et al., 2008). XAD-4, a styrene-based polymer adsorbent, was also used to detoxify corn fiber hydrolysate, decrease the concentration of furfural in it and increase its fermentability (Weil et al., 2002).

Ion exchange resins are the most effective detoxification method to many hydrolysates, especially anion exchange resins because most toxic inhibitors are anions. But they can also partly remove sugars. DeMancilha and Karim (2003) investigated a weak base anion exchange resin on the detoxification of corn stover hydrolysate and found it removed almost all HMF, furfural, acetic acid and only 6% of xylose. For different types of resins, their detoxification efficiency followed the pattern: anion exchange >plain resin >cation exchange (Nivebrandt et al., 2001). Another base resin, Dowex MWA-1, eliminated 2-furancarboxylic acid, 2-furanacetic acid, 5-hydroxymethyl-2-furancarboxylic acid, and ferulic acid which are potential inhibitors in the hydrolysate of green hybrid poplar (Luo et al., 2002). Horvath et al. (2004) tested three strong base (Dowex 1X4, Dowex 2X8, IRA458) and three weak base anion exchangers (IRA67, IRA92 and Duolite A7). And the strong base resins were the most effective in improving fermentation. However, the disadvantage of resins is also obvious. The resins are expensive and the cost to recycle them is also high.

The idea of biodetoxification is to use certain microorganism in removing inhibitors in the hydrolysate. The microorganism must be able to use the inhibitors as substrates or at least convert the inhibitors to non-toxic compounds. Coniochaeta lignaria, a fungus isolated from soil, can successfully removed furfural and HMF (Lopez et al., 2004). C. ligniaria NRRL30616, a bacteria isolated by Nichols et al. (2008), can almost
eliminate all furfural and HMF, and reduce 20% of acetate in hydrolysate. *S. cerevisiae* YGSDC was able to metabolize acetic acid but no sugars (Schneider, 1996). So it can be used to remove acetate which is a common inhibitor in hydrolysate due to its high concentration. *Ureibacillus thermosphaericus*, a thermophilic bacterium, can oxidize furfural and HMF to 2-furancarboxylic acid and 5-hydroxymethyl furancarboxylic acid. The latter two were less toxic than furfural and HMF to yeast (Okuda et al., 2008). However, most microorganisms also consume sugars. So during the biodetoxification, a large portion of sugars is also used. One possible solution is to find some microorganisms that don't consume sugars like *S. cerevisiae* YGSDC. Another possible solution is to extract the useful enzymes and only use the enzymes to detoxify hydrolysate. Another problem is that all new isolated microorganisms works on furan inhibitors. No microorganism has be detected to have the capability of removing toxic phenolic compounds.

Some other detoxification methods were also studied. Liquid-liquid extraction is very effective in extracting phenolic compounds but the solvent residues may be harmful to fermentation (Um et al., 2010). Lignin fragments in hydrolysate were usually negatively charged nanoparticles (200-300nm). Cationic flocculating agent can precipitate the lignin fragments (Hasan et al., 2011). Electrodialysis can be used to remove ions in hydrolysate, especially the high concentration of salt ions due to the pH adjustment (Lynd et al., 2001).
2.5 References


Ezeji, T.C., Hans P. Blaschek, Fermentation of dried distillers’ grains and soluble (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia, Bioresource Technology 2008, 99: 5232–5242


Jennings, E. and Schell, D. Conditioning of dilute-acid pretreated corn stover hydrolysate liquors by treatment with lime or ammonium hydroxide to improve conversion of sugars to ethanol. Bioresource Technology. 2011, 102(2): 1240 –1245


Lynd, L.R., Baskaran, S., and Casten, S. Salt accumulation resulting from base added for 
ph control, and not ethanol, limits growth of thermoanaerobacterium 
thermosaccharolyticum HG-8 at elevated feed xylose concentrations in continuous 

Maddox, I. S., Qureshi N., Gutierrez N.A. Utilization of whey and process technology by 
*Clostridia* In: Woods DR, editor The *Clostridia* and Biotechnology. Butterworth 


Marchal, R., Ropars M., Pourquie J., Fayolle F., Vandecasteele J.P. Large-scale 
enzymatic hydrolysis of agricultural lignocellulosic biomass. Part 2: Conversion 

Marchal, R., Blanchet D., Vandecasteele J.P. Industrial optimization of acetone-butanol 
fermentation: A study of the optimization of Jerusalem artichokes. Applied 
Microbiology and Biotechnology 1985, 23: 92-98.

Martinez, A., Rodriguez, M.E., Wells, M.L., York, S.W., Preston, J.F., Ingram, L.O., 
Detoxification of dilute acid hydrolysates of lignocellulose with lime, 

McMillan, J. D. Pretreatment of lignocellulosic biomass. In Enzymatic ConVersion of 
Meinita, M.N., Yong-Ki Hong, Gwi-Taek Jeong, Detoxification of acidic catalyzed hydrolysate of Kappaphycus alvarezii (cottonii), Bioprocess Biosyst Eng 2012, 35:93–98


Qureshi, N. Agricultural residues and energy crops as potentially economical and novel substrates for microbial production of butanol (a biofuel), Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2010, 5: 1-8


Takagi, M, Inhibition of cellulase by fermentation products. Biotechnol Bioeng 1984, 26:1506–1507


Zverlov, V.V., Berezina O., Velikodvorskaya G.A., Schwarz W.H. Bacterial acetone and butanol production by industrial fermentation in the Soviet union: use of hydrolyzed
### Table 2.1 Properties of butanol (Hastings, 1978)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Butanol</th>
<th>Butanol structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting point(°C)</td>
<td>-89.3</td>
<td></td>
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<tr>
<td>Boiling point(°C)</td>
<td>117.7</td>
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<tr>
<td>Ignition temperature(°C)</td>
<td>35</td>
<td></td>
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<tr>
<td>Flash point(°C)</td>
<td>365</td>
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<tr>
<td>Density at 20°C (g/mL)</td>
<td>0.8098</td>
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</tr>
<tr>
<td>Critical pressure(hPa)</td>
<td>48.4</td>
<td></td>
</tr>
<tr>
<td>Critical temperature(°C)</td>
<td>287</td>
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### Properties of fuels

<table>
<thead>
<tr>
<th></th>
<th>Butanol</th>
<th>Gasoline</th>
<th>Ethanol</th>
<th>Methanol</th>
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<tbody>
<tr>
<td>Energy density(MJ/L)</td>
<td>29.2</td>
<td>32</td>
<td>19.6</td>
<td>16</td>
</tr>
<tr>
<td>Air-fuel ratio</td>
<td>11.2</td>
<td>14.6</td>
<td>9</td>
<td>6.5</td>
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<tr>
<td>Heat of vaporization(MJ/kg)</td>
<td>0.43</td>
<td>0.36</td>
<td>0.92</td>
<td>1.2</td>
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<tr>
<td>Research octane number</td>
<td>96</td>
<td>91-99</td>
<td>129</td>
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<tr>
<td>Motor octane number</td>
<td>78</td>
<td>81-89</td>
<td>102</td>
<td>104</td>
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<tr>
<td>Species / strain</td>
<td>Substrates</td>
<td>Products</td>
<td>pH</td>
<td>Temp. (°C)</td>
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<td>----------</td>
<td>------------</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em></td>
<td>Glucose, xylose, arabinose, cellobiose, mannose, galactose</td>
<td>Acetone, butanol, ethanol, acetate, butyrate, (H_2, CO_2)</td>
<td>5.5 – 6.5</td>
<td>35 ± 1</td>
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<tr>
<td>P262 ATCC 824</td>
<td>Starch</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Lactose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sucrose, fructose, lactose, malto, cellobiose</td>
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<tr>
<td><em>C. carboxidivorans</em></td>
<td>Syngas ((H_2, CO, CO_2))</td>
<td>Acetate, ethanol, butyrate, butanol</td>
<td>5.0 - 6.0</td>
<td>6.2</td>
</tr>
<tr>
<td>P7</td>
<td>H(_2), CO</td>
<td></td>
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<tr>
<td><em>C. saccharoperbutylacetonicum</em></td>
<td>Glucose, starch, maltose Molasses, starch</td>
<td>Acetone, butanol, ethanol, acetate, butyrate, (H_2, CO_2)</td>
<td>6.2</td>
<td>30</td>
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<td>N1-4</td>
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<tr>
<td><em>C. saccharobutylicum</em></td>
<td>Glucose, xylose, arabinose, cellobiose, mannose, galactose</td>
<td>Acetone, butanol, ethanol, acetate, butyrate, (H_2, CO_2)</td>
<td>5.5 – 6.5</td>
<td>35</td>
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<td>262</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. butylicum</em></td>
<td>Glucose, xylose, arabinose, cellobiose, mannose, galactose</td>
<td>Acetone, butanol, ethanol, acetate, butyrate, (H_2, CO_2)</td>
<td>5.5 – 6.5</td>
<td>35</td>
</tr>
<tr>
<td>NRRL 592</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><em>C. beijerinckii</em></td>
<td>Glucose, xylose, arabinose, cellobiose, mannose, galactose</td>
<td>Acetone, butanol, ethanol, acetate, butyrate, (H_2, CO_2)</td>
<td>5.5 – 6.5</td>
<td>35</td>
</tr>
<tr>
<td>BA101</td>
<td>Starch</td>
<td></td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>NCIMB 8052</td>
<td>Lactose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sucrose, fructose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maltodextrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucitol (sorbitol), mannitol</td>
<td></td>
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</tr>
<tr>
<td><em>C. aurantiibutyricum</em></td>
<td>Glucose, xylan, starch, pectin, arabinose, xylose, galactose, mannose</td>
<td>Acetone, butanol, isopropanol, acetate, butyrate</td>
<td>5.5 – 6.8</td>
<td>37</td>
</tr>
<tr>
<td>ATCC 17777</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td><em>C. pasteurianum</em></td>
<td>Glucose</td>
<td>Butanol, ethanol, 1,3-propanediol, acetate, butyrate, lactate</td>
<td>5.0 – 7.0</td>
<td>37</td>
</tr>
<tr>
<td>DSM 525</td>
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<tr>
<td></td>
<td>Glycerol</td>
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Table 2.3 ABE production by solventogenic Clostridia from traditional substrates and renewable lignocellulosic biomass

<table>
<thead>
<tr>
<th>Feedstock</th>
<th>Pretreatment and hydrolysis</th>
<th>Strain</th>
<th>ABE titer (g/L)</th>
<th>ABE yield (g/g)</th>
<th>Productivity (g/L h)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose with corn steep water</td>
<td>None</td>
<td><em>C. beijerinckii</em> NCIMB 8052</td>
<td>19.2</td>
<td>0.40</td>
<td>38.0</td>
<td>Parekh et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. beijerinckii</em> BA101</td>
<td>23.6</td>
<td>0.40</td>
<td>36.0</td>
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<td>Liquefied corn starch</td>
<td>None</td>
<td><em>C. beijerinckii</em> BA101</td>
<td>18.4</td>
<td>0.41</td>
<td>0.15</td>
<td>Ezeji et al., 2007c</td>
</tr>
<tr>
<td>Packing peanuts</td>
<td>None</td>
<td><em>C. beijerinckii</em> BA101</td>
<td>21.7</td>
<td>0.37</td>
<td>0.2</td>
<td>Jesse et al., 2002</td>
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<tr>
<td>Cassava starch</td>
<td>None</td>
<td><em>C. saccharoperbutylaceticum</em> N1-4</td>
<td>21.0</td>
<td>0.41</td>
<td>0.44</td>
<td>Thang et al., 2010</td>
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<tr>
<td>Corn starch</td>
<td>None</td>
<td><em>C. beijerinckii</em> BA101</td>
<td>20.7</td>
<td>0.48</td>
<td>0.31</td>
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<td>Sago starch</td>
<td>Enzyme</td>
<td><em>C. beijerinckii</em> BA101</td>
<td>19.6</td>
<td>0.43</td>
<td>0.27</td>
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<tr>
<td>Corn fiber</td>
<td>Dilute acid + enzyme</td>
<td><em>C. beijerinckii</em> BA101</td>
<td>19.4</td>
<td>0.38</td>
<td>0.40</td>
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<td>Wheat straw</td>
<td>Dilute acid</td>
<td><em>C. beijerinckii</em> BA101</td>
<td>25.0</td>
<td>0.39</td>
<td>0.10</td>
<td>Qureshi et al., 2008a</td>
</tr>
<tr>
<td></td>
<td>Alkaline peroxide + enzymes</td>
<td><em>C. beijerinckii</em> P260</td>
<td>9.3</td>
<td>0.39</td>
<td>0.10</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>22.2</td>
<td>0.41</td>
<td>0.55</td>
<td>Qureshi et al., 2008b</td>
</tr>
<tr>
<td>Distiller’s dried grains and solubles</td>
<td>Dilute acid + AFEX + enzyme</td>
<td><em>C. acetobutylicum</em> 260</td>
<td>4.9-12.9</td>
<td>0.30-0.35</td>
<td>N/A</td>
<td>Ezeji and Blaschek, 2008</td>
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<td></td>
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<td><em>C. acetobutylicum</em> 824</td>
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<td><em>C. saccharobutylicum</em> 262</td>
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<td><em>C. butylicum</em> 592</td>
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<td><em>C. beijerinckii</em> BA101</td>
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<td></td>
<td><em>C. acetobutylicum</em> P260</td>
<td>16.9</td>
<td>N/A</td>
<td>N/A</td>
<td>Wang et al., 2009</td>
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<td>Wheat bran</td>
<td>Dilute acid</td>
<td><em>C. beijerinckii</em> ATCC 55025</td>
<td>11.8</td>
<td>0.32</td>
<td>0.16</td>
<td>Liu et al., 2010</td>
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<tr>
<td>Barley straw</td>
<td>Dilute acid</td>
<td><em>C. beijerinckii</em> P260</td>
<td>26.6</td>
<td>0.43</td>
<td>0.39</td>
<td>Qureshi et al., 2010a</td>
</tr>
<tr>
<td>Corn stover</td>
<td>Dilute acid</td>
<td><em>C. beijerinckii</em> P260</td>
<td>26.3</td>
<td>0.44</td>
<td>0.31</td>
<td>Qureshi et al., 2010b</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>Dilute acid</td>
<td><em>C. beijerinckii</em> P260</td>
<td>14.6</td>
<td>0.39</td>
<td>0.17</td>
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<tr>
<td>Composition (% of Dry Basis)</td>
<td>Current Use</td>
<td>Reference</td>
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<td></td>
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</tr>
<tr>
<td>------------------------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td><strong>Cassava bagasse</strong> (Total fiber)</td>
<td>15-51 41-64 Landfill, burnt</td>
<td>3, 4, 5, 7</td>
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<tr>
<td><strong>Corn fiber</strong></td>
<td>12-32 Animal feed, burnt as fuel, compost, soil conditioner</td>
<td>1, 5, 6</td>
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<tr>
<td><strong>Corn cob</strong></td>
<td>8 45 35 15 5 6 7 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 6</td>
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<td></td>
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<tr>
<td><strong>Corn stover</strong></td>
<td>7-21 38-40 25-28 4 3, 5 7 12-14 --- Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 6, 7</td>
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<td><strong>Rice straw</strong></td>
<td>17-19 28-36 23-28 8 12-14 5, 6 7 7 21 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 6, 7</td>
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<tr>
<td><strong>Wheat straw</strong></td>
<td>10 35-40 20-30 17-19 12-32 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 7, 8</td>
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<td></td>
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<td></td>
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<tr>
<td><strong>Sorghum stalks</strong></td>
<td>11 27 25 8 11 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 7, 8</td>
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<td><strong>Fresh bagasse</strong></td>
<td>18.0 33.4 30 8 18.0 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5</td>
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<td><strong>Sugarcane bagasse</strong></td>
<td>25 40-50 24-25 25 8 10-30 Burnt as fuel, compost, soil conditioner</td>
<td>5, 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Grass</strong></td>
<td>10-30 25-40 25-50 5 20-30 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 9, 10</td>
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<td></td>
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<tr>
<td><strong>Hardwood stems</strong></td>
<td>18-25 40-55 24-40 25 5 20-30 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Softwood stems</strong></td>
<td>25 45-50 25-35 25-35 5 20-30 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 10</td>
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<td><strong>Newspapers</strong></td>
<td>18-30 40-55 25-40 25 5 20-30 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5, 9, 10</td>
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<tr>
<td><strong>Waste papers from chemical pulps</strong></td>
<td>5-10 60-70 10-20 5-10 Landfill, burnt as fuel, compost, soil conditioner</td>
<td>5</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>pretreatment process</th>
<th>advantages</th>
<th>limitations and disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>mechanical comminution</td>
<td>reduces cellulose crystallinity</td>
<td>power consumption usually higher than inherent biomass energy</td>
</tr>
<tr>
<td>steam explosion</td>
<td>causes hemicellulose degradation and lignin transformation; cost-effective</td>
<td>destruction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix; generation of compounds inhibitory to microorganisms</td>
</tr>
<tr>
<td>Ammonia Fiber Explosion (AFEX)</td>
<td>increases accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream processes</td>
<td>not efficient for biomass with high lignin content</td>
</tr>
<tr>
<td>CO₂ explosion</td>
<td>increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds</td>
<td>does not modify lignin or hemicelluloses</td>
</tr>
<tr>
<td>ozonolysis</td>
<td>reduces lignin content; does not produce toxic residues</td>
<td>large amount of ozone required; expensive</td>
</tr>
<tr>
<td>acid hydrolysis</td>
<td>hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure</td>
<td>high cost; equipment corrosion; formation of toxic substances</td>
</tr>
<tr>
<td>Process</td>
<td>Description</td>
<td>Benefits</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Alkaline hydrolysis</td>
<td>Removes hemicelluloses and lignin; increases accessible surface area</td>
<td>Long residence times required; irrecoverable salts formed and incorporated into biomass</td>
</tr>
<tr>
<td>Organosolv</td>
<td>Hydrolyzes lignin and hemicelluloses</td>
<td>Solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost</td>
</tr>
<tr>
<td>Pyrolysis</td>
<td>Produces gas and liquid products</td>
<td>High temperature; ash production</td>
</tr>
<tr>
<td>Pulsed electrical field</td>
<td>Ambient conditions; disrupts plant cells; simple equipment</td>
<td>Process needs more research</td>
</tr>
<tr>
<td>Biological</td>
<td>Degradates lignin and hemicelluloses; low energy requirements</td>
<td>Rate of hydrolysis is very low</td>
</tr>
</tbody>
</table>

(Kumar et al., 2009)
Table 2.6 Inhibition effect of phenolic compounds on cellulases

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition effect</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Ferulic acid</td>
<td>Deactivation effect on ( \beta )-glucosidases</td>
<td>Ximenes et al., 2011</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>cause significant inhibition of the hydrolysis by cellulase</td>
<td>Tejirian et al., 2011</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>Strong inhibition of commercial cellulase</td>
<td>Sineiro et al., 1997</td>
</tr>
<tr>
<td>Substituted phenols (chlorophenols, saligenin, orthiphenyl phenol, and chlorophenyl)</td>
<td>Moderately active against certain fungal cellulases</td>
<td>Mandels et al., 1965</td>
</tr>
<tr>
<td>Gallic, hydroxycinnamic, 4-hydroxybenzoic acids, and vanillin</td>
<td>Cause 20 – 80% deactivation of cellulases and/or ( \beta )-glucosidases after 24 h of pre-incubation</td>
<td>Ximenes et al., 2011</td>
</tr>
</tbody>
</table>
Table 2.7 Inhibition effect of sugars on cellulases (Xiao et al., 2004; Ximenes et al., 2011)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Notably strong inhibition of for cellulase activities</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>Moderate inhibition of cellulase activities</td>
</tr>
<tr>
<td>Xylose</td>
<td>Significantly inhibitory to cellulase activity</td>
</tr>
<tr>
<td>Arabinose</td>
<td>More inhibitory than xylose or D-glucuronic acid to the hydrolysis of celllobiose</td>
</tr>
<tr>
<td>Galactose</td>
<td>Significant inhibition effect on cellulase activity</td>
</tr>
</tbody>
</table>
Table 2.8 Major fermentation inhibitors present in the hydrolysates generated from lignocellulose degradation

<table>
<thead>
<tr>
<th>Categories</th>
<th>Fermentation inhibitors, source of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar degradation products</td>
<td>Furfural (from xylose) 5-hydroxymethyl furfural (HMF) (from hexose) Formic acid (from furfural and HMF) Levulinic acid (from HMF)</td>
</tr>
<tr>
<td>Lignin degradation products</td>
<td>Vanillin, vanillic acid (from guaiacylpropane units) Syringaldehyde, syringic acid (from syringyl propane units) Hydroquinone (1,4-di-hydroxybenzene), 4-hydroxybenzoic acid Catechol (1,2-di-hydroxybenzene) p-Coumaric acid Ferulic acid Glucuronic acid Coniferyl aldehyde</td>
</tr>
<tr>
<td>Lignocellulose structure degradation product</td>
<td>Acetic acid (from the acetyl groups present in the hemicellulose)</td>
</tr>
</tbody>
</table>

(References: Ezeji et al., 2007b; Cho et al., 2009; Mussatto and Roberto, 2004a; Palmqvist and Hahn-Hagerdal, 2000b; Zautsen et al., 2009)
<table>
<thead>
<tr>
<th>Method</th>
<th>Inhibitors removed</th>
<th>Inhibition reduction</th>
<th>Sugar retention</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaporation, flashing</td>
<td>Volatiles</td>
<td>Fair, can also exacerbate</td>
<td>Very good</td>
<td>Larsson et al., 1999; Rodrigues et al., 2001 Walton et al., 2010</td>
</tr>
<tr>
<td>pH adjustment, overliming</td>
<td>Aldehydres, phenols</td>
<td>Effective</td>
<td>Good</td>
<td>Mussatto et al., 2010 Persson et al., 2002 Jennings et al., 2011</td>
</tr>
<tr>
<td>Activated carbon, polymer adsorption</td>
<td>Furans, phenolics, acids</td>
<td>Fair</td>
<td>Good</td>
<td>Qureshi et al., 2007 Berson et al., 2005 Schirmer-Michel et al., 2008 Weil et al., 2002</td>
</tr>
<tr>
<td>Liquid-liquid extraction</td>
<td>Organic acids, phenolics</td>
<td>Moderate</td>
<td>Fair</td>
<td>Um et al., 2010</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>Organic acid anions, metal ions</td>
<td>Effective</td>
<td>Fair</td>
<td>DeMancilha and Karim, 2003) Nivebrandt et al., 2001 Luo et al., 2002 Horvath et al., 2004</td>
</tr>
<tr>
<td>Dialysis</td>
<td>Salts, organic ions</td>
<td>Effective</td>
<td>Good</td>
<td>Martinez et al., 2010 Hasan et al., 2011</td>
</tr>
<tr>
<td>Polymer flocculation</td>
<td>Lignin, lignin derivatives</td>
<td>Not demonstrated</td>
<td>Good</td>
<td>Hasan et al., 2011</td>
</tr>
<tr>
<td>Biodetoxification</td>
<td>Organics</td>
<td>Effective</td>
<td>Poor</td>
<td>Lopez et al., 2004 Nichols et al., 2008 Schneider, 1996 Okuda et al., 2008</td>
</tr>
</tbody>
</table>
Figure 2.1 Metabolic pathways in C. acetobutylicum for the acidogenic and solventogenic phase.

Enzymes are indicated by numbers as follows: (1) enzymes including in glycolysis process (2) pyruvate–ferredoxinoxidoreductase; (3) acetaldehyde dehydrogenase; (4) ethanol dehydrogenase; (5) phosphate acetyltransferase (phosphotransacetyllase); (6) acetate kinase; (7) thiolase (acetyl-CoA acetyltransferase); (8) 3-hydroxybutyryl-CoA dehydrogenase; (9) acetoacetyl-CoA: acetate/butyrate:CoA-transferase; (10) acetoacetate decarboxylase; (11) crotonase; (12) butyryl-CoA dehydrogenase; (13) phosphate butyltransferase (phosphotransbutyrylase); (14) butyrate kinase; (15) butyraldehyde dehydrogenase; (16) butanol dehydrogenase; (17) hydrogenase (Kumar et al., 2011)
Figure 2.2 The endo-exo synergistic action of the cellulolytic enzymes: EGs cleave the internal β-1, 4 bonds. CBHs attack the reducing/nonreducing ends. β-glucosidase produces glucose from cellobiose units (Gokhale et al., 2009)

Figure 2.3 Simplified scheme showing different functional domains of cellobiohydrolase (CBH) (Gokhale et al., 2009)
Chapter 3: Effects of lignocellulosic biomass hydrolysate derived inhibitors on acetone-butanol-ethanol fermentation by *Clostridium spp.*

Abstract

Lignocellulosic biomass is abundant and more economical for use in fermentation to produce butanol, one promising biofuel. However, lignocelluloses need to be thermochemically pretreated and enzymatically hydrolyzed into monosaccharides before fermentation. The pretreatment and hydrolysis processes usually produce some inhibitory compounds that could severely inhibit cell growth and butanol production in acetone-butanol-ethanol (ABE) fermentation. Therefore, the inhibitors in the hydrolysate must be reduced or removed by certain detoxification processes before fermentation. To make the detoxification process more efficient, a better understanding of these inhibitors and their inhibition effects is needed. In this study, the effects of 9 inhibitors derived from the degradation of carbohydrate and lignin on the fermentation kinetics of several solventogenic *C. beijerinckii* and *C. acetobutylicum* strains were investigated at various concentration levels. The inhibitors' effects on butanol and butyraldehyde dehydrogenase activities were also investigated. Among the 9 inhibitors studied, four lignin derived inhibitors (syringaldehyde, ferulic acid, vanillin, and *p*-coumaric acid) were found to strongly inhibit cell growth and butanol and butyraldehyde dehydrogenases.
Keywords: lignocellulose; ABE fermentation; butanol dehydrogenase; butyraldehyde dehydrogenase; Clostridium; inhibitors

3.1. Introduction

Butanol is an important intermediate (Jones et al., 1986) and solvent in the chemical industry. Compared to ethanol, butanol has a higher energy density and lower vapor pressure (Lee et al., 2008), and is thus considered a preferred fuel additive and a potential replacement for gasoline. As the oil price rises, the cost of producing butanol from petrochemical processes also increases dramatically. Therefore, more and more research is being done on ways to produce butanol from fermentation of renewable biomass. It is necessary to reduce the cost of fermentation-derived butanol in order to make it competitive with petrochemically produced butanol. A number of Clostridium mutant strains have been developed to enhance butanol production (Hastings, 1978; Kumar et al., 2011; Qureshi, 2010; Annous et al., 1991; Qureshi et al., 2001). Another most influential economy factor in producing butanol by fermentation is the cost of substrate (Ezeji et al., 2008). Much cheaper substrates were tested for butanol fermentation (Qureshi et al., 2001, 2005, 2007, 2008, 2010a,b; Maddox et al., 1993; Zverlov et al., 2006). Lignocellulosic biomass (Tashiro et al., 2005; Liew et al., 2005; Badr et a., 2001; Ezeji et al., 2007; Lin et al. 1983; Tran et al., 2010; Qureshi et al., 1995; Cho et al., 2009), such as sugarcane bagasse, soybean hull, cotton stalk and corn fibers, are byproducts in the agricultural processing industry and much cheaper than the current industrial substrates such as molasses and corn mesh. However, lignocellulosic biomass cannot be used by the solventogenic Clostridia directly and needs to be hydrolyzed first.
The hydrolysis process usually produces some inhibitory compounds that could severely inhibit cell growth and butanol production (Ezeji et al., 2008). Therefore, the inhibitors in the hydrolysate must be reduced or removed by certain detoxification processes (Martinez et al., 2001; Mussatto et al., 2001; Qureshi et al., 2008) before fermentation. To make the detoxification process more efficient, a better understanding of these inhibitors and their effects on acetone-butanol-ethanol (ABE) fermentation is needed.

The most common potential inhibitors after hydrolysis process (Ezeji et al., 2008; Lee et al., 2010; Lowmeier et al., 1998) can be divided into three main groups: furan derivatives (furfural and 5-hydroxymethyl furfural (HMF)), weak acids (glucuronic acid, formic acid, and levulinic acid), and phenolics ($p$-coumaric acid, ferulic acid, vanillin, hydroquinone, and syringaldehyde). Furan derivatives come mainly from the degradation of monosaccharides, while phenolics are generated from lignin degradation or monosaccharide degradation during acid hydrolysis. HMF’s further breakdown could form some weak acids like formic acid and levulinic acid (Lee et al., 2011). Ezeji et al. studied several inhibitors for their effects on *C. beijerinckii* BA101 (Ezeji et al., 2008). They found that furfural and HMF stimulated the growth of *C. beijerinckii* BA101, while ferulic acid and $p$-coumaric acid were the most toxic compounds to *C. beijerinckii* BA101. Zhang et al. (2012) found that furfural and HMF could be transformed to furfuryl alcohol and HMF alcohol by *C. acetobutylicum* ATCC 824. Cao et al. (2010) studied the effects of furfural, HMF, vanillin, and syringaldehyde on one *Thermoanaerobacterium* strain and reported that the effects depended on the inhibitor's concentration. The
inhibitors present in wood pulping hydrolysate have also been analyzed in a previous study (Lu et al., 2013).

The goal of this study was to understand the inhibition levels and mechanism of main inhibitors derived from lignocellulosic biomass hydrolysates. First, the effects of 9 potential inhibitors on cell growth and butanol production from glucose by several Clostridia strains were investigated in serum bottles and fermentors. These inhibitor's influence on butanol and butyraldehyde dehydrogenase activities were also tested. Then these inhibitors' concentrations in 6 biomass hydrolysates were determined and evaluated. After detoxification by activated carbon, these hydrolysates were used in the ABE fermentation.

3.2. Materials and methods

3.2.1 Strain and inoculum preparation

Spores of C. beijerinckii, C. acetobutylicum, C. tyrobutyricum were stored in a refrigerator at 4 °C in the Clostridia Growth medium (CGM). Spores (2 ml) were heat-shocked at 80°C for 3 min and transferred to 50 ml Reinforced Clostridia Medium (RCM) (Difco, Becton, Dickinson and Company, MD, USA) in a 125 ml serum bottle. The medium was nitrogen-purged for 8 min to remove oxygen. The serum bottle was tightly capped with a rubber stopper and aluminum seal. The mixture was autoclaved at 121 °C for 30 min followed by cooling to 37 °C. The heat-shocked spores were incubated at 37 °C for 12-16 h until cells were highly active. The active culture (5% inoculum) was used as seed culture in fermentation studies.
3.2.2 ABE fermentation in serum bottles

ABE fermentation was studied in serum bottles each containing 50 ml medium to evaluate the effects of various detoxification methods on butanol production. Unless otherwise noted, all fermentation studies were carried out with the P2 medium containing one type of inhibitors, glucose (60 g/L), yeast extract (2 g/L), buffer (0.5 g/L KH₂PO₄ and 0.5 g/L K₂HPO₄), 2.2 g/L ammonium acetate, vitamins (0.001 g/L para-aminobenzoic acid (PABA), 0.001g/L thiamin and 10⁻⁵ g/L biotin), and mineral salts (0.2 g/L MgSO₄·7H₂O, 0.01 g/L MnSO₄·H₂O, 0.01 g/L FeSO₄·7H₂O, 0.01 g/L NaCl). The carbon source solution (containing inhibitors) and P2 stock solution (containing yeast extract, ammonium acetate and buffer, 10-fold concentrated) were autoclaved separately at 121 °C and 15 psig for 30 min for sterilization. Minerals and vitamins were prepared at 200-fold and 1000-fold concentration, respectively, and were filter-sterilized through 0.2 μm membrane filters (25 mm syringe filter, Fisher brand, NJ, USA). Before sterilization, the P2 solutions in serum bottles were purged with nitrogen for 8 min. After sterilization, an appropriate amount of the P2 stock solution was aseptically added into the serum bottle, followed by adding minerals and vitamins. To ensure the medium pH maintained around 5.0 throughout the fermentation, 2 g/L of CaCO₃ was also included in the medium. Actively grown cells were inoculated at 5% (v/v). Batch fermentation was performed at 37 °C with no agitation. Samples were taken periodically for analysis of ABE production.

3.2.3 Butyraldehyde and butanol dehydrogenase activities

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The activities of butyraldehyde dehydrogenase and butanol dehydrogenase were measured by monitoring NADH consumption at 365 nm according to the method described before (Yu et al., 2011) with some modifications. Cells were collected from 50 mL fermentation broth by centrifugation at 4,000 rpm for 10 min using tightly sealed centrifuge tubes purged by nitrogen gas. The cell pellet was washed with Tris-HCl buffer (0.1 M, pH 7.5) once and resuspended in 2.5 mL of the same Tris-HCl buffer. Cell lysis was carried out using Mini BeadBeater (Biospec) with 0.1 mm disruption beads (Zirconia/Silica) for 6 min, stopped every 30 s to cool down the medium on ice. Supernatant was collected by centrifugation at 13,000 rpm for 10 min and used for enzyme activity assay. Enzyme activity was calculated on the basis of a molar NADH extinction coefficient of 3.4 cm\(^{-1}\)mM\(^{-1}\). One unit of enzyme activity was defined as the amount of enzyme converting 1 mmol NADH per minute under the reaction conditions. Protein concentration in cell extract was determined using the Bio-Rad protein assay kit with bovine serum albumin as standard.

3.2.4 Analytical methods

The fermentation products, acetone, butanol, ethanol, acetic acid, and butyric acid were analyzed with a gas chromatograph (GC, Shimadzu GC-2014) equipped with a flame ionization detector and a 30-m fused silica column (0.25 μm film thickness and 0.25 mm ID, Stabilwax-DA). The carrier gas was nitrogen at 1.47 ml/min (linear velocity: 35 cm/s). Samples were diluted 20 times with an internal standard buffer solution containing 0.5 g/L isobutanol, 0.1 g/L isobutyric acid and 1% phosphoric acid (for acidification), and injected (1 μL each) by using an auto-injector (AOC-20i Shimadzu).
The column temperature was held at 80 °C for 3 min, raised to 150 °C at a rate of 30 °C/min, and held at 150 °C for 3.7 min. Both the injector and detector were set at 250 °C.

Cell density was analyzed by measuring the optical density (OD) of cell suspension at 600 nm using a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1). The inhibitors including furfural, HMF, levulinic acid, ferulic acid, p-coumaric acid, and phenolic compounds (syringaldehyde) were analyzed by high performance liquid chromatography (HPLC) using Rezex ROA-Organic Acid H⁺ (8%) column (Phenomenex) at 40 °C and a UV/UIS detector (SPD-20AV, Shimadzu). The eluent was 0.01 N H₂SO₄ at 0.6 ml/min. Furfural, HMF and phenolic compounds were detected at 280 nm. Syringaldehyde was used as the standard for phenolic compounds. levulinic acid, ferulic acid, p-coumaric acid were detected at 200 nm.

3.3. Results and discussion

3.3.1 Effects of individual inhibitors on ABE fermentation

Each inhibitor was added at 1.0 g/L into the P2 medium to test its effect on ABE fermentation of C. beijerinckii strains BA101 and BA101-SV6. Figure 3.1 shows that all tested inhibitors more or less inhibited the growth of C. beijerinckii. Among them, syringaldehyde, ferulic acid, vanillin, and p-coumaric acid had significantly higher inhibition effects. O.D. was only about 2.0 compared to around 9.0 in the control. These four inhibitors also obviously blocked butanol production (Figure 3.1). Almost no butanol was produced under the influence of 1.0 g/L of these inhibitors. Only 3.0~5.0 g/L acids was produced (Figure 3.1). These four inhibitors were derived from partly
breakdown of lignin (Ezeji et al., 2008; Mussatto et al., 2001). According to these results, it would be better to remove lignin from biomass before hydrolysis. The concentration of furan derivatives is usually under 1.0 g/L in lignocellulosic hydrolysates (Lee et al., 2011). Under this concentration, furfural and HMF did not inhibit \( C. \) \textit{beijerinckii}. Therefore, it is unnecessary to remove these two compounds in the detoxification processes. Weak acids (formic acid and levulinic acid) also showed no obvious inhibition effects at 1.0 g/L. The most toxic inhibitors for \( C. \) \textit{beijerinckii} were phenolics (syringaldehyde, ferulic acid, vanillin, and \( p \)-coumaric acid). Then, further studies were done on different concentrations of these four toxic inhibitors (Figure 3.2). Even when the concentration was as low as 0.25 g/L, syringaldehyde, ferulic acid, and vanillin could still inhibit cell growth and butanol production (Figure 3.2). A possible explanation of the toxicity of these inhibitors to cells is that they may destroy cell membrane permeability (Ezeji et al., 2008). Therefore, these phenolic inhibitors were the focus in the following detoxification processes.

Then, these inhibitors’ effects in the fermentation of \( C. \) \textit{acetobutylicum} strains were also tested (Figure 3.3). \( C. \) \textit{acetobutylicum} ATCC 824 showed more tolerance to these 4 lignin-degraded inhibitors. Almost all OD values were close to the level of control, around 5.0. Only formic acid and vanillin inhibited \( C. \) \textit{acetobutylicum} ATCC 824 severely. However, \( C. \) \textit{acetobutylicum} strains produced more acids than \( C. \) \textit{beijerinckii} strains (Figure 3.1). Usually, more than 3.0 g/L total acids could be produced by \( C. \) \textit{acetobutylicum} strains. Syringaldehyde and ferulic acid did not show severe inhibition to butanol production compared to \( C. \) \textit{beijerinckii} strains. Therefore, \( C. \) \textit{acetobutylicum}
strains are more tolerant to these lignin-derived inhibitors than *C. beijerinckii* strains. So *C. acetobutylicum* strains may perform better in ABE fermentation using lignocellulosic hydrolysate as substrates. Further lowering the concentrations of those 4 lignin derived inhibitors was tested on *C. acetobutylicum* ATCC 824 (Figure 3.2). For syringaldehyde, ferulic acid and *p*-coumaric acid, when the concentration decreased, OD and butanol titer increased. When the concentration was 0.25 g/L, there was almost no obvious inhibition for these three inhibitors. But vanillin was still toxic to *C. acetobutylicum* ATCC 824 even at 0.25 g/L.

These 9 inhibitors effects on one mutant of *C. tyrobutyricum* ATCC 25755 (Yu et al., 2011) were also investigated. This strain is engineered to overexpress aldehyde/alcohol dehydrogenase 2 (*adhE2*) and CoA-transferase (*ctfAB*). *adhE2* converts butyryl-CoA to butanol while *ctfAB* converts acetate/butyrate to acetyl-CoA/butyryl-CoA. The metabolic pathway of *C. tyrobutyricum* doesn’t contain the shift from acidogenesis phase to solventogenesis phase like *C. acetobutylicum* and *C. beijerinckii*. Figures 3.1 shows both *C. acetobutylicum* and *C. beijerinckii* still produced a lot of acids when their butanol production was inhibited by some inhibitors. Therefore, these inhibitors may only block the shift process from acidogenesis phase to solventogenesis phase. *C. tyrobutyricum* is used to check this presumption since it doesn’t contain this shift process. Figure 1 shows the results. Its character is very similar to that of *C. beijerinckii*. Those four lignin derived inhibitors, syringaldehyde, ferulic acid, vanillin and *p*-coumaric acid, are still the most toxic to both cell growth and butanol production. Formic acid did not inhibit cell growth but reduced butanol production. Compared to *C. beijerinckii*, *C.*
*tyrobutyricum* showed some resistance to ferulic acid and *p*-coumaric acid. It can still produce about 2.0 g/L butanol in the presence of 1.0 g/L of each inhibitor. So those four most toxic inhibitors may work on a very complex mechanism. They don’t just inhibit the shift process, but they can inhibit both cell growth and butanol production while they have little effect on the acid production.

### 3.3.2 Effects on fermentation kinetics

The inhibitors' effects on fermentation kinetics were further investigated. Figure 3.3 shows the fermentation kinetics of *C. beijerinckii* BA101 with different inhibitors. The kinetics with 1.0 g/L furfural or 1.0 g/L formic acid was very similar to the control without any inhibitor. The OD increased very rapidly to about 6.5 within the first 24 h, became relatively stable and then decreased a little in the end of the fermentation. The OD with 1.0 g/L formic acid was a little lower than the control. It could only reach around 5.5, indicating that 1.0 g/L formic acid was a little toxic to cells and growth. Glucose was consumed about 35 g/L in the first 48 h and then leveled off. Correspondingly, butanol increased very fast in the first 48 h and then leveled off. 1.0 g/L furfural gave a higher butanol titer than the control. 1.0 g/L vanillin and 0.5 g/L ferulic acid were very toxic to *C. beijerinckii* BA101. The fermentation ended very early. The OD almost didn't increase after 24 h and could only reach to ~3.0, less than 50% of the control. The consumption of glucose also leveled off after 24 h. The concentrations of acids and solvents became stable very fast and did not change afterward which means *C. beijerinckii* BA101 cannot detoxify these two inhibitors by itself during the fermentation.
For *C. acetobutylicum* ATCC 824, the fermentation kinetics with 1.0 g/L furfural is similar to the control (Figure 3.4) and gave a slightly higher butanol titer. Different from *C. beijerinckii* BA101, 1.0 g/L formic acid (Figure 7c) was toxic to *C. acetobutylicum* ATCC 824. The OD leveled off at around 3.5 after 24 h, only 70% of the control (5.0). The butanol titer could only reach 7.0 g/L, also about 70% of the control (10.5 g/L). Similar to *C. beijerinckii* BA101, with 1.0 g/L vanillin, all parameters, such as the OD, the glucose concentration and the product concentrations, became stable after 24 h. There was no sign of self-detoxification. But *C. acetobutylicum* ATCC 824 could tolerate 0.5 g/L ferulic acid very well. The OD increased to around 3.5 after 24 h. From 24 h to 48 h, the OD was relatively stable. From 48 h to 72 h, the OD increased again to around 6.0 and then leveled off again. There was also a large increase in the butanol concentration between 48 h and 72 h. So *C. acetobutylicum* ATCC 824 could tolerate 0.5 g/L ferulic acid, but it needs time to build up the tolerance. The fermentation kinetics of *C. tyrobutyricum* ATCC25755 mutant with 1.0 g/L vanillin and 0.5 g/L ferulic acid were also studied. They inhibited cell growth and butanol production from very beginning of the fermentation. The concentrations of solvents and acids leveled off and did not change after 48 h, which means the fermentation stopped due to the toxicity of these inhibitors. With 1.0 g/L vanillin, the OD of *C. tyrobutyricum* ATCC25755 mutant was only about 3.0. The final butanol titer was less than 1.0 g/L. About 2.0 g/L butyric acid and 1.0 g/L acetic acid were produced at the end of the fermentation. Less than 10 g/L glucose was consumed during the fermentation. With 0.5 g/L ferulic acid added in the medium, the OD of *C. tyrobutyricum* ATCC25755 mutant was only about 4.0. The final butanol titer
was about 2.0 g/L. About 3.8 g/L butyric acid and 1.7 g/L acetic acid were produced at the end of the fermentation. About 30 g/L glucose was consumed during the fermentation. This indicates that *C. tyrobutyricum* ATCC25755 mutant has some tolerance to 0.5 g/L ferulic acid, but has almost no resistance to 1.0 g/L vanillin.

The fermentation kinetics confirm the end-point results of the first part. Lignin degraded inhibitors (vanillin and ferulic acid) were the most toxic to *C. acetobutylicum*, *C. beijerinckii* and *C. tyrobutyricum* strains. They inhibited the whole fermentation process from the very beginning of the fermentation.

3.3.3 Effects on butanol dehydrogenase and butyraldehyde dehydrogenase

From the fermentation results in the first two parts, the lignin derived phenolic inhibitors (syringaldehyde, ferulic acid, vanillin, and *p*-coumaric acid) are the most toxic ones to these three *Clostridium* strains. *C. beijerinckii* BA101 and *C. tyrobutyricum* ATCC25755 mutant could hardly grow with 1.0 g/L each of these four inhibitors. But some cells could still survive under this condition. For *C. beijerinckii* BA101, the ODs were 20% of the control; For *C. tyrobutyricum* ATCC25755 mutant, the ODs were 45% of the control. These cells produced a lot of acids, 3.0-5.0 g/L acetic acid and butyric acid. But the butanol titer were very low, less than 2.0 g/L. Therefore, the metabolic pathway of acidogenesis (from glucose to acetyl-CoA and butyryl-CoA, then to acetic acid and butyric acid) was fine in these survived cells. But the metabolic pathway of solventogenesis (from acetyl-CoA and butyryl-CoA to ethanol and butanol) was partly blocked. So these inhibitors may inhibit the activities of two dehydrogenases that convert
butyryl-CoA to butanol. These two dehydrogenases are butanol dehydrogenases and butyraldehyde dehydrogenase.

In this part, the effects of individual inhibitors on these two dehydrogenases were investigated. Total six inhibitors were studied, two sugar degraded inhibitors (furfural and formic acid) and four lignin derived inhibitors (syringaldehyde, ferulic acid, vanillin, and p-coumaric acid). First, 1.0 g/L of each inhibitor was added directly into the enzyme assays to test their in-vitro effects (due to the pH, corresponding sodium salts were added for those acid). The results are shown in Table 3.1. Among those three *Clostridium* strains, *C. beijerinckii* BA101 and *C. tyrobutyricum* ATCC25755 mutant had higher butanol dehydrogenase activity (0.060 U/mg). The butanol dehydrogenase activity in *C. acetobutylicum* ATCC824 was lower (0.022 U/mg). When 1.0 g/L furfural or formic acid was added, the butanol dehydrogenase activity was only a little lower than the control. Among those three strains, the butanol dehydrogenase activity of *C. beijerinckii* BA101 decreased the most, only 50% of the control. When those four lignin degraded inhibitors were added, no butanol dehydrogenase activity was detected in all three strains, which means these four inhibitors can strongly inhibit butanol dehydrogenase. For butyraldehyde dehydrogenase, similar trends can be found. When furfural or formic acid was added, the butyraldehyde dehydrogenase activities were slightly lower than the control. There was one exception. No butyraldehyde dehydrogenase activity was detected in *C. acetobutylicum* ATCC824 when 1.0 g/L formic acid was added. So 1.0 g/L formic acid can strongly inhibit butyraldehyde dehydrogenase in *C. acetobutylicum* ATCC824. This can explain why 1.0 g/L formic acid inhibited butanol production severely during
fermentation. When the four lignin derived inhibitors were added, almost no butyraldehyde dehydrogenase activities was detected in those three strains. There was also one exception. Butyraldehyde dehydrogenase still showed activity (0.25 U/mg, 63% of the control) in *C. beijerinckii* BA101 with 1.0 g/L *p*-coumaric acid.

During the *in-vitro* tests, the enzyme protein was directly exposed to the inhibitor at 1.0 g/L. But in the real fermentation, due to the protection of cell membrane and other metabolic pathways, even 1.0 g/L inhibitor was added in the medium, the dehydrogenases may not be exposed to 1.0 g/L inhibitor. Therefore, the enzymes were extracted after 24 h fermentation with inhibitors and the *in-vivo* effects of inhibitors on the two dehydrogenases were investigated. The results are shown in Table 3.2. With 1.0 g/L furfural or formic acid, the butanol and butyraldehyde dehydrogenase activities were almost the same as the control in all three strains. But for *C. acetobutylicum* ATCC 824, there was still no butyraldehyde dehydrogenase activity with formic acid, which further proves that formic acid can strongly inhibit the dehydrogenase activity in *C. acetobutylicum* ATCC 824. For those four lignin-degradation inhibitors, better than those in Table 1, some strains still showed butanol and butyraldehyde dehydrogenase activities. For *C. beijerinckii* BA101, most of them still totally inhibited butanol and butyraldehyde dehydrogenase activities. But with ferulic acid or *p*-coumaric acid, butyraldehyde dehydrogenase still showed 10% of the activity. For *C. acetobutylicum* ATCC 824, three of them (syringaldehyde, ferulic acid and *p*-coumaric acid) showed almost no effects on butanol dehydrogenase except for vanillin which still totally inhibited the activity. This is one of the reason why *C. acetobutylicum* ATCC 824
showed better tolerance to the lignin-degradation inhibitors than the other two. However, syringaldehyde and vanillin still totally inhibited butanol dehydrogenase while ferulic acid and \( p \)-coumaric acid only partly inhibited butanol dehydrogenase. For \( C.\ tyrobutyricum \) ATCC 25755 mutant, all of them still showed butanol dehydrogenase and butyraldehyde dehydrogenase activities, but lower than the control. Syringaldehyde inhibited butyraldehyde dehydrogenase most (25% of the control). \( p \)-Coumaric acid and vanillin inhibited butanol dehydrogenase most (23% of the control). Butanol dehydrogenase and butyraldehyde dehydrogenase have better tolerance to lignin-derived inhibitors, maybe because they are overexpressed enzymes and unregulated by the cell.

3.4. Conclusion

Sugar-degradation inhibitors (furfural, HMF, formic acid and levulinic acid) are not toxic to \( C.\ beijerinckii, \ C.\ acetobutylicum \) and \( C.\ tyrobutyricum \) strains. On the contrary, lignin derived phenolic inhibitors (syringaldehyde, vanillin, ferulic acid and \( p \)-coumaric acid) are very toxic to \( Clostridia \) strains. Their toxicity has two aspects. First, they can destroy the cell membrane's permeability and lead to cell death. The very low OD in the fermentation is due to the death of cells. Second, they can strongly inhibit butanol and butyraldehyde dehydrogenases. So the \( Clostridia \) strains can only produce a lot of acids, but cannot convert them to butanol.
3.5 References


Ezeji T.C., Hans P. Blaschek, Fermentation of dried distillers’ grains and soluble (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia, Bioresource Technology 2008, 99: 5232–5242


Lu, Congcong; Dong, Jie; Yang, Shang-Tian, Butanol production from wood pulping hydrolysate in an integrated fermentation-gas stripping process, Bioresource Technology (2013), 143, 467-475.


Meinita M.N., Yong-Ki Hong, Gwi-Taek Jeong, Detoxification of acidic catalyzed hydrolysate of Kappaphycus alvarezii (cottonii), Bioprocess Biosyst Eng 2012, 35:93–98


Qureshi N. Agricultural residues and energy crops as potentially economical and novel substrates for microbial production of butanol (a biofuel), Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2010, 5: 1-8


Qureshi N., Saha B.C., Cotta M.A. Butanol production from wheat straw hydrolysate using Clostridium beijerinckii. Bioprocess and Biosystems Engineering, 2007, 30: 419-427


Wang L. and Hongzhang Chen, Acetone-butanol-ethanol Fermentation and Isoflavone Extraction Using Kudzu Roots, Biotechnology and Bioprocess Engineering 2011, 16: 739-745

Yu, Ming-Rui; Zhang, Ya-Li; Tang, I.-Ching; Yang, Shang-Tian, Metabolic engineering of Clostridium tyrobutyricum for n-butanol production, Metabolic Engineering, 2011, 13(4), 373-382.

Zhang Y., Bei Han and Thaddeus Chukwuemeka Ezeji, Biotransformation of furfural and 5-hydroxymethyl furfural (HMF) by Clostridium acetobutylicum ATCC 824 during butanol fermentation, New Biotechnology, 2012, 29(3): 345-351

Table 3.1 The activities of butanol dehydrogenase and butyraldehyde dehydrogenase with 1.0 g/L inhibitors in the enzyme assay

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Butanol dehydrogenase (U/mg)</th>
<th>Butyraldehyde dehydrogenase (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA101</td>
<td>824</td>
</tr>
<tr>
<td>None</td>
<td>0.060±0.016</td>
<td>0.022±0.011</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.045±0.012</td>
<td>0.018±0.011</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.032±0.008</td>
<td>0.011±0.004</td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vanillin</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3.2 The activities of butanol dehydrogenase and butyraldehyde dehydrogenase with 1.0 g/L inhibitors in fermentation medium

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Butanol Dehydrogenase (U/mg)</th>
<th>Butyraldehyde Dehydrogenase (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA101</td>
<td>824</td>
</tr>
<tr>
<td>None</td>
<td>0.040±0.015</td>
<td>0.016±0.006</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.050±0.010</td>
<td>0.017±0.008</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.038±0.011</td>
<td>0.014±0.005</td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td>-</td>
<td>0.022±0.005</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>-</td>
<td>0.010±0.003</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>-</td>
<td>0.017±0.001</td>
</tr>
<tr>
<td>Vanillin</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Significantly different from the control (no inhibitor)
Figure 3.1 Optical densities, butanol and acids production of three *Clostridium* spp. with 1.0 g/L different inhibitors after 72 hr.
Figure 3.2 The fermentation results of *C. beijerinckii* BA101-SV6, *C. acetobutylicum* ATCC 824 and *C. tyrobutyricum* ATCC 25755 mutant after 72 h with different concentrations of phenolic inhibitors.
Figure 3.3 *C. beijerinckii* BA101 Fermentation Kinetics with different inhibitors. (1.0 g/L furfural, 1.0 g/L formic acid, 1.0 g/L vanillin, 0.5 g/L ferulic acid)
Figure 3.4 *C. acetobutylicum* ATCC 824 Fermentation Kinetics with different inhibitors.

(1.0 g/L furfural, 1.0 g/L formic acid, 1.0 g/L vanillin, 0.5 g/L ferulic acid)
Figure 3.5 *C. tyrobutyricum* ATCC 25755 mutant fermentation kinetics with different inhibitors. (1.0 g/L vanillin, 0.5 g/L ferulic acid)
Chapter 4: The impact of acid and alkali pretreatments on lignocellulosic biomass and the following acetone-butanol-ethanol fermentation

Abstract

Butanol is a promising biofuel that has a higher energy density than ethanol and similar octane number with gasoline. Producing butanol from microbial fermentation is a sustainable energy generating strategy. But one challenge is to find more economical substrates to reduce the total cost of butanol fermentation. Lignocellulosic biomass is a type of carbon source that's abundant in nature. The cellulose and hemicellulose contents in it can be hydrolyzed into sugars. So it's a potential cheap substrate for butanol fermentation. In this work, four lignocellulosic biomass, cotton stalk, corn fiber, soybean hull and sugarcane bagasse, were pretreated with acid and then hydrolyzed by enzymes. The obtained hydrolysates were further detoxified to remove the inhibitors in them. The detoxified hydrolysates were then used as carbon source in Acetone-Butanol-Ethanol (ABE) fermentation of Clostridium. All hydrolysates gave very high butanol production. The acid and alkali pretreatments were also compared. Their effects on biomass compositions and subsequent fermentation were also studied.

Keywords: butanol; lignocellulosic biomass; acid pretreatment; detoxification; inhibitors
4.1 Introduction

Butanol, a four carbon alcohol, can be used as fuel or fuel additives. It has higher energy than ethanol and can be directly used by most automobile engines without any modification (Jones et al., 1986). Moreover, butanol can be produced from microbial fermentation which is a more sustainable way to generate energy. However, the cost of butanol fermentation needs to be reduced in order to make it competitive with petrochemical fuels. The substrate cost accounted for over 56% of the butanol production cost (Ezeji et al., 2008). Therefore, choosing cheaper substrates can dramatically reduce the production cost. Lignocellulosic biomass, such as wood, grass, and agriculture residues, are very cheap and abundant in nature. So it is a promising substrate for butanol fermentation.

Extensive research has been done to evaluate lignocellulosic biomass as feedstock for fermentation. Corn fiber is a by-product of the maize wet milling process. Qureshi et al studied it as a substrate to produce butanol after hydrolysis (Qureshi et al., 2007), and obtained 9.3 g/L total ABE (Acetone, butanol and ethanol) was produced. Qureshi et al also used wheat straw as feedstock to produce butanol, and 25.0 g/L ABE was obtained (Qureshi et al., 2010a). Moreover, Qureshi et al investigated barley straw (Qureshi et al., 2010b), maize stover and switchgrass (Zverlov et al., 2006). Thaddeus et al (2008) chose distillers dry grains and soluble (DDGS) as substrate and compared it with different mixed streams of pure sugars. The highest ABE yield in Thaddeus et al’s work was 12.1 g/L. Instead of feed-grade biomass, Zverlov et al investigated agriculture wastes, including hemp waste, corn cobs and sunflower shells (Qureshi et al., 2008).
In this study, four different kinds of lignocellulosic biomass were investigated. Acid pretreatments were first applied to these biomass. The effects of different acid concentrations on the final sugar yield were studied. Inhibitors in the hydrolysates were also measured. Detoxification by activated carbon was also used to remove the inhibitors in the hydrolysates. Finally, acid and alkali pretreatments followed with removing the supernatant before enzymatic hydrolysis were compared, including their influence on biomass compositions and subsequent fermentation.

4.2 Materials and methods

4.2.1 Strain and inoculum preparation

Spores of *C. beijerinckii* BA101, *C. acetobutylicum* ATCC 824, *C. tyrobutyricum* were stored in a refrigerator at 4 °C in the *Clostridia* Growth medium. Spores (2 ml) were heat-shocked at 80 °C for 3 min and transferred to 50 ml RCM growth medium (Difco Reinforced Clostridia Medium, Becton, Dickinson and Company, MD, USA) in a 125 ml serum bottle. The medium was nitrogen-purged for 8 min to remove oxygen. The serum bottle was tightly capped with a rubber stopper and aluminum seal. The mixture was autoclaved at 121 °C for 30 min followed by cooling to 37 °C. The heat-shocked spores were incubated at 37 °C for 12-16 h until cells were highly active. The active culture (5% inoculum) was used as seed culture for all fermentations studied.

4.2.2 ABE fermentation in serum bottles

ABE fermentation was studied in serum bottles each containing 50 ml medium to evaluate the effects of various detoxification methods on butanol production. Unless
otherwise noted, all fermentation studies were carried out with the P2 medium containing one type of inhibitors, glucose (60 g/L), yeast extract (2 g/L), buffer (0.5 g/L KH$_2$PO$_4$ and 0.5 g/L K$_2$HPO$_4$), 2.2 g/L ammonium acetate, vitamins (0.001 g/L para-aminobenzoic acid (PABA), 0.001g/L thiamin and 10$^{-5}$ g/L biotin), and mineral salts (0.2 g/L MgSO$_4$•7H$_2$O, 0.01 g/L MnSO$_4$•H$_2$O, 0.01 g/L FeSO$_4$•7H$_2$O, 0.01 g/L NaCl). The carbon source solution (glucose or lignocellulose hydrolysates) and P2 stock solution (containing yeast extract, ammonium acetate and buffer, 10-fold concentrated) were autoclaved separately at 121°C and 15 psig for 30 min for sterilization. Minerals and vitamins were prepared at 200-fold and 1000-fold concentration, respectively, and were filter-sterilized through 0.2 μm membrane filters (25mm syringe filter, Fisher brand, NJ, USA). Before sterilization, the P2 solutions in serum bottles were purged with nitrogen for 8 min. After sterilization, an appropriate amount of the P2 stock solution was aseptically added into the serum bottle, followed by addition of minerals and vitamins. To ensure the medium pH maintained around 5.0 throughout the fermentation, 2 g/L of CaCO$_3$ was also included in the medium. Actively grown cells were inoculated at 5% (v/v). Batch fermentation was performed at 37°C with no agitation. Samples were taken periodically for analysis of ABE production.

4.2.3 Hydrolysis of biomass

Four lignocellulosic biomasses were hydrolyzed: cotton stalk, sugarcane bagasse (Obtained from South China University of Technology), soybean hull (Minnesota Soybean Processors) and corn fiber (Cargill Inc.). 50 g or 100 g solid particles were suspended in 1.0 L in different concentrations of acid solution (HCl or H$_2$SO$_4$) or just distilled water.
Autoclave at 121 °C, 15 psig for 30 min. Then adjust their pH to 5.0 by NaOH. Add 3.0 g CTec2 (Novozymes) and hydrolyze at 50 °C for 72 h. Centrifuge at 7000 rpm, 25 °C for 15 min to remove the undissolved solid. The supernatant hydrolysate was concentrated by vacuum rotary evaporation at 60 °C to remove ~670 mL water. The concentrated hydrolysate was filter-sterilized through 0.2 μm membrane filters (25 mm syringe filter, Fisher brand, NJ, USA) and stored at 4 °C for further use.

For the processes that remove the acid pretreatment supernatant, 50 g solid particles were suspended in 1.0 L in 0.04 N HCl or H2SO4. Autoclave at 121 °C, 15 psig for 30 min. Centrifuge at 7000 rpm, 25 °C for 15 min and discard the supernatant. Resuspend the solid in 1.0 L distilled water. Centrifuge again at 7000 rpm, 25 °C for 15 min and discard the supernatant. Repeat this washing process twice until the pH of the supernatant had reached about 5.0. Then resuspend the solid in 330 mL distilled water. Take out 5 mL samples for composition analysis. Add 3.0 g CTec2 (Novozymes) into the rest and hydrolyze at 50 °C for 72 h. Centrifuge at 7000 rpm, 25 °C for 15 min to remove the undissolved solid. The supernatant hydrolysate was filter-sterilized through 0.2 μm membrane filters (25 mm syringe filter, Fisher brand, NJ, USA) and stored at 4 °C for further use.

For the processes that remove the alkali pretreatment supernatant, 50 g solid particles were suspended in 1.0 L in 0.04 N NaOH. Keep at 50 °C for 60 min under stirring. Then wash the solid three times by the same method in the acid pretreatment until the pH of supernatant reached about 8.0. Resuspend the solid in 330 mL distilled
water, adjust the pH to about 5.0 with H₂SO₄. The enzymatic hydrolysis was the same as in the above acid pretreatment.

4.2.4 Detoxification of hydrolysate

The pH of hydrolysate was first adjusted to 2.0 with H₂SO₄. Then 2% (w/w) activated carbon was added into the hydrolysate and incubated at pH 2.0, 80 °C for 60 min under stirring. Remove activated carbon by filter paper. Adjust pH back to 6.5 by NaOH. Centrifuge them at 8000 rpm, 25 °C for 15 min. Remove the sediments and keep the clear hydrolysate at 4 °C for further use.

4.2.5 Analytical methods

The compositions of biomass were determined by the methods described by Foster et al. The fermentation products, acetone, butanol, ethanol, acetic acid, and butyric acid were analyzed with a gas chromatograph (GC, Shimadzu GC-2014) equipped with a flame ionization detector and a 30-m fused silica column (0.25 μm film thickness and 0.25 mm ID, Stabilwax-DA). The carrier gas was nitrogen at 1.47 ml/min (linear velocity: 35 cm/s). Samples were diluted 20 times with an internal standard buffer solution containing 0.5 g/L isobutanol, 0.1 g/L isobutyric acid and 1% phosphoric acid (for acidification), and injected (1 μL each) by using an auto-injector (AOC-20i Shimadzu). The column temperature was held at 80 °C for 3 min, raised to 150 °C at a rate of 30 °C/min, and held at 150 °C for 3.7 min. Both the injector and detector were set at 250 °C.

Cell density was analyzed by measuring the optical density (OD) of cell suspension at 600 nm using a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1). The
inhibitors including furfural, HMF, levulinic acid, ferulic acid, \( p \)-coumaric acid, and phenolic compounds (syringaldehyde) were analyzed by high performance liquid chromatography (HPLC) using Rezex ROA-Organic Acid H\(^+\) (8\%) column (Phenomenex) at 40 °C and a UV/UIS detector (SPD-20AV, Shimadzu). The eluent was 0.01 N \( \text{H}_2\text{SO}_4 \) at 0.6 ml/min. Furfural, HMF and phenolic compounds were detected at 280 nm. Syringaldehyde was used as the standard for phenolic compounds. levulinic acid, ferulic acid, \( p \)-coumaric acid were detected at 200 nm.

Acid and alkali pretreated and washed biomass were dried at 120 °C for 24 h. Then the biomass were coated with gold under vacuum. SEM images were taken with Quanta 200.

4.3 Results and discussion

4.3.1 Dilute acid pretreatment with different concentrations of acids

The compositions of cotton stalk, sugarcane bagasse, soybean hull and corn fiber were first analyzed. These four are typical lignocellulosic biomass, their main compositions are cellulose, hemicellulose and lignin. Cellulose accounted for 30%-44%, hemicellulose was 9%-18% and lignin was 13%-28%. Among these four, cotton stalk had the highest cellulose content (44%), lowest hemicellulose (9%) and lignin (13%) content. The composition of soybean hull was very similar to cotton stalk, but it contained much more lignin (28%) than cotton stalk. Compared to cotton stalk and soybean hull, sugarcane bagasse and corn fiber had less cellulose (~30%), but more hemicellulose (18%). Sugarcane bagasse contained more lignin (28%) than corn fiber
(18%). Cellulose and hemicellulose can be converted into monosaccharides, which can be used as carbon source in the fermentation. Therefore, cellulose and hemicellulose are the useful carbon sources for us. Ideally, these four lignocellulosic biomasses can release 48 %-53% total sugars. Lignin cannot be used by *Clostridium* strains. Its degradation may even produce some inhibitory compounds to *Clostridium* strains. So high content of lignin (sugarcane bagasse and soybean hull) may be bad in the following hydrolysis and fermentation processes.

Cotton stalk, sugarcane bagasse, soybean hull and corn fiber were first pretreated with different concentrations of acids and then hydrolyzed by cellulase enzyme (Ctec2, Novozymes). The sugar yields are shown in Table 4.2. All four have similar trends. Increase the HCl concentration from 0 to 0.3 N, the sugar yields gradually increased. Only hemicellulose can release xylose, while cellulose can only produce glucose. Therefore, those that contained more hemicellulose (sugarcane bagasse and corn fiber) released more xylose. Cotton stalk ideally can release 53% total sugars. Increasing HCl concentration only increased the sugar yield from 0.35 g/g to 0.40 g/g. Increasing the HCl concentration helped break down more hemicellulose and release more xylose. The glucose and xylose ratio decreased from 6:1 to 4:1. HCl and H$_2$SO$_4$ showed no difference on sugar yield at 0.04 N. Sugarcane bagasse ideally can release 48% total sugars. Increasing HCl concentration increased the sugar yield from 0.20 g/g to 0.43 g/g, almost reaching its highest yield 0.48 g/g. Increase HCl concentration from 0.04 N to 0.3 N, the glucose production also increased from 21.7 g/L to 36.0 g/L, but xylose production decreased from 11.4 g/L to 6.8 g/L. The glucose and xylose ratio also increased from 2:1
to 6:1. This means xylose began to degrade at high HCl concentration. HCl and H2SO4 showed great difference on sugar yield at 0.04 N. The yield with H2SO4 (0.23 g/g) was much lower than that with HCl (0.33 g/g). For corn fiber, the sugar yield decreased a little bit (from 0.60 g/g to 0.58 g/g) with increasing HCl concentration. The glucose and xylose ratio was around 1:1. The xylose degradation was not as bad as in sugarcane bagasse. The xylose production decreased from 29.1 g/L to 25.0 g/L. HCl and H2SO4 showed a little difference on sugar yield at 0.04 N. The yield with H2SO4 (0.53 g/g) was lower than that with HCl (0.60 g/g). For soybean hull, increasing HCl concentration also increased the total sugar yield from 0.50 g/g to 0.57 g/g. The glucose and xylose ratio was around 3:1. The xylose degradation was not obvious in soybean hull. The xylose production still increased from 11.6 g/L to 15.0 g/L with increasing the HCl concentration. 0.04 N HCl gave much better sugar yield than 0.04 N.

The inhibitors' concentrations after acid pretreatments are shown in Table 4.3. There were 6 types of inhibitors produced after acid pretreatments. Three came from sugar degradation: furfural, HMF and formic acid; the other three came from lignin degradation: ferulic acid, p-coumaric acid and other phenolic compounds (Ezeji et al., 2008; Lee et al., 2011). Generally, increase the acid concentration, the concentrations of these 6 inhibitors also increased. But the concentrations of these 6 inhibitors varied with different biomass. Formic acid was the most easily released inhibitor. It was generated even just using hot water pretreatment. Compared 0.04 N HCl with 0.04 N H2SO4, 0.04 N HCl tended to produce more formic acid except in soybean hull. The previous study (Chapter 3) showed that furfural, HMF and formic acid didn't inhibit the ABE
fermentation of *Clostridium* strains under 1.0 g/L. On the other hand, ferulic acid and p-coumaric acid are very toxic to *Clostridium* strains, they can strongly inhibit the ABE fermentation even at 0.25 g/L. Therefore, when choosing which acid and which concentration are best for each biomass, both Table 4.2 and 4.3 need to be considered. Higher sugar yield is better because this can reduce the substrate cost, but lower inhibitors' level is also needed to have a good butanol production. For cotton stalk, the ferulic acid level exceeded 0.25 g/L even at 0.04 N HCl and the sugar yield didn't increase a lot by increasing HCl concentration (from 0.35 g/g to 0.40 g/g). So it's better just use hot water pretreatment for cotton stalk. For sugarcane bagasse, the ferulic acid level didn't reach 0.25 g/L at 0.04 N HCl. At higher HCl concentrations or 0.04 N H$_2$SO$_4$, ferulic acid all exceeded 0.25 g/L level. On the other hand, the sugar yield with hot water pretreatment was too low (0.20 g/g). So 0.04N HCl was chosen to pretreat sugarcane bagasse later. For corn fiber, the concentrations of formic acid and ferulic acid were very high even at 0.04 N HCl, 1.72 g/L and 2.38 g/L respectively. On the contrary, with 0.04 N H$_2$SO$_4$, there was no ferulic acid detected. The sugar yield with 0.04 N H$_2$SO$_4$ (0.63 g/g) was similar to that with 0.04 N HCl (0.60 g/g). So 0.04 N H$_2$SO$_4$ was chosen to pretreat corn fiber. For soybean hull, the *p*-coumaric acid level exceeded 0.25 g/L at 0.1 N HCl. Among hot water, 0.04 N HCl and 0.04 N H$_2$SO$_4$, 0.04 N HCl has the highest sugar yield (0.50 g/g). Therefore, 0.04 N HCl was used as the pretreatment for soybean hull in the following research. Different biomass released different inhibitor levels even under the same pretreatment conditions. The main reason may be that they have different lignin
compositions and structures. For these four biomass, 0.04 N acid level was enough to obtain high sugar yields with low inhibitors' concentration.

4.3.2 Detoxification and ABE fermentation using the hydrolysate

The hydrolysates obtained by the optimized pretreatments were to be used in ABE fermentation. In order to increase the sugar concentration further, the hydrolysis process was modified. Initial 50 g/L biomass loading was used instead of 100 g/L, then the hydrolysate was further concentrated to remove 2/3 water. So the equivalent initial dry biomass loading was 150 g/L. The sugar yields are shown in Table 4.4. The sugar yield was the same as in Table 4.2, which means there was no sugar degradation during the vacuum concentration process. The initial sugar concentrations were in the range of 52.7 g/L-84.4 g/L, enough to provide sufficient carbon source in the ABE fermentation. The fermentation results of *C. acetobutylicum* ATCC824 using these hydrolysates as carbon sources are shown in Table 4.5. Only sugarcane bagasse and cotton stalk gave relatively good butanol production, 7.3 g/L and 8.4 g/L, respectively. Soybean hull only gave 3.0 g/L butanol titer. The butanol production in corn fiber hydrolysate was totally blocked. But soybean hull and corn fiber still produced a lot of acids, 14.1 g/L and 20.9 g/L, respectively. Only butanol production was inhibited. The inhibitors' concentrations in these hydrolysates were also tested and are given in Table 4.5. Due to the post-hydrolysis concentration, some inhibitors' levels increased to above 0.25 g/L. Ferulic acid concentrations in sugarcane bagasse and soybean hull were 0.29 g/L and 0.32 g/L, respectively. This can explain why butanol production in sugarcane bagasse and soybean hull were partly blocked and lower than the control using pure glucose. In corn fiber
hydrolysate, no ferulic acid was detected, but it contained 1.71 g/L formic acid and 0.19 g/L phenolic compounds. These two together might have caused the total block of butanol production. The inhibitors' levels in cotton stalk hydrolysate were all below the toxic levels. That's why it had the best butanol production among these four hydrolysates.

Activated carbon adsorption is efficient in removing these toxic inhibitors and detoxifying the hydrolysates (Lu et al., 2013). After activated carbon adsorption, the concentrations of inhibitors in the hydrolysates are also shown in Table 4.6. Activated carbon was not very good at adsorbing formic acid. Formic acid in cotton stalk hydrolysate even increased a little bit after the adsorption. This might be due to the side reactions during the adsorption. However, activated carbon worked very well in adsorbing ferulic acid and other phenolic compounds. It could remove all ferulic acid in cotton stalk, sugarcane bagasse and soybean hull hydrolysates. All other phenolic compounds in sugarcane bagasse hydrolysate were also removed. 42% in corn fiber were also removed. In the following fermentation studies, cotton stalk hydrolysates were used directly while the other three hydrolysates were detoxified first by activated carbon absorption.

The fermentation results using these hydrolysates are shown in Table 4.7. Three strains were tested: *C. acetobutylicum* ATCC 824, *C. beijerinckii* BA101 and *C. tyrobutyricum* ATCC 25755 mutant (knocking out Ack genes and overexpressing ctfAB and adhE2 genes). Cotton stalk hydrolysate was the best carbon source. All three strains produced similar butanol titers to the control and some were even a little higher. Corn fiber hydrolysate gave the lowest butanol titer (5.4-6.1 g/L) in all three strains. As shown
in Table 4.6, corn fiber hydrolysate only contained 1.13 g/L formic acid and 0.11 g/L phenolic compounds. There were two possible reasons for the low butanol titer; one was that corn fiber hydrolysate contained some other inhibitors other than those 9 tested; the other is that the 0.11 g/L phenolic compounds contained some very toxic inhibitors. The other two hydrolysates had different performances in different strains. Soybean hull hydrolysate gave very good butanol production in *C. beijerinckii* BA101 (10.0 g/L), but the butanol titer in *C. acetobutylicum* ATCC 824 and *C. tyrobutyricum* ATCC 25755 mutant was lower (7.6 g/L). For sugarcane bagasse hydrolysate, the butanol titer in *C. acetobutylicum* ATCC 824 was 9.6 g/L, but only 7.8 g/L and 7.9 g/L in *C. beijerinckii* BA101 and *C. tyrobutyricum* ATCC 25755 mutant. So ferulic acid is a very toxic inhibitor to these three strains but may not be the only inhibitor in the hydrolysates. The fermentation kinetics of *C. acetobutylicum* ATCC 824 and *C. beijerinckii* BA101 are shown in Figure 4.1 and Figure 4.2. In the first 24 h, only glucose was used for cell growth and almost no xylose was consumed. After 24 h, both glucose and xylose were consumed for acid and solvent production.

4.3.3 Comparison of acid and alkali pretreatment with removing supernatant before enzymatic hydrolysis

The inhibitors mentioned above (furfural, HMF, formic acid, ferulic acid, p-coumaric acid and other phenolic compounds) are mainly produced in the pretreatment process. During the enzyme digestion, the pH (~5.0) and temperature (50 °C) were mild so almost no inhibitor should be generated. The concentrations of the inhibitors were all under 2.0 g/L (Table 4.6), so they could dissolve into water very well. This means the
inhibitors were contained in the liquor after the acid pretreatment. If the liquor containing inhibitors was removed and the biomass solid was washed, then the hydrolysate obtained after enzyme digestion should contain little or no inhibitor. Another common economical pretreatment is using alkali (NaOH). In this part, the effects of these two pretreatments (acid and alkali) on four biomass structures (cotton stalk, sugarcane bagasse, soybean hull and corn fiber) and subsequent ABE fermentation were studied.

Table 4.8 shows the composition changes after acid or alkali pretreatment. Some of the biomass content was either broken up into short fragments and dissolved into water or degraded further and produced the inhibitors. From Table 4.8, acid pretreatment could remove a large portion of hemicellulose, part of lignin and only very small part of cellulose. The hydrogen ion can catalyze the breakage of glycosidic bonds. It more easily attacks the amorphous hemicellulose at low concentration. It can even break the crystalline cellulose into fragments. At a high concentration, the hydrogen ion can break crystal cellulose into glucose. It can also catalyze the breakage of ether bonds in lignin and partly break the lignin structures. In this acid pretreatment, only 0.04 N HCl or 0.04 N H\textsubscript{2}SO\textsubscript{4} was used. The concentration was very low so it mainly broke the hemicellulose part in the biomass. 0.04 N H\textsubscript{2}SO\textsubscript{4} removed 56% of hemicellulose and 31% of lignin in cotton stalk. 0.04 N HCl removed 78% of hemicellulose and 7% of lignin in sugarcane bagasse. It could also remove 90% of hemicellulose and 65% of lignin in soybean hull. 28% of cellulose, 61% of hemicellulose and 50% of lignin in corn fiber were removed by 0.04 N H\textsubscript{2}SO\textsubscript{4}. So 0.04 N hydrogen ions removed 56%-90% of hemicellulose and 7%-65% of lignin in the biomass. Among these four biomass, the lignin in sugarcane bagasse was
the hardest to be broken, only 7% was removed. And the lignin in soybean hull was the easiest to be broken, 65% of lignin was dissolved. The result of corn fiber is different from the others. 28% of cellulose was also dissolved. Possible reason was that the cellulose in corn fiber was not a rigid crystalline fiber. It has a large portion of amorphous structures that can be attacked by the dilute acid. Based on the sugar yields, the acid pretreatment promoted the enzymatic digestion of cellulose and hemicellulose very well. Almost all cellulose and hemicellulose in the biomass were converted into monosaccharides. Compared with acid pretreatment, alkali pretreatment dissolved less hemicellulose, more lignin and almost no cellulose. In cotton stalk, 0.04 N NaOH removed 44% of hemicellulose and 77% of lignin. It dissolved 44% of hemicellulose and 46% of lignin in sugarcane bagasse. It also removed 80% of hemicellulose and 48% of lignin in soybean hull, and 16% of cellulose, 56% of hemicellulose and 78% of lignin in corn fiber. So 0.04 N NaOH dissolved 44%-80% of hemicellulose and 46%-78% of lignin in the biomass. The lignin in cotton stalk and corn fiber was more soluble in 0.04 N NaOH, which removed 77% and 78%, respectively. Less cellulose in corn fiber was dissolved in NaOH, 16% compared with 28% in H₂SO₄. After alkali pretreatment, the rest solid was digested by the enzyme very well. From the sugar yield, almost all cellulose and hemicellulose were hydrolyzed into single sugars.

The SEM images of untreated, acid treated and alkali treated biomass were compared in Figure 4.3. There were no long fibrous structures in untreated cotton stalk. That's why it had a relatively high sugar yield even just using hot water pretreatment. After acid or alkali pretreatment, the structure only became more shattered. Untreated
corn fiber had a mesh structure because it contained a lot of hemicellulose (18%). The hemicellulose formed branches from cellulose fiber and links them to form the grids. After acid pretreatment, there were less branches and it became more like long fiber. After alkali pretreatment, the structure was almost the same. Untreated soybean hull had a hollow fiber structure. After acid pretreatment, almost no hollow fiber could be seen from the image. All the fiber structures were destroyed. After alkali pretreatment, some fibers were still there but with scratches on the fiber, a sign of partly breakage of the fibrous structure. There were still long fiber structures in untreated sugarcane bagasse. 78% of hemicellulose was removed after acid pretreatment and the structure became very amorphous. 44% of hemicellulose and 46% of lignin were removed after alkali pretreatment, and the structure became more scattered and more powdered. Therefore, either acid or alkali pretreatment can destroy the fibrous structures of biomass to some extent, make the structure more fragmented, and promote the enzyme contact in the hydrolysis process.

Removing the liquor and washing the acid or alkali pretreated biomass also removed all the inhibitors produced in the pretreatment process. Actually no inhibitors mentioned above (furfural, HMF, formic acid, ferulic acid, p-coumaric acid and other phenolic compounds) were detected in the hydrolysate. This means all these inhibitors were below 0.01 g/L and had almost no effects on the subsequent ABE fermentation. Then the ABE fermentations of *C. acetobutylicum* ATCC824 using these hydrolysates as carbon sources were performed and are compared with the performance using activated carbon detoxified hydrolysates in Table 4.9. The situation was more complex than
expected. For the acid pretreated with removing supernatant, only cotton stalk and soybean hull hydrolysates gave high butanol titers, 8.2 g/L and 8.4 g/L, respectively. Butanol production in corn fiber and sugarcane bagasse hydrolysates were only 2.9 g/L and 2.3 g/L. For the alkali pretreated with removing supernatant, the final butanol titers in cotton stalk and corn fiber hydrolysates were high, 8.5 g/L and 8.6 g/L. Soybean hull and sugarcane bagasse hydrolysates did not perform as well as the other two, only 5.1 g/L and 5.7 g/L butanol were produced. Therefore, there should be some components or new produced chemicals in some of the biomass that can influence butanol production by *C. acetobutylicum* ATCC824. Corn fiber hydrolysate only gave good butanol production after the alkali pretreatment with removing supernatant. The possible reason is that the alkali pretreatment remove most of lignin in corn fiber (78%) which contained most toxic potential inhibitors for ABE fermentation.

**4.4 Conclusion**

Dilute acid pretreatment is an effective method to promote the enzymatic hydrolysis of cellulose and hemicellulose in biomass. But high concentration of acids catalyzed the further degradation of biomass components and generated large amount of inhibitors to ABE fermentation, especially ferulic acid, exceeding the toxic level that inhibited the butanol production, especially ferulic acid. For corn fiber, soybean hull and sugarcane bagasse, 0.04 N was the best acid level. Activated carbon absorption was effective in removing phenolic inhibitors in the hydrolysate, especially ferulic acid. After the acid and alkali pretreatment, removing the liquor and washing the biomass could
removed all the inhibitors dissolved in the water, but there are still some other inhibitors in the biomass that inhibited butanol production by ABE fermentation.

4.5 Reference


Ezeji T.C., Hans P. Blaschek, Fermentation of dried distillers’ grains and soluble (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia, Bioresource Technology 2008, 99: 5232–5242


Lu, Congcong; Dong, Jie; Yang, Shang-Tian, Butanol production from wood pulping hydrolysate in an integrated fermentation-gas stripping process, Bioresource Technology (2013), 143, 467-475.


Meinita M.N., Yong-Ki Hong, Gwi-Taek Jeong, Detoxification of acidic catalyzed hydrolysate of Kappaphycus alvarezii (cottonii), Bioprocess Biosyst Eng 2012, 35:93–98


Qureshi N. Agricultural residues and energy crops as potentially economical and novel substrates for microbial production of butanol (a biofuel), Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2010, 5: 1-8


Qureshi N., Saha B.C., Cotta M.A. Butanol production from wheat straw hydrolysate using Clostridium beijerinckii. Bioprocess and Biosystems Engineering, 2007, 30: 419-427


Qureshi N., Saha, B.C., Hector R. E., M.A. Removal of fermentation inhibitors from alkaline peroxide pretreated and enzymatically hydrolyzed wheat straw:
Production of butanol from hydrolysate using *Clostridium beijerinckii* in batch reactors, Biomass and Bioenergy, 2008, 32: 1353–1358


Wang L. and Hongzhang Chen, Acetone-butanol-ethanol Fermentation and Isoflavone Extraction Using Kudzu Roots, Biotechnology and Bioprocess Engineering 2011, 16: 739-745
Yu, Ming-Rui; Zhang, Ya-Li; Tang, I.-Ching; Yang, Shang-Tian, Metabolic engineering of Clostridium tyrobutyricum for n-butanol production, Metabolic Engineering (2011), 13(4), 373-382.

Zhang Y., Bei Han and Thaddeus Chukwuemeka Ezeji, Biotransformation of furfural and 5-hydroxymethyl furfural (HMF) by Clostridium acetobutylicum ATCC 824 during butanol fermentation, New Biotechnology, 2012, 29(3): 345-351

Table 4.1 Composition of various lignocellulosic biomass

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton stalk</td>
<td>44%±5%</td>
<td>9%±5%</td>
<td>13%±2%</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>30%±5%</td>
<td>18%±7%</td>
<td>28%±2%</td>
</tr>
<tr>
<td>Soybean hull</td>
<td>40%±4%</td>
<td>10%±6%</td>
<td>23%±2%</td>
</tr>
<tr>
<td>Corn fiber</td>
<td>32%±4%</td>
<td>18%±5%</td>
<td>18%±1%</td>
</tr>
</tbody>
</table>
Table 4.2 Sugar concentrations and yields from biomass with different pretreatment conditions

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Glucose (g/L)</th>
<th>Xylose (g/L)</th>
<th>Yield (g/g dry biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cotton Stalk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot water</td>
<td>29.8±0.5</td>
<td>5.3±0.2</td>
<td>0.35±0.01</td>
</tr>
<tr>
<td>0.04N HCl</td>
<td>29.5±0.6</td>
<td>5.5±0.1</td>
<td>0.35±0.02</td>
</tr>
<tr>
<td>0.04N H₂SO₄</td>
<td>29.6±0.6</td>
<td>5.0±0.1</td>
<td>0.35±0.02</td>
</tr>
<tr>
<td>0.1N HCl</td>
<td>33.1±0.8</td>
<td>6.7±0.3</td>
<td>0.40±0.03</td>
</tr>
<tr>
<td>0.3N HCl</td>
<td>32.0±0.3</td>
<td>8.0±0.1</td>
<td>0.40±0.01</td>
</tr>
<tr>
<td><strong>Sugarcane bagasse</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot water</td>
<td>15.0±0.4</td>
<td>5.1±0.1</td>
<td>0.20±0.01</td>
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<tr>
<td>0.04N HCl</td>
<td>21.7±0.3</td>
<td>11.4±0.5</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>0.04N H₂SO₄</td>
<td>14.7±0.6</td>
<td>8.2±0.7</td>
<td>0.23±0.05</td>
</tr>
<tr>
<td>0.1N HCl</td>
<td>25.0±0.8</td>
<td>10.2±0.2</td>
<td>0.35±0.02</td>
</tr>
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<td>0.3N HCl</td>
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<td>6.8±0.5</td>
<td>0.43±0.03</td>
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<td><strong>Corn fiber</strong></td>
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</tr>
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<td>Hot water</td>
<td>14.0±0.5</td>
<td>4.6±0.1</td>
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<td>29.1±0.8</td>
<td>0.60±0.02</td>
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<td>0.04N H₂SO₄</td>
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<td>0.53±0.01</td>
</tr>
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<td>0.59±0.02</td>
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</tr>
<tr>
<td><strong>Soybean hull</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hot water</td>
<td>33.4±0.5</td>
<td>2.9±0.1</td>
<td>0.36±0.02</td>
</tr>
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<td>0.04N HCl</td>
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<td>11.6±0.3</td>
<td>0.50±0.02</td>
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<td>15.4±0.6</td>
<td>7.2±0.4</td>
<td>0.23±0.04</td>
</tr>
<tr>
<td>0.1N HCl</td>
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<td>12.3±0.2</td>
<td>0.51±0.02</td>
</tr>
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<td>0.3N HCl</td>
<td>41.6±0.2</td>
<td>15.0±0.5</td>
<td>0.57±0.01</td>
</tr>
</tbody>
</table>

a The initial biomass loading was 100 g/L. The results were after enzymatic hydrolysis.
Table 4.3 Inhibitors present in the biomass hydrolysate with different acid pretreatments

<table>
<thead>
<tr>
<th>Pretreatments</th>
<th>Furfural (g/L)</th>
<th>HMF (g/L)</th>
<th>Formic acid (g/L)</th>
<th>Ferulic acid (g/L)</th>
<th>p-Coumaric acid (g/L)</th>
<th>Phenolic (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cotton Stalk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot water</td>
<td>-</td>
<td>-</td>
<td>0.19</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.04N HCl</td>
<td>0.10</td>
<td>-</td>
<td>0.80</td>
<td>0.51</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.04N H₂SO₄</td>
<td>0.07</td>
<td>-</td>
<td>0.75</td>
<td>0.47</td>
<td>0.01</td>
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</tr>
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<td>0.09</td>
<td>3.32</td>
<td>1.57</td>
<td>0.27</td>
<td>0.05</td>
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<td>0.3N HCl</td>
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<td>0.21</td>
<td>4.25</td>
<td>1.86</td>
<td>0.30</td>
<td>0.07</td>
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<td><strong>Sugarcane bagasse</strong></td>
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<td></td>
</tr>
<tr>
<td>Hot water</td>
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<td>0.11</td>
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<td>0.04N HCl</td>
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<td>0.54</td>
<td>0.47</td>
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<td>0.04</td>
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<td>0.1N HCl</td>
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<td>0.59</td>
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<td>1.54</td>
<td>2.71</td>
<td>0.60</td>
<td>0.09</td>
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<td><strong>Corn fiber</strong></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>0.02</td>
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<td>0.71</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.04N HCl</td>
<td>0.04</td>
<td>-</td>
<td>1.72</td>
<td>2.38</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
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<td>-</td>
<td>1.14</td>
<td>-</td>
<td>-</td>
<td>0.13</td>
</tr>
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<td>0.1N HCl</td>
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<td>0.84</td>
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<td>1.12</td>
<td>4.45</td>
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<td>0.72</td>
<td>0.17</td>
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<td><strong>Soybean hull</strong></td>
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<td></td>
</tr>
<tr>
<td>Hot water</td>
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<td>-</td>
<td>0.06</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
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<td>0.04N HCl</td>
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<td>0.52</td>
<td>0.21</td>
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<td>0.04N H₂SO₄</td>
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<td>-</td>
<td>0.65</td>
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<td>-</td>
</tr>
<tr>
<td>0.1N HCl</td>
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<td>0.3N HCl</td>
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<td>1.42</td>
<td>3.16</td>
<td>1.29</td>
<td>0.55</td>
<td>0.12</td>
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</tbody>
</table>

*The initial biomass loading was 100 g/L. The results were after enzymatic hydrolysis.*
Table 4.4 Sugar concentrations and yields of the biomass after hydrolysis

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Pretreatment</th>
<th>Glucose (g/L)</th>
<th>Xylose (g/L)</th>
<th>Yield (g/g dry biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn fiber</td>
<td>0.04N H₂SO₄</td>
<td>43.7±2.9</td>
<td>40.7±1.0</td>
<td>0.53±0.03</td>
</tr>
<tr>
<td>Cotton stalk</td>
<td>Hot Water</td>
<td>44.7±1.9</td>
<td>8.0±2.3</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>0.04N HCl</td>
<td>32.5±0.1</td>
<td>17.1±3.5</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>Soybean hull</td>
<td>0.04N HCl</td>
<td>58.0±2.9</td>
<td>17.4±1.6</td>
<td>0.50±0.03</td>
</tr>
</tbody>
</table>

* The initial biomass loading was 50 g/L, and the hydrolysate was concentrated by 300%.

Table 4.5 *C. acetobutylicum* ATCC824 fermentation results after 72 h using different biomass w/o activated carbon adsorption

<table>
<thead>
<tr>
<th>Pretreatments</th>
<th>Butanol (g/L)</th>
<th>Acids (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not detoxified</td>
<td>Activated carbon treated</td>
</tr>
<tr>
<td>P2 (control)</td>
<td>9.6±0.1</td>
<td>-</td>
</tr>
<tr>
<td>Soybean Hull</td>
<td>0.04N H₂SO₄</td>
<td>3.0±0.2</td>
</tr>
<tr>
<td>Sugarcane Bagasse</td>
<td>0.04N HCl</td>
<td>7.3±0.1</td>
</tr>
<tr>
<td>Corn Fiber</td>
<td>0.04N H₂SO₄</td>
<td>-</td>
</tr>
<tr>
<td>Cotton Stalk</td>
<td>Hot Water</td>
<td>8.4±0.2</td>
</tr>
</tbody>
</table>
Table 4.6 The concentrations of inhibitors present in the biomass hydrolysates before and after activated carbon absorption

<table>
<thead>
<tr>
<th>Activated carbon detoxification</th>
<th>Furfural (g/L)</th>
<th>HMF (g/L)</th>
<th>Formic acid (g/L)</th>
<th>Ferulic acid (g/L)</th>
<th>Phenolics (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton stalk</td>
<td>Before</td>
<td>-</td>
<td>-</td>
<td>0.28±0.09</td>
<td>0.07±0.01</td>
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<tr>
<td></td>
<td>After</td>
<td>-</td>
<td>-</td>
<td>0.68±0.06</td>
<td>-</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>Before</td>
<td>0.30±0.05</td>
<td>-</td>
<td>1.10±0.05</td>
<td>0.29±0.03</td>
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<tr>
<td></td>
<td>After</td>
<td>0.19±0.08</td>
<td>-</td>
<td>0.68±0.04</td>
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<td>Before</td>
<td>0.09±0.02</td>
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<td>1.45±0.08</td>
<td>0.32±0.07</td>
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<tr>
<td></td>
<td>After</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Corn Fiber</td>
<td>Before</td>
<td>0.14±0.04</td>
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<td>1.71±0.06</td>
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<td>After</td>
<td>-</td>
<td>-</td>
<td>1.13±0.03</td>
<td>0.11±0.03</td>
</tr>
</tbody>
</table>

Table 4.7 The fermentation results using biomass hydrolysate as the carbon source for three *Clostridium* strains

<table>
<thead>
<tr>
<th>Biomass</th>
<th>ATCC 824</th>
<th>BA101</th>
<th>C. tyrobutyricum</th>
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<tr>
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<td>Butanol</td>
<td>Acids</td>
<td>Butanol</td>
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<tr>
<td>Control</td>
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<td>4.5±0.2</td>
<td>10.2±0.1</td>
</tr>
<tr>
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<td>10.8±0.2</td>
<td>7.8±0.1</td>
<td>8.9±0.2</td>
</tr>
<tr>
<td>Corn fiber</td>
<td>5.4±0.2</td>
<td>11.7±0.2</td>
<td>5.5±0.2</td>
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<td>Soybean hull</td>
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<td>5.0±0.3</td>
<td>10.0±0.3</td>
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<td>9.6±0.3</td>
<td>4.7±0.2</td>
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Table 4.8 % loss in cellulose, hemicellulose and lignin in biomass after acid or alkali pretreatment

<table>
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<tr>
<th>Biomass</th>
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<th></th>
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<td>Cellulose</td>
<td>Hemi cellulose</td>
<td>lignin</td>
<td>Total loss</td>
<td>Sugar yield (g/g)</td>
<td>Cellulose</td>
<td>Hemi cellulose</td>
<td>lignin</td>
<td>Total loss</td>
<td>Sugar yield (g/g)</td>
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<td>5%</td>
<td>4%</td>
<td>13%</td>
<td>0.47</td>
<td>1%</td>
<td>4%</td>
<td>10%</td>
<td>16%</td>
<td>0.48</td>
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<td>-</td>
<td>14%</td>
<td>2%</td>
<td>20%</td>
<td>0.34</td>
<td>-</td>
<td>8%</td>
<td>13%</td>
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<tr>
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<td>30%</td>
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<td>11%</td>
<td>9%</td>
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<td>5%</td>
<td>10%</td>
<td>14%</td>
<td>31%</td>
<td>0.33</td>
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</table>

\[a\] Percentage is based on the initial dry biomass weight.

\[b\] After enzymatic hydrolysis and based on the dry biomass after removing the supernatant
Table 4.9 *C. acetobutylicum* ATCC824 fermentation results with biomass hydrolysates after acid or alkali pretreatment \(^a\) or activated carbon detoxification

<table>
<thead>
<tr>
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<th>Acid pretreatment</th>
<th>Alkali pretreatment</th>
<th>Activated carbon</th>
</tr>
</thead>
<tbody>
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<td>Butanol (g/L)</td>
<td>Acids (g/L)</td>
<td>Butanol (g/L)</td>
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<td>8.2</td>
<td>7.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Corn fiber</td>
<td>2.9</td>
<td>7.5</td>
<td>8.6</td>
</tr>
<tr>
<td>Soybean hull</td>
<td>8.4</td>
<td>5.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>2.3</td>
<td>8.2</td>
<td>5.7</td>
</tr>
</tbody>
</table>

\(^a\) The liquor containing inhibitors was removed before enzymatic hydrolysis
Figure 4.1 The fermentation kinetics of *C. acetobutylicum* ATCC824 with lignocellulosic hydrolysates after activated carbon adsorption.
Figure 4.2 The fermentation kinetics of *beijerinckii* BA101 with lignocellulosic hydrolysates after activated carbon adsorption
Figure 4.3 SEM images of untreated, acid pretreated or alkali pretreated biomass structures
Chapter 5: The inhibition mechanism of lignin derived phenolic compounds on the metabolic pathways of *Clostridium* spp.

Abstract

Lignocellulosic biomass is a more economical substrate for fermentation to produce butanol, which is a promising biofuel. However, lignocellulosic biomass can not be directly used by most microbes and needs to be hydrolyzed to generate simple sugars. In the hydrolysis process, some inhibitors that are toxic to *Clostridia* may be generated, especially some phenolic compounds. But the inhibition mechanism is still unclear. Four phenolic inhibitors were tested in *C. tyrobutyricum* mutants. They were very toxic to one mutant overexpressing *ctfAB* and *adhE2* genes, but they had no effect on the other mutant overexpressing only *adhE2* gene. Therefore, the phenolic compounds are inhibitors to CoA-transferase expressed by *ctfAB*. This was further confirmed by their toxicity to *C. beijerinckii* and *C. acetobutylicum*. This also explained the toxicity of corn steep liquor as nitrogen source in the butanol fermentation.

Keywords: phenolic inhibitors; *ctfAB*; CoA-transferase; corn steep liquor

5.1 Introduction

Lignocellulosic biomass is being studied as substrate in the Acetone-Butanol-Ethanol fermentation because it is more economical than the current substrates (maize or
sugarcane). Extensive research has been done in using lignocellulosic biomass as butanol fermentation feedstock (Tashiro et al., 2005; Liew et al., 2005; Badr et al., 2001; Ezeji et al., 2007; Lin et al. 1983; Tran et al., 2010; Qureshi et al., 1995; Cho et al., 2009). Lignocellulosic biomass cannot be directly used by Clostridia and the hydrolysis process is needed. In general, pretreatments are also all necessary to break down the rigid structure (Chapter 4). Many inhibitory compounds may be produced during the hydrolysis or pretreatment process (Ezeji et al., 2008; Lee et al., 2010; Lowmeier et al., 1998). However, the inhibition mechanism is still unclear. Ezeji et al. mentioned the phenolic compounds can destroy the permeability of cell membrane (Ezeji et al., 2008). Zhang et al. found that furfural and HMF can be transformed to furfuryl alcohol and HMF alcohol by C. acetobutylicum ATCC 824 (Zhang et al., 2012). Cao et al. studied the effects of furfural, HMF, vanillin, and syringaldehyde on one Thermoanaerobacterium strain and reported that the effects depended on the inhibitor's concentration (Cao et al., 2010). Lowmeier et al. found that furfural and HMF can severely block the xylose metabolic pathway (Lowmeier et al., 1998).

In this paper, four potential phenolic inhibitors (p-coumaric acid, ferulic acid, vanillin and syringaldehyde) were tested in four Clostridium strains (C. beijerinckii, C. acetobutylicum, and two C. tyrobutyricum strains). Their inhibition mechanism was proposed according the different performances on different strains. Corn steep liquor, a byproduct of corn wet milling, was also used as the nitrogen source in the fermentation of Clostridia.
5.2. Materials and methods

5.2.1 Strains and inoculum preparation

Spores of *C. beijerinckii*, *C. acetobutylicum*, *C. tyrobutyricum* were stored in a refrigerator at 4 °C in the *Clostridia* Growth medium (CGM). Spores (2 ml) were heat-shocked at 80 °C for 3 min and transferred to 50 ml RCM growth medium (Difco Reinforced Clostridia Medium, Becton, Dickinson and Company, MD, USA) in a 125 ml serum bottle. The medium was nitrogen-purged for 8 min to remove oxygen. The serum bottle was tightly capped with a rubber stopper and aluminum seal. The mixture was autoclaved at 121 °C for 30 min followed by cooling to 37 °C. The heat-shocked spores were incubated at 37 °C for 12-16 h until cells were highly active. The active culture (5% inoculum) was used as seed culture for all fermentations studied.

5.2.2 ABE fermentation in serum bottles

ABE fermentation was studied in serum bottles each containing 50 ml medium to evaluate the effects of various detoxification methods on butanol production. Unless otherwise noted, all fermentation studies were carried out with the P2 medium containing one type of inhibitors, glucose (60 g/L), yeast extract (2 g/L), buffer (0.5 g/L KH₂PO₄ and 0.5 g/L K₂HPO₄), 2.2 g/L ammonium acetate, vitamins (0.001 g/L para-amino-benzoic acid (PABA), 0.001g/L thiamin and 10⁻⁵g/L biotin), and mineral salts (0.2 g/L MgSO₄·7H₂O, 0.01 g/L MnSO₄·H₂O, 0.01 g/L FeSO₄·7H₂O, 0.01 g/L NaCl). The carbon source (glucose or biomass hydrolysate) solution and P2 stock solution (containing yeast extract, ammonium acetate and buffer, or corn steep liquor and buffer, 10-fold
concentrated) were autoclaved separately at 121 °C and 15 psig for 30 min for sterilization. Minerals and vitamins were prepared at 200-fold and 1000-fold concentration, respectively, and were filter-sterilized through 0.2 μm membrane filters (25 mm syringe filter, Fisher brand, NJ, USA). Before sterilization, the P2 solutions in serum bottles were purged with nitrogen for 8 min. After sterilization, an appropriate amount of the P2 stock solution was aseptically added into the serum bottle, followed by addition of minerals and vitamins. To ensure the medium pH maintained around 5.0 throughout the fermentation, 2 g/L of CaCO₃ was also included in the medium. Actively grown cells were inoculated at 5% (v/v). Batch fermentation was performed at 37 °C with no agitation. Samples were taken periodically for analysis of ABE production.

5.2.3 Hydrolysis of biomass

Four lignocellulosic biomasses were hydrolyzed: cotton stalk, sugarcane bagasse, soybean hull and corn fiber. 50 g solid particles were suspended in 1.0 L in different concentrations of acid solution (HCl or H₂SO₄) or just distilled water. Autoclave at 120 °C, 20 psig for 30 min. Then adjust their pH to 5.0 by NaOH. Add 3.0 g CTec2 (Novozymes) and hydrolyze at 50 °C for 72 h. Centrifuge at 7000 rpm, 25 °C for 15 min to remove the undissolved solid. The supernatant hydrolysate was concentrated by vacuum rotary evaporation at 60 °C to remove 670 mL water. The concentrated hydrolysate was filter-sterilized through 0.2 μm membrane filters (25 mm syringe filter, Fisher brand, NJ, USA) and stored at 4 °C for further use.

5.2.4 Detoxification of hydrolysate
The pH of hydrolysate was first adjusted to 2.0 by H$_2$SO$_4$. Then 2% (w/w) activated carbon was added into the hydrolysate and treated at pH 2.0, 80 °C for 60 min under stirring. Remove activated carbon by filter paper. Adjust pH back to 6.5 by NaOH. Centrifuge them at 8000 rpm, 25 °C for 15 min. Remove the sediments and keep the clear hydrolysate at 4 °C for further use.

5.2.5 Analytical methods

The fermentation products, acetone, butanol, ethanol, acetic acid, and butyric acid were analyzed with a gas chromatograph (GC, Shimadzu GC-2014) equipped with a flame ionization detector and a 30-m fused silica column (0.25 mm film thickness and 0.25 mm ID, Stabilwax-DA). The carrier gas was nitrogen at 1.47 ml/min (linear velocity: 35 cm/s). Samples were diluted 20 times with an internal standard buffer solution containing 0.5 g/L isobutanol, 0.1 g/L isobutyric acid and 1% phosphoric acid (for acidification), and injected (1 μL each) by using an auto-injector (AOC-20i Shimadzu). The column temperature was held at 80 °C for 3 min, raised to 150 °C at a rate of 30 °C/min, and held at 150 °C for 3.7 min. Both the injector and detector were set at 250 °C.

Cell density was analyzed by measuring the optical density (OD) of cell suspension at 600 nm using a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1).

5.3 Results and discussion

5.3.1 Inhibition of phenolic compounds to Clostridium strains

Four phenolic compounds were tested for their inhibition to Clostridium strains (Figure 5.1) : p-coumaric acid, ferulic acid, vanillin and syringaldehyde. Other than the
phenolic group, two of them (p-coumaric acid and ferulic acid) have carboxylic group and the other two (vanillin and syringaldehyde) have a formyl group. These four are mainly the products of lignin degradation and potential inhibitors for ABE fermentation. The effects of 1.0 g/L each of these four inhibitors on O.D., solvent and acid production are shown in Figure 5.2. All four inhibitors inhibited the growth of C. beijerinckii BA101, C. acetobutylicum ATCC824 and C. tyrobutyricum ATCC25755 with ctfAB and adhE2. All of them had a final O.D. of lower than 6.0. On the contrary, these four inhibitors almost had no effect on the growth of C. tyrobutyricum ATCC25755 with only adhE2, which had O.D. reached ~16.0. For butanol production, all four inhibitors strongly inhibited butanol production in C. beijerinckii BA101 and C. tyrobutyricum ATCC25755 (ctfAB-adhE2). The butanol titers were below 2.0 g/L. They were still very toxic even at 0.25 g/L (see Chapter 3). C. acetobutylicum ATCC824 showed some resistance to three of them (p-coumaric acid, ferulic acid and syringaldehyde). The butanol titer reached up to 4.0-7.0 g/L. These four inhibitors still had almost no effect on the butanol titer of C. tyrobutyricum ATCC25755 (adhE2), which gave ~11.0 g/L butanol production in the presence of these inhibitors. For acids production, all four strains gave relatively high acid concentrations, 3.0 g/L-7.0 g/L. In 1.0 g/L p-coumaric acid, vanillin or syringaldehyde, C. acetobutylicum ATCC824 had the highest acid titer, over 5.5 g/L.

The only difference of those two C. tyrobutyricum ATCC25755 mutants was that one overexpressed both ctfAB and adhE2 genes while the other only overexpressed adhE2 genes (Yu et al., 2011). Moreover, both C. beijerinckii BA101 and C. acetobutylicum ATCC824 have ctfAB gene and can express CoA-transferase. Those four
phenolic inhibitors only had severe inhibition effects on *Clostridium* strains with *ctfAB* gene that express CoA-transferase. For *C. tyrobutyricum* ATCC25755 (*adhE2*) without *ctfAB* gene, those four phenolic inhibitors had no toxicity either to the cell growth nor to butanol production. Therefore, the inhibition mechanism of these phenolic inhibitors should work on CoA-transferase. The most important function of CoA-transferase is to convert acetate and butyrate back to acetyl-CoA and butyryl-CoA in the solventogenesis phase (Jones et al., 1986). So acetyl-CoA and butyryl-CoA can be further convert into ethanol and butanol. CoA-transferase is critical in the shift from acidogenesis to solventogenesis phase. Those four inhibitors almost had no effect on acids production in all four strains, so they didn't affect on the acidogenesis phase. This further proves that they influenced CoA-transferase. *p*-Coumaric acid and ferulic acid both have a carboxylic group, so they are potential substrates for CoA-transferase just like acetic acid or butyric acid. They may attach to CoA-transferase and work as competitive inhibitors to CoA-transferase.

5.3.2 *Corn steep liquor as the nitrogen source for ABE fermentation*

Corn steep liquor is the byproduct of corn wet milling and is rich in protein and amino acids. So it is a potential economical nitrogen source for butanol production. When corn steep liquor was used as the only nitrogen source in the fermentation *C. tyrobutyricum* ATCC25755 mutants, very obvious difference was seen between the two mutants. For *C. tyrobutyricum* ATCC25755 with both *ctfAB* and *adhE2* genes, corn steep liquor partly inhibited the butanol production even at 0.25% w/v, only 5.7 g/L. Increasing the concentration of corn steep liquor further decreased the butanol titer. On the other
hand, the acid production was not suppressed by corn steep liquor. The acid concentrations were even higher than the control. For *C. tyrobutyricum* ATCC25755 with only *adhE2* gene, corn steep liquor didn't inhibit butanol production even at 9% (w/v). It even increased the butanol titer in the lower concentration. The highest butanol titer was around 15.0 g/L. This is a similar phenomenon with the previous phenolic inhibitors. Therefore, corn steep liquor must have contained some of those phenolic inhibitors. But due to the complex composition of corn steep liquor, it's very hard to determine the specific phenolic compounds and their concentrations. However, further proofs were obtained when the corn steep liquor was combined with lignocellulosic hydrolysate in the ABE fermentation with three strains (*C. beijerinckii* BA101, *C. acetobutylicum* ATCC824 and *C. tyrobutyricum* ATCC25755 with *ctfAB* and *adhE2*) containing *ctfAB* genes (Table 5.1). Therefore, their butanol production should be inhibited by those four phenolic compounds (p-coumaric acid, ferulic acid, vanillin and syringaldehyde). When corn steep liquor was used as nitrogen source and glucose as carbon source, butanol production was all lower than that using yeast extract and ammonium acetate as nitrogen source. Among those three strains, *C. acetobutylicum* ATCC824 had the highest resistance to the toxicity of corn steep liquor, it still gave 8.0 g/L butanol compared to 9.6 g/L in the control. The butanol titers in *C. beijerinckii* BA101 and *C. tyrobutyricum* ATCC25755 with *ctfAB-adhE2* were only 50% and 70% of the control. The acids production when using corn steep liquor was slightly higher. When combining corn steep liquor with lignocellulosic hydrolysates, the butanol titer was much lower compared with that with yeast extract and ammonium acetate. Especially in corn fiber, soybean hull and
Sugarcane bagasse hydrolysates, almost no butanol was produced. Possible reason is that the phenolic inhibitors in corn steep liquor combined with some other inhibitors in the lignocellulosic hydrolysates showed stronger toxicity to Clostridium strains. When the strain was C. tyrobutyricum ATCC25755 with adhE2, combining corn steep liquor and the lignocellulosic hydrolysates still showed very high butanol production. So the phenolic inhibitors in corn steep liquor were still the most important factor that affects the butanol production. It can inhibit the function of CoA-transferase and block butanol production. So for C. tyrobutyricum ATCC25755 mutant that doesn't contain CoA-transferase, corn steep liquor is a very good and economical nitrogen source for butanol production.

5.4 Conclusion

The phenolic compounds (p-coumaric acid, ferulic acid, vanillin and syringaldehyde) were toxic to Clostridium strains with CoA-transferase but showed almost no effect on strains without CoA-transferase. These phenolic inhibitors may work as competitive inhibitors to CoA-transferase and block the solventogenesis phase that converts acids into solvents. Corn steep liquor is rich in protein and amino acids, and is a potential nitrogen source for butanol production. But the phenolic inhibitors in it may severely inhibit butanol production by solventogenic Clostridia containing ctfAB genes.
5.5 Reference


Ezeji T.C., Hans P. Blaschek, Fermentation of dried distillers’ grains and soluble (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia, Bioresource Technology 2008, 99: 5232–5242


Lu, Congcong; Dong, Jie; Yang, Shang-Tian, Butanol production from wood pulping hydrolysate in an integrated fermentation-gas stripping process, Bioresource Technology (2013), 143, 467-475.


Meinita M.N., Yong-Ki Hong, Gwi-Taek Jeong, Detoxification of acidic catalyzed hydrolysate of Kappaphycus alvarezii (cottonii), Bioprocess Biosyst Eng 2012, 35:93–98


Qureshi N. Agricultural residues and energy crops as potentially economical and novel substrates for microbial production of butanol (a biofuel), Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2010, 5: 1-8

137


Qureshi N., Saha B.C., Cotta M.A. Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. Bioprocess and Biosystems Engineering, 2007, 30: 419-427


Wang L. and Hongzhang Chen, Acetone-butanol-ethanol Fermentation and Isoflavone Extraction Using Kudzu Roots, Biotechnology and Bioprocess Engineering 2011, 16: 739-745

Yu, Ming-Rui; Zhang, Ya-Li; Tang, I.-Ching; Yang, Shang-Tian, Metabolic engineering of Clostridium tyrobutyricum for n-butanol production, Metabolic Engineering (2011), 13(4), 373-382.

Zhang Y., Bei Han and Thaddeus Chukwuemeka Ezeji, Biotransformation of furfural and 5-hydroxymethyl furfural (HMF) by Clostridium acetobutylicum ATCC 824 during butanol fermentation, New Biotechnology, 2012, 29(3): 345-351

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Table 5.1 The fermentation results of different strains combining lignocellulosic hydrolysate with corn steep liquor
Figure 5.1 Chemical structures of phenolic inhibitors
Figure 5.2 The inhibition of phenolic compounds (1.0 g/L each) to the growth and solvents and acids production of four *Clostridium* strains
Figure 5.3 The fermentation results of two *C. tyrobutyricum* ATCC25755 mutants with different concentrations of corn steep liquor as nitrogen source
Chapter 6: Butanol production from non-lignocellulosic biomass

Abstract

The high cost of feedstock is a bottleneck for large-scale butanol fermentation. More economical substrates are needed to reduce the cost of butanol production. Lignocellulosic biomass is a potential substrate. Another possibility is some other agricultural residues. In this study, three agricultural residues were investigated: cassava bagasse, soy molasses and soybean meal. Cassava bagasse and soy molasses are rich carbon source, while soybean meal has plenty of proteins and is a potential nitrogen source. Starch based cassava bagasse only needs enzyme (glucoamylase) hydrolysis. Its hydrolysate contained no toxic inhibitors and performed very well in ABE fermentation. Inulin based Jerusalem artichoke was hydrolyzed only by dilute acid. But its hydrolysate contained high concentration of ferulic acid which is an inhibitor to ABE fermentation. Soy molasses contain mainly oligosaccharides and can be hydrolyzed by α-galactosidase. Soybean meal was also proved to be a good nitrogen source after the acid hydrolysis. ABE fermentation kinetics of three different Clostridium strains using soy molasses as carbon source and soybean meal as nitrogen source were also studied, and C. acetobutylicum ATCC 824 gave the highest butanol titer (8.7 g/L). The detailed cost analysis of large scale butanol plants from different types of biomass were also made, and butanol from soy molasses had the lowest unit cost ($2.71 /gal).
6.1 Introduction

The increasing requirement of energy promotes the research on biofuels. Among the biofuels, butanol has a lot of promising characters, such as high energy density, low vapor pressure and similar octane number with gasoline. In order to reduce the cost of butanol production and make it competitive with petrochemical fuels, cheaper substrates are needed for butanol fermentation (Qureshi et al., 2001, 2005, 2007, 2008, 2010a,b; Maddox et al., 1993; Zverlov et al., 2006). Lignocellulosic biomass is hot candidate because it is cheap and abundant in nature (Tran et al., 2010; Qureshi et al., 1995; Cho et al., 2009). But it has some disadvantages. It cannot be directly used by solventogenic Clostridia. So cellulase enzyme is needed to hydrolyze the lignocellulosic biomass, which increases the production cost. Sometimes pretreatments are also necessary to break down its rigid structures. And the pretreatments can generate inhibitors to Clostridia (Ezeji et al., 2008; Lee et al., 2010; Lowmeier et al., 1998). So other agricultural residues were also studied by researchers (Ezeji et al., 2008; Qureshi et al., 2008; Zverlov et al., 2006).

In this chapter, four biomass were investigated. Three as carbon sources, cassava bagasse, Jerusalem artichoke and soy molasses. The other was as nitrogen source, soybean meal. Cassava bagasse is starch based, Jerusalem artichoke is inulin based biomass, and main components of soy molasses are oligosaccharides, such as stachyose and raffinose. These biomass were first hydrolyzed by acids or enzyme and then used as carbon sources in the ABE fermentation. Soybean meal was also hydrolyzed by acids to
release more amino acids and then used to provide nitrogen source during the ABE fermentation. Cost analysis of large-scale butanol plants from different types of biomass was also made.

6.2 Materials and methods

6.2.1 Strains and inoculum preparation

Spores of *C. beijerinckii* BA101, *C. acetobutylicum* ATCC 824 and ATCC 55025, *C. tyrobutyricum* were stored in a refrigerator at 4 °C in the *Clostridia* Growth medium (CGM). Spores (2 ml) were heat-shocked at 80 °C for 3 min and transferred to 50 ml RCM growth medium (Difco Reinforced Clostridia Medium, Becton, Dickinson and Company, MD, USA) in a 125 ml serum bottle. The medium was nitrogen-purged for 8 min to remove oxygen. The serum bottle was tightly capped with a rubber stopper and aluminum seal. The mixture was autoclaved at 121 °C for 30 min followed by cooling to 37 °C. The heat-shocked spores were incubated at 37 °C for 12-16 h until cells were highly active. The active culture (5% inoculum) was used as seed culture for all fermentations studied.

6.2.2 ABE fermentation in serum bottles and bioreactors

ABE fermentation was studied in serum bottles each containing 50 ml medium to evaluate the effects of various detoxification methods on butanol production. Unless otherwise noted, all fermentation studies were carried out with the P2 medium containing one type of inhibitors, glucose (60 g/L), yeast extract (2 g/L), buffer (0.5 g/L KH₂PO₄ and 0.5 g/L K₂HPO₄), 2.2 g/L ammonium acetate, vitamins (0.001 g/L para-amino-
benzoic acid (PABA), 0.001 g/L thiamin and 10⁻⁵ g/L biotin), and mineral salts (0.2 g/L MgSO₄·7H₂O, 0.01 g/L MnSO₄·H₂O, 0.01 g/L FeSO₄·7H₂O, 0.01 g/L NaCl). The carbon source solution (glucose or biomass hydrolysates) and P2 stock solution (containing yeast extract, ammonium acetate and buffer, 10-fold concentrated) were autoclaved separately at 121°C and 15 psig for 30 min for sterilization. Minerals and vitamins were prepared at 200-fold and 1000-fold concentration, respectively, and were filter-sterilized through 0.2 μm membrane filters (25mm syringe filter, Fisher brand, NJ, USA). Before sterilization, the P2 solutions in serum bottles were purged with nitrogen for 8 min. After sterilization, an appropriate amount of the P2 stock solution was aseptically added into the serum bottle, followed by addition of minerals and vitamins. To ensure the medium pH maintained around 5.0 throughout the fermentation, 2 g/L of CaCO₃ was also included in the medium. Actively grown cells were inoculated at 5% (v/v). Batch fermentation was performed at 37 °C with no agitation. Samples were taken periodically for analysis of ABE production.

In the 500 mL bioreactor fermentation using soy molasses and soybean meal, only 450 mL soy molasses hydrolysate (250 g/L) and 50 mL soybean meal hydrolysate (100 g/L) were mixed into the bioreactor. No other nutrients were added. The bioreactor was then autoclaved at 121 °C for 30 min. After autoclave, the bioreactor was cooled down to 37 °C with continuous nitrogen purging. Then 50 mL seed culture was inoculated into the bioreactor. The fermentation was performed at 37 °C, pH 5.0 (pH 6.0 for C. tyrobutyricum) with 150 rpm agitation. Samples were taken periodically for analysis of ABE production.
6.2.3 Hydrolysis of biomass

Cassava bagasse

50 g solid particles were suspended in 1.0 L in 0.04N H$_2$SO$_4$ or just distilled water. Autoclave at 121 °C, 15 psig for 30 min. Then adjust their pH to 5.0 by NaOH. Add 3.0 g CTec2 (Novozymes) and 0.03 g Distillase® L-400 (glucoamylase, Genencor) hydrolyze at 50 °C for 72 h. Centrifuge at 7000 rpm, 25 °C for 15 min to remove the undissolved solid. The supernatant hydrolysate was concentrated by vacuum rotary evaporation at 60 °C to remove 670 mL water. The concentrated hydrolysate was filter-sterilized through 0.2 μm membrane filters (25 mm syringe filter, Fisher brand, NJ, USA) and stored at 4 °C for further use.

Jerusalem artichoke

50 g solid particles were suspended in 1.0 L in 0.04 N H$_2$SO$_4$ or just distilled water. Autoclave at 121 °C, 15 psig for 30 min. Then adjust their pH to 5.0 by NaOH. Centrifuge at 7000 rpm, 25 °C for 15 min to remove the undissolved solid. The supernatant hydrolysate was concentrated by vacuum rotary evaporation at 60 °C to remove 670 mL water. The concentrated hydrolysate was filter-sterilized through 0.2 μm membrane filters (25 mm syringe filter, Fisher brand, NJ, USA) and stored at 4 °C for further use.

Soy molasses

200 g soy molasses slurry was suspended in 1.0L distilled water. Autoclave at 121 °C, 15 psig for 30 min. Add 10 mL α-galactosidase (Enzyme Development Inc)
hydrolyze at 50°C for 48 h. Then adjust the pH to 5.0 by NaOH. Centrifuge at 7000 rpm, 25 °C for 15 min to remove the undissolved solid. The supernatant hydrolysate was stored at 4 °C for further use.

*Soybean meal*

10 g of soybean meal was suspended in 100 mL dilute acid solution (with various acid concentrations) and then autoclaving at 121°C, 15 psig for 30 minutes. Then, the pH was adjusted to 5.0 and 1 mL α-galactosidase was added into the medium. The enzymatic hydrolysis took 72 h at 50°C under stirring. Centrifuge at 7000 rpm, 25 °C for 15 min to remove the undissolved solid. The supernatant hydrolysate was stored at 4 °C for further use.

6.2.4 Detoxification of hydrolysate

The pH of hydrolysate was first adjusted to 2.0 with H₂SO₄. Then 2% (w/w) activated carbon was added into the hydrolysate and treated at pH 2.0, 80 °C for 60 min under stirring. Remove activated carbon by filtering through filter paper. Adjust pH back to 6.5 by NaOH. Centrifuge them at 8000 rpm, 25 °C for 15 min. Remove the sediments and keep the clear hydrolysate at 4 °C for further use.

6.2.5 Analytical methods

The compositions of biomass were determined by the methods described by Foster et al.(2010) The fermentation products, acetone, butanol, ethanol, acetic acid, and butyric acid were analyzed with a gas chromatograph (GC, Shimadzu GC-2014)
equipped with a flame ionization detector and a 30-m fused silica column (0.25 μm film thickness and 0.25 mm ID, Stabilwax-DA). The carrier gas was nitrogen at 1.47 ml/min (linear velocity: 35 cm/s). Samples were diluted 20 times with an internal standard buffer solution containing 0.5 g/L isobutanol, 0.1 g/L isobutyric acid and 1% phosphoric acid (for acidification), and injected (1 μL each) by using an auto-injector (AOC-20i Shimadzu). The column temperature was held at 80 °C for 3 min, raised to 150 °C at a rate of 30 °C/min, and held at 150 °C for 3.7 min. Both the injector and detector were set at 250 °C.

Cell density was analyzed by measuring the optical density (OD) of cell suspension at 600 nm using a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1). The inhibitors including furfural, HMF, levulinic acid, ferulic acid, p-coumaric acid, and phenolic compounds (syringaldehyde) were analyzed by high performance liquid chromatography (HPLC) using Rezex ROA-Organic Acid H⁺ (8%) column (Phenomenex) at 40 °C and a UV/UIS detector (SPD-20AV, Shimadzu). The eluent was 0.01 N H₂SO₄ at 0.6 ml/min. Furfural, HMF and phenolic compounds were detected at 280 nm. Syringaldehyde was used as the standard for phenolic compounds. levulinic acid, ferulic acid, p-coumaric acid were detected at 200 nm.

6.3 Results and discussion

6.3.1 Cassava bagasse and Jerusalem artichoke as the carbon source for ABE fermentation
Cassava bagasse is the byproduct of starch production from cassava. Jerusalem artichoke is a common wild plant native to eastern North America. The compositions of these two are shown in Table 6.1. Although cassava bagasse is the residue after extracting starch from cassava, 43% of its content was still starch. It also contained 25% cellulose and 9% hemicellulose. Jerusalem artichoke is rich in inulin (54%) and it also contained 15% cellulose. Either starch or inulin can be hydrolyzed and used as carbon sources in the ABE fermentation. Hot water and dilute acid (0.04 N H$_2$SO$_4$) pretreatments were compared for cassava bagasse and Jerusalem artichoke (Table 6.2). Inulin in Jerusalem artichoke was easy to be hydrolyzed, with just hot water pretreatment, which gave a sugar yield of 0.51 g/g. With 0.04 N H$_2$SO$_4$, the sugar yield was 0.75 g/g. No enzyme was needed to further hydrolyze Jerusalem artichoke. Since the sugar yield was much higher for acid treated Jerusalem artichoke, acid pretreatment was used for the following ABE fermentation. For cassava bagasse, the sugar yields were similar either with hot water or 0.04N H$_2$SO$_4$, around 0.45 g/g. Cellulase and amylase were needed to further hydrolyze the cellulose and starch in cassava bagasse. The sugar yields were almost the same, but acid pretreatment had the high risk of generating inhibitors. So hot water pretreatment was chosen in the following ABE fermentation. The inhibitors present in acid pretreated Jerusalem artichoke hydrolysate and hot water pretreated cassava bagasse hydrolysate are listed in Table 6.3. The cassava bagasse hydrolysate was clean, only containing 0.64 g/L formic acid, which was not toxic to most Clostridium strains (Chapter 3). So cassava bagasse hydrolysate did not need to be detoxified before fermentation. Jerusalem artichoke hydrolysate had more inhibitors. It contained 2.19 g/L
formic acid, 0.71 g/L ferulic acid and 0.18 g/L other phenolic compounds. Ferulic acid above 0.25 g/L is toxic to Clostridium strains (Chapter 3). So Jerusalem artichoke hydrolysate needs to be detoxified before fermentation. Activated carbon adsorption was used to remove the inhibitors in Jerusalem artichoke hydrolysate. About 32% of formic acid, 38% of ferulic acid, and all of other phenolic compounds were removed after activated carbon adsorption. But the ferulic acid concentration was still 0.44 g/L, above 0.25 g/L toxic level. These two hydrolysates were used as carbon sources in the ABE fermentation of C. beijerinckii BA101, C. acetobutylicum ATCC 824 and C. tyrobutyricum ATCC 25755 mutant (Table 6.4). Cassava bagasse hydrolysate performed very well. All butanol titers were similar to the control, some were even better than the control, because it almost contained no inhibitors. Butanol production in Jerusalem artichoke hydrolysate was lower than the control for all strains studied. C. acetobutylicum ATCC 824 and C. tyrobutyricum ATCC 25755 mutant gave ~80% of butanol production while C. beijerinckii BA101 only had ~50% butanol titer compared to the control. The lower butanol production was attributed to the high concentration of ferulic acid (0.44 g/L) in Jerusalem artichoke hydrolysate. In my previous study, C. acetobutylicum ATCC 824 showed more resistance to ferulic acid than C. beijerinckii BA101 (see Chapter 3). So C. acetobutylicum ATCC 824 had high butanol production than C. beijerinckii BA101 in Jerusalem artichoke hydrolysate. Another detoxification method needs to be developed for Jerusalem artichoke hydrolysate to further improve the butanol production in the fermentation.

6.3.2 Soy molasses as the carbon source for ABE fermentation
Soy molasses and soybean soluble contain oligosaccharides, mainly stachyose and raffinose, sucrose, glucose and other monosaccharides including galactose, fructose, xylose and arabinose. The exact composition varied with the source of the materials and might change during storage (see Table 6.5). Stachyose (and raffinose) cannot be used by Clostridium. Earlier efforts using acid pretreatment to hydrolyze soy oligosaccharides were not successful – only limited amounts of sugars were released but along with significant amounts of unknown inhibitors that severely inhibited butanol production in ABE fermentation. Later, it was found that soy oligosaccharides present in the molasses can be readily hydrolyzed with α-galactosidase. As can be seen in Figure 6.1, it took only about 0.5 h to hydrolyze all stachyose (and raffinose) present in soybean molasses to galactose and sucrose, which was further hydrolyzed to glucose and fructose in ~3.5 h. A total of 24.3 g/L sugars, mainly glucose, galactose and fructose, were obtained from 80 g/L soy molasses. The conversion was 100% with a sugar yield of 0.30 g/g molasses. For ABE fermentation, 200 g/L initial loading was used, so the sugar concentration at the beginning was 60 g/L.

Soy molasses was used as carbon source in the ABE fermentation by C. acetobutylicum ATCC 824 and C. tyrobutyricum (Δack, adhE2), and the results are shown in Table 6.6. As expected, butanol production from hydrolyzed soy molasses was much better than that from the untreated one. Also, both corn steep liquor and soybean meal hydrolysate appeared to be good nitrogen source with butanol production comparable to that obtained with yeast extract and NH₄-acetate. It is noted that the proteins present in corn steep liquor and soybean meal might adsorb some toxic inhibitors.
in soy molasses. *C. tyrobutyricum* with soybean meal as the nitrogen source gave the highest butanol production and appeared to be better than *C. acetobutylicum* ATCC 824. Up to 8 g/L of butyrate were produced in the fermentation with ATCC 824. A large amount of butyrate was produced, instead of converting into butanol, when the fermentation pH was above 5.5, in which acids were accumulated because solventogenesis in *C. acetobutylicum* usually requires a pH below 5.0. If butyrate can be fully converted into butanol, butanol production would be much higher.

### 6.3.3 Soybean meal as carbon and nitrogen sources for ABE fermentation

Similar to soy molasses, soybean meal also contains a large amount of carbohydrate, mainly oligosaccharides (stachyose and raffinose), sucrose and some monosaccharides (galactose, glucose, fructose). The oligosaccharides present in soybean meal need to be hydrolyzed in order to be utilized by clostridia in butanol fermentation. Similar enzymatic hydrolysis method with α-galactosidase can be used to hydrolyze oligosaccharides present in soybean meal. However, a pretreatment with dilute acid is necessary in order to partially break down the soybean meal solid particles and hydrolyze the proteins in soybean meal. After treating with α-galactosidase, more than 16 g/L of sugars (glucose, galactose, and fructose) were obtained. The highest sugar concentration was 29.2 g/L (pretreated with 0.1 N HCl). If the acid concentration was increased to 0.3 N HCl, the sugars began to degrade and only 20.2 g/L total sugar was obtained. 0.04 N H$_2$SO$_4$ was slightly inferior to 0.04 N HCl in releasing single sugars. The hydrolysis results are summarized in Table 6.7
Soybean meal hydrolysate as both carbon and nitrogen sources were tested in ABE fermentation. No other nutrients were added into the hydrolysates except for some trace minerals. The fermentation results of *C. acetobutylicum* ATCC 824 are shown in Table 6.8. The butanol production was low, with the highest butanol titer of 2.86 g/L, due to the low sugar content in the hydrolysate. No obvious phenolic compounds or weak acid inhibitors were detected in the hydrolysate. So the hydrolysate (pretreated with 0.04 N H$_2$SO$_4$) was concentrated to 48 g/L total sugars and then used in the fermentation. However, the concentrated hydrolysate seemed to be more toxic to the bacteria as less butanol was produced in the fermentation, indicating the presence of inhibitors in the concentrated soybean meal hydrolysate. It can be concluded that soybean meal by itself is not sufficient as both nitrogen and carbon sources for ABE fermentation, although it can provide both of them. Therefore, soybean meal should be used mainly as nitrogen source with additional carbon source such as soy molasses for butanol production in ABE fermentation.

### 6.3.4 Butanol production from soybean meal and soy molasses

Soy molasses, after hydrolysis to sugars, and soybean meal, which is a good nitrogen source, were used together without any additional nutrients supplementation for butanol fermentation. The loading of soy molasses was 200 g/L, and the initial total sugar concentration was ~54 g/L. Three different loadings of soybean meal and four different clostridia strains were tested. The results are shown in Table 6.9. It is clear that 10 g/L soybean meal can provide enough nitrogen source for the fermentation. Increasing the loading to 30 g/L soybean meal appeared to inhibit butanol production. Among the 4
strains studied, *C. acetobutylicum* ATCC 55025 gave the highest butanol titer (8.7 g/L). The fermentation kinetics of three different *Clostridia* strains in 500 mL bioreactors are shown in Figure 6.2. In the bioreactor, the soy molasses loading was increased to 250 g/L while the loading of soybean meal was still 10 g/L. In the fermentation of *C. acetobutylicum* ATCC 55025, glucose and fructose were consumed faster than galactose and xylose. After 72 h, the fermentation consumed 78% glucose, 64% fructose, 77% galactose and 55% xylose. So *C. acetobutylicum* ATCC 55025 could utilize these four sugars simultaneously. The xylose consumption was slower than the other three. In the fermentation of *C. tyrobutyricum* (Δack, adhE2), only glucose and fructose were consumed very rapidly. Almost no galactose and xylose were consumed during the fermentation. After 72 h, 74% glucose, 100% fructose, 7.7% galactose and 27% xylose were consumed. Therefore, *C. tyrobutyricum* (Δack, adhE2) preferred to use fructose and glucose. The consumption rate of fructose was faster than that of glucose. The utilization of xylose was partly inhibited and the consumption of galactose was almost totally blocked in *C. tyrobutyricum* (Δack, adhE2). This can also explain the lower butanol titer in the fermentation. The sugar consumption in the fermentation of *C. acetobutylicum* ATCC 824 showed similar characters as in *C. tyrobutyricum* (Δack, adhE2). The consumption of glucose and fructose was much faster than that of galactose and xylose. After 72 h, 100% glucose, 73% fructose, 62% galactose and 36% xylose were utilized. So only the metabolism of xylose was partly inhibited. Unlike in *C. tyrobutyricum* (Δack, adhE2), the glucose consumption rate was faster than the fructose consumption rate in *C. acetobutylicum* ATCC 824. The butanol production was still partly blocked and ~6.0 g/L
butyric acid was accumulated. So among there three *Clostridia, C. acetobutylicum* ATCC 55025 could co-utilize glucose, fructose, galactose and xylose and gave the highest butanol titer (8.7 g/L).

### 6.4 Conclusion

Cassava bagasse hydrolysate can be a good carbon source for butanol production. It had no toxicity to *Clostridium*. Jerusalem artichoke was easy to be hydrolyzed, but the hydrolysate contained a higher level of ferulic acid, which partly inhibited butanol production. Activated carbon can partly adsorb ferulic acid in Jerusalem artichoke hydrolysate, but not totally removed. Soybean meal with a high protein content (~40%) can be used as a good nitrogen source, replacing yeast extract and corn steep liquor, in ABE fermentation to produce butanol from various biomass feedstock. Soybean meal also has a high carbohydrate content (~30%), which mainly consists of oligosaccharides (stachyose and raffinose). These oligosaccharides are difficult to be used by *Clostridia* and must be pre-hydrolyzed to simple sugars (sucrose, glucose, galactose) preferably by using α-galactosidase. Soybean hull and soy molasses can be used as low-cost carbon source for butanol production after acid pretreatment and enzymatic hydrolysis. Combining the hydrolysates from soybean meal and soybean hull or soy molasses can provide an economical substrate for butanol production. The butanol unit costs from three different types of biomass (cassava bagasse, Jerusalem artichoke and soy molasses) were all lower than the cost from lignocellulosic biomass. Among them, soy molasses had the lowest unit cost ($2.71 /gal).
6.5 Reference


Ezeji T.C., Hans P. Blaschek, Fermentation of dried distillers’ grains and soluble (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia, Bioresource Technology 2008, 99: 5232–5242


Liew S.T., Arbakariya A., Rosfarizan M., Raha A.R. Production of solvent (acetone-
butanol-ethanol) in continuous fermentation by Clostridium saccharobutylicum
DSM 13864 using gelatinised Sago starch as a carbon source. Malaysian J

Lin Y.L., Blaschek H.P. Butanol production by a butanol-tolerant strain of Clostridium
acetobutyricum in extruded corn broth. Appl Environ Microbiol 1983, 45: 966-
973.

Lowmeier, E.M., Sopher, C.R., Lee, H. Intracellular acidification as a mechanism for the
75–81.

Lu, Congcong; Dong, Jie; Yang, Shang-Tian, Butanol production from wood pulping
hydrolysate in an integrated fermentation-gas stripping process, Bioresource
Technology (2013), 143, 467-475.

Maddox I. S., Qureshi N., Gutierrez N.A. Utilization of whey and process technology by
Clostridia In: Woods DR, editor The Clostridia and Biotechnology. Butterworth

Marchal R., Ropars M., Pourquie J., Fayolle F., Vandecasteele J.P. Large-scale
enzymatic hydrolysis of agricultural lignocellulosic biomass. Part 2: Conversion


Meinita M.N., Yong-Ki Hong, Gwi-Taek Jeong, Detoxification of acidic catalyzed hydrolysate of Kappaphycus alvarezii (cottonii), Bioprocess Biosyst Eng 2012, 35:93–98


Qureshi N. Agricultural residues and energy crops as potentially economical and novel substrates for microbial production of butanol (a biofuel), Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2010, 5: 1-8


Qureshi N., Saha B.C., Cotta M.A. Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. Bioprocess and Biosystems Engineering, 2007, 30: 419-427


Wang L. and Hongzhang Chen, Acetone-butanol-ethanol Fermentation and Isoflavone Extraction Using Kudzu Roots, Biotechnology and Bioprocess Engineering 2011, 16: 739-745

Yu, Ming-Rui; Zhang, Ya-Li; Tang, I.-Ching; Yang, Shang-Tian, Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production, Metabolic Engineering (2011), 13(4), 373-382.

Zhang Y., Bei Han and Thaddeus Chukwuemeka Ezeji, Biotransformation of furfural and 5-hydroxymethyl furfural (HMF) by *Clostridium acetobutylicum* ATCC 824 during butanol fermentation, New Biotechnology, 2012, 29(3): 345-351

Table 6.1 The compositions of Cassava bagasse and Jerusalem artichoke

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava bagasse</td>
<td>25%±5% cellulose; 43%±1% starch; 9%±4% hemicellulose; 10%±2% lignin</td>
</tr>
<tr>
<td>Jerusalem artichoke</td>
<td>15%±3% cellulose; 54%±5% inulin; 20%±2% lignin</td>
</tr>
</tbody>
</table>

Table 6.2 The sugar concentrations of the biomass after the hydrolysis

<table>
<thead>
<tr>
<th>Pretreatments</th>
<th>Glucose (g/L)</th>
<th>Xylose and fructose (g/L)</th>
<th>Yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jerusalem artichoke</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot water</td>
<td>20.2±0.5</td>
<td>56.4±1.2</td>
<td>0.51±0.01</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>24.8±3.1</td>
<td>87.2±0.8</td>
<td>0.75±0.03</td>
</tr>
<tr>
<td>Cassava bagasse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot water</td>
<td>59.7±1.5</td>
<td>9.7±3.1</td>
<td>0.46±0.03</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>57.6±2.8</td>
<td>5.9±1.0</td>
<td>0.42±0.03</td>
</tr>
</tbody>
</table>
Table 6.3 The inhibitors’ concentrations in hydrolysates before and after activated carbon absorption

| Activated carbon detoxification | Jerusalem artichoke Before | - | 0.10±0.05 | 2.19±0.08 | 0.71±0.08 | 0.18±0.01 |
|                               | Jerusalem artichoke After  | - | 0.06±0.01 | 1.48±0.06 | 0.44±0.04 | -         |
|                               | Cassava bagasse Before     | - | -         | 0.64±0.08 | -         | -         |
|                               | Cassava bagasse After      | - | -         | 1.09±0.09 | -         | -         |

Table 6.4 The fermentation results using biomass hydrolysates as the carbon source

<table>
<thead>
<tr>
<th>Biomass</th>
<th>ATCC 824</th>
<th>BA101</th>
<th>C. tyrobutyricum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butanol (g/L)</td>
<td>Acids (g/L)</td>
<td>Butanol (g/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acids (g/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Butanol (g/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acids (g/L)</td>
</tr>
<tr>
<td>Control</td>
<td>9.6±0.1</td>
<td>4.5±0.2</td>
<td>10.2±0.1</td>
</tr>
<tr>
<td></td>
<td>3.0±0.2</td>
<td></td>
<td>9.5±0.3</td>
</tr>
<tr>
<td></td>
<td>5.4±0.1</td>
<td></td>
<td></td>
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<tr>
<td>Cassava bagasse</td>
<td>11.2±0.2</td>
<td>6.7±0.1</td>
<td>9.9±0.1</td>
</tr>
<tr>
<td></td>
<td>2.7±0.2</td>
<td></td>
<td>10.1±0.2</td>
</tr>
<tr>
<td></td>
<td>5.1±0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jerusalem artichoke</td>
<td>7.8±0.2</td>
<td>8.4±0.2</td>
<td>5.7±0.2</td>
</tr>
<tr>
<td></td>
<td>3.7±0.3</td>
<td></td>
<td>7.4±0.2</td>
</tr>
<tr>
<td></td>
<td>4.9±0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6.5 Carbohydrate content of soybean soluble and soy molasses.

<table>
<thead>
<tr>
<th></th>
<th>Stachyose</th>
<th>Raffinose</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Others</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean soluble</td>
<td>-</td>
<td>15.7%</td>
<td>5.1%</td>
<td>9.4%</td>
<td>6.7%</td>
<td>36.9%</td>
</tr>
<tr>
<td>Soy molasses - 1</td>
<td>9.0%</td>
<td>5.6%</td>
<td>11%</td>
<td>4.4%</td>
<td>5.1%</td>
<td>35.1%</td>
</tr>
<tr>
<td>Soy molasses - 2</td>
<td>10.5%</td>
<td>-</td>
<td>14.6%</td>
<td>0.5%</td>
<td>1.2%</td>
<td>26.8%</td>
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</table>
Table 6.6 Butanol and acids production from soy molasses by *C. acetobutylicum* ATCC 824 and *C. tyrobutyricum (Δack, adhE2)* after 3 days fermentation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N-source</th>
<th>Butanol (g/L)</th>
<th>Acetate (g/L)</th>
<th>Butyrate (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. acetobutylicum</em> ATCC 824</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>yeast extract, NH₄-acetate</td>
<td>0.6</td>
<td>4.1</td>
<td>4.5</td>
</tr>
<tr>
<td>Enzymes from <em>Aspergillus niger</em> fermentation broth</td>
<td>yeast extract, NH₄-acetate</td>
<td>2.5</td>
<td>6.0</td>
<td>8.0</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>yeast extract, NH₄-acetate</td>
<td>5.0</td>
<td>4.0</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Corn steep liquor (30 g/L)</td>
<td>4.0</td>
<td>5.0</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Soybean meal (10 g/L)</td>
<td>3.6</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td><em>C. tyrobutyricum (Δack, adhE2)</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>Yeast Extract, NH₄-acetate</td>
<td>4.5</td>
<td>4.3</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Corn steep liquor (30 g/L)</td>
<td>4.9</td>
<td>4.2</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Soybean meal (10 g/L)</td>
<td>5.2</td>
<td>3.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>

The loading of soy molasses was 200 g/L.
Table 6.7 Hydrolysis of oligosaccharides present in soybean meal by different acid pretreatments followed with enzymatic hydrolysis with α-galactosidase

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Glucose (g/L)</th>
<th>Galactose + Fructose (g/L)</th>
<th>Total sugar (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04 N HCl</td>
<td>8.8</td>
<td>11.1</td>
<td>19.9</td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>13.7</td>
<td>15.5</td>
<td>29.2</td>
</tr>
<tr>
<td>0.3 N HCl</td>
<td>7.6</td>
<td>12.6</td>
<td>20.2</td>
</tr>
<tr>
<td>0.04 N H$_2$SO$_4$</td>
<td>7.3</td>
<td>9.2</td>
<td>16.5</td>
</tr>
</tbody>
</table>

* The loading of soybean meal was 100 g/L

Table 6.8 Fermentation results of *C. acetobutylicum* ATCC 824 using soybean meal hydrolysates pretreated with various acid concentrations followed with enzymatic hydrolysis with α-galactosidase

<table>
<thead>
<tr>
<th>Acid pretreatment</th>
<th>Initial sugar (g/L)</th>
<th>Final butanol (g/L)</th>
<th>Acids (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04 N HCl</td>
<td>19.9</td>
<td>0.89</td>
<td>5.3</td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>29.2</td>
<td>0.79</td>
<td>4.2</td>
</tr>
<tr>
<td>0.3 N HCl</td>
<td>20.2</td>
<td>1.43</td>
<td>4.6</td>
</tr>
<tr>
<td>0.04 N H$_2$SO$_4$</td>
<td>16.5</td>
<td>2.86</td>
<td>4.3</td>
</tr>
<tr>
<td>0.04 N H$_2$SO$_4$, concentrated 3-fold</td>
<td>48.0</td>
<td>0.12</td>
<td>7.2</td>
</tr>
</tbody>
</table>
Table 6.9 Butanol production from soy molasses hydrolysate as the carbon source and soybean meal hydrolysate as the nitrogen source

<table>
<thead>
<tr>
<th>Strain</th>
<th>Soybean meal (g/L)</th>
<th>Butanol (g/L)</th>
<th>Acids (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. acetobutylicum</em> ATCC 824</td>
<td>30</td>
<td>2.2</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.4</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.6</td>
<td>8.9</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> BA101</td>
<td>10</td>
<td>1.7</td>
<td>5.3</td>
</tr>
<tr>
<td><em>C. tyrobutyricum</em> (Δack, adhE2)</td>
<td>10</td>
<td>5.2</td>
<td>7.1</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em> ATCC 55025</td>
<td>10</td>
<td>8.7</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 6.10 Fermentation Parameters based on *C. tyrobutyricum* (Δack, adhE2)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butanol final titer</td>
<td>20 g/L</td>
</tr>
<tr>
<td>Butanol productivity</td>
<td>1.0 g/L·h</td>
</tr>
<tr>
<td>Butanol yield</td>
<td>0.3 g/g sugars</td>
</tr>
<tr>
<td>Butanol, Acetone, Ethanol ratio</td>
<td>20:1:2</td>
</tr>
<tr>
<td>Annual butanol production</td>
<td>~7.5 Million Gal</td>
</tr>
</tbody>
</table>
Table 6.11 Hydrolysis parameters of different biomasses

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Loading (w/w)</th>
<th>Temperature (°C)</th>
<th>Catalyst</th>
<th>Sugar yield (g/g biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn starch</td>
<td>10%</td>
<td>60</td>
<td>No</td>
<td>0.8</td>
</tr>
<tr>
<td>Lignocellulose*a</td>
<td>20%</td>
<td>120</td>
<td>0.04N HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Cassava bagasse</td>
<td>15%</td>
<td>50</td>
<td>5% cellulase</td>
<td></td>
</tr>
<tr>
<td>Jerusalem artichoke</td>
<td>20%</td>
<td>120</td>
<td>0.04N H₂SO₄</td>
<td>0.7</td>
</tr>
<tr>
<td>Soy molasses</td>
<td>20%</td>
<td>50</td>
<td>1% α-galactosidase</td>
<td>0.3b</td>
</tr>
</tbody>
</table>

*a The composition of cotton stalk is used to stand for lignocellulose biomass. And its hydrolysis contains two steps: HCl pretreatment and cellulose hydrolysis

*b Soy molasses is a thick liquid, so this sugar yield is g sugars/g liquid

Figure 6.1 Hydrolysis of soybean molasses by α-galactosidase (0.1% v/v) at pH 5.0, 50 °C

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Figure 6.2 The fermentation kinetics of different *Clostridia* with soy molasses and soybean meal hydrolysates: (a) *C. acetobutylicum* ATCC 55025; (b) *C. tyrobutyricum* (*Δ*ack, *adhE2*); (c) *C. acetobutylicum* ATCC 824
Figure 6.3 Unit production costs by using different raw materials

(The raw material costs are: Corn starch $150/MT; Lignocellulose $50/MT; Cassava bagasse $20/MT; Jerusalem artichoke $50/MT; Soy molasses $10/MT)

Figure 6.4 Capital costs by using different raw materials
Figure 6.5 Operating costs by using different raw materials

Figure 6.6 Sensitivity analysis of unit production cost for the variation in the cost of raw materials.
Figure 6.7 Sensitivity analysis of unit production cost for the variation in the yield of butanol.

Figure 6.8 Pretreatment of corn starch process.
Figure 6.9 Pretreatment of lignocellulose hydrolysis process

Figure 6.10 Pretreatment of cassava bagasse
Figure 6.11 Pretreatment of Jerusalem artichoke

Figure 6.12 Pretreatment of soy molasses
Figure 6.13 Fermentation Section

Figure 6.14 Separation of acetone, ethanol and butanol
Chapter 7: Conclusions and Recommendations

7.1 Conclusions

In this study, high butanol production from different types of lignocellulosic biomass and agriculture residues was successfully obtained. The inhibitory compounds produced from the hydrolysis process of lignocellulosic biomass were investigated in detail. The inhibitors' effects on C. beijerinckii, C. acetobutylicum and C. tyrobutyricum strains were tested at different concentration. Lignin degraded phenolic inhibitors were the most toxic ones. They can strongly inhibit the activities of butanol and butyraldehyde dehydrogenases. They also have negative effects on CoA-transferase. Four lignocellulosic biomass, cotton stalk, sugarcane bagasse, soybean hull and corn fiber, and three non- lignocellulosic agriculture residues, cassava bagasse, Jerusalem artichoke and soy molasses were all successfully hydrolyzed and detoxified. The detoxified hydrolysates gave high butanol production as carbon sources in ABE fermentation. The important findings in this project were summarized as follows.

7.1.1 Effects of lignocellulosic biomass hydrolysate derived inhibitors on ABE fermentation

Sugar-degradation inhibitors (furfural, HMF, formic acid and levulinic acid) are not toxic to C. beijerinckii, C. acetobutylicum and C. tyrobutyricum strains below 1.0 g/L.
On the contrary, lignin derived phenolic inhibitors (syringaldehyde, vanillin, ferulic acid and \( p \)-coumaric acid) are very toxic to \textit{Clostridia} strains even at 0.25 g/L. Their toxicity has two aspects. First, they can destroy the cell membrane's permeability and lead to cell death. The very low OD in the fermentation is due to the death of cells. Second, they can strongly inhibit butanol and butyraldehyde dehydrogenases. So the \textit{Clostridia} strains can only produce a lot of acids, but cannot convert them to butanol.

\textbf{7.1.2 Butanol production from lignocellulosic biomass and agriculture residues}

Dilute acid pretreatment is an effective method to promote the enzymatic hydrolysis of cellulose and hemicellulose in biomass. But high concentration of acids catalyzed the further degradation of biomass components and generated large amount of inhibitors, especially ferulic acid to ABE fermentation, exceeding the toxic level that inhibited the butanol production, especially ferulic acid. For corn fiber, soybean hull and sugarcane bagasse, 0.04 N was the best acid level. Activated carbon absorption was effective in removing phenolic inhibitors in the hydrolysate, especially ferulic acid. The acid and alkali pretreatments were also compared. The acid pretreatment removed 56\%-90\% of hemicellulose and 7\%-65\% of lignin in the biomass at 0.04 N hydrogen ions while 0.04 N NaOH dissolved 44\%-80\% of hemicellulose and 46\%-78\% of lignin in the biomass. Therefore, both acid and alkali pretreatments can remove part of hemicellulose and lignin in the biomass. The dissolving capability depends on the compositions of the biomass.

Cassava bagasse hydrolysate can be a good carbon source for butanol production. It had no toxicity to \textit{Clostridia}. Jerusalem artichoke was easy to be hydrolyzed, but the
hydrolysate contained a higher level of ferulic acid, which partly inhibited the butanol production. Activated carbon can adsorb 38% of ferulic acid in Jerusalem artichoke hydrolysate, but not totally removed. Soybean meal with a high protein content (~40%) can be used as a good nitrogen source, replacing yeast extract and corn steep liquor, in ABE fermentation to produce butanol from various biomass feedstocks. Soybean meal also has a high carbohydrate content (~30%), which mainly consists of oligosaccharides (stachyose and raffinose). These oligosaccharides are difficult to be used by clostridia and must be pre-hydrolyzed to simple sugars (sucrose, glucose, galactose) preferably by using α-galactosidase. Soy molasses can be used as low-cost carbon source for butanol production after enzymatic hydrolysis. Combining the hydrolysates from soybean meal and soy molasses can provide an economical substrate for butanol production.

7.1.3 The inhibition mechanism of phenolic inhibitors to the metabolic pathway of Clostridia

The phenolic compounds (p-coumaric acid, ferulic acid, vanillin and syringaldehyde) were toxic to Clostridium strains with CoA-transferase but showed almost no effects on strains without CoA-transferase. These phenolic inhibitors may work as competitive inhibitors to CoA-transferase and block the solventogenesis phase that converts acids into solvents. Corn steep liquor is rich in protein and amino acids, and is a potential nitrogen source for butanol production. But the phenolic inhibitors in it may severely inhibit butanol production by solventogenic Clostridia containing ctfAB genes.
7.2 Recommendations

Although seven different types of biomass were successfully hydrolyzed, detoxified and used in ABE fermentation, giving high butanol production, many areas still require further research before butanol fermentation can be commercialized and competitive with petrochemically-derived butanol. Some recommendations about further work were listed as follows.

7.2.1 Combined Effects of lignocellulosic biomass hydrolysate derived inhibitors

In this research, the inhibitors' effects on ABE fermentation were studied. Four sugar-degradation inhibitors (furfural, HMF, formic acid and levulinic acid) and four lignin derived inhibitors (syringaldehyde, vanillin, ferulic acid and p-coumaric acid) were tested on three solventogenic Clostridia (C. beijerinckii, C. acetobutylicum and C. tyrobutyricum). Some other inhibitors with larger molecule weights like lignin fragments can also be produced in the hydrolysis of biomass. The inhibition effects of these larger inhibitors need further study. Other Clostridia such as C. saccharobutylicum and C. aurantibutyricum can also be tested for their tolerance to inhibitors.

Individual inhibitor was used in the inhibition test of ABE fermentation. The inhibition effects may be different if two or more inhibitors were combined together. The combined inhibitors will be more like the situations in the real biomass hydrolysate. So they may be closer to the real inhibition effects in the real biomass hydrolysate. The combinations of different inhibitors may be huge. So statistical experiment design tools are necessary. Another approach is that first find the inhibitors' types and concentrations
in the real biomass hydrolysate and then only combine the inhibitors existing in the hydrolysate.

7.2.2 Optimizations of hydrolysis processes

Detailed acid and alkali pretreatments were studied in this research. Other pretreatments such as steam explosion, ammonia explosion or biological degradation can also be evaluated. The biomass change after pretreatment is the key to find a better pretreatment for each biomass. In this study, after removing the acid or alkali pretreatment supernatant, some of the biomass hydrolysates were still very toxic to Clostridium strains. What caused this inhibition is still unclear and needs further study.

Other detoxification method can also be investigated. Lignin derived phenolic inhibitors were found to be the toxic inhibitors to solventogenic Clostridia. So an effective detoxification method must have the ability be removed all the phenolic inhibitors. Overliming is the most cost effective detoxification method. But its capability of removing phenolic compounds is still unclear. Ion exchange resins, especially anion exchange resins showed promising efficiency in removing phenolic compounds. Although activated carbon can adsorb the phenolic compounds effectively, it can also remove certain portion of sugar. So other adsorbents that only remove phenolic compounds can be tried in the detoxification process. Most inhibitors are produced in the pretreatments and soluble in water, so another detoxification idea is that the inhibitors can be removed together with the supernatants after the pretreatments. Removing the
pretreatment supernatants and washing the biomass solid can remove most of the soluble inhibitors.

The compositions of biomass have great impact on the pretreatments and the final components of hydrolysate. Different types of lignocellulosic biomass also have different cellulose, hemicellulose and lignin contents. Even for one biomass, its compositions may vary with different harvest seasons or different places. This composition difference induces different types of inhibitors in the hydrolysate and different detoxification methods. Therefore, it is necessary to evaluate more biomass with different compositions. This can further prove the diversity of substrates used in ABE fermentation and feasibility of large-scale butanol production in different regions.

7.2.3 The inhibition mechanism of phenolic inhibitors to the metabolic pathway of Clostridia

From this project, the phenolic compounds can inhibit the function of CoA-transferase. So Clostridium strains that don't contain this CoA-transferase are preferred when biomass hydrolysate is used as the substrates. Metabolic engineering that knock out the ctfAB genes which express CoA-transferase or even block the entire acid production phase is also a possible way to solve the inhibition. Another possible solution is to find an effective detoxification method to remove the phenolic compounds from biomass hydrolysate before the fermentation.

Further study about phenolic inhibitors' effects to the metabolic pathway of solventogenic Clostridia is also needed. The function of phenolic compounds as
competitive inhibitors to CoA-transferase needs further proofs. It's better to isolate CoA-transferase and test the effects of phenolic compounds directly on it. Our study also showed phenolic compounds can inhibit butanol and butyraldehyde dehydrogenases. Therefore, it seems phenolic compounds have several different inhibition points in the metabolic pathway of solventogenic Clostridia. A complete understanding of phenolic compounds' inhibition mechanism can guide the detoxification process and strain developments.
Bibliography


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Jennings, E. and Schell, D. Conditioning of dilute-acid pretreated corn stover hydrolysate liquors by treatment with lime or ammonium hydroxide to improve conversion of sugars to ethanol. Bioresource Technology. 2011, 102(2): 1240 – 1245


Lynd, L.R., Baskaran, S., and Casten, S. Salt accumulation resulting from base added for pH control, and not ethanol, limits growth of thermoanaerobacterium thermosaccharolyticum HG-8 at elevated feed xylose concentrations in continuous culture. Biotechnol. Prog. 2001, 17: 118 – 125


Meinita, M.N., Yong-Ki Hong, Gwi-Taek Jeong, Detoxification of acidic catalyzed hydrolysate of Kappaphycus alvarezii (cottonii), Bioprocess Biosyst Eng 2012, **35**:93–98


Nair, R.V., E.M. Green, D.E. Watson, G.N. Bennett, E.T. Papouskis (1999). Regulation of the sol locus genes for butanol and acetone formation in *Clostridium*


Takagi, M, Inhibition of cellulase by fermentation products. Biotechnol Bioeng 1984, 26:1506–1507


Appendix A. Cost analysis of butanol plants using soybean hull or soy molasses and soybean meal as substrates
Two plants are designed for butanol production. One uses soybean hull and soybean meal as substrates, and the other uses soy molasses and soybean meal as substrates. The process flowsheets for these two plants are shown in Figures A.1-3, respectively. In general, the plant can be divided into three sections: Pretreatment section, Fermentation section and Separation section.

In the Pretreatment section (Figure A.1), soybean hull is washed, dispensed in water (P-2) and grinded into fine powder (P-3). Then, it is pumped into acid pretreatment reactor (P-4). In this reactor, 90% of hemicellulose in soybean hull is hydrolyzed with 0.04 N HCl at 121 °C, 15 psig. Then, 90% of cellulose is hydrolyzed by cellulase in the following reactor (P-5, pH 4.5, 50°C). The hydrolysate is filtered (P-7) to remove ash, lignin and other undissolved solids. The clear sugar solution is sent to the storage tank (P-8) for use in the Fermentation section. The treatment of soybean meal is similar. The only difference is that it doesn’t need the enzymatic hydrolysis reactor. The pretreatment process for soy molasses (Figure A.2) is simpler than that for soybean hull. Soy molasses does not need to be washed, grinded and acid hydrolyzed. It only needs the enzymatic hydrolysis reactor.

In the Fermentation section (Figure A.3), the seed culture is prepared in the seed fermentor (P-14) and then pumped into the main fermentor (P-1). The fermentation broth containing butanol is sent to the storage tank (P-15) for butanol separation. In the Separation section, distillation is used to separate different solvents. The first two columns (P-19, P-20) are used to remove acetone and ethanol from the solution. The
purification of butanol needs two stages (P-21, P-23) because butanol-water system has an azeotropic point. At last, the main product is 99.9% butanol.

Cost analysis is based on an annual butanol production of 7.6 MM gal (23,000 ton) at each plant with the following fermentation parameters: butanol yield, 0.30 g/g sugar; volumetric productivity, 5.5 g/L·h; butanol titer, 22 g/L. The economic analysis for the plant using soybean hull and soybean meal is summarized in Table A.1. The total capital investment is $13.7 MM. The annual operating cost is $29.6 MM. The production cost is $3.90/gal butanol. The major equipment is listed in Table A.2. The most costly pieces of equipment are 4 main fermentors (FR-101) and 6 enzyme hydrolysis reactors (R-102). The Pretreatment section accounts for 43% of the equipment cost, and the Fermentation section accounts for 22%. Table A.3 lists the other capital investments and Table A.4 lists the material costs. Soybean meal accounts for 51% of the total cost. Table A.5 lists the annual operating cost. About 1/3 comes from raw materials, 1/3 from labor and 1/3 from utilities. Table A.6 shows the utility cost summary. About 73% comes from steam, which is mainly used in the Separation section. So if the butanol separation method is improved such as using gas stripping or pervaporation, this steam cost can be significantly reduced. The steam cost can also be reduced if the plant has its own utility section, which means burning part of soybean hull to produce steam.

Tables A.7-12 shows the detailed cost analysis for the plant using soy molasses and soybean meal. Compared to soybean hull plant, this plant has less total capital investment ($10 MM). One reason is that soy molasses does not need washing, grinding and acid pretreatment. The other reason is that its enzymatic hydrolysis is much faster
than soybean hull, so the total volume of the enzymatic hydrolysis reactors is much smaller. Soybean hull needs six 200 m$^3$ reactors, while soy molasses only needs two 150 m$^3$ reactors. The operating cost is also reduced because there are fewer enzyme reactors in the Pretreatment section. The unit product cost is $3.54/gal butanol.

In conclusion, soybean hull, soy molasses and soybean meal can be used as economical substrates for industrial production of butanol. The projected butanol production cost from these substrates is significantly lower than the current market price (~$6.75/gal) and the process should be feasible and economically attractive for industrial application.
Table A.1 Executive summary (2013 prices) - Soybean hull

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Capital Investment</td>
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<td>$</td>
</tr>
<tr>
<td>Capital Investment Charged to This Project</td>
<td>13,753,000.00</td>
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</tr>
<tr>
<td>Operating Cost</td>
<td>29,648,000.00</td>
<td>$/yr</td>
</tr>
<tr>
<td>Main Revenue</td>
<td>36,855,000.00</td>
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<tr>
<td>Other Revenues</td>
<td>1,835,835.00</td>
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</tr>
<tr>
<td>Total Revenues</td>
<td>38,690,000.00</td>
<td>$/yr</td>
</tr>
<tr>
<td>Cost Basis Annual Rate</td>
<td>7,605,000</td>
<td>gal(STP) Bu/yr</td>
</tr>
<tr>
<td>Unit Production Cost</td>
<td>3.90</td>
<td>$/gal(STP) Bu</td>
</tr>
<tr>
<td>Unit Production Revenue</td>
<td>5.09</td>
<td>$/gal(STP) Bu</td>
</tr>
<tr>
<td>Gross Margin</td>
<td>23.37</td>
<td>%</td>
</tr>
<tr>
<td>Return On Investment</td>
<td>46.87</td>
<td>%</td>
</tr>
<tr>
<td>Payback Time</td>
<td>2.13</td>
<td>years</td>
</tr>
<tr>
<td>IRR (After Taxes)</td>
<td>27.89</td>
<td>%</td>
</tr>
<tr>
<td>NPV (at 7.0% Interest)</td>
<td>48,090,000.00</td>
<td>$</td>
</tr>
</tbody>
</table>

Bu = Total Flow of Stream '>99.9% Butanol'
Table A.2 Major equipment specifications and cost (2013 prices) - Soybean hull

<table>
<thead>
<tr>
<th>Quantity/</th>
<th>Name</th>
<th>Description</th>
<th>Unit Cost ($)</th>
<th>Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staggered</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 / 0 / 0</td>
<td>GR-101</td>
<td>Grinder</td>
<td>60,000.00</td>
<td>60,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Size/Capacity = 100000.00 kg/h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 / 0 / 0</td>
<td>GP-101</td>
<td>Gear Pump</td>
<td>12,000.00</td>
<td>12,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pump Power = 3.78 kW</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 / 0 / 0</td>
<td>BF-101</td>
<td>Belt Filter</td>
<td>80,000.00</td>
<td>80,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Belt Width = 31.00 m</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>GR-102</td>
<td>Grinder</td>
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<td></td>
<td>Size/Capacity = 10000.00 kg/h</td>
<td></td>
<td></td>
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<tr>
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<td>GP-102</td>
<td>Gear Pump</td>
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<td>2,000.00</td>
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<tr>
<td></td>
<td></td>
<td>Pump Power = 0.39 kW</td>
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<td>1 / 0 / 0</td>
<td>BF-102</td>
<td>Belt Filter</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Belt Width = 3.33 m</td>
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<tr>
<td>1 / 0 / 0</td>
<td>C-101</td>
<td>Distillation Column</td>
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<tr>
<td></td>
<td></td>
<td>Column Volume = 9481.82 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 / 0 / 0</td>
<td>C-102</td>
<td>Distillation Column</td>
<td>57,000.00</td>
<td>114,000.00</td>
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<tr>
<td></td>
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Continued
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<thead>
<tr>
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<th>Material 2</th>
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<tbody>
<tr>
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<td>34,000.00</td>
<td>34,000.00</td>
</tr>
<tr>
<td></td>
<td>Column Volume = 7319.60 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-104</td>
<td>Decanter Tank</td>
<td>38,000.00</td>
<td>38,000.00</td>
</tr>
<tr>
<td></td>
<td>Vessel Volume = 1108.79 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-104</td>
<td>Distillation Column</td>
<td>31,000.00</td>
<td>31,000.00</td>
</tr>
<tr>
<td></td>
<td>Column Volume = 1438.95 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-101</td>
<td>Stirred Reactor</td>
<td>80,000.00</td>
<td>80,000.00</td>
</tr>
<tr>
<td></td>
<td>Vessel Volume = 52.95 m³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-102</td>
<td>Stirred Reactor</td>
<td>60,000.00</td>
<td>360,000.00</td>
</tr>
<tr>
<td></td>
<td>Vessel Volume = 190.08 m³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-103</td>
<td>Stirred Reactor</td>
<td>50,000.00</td>
<td>50,000.00</td>
</tr>
<tr>
<td></td>
<td>Vessel Volume = 11060.47 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-101</td>
<td>Flat Bottom Tank</td>
<td>70,000.00</td>
<td>70,000.00</td>
</tr>
<tr>
<td></td>
<td>Vessel Volume = 154.92 m³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFR-101</td>
<td>Seed Fermentor</td>
<td>48,000.00</td>
<td>48,000.00</td>
</tr>
<tr>
<td></td>
<td>Vessel Volume = 15.49 m³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FR-101</td>
<td>Fermentor</td>
<td>70,000.00</td>
<td>280,000.00</td>
</tr>
<tr>
<td></td>
<td>Vessel Volume = 154.97 m³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-102</td>
<td>Flat Bottom Tank</td>
<td>70,000.00</td>
<td>70,000.00</td>
</tr>
<tr>
<td></td>
<td>Vessel Volume = 152.48 m³</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unlisted Equipment</td>
<td>361,000.00</td>
<td></td>
</tr>
</tbody>
</table>
Table A.3 Fixed capital estimate summary (2013 prices in $) - Soybean hull

A. Total Plant Direct Cost (TPDC) (physical cost)

1. Equipment Purchase Cost 1,805,000.00
2. Installation 641,000.00
3. Process Piping 632,000.00
4. Instrumentation 722,000.00
5. Insulation 54,000.00
6. Electrical 181,000.00
7. Buildings 812,000.00
8. Yard Improvement 271,000.00
9. Auxiliary Facilities 722,000.00

TPDC 5,839,000.00

B. Total Plant Indirect Cost (TPIC)

10. Engineering 1,460,000.00
11. Construction 2,044,000.00

TPIC 3,504,000.00

C. Total Plant Cost (TPC = TPDC+TPIC)

TPC 9,343,000.00

D. Contractor's Fee & Contingency (CFC)

12. Contractor's Fee 467,000.00

Continued
Table A.3 Continued

13. Contingency  
\[ \text{CFC} = 12+13 \]
\[ 934,000.00 \]
\[ 1,401,000.00 \]

E. Direct Fixed Capital Cost (DFC = TPC+CFC)

DFC  
\[ 10,744,000.00 \]

---

Table A.4 Material costs - Soybean hull

<table>
<thead>
<tr>
<th>Bulk Material</th>
<th>Unit Cost ($)</th>
<th>Annual Amount</th>
<th>Annual Cost ($)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>soybean hull</td>
<td>20.00</td>
<td>158,400.00 MT</td>
<td>3,168,000.00</td>
<td>32.85</td>
</tr>
<tr>
<td>soybean meal</td>
<td>280.00</td>
<td>15,714.00 MT</td>
<td>4,888,972.00</td>
<td>50.70</td>
</tr>
<tr>
<td>HCl</td>
<td>0.10</td>
<td>25.00 MT</td>
<td>2,455.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Cellulase</td>
<td>2.00</td>
<td>792.00 MT</td>
<td>1,584,000.00</td>
<td>16.43</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td>9,643,427.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>
### Table A.5 Annual operating cost (2013 prices) - Soybean hull

<table>
<thead>
<tr>
<th>Cost Item</th>
<th>$</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Materials</td>
<td>9,643,000.00</td>
<td>32.53</td>
</tr>
<tr>
<td>Labor-Dependent</td>
<td>8,778,000.00</td>
<td>29.61</td>
</tr>
<tr>
<td>Facility-Dependent</td>
<td>2,025,000.00</td>
<td>6.83</td>
</tr>
<tr>
<td>Laboratory/QC/QA</td>
<td>439,000.00</td>
<td>1.48</td>
</tr>
<tr>
<td>Utilities</td>
<td>8,763,000.00</td>
<td>29.56</td>
</tr>
<tr>
<td>TOTAL</td>
<td>29,648,000.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

### Table A.6 Utilities cost (2013 prices) - Soybean hull

<table>
<thead>
<tr>
<th>Utility</th>
<th>Unit Cost ($)</th>
<th>Annual Amount</th>
<th>Ref. Units</th>
<th>Annual Cost ($)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std Power</td>
<td>0.05</td>
<td>2,179,998.00</td>
<td>kW-h</td>
<td>109,000.00</td>
<td>1.24</td>
</tr>
<tr>
<td>Steam</td>
<td>12.00</td>
<td>535,443.00</td>
<td>MT</td>
<td>6,425,316.00</td>
<td>73.32</td>
</tr>
<tr>
<td>Cooling Water</td>
<td>0.05</td>
<td>44,580,414.00</td>
<td>MT</td>
<td>2,229,021.00</td>
<td>25.44</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td></td>
<td>8,763,337.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>
Table A.7 Executive summary (2013 prices) - Soy malasses

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Capital Investment</td>
<td>10,504,000.00</td>
<td>$</td>
</tr>
<tr>
<td>Capital Investment Charged to This Project</td>
<td>10,504,000.00</td>
<td>$</td>
</tr>
<tr>
<td>Operating Cost</td>
<td>26,136,000.00</td>
<td>$/yr</td>
</tr>
<tr>
<td>Main Revenue</td>
<td>35,736,000.00</td>
<td>$/yr</td>
</tr>
<tr>
<td>Other Revenues</td>
<td>1,780,110.00</td>
<td>$/yr</td>
</tr>
<tr>
<td>Total Revenues</td>
<td>37,516,000.00</td>
<td>$/yr</td>
</tr>
<tr>
<td>Cost Basis Annual Rate</td>
<td>7,374,334.44</td>
<td>gal(STP) Bu/yr</td>
</tr>
<tr>
<td>Unit Production Cost</td>
<td>3.54</td>
<td>$/gal(STP) Bu</td>
</tr>
<tr>
<td>Unit Production Revenue</td>
<td>5.09</td>
<td>$/gal(STP) Bu</td>
</tr>
<tr>
<td>Gross Margin</td>
<td>30.33</td>
<td>%</td>
</tr>
<tr>
<td>Return On Investment</td>
<td>72.15</td>
<td>%</td>
</tr>
<tr>
<td>Payback Time</td>
<td>1.39</td>
<td>years</td>
</tr>
<tr>
<td>IRR (After Taxes)</td>
<td>40.39</td>
<td>%</td>
</tr>
<tr>
<td>NPV (at 7.0% Interest)</td>
<td>64,513,000.00</td>
<td>$</td>
</tr>
</tbody>
</table>

Bu = Total Flow of Stream '>99.9% Butanol'
<table>
<thead>
<tr>
<th>Quantity/</th>
<th>Name</th>
<th>Description</th>
<th>Unit Cost ($)</th>
<th>Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BF-101</td>
<td>Belt Filter</td>
<td>80,000.00</td>
<td>80,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Belt Width = 60.00 m</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GR-102</td>
<td>Grinder</td>
<td>30,000.00</td>
<td>30,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Size/Capacity = 10000.00 kg/h</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GP-102</td>
<td>Gear Pump</td>
<td>2,000.00</td>
<td>2,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pump Power = 0.39 kW</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BF-102</td>
<td>Belt Filter</td>
<td>40,000.00</td>
<td>40,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Belt Width = 3.33 m</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-101</td>
<td>Distillation Column</td>
<td>47,000.00</td>
<td>47,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Column Volume = 10882.94 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-102</td>
<td>Distillation Column</td>
<td>61,000.00</td>
<td>122,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Column Volume = 13657.85 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-103</td>
<td>Distillation Column</td>
<td>36,000.00</td>
<td>36,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Column Volume = 8318.65 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V-104</td>
<td>Decanter Tank</td>
<td>38,000.00</td>
<td>38,000.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vessel Volume = 1095.06 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-104</td>
<td>Distillation Column</td>
<td>32,000.00</td>
<td>32,000.00</td>
</tr>
</tbody>
</table>

Continued
Table A.8 Continued

<table>
<thead>
<tr>
<th>Column Volume = 1556.40 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 / 0 / 0 R-102 Stirred Reactor</td>
</tr>
<tr>
<td>Vessel Volume = 105.13 m³</td>
</tr>
<tr>
<td>1 / 0 / 0 R-103 Stirred Reactor</td>
</tr>
<tr>
<td>Vessel Volume = 11060.47 L</td>
</tr>
<tr>
<td>1 / 0 / 0 V-101 Flat Bottom Tank</td>
</tr>
<tr>
<td>Vessel Volume = 177.11 m³</td>
</tr>
<tr>
<td>1 / 0 / 0 SFR-101 Seed Fermentor</td>
</tr>
<tr>
<td>Vessel Volume = 17.71 m³</td>
</tr>
<tr>
<td>4 / 0 / 0 FR-101 Fermentor</td>
</tr>
<tr>
<td>Vessel Volume = 177.16 m³</td>
</tr>
<tr>
<td>1 / 0 / 0 V-102 Flat Bottom Tank</td>
</tr>
<tr>
<td>Vessel Volume = 174.74 m³</td>
</tr>
<tr>
<td>Unlisted Equipment</td>
</tr>
<tr>
<td>TOTAL</td>
</tr>
</tbody>
</table>
Table A.9 Fixed capital estimate summary (2013 prices in $) - Soy molasses

<table>
<thead>
<tr>
<th>A. Total Plant Direct Cost (TPDC) (physical cost)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Equipment Purchase Cost</td>
<td>1,335,000.00</td>
</tr>
<tr>
<td>2. Installation</td>
<td>451,000.00</td>
</tr>
<tr>
<td>3. Process Piping</td>
<td>467,000.00</td>
</tr>
<tr>
<td>4. Instrumentation</td>
<td>534,000.00</td>
</tr>
<tr>
<td>5. Insulation</td>
<td>40,000.00</td>
</tr>
<tr>
<td>6. Electrical</td>
<td>134,000.00</td>
</tr>
<tr>
<td>7. Buildings</td>
<td>601,000.00</td>
</tr>
<tr>
<td>8. Yard Improvement</td>
<td>200,000.00</td>
</tr>
<tr>
<td>9. Auxiliary Facilities</td>
<td>534,000.00</td>
</tr>
<tr>
<td>TPDC</td>
<td>4,296,000.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Total Plant Indirect Cost (TPIC)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10. Engineering</td>
<td>1,074,000.00</td>
</tr>
<tr>
<td>11. Construction</td>
<td>1,503,000.00</td>
</tr>
<tr>
<td>TPIC</td>
<td>2,577,000.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Total Plant Cost (TPC = TPDC+TPIC)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC</td>
<td>6,873,000.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D. Contractor's Fee &amp; Contingency (CFC)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>12. Contractor's Fee</td>
<td>344,000.00</td>
</tr>
<tr>
<td>13. Contingency</td>
<td>687,000.00</td>
</tr>
<tr>
<td></td>
<td>Continued</td>
</tr>
</tbody>
</table>
Table A.9 Continued

CFC = 12+13

1,031,000.00

E. Direct Fixed Capital Cost (DFC = TPC+CFC)

DFC

7,904,000.00

Table A.10 Materials cost – Soy molasses

<table>
<thead>
<tr>
<th>Bulk Material</th>
<th>Unit</th>
<th>Annual Amount</th>
<th>Annual Cost  ($)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>soybean molasses</td>
<td>0.05</td>
<td>39895306.00</td>
<td>1,994,765.00</td>
<td>25.98</td>
</tr>
<tr>
<td>soybean meal</td>
<td>280.00</td>
<td>15715.00</td>
<td>4,888,972.00</td>
<td>63.67</td>
</tr>
<tr>
<td>a-galactosidase</td>
<td>1.00</td>
<td>792.00</td>
<td>792,000.00</td>
<td>10.32</td>
</tr>
<tr>
<td>HCl</td>
<td>0.10</td>
<td>24.00</td>
<td>2,376.00</td>
<td>0.03</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td>7,678,113.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

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### Table A.11 Annual operating cost (2013 prices) – Soy molasses

<table>
<thead>
<tr>
<th>Cost Item</th>
<th>$</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Materials</td>
<td>7,678,000.00</td>
<td>29.38</td>
</tr>
<tr>
<td>Labor-Dependent</td>
<td>7,783,000.00</td>
<td>29.78</td>
</tr>
<tr>
<td>Facility-Dependent</td>
<td>1,490,000.00</td>
<td>5.70</td>
</tr>
<tr>
<td>Laboratory/QC/QA</td>
<td>389,000.00</td>
<td>1.49</td>
</tr>
<tr>
<td>Utilities</td>
<td>8,796,000.00</td>
<td>33.65</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>26,136,000.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

### Table A.12 Utilities cost (2013 prices) - Soy molasses

<table>
<thead>
<tr>
<th>Utility</th>
<th>Unit Cost</th>
<th>Annual Amount</th>
<th>Ref. Units</th>
<th>Annual Cost ($)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std Power</td>
<td>0.05</td>
<td>1022497.00</td>
<td>kW-h</td>
<td>51125.00</td>
<td>0.58</td>
</tr>
<tr>
<td>Steam</td>
<td>12.00</td>
<td>541082.00</td>
<td>MT</td>
<td>6492982.00</td>
<td>73.82</td>
</tr>
<tr>
<td>Cooling Water</td>
<td>0.05</td>
<td>45036648.00</td>
<td>MT</td>
<td>2251832.00</td>
<td>25.60</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td></td>
<td></td>
<td>8795939.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>
Figure A.1 The acid pretreatment and hydrolysis processes for soybean hull and soybean meal
Figure A.2 The pretreatment and hydrolysis processes for soy molasses and soybean meal.
Figure A.3 Process flowsheet for butanol fermentation and separation.
Appendix B. Analytical procedures of inhibitors by high performance liquid chromatograph
The inhibitors including furfural, HMF, levulinic acid, ferulic acid, \(p\)-coumaric acid, and phenolic compounds (syringaldehyde) were analyzed by high performance liquid chromatography (HPLC) using Rezex ROA-Organic Acid H\(^+\) (8\%) column (Phenomenex) at 40 °C and a UV/UIS detector (SPD-20AV, Shimadzu). Samples were centrifuged at 13.2 g for 5 min in microcentrifuge tubes and diluted 20 times with distilled water prior to analysis on HPLC. The eluent was 0.01 N H\(_2\)SO\(_4\) at 0.6 ml/min. Furfural, HMF and phenolic compounds were detected at 280 nm. Syringaldehyde was used as the standard for phenolic compounds. Levulinic acid, ferulic acid, \(p\)-coumaric acid were detected at 200 nm. 15μL sample was injected by an automatic injector (SIL-10Ai) and the running time was set at 60 min. Peak height was used to calculate concentration of sugars in the sample based on the peak height of standard sample. HPLC chromatogram of the 1.0 g/L standard inhibitor sample is shown in Figure B.1-B.8.

![HPLC chromatogram of 1.0 g/L hydroquinone at 280 nm](image-url)
Figure B.2 HPLC chromatogram of 1.0 g/L furfural at 280 nm

Figure B.2 HPLC chromatogram of 1.0 g/L HMF at 280 nm
Figure B.4 HPLC chromatogram of 1.0 g/L syringaldehyde at 280 nm

Figure B.5 HPLC chromatogram of 1.0 g/L levulinic acid at 200 nm
Figure B.6 HPLC chromatogram of 1.0 g/L p-coumaric acid at 200 nm

Figure B.6 HPLC chromatogram of 1.0 g/L formic acid at 200 nm